Stefan Magez · Magdalena Radwanska
Editors

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Introduction

'Trypanosomiasis' in general refers to a collection of parasitic diseases caused by both extracellular and intracellular protozoan parasites. This book will focus mainly on the extracellular trypanosome species that cause infections in human and animals and include *T. brucei brucei*, *T. brucei rhodesiense*, *T. brucei gambiense*, *T. congolense*, *T. evansi* and *T. vivax*. Often, the diseases caused by these parasites are referred to as 'African Trypanosomiasis'. However, while it is correct to state that historically these infections have mostly confined to the sub-Saharan region of the African continent, both *T. evansi* and *T. vivax* have moved now beyond the borders of the African continent and infect large regions of Asia and South America, as they no longer need the African tsetse fly as an obligatory host for disease transmission.

To date, most knowledge on the biology of trypanosomes and their interaction with both the mammalian and the insect vector–host comes from experimental data obtained with *Trypanosoma brucei brucei*. Hence, the various authors involved in the writing of this manuscript have focused on this parasite and where needed have made links to either *T. congolense*, *T. evansi* or *T. vivax*. The last chapter of this book specifically deals with the latter, focusing on *T. vivax* infections in South America and the emerging devastating problems these infections are bringing to the new world.

As for transmission, a full chapter is dedicated to the tsetse fly as a vector crucial in the life cycle of most trypanosomes on the African continent. Interesting to observe here is the fact that the adaptations and different life stages of the trypanosome seen in its insect vector–host are much more diverse than those seen in the mammalian host. In a classic view, the trypanosome needs the tseste fly to be transmitted from one mammal to the next. However, from the entomology point of view, one could argue that the trypanosome is perfectly adapted to life inside the tsetse fly (even allowing exchange of genetic material between individual trypanosomes) and that it is capable of using any mammalian infection to ensure passage from one fly to the next.

Combined, the authors of this manuscript have attempted to bring together all the recent knowledge on (1) the cell biology of the protozoan parasite, (2) the biology of the parasite–fly interaction, (3) the parasite cell surface that provides the interface between the parasite and its hosts, (4) the innate immunity of the mammalian host, (5) the adaptive immunity, (6) specific aspects of host defense system

in the context of human infections and (7) the parasite proteome/secretome in view of new understandings with respect to parasite–host interactions. Finally, the work also covers (8) the current state of diagnostics and (9) drug treatment strategies for trypanosomiasis, while ending with a specific discussion on (10) Animal African Trypanosomiasis and (11) the situation of Animal Trypanosomiasis in South America.

Together, the topics covered here include both Human African Trypanosomaisis, also called Sleeping Sickness, as well as a variety of Animal trypanosome infections known under the names of *nagana*, *surra*, *dourine*, *derrengadera* or *mal de cadeiras*. It is now about 120 years ago that Sir Bruce made the link between the trypanosome and the disease trypanosomiasis, while establishing the parasitic cause of *nagana* (the Zulu expression for 'being in low spirit') in animal infections present in the Southern Africa Zulu lands. To date, we do understand a great deal of the parasite's biology, its transmission and its interaction with transmission vectors. While recent efforts to control Human Trypanosomiasis have been quite successful, Animal trypanosomiasis however is far from being controlled and is even rapidly extending in territories both in South America and Asia. With no real limitations to the mammalian host repertoire, controlling Animal trypanosomiasis is going to be a very big challenge for the future if one wants to limit the economic damage caused by *T. congolense*, *T. vivax* and *T. evansi*. Finally, it is important to note that in recent years, Human Trypanosomiasis has been reported, albeit extremely rare, on the Indian subcontinent. Indeed, when specific human conditions occur, *T. evansi* has been shown to be able to cause lethal infections in human. This should serve as a reminder that parasite evolution is an ever ongoing feature and that as long as Animal Trypanosomiasis is not irradicated, the next Human form of Trypanosomiais could be around the corner. While the most likely candidate to cause this new form of the disease would be *T. evansi*, the vast and rapid spread of *T. vivax* on the South-American continent, its journey up north towards Central-America and the adaptation to new mammalian host species and modes of transmission (including by vampire bats) are equal reasons for concern. Hopefully, persistent future research into basic biology of trypanosomiasis can yield the discovery of new drug targetable biochemical pathways crucial for parasite survival. In addition, new sensitive, specific, cheap and easy-to-use field diagnostic tools will have to be developed to aid in a final solution for trypanosomiasis, a disease that till today mostly affects rural communities that do not always have access to the knowledge and financial means to make trypanosomiasis irradication a top priority.

Acknowledgement

This book came together by the combined effort of a number of trypanosome experts who all joined the Colloquium on Trypanosomiasis, held on September 14th, 2012 at the Institute for Tropical Medicine, Antwerp, Belgium. In addition to the contributing authors, there are four senior Research Professors who all in their own way did provide tremendous input into the field of trypanosome research and moreover have paved the way for a new generation of research to take on new tasks in this intriguing field of trypanosome biology.

Hereby, we would like to thank Prof. Patrick De Baetselier, Prof. Dave Barry, Prof. Sam Black and Prof. Etienne Pays for the help, support and mentorship all of them have provided over the last decades.

Contents

Cell Biology for Immune Evasion: Organizing Antigenic Variation, Surfaces, Trafficking, and Cellular Structures in *Trypanosoma brucei*

1

Ka Fai Leung, Paul T. Manna, Cordula Boehm, Luke Maishman, and Mark C. Field

Abstract

For any pathogen to maintain an infection for a protracted period, there is a necessity for precise adaptation to the host environment to avoid the twin perils of elimination by the host defense system or by death of the host from a fatal impact on host physiology. There is also a need to maintain a sufficiently robust infection, and hence cell number, so that the probability of transmission is maximized, but again avoiding overwhelming host resources. For African trypanosomes, which, in the case of *Trypanosoma brucei* gambiense, can survive within certain mammalian hosts for many years or even decades, these constraints are clearly very well met. Here we will consider several cellular systems and current thinking on how these contribute toward immune evasion and survival; specific areas are maintaining the parasite surface proteome, motility, and control of gene expression of virulence-associated surface molecules. Our focus is essentially restricted to African trypanosomes, due in part to the overwhelmingly greater understanding we have of the cell biology of these trypanosomatids.

1.1 Introduction

Trypanosomes are members of the Excavata supergroup, a likely early branching group of Eukaryotes, which may even lie close to the root of the eukaryotic lineage. This accident of evolution makes the host of unique or unusual features in these organisms easier to comprehend as they have had some billion years to go their own way. The trypanosomes however that interest us the most are those who have

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completed a circular journey, and have returned to humans, our livestock, and crop plants to share an intimate, and frequently damaging, relationship. Presently our understanding of these organisms is, rightly, biased towards those that do directly infect or impact humans, although interest in the nonparasitic species is increasing with a welcome resurgence in protistology. It is still too early to make sweeping conclusions, but initial data suggest that much of the biology of trypanosomes is highly conserved across the group.

African trypanosomes, *Trypanosoma brucei* spp. are the causative agent of human African trypanosomiasis and nagana, a related disease in cattle. The many *Leishmania* species and the South American trypanosome, *T. cruzi*, cause leishmaniasis of varying severity and Chagas' disease, respectively, while additional species such as Phytomonas are important plant pathogens. Hence the reach of these parasites is both deep in terms of the levels of morbidity that they can inflict and broad in geographical terms. Many of the unusual biological features, such as RNA editing of mitochondrial transcripts, a surface dominated by molecules using a glycosylphosphatidylinositol anchor, a subpellicular microtubule cytoskeleton dictating cell morphology, and polycistronic transcription/trans-splicing appear to be shared between all of these taxa. It is probably a fair statement that the levels of similarity between these organisms far outweigh the differences, making it a significant challenge to understand the huge differences in life cycle, host range, and pathology, and that sequence data has so far failed to yield clear clues as to where these differences lie (albeit with massive contributions towards understanding within taxon disease mechanisms such as antigenic variation or cell invasion).

In terms of cell biology, multiple areas of trypanosome biology offer important potential insights into disease mechanisms, evolutionary processes, and the basic biology of a highly diverse lineage, with a global impact on ecosystems. These encompass the cell surface, endomembrane system, cytoskeleton, organization of the nucleus, and many metabolic processes. In large part, due to the development of a sophisticated toolbox for the dissection of cellular processes has been vastly superior for *T. brucei*, much of which is predicated upon the highly efficient RNA interference system that this organism possesses, and which most *Leishmania* species and *T. cruzi* lack. Success, of course, begets success, and the consequence is that *T. brucei* serves as the go-to model for much of the cell biology addressing trypanosomes in general, with our understanding now breaching great depths of detailed mechanistic insight. In some ways this is a curious state of affairs as *Leishmania* and *T. cruzi* are, at the time of writing, a rather more serious public health threat.

This review will focus exclusively on *T. brucei*, although much of what is described is applicable across the trypanosomatids. We have selected several cellular areas where significant advances have been made in the last few years, and specifically the cytoskeleton, surface, endomembrane system, and nuclear organization. We apologize in advance to those whose specialized areas may not have been included, but many of these are addressed elsewhere in this volume.

1.2 Organizing Cell Shape, Structure, and Motility

1.2.1 Cytoskeleton and Morphology

The pathogenesis, growth, life cycle progression, and morphology of trypanosomes are intimately interconnected biological phenomena. All trypanosomatids have a highly organized morphology, and perhaps even more remarkably, a precise series of morphotypes which accompany life cycle progression. In the mammalian bloodstream form (BSF) of *T. brucei* the cell body is a biogivoid (a sharpened ellipse), with a mean length of ~15 μm and a girth of ~3 μm, although these parameters are variable. This morphology is maintained by a subpellicular array of microtubules that are both cross-linked with each other and attached to the plasma membrane very reminiscent of the ribs of a Zeppelin airship; the dynamic plus end of these microtubules are all gathered at the posterior end of the cell. This microtubule structure is important both in stabilizing within stage morphology as well as facilitating rapid and efficient remodeling during differentiation. Microtubule-associated proteins and other factors required for the assembly and maintenance of microtubules and the flagellum, including intraflagellar transport (IFT), are well conserved in the trypanosome genome (Berriman et al. 2005; van Dam et al. 2013). Conversely the actin system is under-represented and has only one known role, endocytosis in the BSF (Garcia-Salcedo et al. 2004). Interestingly morphological heterogeneity has been recognized for over a century, being imaginatively used by David Bruce as a tool for strain definition (Bruce 1915).

African trypanosomes spend the majority of their life cycle as a trypomastigote, with only a short period in the tsetse fly vector recognized as the epimastigote forms. In both of these forms a single flagellum, with a canonical 9 + 2 microtubule configuration, emerges from the cell, in trypomastigotes near the posterior end and more centrally in epimastigotes. The flagellum is attached lengthwise as far as at the anterior cell tip, and for functions that are unclear, extends in both of these stages beyond the cell body. The structure of the flagellum and its integration with other organelles of the trypanosome cell has been advanced recently by the publication of several 3D tomograms and high resolution EM data (Lacomble et al. 2009, 2010; Gadelha et al. 2009). A set of four microtubules are located beneath the flagellum and associated with the flagellar attachment zone (FAZ) and have opposite polarity to the remaining subpellicular microtubules; these originate near the basal body and follow a helical route around the flagellar pocket and also pass through two pocket-associated structures, the flagellar pocket collar (FPC) and neck. A filament of unknown composition is associated with this microtubule quartet as it emerges from the flagellar pocket neck (Lacomble et al. 2009, 2010). The FAZ is clearly responsible for flagellum attachment to the cell body and is unique to Euglenids. A second major component of the trypanosomatid flagellum is the paraflagellar rod (PFR), a quasicrystalline structure that is connected directly to the axoneme, likely via dynein bridges (Hughes et al. 2012). The bulk of PFR mass is made up of two proteins PFR-A and PFR-C; recent work has elucidated a basic 3D repeat structure to the PFR which comprises a series of overlapping laths or strips; the somewhat

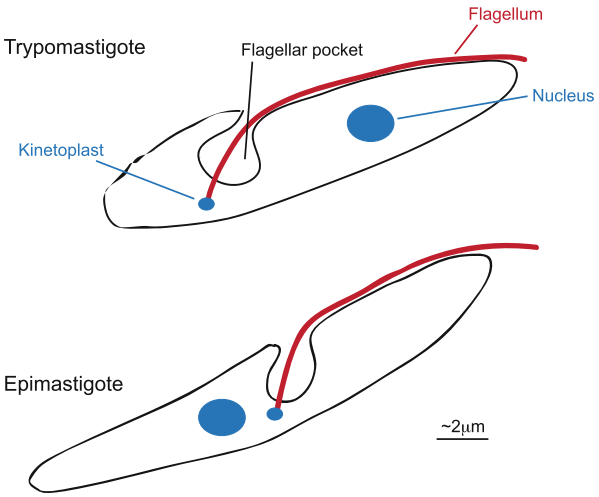

Fig. 1.1 Major morphological forms in the African trypanosome life cycle. The predominant form is the trypomastigote, where the kinetoplast is positioned close to the posterior of the cell. The precise positioning is itself dependent on the life stage, with the kinetoplast being closer to the posterior of the cell for example in the BSF compared to the procyclic form. A secondary form, the epimastigote, has the kinetoplast/flagellar pocket positioned substantially more centrally in the cell. Potential reasons for these morphological changes are discussed in the text, but may have roles in immune evasion, motility, and interactions with the host

open nature of this structure is well suited to providing a semirigid scaffold against which the axoneme may act, and also potentially acts as a spring, storing some of the energy of each flagellum beat (Hughes et al. 2012) (Fig. 1.1).

The flagellar pocket is a flask-shaped invagination of the plasma membrane where the flagellum enters the cell and which is the site of all known exo- and endocytic traffic. Where the flagellum transits into the pocket is the FPC, an electron-dense structure with presumed cytoskeletal barrier functions, while the neck is a region where the surface and pocket membranes come into very close apposition (Gadelha et al. 2009). A small furrow within this region has been suggested to act as a conduit for the uptake of solute macromolecules; fluid phase material does appear preferentially associated with this furrow, and the possibility that flagellar beating could utilize such a restriction to augment interchange between luminal and extracellular volumes by a peristalsis-like mechanism is evident. However, formal evidence remains to be obtained.

Each of the pocket-associated subregions can be viewed as a distinct membrane subdomain, and together with the flagellum membrane, bulk plasma membrane, and also the FAZ-associated membrane, serve to differentiate the trypanosome surface into putative functionally specialized subdomains; it is clear that specific polypeptides are associated with each of these domains, but the mechanisms of targeting and assembly for the most part remain to be determined.

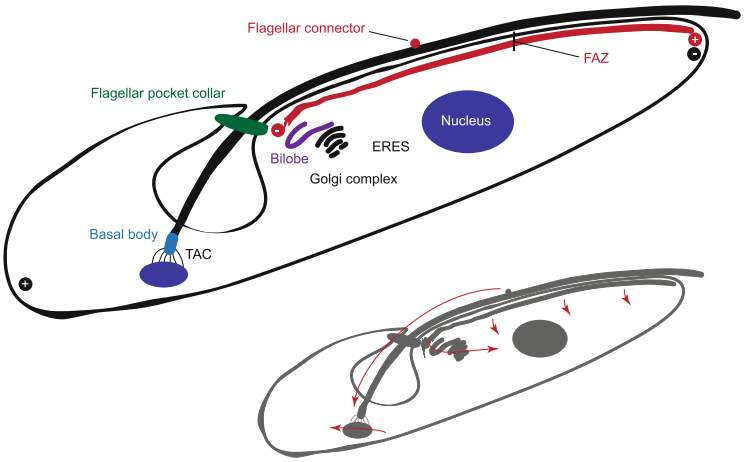

Fig. 1.2 Spatial organizers within the trypanosome cell. A schematic of a trypomastigote trypanosome is shown; the nucleus is somewhat more anterior for clarity. Principle features are the presence of a polarized microtubule subpellicular array, with polarity shown by the "+" and "−" in *black*. A second microtubule structure is the flagellar attachment zone (FAZ), which consists of proteinaceous linkers between the flagellum and plasma membrane, together with a quartet of reverse polarity microtubules (*red*). The FAZ connects with the flagellar collar (*green*), which is required for the formation of the flagellar pocket and also positioning of the flagellum/basal body, and also with the bilobe structure (*magenta*) that subtends the Golgi complex (*black*). In some manner ER exit sites (ERES) are coordinated with the Golgi complex. Finally the flagellum is attached to the basal body and also the kinetoplast via the tripartite attachment complex (TAC) and also the new flagellum (not shown) attaches to the old during cytokinesis via the flagellar connector (*red*). In *gray* is a representation of the same cell, with putative connections between the organizers and other structures. These are arbitrarily shown as being unidirectional but the interconnectedness of the system means that mechanical information may be transmitted bidirectionally. See text for details

1.2.2 Interplay Between Cytoskeleton and Organellar Positioning

The level of integration of the cytoskeleton with other intracellular organelles is at a remarkable level in trypanosomes. As many organelles are single copy, including the kinetoplast, mitochondrion, Golgi complex, nucleus, and terminal lysosome, there is a vital requirement for accurate segregation at cell division. Somewhat paradoxically in this regard, trypanosomes seem to lack an obvious cytoplasmic cytoskeleton equivalent to the microtubules of mammalian cells, which has presented a significant conundrum. Recent work however is suggesting a remarkable level of integration between organelles and the flagellum, essentially anchoring these structures to a specific frame of reference as well as a microtubule organizing center (Fig. 1.2).

It has been clear that the basal body and kinetoplast replicate in a coordinate manner, and the description of a physical connection between these two structures goes a considerable way to a mechanistic explanation (Ogbadoyi et al. 2003). Fibers span the gap between the BB and kinetoplast, traversing the double mitochondrial

membrane; these fibers clearly replicate in a coordinate fashion with the BB and kinetoplast and the stability of this association likely accounts for the ready biochemical isolation of flagella with an attached kinetoplast; this structure has been termed the tripartite attachment complex (TAC). One TAC protein, p166, has been identified and appears essential for segregation of the kinetoplast (Zhao et al. 2008), while additional proteins are clearly present but await molecular characterization (Bonhivers et al. 2008b). Further, the FAZ itself appears connected to the endoplasmic reticulum (ER) in some manner, with a close juxtaposition of a subset of ER membrane with the FAZ microtubule quartet, particularly apparent in 3D tomograms (Lacomble et al. 2012). Knockdown of TbVAP (a homolog of *V*AMP-*a*ssociated *p*rotein) leads to loss of this ER-FAZ co-ordination, but in procyclic cells at least this association does not appear to affect proliferation or cell cycle progression, so that the precise role of this tight level of structural coordination remains unclear.

Replication of the flagellum is a highly controlled process and requires precise and coordinated elongation of FAZ, axoneme and PFR, as well as the new flagellar membrane. Given that IFT is well conserved in trypanosomes, and that two flagellar proteomes have now been reported, it can be anticipated that a number of the proteins that assist in coordination of biosynthesis will be soon identified (Broadhead et al. 2006; Oberholzer et al. 2011). However, one structure, the flagellar connector, has been demonstrated to coordinate the timing of flagellar growth and segregation of the BB and kinetoplast. Interestingly, this structure acts as a physical connection between the tip of the new flagellum and the axonemal membrane of the old and slides towards the anterior end of the cell as the new flagellum grows. The connector appears in some manner to pull along the flagellum membrane as defects in axonemal assembly result in empty membrane tubules, suggesting that synthesis of the flagellar membrane does not depend on accurate axonemal synthesis. Significantly, at a point about midway along the old flagellum the connector ceases to migrate and instead the two BB/kinetoplast complexes associated with the old and new flagellum move apart as the new flagellum continues to elongate (Davidge et al. 2006). The ongoing axonemal construction may provide some of the mechanical energy required for segregation of the posterior flagellum/flagellum-associated elements, but currently what causes cessation of flagellar connector migration is unknown.

A further cytoplasmic structure, the bilobe, has recently moved closer to the center of thinking in terms of replication and may act as an organizer of several events. The trypanosome Golgi complex is a single stack in the vast majority of cells, and replicates very early in cell division, at a point essentially coincident with kinetoplast/BB replication, and clearly indicating a coordinated process (Field et al. 2000). Replication is by a complex mechanism of binary fission and generation of new material, and appears to be template encoded, in part due to the bilobe structure. Originally visualized as a structure that contains centrin2 in addition to the BB, the bilobe also contains a leucine-rich repetitive protein, LRRP1, and a second highly repetitive protein comprised of multiple MORN repeats, TbMORN (Morriswood et al. 2009). All of these components are required for both synthesis of

the bilobe as well as Golgi replication. The distribution of TbMORN suggests a mechanism for bilobe function; during mitosis the new Golgi is built on the vacant second lobe of the bilobe. More significantly, the bilobe, as visualized via TbMORN, is positioned very close to the FAZ. Further, coimmunoprecipitation suggests a physical connection between the bilobe, the FAZ, and the flagellar pocket neck as BILBO-1, a component of the FPC (see below), is recovered in these preparations and also localizes extremely close to the bilobe (Zhou et al. 2010). The bilobe then is clearly a complex and important structure within the trypanosome cell, and very recently, using a novel "BioID" proteomics approach, was shown to contain a cohort of large coiled-coil proteins, several with little apparent sequence relationships outside of the trypanosomatids (Morriswood et al. 2013). Replication of the bilobe appears controlled, or coordinated at least, by the single polo-like kinase (PLK) of *T. brucei* which, despite the deep divergence, demonstrates a mode of action that is likely homologous to higher eukaryotes, specifically in that a mobile kinase facilitates accurate temporal coordination of organelle replication, and which may extend to flagellar events as well (Ikeda and de Graffenried 2012). Association of PLK with the bilobe and FAZ provides an elegant solution to ensuring that the trypanosome cell and its many single copy organelles divide correctly. Given also an apparent coordination between the Golgi complex and ER exit sites (ERES) (Bangs 2011), the reach of the flagellum now extends all the way to early exocytic processes in at least two ways, i.e., via the FAZ/TbVAP and also via the bilobe/Golgi complex.

Another essential structural feature of trypanosome cells is the flagellar neck collar, which is represented by BILBO-1. In trypanosomes BILBO-1 is seen as a ring surrounding the flagellar pocket neck and is also capable of forming rings in the apparent absence of other proteins following heterologous expression in mammalian cells (Bonhivers et al. 2008a). Knockdown of BILBO-1 eliminates the flagellar pocket neck and demonstrates that anchoring the flagellar pocket around the flagellum is essential. Not only does intracellular transport to and from the plasma membrane become compromised in BILBO-1 knockdown cells, likely due to dilution of docking factors into the bulk plasma membrane and misplacement of the Golgi complex and post-Golgi transport, but these cells also suggest that the flagellar neck collar also contributes to positioning of the flagellum, presumably through anchoring within the subpellicular array to a specific position. Importantly, there is no endocytic activity associated with the plasma membrane in the area around the new pocket-less flagellum, suggesting that the pocket itself drives vesicle formation rather than the positioning of the basal body/centrosome as seen for example in the formation of an immune cell synapse, another example of highly polarized endocytic and exocytic trafficking (Griffiths et al. 2010).

Precise positioning of the daughter nuclei is also an essential event in cell division, but the absence of any evidence for the LINK complex from trypanosomes (Field et al. 2012), and an observable microtubule cytoskeleton suggests a divergent mechanism. The LINK complex in higher eukaryotes spans the double membranes of the nuclear envelope and interacts with both the microtubule cytoskeleton and the nuclear lamina. A number of gene products have been implicated in nuclear

positioning in trypanosomes but the vast majority also invoke additional morphological defects, with the result that the specificity of the role is difficult to assess. Recently the *T. brucei* ortholog of AIR9 from *Arabidopsis thaliana* was described, and which seems to play a role in positioning the nucleus, and also the cytokinesis furrow (May et al. 2012). However, a direct connection between AIR9 and events at the nuclear envelope remains lacking as TbAIR9 is clearly predominantly located at the subpellicular array, and there are likely several molecular links that need to be characterized before the full connection between nuclear positioning and the cytoskeleton can be made. The very common cytokinesis defect that results from many knockdown studies may simply reflect the huge complexity of cell division in trypanosomes, together with the highly integrated nature of the multiple organelle replication mechanisms. By analogy, it does not matter which leg you remove from a three-legged stool, the result is still pretty much the same.

1.2.3 Replication and Change of Size

One of the important functions of the trypanosome cytoskeleton is to facilitate both conservation of cellular structure within a given life cycle stage and also to allow large changes to morphology during transitions between those life stages. Recent work has uncovered a new replication step in the tsetse fly, whereby the epimastigote forms initially appear to divide in a symmetric manner, but then go on to an asymmetric division mode, where one of the daughters is much smaller than the other. The smaller daughter has regained the trypomastigote morphology, presumably in readiness for reinfection of the mammalian host (Rotureau et al. 2012). In most transitions, i.e., stumpy to procyclic and procyclic to mesocyclic, alterations to cell volume are restricted to remodeling of the posterior of the cell, i.e., the plus end and where tubulin monomer turnover is at its most dynamic; these are also all examples of homologous morphotype transitions, i.e., trypomastigote to trypomastigote. By contrast, in the case of the epimastigote asymmetric division, subpellicular array alterations appear rather more extensive and new tubulin subunits are incorporated throughout the subpellicular corset (Rotureau et al. 2011). Precisely what the nature of this mechanistic switch is, and how it is regulated, is unknown, but these findings differentiate within and between morphotype transitions.

1.2.4 Motility, Swimming, and Immune Avoidance

Alterations between distinct morphological forms of course beg the question of what the role of these changes is within life cycle progression. Obvious possibilities include motility, immune evasion, signaling, and modifications to accommodate size constraints from the environment. The elongated forms of procyclic, BSFs, and mesocyclics are clearly needed for migration, and these highly motile forms are well suited to such a role. The smaller size of the metacyclics and the adherent

abilities of the epimastigotes are likely adaptations for invasion of the salivary glands and for maintaining a precarious position close to the salivary glands, respectively (Rotureau et al. 2011). Motility has a clear role in capping of antibody recognizing surface epitopes and delivery of these immune complexes to the endocytic system where the antibody may be degraded (Pal et al. 2003; Engstler et al. 2007). This is now an accepted aspect of the trypanosome immune evasion repertoire.

The mode of swimming and undulation of the flagellum has recently been proposed to incorporate a perversion or kink in the left-handed helical twist (Hill 2010). This prevents the trypanosome from essentially swimming with a simple rotary motion and likely underlies the tumbling phenomenon well known to anyone who has watched cultured trypanosomes for any period of time, whereby cells will rapidly change direction between periods of more linear travel. This may be due to the kink becoming compressed and then relaxing, resulting in a change of polarity to the flagellum beat. However, this model has been contested more recently (Heddergott et al. 2012).

Recent analysis of the motility of trypanosomes using 3D microscopy has resolved three modes of motility; swimming, tumbling, and transitional, i.e., switching between phases. Individual cells display all three modes of motion. The frequency of tumbling is greater in the BSF (Weiße et al. 2012). The role of this tumbling is unclear; it may serve to release energy at periodic intervals, which may have advantages in navigation around obstacles or through narrow capillaries, and this may also be influenced by viscosity (Heddergott et al. 2012). The potency of this tumbling within turbulent flow in the mammalian bloodstream in terms of vectoral movement would seem to be limited. However, tumbling may be more important in slow-flowing lymphatic or central nervous system compartments or even important in diapedesis across the blood–brain barrier: these systems have not been studied as yet. Also, interactions between trypanosomes and solid objects such as erythrocytes may be important in terms of velocity and reaching a threshold velocity sufficient to cap antibody (Heddergott et al. 2012). Further, antibody capping toward the posterior end of the trypanosome may be assisted by tumbling, which could prevent capped antibody-variant surface glycoprotein (VSG) complexes from accumulating on the cell surface and particularly at the posterior pole of the cell, where they would be unable to enter the flagellar pocket efficiently (Engstler et al. 2007).

1.2.5 Developmental Programming; Clockwork Machines

Despite the inherent complexities encompassed within all of these remodeling steps and their potential involvement in adaptations to specific environments and needs, it is remarkable to what simplistic levels the control of life cycle progression can be reduced. In a somewhat startling discovery, it has recently been demonstrated that the entire insect portion of the life cycle can be recapitulated in vitro, simply by the overexpression of TbRBP6, a RNA-binding protein that may function as a high

level regulator of the transcriptome (Kolev et al. 2012). Significantly, this finding potentially excludes much of the requirement for environmental stimuli, with the mesocyclic, epimastigote, and metacyclic stages all emerging in the correct temporal order. This suggests an intrinsic program that is capable of being run in a fully synthetic environment, driving a rigid developmental progression (Koumandou et al. 2008). How such an automatic system has evolved in response to selective pressure is a fascinating question and one of potential major significance in understanding protist development.

1.3 Organizing the Surface

During both transmission between hosts and within a host, life cycle progression is accompanied by wholesale changes to the surface proteome, which are presumed to be intimately linked with requirements for immune evasion, survival, and transmission. Currently, characterization of the African trypanosome surface proteome is poor; while proteomic data are available, little systematic validation is in place (Bridges et al. 2008). Numerous transporters for nucleotides, nucleosides, and hexoses have been described, presumed to reside on the plasma membrane, but again our understanding of the cell biology of these molecules is primitive. Data for other trypanosomatids are even more fragmentary. Here we will focus on a small number of known surface players.

1.3.1 Variant Surface Glycoprotein

Trypanosomatid surfaces are unusual in the high copy number of GPI-anchored proteins (see also Chap. 10). In *T. brucei* BSF ~10^7 copies of the glycosylphosphatidylinositol (GPI)-anchored VSG are expressed at the surface as homodimers, representing ~90 % of total surface proteins. Despite a role in antigenic variation, VSGs themselves are highly antigenic and anti-VSG antibodies are rapidly and efficiently generated (Baral 2010). To circumvent this, African trypanosomes have evolved a rapid endocytic system, such that antibodies that cap VSG are rapidly endocytosed and degraded in the lysosome; the system appears to have been modified to facilitate this rapid flux, specifically by dispensing with the AP-2 complex and dynamin, normally essential factors for endocytosis (Barry 1979; O'Beirne et al. 1998; Pal et al. 2003; Engstler et al. 2007; Manna et al. 2013).

VSGs have a very long half-life (~33 h) suggesting that they are mostly trafficked through the recycling pathway (Seyfang et al. 1990; Koumandou et al. 2013). In addition to its role in antigenic variation and immune evasion, the VSG coat is also thought to shield most underlying invariant epitopes of other surface molecules from immune attack. The VSG layer is ~15 nm in thickness and the dense packing of these molecules can preclude antibody access to embedded epitopes (Vickerman and Luckins 1969; Schwede et al. 2011). While there is certainly strong evidence to support this model, we cannot exclude the possibility

that some surface epitopes may be accessible by the immune response (Chung et al. 2004). Indeed, recent structural data suggest that the haptoglobin–hemoglobin receptor (HpHbR) is also of sufficient size to project beyond the VSG monolayer, allowing for recognition of specific soluble ligands, without a need to disrupt the VSG coat (Higgins et al. 2013).

Each VSG monomer (~45–55 kDa) has an N-terminal domain consisting of two major α-helices forming a coiled-coil extending perpendicular to the cell surface (Freymann et al. 1990). So far, only two structures for N-terminal domains of two distinct VSGs (MITat1.2 and ILTat1.24) have been solved and although the sequence identity between the two is only 16 %, the structures are highly similar indicating that this is likely to be shared with other VSGs and that conservation of this structure is important for their function (Blum et al. 1993). The C-terminal domain of MITat1.2 comprises a core consisting of a short α-helix followed by a 3_{10}-helix and an antiparallel β-sheet (Chattopadhyay et al. 2005). Within this core multiple cysteines involved in disulphide bond formation and an N-glycosylation site are conserved in different VSGs. More recently, the structure of the GPI-anchored domain of VSG has been solved and reveals that the core glycan of the GPI anchor is in close association with a groove on the surface of the GPI-anchored domain (Jones et al. 2008). Knowledge of the full VSG structure will enable us to better understand how the surface coat is arranged relative to other surface molecules and how it prevents access of invariant epitopes from the immune system. Interestingly, evolution of VSGs is complex and has likely followed distinct pathways for the various African trypanosome lineages; for *T. brucei* this appears to have involved frequent recombination and the generation of new variants. This may also have facilitated neofunctionalization of VSGs (Jackson et al. 2012).

1.3.2 Invariant Surface Glycoproteins

Invariant surface glycoproteins (ISG)65 and ISG75 are developmentally regulated type I transmembrane domain proteins expressed in BSF *T. brucei* (Ziegelbauer et al. 1992; Ziegelbauer and Overath 1992). Although not as abundant as VSG, they are the major transmembrane domain proteins on the trypanosome surface (~70,000 and ~50,000 copies per cell, respectively). There are six members in each family in the 927 genome strain arranged in a tandem array. ISG65 family members are modified by either two or three N-glycan moieties, whereas ISG75 has only one. Both ISG65 and ISG75 are found in approximately equal proportions on the cell surface and in early and recycling endosomes. Evidence suggests that ISG65 may be monomeric, while ISG75 is likely an oligomer (Leung et al. 2011). ISG expression is strictly restricted to the BSF, and multiple mechanisms appear to ensure that no ISG polypeptide is expressed at the procyclic stage surface (Leung et al. 2011).

Infected animals are able to mount immune responses to ISGs as antibodies against these molecules can be detected in sera (Ziegelbauer and Overath 1993;

Giroud et al. 2009). Recent work has demonstrated that endocytosis and turnover of ISG65 and ISG75 are dependent on ubiquitination of key lysine residues within the cytoplasmic domain, implicating a ubiquitin-dependent pathway for uptake of surface transmembrane proteins; the turnover of ISGs is substantially faster than for VSG, indicating a need to efficiently sort ISG and VSG from each other. The absence of orthologs of higher eukaryote ubiquitin ligases from the trypanosome genome suggests that there may exist significant mechanistic differences between the system in trypanosomes and mammals (Chung et al. 2004, 2008; Leung et al. 2011). Further, in higher eukaryotes, cell surface membrane proteins and mitogenic receptors are endocytosed and downregulated by ubiquitination, which together with the short half-life suggests that ISGs may also function as receptors (Chung et al. 2004). This is supported by recent studies where, using an RNAi target-sequencing (RIT-seq) screen, it was shown that ISG75 is involved in suramin drug action, most likely through binding and uptake (Alsford et al. 2012). However, suramin is obviously not a natural ligand, making it unclear what is the physiological binding partner of ISGs.

1.3.3 Procyclins

BSF parasites are ingested by the tsetse fly, and these forms rapidly differentiate into procyclic forms in the insect midgut. The VSG coat is rapidly replaced by procyclin, a GPI-anchored protein present at ~10^6 copies per cell, making it the dominant surface protein (Roditi et al. 1989). Procyclin is characterized by protease-resistant dipeptide (EP) or pentapeptide (GPEET) repeats. Both EP and GPEET procyclins are expressed by early procyclic forms, with GPEET being repressed in late procyclic forms (~7 days postinfection) (Vassella et al. 2000; Acosta-Serrano et al. 2001). Procyclins are mainly unstructured due to the abundance of proline repeats. There are three closely related EP isoforms termed EP1, EP2, and EP3, which differ from each other in a few amino acid residues and in the presence (EP1 and EP3) or absence (EP2) of an N-glycosylation site. In addition to being N-glycosylated, GPEET1 procyclin is also phosphorylated (Mehlert et al. 1999). Aside from these differences, all procyclins share similar GPI anchors with a large side chain consisting of branched poly-N-lactosamine units terminating in sialic acid residues (Treumann et al. 1997). The precise function of procyclin is not known, but they were thought to play a role in protecting the parasite against trypanolytic factors (TLFs) in the tsetse fly (Ferguson et al. 1993). Interestingly, trypanosomes isolated from tsetse flies possess truncated procyclins lacking the N-terminal domains, although the reason for this is unclear (Acosta-Serrano et al. 2001). It is possible that the removal of the N-terminal domains exposes the anionic repeats to protect the parasite from proteolytic hydrolases in the tsetse midgut. Other work has demonstrated that deletion of all the EP procyclin genes severely impairs the establishment of infections in the tsetse fly midgut (Ruepp et al. 1997), while impairment of surface procyclin expression due to disruption in GPI synthesis resulted in a significant reduction in trypanosome numbers in the fly midgut

(Nagamune et al. 2000), suggesting that they have a role in establishing an infection in the insect vector. However, this role is nonessential as procyclin null parasites are able to transmit through the tsetse fly (Vassella et al. 2009), albeit with decreased efficiency. A compensatory mechanism, whereby levels of surface GPI-anchored glycolipids are increased in the procyclin nulls may provide a backup protective mechanism, circumventing the need for procyclin per se (Vassella et al. 2003).

1.3.4 Brucei Alanine-Rich Protein

For many years, a simple "two coat" model was ascribed for the life cycle of *T. brucei*. Specifically VSG was present on the surface of all mammalian infective forms, i.e., BSFs and insect stage metacyclics, and procyclin in all other stages. However, a third coat, brucei alanine-rich protein (BARP), predominates in the epimastigote stage in infected tsetse flies, where VSG and procyclin proteins are not expressed (Urwyler et al. 2007); this also opens up the possibility that the remodeling of the surface of *T. brucei* during the life cycle is in fact substantially more complex that originally envisaged. BARP is modified by at least one N-glycan and it is also GPI anchored. While the function of BARP is unknown, in *T. congolense*, the epimastigote form in the proboscis of the tsetse fly expresses a distantly related protein "glutamic acid and alanine-rich protein" or GARP (Bütikofer et al. 2002). These data suggest that these proteins may be involved in the targeting of trypanosomes to specific tissues or have other location-specific functions, for example, in adhesion (Urwyler et al. 2007).

1.3.5 Transferrin Receptor

The *T. brucei* transferrin receptor (TfR) is a heterodimer encoded by expression site-associated gene 6 (ESAG6) and ESAG7 and only expressed in the bloodstream stage. It is present at ~2,300 copies per cell (Salmon et al. 1994; Steverding et al. 1995) and resides at the flagellar pocket (Steverding et al. 1994), thus rendering the receptor less accessible to immune effectors. Through the TfR, the parasite scavenges transferrin from the host (Steverding 1998). Unlike in vertebrates, where the TfR is a transmembrane protein, the trypanosome TfR is anchored to the plasma membrane via a GPI anchor attached to the ESAG6 gene product, while ESAG7 is membrane associated via noncovalent binding to ESAG6. The TfR bears some structural similarity to VSG. ESAG6 and ESAG7 genes are associated with VSG expression sites such that only one dominant TfR is expressed, depending on which site is active. This switching also means that different ESAG6/ESAG7 pairs with distinct binding affinities for transferrin may be expressed (Steverding 2003; van Luenen et al. 2005). However the relevance of this is unclear as the concentration of transferrin within the serum of most mammals is sufficiently high to saturate most versions of the trypanosome TfR. Finally, the mammalian and trypanosome TfRs provide a spectacular example of convergent evolution and neofunctionalization.

There is no evolutionary relationship between these two receptors, and the trypanosome TfR clearly arose from a protoVSG during adaptation to the mammalian host (Jackson et al. 2012).

1.3.6 Lipoprotein Receptors

Sterols are essential for trypanosomes and are obtained from the host in the form of low and high density lipoproteins (LDL and HDL). LDL uptake occurs via receptor-mediated endocytosis (Coppens et al. 1987). There appears to be two receptor classes, a high-copy low affinity site and a low-copy high affinity site (Coppens et al. 1988). Manipulation of surface trafficking suggests that both classes of binding site are likely to be the same protein, with the change in affinity due to oligomerization (Pal et al. 2002).

HDL is taken up via the HpHbR, which likely evolved to mediate haem uptake (Widener et al. 2007; Vanhollebeke et al. 2008). As with VSG and TfR, HpHbR is attached to the plasma membrane via a GPI anchor. The crystal structure of *T. congolense* HpHbR revealed the protein to be more elongated than VSG, suggesting that the receptor is unlikely to be completely shielded from immune effectors (Higgins et al. 2013). However, it is likely that the low copy number coupled with its restricted location within the flagellar pocket both contribute to its evasion from the immune system. Uptake of HDL by HpHbR is a Trojan horse, as trypanosome lytic factor (TLF)1, now know to be apoL1, and responsible for innate immunity against *T. brucei* (Widener et al. 2007; Vanhollebeke et al. 2008), is a constituent of HDL particles.

HDL is composed of apolipoprotein L1 (apoL1), apoA1, and haptoglobin-related protein (Hpr) in complex with hemoglobin (Hb) (Bullard et al. 2012). Binding of the Hpr–Hb component of HDL to HpHbR results in endocytosis of HDL and thus also TLF1. The low pH of endosomal compartments causes release of apoL1 from the receptor and delivery and insertion into the lysosomal membrane, leading to osmotic swelling and cell lysis (Pays et al. 2006). Resistance of *T. b. rhodesiense* to TLF is conferred by the serum resistance-associated (SRA) protein, which binds and inactivates TLF1 via recognition of apoL1 (De Greef et al. 1989; Xong et al. 1998). Despite also being immune to TLF, *T. b. gambiense* lacks the SRA gene suggesting that protection from TLF is achieved via a different mechanism; this seems to involve decreased affinity and/or expression of the HpHbR for TLF, as well as mechanisms likely unrelated to the HpHbR (Kieft et al. 2010; Capewell et al. 2011). Significantly, similarly to the TfR, SRA is also a VSG-related protein and therefore represents an additional example of neofunctionalization of the VSG family.

1.3.7 Adenylate Cyclases

In addition to the ESAG6/ESAG7, a third expression site-associated gene, ESAG4 also has an important presence on the BSF surface. ESAG4 is an adenylate cyclase (Pays et al. 1989), and one of over 80 adenylate cyclases present in the *T. brucei* genome. The other adenylate cyclases are present within the core of megabase chromosomes and are constitutively transcribed throughout the life cycle (Alexandre et al. 1990, 1996). All share a similar receptor-like structure, possessing a large highly variable extracellular domain and a conserved intracellular catalytic domain. Deletion of ESAG4 does not affect cell viability or growth suggesting that it is nonessential (Salmon et al. 2012a, b). The lack of a phenotype is most likely compensated by the abundance of other adenylate cyclases in the family. Indeed, RNAi knockdown of two ESAG4-like (ESAG4L) cyclases in cells lacking ESAG4 resulted in a decrease in total adenylate cyclase activity as well as defective cytokinesis (Salmon et al. 2012a, b). However, decreased adenylate cyclase activity also leads to an inability of the parasite to control the innate immune response of the host (Salmon et al. 2012b). Significantly direct contact between myeloid cells and parasites appears to reduce TNF expression, by itself contributing to parasitaemia control, such that parasites inhibit TNF production and this effect appears mediated via adenylate cyclase activity and the action of protein kinase A (Salmon et al. 2012b).

1.3.8 Transporters

The transport of glucose is mediated by the trypanosome hexose transporter (THT) family, conserved among kinetoplastids and homologous to the glucose transporters of humans, yeast, and *Escherichia coli* (Bringaud and Baltz 1992). Two classes have been identified: THT1 expressed only in BSFs and THT2, preferentially expressed in procyclics (Bringaud and Baltz 1993). Despite similar topology to the mammalian glucose transporter GLUT1 (both predicted with 12 transmembrane helices), sequence identity is only 19 %. It is thought that THT1 controls glycolytic flux and hence ATP synthesis in BSFs.

Trypanosomes are unable to synthesize their own purines and rely on scavenging through nucleoside and nucleobase transporters. Two classes are known; P1 transporters, which import adenosine, guanosine, and inosine, and P2 transporters which take up adenosine and adenine (Carter and Fairlamb 1993; de Koning et al. 1998). P1 are present in both procyclic and BSFs, while P2 are restricted to BSFs. The best characterized is the P2 adenosine transporter TbAT1, also involved in uptake of melaminophenyl arsenical drugs such as melarsoprol and diamidines such as pentamidine. Disruption of TbAT1 activity leads to drug resistance (Mäser et al. 1999; Lanteri et al. 2006; Baker et al. 2013). In contrast, pyrimidine uptake is nonessential as trypanosomes are able to synthesize pyrimidines. However, a number of pyrimidine transporters have been identified in *T. b. brucei*, and there are clear differences between the mammalian and insect forms. The presence of

pyrimidine transporters may suggest that pyrimidine scavenging could be advantageous to parasite growth under certain environmental conditions (Gudin et al. 2006; Ali et al. 2013).

Other transporters recently identified include the carboxylate surface transporter family or protein associated with differentiation (PAD) proteins that are part of an eight-gene array. Importantly, PAD1 is the first molecular marker to be identified that is specifically expressed in short stumpy cells, while PAD2 is upregulated in procyclic forms (Dean et al. 2009). Further, recent evidence implicates the aquaporins in drug interactions, suggesting that these transporters are also of therapeutic interest (Alsford et al. 2012).

1.4 Organizing the Endomembrane System

The directed flow of material between intracellular membrane-bound organelles and the plasma membrane is an essential process in compartmentalized eukaryotic cells. Accordingly, the broad organization of the endomembrane system and the molecular machineries involved arose early in the evolution of the eukaryotes (Dacks and Field 2007). It is therefore unsurprising that *T. brucei* possesses endomembrane compartments largely typical of those seen in other eukaryotes, including the endoplasmic reticulum, Golgi apparatus, various classes of endosome, and the terminal lytic lysosome. This familiarity is further reflected in the broad conservation of protein families involved in intracellular membrane transport, namely the Rabs, SNAREs, SM proteins, tethering complexes, and vesicle coat proteins (Elias et al. 2012; Koumandou et al. 2007). Nevertheless, closer inspection reveals evolutionary divergence arising via subtle remodeling or sculpting of pre-existing pathways and components rather than via large-scale innovation (Dacks and Field 2007; Dacks et al. 2009; Elias et al. 2012). Many endomembrane structures are present in single copy numbers, for example, the Golgi, ER–Golgi junction, and the lysosome, and their positioning is tightly controlled (Field et al. 2009). The endomembrane system is highly polarized toward a specialized invagination of the plasma membrane at the cell posterior, the flagellar pocket, and mostly restricted to the area between this organelle and the centrally located nucleus. The flagellar pocket is the sole site for exocytosis and endocytosis in the trypanosome plasma membrane and as such the streamlining and polarization of the endomembrane system with respect to this organelle likely increases the efficiency of transport of the major surface protein families VSG and procyclin (Field and Carrington 2009).

Perhaps owing to the streamlined and efficient organization of membrane transport in *T. brucei*, the parasite is extremely sensitive to any perturbation of this system (Allen et al. 2003; Hall et al. 2004b; Huang et al. 2011; Tazeh et al. 2009). This is particularly true in the mammalian bloodstream stage of the parasite life cycle, during which endocytic and exocytic membrane flux is dramatically upregulated, by as much as tenfold with respect to the insect stage (Engstler et al. 2004). This developmental remodeling is suggested to have direct relevance to

immune evasion and the persistence of parasitemia (Natesan et al. 2007). Further, it is now clear that the trypanosome endomembrane system has been targeted by the evolution of the host defense and, unwittingly, by human chemotherapeutics. Below we briefly describe the peculiarities of membrane trafficking in *T. brucei*, with special attention to the involvement of this system in the persistence of parasitemia and the actions of trypanocidal molecules. More comprehensive reviews of membrane trafficking in *T. brucei* are available (Field and Carrington 2009; Field et al. 2009; Silverman and Bangs 2012).

1.4.1 Transport Through the Endoplasmic Reticulum

The efficient synthesis and rapid delivery of secretory vesicles to the surface is essential in trypanosomes since the maintenance of the VSG coat is vital to the survival of the parasite in the mammalian bloodstream. The ER, as the site of GPI anchor addition and N-glycosylation, plays a central role in this process. In trypanosomes, as in other cells, protein translocation across the ER membrane either occurs co-translationally via the signal recognition particle (SRP) or by a post-translational mechanism via cytosolic chaperones. However the poor translocation competence of *T. brucei* proteins when expressed in heterologous systems (Al-Qahtani et al. 1998) suggests divergent ER-targeting mechanisms and indeed *T. brucei* does have an unusual SRP. In addition to the ubiquitous 7SL RNA *T. brucei* encodes a short tRNA-like molecule termed sRNA-76 (Beja et al. 1993) but only has a limited set of proteins (Liu et al. 2003; Lustig et al. 2005). Also the chaperone-dependent pathway and the Sec61 membrane channel itself are simplified with several components found in higher eukaryotes apparently missing in the parasite (Goldshmidt et al. 2008).

Maybe the most unique feature of protein biosynthesis in trypanosomatids is the unusual N-glycosylation. In higher eukaryotes $Glc_3Man_9GlcNAc_2$-PP-Dol is transferred by oligosaccharyltransferase (OST), to Asn residues within Asn-X-Ser/Thr sequences (reviewed in Schwarz and Aebi 2011). Trypanosomatids transfer the unglycosylated $Man_{9–5}GlcNAc_2$, that is processed species dependently into structures ranging from $Man_5GlcNAc_2$ to $Man_9GlcNAc_2$ (reviewed in Mehlert et al. 1999), probably arising from an inability to synthesize the glucosyl donor dolichol-P-Glc due to loss of the biosynthetic gene products (de la Canal and Parodi 1987; Manthri et al. 2008). OST in higher eukaryotes is a multimeric enzyme complex, but only a single subunit is found in trypanosomatids (Kelleher and Gilmore 2006) likely due to the transfer of a single dominant glycan molecule (Castro et al. 2006). In higher eukaryotes a calnexin (CNX)-/calreticulin (CRT)-dependent chaperone pathway removes the first two glucose residues of the polypeptide-bound glycan by action of glucosidase I and II, resulting in $GlcMan_9GlcNAc_2$ which is bound by the membrane-associated CNX or the soluble paralog CRT to prevent aggregation and misfolding (Moremen and Molinari 2006). Again in trypanosomes this pathway seems to be simplified with some of the key components missing from the parasite genome (Field et al. 2010).

Natively folded proteins and assembled multimeric complexes are selectively segregated at organized membrane domains called ERES, where cargo molecules are packaged in COPII-coated vesicles and directed to the Golgi complex (reviewed in Zanetti et al. 2011). Homologs of the complete COPII exit machinery are found in trypanosomes (He et al. 2004). Terminally misfolded proteins are destined for ER-associated degradation (ERAD) (Caramelo and Parodi 2007; Hebert and Molinari 2007). In higher eukaryotes Mannosidase I removes terminal mannose residues and the resultant intermediates are recognized by the ER degradation-enhancing α-mannosidase-like protein (EDEM). Furthermore BiP and its two interaction partners Scj1 and Jem1 are involved in directing misfolded soluble proteins for degradation to the proteasome (Plemper et al. 1997; Nishikawa et al. 2001). Homologs of the mannose-binding protein, BIP, Scj1, and Jem1 are present in trypanosomes, suggesting a functional ERAD system. Further, evidence suggests that VSG is synthesized in excess of requirements and that the surplus is degraded via the proteasome, mirroring the ERAD system of higher eukaryotes (Field et al. 2010).

1.4.2 Delivery to the Golgi

Trypanosomes possess a single Golgi apparatus built of stacked, flattened cisternae highly similar to the structure described in higher eukaryotes (Grab et al. 1997). Comparative genomics has identified the complete conserved octameric Golgi complex (COG) that mediates intra-Golgi transport in the parasite genome (Koumandou et al 2007). Interestingly in insect stage trypanosomes the Golgi is almost twice the size compared to parasites living in the mammalian bloodstream. This may result from differential demand on the secretory system between the two life cycle stages and is accompanied by altered distribution of several Golgi-resident Rab proteins (Field and Carrington 2004). The single Golgi apparatus has the consequence that faithful replication and segregation during mitosis is essential. Unlike other eukaryotic organisms, Golgi biosynthesis in trypanosomes occurs by binary fission early in the cell cycle and depends on the bilobe (discussed above) and a class III PI-3-kinase TbVps34 (Field et al. 2000; He et al. 2004; Hall et al. 2006).

Retrograde transport and recycling of transmembrane receptors from endosomes to the Golgi is vital in higher eukaryotes (Pavelka et al. 2008; Sannerud et al. 2003). Orthologs of all retromer and COPI complex components were identified by comparative genomics in *T. brucei* (Koumandou et al. 2011). Several findings suggest that retromer plays a special role in trypanosomatids. All retromer components are significantly upregulated in the BSF and knockdown of cargo selection subunits is lethal in this life cycle stage (Koumandou et al. 2011). This suggests that in the BSF recycling is the main trafficking route required to maintain the VSG surface coat, while in the insect form the lysosomal route is more active (Field et al. 2007a).

1.4.3 Post-Golgi Trafficking

Post-Golgi trafficking and the secretory pathway in trypanosomatids is less well understood than the endocytic system. However comparative genomics and phylogenetic analyses have identified key exocytic components. The exocyst, an octameric complex responsible for targeting secretory vesicles to the plasma membrane, is highly conserved among eukaryotes but only partially found in the parasite genome (Koumandou et al. 2007). SNAREs, the coiled coil proteins that facilitate the fusion of secretory vesicles with the plasma membrane, are also not well characterized in trypanosomes. However a bioinformatic analysis of the SNARE repertoire in the closely related kinetoplastid *Leishmania major* revealed a complexity comparable with those of metazoans such as *Drosophila melanogaster*. Interestingly, certain types of SNAREs found in metazoans and yeast are absent in *Leishmania*, while species specific SNAREs are found as well as SNAREs with unusual localizations (Besteiro et al. 2006).

1.4.4 Accessing the Flagellar Pocket

The flagellar pocket is segregated from the bulk plasma membrane and extracellular milieu by a tight junction-like structure, the FPC, encircling the distal neck of the organelle. A channel runs through the FPC in which soluble endocytic cargoes accumulate when endocytosis is blocked by low temperature incubation (Gadelha et al. 2009). How this channel is formed and whether trypanosome surface proteins are restricted from accessing the pocket via this route has not been investigated, but it raises the possibility of a selective barrier controlling access to the pocket. Currently only a single component of the FPC itself has been identified, BILBO-1 (Bonhivers et al. 2008b see above).

1.4.5 Clathrin-Mediated Endocytosis

Uptake of material from the pocket is entirely dependent upon the classical clathrin-mediated endocytosis (CME) pathway and in bloodstream stage parasites is extremely rapid, with one entire cell surface pool equivalent of VSG being turned over approximately every 12 min (Allen et al. 2003; Engstler et al. 2004). Inhibition of clathrin-mediated endocytosis is rapidly lethal in BSFs, leading to dramatic enlargement of the flagellar pocket due to an imbalance between delivery and removal of membrane at this organelle (Allen et al. 2003). This rapid endocytic activity aids in clearance of host antibody from the cell surface and immune evasion (Field and Carrington 2009). Indeed, bloodstream stage parasites take advantage of hydrodynamic flow-mediated sorting to selectively deliver antibody-bound surface molecules, having higher drug versus free counterparts, to the pocket for rapid endocytic uptake and subsequent degradation, effectively clearing their surface of host antibody (Engstler et al. 2007). Importantly, inhibition of sorting and selective

uptake of antibody-bound VSG increases the susceptibility of trypanosomes to complement-mediated lysis.

Detailed analyses of CME in yeast and mammalian cells have revealed conserved protein modules underlying key stages of clathrin-coated pit (CCP) formation (McMahon and Boucrot 2011). Possibly owing to the unusual restriction of endocytosis to the flagellar pocket, the earliest stages of endocytic pit formation display considerable divergence in *T. brucei*. In yeast and mammalian cells the demarcation of sites for CCP formation occurs via phosphatidylinositol-4,5-bisphosphate-mediated recruitment of EH domain-containing proteins such as the mammalian epidermal growth factor receptor substrate 15 (Eps15) and Eps15-related (Eps15R) or the yeast orthologs Ede1 and Pan1, which in turn recruit cargo adaptors and clathrin. While little is known about the lipid composition of the flagellar pocket membrane, these protein families, broadly conserved across the eukaryotes, are notably absent from the kinetoplastids, suggesting divergent mechanisms of endocytic pit initiation (Field et al. 2007a, b). Secondly, in a major departure from what is seen in other eukaryotes there is no evidence for sorting or concentration of cargoes into CCPs of BSF *T. brucei* (Field and Carrington 2009). This possibly reflects the relative homogeneity and abundance of the VSG surface coat combined with the GPI-anchored nature of this molecule precluding interactions with cytosolic cargo adaptor proteins. In line with this suggestion, the major endocytic cargo-binding clathrin adaptor complex, the assembly polypeptide 2 (AP2) complex is absent from the genomes of all salivarian trypanosomes expressing forms of VSG on their surface (Field et al. 2007a, b; Manna et al. 2013). In the absence of AP2, recruitment of clathrin to the membrane relies upon non-cargo-selective adaptor proteins such as EpsinR and CALM, of which there are clear orthologs present in the genome of *T. brucei*. RNAi depletion of TbEpsinR results in reduced endocytic capacity (Gabernet-Castello et al. 2009) and this defect is exacerbated by concomitant depletion of the CALM homolog (PM and MCF, unpublished observations) suggesting some degree of redundancy in the mechanism of clathrin recruitment but confirming the involvement of these two proteins.

While a lack of key components of the CME machinery such as AP2 and Eps15 intuitively suggests that this process is simplified in *T. brucei*, it is possible that significant mechanistic or sequence divergence obscures the true complexity of this process from comparative approaches. A recent study has employed immunoprecipitation and mass spectrometry to identify, de novo, clathrin interaction partners in *T. brucei* (Adung'a et al. 2013). Several trypanosome-specific proteins, termed TbCAPs, with clear roles in CCP formation were identified, demonstrating previously unrecognized complexity in this system and highlighting the importance of approaches extending beyond comparative in silico analysis.

The later steps in the CCP life cycle, i.e., invagination, budding, and uncoating are common to clathrin function at other intracellular sites and also appear to occur by more standard mechanisms in *T. brucei* involving actin and the uncoating ATPase Hsc70 (Adung'a et al. 2013; Garcia-Salcedo et al. 2004).

1.4.6 Retrograde Transport

Post-endocytic retrograde transport is similar to that seen in other organisms, with cargoes passing through early sorting endosomes to late endosomes and multivesicular bodies en route to the terminal degradative lysosome. Multiple cargo sorting and concentration steps exist along this pathway together with opportunities for recycling of cargoes back to the cell surface or the Golgi. Cargo sorting and concentration is dependent upon the action of Rab proteins in other organisms and this is equally true for *T. brucei*. Numerous Rabs have been characterized in *T. brucei* and their functions are generally conserved when compared with homologous Rabs in other species, for example, the two Rab5 homologs in *T. brucei* are important for early endocytic cargo transport (Hall et al. 2004a), with Rab7 mediating delivery to late endosomes (Silverman et al. 2011) and Rab11 driving recycling to the cell surface (Grunfelder et al. 2003) (discussed in more detail below).

Transport from early to late endosomes is mediated by the budding of small clathrin-coated vesicles 50–60 nm in diameter away from the early sorting endosomes. These small vesicles are depleted of VSG, which predominantly undergoes recycling to the cell surface (Grunfelder et al. 2003). From the late endosome the major retrograde trafficking pathways are ESCRT-mediated transport to the lysosome via selection of ubiquitinated cargoes into inward budding vesicles and retromer-mediated transport via outward budding tubules to the Golgi. Comparative genomics and functional studies have demonstrated the conserved nature of these transport pathways in *T. brucei* (Koumandou et al. 2011; Leung et al. 2008). However, it was recently demonstrated that the regulation of both retromer and ESCRT function depends upon the action of the conserved Rab28 protein in *T. brucei*, an unexpected finding with potential relevance beyond this organism (Lumb et al. 2011).

1.4.7 Recycling

As for CME, the recycling of cargoes back to the cell surface has features that are both familiar and divergent. As in mammalian cells, Rab4 is involved in recycling in procyclic-form parasites but this pathway is downregulated in BSF along with a corresponding increase in the activity of the Rab11 recycling pathway (Hall et al. 2004b, 2005; Natesan et al. 2007; Pal et al. 2003). In mammalian cells cargo recycling via the Rab11 recycling pathway is significantly slower than the Rab4-dependent pathway. Considering the upregulation of endocytic trafficking in the BSF parasite, it seems counterproductive to restrict transport to the slower Rab11 pathway. However, as endocytic cycling of the VSG coat facilitates removal of host antibody, a longer transit time may improve the efficiency of this process by exposing VSG–antibody complexes to a lower pH environment and for a longer time. It is well established that VSGs are recycled back to the flagellar pocket via Rab11-dependent recycling endosomes, but this recycling pathway was recently

shown to be important also in the regulation of ISG65 and ISG75 levels in BSF trypanosomes (Koumandou et al. 2013). A recent analysis of Rab11 interaction partners highlighted a conserved interaction with the exocyst component Sec15 at the flagellar pocket and two novel interaction partners TbRBP74 and TbAZI1 (Gabernet-Castello et al. 2011). Of these two proteins, TbRBP74 colocalizes with Rab11 at endosomes and is restricted to trypanosomatids, suggesting divergence in the molecular basis for cargo recycling.

1.4.8 Transport to Glycosomes

In all other organisms glycolysis is essentially cytosolic. In trypanosomatids this pathway is partly compartmentalized in special organelles termed glycosomes, which are bounded by a single phospholipid bilayer. The seven enzymes involved in the conversion of glucose into 3-phosphoglycerate are localized in the glycosome, those catalyzing the conversion of 3-phosphoglycerate to pyruvate reside in the cytosol (Opperdoes and Borst 1977). This unique compartmentalization likely plays a role in the metabolic adaptation to the two life cycle stages of the parasite. In the glucose-rich environment of the mammalian bloodstream, ATP is solely synthesized by glycolysis and mitochondrial systems are repressed. In the insect form glycolysis is not essential, procyclic trypanosomes grown in glucose-depleted media increase the consumption rate of L-proline, their main carbon source (reviewed in Michels et al. 2006). Glycosomal import is mediated by PTS1 and PTS2, two peptide-targeting signals that are highly conserved across species (Hettema et al. 1999). Additional targeting signals that work independently or in combination with PTS1 or PTS2 have been suggested (reviewed in Sommer and Wang 1994).

1.4.9 Transport to the Flagellum

Soluble proteins that build the eukaryotic flagellum are synthesized in the cytoplasm and transported to the flagellum, but flagellar targeting is not fully understood yet. In trypanosomes the first flagellar import signal discovered was the tripeptide HLA that targets PFRA and ARP to the flagellum (Ersfeld and Gull 2001). A second signal sequence was found in the two flagellar adenylate kinases of *T. brucei*. Compared to other adenylate kinases, TbADKA and TbADKB possess an additional 55 amino acids at their N terminus containing a conserved motif YLX4IPXLXE, followed by two conserved proline residues (Pullen et al. 2004). But, since not all flagellar proteins contain these motifs, additional targeting signals must exist. The finding that the flagellar membrane has increased concentrations of sterols and saturated fatty acids led to the theory that protein association with lipid rafts plays a role in targeting or retaining flagellar membrane proteins (Tyler et al. 2009).

Membrane proteins are delivered to the base of the flagellum via vesicles formed by the BBSome coat complex functioning in combination with the Arf-like GTPase Arl6 (Jin et al. 2010). The genome of *T. brucei* encodes all ancestral subunits of the BBSome complex (Hodges et al. 2010) and trypanosome Arl6 colocalizes with the BBSome component BBS1 at the base of the flagellum and is involved in flagellum extension (Price et al. 2012). From the base of the flagellum cargoes are transported along the axoneme by IFT particles, multiprotein complexes powered by dynein and kinesin motors (Emmer et al. 2010). The genome of *T. brucei* encodes a full set of IFT proteins which are essential for flagellum formation (Absalon et al. 2008a, b; van Dam et al. 2013).

1.4.10 Trypanocides Acting Via the Endomembrane System

While *T. b. gambiense* and *T. b. rhodesiense* are able to infect humans, the sympatric *T. b. brucei* is not. Exposure to human serum is rapidly lethal for *T. b. brucei* due to the action of the TLF (discussed above). Critically, killing by TLF is fully dependent on an active endocytic system, and the high rate of endocytosis in the BSF may account for the efficiency (and evolution) of the TLF system in higher primates (Chap. 6).

A further example of trypanocidal activity being mediated via the endosomal system comes from a recent genome-wide RNAi screen to identify mechanisms of anti-trypanosomal drug action and the potential for emergence of resistance (Alsford et al. 2012). This screen identified a potential cell surface receptor for suramin in the ISG75 protein along with implicating several putative endosomal and membrane trafficking proteins in its mechanism of action. In particular all components of the cytosolic vesicle coat complex AP1 were identified together with multiple lysosomal proteins, including a putative lysosomal phospholipase (p67), a lysosomal protein of unknown function (GLP-1) and a lysosomal transporter of the major facilitator superfamily. Further work is needed to clarify the exact mechanism of action of suramin, but these data strongly implicate endocytic trafficking to the lysosome as being a key factor in the efficacy of this drug.

1.5 Organizing the Nucleus

The eukaryotic nucleus is bound by a double membrane, the nuclear envelope (NE), perforated by nuclear pore complexes (NPCs), allowing selective bidirectional transport. In many taxa there is a nucleoskeleton beneath the NE that has dual roles in both nuclear structure and chromatin organization. Spatial and temporal nuclear organization is crucial for regulating transcription, mRNA processing, and the assembly of ribosomes. In mammalian nuclei this includes packaging of chromatin into more open transcriptionally active euchromatin or less active heterochromatin, the occupancy of specific territories by chromosomes and the presence of subnuclear compartments or bodies. Many of these features likely exist in

trypanosomes, but may be quite divergent from mammalian systems, as expected from their evolutionary separation. The situation is further complicated by closed mitosis in trypanosomes, meaning that subnuclear organization must be successfully maintained and replicated during the entire process of nuclear division (Mao et al. 2011; Daniels et al. 2010).

1.5.1 NPCs, Chromosomes, and the Lamina of Trypanosomes

In spite of great divergence at the primary sequence level, the trypanosome NPC shares a similar number of nucleoporins to the yeast and mammalian counterpart, with great similarity at the level of protein fold arrangements and NPC architecture and modularity (DeGrasse et al. 2009). The structure contains multiple copies of over 20 distinct proteins, and both knockdown and proteomic data suggest that trypanosomal NPCs are held in position by a lamina-like nucleoskeleton, similar to mammalian cells (DuBois et al. 2012, S. Obado, M.P. Rout, MCF and B.T. Chait, unpublished data). Trypanosome NPCs appear to be remain regularly spaced during the cell cycle, with no evidence for a major reorganization or alteration in NPC composition accompanying mitosis. A single trypanosome nucleoporin, NUP92, does appear to disengage from the NPC, but otherwise there is no obvious cell-cycle-dependent NPC modulation (deGrasse et al. 2009, J. Holden and MCF, unpublished data). Electron-dense material at the nuclear periphery is thought to be heterochromatin and is not found in the proximity of the NPCs (Rout and Field 2001), suggesting higher rates of transcriptional activity in these areas.

The lamina, which in mammalian cells has multiple roles in organization of the nucleus and heterochromatin (Kind and van Steensel 2010), together with control of expression of developmental genes, appears to be supported by a highly divergent set of proteins, which include NUP-1, a highly repetitive protein that may form a network at the nuclear periphery analogous to lamins in mammals (DuBois et al. 2012). The trypanosomal lamina is extremely divergent and may have hard-to-foresee roles in heterochromatin organization and chromosome organization in general—it has already been hinted that TbNUP-1 may be involved in chromosome segregation during mitosis (Field et al. 2012). Further elucidation of the trypanosomal nucleoskeleton is therefore essential for decoding the organization of heterochromatin and euchromatin in these cells.

Unlike their mammalian counterparts, yeast chromosomes do not occupy spatially limited chromosome territories (Meaburn and Misteli 2007). Likewise, no such territories have been identified in trypanosomes, though little direct work has been done in this area. It has been argued that the chromosome territories model is inappropriate for the significantly smaller yeast or trypanosome nucleus, as genetic loci can move by 1 μm in several minutes (Misteli 2005), about half of the diameter of the trypanosome nucleus, but given the very distinct mode of transcriptional regulation between trypanosomes and higher eukaryotes the potential for high-order nuclear architecture participating in control of gene expression deserves some attention. Overall, these data suggest that while much of the nucleocytoplasmic

transport system is conserved with higher eukaryotes, the organization of the nuclear periphery, and potentially how this relates to chromatin organization and higher order control is rather divergent.

1.5.2 Roles of Heterochromatin in Trypanosomes

Ultrastructural analysis of the trypanosome nucleus demonstrates a clear distinction between electron-lucent and electron-dense regions which tend to be located toward the nuclear periphery (Daniels et al. 2010); these regions are assumed to correspond to transcriptionally active euchromatin and silent heterochromatin, respectively. This view is supported by the observation that histone H1 depletion leads to chromatin becoming generally more accessible in BSF cells (Povelones et al. 2012). This arrangement of chromatin, which is employed in multicellular organisms to enable tissue-specific expression, for example, has been suggested to contribute to stage-specific expression in trypanosomes, where a very high degree of repression is likely required (Field et al. 2012). Clearly, for African trypanosomes this implies control of VSG and procyclin expression.

It has been argued that the presence of non-coregulated genes in the same polycistronic transcription unit (PTU) means that long-range silencing of the main body of megabase chromosomes by chromatin organization is unlikely (Daniels et al. 2010). Instead, in BSF trypanosomes heterochromatin is thought to comprise transcriptionally silent minichromosomes and the telomeric ends of the megabase chromosomes, including inactive VSG expression sites that are located at the nuclear periphery (Navarro and Gull 2001). This view is further supported by nucleosome occupancy; nucleosomes are enriched at silent VSG expression sites when compared to active expression sites. The overall highest density of nucleosomes is at the silent repeat arrays found upstream of expression sites and on minichromosomes, suggesting that these are highly repressed loci and that that repression is due to extreme heterochromatinization (Stanne and Rudenko 2010; Figueiredo and Cross 2010). Furthermore, depletion of histone H1, the histone methyltransferase DOT1B, the chromatin-remodeling protein TbISIWI, and NUP-1 all lead to partial expression site B-ES derepression (Povelones et al. 2012; Stanne et al. 2011; Figueiredo et al. 2008; DuBois et al. 2012), all of which suggest that maintenance of heterochromatin and positional information form part of an integrated mechanism for expression site repression. The transcriptional state of the procyclin locus also correlates with its nuclear position. The loci are peripheral to the nucleolus when transcribed in the PCF and lose their nucleolar association in the BSF; while not formally proven the procyclin loci are suspected to move to the nuclear periphery when inactive (Landeira and Navarro 2007; Field et al. 2012).

1.5.3 Subcompartmentalization of the Trypanosome Nucleus

Several subnuclear structures or "nuclear bodies" are known in higher eukaryotes, and trypanosomes possess equivalents of several of these as well as at least one lineage-specific nuclear compartment, the expression site body (ESB).

The nucleolus is the site of rRNA transcription, pre-rRNA processing and assembly of the ribosomal subunits, as well as some other roles in nuclear RNP and RNA processing and cell cycle regulation (Pederson 2011). RNA pol I is found exclusively in the nucleolus in most eukaryotic cells and considered a defining component (Mao et al. 2011). The trypanosome nucleolus is comparatively small, with a maximum of 1 μm diameter (Ersfeld et al. 1999). Distinct from the tripartite structure of mammalian cells, the trypanosome nucleolus has a simpler bipartite structure that has been proposed to extend to all non-amniotes (Thiry and Lafontaine 2005). This resolves as a fibrillar center and a granular compartment (Ogbadoyi et al. 2000). There is also evidence of trypanosomal nucleolar subcompartmentalization from immunofluorescence; TbNopp140 is not homogenously distributed throughout the nucleolus and moreover the TbNopp140 nucleolar subcompartments separate further on depletion of the Nopp140-like protein TbNoLP (Kelly et al. 2006). Furthermore, the ribosomal genes and the largest subunit of pol I concentrate at the nucleolar periphery (Landeira and Navarro 2007), suggesting a distinct arrangement to mammalian center-to-edge nucleolar ribosomal biogenesis. Other nucleolar processes appear conserved, and include assembly of SRPs, which is also a function of the mammalian nucleolus (Daniels et al. 2010).

The ESB is a trypanosome-specific subnuclear compartment of great importance for antigenic variation; it appears to be the sole site of transcription of the active VSG expression site (Navarro and Gull 2001). This unique feature was first identified as an extra-nucleolar concentration of RNA pol I (Navarro and Gull 2001). In spite of many attempts a factor specific to the ESB remains to be identified (Daniels et al. 2010). However, the absence of the nucleolar marker fibrillarin indicates that the ESB has a distinct composition to the nucleolus, while the observation that the expression site within the ESB is specifically DNase resistant, unlike nearby DNA repeats, suggests that the ESB is a true subcompartment (Navarro and Gull 2001). When rapid expression site switching is selected for, the relevant expression sites are observed to be in close proximity, presumably in an intermediate of in situ switching (Chaves et al. 1999). Mechanisms by which the singularity of the ESB may be achieved include the presence of an enhancer-like DNA element required for ESB formation, similar to the cis-acting DNA region that ensures the one odorant receptor/one olfactory neuron rule in mice (Serizawa et al. 2003; Daniels et al. 2010) or the existence of an essential protein component that aggregates at the ESB in a self-reinforcing process (Daniels et al. 2010). Depletion of the telomeric protein RPA1 leads to more than one extranucleolar concentration of pol I, accompanied by expression site derepression, suggesting that telomeric chromatin structure is also required for maintenance of the single ESB (Yang et al. 2009).

Unlike murine olfactory neurons, the specificity of the ESB must be maintained through the BSF cell cycle as each daughter ESB inherits the active expression site and the associated transcriptional activity in the vast majority of mitotic events (Navarro and Gull 2001). Furthermore, it is possible to induce formation of two ESBs in post-S-phase cells by depletion of the cohesin subunit TbSCC1 (Landeira et al. 2009). This suggests that the ESB is a self-assembling suborganelle, rather than a determined aspect of nuclear architecture—presumably the two ESBs in TbSCC1 depleted cells are caused by premature separation of the chromatids carrying the active expression site.

Processing of trypanosomal pre-mRNA by the addition of a 5′ spliced leader mini exon generates a huge demand for spliced leader transcripts, which are transcribed by pol II. Unsurprisingly, the largest pol II subunit is found concentrated at the genomic tandem repeat SL RNA locus (Dossin Fde and Schenkman 2005), which appear similar to nuclear bodies. It has been suggested that protein coding genes in PTUs are also transcribed at these sites (Daniels et al. 2010), but PTUs reside throughout the nucleoplasm and tubulin gene loci do not colocalize with the spliced leader pol II concentrations (Dossin Fde and Schenkman 2005), suggesting that this would require considerable chromosome movement. Based on the colocalization of spliced leader RNA transcripts with an snRNP (SmE) and a RNA (SLA1) involved in its modification and processing, it has been proposed that there are spatially defined spliced leader RNP factories within the nucleoplasm (Tkacz et al. 2007). However, there remains debate as to whether spliced leader RNA processing also involves a cytoplasmic step (Zeiner et al. 2003; Biton et al. 2006).

An additional nuclear subcompartment that is retained by trypanosomes is nuclear speckles, structures of the interchromatin space that contain many pre-mRNA splicing factors. In trypanosomes there is evidence for speckle-like organization of splicing components, including Prp31, SmE, SSm 2–1, and U2 snRNA (Liang et al. 2006; Tkacz et al. 2007).

By contrast, Cajal bodies are subcompartments involved in RNP biogenesis, characterized by the presence of coilin and Nopp40. The exclusive localization of TbNopp140 to the nucleolus and lack of a trypanosomal coilin homolog in the genome imply the absence of true Cajal body homologs in trypanosomes (Daniels et al. 2010); though it has been suggested that some Cajal body functions are carried out by analogous structures to those found in yeast (Hury et al. 2009).

Overall, the trypanosome nucleus bears much that is conserved with higher eukaryotes, but there is clear evidence for substantial differences, including the lamina, arrangement of subcompartments, and the mechanisms of control of the ESB and telomeric repression. The presence of trypanosome-specific proteins implicated as part of these processes suggests that a careful molecular dissection of these functions and structures is required to progress our understanding.

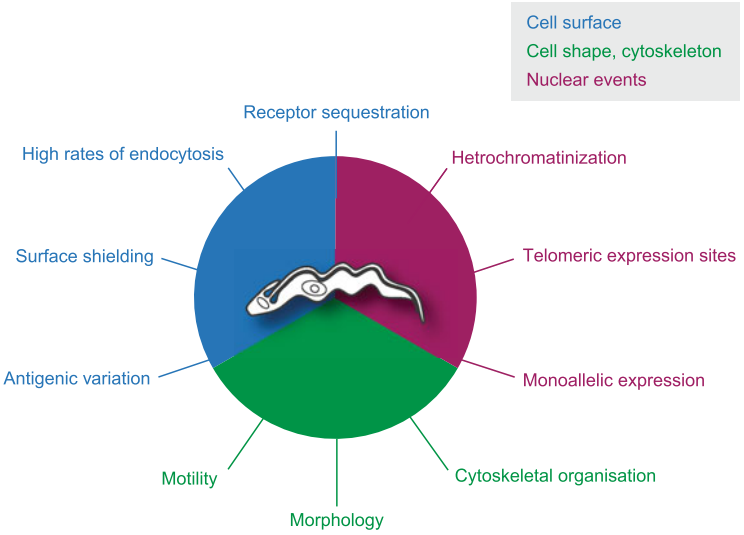

Fig. 1.3 Major aspects of cell biology impacting virulence in African trypanosomes. The various cellular systems are grouped into surface (*blue*), morphology and cytoskeleton (*green*), and nuclear functions. Individual aspects are explored and considered in the text

1.6 Going Forward

We are now 8 years post the formal publication of the *T. brucei* 927 genome (Berriman et al. 2005) and many researchers enjoyed access to much of the sequence prior to then. A decade of postgenomic era research has yielded massively improved insights into the cell biology of African trypanosomes and coupled with falling sequencing costs facilitates improved understanding of the adaptations that accompany evolution of a specific mode of parasitism (Manna et al. 2013). What remains to be done? It is clear from many areas that the era of the candidate-based approach, i.e., analysis of clear or likely orthologs of gene products in higher eukaryotes, is drawing to a close, and returns are diminishing. The last 2 years have seen some major advances in interactome/proteomic studies, as well as a true genome-wide screening strategy becoming part of the toolbox (Alsford et al. 2012). This now provides potential entry into truly parasite-specific functions, as well as the opportunity to integrate data from multiple areas and to forge new connections (Fig. 1.3).

A great deal is now known about membrane trafficking in *T. brucei* but there are still some large gaps. In higher eukaryotes the cytoskeleton plays a fundamental role in endocytosis and intracellular vesicle transport, but cytoskeletal organization in trypanosomes is distinct, and cytosolic tubulin appears to be essentially absent. Further, the functions of actin and myosin are not fully understood, and surprisingly both are only essential in the BSF where depletion results in a significant reduction of endocytic activity (Garcia-Salcedo et al. 2004; Spitznagel et al. 2010). The Arp2/

3 complex, which stimulates actin polymerization is present but several Arp2/3 activation factors are missing (Berriman et al. 2005) suggesting a specific actin polymerization mechanism. This then raises the question of how vesicle movement is directed and regulated in trypanosomes, and the relationship between the various cytoskeletal organizers, endomembrane organellar function, and cell division/remodeling.

Another key question concerns the demarcation of specific organellar membranes by lipids, specifically the seven phosphorylated phosphatidylinositol (PtdIns) derivatives arising through phosphorylation of the inositol ring at positions 3, 4, and 5. In many eukaryotes different PtdIns phosphates (PIPs) are enriched at the cytoplasmic face of specific organelles and preferentially recruit cytosolic proteins harboring corresponding lipid-binding domains such as PX, PH, or FYVE domains. The conservation of the PIP-binding A/ENTH domains of TbCALM and TbEpsinR hints at a conserved role for $PI(4,5)P_2$ in endocytosis in *T. brucei*. PI(3)P localizes to the trypanosome endosomes, as seen in other systems (Hall et al. 2006). These PIPs are formed by the actions of various phosphatidylinositol (PI) kinases, of which there are clear orthologs in the *T. brucei* genome. Where this has been studied, depletion of these kinases by RNAi leads to dramatic effects on the organization of the endomembrane system, demonstrating a conserved role of PIPs in membrane trafficking (Hall et al. 2006; Rodgers et al. 2007).

We also remain quite ignorant of events within the endosomal system or of a detailed description of the cell surface proteome. Antibody and transferrin are degraded within parasite endosomes, and by contrast apoLI, in TLF-sensitive parasites, is delivered to the lysosome. But the precise compartments where uncoupling between receptors and ligands, the proteases involved in degradation, the fates of fragments, and the mechanisms of sorting are understood in only the broadest of terms. As these processes are at the heart of immune evasion mechanisms, these are critical gaps to knowledge, and areas where therapeutic gain is possible. Further, while it is clear that there are a great many proteins located at the plasma membrane, a detailed high quality catalog of location, copy number, sorting mechanisms, and potential functions of many surface proteins remain essentially undocumented, with the result that our understanding of sensing mechanisms and how the parasite interacts with the host remains fragmentary. Improved imaging and re-evaluation of much of the designation of specific organelles, together with more precise proteomics and gene product localizations are likely needed here.

Our discussion also touched briefly on the cell biology of gene expression and how organization of the nucleus is likely a highly important aspect of control of antigenic variation and possibly also development. At present we have a rather disjointed view of how all of the various processes fit together, with ideas for subnuclear compartments, heterochromatinization, and transcriptional control all essentially occupying distinct territories. This example is almost a microcosm of the overall situation, where similar lack of integration is apparent when considering the cell surface, the endomembrane system, and how signaling pathways operate. Arguably the next frontier will be to exploit new technologies in imaging, genetic

screening, and interactome mapping to more fully interrogate these systems and to bring a deeper and more holistic understanding to trypanosome cell biology and cellular organization.

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References

Absalon S, Blisnick T, Bonhivers M, Kohl L, Cayet NG, Toutirais GR, Buisson J, Robinson D, Bastin P (2008a) Flagellum elongation is required for correct structure, orientation and function of the flagellar pocket in Trypanosoma brucei. J Cell Sci 121(22):3704–3716

Absalon S, Blisnick T, Kohl L, Toutirais GR, Dore GN, Julkowska D, Tavenet A, Bastin P (2008b) Intraflagellar transport and functional analysis of genes required for flagellum formation in trypanosomes. Mol Biol Cell 19(3):929–944

Acosta-Serrano A, Vassella E, Liniger M, Kunz Renggli C, Brun R, Roditi I, Englund PT (2001) The surface coat of procyclic Trypanosoma brucei: programmed expression and proteolytic cleavage of procyclin in the tsetse fly. Proc Natl Acad Sci USA 98(4):1513–1518

Adung'a VO, Gadelha C, Field MC (2013) Proteomic analysis of clathrin interactions in trypanosomes reveals dynamic evolution of endocytosis. Traffic 14(4):440–457

Alexandre S, Paindavoine P, Tebabi P, Pays A, Halleux S, Steinert M, Pays E (1990) Differential expression of a family of putative adenylate/guanylate cyclase genes in Trypanosoma brucei. Mol Biochem Parasitol 43(2):279–288

Alexandre S, Paindavoine P, Hanocq-Quertier J, Paturiaux-Hanocq F, Tebabi P, Pays E (1996) Families of adenylate cyclase genes in Trypanosoma brucei. Mol Biochem Parasitol 77 (2):173–182

Ali JA, Creek DJ, Burgess K, Allison HC, Field MC, Mäser P, De Koning HP (2013) Pyrimidine salvage in Trypanosoma brucei bloodstream forms and the trypanocidal action of halogenated pyrimidines. Mol Pharmacol 83(2):439–453

Allen CL, Goulding D, Field MC (2003) Clathrin-mediated endocytosis is essential in Trypanosoma brucei. EMBO J 22(19):4991–5002

Al-Qahtani A, Teilhet M, Mensa-Wilmot K (1998) Species-specificity in endoplasmic reticulum signal peptide utilization revealed by proteins from Trypanosoma brucei and leishmania. Biochem J 331(2):521–529

Alsford S, Eckert S, Baker N, Glover L, Sanchez-Flores A, Leung KF, Turner DJ, Field MC, Berriman M, Horn D (2012) High-throughput decoding of antitrypanosomal drug efficacy and resistance. Nature 482(7384):232–236

Baker N, de Koning HP, Mäser P, Horn D (2013) Drug resistance in African trypanosomiasis: the melarsoprol and pentamidine story. Trends Parasitol 29(3):110–118

Bangs JD (2011) Replication of the ERES:Golgi junction in bloodstream-form African trypanosomes. Mol Microbiol 82(6):1433–1443

Baral TN (2010) Immunobiology of African trypanosomes: need of alternative interventions. J Biomed Biotechnol 2010:389153

Barry JD (1979) Capping of variable antigen on Trypanosoma brucei, and its immunological and biological significance. J Cell Sci 37:287–302

Beja O, Ullu E, Michaeli S (1993) Identification of a tRNA-like molecule that copurifies with the 7sl rna of Trypanosoma brucei. Mol Biochem Parasitol 57(2):223–229

Berriman M, Ghedin E, Hertz-Fowler C, Blandin G, Renauld H, Bartholomeu DC, Lennard NJ, Caler E, Hamlin NE, Haas B, Böhme U, Hannick L, Aslett MA, Shallom J, Marcello L, Hou L, Wickstead B, Alsmark UC, Arrowsmith C, Atkin RJ, Barron AJ, Bringaud F, Brooks K,

Carrington M, Cherevach I, Chillingworth TJ, Churcher C, Clark LN, Corton CH, Cronin A, Davies RM, Doggett J, Djikeng A, Feldblyum T, Field MC, Fraser A, Goodhead I, Hance Z, Harper D, Harris BR, Hauser H, Hostetler J, Ivens A, Jagels K, Johnson D, Johnson J, Jones K, Kerhornou AX, Koo H, Larke N, Landfear S, Larkin C, Leech V, Line A, Lord A, Macleod A, Mooney PJ, Moule S, Martin DM, Morgan GW, Mungall K, Norbertczak H, Ormond D, Pai G, Peacock CS, Peterson J, Quail MA, Rabbinowitsch E, Rajandream MA, Reitter C, Salzberg SL, Sanders M, Schobel S, Sharp S, Simmonds M, Simpson AJ, Tallon L, Turner CM, Tait A, Tivey AR, Van Aken S, Walker D, Wanless D, Wang S, White B, White O, Whitehead S, Woodward J, Wortman J, Adams MD, Embley TM, Gull K, Ullu E, Barry JD, Fairlamb AH, Opperdoes F, Barrell BG, Donelson JE, Hall N, Fraser CM, Melville SE, El-Sayed NM (2005) The genome of the African trypanosome Trypanosoma brucei. Science 309(5733):416–422

Besteiro S, Coombs GH, Mottram JC (2006) The SNARE protein family of Leishmania major. BMC Genomics 7:250

Biton M, Mandelboim M, Arvatz G, Michaeli S (2006) RNAi interference of XPO1 and Sm genes and their effect on the spliced leader RNA in Trypanosoma brucei. Mol Biochem Parasitol 150 (2):132–143

Blum ML, Down JA, Gurnett AM, Carrington M, Turner MJ, Wiley DC (1993) A structural motif in the variant surface glycoproteins of Trypanosoma brucei. Nature 362(6421):603–609

Bonhivers M, Landrein N, Decossas M, Robinson DR (2008a) A monoclonal antibody marker for the exclusion-zone filaments of Trypanosoma brucei. Parasit Vectors 1(1):21

Bonhivers M, Nowacki S, Landrein N, Robinson DR (2008b) Biogenesis of the trypanosome endo-exocytotic organelle is cytoskeleton mediated. PLoS Biol 6(5):e105

Bridges DJ, Pitt AR, Hanrahan O, Brennan K, Voorheis HP, Herzyk P, de Koning HP, Burchmore RJ (2008) Characterisation of the plasma membrane subproteome of bloodstream form Trypanosoma brucei. Proteomics 8(1):83–99

Bringaud F, Baltz T (1992) A potential hexose transporter gene expressed predominantly in the bloodstream form of Trypanosoma brucei. Mol Biochem Parasitol 52(1):111–121

Bringaud F, Baltz T (1993) Differential regulation of two distinct families of glucose transporter genes in Trypanosoma brucei. Mol Cell Biol 13(2):1146–1154

Broadhead R, Dawe HR, Farr H, Griffiths S, Hart SR, Portman N, Shaw MK, Ginger ML, Gaskell SJ, McKean PG, Gull K (2006) Flagellar motility is required for the viability of the bloodstream trypanosome. Nature 440(7081):224–227

Bruce D (1915) The croonian lectures on trypanosomes causing disease in man and domestic animals in central Africa: delivered before the royal college of physicians of London. Br Med J 2(2845):48–53

Bullard W, Kieft R, Capewell P, Veitch NJ, Macleod A, Hajduk SL (2012) Haptoglobin-hemoglobin receptor independent killing of African trypanosomes by human serum and trypanosome lytic factors. Virulence 3(1):72–76

Bütikofer P, Vassella E, Boschung M, Renggli CK, Brun R, Pearson TW, Roditi I (2002) Glycosylphosphatidylinositol-anchored surface molecules of Trypanosoma congolense insect forms are developmentally regulated in the tsetse fly. Mol Biochem Parasitol 119(1):7–16

Capewell P, Veitch NJ, Turner CM, Raper J, Berriman M, Hajduk SL, MacLeod A (2011) Differences between Trypanosoma brucei gambiense groups 1 and 2 in their resistance to killing by trypanolytic factor 1. PLoS Negl Trop Dis 5(9):e1287

Caramelo JJ, Parodi AJ (2007) How sugars convey information on protein conformation in the endoplasmic reticulum. Semin Cell Dev Biol 18(6):732–742

Carter NS, Fairlamb AH (1993) Arsenical-resistant trypanosomes lack an unusual adenosine transporter. Nature 361(6408):173–176

Castro O, Movsichoff F, Parodi AJ (2006) Preferential transfer of the complete glycan is determined by the oligosaccharyltransferase complex and not by the catalytic subunit. Proc Natl Acad Sci 103(40):14756–14760

Chattopadhyay A, Jones NG, Nietlispach D, Nielsen PR, Voorheis HP, Mott HR, Carrington M (2005) Structure of the C-terminal domain from Trypanosoma brucei variant surface glycoprotein MITat1.2. J Biol Chem 280(8):7228–7235

Chaves I, Rudenko G, Dirks-Mulder A, Cross M, Borst P (1999) Control of variant surface glycoprotein gene-expression sites in Trypanosoma brucei. EMBO J 18(17):4846–4855

Chung WL, Carrington M, Field MC (2004) Cytoplasmic targeting signals in transmembrane invariant surface glycoproteins of trypanosomes. J Biol Chem 279(52):54887–54895

Chung WL, Leung KF, Carrington M, Field MC (2008) Ubiquitylation is required for degradation of transmembrane surface proteins in trypanosomes. Traffic 9(10):1681–1697

Coppens I, Opperdoes FR, Courtoy PJ, Baudhuin P (1987) Receptor-mediated endocytosis in the bloodstream form of Trypanosoma brucei. J Protozool 34(4):465–473

Coppens I, Baudhuin P, Opperdoes FR, Courtoy PJ (1988) Receptors for the host low density lipoproteins on the hemoflagellate Trypanosoma brucei: purification and involvement in the growth of the parasite. Proc Natl Acad Sci USA 85(18):6753–6757

Dacks JB, Field MC (2007) Evolution of the eukaryotic membrane-trafficking system: origin, tempo and mode. J Cell Sci 120(17):2977–2985

Dacks JB, Peden AA, Field MC (2009) Evolution of specificity in the eukaryotic endomembrane system. Int J Biochem Cell Biol 41(2):330–340

Daniels JP, Gull K, Wickstead B (2010) Cell biology of the trypanosome genome. Microbiol Mol Biol Rev 74(4):552–569

Davidge JA, Chambers E, Dickinson HA, Towers K, Ginger ML, McKean PG, Gull K (2006) Trypanosome IFT mutants provide insight into the motor location for mobility of the flagella connector and flagellar membrane formation. J Cell Sci 119(Pt 19):3935–3943

De Greef C, Imberechts H, Matthyssens G, Van Meirvenne N, Hamers R (1989) A gene expressed only in serum-resistant variants of Trypanosoma brucei rhodesiense. Mol Biochem Parasitol 36 (2):169–176

de Koning HP, Watson CJ, Jarvis SM (1998) Characterization of a nucleoside/proton symporter in procyclic Trypanosoma brucei brucei. J Biol Chem 273(16):9486–9494

de la Canal L, Parodi AJ (1987) Synthesis of dolichol derivatives in trypanosomatids. Characterization of enzymatic patterns. J Biol Chem 262(23):11128–11133

Dean S, Marchetti R, Kirk K, Matthews KR (2009) A surface transporter family conveys the trypanosome differentiation signal. Nature 459(7244):213–217

DeGrasse JA, DuBois KN, Devos D, Siegel TN, Sali A, Field MC, Rout MP, Chait BT (2009) Evidence for a shared nuclear pore complex architecture that is conserved from the last common eukaryotic ancestor. Mol Cell Proteomics 8(9):2119–2130

Dossin Fde M, Schenkman S (2005) Actively transcribing RNA polymerase II concentrates on spliced leader genes in the nucleus of Trypanosoma cruzi. Eukaryot Cell 4(5):960–970

DuBois KN, Alsford S, Holden JM, Buisson J, Swiderski M, Bart JM, Ratushny AV, Wan Y, Bastin P, Barry JD, Navarro M, Horn D, Aitchison JD, Rout MP, Field MC (2012) NUP-1 Is a large coiled-coil nucleoskeletal protein in trypanosomes with lamin-like functions. PLoS Biol 10(3):e1001287

Elias M, Brighouse A, Gabernet-Castello C, Field MC, Dacks JB (2012) Sculpting the endomembrane system in deep time: high resolution phylogenetics of rab gtpases. J Cell Sci 125(10):2500–2508

Emmer BT, Maric D, Engman DM (2010) Molecular mechanisms of protein and lipid targeting to ciliary membranes. J Cell Sci 123(4):529–536

Engstler M, Thilo L, Weise F, Grunfelder CG, Schwarz H, Boshart M, Overath P (2004) Kinetics of endocytosis and recycling of the gpi-anchored variant surface glycoprotein in Trypanosoma brucei. J Cell Sci 117(7):1105–1115

Engstler M, Pfohl T, Herminghaus S, Boshart M, Wiegertjes G, Heddergott N, Overath P (2007) Hydrodynamic flow-mediated protein sorting on the cell surface of trypanosomes. Cell 131 (3):505–515

Ersfeld K, Gull K (2001) Targeting of cytoskeletal proteins to the flagellum of Trypanosoma brucei. J Cell Sci 114(1):141–148

Ersfeld K, Melville SE, Gull K (1999) Nuclear and genome organization of Trypanosoma brucei. Parasitol Today 15(2):58–63

Ferguson MA, Murray P, Rutherford H, McConville MJ (1993) A simple purification of procyclic acidic repetitive protein and demonstration of a sialylated glycosyl-phosphatidylinositol membrane anchor. Biochem J 291(Pt 1):51–55

Field MC, Carrington M (2004) Intracellular membrane transport systems in Trypanosoma brucei. Traffic 5(12):905–913

Field MC, Carrington M (2009) The trypanosome flagellar pocket. Nat Rev Microbiol 7 (11):775–786

Field H, Sherwin T, Smith AC, Gull K, Field MC (2000) Cell-cycle and developmental regulation of TbRAB31 localisation, a GTP-locked Rab protein from Trypanosoma brucei. Mol Biochem Parasitol 106(1):21–35

Field M, Gabernet-Castello C, Dacks J (2007a) Reconstructing the evolution of the endocytic system: insights from genomics and molecular cell biology. In: Gáspár J (ed) Eukaryotic membranes and cytoskeleton. Springer, New York, pp 84–96

Field MC, Natesan SKA, Gabernet-Castello C, Koumandou VL (2007b) Intracellular trafficking in the trypanosomatids. Traffic 8(6):629–639

Field MC, Lumb JH, Adung'a VO, Jones NG, Engstler M (2009) Macromolecular trafficking and immune evasion in African trypanosomes. In: Kwang WJ (ed) International review of cell and molecular biology. Academic, Boston, MA, pp 1–67

Field MC, Sergeenko T, Wang YN, Böhm S, Carrington M (2010) Chaperone requirements for biosynthesis of the trypanosome variant surface glycoprotein. PLoS One 5(1):e8468

Field MC, Horn D, Alsford S, Koreny L, Rout MP (2012) Telomeres, tethers and trypanosomes. Nucleus 3(6):478–486

Figueiredo LM, Cross GA (2010) Nucleosomes are depleted at the VSG expression site transcribed by RNA polymerase I in African trypanosomes. Eukaryot Cell 9(1):148–154

Figueiredo LM, Janzen CJ, Cross GA (2008) A histone methyltransferase modulates antigenic variation in African trypanosomes. PLoS Biol 6(7):e161

Freymann D, Down J, Carrington M, Roditi I, Turner M, Wiley D (1990) 2.9 A resolution structure of the N-terminal domain of a variant surface glycoprotein from Trypanosoma brucei. J Mol Biol 216(1):141–160

Gabernet-Castello C, Dacks JB, Field MC (2009) The single enth-domain protein of trypanosomes; endocytic functions and evolutionary relationship with epsin. Traffic 10 (7):894–911

Gabernet-Castello C, DuBois KN, Nimmo C, Field MC (2011) Rab11 function in Trypanosoma brucei: identification of conserved and novel interaction partners. Eukaryot Cell 10 (8):1082–1094

Gadelha C, Rothery S, Morphew M, McIntosh JR, Severs NJ, Gull K (2009) Membrane domains and flagellar pocket boundaries are influenced by the cytoskeleton in African trypanosomes. Proc Natl Acad Sci USA 106(41):17425–17430

Garcia-Salcedo JA, Perez-Morga D, Gijon P, Dilbeck V, Pays E, Nolan DP (2004) A differential role for actin during the life cycle of Trypanosoma brucei. EMBO J 23(4):780–789

Giroud C, Ottones F, Coustou V, Dacheux D, Biteau N, Miezan B, Van Reet N, Carrington M, Doua F, Baltz T (2009) Murine models for Trypanosoma brucei gambiense disease progression–from silent to chronic infections and early brain tropism. PLoS Negl Trop Dis 3 (9):e509

Goldshmidt H, Sheiner L, Butikofer P, Roditi I, Uliel S, Gunzel M, Engstler M, Michaeli S (2008) Role of protein translocation pathways across the endoplasmic reticulum in Trypanosoma brucei. J Biol Chem 283(46):32085–32098

Grab DJ, Webster P, Verjee Y, Lonsdale-Eccles J (1997) Golgi-associated phosphohydrolases in Trypanosoma brucei brucei. Mol Biochem Parasitol 86(1):127–132

Griffiths GM, Tsun A, Stinchcombe JC (2010) The immunological synapse: a focal point for endocytosis and exocytosis. J Cell Biol 189(3):399–406

Grunfelder CG, Engstler M, Weise F, Schwarz H, Stierhof Y-D, Morgan GW, Field MC, Overath P (2003) Endocytosis of a glycosylphosphatidylinositol-anchored protein via clathrin-coated vesicles, sorting by default in endosomes, and exocytosis via rab11-positive carriers. Mol Biol Cell 14(5):2029–2040

Gudin S, Quashie NB, Candlish D, Al-Salabi MI, Jarvis SM, Ranford-Cartwright LC, de Koning HP (2006) Trypanosoma brucei: a survey of pyrimidine transport activities. Exp Parasitol 114 (2):118–125

Hall B, Allen CL, Goulding D, Field MC (2004a) Both of the rab5 subfamily small GTPases of Trypanosoma brucei are essential and required for endocytosis. Mol Biochem Parasitol 138 (1):67–77

Hall BS, Pal A, Goulding D, Field MC (2004b) Rab4 is an essential regulator of lysosomal trafficking in trypanosomes. J Biol Chem 279(43):45047–45056

Hall BS, Pal A, Goulding D, Acosta-Serrano A, Field MC (2005) Trypanosoma brucei: TbRab4 regulates membrane recycling and expression of surface proteins in procyclic forms. Exp Parasitol 111(3):160–171

Hall BS, Gabernet-Castello C, Voak A, Goulding D, Natesan SK, Field MC (2006) Tbvps34, the trypanosome orthologue of vps34, is required for Golgi complex segregation. J Biol Chem 281 (37):27600–27612

Hebert DN, Molinari M (2007) In and out of the ER: protein folding, quality control, degradation, and related human diseases. Physiol Rev 87(4):1377–1408

He CY, Ho HH, Malsam J, Chalouni C, West CM, Ullu E, Toomre D, Warren G (2004) Golgi duplication in Trypanosoma brucei. J Cell Biol 165(3):313–321

Heddergott N, Krüger T, Babu SB, Wei A, Stellamanns E, Uppaluri S, Pfohl T, Stark H, Engstler M (2012) Trypanosome motion represents an adaptation to the crowded environment of the vertebrate bloodstream. PLoS Pathog 8(11):e1003023

Hettema EH, Distel B, Tabak HF (1999) Import of proteins into peroxisomes. Biochim Biophys Acta 1451(1):17–34

Higgins MK, Tkachenko O, Brown A, Reed J, Raper J, Carrington M (2013) Structure of the trypanosome haptoglobin-hemoglobin receptor and implications for nutrient uptake and innate immunity. Proc Natl Acad Sci USA 110(5):1905–1910

Hill KL (2010) Parasites in motion: flagellum-driven cell motility in African trypanosomes. Curr Opin Microbiol 13(4):459–465

Hodges ME, Scheumann N, Wickstead B, Langdale JA, Gull K (2010) Reconstructing the evolutionary history of the centriole from protein components. J Cell Sci 123(9):1407–1413

Huang G, Fang J, Sant'Anna C, Li Z-H, Wellems DL, Rohloff P, Docampo R (2011) Adaptor protein-3 (ap-3) complex mediates the biogenesis of acidocalcisomes and is essential for growth and virulence of Trypanosoma brucei. J Biol Chem 286(42):36619–36630

Hughes LC, Ralston KS, Hill KL, Zhou ZH (2012) Three-dimensional structure of the Trypanosome flagellum suggests that the paraflagellar rod functions as a biomechanical spring. PLoS One 7(1):e25700

Hury A, Goldshmidt H, Tkacz ID, Michaeli S (2009) Trypanosome spliced-leader-associated RNA (SLA1) localization and implications for spliced-leader RNA biogenesis. Eukaryot Cell 8 (1):56–68

Ikeda KN, de Graffenried CL (2012) Polo-like kinase is necessary for flagellum inheritance in Trypanosoma brucei. J Cell Sci 125(Pt 13):3173–3184

Jackson AP, Berry A, Aslett M, Allison HC, Burton P, Vavrova-Anderson J, Brown R, Browne H, Corton N, Hauser H, Gamble J, Gilderthorp R, Marcello L, McQuillan J, Otto TD, Quail MA, Sanders MJ, van Tonder A, Ginger ML, Field MC, Barry JD, Hertz-Fowler C, Berriman M (2012) Antigenic diversity is generated by distinct evolutionary mechanisms in African trypanosome species. Proc Natl Acad Sci USA 109(9):3416–3421

Jin H, White SR, Shida T, Schulz S, Aguiar M, Gygi SP, Bazan JF, Nachury MV (2010) The conserved Bardet-Biedl syndrome proteins assemble a coat that traffics membrane proteins to cilia. Cell 141(7):1208–1219

Jones NG, Nietlispach D, Sharma R, Burke DF, Eyres I, Mues M, Mott HR, Carrington M (2008) Structure of a glycosylphosphatidylinositol-anchored domain from a trypanosome variant surface glycoprotein. J Biol Chem 283(6):3584–3593

Kelleher DJ, Gilmore R (2006) An evolving view of the eukaryotic oligosaccharyltransferase. Glycobiology 16(4):47R–62R

Kelly S, Singleton W, Wickstead B, Ersfeld K, Gull K (2006) Characterization and differential nuclear localization of Nopp140 and a novel Nopp140-like protein in trypanosomes. Eukaryot Cell 5(5):876–879

Kieft R, Capewell P, Turner CM, Veitch NJ, MacLeod A, Hajduk S (2010) Mechanism of Trypanosoma brucei gambiense (group 1) resistance to human trypanosome lytic factor. Proc Natl Acad Sci USA 107(37):16137–16141

Kind J, van Steensel B (2010) Genome-nuclear lamina interactions and gene regulation. Curr Opin Cell Biol 22(3):320–325

Kolev NG, Ramey-Butler K, Cross GA, Ullu E, Tschudi C (2012) Developmental progression to infectivity in Trypanosoma brucei triggered by an RNA-binding protein. Science 338 (6112):1352–1353

Koumandou VL, Dacks J, Coulson R, Field M (2007) Control systems for membrane fusion in the ancestral eukaryote; evolution of tethering complexes and sm proteins. BMC Evol Biol 7(1):29

Koumandou VL, Natesan SK, Sergeenko T, Field MC (2008) The trypanosome transcriptome is remodelled during differentiation but displays limited responsiveness within life stages. BMC Genomics 9:298

Koumandou VL, Klute MJ, Herman EK, Nunez-Miguel R, Dacks JB, Field MC (2011) Evolutionary reconstruction of the retromer complex and its function in Trypanosoma brucei. J Cell Sci 124(9):1496–1509

Koumandou VL, Boehm C, Horder KA, Field MC (2013) Evidence for recycling of invariant surface transmembrane domain proteins in African trypanosomes. Eukaryot Cell 12 (2):330–342

Lacomble S, Vaughan S, Gadelha C, Morphew MK, Shaw MK, McIntosh JR, Gull K (2009) Three-dimensional cellular architecture of the flagellar pocket and associated cytoskeleton in trypanosomes revealed by electron microscope tomography. J Cell Sci 122(Pt 8):1081–1090

Lacomble S, Vaughan S, Gadelha C, Morphew MK, Shaw MK, McIntosh JR, Gull K (2010) Basal body movements orchestrate membrane organelle division and cell morphogenesis in Trypanosoma brucei. J Cell Sci 123(Pt 17):2884–2891

Lacomble S, Vaughan S, Deghelt M, Moreira-Leite FF, Gull K (2012) A Trypanosoma brucei protein required for maintenance of the flagellum attachment zone and flagellar pocket ER domains. Protist 163(4):602–615

Landeira D, Navarro M (2007) Nuclear repositioning of the VSG promoter during developmental silencing in Trypanosoma brucei. J Cell Biol 176(2):133–139

Landeira D, Bart JM, Van Tyne D, Navarro M (2009) Cohesin regulates VSG monoallelic expression in trypanosomes. J Cell Biol 186(2):243–254

Lanteri CA, Stewart ML, Brock JM, Alibu VP, Meshnick SR, Tidwell RR, Barrett MP (2006) Roles for the Trypanosoma brucei P2 transporter in DB75 uptake and resistance. Mol Pharmacol 70(5):1585–1592

Leung KF, Dacks JB, Field MC (2008) Evolution of the multivesicular body escrt machinery; retention across the eukaryotic lineage. Traffic 9(10):1698–1716

Leung KF, Riley FS, Carrington M, Field MC (2011) Ubiquitylation and developmental regulation of invariant surface protein expression in trypanosomes. Eukaryot Cell 10(7):916–931

Liang XH, Liu Q, Liu L, Tschudi C, Michaeli S (2006) Analysis of spliceosomal complexes in Trypanosoma brucei and silencing of two splicing factors Prp31 and Prp43. Mol Biochem Parasitol 145(1):29–39

Liu L, Ben-Shlomo H, Y-x X, Stern MZ, Goncharov I, Zhang Y, Michaeli S (2003) The trypanosomatid signal recognition particle consists of two RNA molecules, a 7SL RNA homologue and a novel tRNA-like molecule. J Biol Chem 278(20):18271–18280

Lumb JH, Leung KF, DuBois KN, Field MC (2011) Rab28 function in trypanosomes: interactions with retromer and escrt pathways. J Cell Sci 124(22):3771–3783

Lustig Y, Goldshmidt H, Uliel S, Michaeli S (2005) The Trypanosoma brucei signal recognition particle lacks the alu-domain-binding proteins: purification and functional analysis of its binding proteins by rnai. J Cell Sci 118(19):4551–4562

Manna PT, Kelly S, Field MC (2013) Adaptin evolution in kinetoplastids and emergence of the variant surface glycoprotein coat in african trypanosomatids. Mol Phylogenet Evol 67 (1):123–128

Manthri S, Guther MLS, Izquierdo L, Acosta-Serrano A, Ferguson MAJ (2008) Deletion of the tbalg3 gene demonstrates site-specific n-glycosylation and n-glycan processing in Trypanosoma brucei. Glycobiology 18(5):367–383

Mao YS, Zhang B, Spector DL (2011) Biogenesis and function of nuclear bodies. Trends Genet 27 (8):295–306

Mäser P, Sütterlin C, Kralli A, Kaminsky R (1999) A nucleoside transporter from Trypanosoma brucei involved in drug resistance. Science 285(5425):242–244

May SF, Peacock L, Almeida Costa CI, Gibson WC, Tetley L, Robinson DR, Hammarton TC (2012) The Trypanosoma brucei AIR9-like protein is cytoskeleton-associated and is required for nucleus positioning and accurate cleavage furrow placement. Mol Microbiol 84(1):77–92

McMahon HT, Boucrot E (2011) Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nat Rev Mol Cell Biol 12(8):517–533

Meaburn KJ, Misteli T (2007) Cell biology: chromosome territories. Nature 445(7126):379–781

Mehlert A, Treumann A, Ferguson MA (1999) Trypanosoma brucei GPEET-PARP is phosphorylated on six out of seven threonine residues. Mol Biochem Parasitol 98(2):291–296

Misteli T (2005) Concepts in nuclear architecture. Bioessays 27(5):477–487

Michels PA, Bringaud F, Herman M, Hannaert V (2006) Metabolic functions of glycosomes in trypanosomatids. Biochim Biophys Acta 1763(12):1463–1477

Moremen KW, Molinari M (2006) N-linked glycan recognition and processing: the molecular basis of endoplasmic reticulum quality control. Curr Opin Struct Biol 16(5):592–599

Morriswood B, He CY, Sealey-Cardona M, Yelinek J, Pypaert M, Warren G (2009) The bilobe structure of Trypanosoma brucei contains a MORN-repeat protein. Mol Biochem Parasitol 167 (2):95–103

Morriswood B, Havlicek K, Demmel L, Yavuz S, Sealey-Cardona M, Vidilaseris K, Anrather D, Kostan J, Djinovic-Carugo K, Roux KJ, Warren G (2013) Novel bilobe components in Trypanosoma brucei identified using proximity-dependent biotinylation. Eukaryot Cell 12 (2):356–367

Nagamune K, Nozaki T, Maeda Y, Ohishi K, Fukuma T, Hara T, Schwarz RT, Sutterlin C, Brun R, Riezman H, Kinoshita T (2000) Critical roles of glycosylphosphatidylinositol for Trypanosoma brucei. Proc Natl Acad Sci USA 97(19):10336–10341

Natesan SKA, Peacock L, Matthews K, Gibson W, Field MC (2007) Activation of endocytosis as an adaptation to the mammalian host by trypanosomes. Eukaryot Cell 6(11):2029–2037

Navarro M, Gull K (2001) A pol I transcriptional body associated with VSG mono-allelic expression in Trypanosoma brucei. Nature 414(6865):759–763

Nishikawa S-I, Fewell SW, Kato Y, Brodsky JL, Endo T (2001) Molecular chaperones in the yeast endoplasmic reticulum maintain the solubility of proteins for retrotranslocation and degradation. J Cell Biol 153(5):1061–1070

O'Beirne C, Lowry CM, Voorheis HP (1998) Both IgM and IgG anti-VSG antibodies initiate a cycle of aggregation-disaggregation of bloodstream forms of Trypanosoma brucei without damage to the parasite. Mol Biochem Parasitol 91(1):165–193

Oberholzer M, Langousis G, Nguyen HT, Saada EA, Shimogawa MM, Jonsson ZO, Nguyen SM, Wohlschlegel JA, Hill KL (2011) Independent analysis of the flagellum surface and matrix

proteomes provides insight into flagellum signaling in mammalian-infectious Trypanosoma brucei. Mol Cell Proteomics 10(10):M111.010538
Ogbadoyi E, Ersfeld K, Robinson D, Sherwin T, Gull K (2000) Architecture of the Trypanosoma brucei nucleus during interphase and mitosis. Chromosoma 108(8):501–513
Ogbadoyi EO, Robinson DR, Gull K (2003) A high-order trans-membrane structural linkage is responsible for mitochondrial genome positioning and segregation by flagellar basal bodies in trypanosomes. Mol Biol Cell 14(5):1769–1779
Opperdoes FR, Borst P (1977) Localization of nine glycolytic enzymes in a microbody-like organelle in Trypanosoma brucei: the glycosome. FEBS Lett 80(2):360–364
Pal A, Hall BS, Nesbeth DN, Field HI, Field MC (2002) Differential endocytic functions of Trypanosoma brucei Rab5 isoforms reveal a glycosylphosphatidylinositol-specific endosomal pathway. J Biol Chem 277(11):9529–9539
Pal A, Hall BS, Jeffries TR, Field MC (2003) Rab5 and Rab11 mediate transferrin and anti-variant surface glycoprotein antibody recycling in Trypanosoma brucei. Biochem J 374(Pt 2):443–451
Pays E, Tebabi P, Pays A, Coquelet H, Revelard P, Salmon D, Steinert M (1989) The genes and transcripts of an antigen gene expression site from T. brucei. Cell 57(5):835–845
Pays E, Vanhollebeke B, Vanhamme L, Paturiaux-Hanocq F, Nolan DP, Pérez-Morga D (2006) The trypanolytic factor of human serum. Nat Rev Microbiol 4(6):477–486
Pederson T (2011) The nucleolus. Cold Spring Harb Perspect Biol 3(3):pii: a000638
Plemper RK, Bohmler S, Bordallo J, Sommer T, Wolf DH (1997) Mutant analysis links the translocon and bip to retrograde protein transport for er degradation. Nature 388 (6645):891–895
Povelones ML, Gluenz E, Dembek M, Gull K, Rudenko G (2012) Histone H1 plays a role in heterochromatin formation and VSG expression site silencing in Trypanosoma brucei. PLoS Pathog 8(11):e1003010
Pavelka M, Neumüller J, Ellinger A (2008) Retrograde traffic in the biosynthetic-secretory route. Histochem Cell Biol 129(3):277–288
Price HP, Hodgkinson MR, Wright MH, Tate EW, Smith BA, Carrington M, Stark M, Smith DF (2012) A role for the vesicle-associated tubulin binding protein arl6 (bbs3) in flagellum extension in Trypanosoma brucei. Biochim Biophys Acta 1823(7):1178–1191
Pullen TJ, Ginger ML, Gaskell SJ, Gull K (2004) Protein targeting of an unusual, evolutionarily conserved adenylate kinase to a eukaryotic flagellum. Mol Biol Cell 15(7):3257–3265
Rodgers MJ, Albanesi JP, Phillips MA (2007) Phosphatidylinositol 4-kinase iii-β is required for golgi maintenance and cytokinesis in Trypanosoma brucei. Eukaryot Cell 6(7):1108–1118
Roditi I, Schwarz H, Pearson TW, Beecroft RP, Liu MK, Richardson JP, Bühring HJ, Pleiss J, Bülow R, Williams RO et al (1989) Procyclin gene expression and loss of the variant surface glycoprotein during differentiation of Trypanosoma brucei. J Cell Biol 108(2):737–746
Rotureau B, Subota I, Bastin P (2011) Molecular bases of cytoskeleton plasticity during the Trypanosoma brucei parasite cycle. Cell Microbiol 13(5):705–716
Rotureau B, Subota I, Buisson J, Bastin P (2012) A new asymmetric division contributes to the continuous production of infective trypanosomes in the tsetse fly. Development 139 (10):1842–1850
Rout MP, Field MC (2001) Isolation and characterization of subnuclear compartments from Trypanosoma brucei. Identification of a major repetitive nuclear lamina component. J Biol Chem 276(41):38261–38271
Ruepp S, Furger A, Kurath U, Renggli CK, Hemphill A, Brun R, Roditi I (1997) Survival of Trypanosoma brucei in the tsetse fly is enhanced by the expression of specific forms of procyclin. J Cell Biol 137(6):1369–1379
Sommer JM, Wang CC (1994) Targeting proteins to the glycosomes of African trypanosomes. Annu Rev Microbiol 48:105–138
Sannerud R, Saraste J, Goud B (2003) Retrograde traffic in the biosynthetic-secretory route: pathways and machinery. Curr Opin Cell Biol 15(4):438–445

Salmon D, Geuskens M, Hanocq F, Hanocq-Quertier J, Nolan D, Ruben L, Pays E (1994) A novel heterodimeric transferrin receptor encoded by a pair of VSG expression site-associated genes in T. brucei. Cell 78(1):75–86

Salmon D, Bachmaier S, Krumbholz C, Kador M, Gossmann JA, Uzureau P, Pays E, Boshart M (2012a) Cytokinesis of Trypanosoma brucei bloodstream forms depends on expression of adenylyl cyclases of the ESAG4 or ESAG4-like subfamily. Mol Microbiol 84(2):225–242

Salmon D, Vanwalleghem G, Morias Y, Denoeud J, Krumbholz C, Lhommé F, Bachmaier S, Kador M, Gossmann J, Dias FB, De Muylder G, Uzureau P, Magez S, Moser M, De Baetselier P, Van Den Abbeele J, Beschin A, Boshart M, Pays E (2012b) Adenylate cyclases of Trypanosoma brucei inhibit the innate immune response of the host. Science 337 (6093):463–466

Schwarz F, Aebi M (2011) Mechanisms and principles of n-linked protein glycosylation. Curr Opin Struct Biol 21(5):576–582

Schwede A, Jones N, Engstler M, Carrington M (2011) The VSG C-terminal domain is inaccessible to antibodies on live trypanosomes. Mol Biochem Parasitol 175(2):201–204

Serizawa S, Miyamichi K, Nakatani H, Suzuki M, Saito M, Yoshihara Y, Sakano H (2003) Negative feedback regulation ensures the one receptor-one olfactory neuron rule in mouse. Science 302(5653):2088–2094

Seyfang A, Mecke D, Duszenko M (1990) Degradation, recycling, and shedding of Trypanosoma brucei variant surface glycoprotein. J Protozool 37(6):546–552

Silverman JS, Bangs JD (2012) Form and function in the trypanosomal secretory pathway. Curr Opin Microbiol 15(4):463–468

Silverman JS, Schwartz KJ, Hajduk SL, Bangs JD (2011) Late endosomal rab7 regulates lysosomal trafficking of endocytic but not biosynthetic cargo in Trypanosoma brucei. Mol Microbiol 82(3):664–678

Spitznagel D, O'Rourke JF, Leddy N, Hanrahan O, Nolan DP (2010) Identification and characterization of an unusual class I myosin involved in vesicle traffic in Trypanosoma brucei. PLoS One 5(8):e12282

Stanne TM, Rudenko G (2010) Active VSG expression sites in Trypanosoma brucei are depleted of nucleosomes. Eukaryot Cell 9(1):136–147

Stanne TM, Kushwaha M, Wand M, Taylor JE, Rudenko G (2011) TbISWI regulates multiple polymerase I (Pol I)-transcribed loci and is present at Pol II transcription boundaries in Trypanosoma brucei. Eukaryot Cell 10(7):964–976

Steverding D (1998) Bloodstream forms of Trypanosoma brucei require only small amounts of iron for growth. Parasitol Res 84(1):59–62

Steverding D (2003) The significance of transferrin receptor variation in Trypanosoma brucei. Trends Parasitol 19(3):125–127

Steverding D, Stierhof YD, Chaudhri M, Ligtenberg M, Schell D, Beck-Sickinger AG, Overath P (1994) ESAG 6 and 7 products of Trypanosoma brucei form a transferrin binding protein complex. Eur J Cell Biol 64(1):78–87

Steverding D, Stierhof YD, Fuchs H, Tauber R, Overath P (1995) Transferrin-binding protein complex is the receptor for transferrin uptake in Trypanosoma brucei. J Cell Biol 131 (5):1173–1182

Tazeh NN, Silverman JS, Schwartz KJ, Sevova ES, Sutterwala SS, Bangs JD (2009) Role of AP-1 in developmentally regulated lysosomal trafficking in Trypanosoma brucei. Eukaryot Cell 8 (9):1352–1361

Thiry M, Lafontaine DL (2005) Birth of a nucleolus: the evolution of nucleolar compartments. Trends Cell Biol 15(4):194–199

Tkacz ID, Lustig Y, Stern MZ, Biton M, Salmon-Divon M, Das A, Bellofatto V, Michaeli S (2007) Identification of novel snRNA-specific Sm proteins that bind selectively to U2 and U4 snRNAs in Trypanosoma brucei. RNA 13(1):30–43

Treumann A, Zitzmann N, Hülsmeier A, Prescott AR, Almond A, Sheehan J, Ferguson MA (1997) Structural characterisation of two forms of procyclic acidic repetitive protein expressed by procyclic forms of Trypanosoma brucei. J Mol Biol 269(4):529–547

Tyler KM, Fridberg A, Toriello KM, Olson CL, Cieslak JA, Hazlett TL, Engman DM (2009) Flagellar membrane localization via association with lipid rafts. J Cell Sci 122(6):859–866

Urwyler S, Studer E, Renggli CK, Roditi I (2007) A family of stage-specific alanine-rich proteins on the surface of epimastigote forms of Trypanosoma brucei. Mol Microbiol 63(1):218–228

van Dam TPJ, Townsend MJ, Turk M, Schlessinger A, Sali A, Field MC, Huynen M (2013) Intraflagellar transport is a modular complex that evolved from a coatomer-like protein complex. Proc Natl Acad Sci USA 110(17):6943–6948

van Luenen HG, Kieft R, Mussmann R, Engstler M, ter Riet B, Borst P (2005) Trypanosomes change their transferrin receptor expression to allow effective uptake of host transferrin. Mol Microbiol 58(1):151–165

Vanhollebeke B, De Muylder G, Nielsen MJ, Pays A, Tebabi P, Dieu M, Raes M, Moestrup SK, Pays E (2008) A haptoglobin-hemoglobin receptor conveys innate immunity to Trypanosoma brucei in humans. Science 320(5876):677–681

Vassella E, Den Abbeele JV, Bütikofer P, Renggli CK, Furger A, Brun R, Roditi I (2000) A major surface glycoprotein of Trypanosoma brucei is expressed transiently during development and can be regulated post-transcriptionally by glycerol or hypoxia. Genes Dev 14(5):615–626

Vassella E, Bütikofer P, Engstler M, Jelk J, Roditi I (2003) Procyclin null mutants of Trypanosoma brucei express free glycosylphosphatidylinositols on their surface. Mol Biol Cell 14 (4):1308–1318

Vassella E, Oberle M, Urwyler S, Renggli CK, Studer E, Hemphill A, Fragoso C, Bütikofer P, Brun R, Roditi I (2009) Major surface glycoproteins of insect forms of Trypanosoma brucei are not essential for cyclical transmission by tsetse. PLoS One 4(2):e4493

Vickerman K, Luckins AG (1969) Localization of variable antigens in the surface coat of Trypanosoma brucei using ferritin conjugated antibody. Nature 224(5224):1125–1126

Weiße S, Heddergott N, Heydt M, Pflästerer D, Maier T, Haraszti T, Grunze M, Engstler M, Rosenhahn A (2012) A quantitative 3D motility analysis of Trypanosoma brucei by use of digital in-line holographic microscopy. PLoS One 7(5):e37296

Widener J, Nielsen MJ, Shiflett A, Moestrup SK, Hajduk S (2007) Hemoglobin is a co-factor of human trypanosome lytic factor. PLoS Pathog 3(9):1250–1261

Xong HV, Vanhamme L, Chamekh M, Chimfwembe CE, Van Den Abbeele J, Pays A, Van Meirvenne N, Hamers R, De Baetselier P, Pays E (1998) A VSG expression site-associated gene confers resistance to human serum in Trypanosoma rhodesiense. Cell 95(6):839–846

Yang X, Figueiredo LM, Espinal A, Okubo E, Li B (2009) RAP1 is essential for silencing telomeric variant surface glycoprotein genes in Trypanosoma brucei. Cell 137(1):99–109

Zanetti G, Pahuja KB, Studer S, Shim S, Schekman R (2011) Copii and the regulation of protein sorting in mammals. Nat Cell Biol 14(1):20–28 (Erratum in 2012)

Zeiner GM, Sturm NR, Campbell DA (2003) Exportin 1 mediates nuclear export of the kinetoplastid spliced leader RNA. Eukaryot Cell 2(2):222–230

Zhao Z, Lindsay ME, Roy Chowdhury A, Robinson DR, Englund PT (2008) p166, a link between the trypanosome mitochondrial DNA and flagellum, mediates genome segregation. EMBO J 27(1):143–154

Zhou Q, Gheiratmand L, Chen Y, Lim TK, Zhang J, Li S, Xia N, Liu B, Lin Q, He CY (2010) A comparative proteomic analysis reveals a new bi-lobe protein required for bi-lobe duplication and cell division in Trypanosoma brucei. PLoS One 5(3):e9660

Ziegelbauer K, Overath P (1992) Identification of invariant surface glycoproteins in the blood-stream stage of Trypanosoma brucei. J Biol Chem 267(15):10791–10796

Ziegelbauer K, Overath P (1993) Organization of two invariant surface glycoproteins in the surface coat of Trypanosoma brucei. Infect Immun 61(11):4540–4545

Ziegelbauer K, Multhaup G, Overath P (1992) Molecular characterization of two invariant surface glycoproteins specific for the bloodstream stage of Trypanosoma brucei. J Biol Chem 267 (15):10797–10803

The Biology of Tsetse–Trypanosome Interactions

2

Guy Caljon, Linda De Vooght, and Jan Van Den Abbeele

Abstract

Human African trypanosomiasis (HAT) or sleeping sickness is a disease caused by human-pathogenic protozoan parasites of the *Trypanosoma brucei* species. These parasites are transmitted by the tsetse fly vector in which parasites have to surmount several natural bottlenecks to eventually reach the insect salivary glands and to differentiate into a final stage that is infective for a new vertebrate host. During the development in tsetse flies, trypanosomes change their surface properties, traverse physical barriers and achieve colonization of various tissues while going through complex cellular proliferation and differentiation programs. The general tsetse fly physiology and immunology, the blood feeding machinery and symbiotic relationships are important parameters that affect the parasite transmissibility.

2.1 Introduction

Tsetse flies encompass 33 species and subspecies (Elsen et al. 1990; Leak 1999) that are all restricted to the African continent and are limited by several natural borders, such as the Sahara and Somali deserts in the North and Kalahari and Namibian deserts in the South (reviewed in Leak 1999; Despommier and Hotez 2006). Tsetse flies belong to the order of *Diptera* and based on their reproduction through adenotropic viviparity (Leak 1999; Gooding and Krafsur 2005), tsetse flies are categorized as members of the superfamily *Hippoboscidea*, the family of *Glossinidae* and genus of *Glossina*. Tsetse flies are characterized by a distinct proboscis, antenna with branched arista hairs and wings that fold at rest and that have a characteristic "hatchet" cell (Fig. 2.1). Three subgenera were identified

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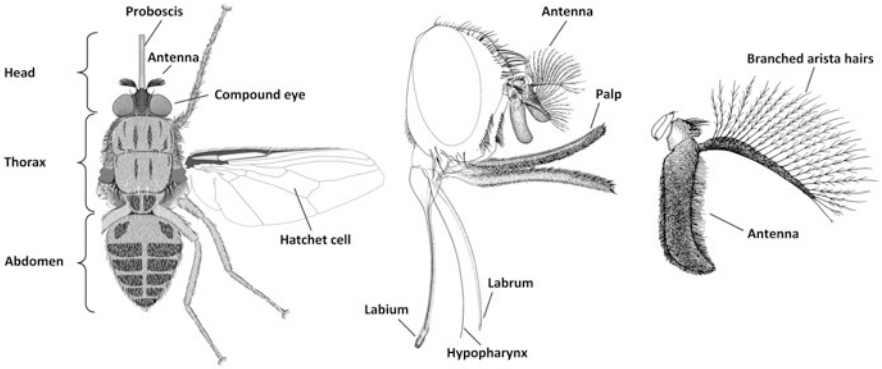

Fig. 2.1 Anatomy of the tsetse fly highlighting the characteristic wing pattern, the proboscis with a detail of the mouthparts and the antennal structure. Compiled figures reproduced with permission of ITM

within the *Glossina* genus: (1) *Morsitans*, (2) *Palpalis* and (3) *Fusca*. One *Glossina* species, *G. austeni*, previously classified under the *Morsitans* group is now under a distinct subgenus, *Machadomyia* (reviewed in Gooding and Krafsur 2005). The current taxonomy is based on morphology, structural differences of the male and female genitalia and DNA sequence data of tsetse fly. The current taxonomy is also supported by 16S rDNA sequences of the maternally transmitted primary endosymbiont *Wigglesworthia glossinidia* (Chen et al. 1999).

Tsetse flies occur in a broad range of habitats distributed throughout the sub-Saharan continent [distribution maps are available on http://ergodd.zoo.ox.ac.uk/tseweb/distributions.htm, reviewed in Leak (1999) and Krafsur (2009)]. Flies of the *Morsitans* group mainly occur in the East African savannah woodlands and lowland forests and in the narrow savannah belt next to the rain forest in West Africa. *G. austeni* of the *Machadomyia* subgenus occurs discontinuously along the East African coast from Kwazulu-Natal (South Africa) to Somalia. Tsetse flies of the *Palpalis* group are predominantly found in the lowland rainforests of Western Africa, from Guinea to Cameroon. They are considered as "riverine species" due to their particular occurrence along rivers and streams. *G. tachinoides* of the *Palpalis* group has been described to advance into Eastern Africa, probably as a result of agricultural development and human population growth. Except *G. longipennis* that discontinuously occurs in very dry habitats in East Africa, tsetse flies of the *Fusca* group live in moist West African forests and savannah.

All tsetse flies are obligate blood-feeding insects, implying that both male and female flies require a blood meal every 3–4 days (Leak 1999). They rely on a pool feeding strategy which involves the laceration of the skin with their proboscis and blood ingestion from a superficial lesion. Once the skin is pierced, the proboscis is often partially withdrawn before being thrust again at a slightly different angle to probe for suitable blood vessels and to enhance the blood pool formation (Lehane 2005). The expected lifespan of the adult tsetse fly stage is approximately 3–4

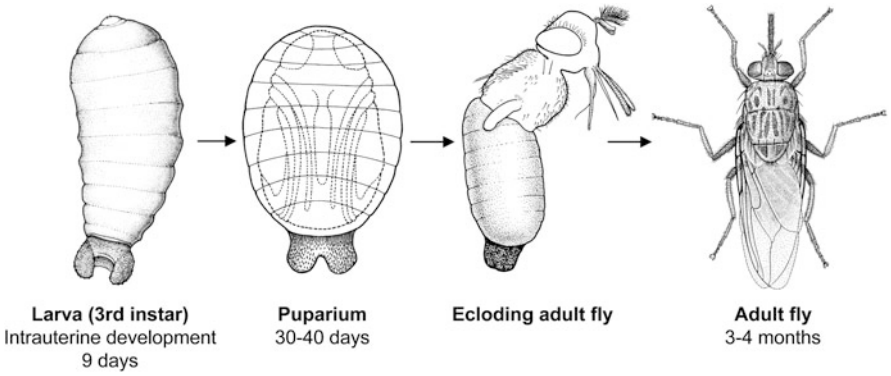

Fig. 2.2 The *Glossina* life cycle illustrating the development from an intrauterine larva into an adult fly. Compiled figures reproduced with permission of ITM

months. Female flies are viviparous (Leak 1999; Gooding and Krafsur 2005) and generally mate once or twice (Bonomi et al. 2011), storing spermatozoites in spermatheca that allows the fertilization of one egg at the time. The egg embryonates in the female's reproductive tract and larvae develop inside the insect through feeding on material ("milk") secreted by the reproductive accessory glands. This secretion is rich in lipids and contains milk gland proteins (including lipocalin-like proteins and transferrin) as well the symbiotic micro-organisms (*Sodalis glossinidius* and *Wigglesworthia glossinidia*). Specific gene silencing of several of these proteins results in a reduced fecundity or delayed gestation and pupal abnormalities (lower masses and hatch rates) (Attardo et al. 2008; Benoit et al. 2012). Approximately 9 days after fertilization, a single mature larva is deposited on the ground and moves into the loose soil, where rapid pupation takes place within about 15 min. Depending on environmental parameters such as temperature and humidity, adult flies emerge after 30–40 days (Fig. 2.2). Due to this viviparity, tsetse flies produce only a limited number of offspring, with 8–10 larvae deposited at a 9–11 days interval in a well-managed laboratory colony. In nature, reproductive efficiency is estimated to be much lower, with an average over all seasons of two offspring that manage to reproduce per fly (Gooding and Krafsur 2005).

2.2 Tsetse Flies as Invertebrate Hosts for Trypanosomes

Transmission of several pathogenic *Trypanosoma* species within the vertebrate host population primarily occurs through the bite of male and female tsetse flies. Only some specific species are important for trypanosome transmission to humans. Tsetse flies of the *Morsitans* group (*G. morsitans ssp.* and *G. pallidipes*) transmit *T. b. rhodesiense* infections in East Africa. In contrast, tsetse flies of the *Palpalis* group (*G. palpalis ssp.*, *G. fuscipes ssp.* and *G. tachinoides*) are mainly responsible for the transmission of *T. b. gambiense* infections in West and Central Africa.

Infection rates of *T. brucei ssp*., the causative agents of HAT and *nagana* in cattle, in tsetse fly salivary glands are very low and in the range of 0.1 % based on a number of studies (Otieno and Darji 1979; Morlais et al. 1998; Msangi et al. 1998). These field data support observations of low tsetse fly infection efficiencies in laboratory conditions. Collectively, a high percentage of tsetse flies is refractory to trypanosome infection. Important factors influencing this phenomenon are believed to be the immune status of the fly at the time of parasite acquisition and the induction efficiency of innate immune responses.

2.3 The Tsetse Fly Immune System

The tsetse fly is a haven for micro-organisms and its alimentary tract continuously faces challenges with various micro-organisms including commensals, symbionts and opportunistic microbes or pathogens. The recognition, elimination, control or toleration of these organisms relies on complex and tightly regulated immune responses that balance between mounting anti-microbial effectors and controlling pathogenicity. Physical barriers including the cuticle, peritrophic matrix, epithelial layers and basal membranes are the first line of defence against micro-organisms. Based on extensive research in the *Drosophila* model system, a significant innate immune system was uncovered in insects including tsetse flies (reviewed in Lehane et al. 2004; Welburn and Maudlin 1999). Similar as for vertebrates, the insect innate immune system relies on both cell- and molecule-based (humoral) mechanisms. The cellular arm involves macrophage-like cells that can phagocytose the infecting micro-organism. The molecule arm involves pattern recognition molecules that recognize pathogen-associated molecular patterns (PAMPs), antimicrobial peptides (AMPs), lectins, proteases, reactive oxygen species (ROS) and nitric oxide (NO). In the induction of innate immune responses against fungi, Gram-positive and Gram-negative bacteria and parasites, three immunity pathways can be implicated: Toll, IMD and Jak/Stat. Involvement of cell-based trypanosome clearance has not yet been documented as trypanosomes do not enter the tsetse fly hemocoel, while there are a number of reports documenting the contribution of humoral anti-parasite responses.

In immune-challenged tsetse flies, the induction of three AMPs was detectable in the hemolymph: cecropin, attacin and defensin (Boulanger et al. 2002). The diptericin AMP was found to be constitutively expressed in the fat body and gut tissue of *Glossina*, possibly reflecting the immune response against the Gram-negative endosymbionts (Hu and Aksoy 2006; Hao et al. 2001). Using RNA interference (RNAi), attacin driven through the IMD pathway was identified as an important effector in trypanosome midgut elimination (Hu and Aksoy 2006). As such, attacin expression could be a critical determinant of refractoriness/susceptibility of individual tsetse flies to trypanosome infection. The higher susceptibility of starved tsetse flies to trypanosome infection correlated to lower baseline and trypanosome-induced expression levels of the antimicrobial peptides attacin and cecropin in starved flies (Akoda et al. 2009). Furthermore, midgut extracts from

refractory flies display more erythrocyte agglutinating activity than those from susceptible ones, suggesting that lectins might be involved in parasite clearance (Maudlin and Welburn 1987; Ibrahim et al. 1984). An initial support for this hypothesis was that addition of the lectin-inhibitory sugars *N*-acetyl glucosamine and D-glucosamine to infective feeds increases midgut infection rates (Maudlin and Welburn 1987; Welburn et al. 1994). However, an alternative explanation is that glucosamine exerts a lectin-independent activity that could relate to the rate of blood digestion, parasite growth and interaction with reactive oxygen species (Peacock et al. 2006; MacLeod et al. 2007).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been suggested to play a role in the refractoriness of tsetse to trypanosome infection. ROS are oxygen-derived radical species that exert a cytotoxic activity by oxidizing lipids, proteins and nucleic acids. ROS are produced in insects by dual oxidase (DUOX), a transmembranal protein of the NADPH oxidase family. ROS can also induce NO production by nitric oxide synthase (NOS). Here, NO could represent an important innate immune effector and messenger. NO can exert a direct antimicrobial activity by causing DNA damage by nucleotide deamination and by inactivation of enzymes by S-nitrosylation. In addition, NO released in the gut can trigger AMP production in hemocytes and the fat body which could be an important aspect of inter-tissue immunological communication (Wu et al. 2012). Dietary supplementation of tsetse flies with antioxidants such as glutathione, L- and D-cysteine, *N*-acetyl-cysteine, ascorbic acid and uric acid in the infective blood meal leads to considerable increases in midgut trypanosome infections suggesting that antioxidants detoxify free radicals within the gut, thereby promoting trypanosome survival and establishment (MacLeod et al. 2007). In addition, hydrogen peroxide (H_2O_2) levels have been shown to be significantly increased in the midgut of tsetse following trypanosome challenge, suggesting that this activity might be involved in clearing trypanosomes early in the infection process (Hao et al. 2003). In tsetse flies, bacterial challenge results in a pronounced activation of NOS and release of NO in the proventriculus. However, ingestion of bloodstream trypanosome parasites only elicited an increase of ROS and feeding experiments with the NOS substrate L-arginine or the NOS inhibitor L-NAME did not modify trypanosome establishment rates in the tsetse fly midgut. However, L-NAME disturbed the procyclic midgut trypanosomes in their upstream maturation process suggesting an agonistic role of NO in this part of trypanosome development in the tsetse fly (MacLeod et al. 2007).

Comparison of trypanosome-susceptible tryptophan oxygenase-deficient (salmon eye colour mutant) versus wildtype flies, revealed a particular tsetse midgut protein rich in Glu/Pro (EP)-repeats (EP protein) to be differentially represented in the midgut proteome (Haddow et al. 2005). RNAi-mediated reduction in EP protein levels in the midgut significantly increased midgut infection rates (Haines et al. 2010). Concomitantly, starved flies that are sensitive to trypanosome infection have lower EP levels than the well-fed counterparts.

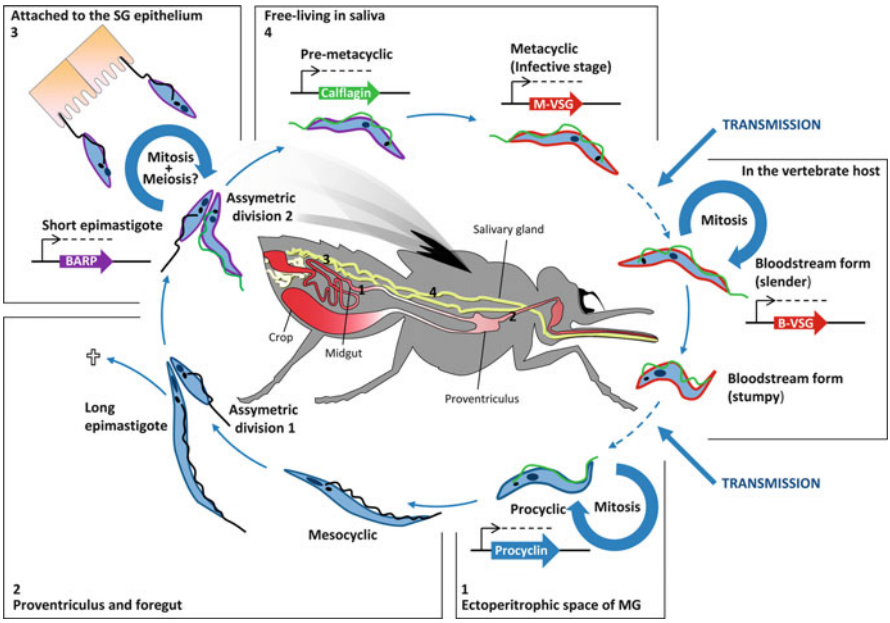

Fig. 2.3 Life cycle of the trypanosome in the vertebrate and invertebrate (tsetse fly) host: the complex cellular proliferation and differentiation events with indication of changes in surface properties, traversal of physical barriers and colonization of the various tissues. *SG* salivary gland, *MG* midgut, *B-VSG* and *M-VSG* bloodstream and metacyclic variant-specific glycoprotein, *BARP brucei* alanine rich protein. Tsetse fly figure reproduced with permission of ITM

Concerning the trypanosomal colonization of the salivary gland, no information on regulatory salivary components or immune responsiveness of this tissue is available yet.

2.4 The Trypanosome Journey in the Tsetse Fly

Tsetse flies can get infected through feeding on a parasitized host and subsequently accommodate the trypanosome during their entire adult lifespan (Fig. 2.3; reviewed in Dyer et al. 2013; Sharma et al. 2009). In the case of *T. brucei ssp*., engorged trypanosomes have to colonize the midgut and undergo differentiation into a proliferative, insect-adapted procylic form. The first 3 days after the infective blood meal appear to be the most stringent for successful infection initiation. This requires the parasites to resist the digestive enzymes and innate immune responses present in the vector and to gain access of the ectoperitrophic space by penetrating the peritrophic matrix that lines up the gut epithelium. Tsetse flies have a type II peritrophic matrix that is composed of proteins, glycoproteins and chitin microfibrils in a proteoglycan matrix and that is produced by a specialized region of the anterior midgut (cardia). After a successful colonization of the tsetse midgut,

trypanosomes need to gain salivary gland access to ensure their transmission to a new host. This does not depend on entering the hemocoel but requires a directional migration upstream the alimentary tract, accompanied by differentiation of the procyclic to a mesocyclic form in the anterior midgut. Recently, "social motility" behaviour of procyclic trypanosomes was observed in vitro revealing the recruitment of parasites into multicellular communities accompanied by their polarized movement. It remains to be elucidated whether this striking phenomenon plays a role in tissue colonization, resistance to host responses and migration in vivo (Oberholzer et al. 2010). After migration from the anterior midgut to the foregut, oesophagus and the mouthparts, *T. brucei* parasites have to enter the lumen of the salivary gland as highly motile tetraploid (4N) epimastigote forms that undergo an asymmetrical cell division, yielding a short and a long epimastigote daughter cell (2N) (Van Den Abbeele et al. 1999). This represents a severe bottleneck in the completion of the trypanosome life cycle as it is believed that only very few parasites (<5) manage to reach the salivary gland (Oberle et al. 2010). The short epimastigote further replicates and attaches to the epithelium through intricate membrane and cytoskeletal connections between the parasite flagellum and the epithelial cell membrane (Tetley and Vickerman 1985). The short epimastigote gives rise to free VSG-coated metacyclic forms by a second asymmetric division and accompanied by a complex cascade of cellular and metabolic changes (Rotureau et al. 2012). These forms are uniquely adapted to survival in the mammalian bloodstream and are injected into a new host through the bite of the infected tsetse fly. Upon transmission to the vertebrate host, trypanosomes will transform into actively proliferating (long slender) forms to allow a systemic colonization of the host. Through a mechanism of quorum-sensing, trypanosomes in the bloodstream become arrested in the G1/G0 stage, undergo morphological changes into a short stumpy form and exhibit mitochondrial biogenesis (Vassella et al. 1997). These changes are considered to pre-adapt the trypanosome to uptake, survival and subsequent differentiation in the tsetse fly. During the complex life cycle of the parasite in the insect and vertebrate host, trypanosomes undergo several metabolic changes for the energy acquisition from different available sources and modify mechanisms for the uptake of host nutrients. In the fly, trypanosomes utilize amino acids (e.g. proline) as primary energy sources, while trypanosomes in the vertebrate hosts metabolize glucose via glycolysis in a unique organelle, the glycosome (Vickerman et al. 1988; Visser et al. 1981).

2.5 Colonization of the Tsetse Fly Midgut

When *T. brucei* short stumpy forms are ingested, they end up in the hostile environment of the tsetse fly alimentary tract. The midgut and especially the proventriculus are environments with a strongly alkaline pH varying between 9.0 and 10.6 in the proventriculus and 7.9–9.5 in the midgut. In addition, the posterior part of the midgut exhibits high proteolytic activity due to the presence of trypsin, chymotrypsin and other proteases and peptidases. Midgut trypsin was suggested to

induce differentiation of short stumpy trypanosomes into procyclic forms as has been illustrated with a transgenic procycline-reporter strain of *T. brucei* (Sbicego et al. 1999). However, no in vivo evidence for this could be obtained from artificial feeding experiments with trypsin and specific inhibitors and an antibody (Welburn and Maudlin 1999). Comparison of trypanosome lines with differential ability to transform into procyclics, resulted in the identification of two homologues of the carboxylate transporter family, PAD1 and PAD2, as the transducers of the citrate and cis-aconitate differentiation trigger. PAD1 expression is readily upregulated in stumpy trypanosomes, while PAD2 is thermoregulated and responds to temperatures compatible with parasite uptake in the insect (Dean et al. 2009).

Upon differentiation of the blood stream trypanosomes into procyclic forms, the variant-specific surface glycoprotein (VSG) coat is shed and replaced by another set of GPI-anchored proteins, the procyclins. Based upon the presence of specific internal amino acid repeats, procyclins are subdivided into two classes: the EP procyclins with Glu–Pro repeats and the GPEET procyclins containing Gly–Pro–Glu–Glu–Thr repeats (reviewed in Roditi et al. 1998; Roditi and Liniger 2002). During the initial midgut colonization, both procyclins are expressed simultaneously and both are susceptible for N-terminal cleavage by proteolytic enzymes present in the tsetse midgut (Acosta-Serrano et al. 2001). Coinciding with the penetration of the peritrophic matrix, GPEET expression is repressed (Vassella et al. 2000), while EP expression persists until the epimastigote stage in the salivary glands. Major disruption of the surface architecture in trypanosomes deficient in GPI anchoring of proteins severely impaired their ability to colonize tsetse fly midguts suggesting the importance of the procyclins in midgut establishment (Guther et al. 2006; Lillico et al. 2003). EP procyclin but not GPEET procyclin deletion mutants exhibited a reduced ability to colonize the midgut (Ruepp et al. 1997). Strikingly, procyclin null mutants were still able to complete their life cycle, but it should be noted that they achieved much lower densities in the salivary gland and seemed to only weakly adhere to the gland epithelium (Vassella et al. 2009). In addition, this mutant suffered a reduced fitness as compared to wildtype parasites during co-infection experiments. A membrane-spanning surface phosphoprotein, PSSA-2, that is present in different insect trypanosome stages, plays an important role in completion of trypanosome life cycle in the tsetse fly. This was exemplified with a null-mutant that established well in the midgut but was seriously compromised in colonizing the salivary glands (Fragoso et al. 2009).

Recently, a master regulator of the parasite maturation process, RNA-binding protein 6 (RBP6), has been identified and overexpression in procyclic trypanosomes is sufficient to reproduce in vitro all developmental stages that occur in the tsetse fly up to metacyclics (Kolev et al. 2013). Although the in vivo trigger is unknown, differential parasite transcriptome analyses detected the *RBP6* induction in the proventriculus. Another set of hypothetical RNA-binding proteins, Alba proteins (Alba3/4), are likely to contribute to the negative regulation of trypanosome differentiation in the midgut. This was elucidated from cellular differentiation of cultured procyclic trypanosomes observed after RNAi and an impaired in vivo differentiation by Alba overexpression (Subota et al. 2011).

2.6 Colonization the Tsetse Fly Salivary Gland

In the final phase, short epimastigotes resulting from an asymmetric division attach to the salivary gland epithelium and transform into metacyclic trypanosomes that express metacyclic VSG (M-VSG). Attachment of the epimastigote to the epithelium of the salivary gland is achieved by the insertion of the parasite's flagellum between the microvilli of the epithelial lining. Through this interaction, the immature parasite is prevented from being ejected with the tsetse's saliva thereby ensuring the permanent infection status of the tsetse fly. Detailed ultrastructural studies describe this *T. brucei*–tsetse junctional complex as 'hemi-desmosome'-like attachment plaques adhering the flagellar outgrowths of the epimastigote parasite to the microvilli at the apical surface of the insect epithelial cells (Tetley and Vickerman 1985). Recently, epimastigotes were demonstrated to undergo two distinct modes of proliferation occurring simultaneously in the salivary glands. Rapid colonization of the glands relies on a cycle that produces two equivalent cells that are attached to the epithelium. The second mode is more frequent at later stages of infection and involves an asymmetric division that produces a pre-metacyclic daughter cell that further matures into the infective metacyclic form (Rotureau et al. 2012). The cells that result from this asymmetric division seem to pre-adapt for infection of the vertebrate host as they express calflagins, calcium-binding proteins, that are highly present on bloodstream trypanosomes but absent in epimastigotes (Rotureau et al. 2012). A protein that is specifically expressed at the surface of the epimastigote trypanosome stage in the tsetse salivary glands is the GPI-anchored *brucei* alanine-rich protein (BARP) (Urwyler et al. 2007). Although the function is not known, it is unlikely that this protein is playing an active role in the parasite attachment with the salivary gland epithelium since it is covering the whole surface of the parasite, whereas the tight trypanosome binding to the epithelial cells is restricted to only the tip of the parasite flagellum.

The epimastigote stage is also of particular interest in terms of genetic exchange between trypanosomes. Coinfection of tsetse flies with parasites that exclusively express either a green or red fluorescent protein (GFP or RFP) resulted in the observation of short epimastigote hybrids as soon as they invaded the salivary glands and before attachment to the gland epithelium. Hybrids appeared to be diploid, triploid or tetraploid (Gibson et al. 2008). Meiosis is likely part of this process as the expression of three different homologs of meiosis-specific genes has been documented to occur in the short free-living epimastigotes. However, haploid stages could not yet be found despite the presence of clearly identifiable meiotic trypanosomes during the salivary gland colonization (Peacock et al. 2011).

Upon differentiation into metacyclic forms, an M-VSG coat is expressed that is individually homogenous as each trypanosome only transcribes a single *M-VSG* gene (Tetley et al. 1987). Different from the bloodstream VSG expression, transcription occurs from a monocistronic unit where the VSG gene is not preceded by expression site-associated genes (ESAGs). In the infective trypanosome dose, up to 27 different M-VSG coats are expressed (Turner et al. 1988), enhancing the likelihood of successful re-infection of a previously infected host. Switching from

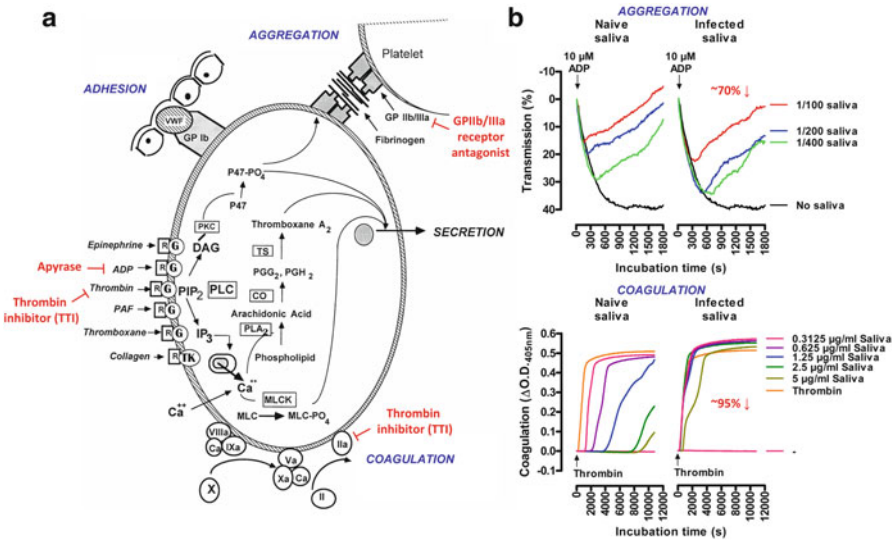

Fig. 2.4 (**a**) Overview of the anti-platelet and anti-coagulant activities documented for tsetse flies. Figure modified from (Rao 1998) (**b**) Effects of trypanosome infection on the ability of different saliva concentrations to inhibit platelet aggregation and plasma coagulation

M-VSG to bloodstream VSG (B-VSG) expression occurs within a few days following infection of a mammalian host.

2.7 Tsetse Fly Saliva to the Benefit of Trypanosome Transmission

Tsetse flies rely on a broad repertoire of physiologically active saliva components to efficiently acquire a blood meal (Fig. 2.4a). Anti-hemostatic components primarily interfere with host responses such as vasoconstriction (Elsen et al. 1990; Leak 1999), primary hemostasis through the adherence and aggregation of thrombocytes (Despommier and Hotez 2006; Gooding and Krafsur 2005) and a secondary hemostatic cascade mainly relying on serine proteases such as thrombin (Chen et al. 1999; Krafsur 2009). A component that underwent thorough functional analysis is the tsetse thrombin inhibitor (TTI, 3.5 kDa), characterized as an extremely potent inhibitor of thrombinase activity (Cappello et al. 1996). Additionally, an inhibitor (11–13 kDa) of thrombin's serine proteolytic and esterolytic properties has been detected (Parker and Mant 1979) and an apyrase with fibrinogen receptor (GPIIb/IIIa) antagonistic properties has been shown to inhibit platelet aggregation and to even cause thrombocyte disaggregation (Mant and Parker 1981; Caljon et al. 2010). These components are likely to play a crucial role in the blood-feeding process by ensuring persistent blood flux at the feeding site and preserving mouthpart, crop and digestive function by avoiding clot formation.

Comparison of these two major anti-haemostatic activities, i.e. anti-platelet aggregation and anti-coagulation activity, in infected versus non-infected tsetse flies, revealed a significant suppression as a result of the trypanosome-infection status (Van Den Abbeele et al. 2010). This effect was mainly related to the parasite-induced reduction in salivary gland gene transcription, resulting in a strong decrease in protein content and related biological activities. Apyrase activity that relates to inhibition of platelet aggregation was reduced by approximately 70 %. Strikingly, the anti-thrombin activity and inhibition of thrombin-induced coagulation was even more severely hampered as a result of the trypanosome infection. While naive tsetse saliva strongly inhibited human thrombin activity and thrombin-induced plasma coagulation, activity in saliva from *T. brucei*-infected flies was reduced to less than 5 %. As a result, salivary gland *Trypanosoma brucei*-infected flies display a significantly prolonged overall feeding time due to an inefficient probing phase, thereby enhancing the likelihood of interrupted feeding and infecting multiple hosts during a single blood meal cycle.

By performing experimental infections using purified *T. brucei* parasites and harvested tsetse fly saliva, another important contribution of saliva to trypanosome transmission was demonstrated. Co-injection of parasites with salivary compounds can significantly enhance the early onset of a trypanosome infection (Caljon et al. 2006a). This effect was correlated with a reduced inflammatory gene transcription (IL-6 and IL-12 mRNA levels) at the skin site where the infection was initiated. Although it was not formally demonstrated, this immune modulatory effect could have significant effects on the minimal infective dose and thereby contribute to high infection success rates upon exposure to a trypanosome-infected fly in the field. Indeed, it was experimentally demonstrated in mice that a single fly can infect up to seven hosts in one blood-feeding cycle (Jenni et al. 1980). By analysing the immunogenicity profile, it became clear that tsetse fly saliva has a strong propensity to induce Th2 cellular responses and to moreover display the ability to suppress B and T cell responses against heterologous antigen (Caljon et al. 2006b). Nevertheless, the tsetse fly–host interaction results in the elicitation of saliva-specific IgG responses that are mainly reactive against the highly abundant 43–45 kDa Tsal family of nucleic acid-binding proteins (Caljon et al. 2006b, 2012). Also IgE responses could be detected, mainly against TAg5 that we have documented to be a potent allergen with high similarity to vespid toxin allergens and the propensity to induce anaphylactic reactions (Caljon et al. 2009). Interestingly, repeated exposure to tsetse fly bites and acquired immunity to tsetse fly saliva did not inhibit infection rates but rather enhanced the parasitemia onset in mice (Caljon et al. 2006a).

2.8 The Tsetse–Trypanosome–Symbiont Tripartite

Next to being a vector for trypanosomes, tsetse flies harbour several endosymbiotic organisms (reviewed in Aksoy 2000, 2003; Rio et al. 2004). Two Gram-negative symbionts, found in all tsetse flies, are members of the *Enterobacteriaceae*: the anciently associated obligate mutualist [primary (P) symbiont] *Wigglesworthia*

glossinidia and the more recently established commensal [secondary (S)] *Sodalis glossinidius*. A third facultative endosymbiont that occurs in some natural tsetse populations is an α-Proteobacterium (Gram-negative) of the genus *Wolbachia* (O'Neill et al. 1993). Given the viviparous reproductive biology of *Glossina* species, all three endosymbionts are maternally transmitted. *Wigglesworthia* and *Sodalis* are transmitted to the intrauterine developing larvae, while *Wolbachia* parasitizes developing oocytes in the ovary.

Wigglesworthia is found intracellularly within an organ immediately adjacent to tsetse's midgut called the bacteriome, as well as extracellularly within maternal milk gland secretions (Attardo et al. 2008). This enterobacterium is believed to supplement tsetse's nutritionally restricted blood diet with vitamins (Akman et al. 2002), and without *Wigglesworthia*, females are reproductively sterile (Pais et al. 2008). Recent studies also indicate an immunologic role for *Wigglesworthia* in tsetse. It was shown that when *Wigglesworthia* is absent from tsetse during the maturation of immature larval stages, subsequent adults are characterized by an underdeveloped cellular immune system and exhibit unusual susceptibility for trypanosome infections (Weiss et al. 2011).

S. glossinidius displays a wide tissue tropism and is harboured both intra- and extracellularly in the tsetse fly midgut, muscle, fat body, milk glands and salivary glands (Cheng and Aksoy 1999). In contrast to *Wigglesworthia*, the functional contributions for *Sodalis* are less well known. *Sodalis* appears to be present in all lab-colony flies, whereas a varying prevalence (0–75 %) has been observed in wild tsetse flies (Geiger et al. 2009; Farikou et al. 2010). However, experimental elimination of *Sodalis* resulted in a reduction of host longevity (Dale and Welburn 2001) suggesting a mutualistic function for this symbiosis in tsetse. *Sodalis* represents a bacterium in the early/intermediate state of the transition towards symbiosis evidenced by the absence of phylogenetic congruence in the evolution of *Sodalis* and tsetse (Chen et al. 1999) and a significantly reduced coding capacity of its genome (Toh et al. 2006). Moreover, *Sodalis* still has the capacity to grow in vitro in cell-free media (Welburn et al. 1987), supporting a recent symbiotic interaction. Although its actual role in the ability of tsetse flies to acquire and transmit the parasite still remains controversial, *Sodalis* infections have been suggested to be associated with an enhanced susceptibility of the tsetse host towards trypanosomes, an effect that varies with the genotype of the symbiont (Dale and Welburn 2001; Farikou et al. 2010; Geiger et al. 2007).

Wolbachia infects tsetse germline tissues (Cheng et al. 2000) and is involved in cytoplasmic incompatibility, a phenomenon of early cell death during embryogenesis due to imprinting differences between the oocyte and the sperm cell. Female *Wolbachia*-infected flies have a reproductive advantage as they can successfully produce progeny with both a naive and infected individual.

Maintaining these long-term symbiotic relationships requires both the host and the symbiont to constrain adaptive interactions. Indeed, endosymbionts are maintained within their insect hosts in spite of the broad spectrum of defence mechanisms against microbial intruders. In tsetse, both host- and symbiont-mediated adaptations have contributed to tolerance to microbial fauna. The *S. glossinidius*

PhoP–PhoQ system, which controls various virulence mechanisms including resistance to antimicrobial peptides (AMPs) through lipopolysaccharide (LPS) modifications, has undergone sensory adaptations allowing *S. glossinidius* to constitutively express genes that facilitate resistance to host-derived AMPs (Pontes et al. 2011).

Homeostasis between *Wigglesworthia* and the tsetse fly is maintained through the peptidoglycan recognition protein LB (PGRP-LB), expressed at high levels in *Wigglesworthia*-associated tissues. Tsetse PGRP-LB scavenges immunogenic peptidoglycan released by *Wigglesworthia* thereby protecting the symbiont from host damage by preventing immune activation. Additionally, increased PGRP-LB levels correlate with refractoriness to trypanosome infection, suggesting a dual role for PGRP-LB in tsetse flies by mediating the immune response towards symbionts on the one hand and enhancing parasite resistance on the other hand (Wang et al. 2009; Weiss et al. 2011).

2.9 Going Forward

Although some research on tsetse fly immune responses and biotic and abiotic factors that affect trypanosome development has been documented in literature, the molecular basis of the trypanosome life cycle in the tsetse fly still represents a genuine black box. The successfulness of trypanosome development in its insect vector is the outcome of the parasite molecular communication and adaptive capabilities to the complex and variable tsetse fly micro-environments. Several trypanosome- and tsetse fly-derived factors and molecular events are suggested to control trypanosome infection within the tsetse fly. Survival in and colonization of the tsetse fly midgut and proventriculus are the first crucial bottlenecks for the trypanosome to overcome. Tsetse fly-derived factors that have been demonstrated to be involved during this part of parasite development are the immune deficiency (IMD) pathway-regulated antimicrobial peptide attacin (Hu and Aksoy 2006) and an immune-responsive midgut EP-protein (Haines et al. 2010). Oxidative stress has also been suggested to play an important role in trypanosome establishment and development (MacLeod et al. 2007). However, many questions regarding the molecular mechanisms underlying the innate immune response of the tsetse fly towards trypanosomes remain. Furthermore, the alimentary tract of the tsetse fly represents a site of multi-taxon interactions that includes not only the arthropod vector and the pathogen but also the gut microbiome. Recent identification of regulators [RBP6 and Alba3/4 proteins (Subota et al. 2011; Kolev et al. 2013)] of insect stage parasite differentiation might pave the way to the discovery of the specific triggers which could eventually contribute to new modes of intervention. Besides trypanosome cell biological research, this requires intensive research upon the tsetse fly vector, which is currently hampered by (1) the viviparous reproductive features of tsetse flies that preclude large mutagenesis screens and transgenic approaches similar to those applied in *Drosophila* and mosquito functional research, (2) the efficiency of protein silencing by RNA interference is target-

dependent and only moderate (40–75 %) in tissues such as the salivary glands and (3) no in vitro tsetse insect cell culture or tissue explants techniques have been established. The only way to obtain novel insights into this part of the parasite life cycle is to profoundly study the tsetse fly environment and to experimentally explore trypanosome development in vivo, i.e. within the tsetse fly. The recent tsetse fly genome sequencing under impulse of the WHO-TDR International *Glossina* Genome Initiative (IGGI) will further advance this research on this under studied disease vector.

Genetically modified disease vectors that are rendered resistant (refractory) to pathogen transmission could provide unique tools for developing or complementing new and existing control strategies. One of these strategies focuses on reducing vector competence by utilizing genetically modified insect symbionts to express proteins that interfere with pathogen transmission, an approach also known as paratransgenesis. A paratransgenic approach in tsetse flies is currently of high interest, since tsetse flies are not amenable to germ-line transformation. This technology to render tsetse flies resistant to trypanosome infection would strongly complement the current Sterile Insect Technique (SIT) that has been proven effective in eradicating tsetse in isolated pockets. Indeed, as SIT involves the massive release of sterilized males, the accompanied temporary increase in the number of potential vectors for trypanosomes could potentially contribute to an increase in disease transmission. Therefore, the release of trypanosome-refractory tsetse flies would render this approach less controversial especially in regions where HAT occurs (Van Den Abbeele et al. 2013).

Therefore, a new line of research in the fight against African trypanosomiasis is focusing on the use of *Sodalis* as a delivery system for anti-trypanosomal components to control parasite development in the fly (Rotureau et al. 2012). *S. glossinidius* is ideally suited as a paratransgenic platform organism since it (1) resides in different tsetse tissues (midgut, hemolymph and salivary glands) that are in close proximity to pathogenic trypanosomes; (2) can be cultured and genetically modified in vitro; (3) can be re-introduced into the tsetse; (4) is maternally transmitted to the offspring and (5) is restricted to the tsetse host niche ensuring that this symbiont is a safe candidate for use in the paratransgenic strategy.

Very recently, an expression system that allows *Sodalis* to constitutively express and extracellularly release functional nanobodies was developed (De Vooght et al. 2012). These recombinant *S. glossinidius* strains were not affected in their growth, suggesting that they may be competitive with endogenous microbiota in the tsetse fly. These results are an important first step in the development of a *Sodalis* expression system that will allow the delivery of nanobodies to study or interfere with the complex trypanosome life cycle inside the tsetse fly.

References

Acosta-Serrano A, Vassella E, Liniger M, Kunz Renggli C, Brun R, Roditi I, Englund PT (2001) The surface coat of procyclic *Trypanosoma brucei*: programmed expression and proteolytic cleavage of procyclin in the tsetse fly. Proc Natl Acad Sci U S A 98(4):1513–1518

Akman L, Yamashita A, Watanabe H, Oshima K, Shiba T, Hattori M, Aksoy S (2002) Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglesworthia glossinidia*. Nat Genet 32(3):402–407

Akoda K, Van den Bossche P, Marcotty T, Kubi C, Coosemans M, De Deken R, Van den Abbeele J (2009) Nutritional stress affects the tsetse fly's immune gene expression. Med Vet Entomol 23(3):195–201

Aksoy S (2000) Tsetse–A haven for microorganisms. Parasitol Today 16(3):114–118

Aksoy S (2003) Control of tsetse flies and trypanosomes using molecular genetics. Vet Parasitol 115(2):125–145

Attardo GM, Lohs C, Heddi A, Alam UH, Yildirim S, Aksoy S (2008) Analysis of milk gland structure and function in *Glossina morsitans*: milk protein production, symbiont populations and fecundity. J Insect Physiol 54(8):1236–1242

Benoit JB, Attardo GM, Michalkova V, Takac P, Bohova J, Aksoy S (2012) Sphingomyelinase activity in mother's milk is essential for juvenile development: a case from lactating tsetse flies. Biol Reprod 87(1):17, 11–10

Bonomi A, Bassetti F, Gabrieli P, Beadell J, Falchetto M, Scolari F, Gomulski LM, Regazzini E, Ouma JO, Caccone A, Okedi LM, Attardo GM, Guglielmino CR, Aksoy S, Malacrida AR (2011) Polyandry is a common event in wild populations of the Tsetse fly *Glossina fuscipes fuscipes* and may impact population reduction measures. PLoS Negl Trop Dis 5(6):e1190

Boulanger N, Brun R, Ehret-Sabatier L, Kunz C, Bulet P (2002) Immunopeptides in the defense reactions of *Glossina morsitans* to bacterial and *Trypanosoma brucei brucei* infections. Insect Biochem Mol Biol 32(4):369–375

Caljon G, Van Den Abbeele J, Stijlemans B, Coosemans M, De Baetselier P, Magez S (2006a) Tsetse fly saliva accelerates the onset of *Trypanosoma brucei* infection in a mouse model associated with a reduced host inflammatory response. Infect Immun 74(11):6324–6330

Caljon G, Van Den Abbeele J, Sternberg JM, Coosemans M, De Baetselier P, Magez S (2006b) Tsetse fly saliva biases the immune response to Th2 and induces anti-vector antibodies that are a useful tool for exposure assessment. Int J Parasitol 36(9):1025–1035

Caljon G, Broos K, De Goeyse I, De Ridder K, Sternberg JM, Coosemans M, De Baetselier P, Guisez Y, Den Abbeele JV (2009) Identification of a functional Antigen5-related allergen in the saliva of a blood feeding insect, the tsetse fly. Insect Biochem Mol Biol 39(5–6):332–341

Caljon G, De Ridder K, De Baetselier P, Coosemans M, Van Den Abbeele J (2010) Identification of a tsetse fly salivary protein with dual inhibitory action on human platelet aggregation. PLoS One 5(3):e9671

Caljon G, De Ridder K, Stijlemans B, Coosemans M, Magez S, De Baetselier P, Van Den Abbeele J (2012) Tsetse salivary gland proteins 1 and 2 are high affinity nucleic acid binding proteins with residual nuclease activity. PLoS One 7(10):e47233

Cappello M, Bergum PW, Vlasuk GP, Furmidge BA, Pritchard DI, Aksoy S (1996) Isolation and characterization of the tsetse thrombin inhibitor: a potent antithrombotic peptide from the saliva of *Glossina morsitans morsitans*. Am J Trop Med Hyg 54(5):475–480

Chen X, Li S, Aksoy S (1999) Concordant evolution of a symbiont with its host insect species: molecular phylogeny of genus *Glossina* and its bacteriome-associated endosymbiont, *Wigglesworthia glossinidia*. J Mol Evol 48(1):49–58

Cheng Q, Aksoy S (1999) Tissue tropism, transmission and expression of foreign genes *in vivo* in midgut symbionts of tsetse flies. Insect Mol Biol 8(1):125–132

Cheng Q, Ruel TD, Zhou W, Moloo SK, Majiwa P, O'Neill SL, Aksoy S (2000) Tissue distribution and prevalence of *Wolbachia* infections in tsetse flies, *Glossina* spp. Med Vet Entomol 14 (1):44–50

Dale C, Welburn SC (2001) The endosymbionts of tsetse flies: manipulating host-parasite interactions. Int J Parasitol 31(5–6):628–631

De Vooght L, Caljon G, Stijlemans B, De Baetselier P, Coosemans M, Van den Abbeele J (2012) Expression and extracellular release of a functional anti-trypanosome Nanobody(R) in *Sodalis glossinidius*, a bacterial symbiont of the tsetse fly. Microb Cell Fact 11:23

Dean S, Marchetti R, Kirk K, Matthews KR (2009) A surface transporter family conveys the trypanosome differentiation signal. Nature 459(7244):213–217

Despommier G, Hotez K (2006) Parasitic diseases, 5th edn. Apple Trees Productions, New York, NY

Dyer NA, Rose C, Ejeh NO, Acosta-Serrano A (2013) Flying tryps: survival and maturation of trypanosomes in tsetse flies. Trends Parasitol 29(4):188–196

Elsen P, Amoudi MA, Leclercq M (1990) First record of *Glossina fuscipes fuscipes* Newstead, 1910 and *Glossina morsitans submorsitans* Newstead, 1910 in southwestern Saudi Arabia. Ann Soc Belg Med Trop 70(4):281–287

Farikou O, Njiokou F, Mbida Mbida JA, Njitchouang GR, Djeunga HN, Asonganyi T, Simarro PP, Cuny G, Geiger A (2010) Tripartite interactions between tsetse flies, *Sodalis glossinidius* and trypanosomes—an epidemiological approach in two historical human African trypanosomiasis foci in Cameroon. Infect Genet Evol 10(1):115–121

Fragoso CM, Schumann Burkard G, Oberle M, Renggli CK, Hilzinger K, Roditi I (2009) PSSA-2, a membrane-spanning phosphoprotein of *Trypanosoma brucei*, is required for efficient maturation of infection. PLoS One 4(9):e7074

Geiger A, Ravel S, Mateille T, Janelle J, Patrel D, Cuny G, Frutos R (2007) Vector competence of *Glossina palpalis gambiensis* for *Trypanosoma brucei* s.l. and genetic diversity of the symbiont *Sodalis glossinidius*. Mol Biol Evol 24(1):102–109

Geiger A, Fardeau ML, Grebaut P, Vatunga G, Josenando T, Herder S, Cuny G, Truc P, Ollivier B (2009) First isolation of *Enterobacter*, *Enterococcus*, and *Acinetobacter* spp. as inhabitants of the tsetse fly (*Glossina palpalis palpalis*) midgut. Infect Genet Evol 9(6):1364–1370

Gibson W, Peacock L, Ferris V, Williams K, Bailey M (2008) The use of yellow fluorescent hybrids to indicate mating in *Trypanosoma brucei*. Parasit Vectors 1(1):4

Gooding RH, Krafsur ES (2005) Tsetse genetics: contributions to biology, systematics, and control of tsetse flies. Annu Rev Entomol 50:101–123

Guther ML, Lee S, Tetley L, Acosta-Serrano A, Ferguson MA (2006) GPI-anchored proteins and free GPI glycolipids of procyclic form *Trypanosoma brucei* are nonessential for growth, are required for colonization of the tsetse fly, and are not the only components of the surface coat. Mol Biol Cell 17(12):5265–5274

Haddow JD, Haines LR, Gooding RH, Olafson RW, Pearson TW (2005) Identification of midgut proteins that are differentially expressed in trypanosome-susceptible and normal tsetse flies (*Glossina morsitans morsitans*). Insect Biochem Mol Biol 35(5):425–433

Haines LR, Lehane SM, Pearson TW, Lehane MJ (2010) Tsetse EP protein protects the fly midgut from trypanosome establishment. PLoS Pathog 6(3):e1000793

Hao Z, Kasumba I, Lehane MJ, Gibson WC, Kwon J, Aksoy S (2001) Tsetse immune responses and trypanosome transmission: implications for the development of tsetse-based strategies to reduce trypanosomiasis. Proc Natl Acad Sci U S A 98(22):12648–12653

Hao Z, Kasumba I, Aksoy S (2003) Proventriculus (cardia) plays a crucial role in immunity in tsetse fly (Diptera: Glossinidiae). Insect Biochem Mol Biol 33(11):1155–1164

Hu C, Aksoy S (2006) Innate immune responses regulate trypanosome parasite infection of the tsetse fly *Glossina morsitans morsitans*. Mol Microbiol 60(5):1194–1204

Ibrahim EA, Ingram GA, Molyneux DH (1984) Haemagglutinins and parasite agglutinins in haemolymph and gut of *Glossina*. Tropenmed Parasitol 35(3):151–156

Jenni L, Molyneux DH, Livesey JL, Galun R (1980) Feeding behaviour of tsetse flies infected with salivarian trypanosomes. Nature 283(5745):383–385

Kolev NG, Ramey-Butler K, Cross GA, Ullu E, Tschudi C (2013) Developmental progression to infectivity in *Trypanosoma brucei* triggered by an RNA-binding protein. Science 338 (6112):1352–1353

Krafsur ES (2009) Tsetse flies: genetics, evolution, and role as vectors. Infect Genet Evol 9 (1):124–141

Leak SGA (1999) Tsetse biology and ecology: their role in the epidemiology and control of trypanosomosis. Cabi, Wallingford, UK

Lehane MJ (2005) The biology of blood-sucking in insects. Cambridge University Press, Cambridge

Lehane MJ, Aksoy S, Levashina E (2004) Immune responses and parasite transmission in blood-feeding insects. Trends Parasitol 20(9):433–439

Lillico S, Field MC, Blundell P, Coombs GH, Mottram JC (2003) Essential roles for GPI-anchored proteins in African trypanosomes revealed using mutants deficient in GPI8. Mol Biol Cell 14 (3):1182–1194

MacLeod ET, Maudlin I, Darby AC, Welburn SC (2007) Antioxidants promote establishment of trypanosome infections in tsetse. Parasitology 134(6):827–831

Mant MJ, Parker KR (1981) Two platelet aggregation inhibitors in tsetse (*Glossina*) saliva with studies of roles of thrombin and citrate in *in vitro* platelet aggregation. Br J Haematol 48 (4):601–608

Maudlin I, Welburn SC (1987) Lectin mediated establishment of midgut infections of *Trypanosoma congolense* and *Trypanosoma brucei* in *Glossina morsitans*. Trop Med Parasitol 38(3):167–170

Morlais I, Grebaut P, Bodo JM, Djoha S, Cuny G (1998) Characterization of trypanosome infections by polymerase chain reaction (PCR) amplification in wild tsetse flies in Cameroon. Parasitology 116(6):547–554

Msangi AR, Whitaker CJ, Lehane MJ (1998) Factors influencing the prevalence of trypanosome infection of *Glossina pallidipes* on the Ruvu flood plain of Eastern Tanzania. Acta Trop 70 (2):143–155

Oberholzer M, Lopez MA, McLelland BT, Hill KL (2010) Social motility in african trypanosomes. PLoS Pathog 6(1):e1000739

Oberle M, Balmer O, Brun R, Roditi I (2010) Bottlenecks and the maintenance of minor genotypes during the life cycle of *Trypanosoma brucei*. PLoS Pathog 6(7):e1001023

O'Neill SL, Gooding RH, Aksoy S (1993) Phylogenetically distant symbiotic microorganisms reside in *Glossina* midgut and ovary tissues. Med Vet Entomol 7(4):377–383

Otieno LH, Darji N (1979) The abundance of pathogenic African trypanosomes in the salivary secretions of wild *Glossina pallidipes*. Ann Trop Med Parasitol 73(6):583–588

Pais R, Lohs C, Wu Y, Wang J, Aksoy S (2008) The obligate mutualist *Wigglesworthia glossinidia* influences reproduction, digestion, and immunity processes of its host, the tsetse fly. Appl Environ Microbiol 74(19):5965–5974

Parker KR, Mant MJ (1979) Effects of tsetse (*Glossina morsitans morsitans* Westw.) (Diptera: Glossinidae) salivary gland homogenate on coagulation and fibrinolysis. Thromb Haemost 42 (2):743–751

Peacock L, Ferris V, Bailey M, Gibson W (2006) Multiple effects of the lectin-inhibitory sugars D-glucosamine and N-acetyl-glucosamine on tsetse-trypanosome interactions. Parasitology 132(5):651–658

Peacock L, Ferris V, Sharma R, Sunter J, Bailey M, Carrington M, Gibson W (2011) Identification of the meiotic life cycle stage of *Trypanosoma brucei* in the tsetse fly. Proc Natl Acad Sci U S A 108(9):3671–3676

Pontes MH, Smith KL, De Vooght L, Van Den Abbeele J, Dale C (2011) Attenuation of the sensing capabilities of PhoQ in transition to obligate insect-bacterial association. PLoS Genet 7 (11):e1002349

Rao AK (1998) Congenital disorders of platelet function: disorders of signal transduction and secretion. Am J Med Sci 316(2):69–76

Rio RV, Hu Y, Aksoy S (2004) Strategies of the home-team: symbioses exploited for vector-borne disease control. Trends Microbiol 12(7):325–336

Roditi I, Liniger M (2002) Dressed for success: the surface coats of insect-borne protozoan parasites. Trends Microbiol 10(3):128–134

Roditi I, Furger A, Ruepp S, Schurch N, Butikofer P (1998) Unravelling the procyclin coat of *Trypanosoma brucei*. Mol Biochem Parasitol 91(1):117–130

Rotureau B, Subota I, Buisson J, Bastin P (2012) A new asymmetric division contributes to the continuous production of infective trypanosomes in the tsetse fly. Development 139 (10):1842–1850

Ruepp S, Furger A, Kurath U, Renggli CK, Hemphill A, Brun R, Roditi I (1997) Survival of *Trypanosoma brucei* in the tsetse fly is enhanced by the expression of specific forms of procyclin. J Cell Biol 137(6):1369–1379

Sbicego S, Vassella E, Kurath U, Blum B, Roditi I (1999) The use of transgenic *Trypanosoma brucei* to identify compounds inducing the differentiation of bloodstream forms to procyclic forms. Mol Biochem Parasitol 104(2):311–322

Sharma R, Gluenz E, Peacock L, Gibson W, Gull K, Carrington M (2009) The heart of darkness: growth and form of *Trypanosoma brucei* in the tsetse fly. Trends Parasitol 25(11):517–524

Subota I, Rotureau B, Blisnick T, Ngwabyt S, Durand-Dubief M, Engstler M, Bastin P (2011) ALBA proteins are stage regulated during trypanosome development in the tsetse fly and participate in differentiation. Mol Biol Cell 22(22):4205–4219

Tetley L, Vickerman K (1985) Differentiation in *Trypanosoma brucei*: host-parasite cell junctions and their persistence during acquisition of the variable antigen coat. J Cell Sci 74:1–19

Tetley L, Turner CM, Barry JD, Crowe JS, Vickerman K (1987) Onset of expression of the variant surface glycoproteins of *Trypanosoma brucei* in the tsetse fly studied using immunoelectron microscopy. J Cell Sci 87(2):363–372

Toh H, Weiss BL, Perkin SA, Yamashita A, Oshima K, Hattori M, Aksoy S (2006) Massive genome erosion and functional adaptations provide insights into the symbiotic lifestyle of *Sodalis glossinidius* in the tsetse host. Genome Res 16(2):149–156

Turner CM, Barry JD, Maudlin I, Vickerman K (1988) An estimate of the size of the metacyclic variable antigen repertoire of *Trypanosoma brucei rhodesiense*. Parasitology 97(2):269–276

Urwyler S, Studer E, Renggli CK, Roditi I (2007) A family of stage-specific alanine-rich proteins on the surface of epimastigote forms of *Trypanosoma brucei*. Mol Microbiol 63(1):218–228

Van Den Abbeele J, Claes Y, van Bockstaele D, Le Ray D, Coosemans M (1999) *Trypanosoma brucei* spp. development in the tsetse fly: characterization of the post-mesocyclic stages in the foregut and proboscis. Parasitology 118(5):469–478

Van Den Abbeele J, Caljon G, De Ridder K, De Baetselier P, Coosemans M (2010) *Trypanosoma brucei* modifies the tsetse salivary composition, altering the fly feeding behavior that favors parasite transmission. PLoS Pathog 6(6):e1000926

Van Den Abbeele J, Bourtzis K, Weiss B, Cordon-Rosales C, Miller W, Abd-Alla AM, Parker A (2013) Enhancing tsetse fly refractoriness to trypanosome infection—a new IAEA coordinated research project. J Invertebr Pathol 112(Suppl):S142–S147

Vassella E, Reuner B, Yutzy B, Boshart M (1997) Differentiation of African trypanosomes is controlled by a density sensing mechanism which signals cell cycle arrest via the cAMP pathway. J Cell Sci 110(21):2661–2671

Vassella E, Den Abbeele JV, Butikofer P, Renggli CK, Furger A, Brun R, Roditi I (2000) A major surface glycoprotein of *Trypanosoma brucei* is expressed transiently during development and can be regulated post-transcriptionally by glycerol or hypoxia. Genes Dev 14(5):615–626

Vassella E, Oberle M, Urwyler S, Renggli CK, Studer E, Hemphill A, Fragoso C, Butikofer P, Brun R, Roditi I (2009) Major surface glycoproteins of insect forms of *Trypanosoma brucei* are not essential for cyclical transmission by tsetse. PLoS One 4(2):e4493

Vickerman K, Tetley L, Hendry KA, Turner CM (1988) Biology of African trypanosomes in the tsetse fly. Biol Cell 64(2):109–119

Visser N, Opperdoes FR, Borst P (1981) Subcellular compartmentation of glycolytic intermediates in *Trypanosoma brucei*. Eur J Biochem 118(3):521–526

Wang J, Wu Y, Yang G, Aksoy S (2009) Interactions between mutualist *Wigglesworthia* and tsetse peptidoglycan recognition protein (PGRP-LB) influence trypanosome transmission. Proc Natl Acad Sci U S A 106(29):12133–12138

Weiss BL, Wang J, Aksoy S (2011) Tsetse immune system maturation requires the presence of obligate symbionts in larvae. PLoS Biol 9(5):e1000619

Welburn SC, Maudlin I (1999) Tsetse-trypanosome interactions: rites of passage. Parasitol Today 15(10):399–403

Welburn SC, Maudlin I, Ellis DS (1987) *In vitro* cultivation of rickettsia-like-organisms from *Glossina* spp. Ann Trop Med Parasitol 81(3):331–335

Welburn SC, Maudlin I, Molyneux DH (1994) Midgut lectin activity and sugar specificity in teneral and fed tsetse. Med Vet Entomol 8(1):81–87

Wu SC, Liao CW, Pan RL, Juang JL (2012) Infection-induced intestinal oxidative stress triggers organ-to-organ immunological communication in *Drosophila*. Cell Host Microbe 11 (4):410–417

Withstanding the Challenges of Host Immunity: Antigenic Variation and the Trypanosome Surface Coat

3

James Peter John Hall and Lindsey Plenderleith

Abstract

Prolonged survival in the face of host immunity has been a major force shaping the biology and evolution of the African trypanosomes, and nowhere are the effects of this force more apparent than in the antigenic variation of the trypanosome variant surface glycoprotein (VSG) coat. The coat protects the trypanosome within it from immune effectors, and spontaneous and stochastic events occurring at the molecular level cause individual trypanosomes to change the VSG variant they are expressing. The consequence of this switching at the population level is a diverse population that can pre-empt the specific immune responses that arise against VSG. The template for changes to VSG is an extensive archive of silent *VSG* genes and pseudogenes. *VSG* from the archive are activated not only as full-length genes but also through the combination of segments to form mosaic *VSG* genes, a process that augments the potential for antigenic variation by introducing combinatorial variation and allowing *VSG* pseudogenes to be used. The main part of the archive occupies subtelomeres and so is itself prone to mutation and rapid evolution, which are important features when superinfection or reinfection of partially immune hosts is necessary. The antigenic variation 'diversity phenotype' is thus a multifaceted one, enlisting and coordinating fundamental mechanisms of cell biology to bring about a process

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that unfolds across populations, thereby facilitating the success of the African trypanosomes.

3.1 Introduction

The success of African salivarian trypanosomes depends on their survival in mammalian blood. It is only blood-borne trypanosomes that can be transmitted by biting insects, and so traits prolonging persistence in this habitat are likely to be beneficial, because they increase opportunities for transmission. Blood is chemically stable and rich in nutrients but is intensely hostile to foreign bodies, thanks to a sophisticated immune system that deploys both immediate, non-specific 'innate' responses and specific 'adaptive' responses to efficiently eradicate pathogens. Unusually amongst chronic pathogens, African trypanosomes do not invade host cells to escape from immunity. Instead, they are extracellular during the mammalian phase of their life cycle. Protecting each parasite from host immunity is a dense, uniform, glycoprotein 'coat' that covers its entire surface, made from approximately six million dimers (Jackson et al. 1985) of the major African trypanosome variable antigen: variant surface glycoprotein (VSG).

The VSG surface coat is critical for a trypanosome to survive in the blood—in its absence or dysfunction, parasites are killed by factors present in naive serum (Ferrante and Allison 1983; Mosser and Roberts 1982). An intact VSG coat obstructs immune effectors, preventing them from accessing the cell membrane or invariant surface antigens (Overath et al. 1994), and in doing so it forms the most immediate interface between parasite and host during infection. Although the coat shields the parasite surface, VSG itself is highly immunogenic, and adaptive responses against the antigen are rapidly mounted by the host (Black et al. 2010). A mechanism to withstand the challenges of adaptive immunity operates at the population level: a single host can maintain a population of millions, if not billions, of trypanosomes (Barry 1986), and as an individual trypanosome multiplies, it can switch to express a different VSG. The diversity that unfolds across the burgeoning population pre-empts specific immune responses, making the infection resilient to eradication. As different VSG variants are targeted and neutralised by immunity, the population is replenished by the proliferation of the survivors, which undergo further VSG switches and are further shaped and selected by immunity to yield the patterns of antigenic variation of which the African trypanosomes are a paradigm. The VSG coat—and its variation—represents a remarkable example of biological diversity that harnesses genome biology, cellular processes and population dynamics to enable African trypanosomes to survive despite a precarious life cycle that is balanced between a broad range of host mammals and the insects that feed on them.

Compared with general mutation of antigens caused by background cellular processes, true antigenic variation is a system that has evolved under pressure from adaptive immunity to present an ever-changing 'diversity phenotype'. With the African trypanosomes, we can clearly infer that such selection has occurred by

identifying distinct processes and resources specifically associated with introducing dramatic change in the expressed antigen: (1) exclusive, tightly regulated expression of VSG from a dedicated locus; (2) mechanisms to vary the expressed *VSG* gene and (3) an archive of silent *VSG* genes that can be utilised as source material for this variation. Importantly, the antigenic variation diversity phenotype is a programme emerging from a trypanosome *genome*, one that becomes manifest in the behaviour of a population of clonally related parasites. Selection favours those genomes that maximise their overall transmission and propagation, and strategies that might appear counterintuitive or hugely wasteful in terms of the death of individual parasites can be highly effective at maximising the transmission, and hence success, of the genomes that give rise to those strategies (Reece et al. 2011). Similar examples abound across biology (West et al. 2006), one being the transmission of cellular slime moulds such as *Dictyostelium discoidium*. Upon starvation, unicellular *D. discoidium* amoebae unite to form a fruiting body, a process that is necessary for the effective dispersal of spores. However, only some cells are transmitted; the others perish in the construction of the fruiting body, losses which are accommodated—indeed, selected for—at the population level (Hudson et al. 2002). An understanding of the multiple levels at which selection acts means we must consider not only the specific molecular events that occur within individual trypanosomes during infection but also the behaviours of the trypanosome population that emerge from a genome's programme (Marcello and Barry 2007b), and how the patterns of diversity that appear interact with other key pressures on trypanosome biology and natural history.

In this chapter we will discuss the antigenic variation phenotype, the molecular processes that underpin it and the highly successful survival strategy that emerges. First, the processes occurring in individual trypanosomes will be covered. Then we will discuss how these features play out over the course of infection. The broader evolutionary trajectories of the genes involved will then be discussed, followed by an overview of how this phenotype might interact with other key features of the trypanosomes' natural histories. Finally, we will consider the outstanding questions posed by antigenic variation, and what approaches might be best suited to addressing them.

Although all species of African salivarian trypanosome possess VSG, undergo antigenic variation and are subject to the pressures imposed by mammalian adaptive immunity, it is likely that differences exist in the patterns and structures involved. This chapter will focus on the model organism *Trypanosoma brucei*, which is by far the best characterised. Features of the *T. brucei* antigenic variation system are likely to apply also in the *T. brucei* derivatives *T. evansi* and *T. equiperdum*. Where data are sufficient, related species such as *T. congolense* and *T. vivax* will be covered, but the details of antigenic variation in these parasites are less clear.

3.2 How Does the Structure of the VSG Coat Relate to Its Function?

At the level of the individual, a key feature of VSG is its ability to form an effective barrier. VSG must therefore possess a particular structure, one that confers depth and density on an intact coat. Yet at the level of the population, different VSG coats must vary sufficiently from one another to enable antigenic variation. These demands have been met by a conserved tertiary fold that can be formed by widely divergent VSG amino acid sequences (Blum et al. 1993; Carrington et al. 1991). In this way, functional barrier-forming variants can be completely antigenically distinct from one another, allowing evasion of both non-specific innate immunity and specific adaptive immunity.

The VSG dimer forms an elongated structure that stands perpendicular to the membrane, the overall effect being a deep coat within which invariant surface molecules are buried (Vickerman 1969), as shown in Fig. 3.1. The VSG N-terminal domain (NTD) forms the exposed, membrane-distal part of the antigen (Schwede et al. 2011). This domain is essentially a pair of long helical elements that mediate dimerisation (Cohen et al. 1984), from which hang numerous loops and smaller units of secondary structure. In the case of the *brucei* clade, VSG also possesses a C-terminal domain (CTD) comprising one or two small subdomains, which may act to extend the molecule further and increase packing density of the coat (Chattopadhyay et al. 2005; Jones et al. 2008), but are apparently absent from *T. congolense* and *T. vivax* (Jackson et al. 2012). Both the NTD and the CTD are reinforced by a number of disulphide bridges between highly conserved cysteine residues (Allen and Gurnett 1983; Bussler et al. 1998); these probably strengthen the molecule against the vigorous physical forces it is likely to encounter (O'Beirne et al. 1998). VSG are anchored in the membrane by means of a glycosylphosphatidylinositol (GPI) group, which can be distinguished from the mammalian host GPI anchors by its shorter dimyristoylglycerol lipid moiety and (for most VSG) its possession of galactose residues (Ferguson 1991); and VSG are often also N-glycosylated elsewhere (Mehlert et al. 2002). Glycosylation may enhance the barrier function of an intact surface coat, and contribute to the structure and accessibility of epitopes, as it does for many other variable antigens such as influenza haemagglutinin (Caton et al. 1982). The lack of a transmembrane domain results in a coat that is exceptionally fluid: VSG dimers can flex and spin in place, move freely across the surface of the parasite (Engstler et al. 2007) and constitutively recycle through the endosomal system (Pal et al. 2003; Seyfang et al. 1990), creating a bustling, dynamic structure. This fluidity may facilitate efficient function of other surface molecules such as transferrin (Pal et al. 2003) and may assist in the rapid turnover of the coat that occurs during switching. There is also evidence that coat fluidity can assist the clearance of surface-bound antibodies when these are present at low concentration, providing a further, complementary means of immune escape (Engstler et al. 2007).

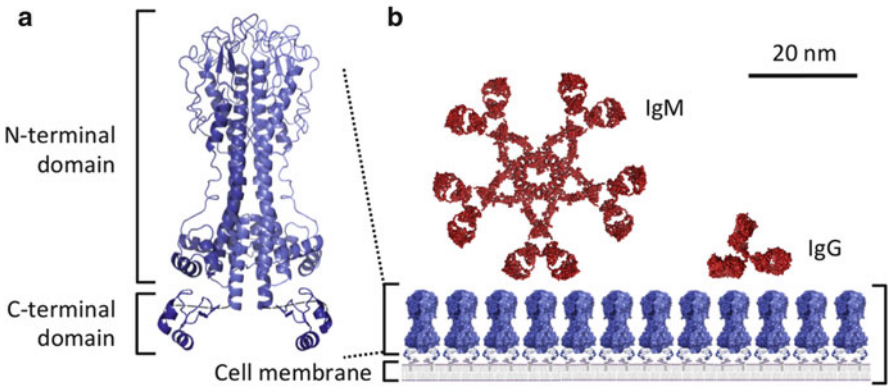

Fig. 3.1 (**a**) Each VSG dimer is an extended structure consisting of an N-terminal domain (NTD) and a C-terminal domain consisting of one or two subdomains. The membrane-distal end of the NTD is the region of the glycoprotein exposed to immune effectors on an assembled surface coat. (**b**) The assembled surface coat sterically hinders access of host immune effectors to the cell membrane or invariant surface proteins. Images were assembled using Protein Data Bank structures 1vsg, 1xu6, 1rcj and 1igt, were visualised using Pymol (Schrödinger, LLC) and were inspired by Engstler et al. (2007)

3.3 VSG Production Is a Tightly Regulated Process

It is presumably important that parasites maintain coat density whilst undergoing growth and replication, and VSG production is a principal aspect of the biology of the proliferating bloodstream-form trypanosome (Smith et al. 2009). The large surface area covered by an intact coat requires the production of huge quantities of the coat protein—in fact, approximately 10 % of *T. brucei* total soluble protein is VSG (Cross 1990). VSG is transcribed from a specialised locus, the active expression site (ES), one reason for which may be the large volume of VSG mRNA required by such high expression. There are two types of expression site, metacyclic (MES), of which there are approximately 20 in a *T. brucei* genome (Horn and Barry 2005), and bloodstream (BES), which number up to 23 (Young et al. 2008). Each ES contains a single intact *VSG* gene and a number of expression site-associated genes (ESAGs) (McCulloch and Horn 2009); several ESAGs have functions that have been experimentally associated with survival in the mammalian blood, such as iron acquisition (Bitter et al. 1998) and immune system modulation (Salmon et al. 2012), and ESAG content varies between expression sites (Hertz-Fowler et al. 2008). In fact MES possess no ESAGs at all and thus metacyclic *VSG* are unique in being the only trypanosome genes transcribed from their own promoter (Graham and Barry 1995). Critically, the expression sites are under strict control: only one is active at any time. The actively transcribed VSG itself is at the promoter-distal end of the expression site, proximal to the telomere repeats (Aline and Stuart 1989). Upstream of the *VSG*, and separating it from the rest of the expression site, is a set of 70-bp AT-rich imperfect repeats (Campbell et al. 1984). Transcription of the

active expression site is initiated at a single promoter, and separate mRNA molecules for each of the genes in the ES are produced by trans-splicing of this polycistronic transcript with a 5′ 'spliced leader' sequence that is common to all trypanosome mRNAs (Cully et al. 1985; Parsons et al. 1984). Newly translated VSG is folded in the endoplasmic reticulum with the aid of numerous chaperones (Field et al. 2010) and undergoes post-translational modification such as signal peptide cleavage (McConnell et al. 1981) and glycosylation (Ferguson et al. 1986; Mehlert et al. 2002). Mature VSG reaches the surface at the 'flagellar pocket', an invagination of the plasma membrane at the base of the flagellum where all endocytosis and exocytosis takes place (Overath et al. 1997), from where it diffuses to spread across the entire parasite surface.

3.4 Only One VSG Is Transcribed at a Time

Because each ES contains just one intact *VSG*, and because just one ES is active at any time, each individual trypanosome transcribes only one *VSG*. Exclusive expression is thought to be a strongly selected trait: simultaneous expression of multiple *VSG* would result in a heterogeneous coat, exposing all of the expressed VSG to the immune system, and resulting in a shorter infection than could have been achieved, had the different variants been expressed exclusively and consecutively (Morrison et al. 2005). An example of the importance of exclusive expression in prolonging infection can be found in another antigenically variant pathogen, *Giardia lamblia*, where disruption of monoallelic expression resulted in immune responses developing against all exposed antigens (Rivero et al. 2010). It has proved possible to generate parasites that simultaneously express two different VSG from one expression site (Muñoz-Jordán et al. 1996), a result indicating that the presence of just one *VSG* in an expression site is most likely a consequence of the switching mechanism rather than a demand enforced by it. On the other hand, the exclusive activation of a single ES—monoallelic expression—is much more strictly maintained, and attempts to generate lines expressing multiple ES result only in unstable clones switching rapidly between ES (Ulbert et al. 2002). How is it that one, and only one, ES is active at a time? One hypothesis has been built on the identification of a single multicomponent complex that drives transcriptional elongation from the active ES, termed the expression site body (ESB) (Navarro and Gull 2001). The ESB associates closely with the mitotic machinery, which could explain the heritability of the exclusive ESB–ES association (Landeira et al. 2009). However, the presence of a single ESB could be taken as a consequence of monoallelic expression as much as its cause. There are many questions about the mechanism that remain unclear, not least how a single ESB is maintained (Horn and McCulloch 2010). At the same time, complementary processes silence the inactive ES, and a number of epigenetic control pathways have been implicated (Alsford et al. 2012). There are numerous factors for which depletion has been shown to allow some transcription from inactive ES, including chromatin remodelling factors (Hughes et al. 2007; Wang et al. 2010), histones (Povelones et al. 2012), histone chaperones (Alsford et al. 2012) and DNA

replication factors (Benmerzouga et al. 2013; Tiengwe et al. 2012), to list only a few. However, in no case has derepression led to a level of transcription comparable to that from the active ES. The physical location of inactive ES at the nuclear periphery has also been proposed to contribute to their repression (DuBois et al. 2012; Navarro et al. 2007). The number of candidate factors associated with monoallelic expression, alongside the close physical proximity of these systems in a living cell, raises the possibility that monoallelic expression and its maintenance is a non-linear network, with many feedback loops and redundancies that may require rethinking of whether there can be a single necessary and sufficient causative agent. Resolving this problem is an important current question in trypanosome biology.

However monoallelic expression is ultimately achieved, it is flexible. The property of exclusive expression is frequently transferred to another *VSG* by switching, and it is to this that we now turn.

3.5 The VSG Coat Is Switched Frequently, Spontaneously and Stochastically

An individual parasite will occasionally change its expressed *VSG* gene, causing the replacement of its VSG surface coat with one composed of the new variant. This process is frequent, occurring within a lineage as often as once per 100 divisions (Turner and Barry 1989). It is spontaneous, occurring in culture (Horn and Cross 1997) and in immunodeficient animals (Magez et al. 2008). It is also stochastic: switching does not appear to be a deterministic process and is best understood probabilistically (Lythgoe et al. 2007). Emerging from these discrete switching events is abundant antigen 'richness' across the resident trypanosome population.

The primary source of this diversity is within the genome of the infecting trypanosomes, in the form of an extensive archive of 'silent' *VSG* genes. Genes in the silent archive are found in one of two locus types in addition to silent ES: minichromosomes and *VSG* arrays. Minichromosomal *VSG* are located close to the telomeres of the short (30–150 kb), linear minichromosomes, which otherwise consist primarily of repeats of a 177-bp motif (Weiden et al. 1991; Wickstead et al. 2004). It is thought that most minichromosomes contain a *VSG* proximal to each telomere, giving a total of approximately 200 minichromosomal *VSG* (Barry et al. 2005; Van der Ploeg et al. 1984). Most silent *VSG*, however, are found in tandem arrays in the subtelomeres of the parasite's standard diploid ('megabase') chromosomes. The first trypanosome strain to be comprehensively sequenced has a comparatively small *VSG* archive, yet its *VSG* arrays are still thought to contain between 1,000 and 2,000 *VSG* altogether (Berriman et al. 2005). A large part of the archive is annotated in this strain, in the current TREU 927 genome assembly available from TriTrypDB (Aslett et al. 2010), but it is likely that a significant fraction remains missing from the current archive annotation, due to poor coverage of minichromosomes, and to the fact that in most cases only one of each pair of homologous chromosomes is represented. Figure 3.2a illustrates the subtelomeric arrays annotated in the current version of the TREU 927 genome.

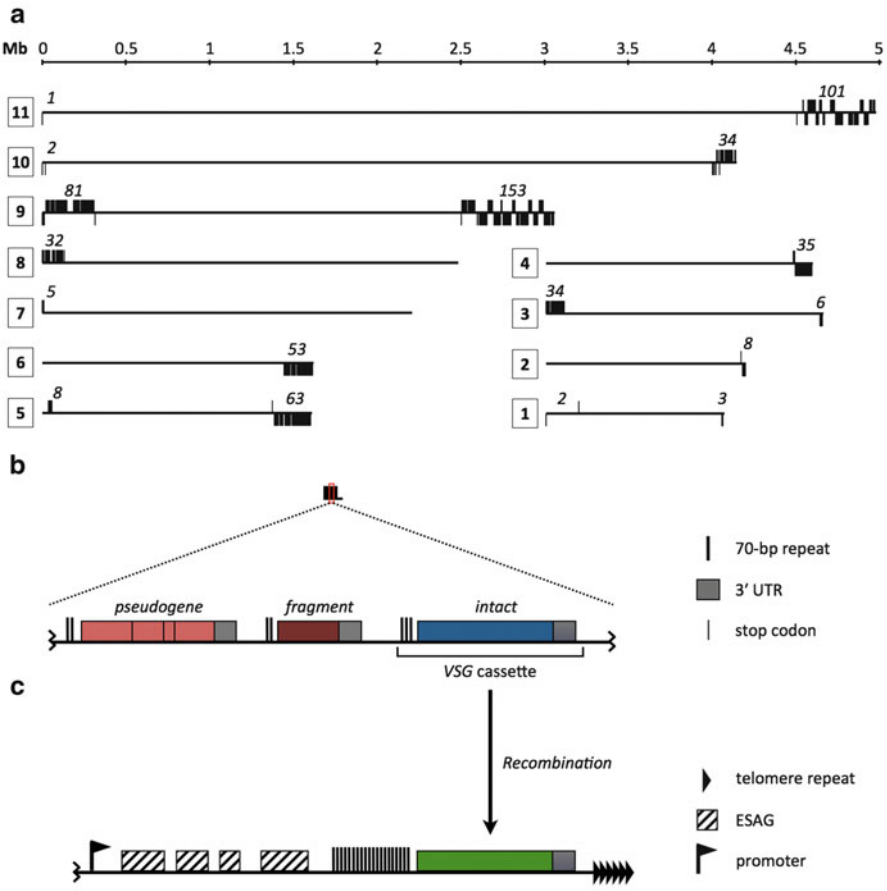

Fig. 3.2 (**a**) Subtelomeric *VSG* arrays are found across the *T. brucei* genome. Chromosome numbers are shown in *boxes*, numbers in *italics* above subtelomeric arrays indicate the number of genes in each in the current version of the TREU 927 genome assembly. *Lines* projecting above the line of the chromosome represent VSG genes, pseudogenes or gene fragments on the forward strand; those projecting below represent the same on the reverse strand. For chromosome 11, the assembly consists of a main contiguation and two much smaller fragments: only the main contiguation is shown, although the fragments not shown also contain VSG. (**b**) Stylised structure of part of a *VSG* array, showing *VSG* cassettes with features with homology with expression sites. The *box outline* indicates the approximate scale in (**a**) of the part of the array shown. (**c**) Stylised representation of the principal features of a telomeric VSG expression site

VSG in both locus types usually exist as cassettes with sequences at either end of the cassette that are homologous to those found in expression sites—AT-rich 70-bp repeats at the 5′ end and a conserved VSG-specific sequence at the 3′ end (Fig. 3.2b, Marcello and Barry 2007a; Pays et al. 1981; Van der Ploeg et al. 1984). A striking

feature of silent *VSG* that was revealed by the genome project is that only a small proportion of the array *VSG* (4.5 %) encode full-length, intact proteins (Berriman et al. 2005). This small proportion is in contrast with the minichromosomal *VSG*, which are thought to be mainly intact (Taylor and Rudenko 2006). The remainder of the array *VSG* are considered atypical due to predictions of inconsistent folding or post-translational modifications (9.5 %); or are pseudogenes, either containing frameshifts or stop codons (65 %), or encoding only one of the two necessary VSG domains (21 %) (Berriman et al. 2005; Marcello and Barry 2007a). These non-intact genes are nonetheless utilised during infection as substrates for 'mosaic' *VSG* gene assembly, as discussed below.

There are broadly two mechanisms by which this silent archive can be accessed, and hence switching can be achieved: transcriptional switching and recombinatorial switching. Transcriptional switching does not require any genetic rearrangement. As *T. brucei* has multiple expression sites, a different VSG will become expressed when the property of exclusive expression is transferred from one expression site to another. Transcriptional switching occurs rapidly in the bloodstream form. A simple model would involve the ESB moving to a different ES (Navarro and Gull 2001), but the triggers and mechanism of this process are vague. Furthermore, it appears that other factors are at work: the activation of a new ES and the silencing of the old one are separable processes, with chromatin remodelling required to silence the old ES (Figueiredo et al. 2008). The role of transcriptional switching in natural infections is unclear—only a small subset of *VSG* are present in ES and hence accessible in situ, and analyses of field-relevant trypanosome lines shows that over the course of a single infection transcriptional switching is a minor process compared with recombinatorial mechanisms (Robinson et al. 1999). Instead, it has been proposed that transcriptional switching is primarily a means of accessing different ESAG collections: the ESAGs present in different ES could evolve to the peculiarities of different host species (Bitter et al. 1998; Salmon et al. 2012; Young et al. 2008).

Recombinatorial switching involves activation of a silent *VSG* by its copying—or complete migration—into an active expression site. This process occurs by multiple mechanisms, the most important being gene conversion. In its best-understood form, *VSG* gene conversion co-opts the ancient DNA repair mechanism of homologous recombination to delete the existing ES-occupying *VSG* and replace it with a different *VSG* from elsewhere in the genome (Morrison et al. 2009). The trigger for recombination is thought to be damage to the active ES, for example a double-stranded break in the DNA (Boothroyd et al. 2009): a likely occurrence, given the extremely exposed nature of the DNA in the active ES and the physical instability of the AT-rich 70-bp repeats just upstream (Lin et al. 2009; Stanne and Rudenko 2010). Homologous recombination relies on similarities between DNA sequences, and rates of recombination are reduced as the length and degree of homology between substrate and template decrease (Barnes and McCulloch 2007). Sequence examination of ES which had undergone gene conversion revealed that

the 5′ boundary of recombination was usually located in the 70-bp repeat regions located upstream of the ES *VSG* (Liu et al. 1983), and the 3′ boundary of recombination was usually in a region spanning the 3′ end of the *VSG* (Bernards et al. 1981; Liu et al. 1985; Michels et al. 1983; Timmers et al. 1987). Fittingly, these regions correspond with the conserved boundaries of the '*VSG* cassette' (Fig. 3.2c). A straightforward model of recombinatorial switching therefore sees an archival *VSG* cassette replacing the telomere-proximal *VSG*-containing section of the ES, from the 70-bp repeat region to the 3′ UTR of the *VSG* or beyond (Morrison et al. 2009). An additional pathway of recombinatorial switching is available to *VSG* present at telomeres, such as those of the minichromosomes. Here, classical recombination acts to reciprocally exchange chromosome ends (Pays et al. 1985), swapping the active ES *VSG* with another telomere-proximal *VSG*. Trypanosomes deficient in the key homologous recombination enzyme RAD51 show a greatly reduced rate of switching (McCulloch and Barry 1999). Yet residual recombination events can still occur, by a process that requires only very short regions of homology, and can tolerate mismatches (Conway et al. 2002). There are clearly further mechanisms at work, occurring at lower frequency and with greater flexibility.

Recombinatorial switching does not always include the entire *VSG* and can combine part of the archive gene with part of another. Such 'segmental gene conversion' can occur anywhere in the *VSG*, including in the antigenically important NTD, and generates a mosaic *VSG* (Kamper and Barbet 1992; Roth et al. 1989; Thon et al. 1990). A mosaic *VSG* has multiple donors, each of which can contribute multiple segments to form an expressed *VSG*: genes comprising 15 segments from four different donors have been observed (Kamper and Barbet 1992). Donors to the same mosaic *VSG* show sequence similarity to one another, but the size of contributed segments can be very small, and the long regions of near-perfect identity that are required for classical homologous recombination in *T. brucei* do not seem to be necessary (Barnes and McCulloch 2007; Hall et al. 2013). The presence of mosaic genes is not merely an inconsequential by-product of high recombination in a region with multiple homologous genes but has two important features that could contribute to antigenic variation. Firstly, assembly of mosaic genes from fragments of pseudogenes allows accommodation of damaged *VSG*, and access to epitopes encoded in the archive that would otherwise be unable to contribute to antigenic variation, a feature that may be crucial in the context of an archive under a hypermutation protocol (see below). Secondly, use of mosaics introduces an extra level into antigenic variation: that of recombinational variability. The construction of mosaics in each new infection allows donors to combine in multiple different ways, potentially increasing many-fold the antigenic profiles obtainable from the archive. Related *T. brucei* mosaic VSG have been shown experimentally to be antigenically distinct (Hall et al. 2013), and other antigenically variant pathogens *Anaplasma spp.* and *Borrelia burgdorferi*, leverage segmental gene conversion to generate huge numbers of antigen variants (Coutte et al. 2009; Zhuang et al. 2007).

Yet mosaic *VSG* formation is also likely to be a risky process. Premature stop codons could easily be introduced into the mosaic by out-of-frame recombination events or involvement of damaged donor regions. The translated mosaic may not fold correctly, may lack key structural features such as conserved cysteines or glycosylation signals or may be incapable of forming an effective coat. Although it is possible that as-yet-undefined cellular mechanisms exist to recognise and resolve such errors, the likelihood is that the generation of VSG by assembly of mosaics is hugely inefficient at the level of an individual trypanosome. However, at the level of the population, which can number in the hundreds of billions within a host (Barry et al. 2012), these risks can be accommodated, allowing mosaic formation to greatly enhance the potential for antigenic variation. Indeed, it appears that mosaic VSG are abundant once beyond the early stages of infection (Marcello and Barry 2007a).

Each switching process requires the convergence of different factors, and as such each has a different chance of occurring to a given silent *VSG*. The resultant variation in activation probability between silent *VSG* is the basis of an overall hierarchy of expression across infection. Those *VSG* that are readily activated tend to be important earlier on in infection; later on, they are still likely to be frequently re-activated, but parasites that express them would be rapidly eliminated if the immune responses previously raised against their coats were still effective (Morrison et al. 2005). Other *VSG*, which perhaps require complex segmental gene conversion events to be utilised, are less likely to be activated, and so they become important later on in infection, once immune responses have appeared against the easily-activated *VSG*. ES *VSG*, resident in telomeric sites, appear to be preferentially activated early in infection. Such early activation is probably because telomeres tend to interact with one another, which promotes recombination (Barry et al. 2003), and because, compared with the archive *VSG* cassettes, the inactive ES provides more sequence that is homologous to the active ES (Hertz-Fowler et al. 2008). Intact array and minichromosomal *VSG* are usually activated somewhat later, because they have shorter stretches of more variable homology to the active ES (Liu et al. 1985; Robinson et al. 1999). However, only a single step is needed to activate an intact *VSG*, so such genes are usually expressed earlier in infection than are mosaics composed of pseudogenic *VSG* segments, which require assembly by an inefficient process of segmental conversion and hence appear later (Roth et al. 1989). The hierarchy is flexible, with previously 'late' *VSG* able to occupy more easily-activated genomic locations and thus taking an earlier position in the hierarchy (Laurent et al. 1984). The significance of and selection pressures acting on hierarchy in expression are unclear; hypotheses explaining its importance include a need to co-ordinate expression somewhat across an infection population, so as to exhaust neither archive (Morrison et al. 2005) nor host (Turner 1999). Nevertheless, hierarchies have been identified in a number of antigenic variation systems, with *Borrelia hermsii* (Barbour et al. 2006) and *Plasmodium falciparum* (Recker et al. 2011) both exhibiting clear trends in the patterns of variants that appear.

The molecular processes described in this section, summarised in Fig. 3.3, underlie the observed patterns of frequent, spontaneous, stochastic switching, the

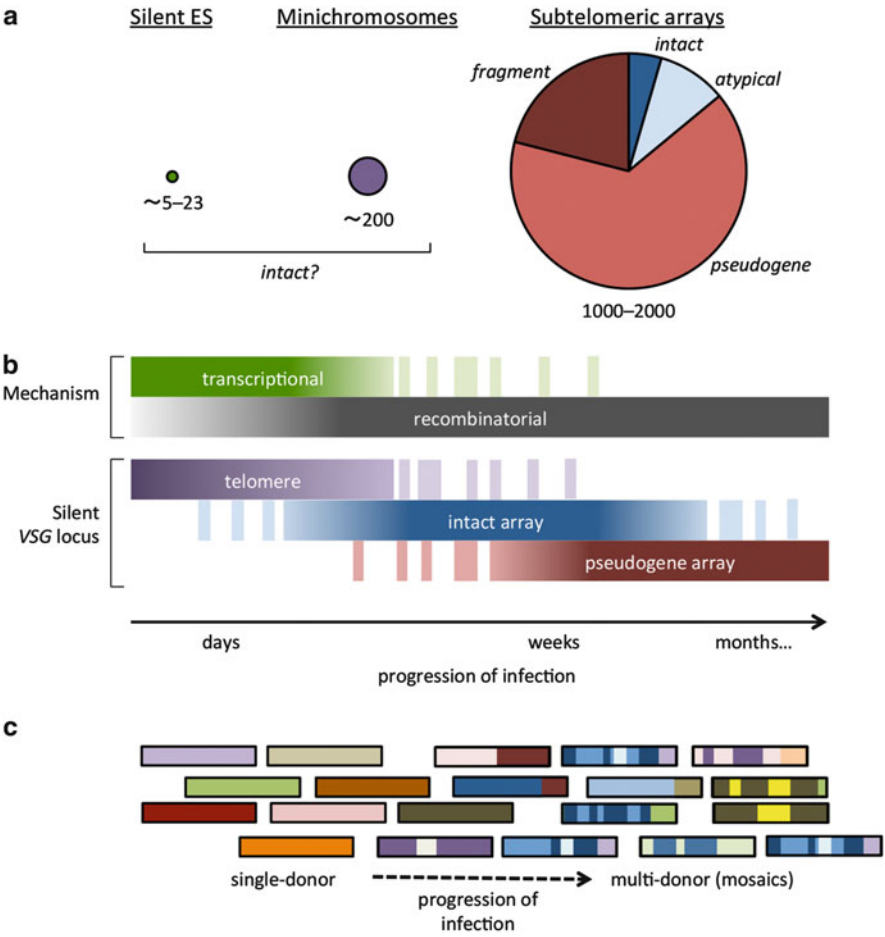

Fig. 3.3 (**a**) Several different locus types contain the silent *VSG* archive. *Circles* indicate archive *VSG* present at silent ES, minichromosomes and in subtelomeric arrays. The areas of the *circles* are approximately proportional to the relative number of *VSG* at each location type. The approximate number of *VSG* present at each location is given below each *circle*; the exact number varies between strains. In the case of the subtelomeric arrays, the *circle* is divided proportionally according to the intactness of silent *VSG* (after Marcello and Barry 2007a). Silent ES and minichromosomal *VSG* are thought to be largely intact. (**b**) *VSG* is expressed according to a hierarchy. For each process, intensity of *shading* indicates the relative importance in activating novel *VSG* over the course of infection. Recombinatorial switching assumes prominence over transcriptional switching beyond the early stages of infection. Telomere-resident *VSG* in silent ES and minichromosomes are activated more readily than those in the *VSG* arrays. Pseudogenic *VSG* require low-probability segmental gene conversion events to be accessed and tend not to appear until the chronic stage of infection in the form of mosaic *VSG*. This diagram was inspired by Morrison et al. (2009). (**c**) Expressed VSG are diverse, and mosaic *VSG* become increasingly predominant as infections progress and immunity neutralises readily activated single-donor VSG. *Shaded rectangles* are a stylised representation of expressed *VSG* that might be sampled at different points of infection. Different *shades* represent different donors. Note that it is likely that the total population of expressed *VSG* is much richer at any given point than indicated here

consequence of which is vast sustained diversity amongst the trypanosome population. Calculations based on total population size in a cow and the estimated *VSG* switch rate suggest that by the first peak of parasitaemia, more than 10^8 switches will have occurred (Barry et al. 2012). Experimental studies of chronic infections of mice have identified at least 15 distinct variants within individual samples, with many more likely to be present (Hall et al. 2013).

3.6 Host Immune Responses Shape Expressed Diversity

Host responses shape expressed VSG diversity, resulting in the patterns of antigenic variation. Antibodies against VSG clearly play a central role in the host adaptive response to trypanosome infection (Guirnalda et al. 2007; Magez et al. 2008), killing parasites by fixing complement, which can lyse parasites directly (at least in vitro, Van Meirvenne et al. 1995) or activate parasite-killing macrophages (Guirnalda et al. 2007; Pan et al. 2006). Immune responses to VSG are expected to constantly select for novelty in VSG expression, giving a directionality to the progression of antigenic variation as variants are successively neutralised, and any parasites that re-activate them are quickly eradicated (Barry and McCulloch 2001). However, trypanosome infection can have a substantial suppressive effect on host immunity (Askonas et al. 1979). *T. brucei* infections of mice can induce apoptosis in marginal zone B-cells, rendering hosts susceptible to re-challenge with previously encountered antigens, including VSG coats (Radwanska et al. 2008). It is possible that this phenomenon is unusually exaggerated in hosts such as mice that sustain exceptionally high parasitaemia for their body mass (La Greca and Magez 2011). In other hosts B-cell dysfunction and its consequences may be less extreme, manifesting perhaps in the occasional reappearance of 'early' variants in the chronic stage of infection, as such variants might not be effectively eliminated if they are re-activated. If, on the other hand, absolute B-cell dysregulation is widespread amongst natural hosts, the selection pressures favouring the evolution of the elaborate system of trypanosome antigenic variation would be mysterious, since parasites would only need to possess a handful of distinct antigens if they were able to abrogate immunological memory altogether. Investigations on a broader range of hosts are required to resolve the relative contributions of antigenic variation and immunosuppression to trypanosome persistence.

3.7 Antigenic Variation Interacts Closely with Trypanosome Transmission

Alongside the extrinsic force of the immune system, trypanosomes have an intrinsic mechanism of population control (Magez et al. 2008; Seed and Sechelski 1988), linked to transmission. Trypanosomes have a complex life cycle, encompassing numerous host species, and their success relies on efficient infection of feeding

tsetse flies and efficient establishment of infection in new hosts. In fact, prolonged bloodstream presence is necessary only in so far as it enables transmission, and mechanisms that promote transmission are therefore likely to be under strong selection. Infection of a tsetse fly requires viable transmission form ('short stumpy') parasites to be taken up in the blood meal of a feeding fly (Gibson and Bailey 2003). Stumpy forms are generated by irreversible differentiation of resident proliferative form ('long slender') parasites, from which they differ in a number of ways (MacGregor et al. 2012), most notably in that the stumpy form does not replicate and has a half-life of only 48–72 h (Turner et al. 1995). As a consequence, stumpy forms do not switch *VSG*, in fact, *VSG* transcription in stumpy forms is suppressed below the limit of detectability (Amiguet-Vercher et al. 2004). Stumpy form differentiation is triggered by an as-yet-unidentified, soluble, parasite-produced signal, 'stumpy induction factor' (SIF) (Vassella et al. 1997), which has a density-dependent effect on the population, with two consequences: providing a plentiful source of stumpy forms that maximise tsetse infectivity (MacGregor et al. 2011) and creating a negative feedback loop that stabilises total parasitaemia, preventing early death of the host (Seed et al. 2003). Every host has a carrying capacity and the limitations imposed are predicted to have a crucial role in the dynamics of antigenic variation. For example, carrying capacity varies between hosts, with some species—those with larger blood volumes, for example—able to harbour much larger populations of parasites than others. The larger the parasite population size, the greater the chance of a particular switch event occurring. Therefore, for large populations an acceleration of the kinetics of antigenic variation occurs (Barry 1986), as variants become activated sooner (Gjini et al. 2010).

Stumpy form parasites are abundant during infection (MacGregor et al. 2011). A high rate of differentiation reduces the population of parasites undergoing VSG switching to a smaller set of 'stem cell-like' slender forms, with important consequences for the dynamics of antigenic variation. If the effects of SIF are variant-independent, differentiation could maintain rarer variants at low abundance in the slender form. Given that there is likely to be a lower threshold to the size of inoculum necessary for induction of specific immunity (Morrison et al. 1982), these subpopulations could be suppressed 'below the radar' of immune sensitivity by differentiation (Gjini et al. 2010) until a dominant VSG expressor is eliminated by an immune response. This event opens a space in the host's carrying capacity, allowing one or more of these cryptic slender form subpopulations to expand to form part of the next wave of parasitaemia. As the expressed VSG diversity increases, relative to the sensitivity of the immune response, the greater the role of differentiation in controlling parasitaemia, since the number of different variants at low concentration cannot all induce sufficiently powerful specific immune responses for their rapid elimination. Similarly, the larger the size of the trypanosome population, the greater the likelihood that harder-to-activate variants will appear in an infection, since switching is parasite intrinsic. Increasing the number of variants that become activated tilts the balance of infection towards differentiation-based control (Gjini et al. 2010), increasing opportunities for tsetse transmission, but also risking premature host death as a consequence of persistent high

parasitaemia (Seed et al. 2003). Negotiating this trade-off may have been important in shaping the expression hierarchy (Gjini et al. 2010). In this vein it is interesting to note that both rates of VSG switching and production of transmission forms are greatly reduced in extensively syringe-passaged trypanosomes, a phenotype that can be reversed by passing through a tsetse fly (Turner 1997). Undergoing switching may impose a cost arising from risky recombination events; by artificially changing the life cycle to one where the ability to sustain prolonged infection confers no benefit, frequent syringe-passaging or in vitro culture likely select for parasites that have turned down the switch rate. Whether and how the changes in life cycle undertaken by non-tsetse transmitted trypanosomes impact on their patterns of antigenic variation would be an interesting subject for further investigation on this topic.

3.8 Antigenic Variation Can Promote Superinfection and Reinfection

A trypanosome genome does not exist in isolation. Although tsetse flies have a broad host range, the high levels of infection found in wild populations (e.g. Njiokou et al. 2006) suggests that they are likely to feed on hosts which have previously been infected or are already harbouring a trypanosome infection and thus which already are likely to have pre-circulating responses or immunological memory to many different VSG. Indeed, many tsetse flies are infected with more than one trypanosome genotype (Balmer and Caccone 2008; Macleod et al. 2001). Trypanosomes are therefore likely to encounter other trypanosome clones, both directly within an individual host, and—where destruction of immunological memory is not absolute (Radwanska et al. 2008)—indirectly through the immunological memories of hosts. These interactions between strains are likely to be a major pressure on trypanosomes, shaping life cycle features including antigenic variation and the *VSG* archive.

The effect of this pressure is apparent amongst VSG-expressing metacyclic trypanosomes entering a host in the bite of a tsetse fly. Establishing a successful infection in a previously infected, partially immune host requires the population of infecting parasites to evade circulating immunity, which may explain why metacyclic trypanosomes express VSG from specialised metacyclic ES (Barry et al. 1998). Each developing metacyclic trypanosome activates one of dozens of metacyclic ES, apparently at random, and so the metacyclic population shows diversity in VSG expression: as many as 27 different antibodies were required to neutralise all the metacyclic trypanosomes from a single tsetse fly (Turner et al. 1988). Moreover, the *VSG* present in the metacyclic expression sites undergo gradual turnover (Barry et al. 1983), facilitating superinfection and reinfection of previously infected hosts.

The diversifying selection imposed by host immunity is expected to favour such strain specificity, and indeed, this pressure extends beyond the metacyclic VSG. The stochastic formation of mosaic VSG over the course of infection could be

viewed as a mechanism of generating infection-unique variants. On a broader timescale, the complement of archive *VSG* is itself likely to develop strain specificity as the component genes evolve and diversify, as described further below.

Inter-clone competition may have acted in other ways to shape antigenic variation. Within a host, it is likely that there is competition for resources or carrying capacity, particularly if different clones use the same SIF signalling pathways (MacGregor and Matthews 2012). Under these conditions, clones that frequently undertake inefficient switching events such as mosaic construction will suffer a disadvantage when competing against clones that switch only to intact genes or switch only rarely. A successful antigenic variation protocol, when in competition with others, will need to balance efficiency of switching with scope of variability as an infection progresses. The battery of easily accessible minichromosomal *VSG* deployed early in infection may represent a compromise, allowing risky mosaic *VSG* construction to be relegated to the chronic stage of infection. Antigenic variation is not just a battle between a parasite and its host, and the effects of inter-strain competition on the diversity phenotype represent an interesting subject for further study.

3.9 The VSG Archive Is Adapted to Promote Rapid Change of the Repertoire

Given that uniqueness and diversity in the *VSG* archive are likely to be favoured, the trypanosome genome itself is in a constant state of flux. While the 'trypanosome genome' is an extremely useful resource (Berriman et al. 2005), it represents only a snapshot of the genome at a single point in time. The genomes of kinetoplastids are extremely plastic: for example *Leishmania* species display considerable variation in chromosome and gene copy number (Rogers et al. 2011), and the sizes of homologous chromosomes can vary considerably between *T. brucei* strains (Melville et al. 2000). In trypanosomes, this variability has been co-opted in the evolution of the *VSG* archive. Diversity within the archive is ancient, as evidenced by the fact that multiple contemporary *VSG* lineages are shared between African trypanosome species (Jackson et al. 2012). However, the archive continues to evolve rapidly, such that differences in *VSG* archives may account for most of the difference in chromosome size between strains. For example, in one examined strain, one copy of chromosome 1 has over half of its length devoted to *VSG*— around 3 Mb, potentially 600 *VSG* —but in the genome strain there are fewer than 10 *VSG* currently annotated in the haploid chromosome 1 assembly (Berriman et al. 2005; Callejas et al. 2006; Marcello and Barry 2007a).

Genome variability is apparent not only in the size of the archive but also in the sequence of *VSG* genes comprising it. Strains that have minor differences in housekeeping genes can have large differences in their *VSG* repertoires, and repertoires appear to have diverged to become strain specific (Bernards et al. 1986; Hutchinson et al. 2007). However, it is not immediately obvious how selection could promote these changes in the genome. An individual *VSG* gene

will be invisible to selection unless it is expressed, which most are not; and the size of the archive means there is a degree of redundancy: any individual gene can be lost with probably little effect. It therefore seems unlikely that the rapid rate of change is generally the result of strong selection on individual *VSG* genes. More likely is the hypothesis that 'second-order selection' (Caporale 2003) for diversity in expressed VSG has promoted the evolution of *mechanisms* that generate mutations of various sorts in *VSG* genes, resulting in the observed hyperevolution (Barry et al. 2012).

An important adaptation promoting the evolution of diversity in *VSG* genes is their location in subtelomeres. Subtelomeres are the transitionary regions between the low-complexity telomere repeats at the tips of linear chromosomes, and core regions, in which gene content, order and intergenic sequence are shared between homologous chromosome partners. A key feature of subtelomeres is their rapid rate of change compared with chromosome cores (Riethman et al. 2005). Elevated rates of ectopic and homologous recombination in the subtelomeres promote diversity by allowing subtelomeric genes to exchange sequence in a process of segmental conversion; and by duplicating genes (Linardopoulou et al. 2005; Mefford and Trask 2002). Gene duplication may lead to relaxation of selection on one of the copies and open the possibility of neofunctionalisation, as has been studied in subtelomeric families of disaccharide utilisation genes in yeast (Brown et al. 2010).

Most subtelomere sequence is non-coding, but these regions are frequently home to members of large, highly diverse families. *T. brucei* is only one example of a pathogen that has located in subtelomeres gene families that are important to a diversity phenotype: other subtelomeric gene families include the *var*, *rif* and *stevor* antigenic variation gene families of *P. falciparum* (Duffy and Tham 2007; Gardner et al. 2002; Hernandez-Rivas et al. 1997; Kyes et al. 1999; Scherf et al. 2008); the *vir* superfamily of *Plasmodium vivax* (Fernandez-Becerra et al. 2009); the major surface glycoproteins used in antigenic variation in *Pneumocystis carinii* (Stringer and Keely 2001); and various surface protein genes in *T. cruzi* (Moraes Barros et al. 2012).

Partitioning of *VSG* genes in a different environment from chromosome cores provides a means for *VSG* to be subject to different mutational activities from core genes, for which a high mutation rate could be catastrophic. Specifically, the location of *VSG* in the subtelomeres means that these genes are exposed to the hypermutational environment of these regions, which influences the evolution of *VSG* in several ways (Barry et al. 2003). Firstly, the high subtelomeric recombination rate will likely promote duplication of *VSG*. The duplication of intact genes might be a mechanism for replenishing the archive, compensating for the degeneration of pseudogenes and their eventual loss from the usable archive (Nei and Rooney 2005). Other mutagenic processes acting on the two gene copies could then eventually produce two antigenically distinct VSG. The frequent occurrence of duplications also has an effect beyond creating more *VSG* genes, because it results in the presence of subfamilies in the archive: *VSG* genes have very low identity to most others in the archive, but around 40 % of genes in the TREU 927 archive have a high-identity partner, presumably due to a recent duplication (Marcello and Barry 2007a).

This subfamily structure is thought to be key to providing multiple donors of sufficient identity to each other to assemble mosaic *VSG* genes, allowing evolving diversity in pseudogenes to be accessed. The existence of closely related genes within the archive, combined with the high level of ectopic recombination in the subtelomere, can also generate new combinations of *VSG* sequence through a process of segmental conversion (Gjini et al. 2012b). There is some debate as to whether recombination is capable of increasing diversity (Martinsohn et al. 1999), but it is possible that an effect of ectopic recombination in *VSG* arrays may be to assemble antigenically novel genes in a process analogous to the generation of mosaic *VSG* during infection. Interestingly, it appears that recombination between *VSG* has been less important in shaping the *T. congolense* and *T. vivax* archives, suggesting that antigenic diversity is generated by distinct mechanisms in each species (Jackson et al. 2012).

Secondly, comparison of genes within the TREU 927 *VSG* archive has been used to infer the events of *VSG* evolution, and these analyses have suggested that smaller scale mutational processes are also important, namely point mutation and short insertion–deletions (Gjini et al. 2012b; Marcello and Barry 2007a). Analysis in our laboratory of the *VSG* archives of sequential isolates of one trypanosome strain has indicated that substitution mutation processes are both qualitatively and quantitatively different in *VSG* from cores, with substitution mutations accumulating at silent sites at a rate several times higher in *VSG* than in core genes (L.P., T. Otto, M. Berriman, and J.D. Barry, unpublished). The role that such small mutations might play in antigenic variation is unclear. The scale and rate of point mutations indicate that point mutation is unlikely to produce antigenically novel sequence rapidly enough to make a substantial contribution to antigenic variation in a single infection of a single host (Graham and Barry 1996). However, the accumulation of small mutations over a longer timescale could introduce new diversity to the archive and might give an advantage at the scale of the parasite population if it allowed infection of a previously infected host.

As noted above, *VSG* archives vary considerably between strains. The occurrence of changes at the sequence level is a key feature of *VSG* evolution, but other factors are also thought to be involved in building archive strain specificity. One hypothesis is that trypanosomes have ceased to exchange sequence between telomeres and subtelomeres during meiosis. Meiotic recombination tends to homogenise gene sequences between homologous chromosomes, and the removal of this homogenising effect would promote the divergence of archives between strains as they accumulated different changes (Hutchinson et al. 2007). A second possible factor is the operation of population bottlenecks: migration to the tsetse salivary glands appears to be a severe bottleneck in the parasite life cycle (Oberle et al. 2010; Van Den Abbeele et al. 1999), and as such could rapidly fix different variants in different populations.

The subtelomeric *VSG* arrays, therefore, represent a large repository of information available for the trypanosome to use in antigenic variation, but their role goes considerably beyond that of an inanimate archive. Rather, the arrays are potent generators of diversity, driven by an active hypermutation protocol. The mutations

that occur under this protocol are likely to introduce pseudogenicity, which imposes a requirement for the formation of mosaic *VSG* during infection, if the full range of antigens is to be exploited. Additionally, the accumulation of mutations contributes to strain specificity. Thus, the structure and evolution of the *VSG* archive plays a key role in antigenic variation at several levels (Gjini et al. 2012a).

3.10 Going Forward

In summary, African trypanosome antigenic variation is a genome strategy for prolonged infection and transmission, a diversity phenotype that emerges at the population level amongst clonally related parasites. The antigenic variation system operates through an immunogenic surface coat of VSG, which shields invariant surface molecules and protects the cell from the innate immune system. Expression can readily switch to a different VSG resulting in a diverse population that pre-empts adaptive immunity. The diversity phenotype emerges at the population level from the operation of a complex network of molecular actors that maintain strict monoallelic expression and effect switching between VSG in a frequent, spontaneous and stochastic manner. The *VSG* repertoire, although containing a huge number of silent *VSG*, contains many pseudogenes. The construction of mosaic *VSG* allows use of these pseudogenes and may also be important for introducing combinatorial diversity into the system and allowing the generation of infection-specific variants. In the longer term, the evolution of diversity is promoted by adaptations that shape the *VSG* archive, notably the location of the main part of the archive in hypermutational subtelomeres. Antigenic variation is likely to have a profound influence in the dynamics of infection and superinfection, which are determined by the interaction of many factors including the probabilistic order of VSG expression, differentiation, inter-strain competition and the action of immune effectors.

African trypanosome antigenic variation represents a highly successful survival strategy and as such is an inherently interesting biological phenomenon. Currently, a considerable amount of detail is being elucidated in terms of understanding the molecular mechanisms of antigenic variation, with demonstration of involvement for numerous proteins (e.g. reviewed in Alsford et al. 2012; Morrison et al. 2009). In the future, perhaps we can look forward to the integration of these data into a more complete model of the mechanisms of antigenic variation from the molecular level upwards. Important to the development of any model would be a consideration of low frequency events which are likely to occur in the large populations in which antigenic variation is made manifest. For example, elucidating the molecular details and temporal dynamics of mosaic VSG assembly would provide a key to the processes facilitating chronic infection. Further illumination would be given by a better understanding of the infection biology and antigenic variation systems of non-*brucei* African trypanosomes, which are beginning to be less neglected (e.g. Chamond et al. 2010; Coustou et al. 2010; Greif et al. 2013). Such comparative studies, along with the wider availability of genome data, are also yielding insights into the evolution of the *VSG* archive (Jackson et al. 2012); further research on these

patterns will be useful in inferring the selective pressures that have shaped this system in related species. Increasing availability and sensitivity of deep sequencing techniques will be invaluable in dissecting the composition of the trypanosome population over the course of infection, which could become a powerful tool in the study of infection dynamics. Finally, it will be important to resolve the how antigenic variation and immune suppression interact to prolong infection, as the details and significance of the latter become better understood. Because it is so closely allied to various fundamental aspects of parasite biology and dynamics of population diversity, future studies of trypanosome antigenic variation are likely to provide insights not only in understanding this specific system but also into broader questions associated with fields as broad-ranging as gene expression, DNA recombination, host–parasite co-evolution and biological diversity.

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References

Aline RF, Stuart K (1989) Trypanosoma brucei: conserved sequence organization 3′ to telomeric variant surface glycoprotein genes. Exp Parasitol 68:57–66

Allen G, Gurnett LP (1983) Locations of the six disulphide bonds in a variant surface glycoprotein (VSG 117) from Trypanosoma brucei. Biochem J 209:481–487

Alsford S, duBois K, Horn D, Field MC (2012) Epigenetic mechanisms, nuclear architecture and the control of gene expression in trypanosomes. Expert Rev Mol Med 14:e13. doi:10.1017/erm.2012.7

Amiguet-Vercher A, Pérez-Morga D, Pays A et al (2004) Loss of the mono-allelic control of the VSG expression sites during the development of Trypanosoma brucei in the bloodstream. Mol Microbiol 51:1577–1588. doi:10.1111/j.1365-2958.2003.03937.x

Askonas BA, Corsini AC, Clayton CE, Ogilvie BM (1979) Functional depletion of T- and B-memory cells and other lymphoid cell subpopulations-during trypanosomiasis. Immunology 36:313–321

Aslett M, Aurrecoechea C, Berriman M et al (2010) TriTrypDB: a functional genomic resource for the Trypanosomatidae. Nucleic Acids Res 38:D457–D462. doi:10.1093/nar/gkp851

Balmer O, Caccone A (2008) Multiple-strain infections of Trypanosoma brucei across Africa. Acta Trop 107:275–279. doi:10.1016/j.actatropica.2008.06.006

Barbour AG, Dai Q, Restrepo BI et al (2006) Pathogen escape from host immunity by a genome program for antigenic variation. Proc Natl Acad Sci USA 103:18290–18295. doi:10.1073/pnas.0605302103

Barnes RL, McCulloch R (2007) Trypanosoma brucei homologous recombination is dependent on substrate length and homology, though displays a differential dependence on mismatch repair as substrate length decreases. Nucleic Acids Res 35:3478–3493. doi:10.1093/nar/gkm249

Barry JD (1986) Antigenic variation during Trypanosoma vivax infections of different host species. Parasitology 92(Pt 1):51–65

Barry JD, McCulloch R (2001) Antigenic variation in trypanosomes: enhanced phenotypic variation in a eukaryotic parasite. Adv Parasitol 49:1–70

Barry JD, Crowe JS, Vickerman K (1983) Instability of the Trypanosoma brucei rhodesiense metacyclic variable antigen repertoire. Nature 306:699–701

Barry JD, Graham SV, Fotheringham M et al (1998) VSG gene control and infectivity strategy of metacyclic stage Trypanosoma brucei. Mol Biochem Parasitol 91:93–105
Barry JD, Ginger ML, Burton P, McCulloch R (2003) Why are parasite contingency genes often associated with telomeres? Int J Parasitol 33:29–45
Barry JD, Marcello L, Morrison LJ et al (2005) What the genome sequence is revealing about trypanosome antigenic variation. Biochem Soc Trans 33:986–989. doi:10.1042/BST20050986
Barry JD, Hall JPJ, Plenderleith L (2012) Genome hyperevolution and the success of a parasite. Ann N Y Acad Sci 1267:11–17. doi:10.1111/j.1749-6632.2012.06654.x
Benmerzouga I, Concepción-Acevedo J, Kim H-S et al (2013) Trypanosoma brucei Orc1 is essential for nuclear DNA replication and affects both VSG silencing and VSG switching. Mol Microbiol 87:196–210. doi:10.1111/mmi.12093
Bernards A, Van der Ploeg LH, Frasch AC et al (1981) Activation of trypanosome surface glycoprotein genes involves a duplication-transposition leading to an altered 3′ end. Cell 27:497–505
Bernards A, Van der Ploeg LH, Gibson WC et al (1986) Rapid change of the repertoire of variant surface glycoprotein genes in trypanosomes by gene duplication and deletion. J Mol Biol 190:1–10
Berriman M, Ghedin E, Hertz-Fowler C et al (2005) The genome of the African trypanosome Trypanosoma brucei. Science 309:416–422. doi:10.1126/science.1112642
Bitter W, Gerrits H, Kieft R, Borst P (1998) The role of transferrin-receptor variation in the host range of Trypanosoma brucei. Nature 391:499–502. doi:10.1038/35166
Black SJ, Guirnalda P, Frenkel D et al (2010) Induction and regulation of Trypanosoma brucei VSG-specific antibody responses. Parasitology 137:2041–2049
Blum ML, Down JA, Gurnett AM et al (1993) A structural motif in the variant surface glycoproteins of Trypanosoma brucei. Nature 362:603–609. doi:10.1038/362603a0
Boothroyd CE, Dreesen O, Leonova T et al (2009) A yeast-endonuclease-generated DNA break induces antigenic switching in Trypanosoma brucei. Nature 459:278–281. doi:10.1038/nature07982
Brown CA, Murray AW, Verstrepen KJ (2010) Rapid expansion and functional divergence of subtelomeric gene families in yeasts. Curr Biol 20:895–903. doi:10.1016/j.cub.2010.04.027
Bussler H, Linder M, Linder D, Reinwald E (1998) Determination of the disulfide bonds within a B domain variant surface glycoprotein from Trypanosoma congolense. J Biol Chem 273:32582–32586
Callejas S, Leech V, Reitter C, Melville S (2006) Hemizygous subtelomeres of an African trypanosome chromosome may account for over 75% of chromosome length. Genome Res 16:1109–1118. doi:10.1101/gr.5147406
Campbell DA, van Bree MP, Boothroyd JC (1984) The 5′-limit of transposition and upstream barren region of a trypanosome VSG gene: tandem 76 base-pair repeats flanking (TAA)90. Nucleic Acids Res 12:2759–2774
Caporale LH (2003) Natural selection and the emergence of a mutation phenotype: an update of the evolutionary synthesis considering mechanisms that affect genome variation. Annu Rev Microbiol 57:467–485. doi:10.1146/annurev.micro.57.030502.090855
Carrington M, Miller N, Blum ML et al (1991) Variant specific glycoprotein of Trypanosoma brucei consists of two domains each having an independently conserved pattern of cysteine residues. J Mol Biol 221:823–835
Caton AJ, Brownlee GG, Yewdell JW, Gerhard WU (1982) The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell 31:417–427
Chamond N, Cosson A, Blom-Potar MC et al (2010) Trypanosoma vivax infections: pushing ahead with mouse models for the study of Nagana. I. Parasitological, hematological and pathological parameters. PLoS Negl Trop Dis 4:e792
Chattopadhyay A, Jones NG, Nietlispach D et al (2005) Structure of the C-terminal domain from Trypanosoma brucei variant surface glycoprotein MITat1.2. J Biol Chem 280:7228–7235. doi:10.1074/jbc.M410787200

Cohen C, Reinhardt B, Parry DA et al (1984) Alpha-helical coiled-coil structures of Trypanosoma brucei variable surface glycoproteins. Nature 311:169–171
Conway C, Proudfoot C, Burton P et al (2002) Two pathways of homologous recombination in Trypanosoma brucei. Mol Microbiol 45:1687–1700
Coustou V, Guegan F, Plazolles N, Baltz T (2010) Complete in vitro life cycle of Trypanosoma congolense: development of genetic tools. PLoS Negl Trop Dis 4:e618. doi:10.1371/journal.pntd.0000618
Coutte L, Botkin DJ, Gao L, Norris SJ (2009) Detailed analysis of sequence changes occurring during vlsE antigenic variation in the mouse model of Borrelia burgdorferi infection. PLoS Pathog 5:e1000293. doi:10.1371/journal.ppat.1000293
Cross GAM (1990) Cellular and genetic aspects of antigenic variation in trypanosomes. Annu Rev Immunol 8:83–110. doi:10.1146/annurev.iy.08.040190.000503
Cully DF, Ip HS, Cross GA (1985) Coordinate transcription of variant surface glycoprotein genes and an expression site associated gene family in Trypanosoma brucei. Cell 42:173–182. doi:10.1016/S0092-8674(85)80113-6
DuBois KN, Alsford S, Holden JM et al (2012) NUP-1 Is a large coiled-coil nucleoskeletal protein in trypanosomes with lamin-like functions. PLoS Biol 10:e1001287. doi:10.1371/journal.pbio.1001287
Duffy MF, Tham W-H (2007) Transcription and coregulation of multigene families in Plasmodium falciparum. Trends Parasitol 23:183–186. doi:10.1016/j.pt.2007.02.010, discussion 186–7
Engstler M, Pfohl T, Herminghaus S et al (2007) Hydrodynamic flow-mediated protein sorting on the cell surface of trypanosomes. Cell 131:505–515. doi:10.1016/j.cell.2007.08.046
Ferguson M (1991) Lipid anchors on membrane proteins. Curr Opin Struct Biol 1:522–529
Ferguson MA, Duszenko M, Lamont GS et al (1986) Biosynthesis of Trypanosoma brucei variant surface glycoproteins. N-glycosylation and addition of a phosphatidylinositol membrane anchor. J Biol Chem 261:356–362
Fernandez-Becerra C, Yamamoto MM, Vêncio RZN et al (2009) Plasmodium vivax and the importance of the subtelomeric multigene vir superfamily. Trends Parasitol 25:44–51. doi:10.1016/j.pt.2008.09.012
Ferrante A, Allison AC (1983) Alternative pathway activation of complement by African trypanosomes lacking a glycoprotein coat. Parasite Immunol 5:491–498
Field MC, Sergeenko T, Wang Y-N et al (2010) Chaperone requirements for biosynthesis of the trypanosome variant surface glycoprotein. PLoS One 5:e8468. doi:10.1371/journal.pone.0008468
Figueiredo LM, Janzen CJ, Cross GAM (2008) A histone methyltransferase modulates antigenic variation in African trypanosomes. PLoS Biol 6:e161. doi:10.1371/journal.pbio.0060161
Gardner MJ, Hall N, Fung E et al (2002) Genome sequence of the human malaria parasite Plasmodium falciparum. Nature 419:498–511. doi:10.1038/nature01097
Gibson W, Bailey M (2003) The development of Trypanosoma brucei within the tsetse fly midgut observed using green fluorescent trypanosomes. Kinetoplastid Biol Dis 2:1
Gjini E, Haydon DT, Barry JD, Cobbold CA (2010) Critical interplay between parasite differentiation, host immunity, and antigenic variation in trypanosome infections. Am Nat 176:424–439. doi:10.1086/656276
Gjini E, Haydon DT, Barry JD, Cobbold CA (2012a) Linking the antigen archive structure to pathogen fitness in African trypanosomes. Proc Roy Soc B Biol Sci 280:20122129. doi:10.1098/rspb.2012.2129
Gjini E, Haydon DT, Barry JD, Cobbold CA (2012b) The impact of mutation and gene conversion on the local diversification of antigen genes in African trypanosomes. Mol Biol Evol 29:3321–3331. doi:10.1093/molbev/mss166
Graham SV, Barry JD (1995) Transcriptional regulation of metacyclic variant surface glycoprotein gene expression during the life cycle of Trypanosoma brucei. Mol Cell Biol 15:5945–5956
Graham VS, Barry JD (1996) Is point mutagenesis a mechanism for antigenic variation in Trypanosoma brucei? Mol Biochem Parasitol 79:35–45

Greif G, de Leon MP, Lamolle G et al (2013) Transcriptome analysis of the bloodstream stage from the parasite Trypanosoma vivax. BMC Genomics 14:149. doi:10.1186/1471-2164-14-149

Guirnalda P, Murphy NB, Nolan D, Black SJ (2007) Anti-Trypanosoma brucei activity in Cape buffalo serum during the cryptic phase of parasitemia is mediated by antibodies. Int J Parasitol 37:1391–1399. doi:10.1016/j.ijpara.2007.04.019

Hall JPJ, Wang H, Barry JD (2013) Mosaic VSGs and the scale of Trypanosoma brucei antigenic variation. PLoS Pathog 9(7): e1003502. doi: 10.1371/journal.ppat.1003502

Hernandez-Rivas R, Mattei D, Sterkers Y et al (1997) Expressed var genes are found in Plasmodium falciparum subtelomeric regions. Mol Cell Biol 17:604–611

Hertz-Fowler C, Figueiredo LM, Quail MA et al (2008) Telomeric expression sites are highly conserved in Trypanosoma brucei. PLoS One 3:e3527. doi:10.1371/journal.pone.0003527

Horn D, Barry JD (2005) The central roles of telomeres and subtelomeres in antigenic variation in African trypanosomes. Chromosome Res 13:525–533. doi:10.1007/s10577-005-0991-8

Horn D, Cross GA (1997) Analysis of Trypanosoma brucei vsg expression site switching in vitro. Mol Biochem Parasitol 84:189–201

Horn D, McCulloch R (2010) Molecular mechanisms underlying the control of antigenic variation in African trypanosomes. Curr Opin Microbiol 13:700–705. doi:10.1016/j.mib.2010.08.009

Hudson RE, Aukema JE, Rispe C, Roze D (2002) Altruism, cheating, and anticheater adaptations in cellular slime molds. Am Nat 160:31–43. doi:10.1086/340613

Hughes K, Wand M, Foulston L et al (2007) A novel ISWI is involved in VSG expression site downregulation in African trypanosomes. EMBO J 26:2400–2410. doi:10.1038/sj.emboj.7601678

Hutchinson OC, Picozzi K, Jones NG et al (2007) Variant surface glycoprotein gene repertoires in Trypanosoma brucei have diverged to become strain-specific. BMC Genomics 8:234. doi:10.1186/1471-2164-8-234

Jackson DG, Owen MJ, Voorheis HP (1985) A new method for the rapid purification of both the membrane-bound and released forms of the variant surface glycoprotein from Trypanosoma brucei. Biochem J 230:195–202

Jackson AP, Berry A, Aslett M et al (2012) Antigenic diversity is generated by distinct evolutionary mechanisms in African trypanosome species. Proc Natl Acad Sci USA 109:3416. doi:10.1073/pnas.1117313109

Jones NG, Nietlispach D, Sharma R et al (2008) Structure of a glycosylphosphatidylinositol-anchored domain from a trypanosome variant surface glycoprotein. J Biol Chem 283:3584–3593. doi:10.1074/jbc.M706207200

Kamper SM, Barbet AF (1992) Surface epitope variation via mosaic gene formation is potential key to long-term survival of Trypanosoma brucei. Mol Biochem Parasitol 53:33–44

Kyes SA, Rowe JA, Kriek N, Newbold CI (1999) Rifins: a second family of clonally variant proteins expressed on the surface of red cells infected with Plasmodium falciparum. Proc Natl Acad Sci USA 96:9333–9338

La Greca F, Magez S (2011) Vaccination against trypanosomiasis: can it be done or is the trypanosome truly the ultimate immune destroyer and escape artist? Hum Vaccin 7:1225–1233. doi:10.4161/hv.7.11.18203

Landeira D, Bart J-M, Van Tyne D, Navarro M (2009) Cohesin regulates VSG monoallelic expression in trypanosomes. J Cell Biol 186:243–254. doi:10.1083/jcb.200902119

Laurent M, Pays E, Van der Werf A et al (1984) Translocation alters the activation rate of a trypanosome surface antigen gene. Nucleic Acids Res 12:8319–8328

Lin Y, Hubert L, Wilson JH (2009) Transcription destabilizes triplet repeats. Mol Carcinog 48:350–361. doi:10.1002/mc.20488

Linardopoulou EV, Williams EM, Fan Y et al (2005) Human subtelomeres are hot spots of interchromosomal recombination and segmental duplication. Nature 437:94–100. doi:10.1038/nature04029

Liu AY, Van der Ploeg LH, Rijsewijk FA, Borst P (1983) The transposition unit of variant surface glycoprotein gene 118 of Trypanosoma brucei. Presence of repeated elements at its border and absence of promoter-associated sequences. J Mol Biol 167:57–75

Liu AY, Michels PA, Bernards A, Borst P (1985) Trypanosome variant surface glycoprotein genes expressed early in infection. J Mol Biol 182:383–396

Lythgoe KA, Morrison LJ, Read AF, Barry JD (2007) Parasite-intrinsic factors can explain ordered progression of trypanosome antigenic variation. Proc Natl Acad Sci USA 104: 8095–8100. doi:10.1073/pnas.0606206104

MacGregor P, Matthews KR (2012) Identification of the regulatory elements controlling the transmission stage-specific gene expression of PAD1 in Trypanosoma brucei. Nucleic Acids Res 40:7705–7717. doi:10.1093/nar/gks533

MacGregor P, Savill NJ, Hall D, Matthews KR (2011) Transmission stages dominate trypanosome within-host dynamics during chronic infections. Cell Host Microbe 9:310–318. doi:10.1016/j.chom.2011.03.013

MacGregor P, Szöőr B, Savill NJ, Matthews KR (2012) Trypanosomal immune evasion, chronicity and transmission: an elegant balancing act. Nat Rev Microbiol 10(6):431–438. doi:10.1038/nrmicro2779

Macleod A, Tait A, Turner CM (2001) The population genetics of Trypanosoma brucei and the origin of human infectivity. Philos Trans R Soc Lond B Biol Sci 356:1035–1044. doi:10.1098/rstb.2001.0892

Magez S, Schwegmann A, Atkinson R et al (2008) The role of B-cells and IgM antibodies in parasitemia, anemia, and VSG switching in Trypanosoma brucei-infected mice. PLoS Pathog 4:e1000122. doi:10.1371/journal.ppat.1000122

Marcello L, Barry JD (2007a) Analysis of the VSG gene silent archive in Trypanosoma brucei reveals that mosaic gene expression is prominent in antigenic variation and is favored by archive substructure. Genome Res 17:1344–1352. doi:10.1101/gr.6421207

Marcello L, Barry JD (2007b) From silent genes to noisy populations-dialogue between the genotype and phenotypes of antigenic variation. J Eukaryot Microbiol 54:14–17. doi:10.1111/j.1550-7408.2006.00227.x

Martinsohn JT, Sousa AB, Guethlein LA, Howard JC (1999) The gene conversion hypothesis of MHC evolution: a review. Immunogenetics 50:168–200

McConnell J, Gurnett AM, Cordingley JS et al (1981) Biosynthesis of Trypanosoma brucei variant surface glycoprotein. I. Synthesis, size, and processing of an N-terminal signal peptide. Mol Biochem Parasitol 4:225–242

McCulloch R, Barry JD (1999) A role for RAD51 and homologous recombination in Trypanosoma brucei antigenic variation. Genes Dev 13:2875–2888

McCulloch R, Horn D (2009) What has DNA sequencing revealed about the VSG expression sites of African trypanosomes? Trends Parasitol 25:359–363. doi:10.1016/j.pt.2009.05.007

Mefford HC, Trask BJ (2002) The complex structure and dynamic evolution of human subtelomeres. Nat Rev Genet 3:91–102. doi:10.1038/nrg727

Mehlert A, Bond CS, Ferguson MAJ (2002) The glycoforms of a Trypanosoma brucei variant surface glycoprotein and molecular modeling of a glycosylated surface coat. Glycobiology 12: 607–612

Melville SE, Leech V, Navarro M, Cross GA (2000) The molecular karyotype of the megabase chromosomes of Trypanosoma brucei stock 427. Mol Biochem Parasitol 111:261–273

Michels PA, Liu AY, Bernards A et al (1983) Activation of the genes for variant surface glycoproteins 117 and 118 in Trypanosoma brucei. J Mol Biol 166:537–556

Moraes Barros RR, Marini MM, Antônio CR et al (2012) Anatomy and evolution of telomeric and subtelomeric regions in the human protozoan parasite Trypanosoma cruzi. BMC Genomics 13:229. doi:10.1186/1471-2164-13-229

Morrison WI, Black SJ, Paris J et al (1982) Protective immunity and specificity of antibody responses elicited in cattle by irradiated Trypanosoma brucei. Parasite Immunol 4:395–407

Morrison LJ, Majiwa P, Read AF, Barry JD (2005) Probabilistic order in antigenic variation of Trypanosoma brucei. Int J Parasitol 35:961–972. doi:10.1016/j.ijpara.2005.05.004

Morrison LJ, Marcello L, McCulloch R (2009) Antigenic variation in the African trypanosome: molecular mechanisms and phenotypic complexity. Cell Microbiol 11:1724–1734. doi:10.1111/j.1462-5822.2009.01383.x

Mosser DM, Roberts JF (1982) Trypanosoma brucei: recognition in vitro of two developmental forms by murine macrophages. Exp Parasitol 54:310–316

Muñoz-Jordán JL, Davies KP, Cross GAM (1996) Stable expression of mosaic coats of variant surface glycoproteins in Trypanosoma brucei. Science 272:1795–1797

Navarro M, Gull K (2001) A pol I transcriptional body associated with VSG mono-allelic expression in Trypanosoma brucei. Nature 414:759–763. doi:10.1038/414759a

Navarro M, Peñate X, Landeira D (2007) Nuclear architecture underlying gene expression in Trypanosoma brucei. Trends Microbiol 15:263–270. doi:10.1016/j.tim.2007.04.004

Nei M, Rooney AP (2005) Concerted and birth-and-death evolution of multigene families. Annu Rev Genet 39:121–152. doi:10.1146/annurev.genet.39.073003.112240

Njiokou F, Laveissière C, Simo G et al (2006) Wild fauna as a probable animal reservoir for Trypanosoma brucei gambiense in Cameroon. Infect Genet Evol 6:147–153. doi:10.1016/j.meegid.2005.04.003

O'Beirne C, Lowry CM, Voorheis HP (1998) Both IgM and IgG anti-VSG antibodies initiate a cycle of aggregation-disaggregation of bloodstream forms of Trypanosoma brucei without damage to the parasite. Mol Biochem Parasitol 91:165–193

Oberle M, Balmer O, Brun R, Roditi I (2010) Bottlenecks and the maintenance of minor genotypes during the life cycle of Trypanosoma brucei. PLoS Pathog 6:e1001023. doi:10.1371/journal.ppat.1001023

Overath P, Chaudhri M, Steverding D, Ziegelbauer K (1994) Invariant surface proteins in bloodstream forms of Trypanosoma brucei. Parasitol Today (Regul Ed) 10:53–58

Overath P, Stierhof YD, Wiese M (1997) Endocytosis and secretion in trypanosomatid parasites – Tumultuous traffic in a pocket. Trends Cell Biol 7:27–33. doi:10.1016/S0962-8924(97)10046-0

Pal A, Hall BS, Jeffries TR, Field MC (2003) Rab5 and Rab11 mediate transferrin and anti-variant surface glycoprotein antibody recycling in Trypanosoma brucei. Biochem J 374:443–451. doi:10.1042/BJ20030469

Pan W, Ogunremi O, Wei G et al (2006) CR3 (CD11b/CD18) is the major macrophage receptor for IgM antibody-mediated phagocytosis of African trypanosomes: diverse effect on subsequent synthesis of tumor necrosis factor alpha and nitric oxide. Microbes Infect 8:1209–1218. doi:10.1016/j.micinf.2005.11.009

Parsons M, Nelson RG, Watkins KP, Agabian N (1984) Trypanosome mRNAs share a common 5′ spliced leader sequence. Cell 38:309–316

Pays E, Lheureux M, Steinert M (1981) Analysis of the DNA and RNA changes associated with the expression of isotypic variant-specific antigens of trypanosomes. Nucleic Acids Res 9:4225–4238

Pays E, Guyaux M, Aerts D et al (1985) Telomeric reciprocal recombination as a possible mechanism for antigenic variation in trypanosomes. Nature 316:562–564

Povelones ML, Gluenz E, Dembek M et al (2012) Histone H1 plays a role in heterochromatin formation and VSG expression site silencing in Trypanosoma brucei. PLoS Pathog 8:e1003010. doi:10.1371/journal.ppat.1003010

Radwanska M, Guirnalda P, De Trez C et al (2008) Trypanosomiasis-induced B cell apoptosis results in loss of protective anti-parasite antibody responses and abolishment of vaccine-induced memory responses. PLoS Pathog 4:e1000078. doi:10.1371/journal.ppat.1000078

Recker M, Buckee CO, Serazin A et al (2011) Antigenic variation in Plasmodium falciparum malaria involves a highly structured switching pattern. PLoS Pathog 7:e1001306. doi:10.1371/journal.ppat.1001306

Reece SE, Pollitt LC, Colegrave N, Gardner A (2011) The meaning of death: evolution and ecology of apoptosis in protozoan parasites. PLoS Pathog 7:e1002320. doi:10.1371/journal.ppat.1002320

Riethman H, Ambrosini A, Paul S (2005) Human subtelomere structure and variation. Chromosome Res 13:505–515. doi:10.1007/s10577-005-0998-1

Rivero FD, Saura A, Prucca CG et al (2010) Disruption of antigenic variation is crucial for effective parasite vaccine. Nat Med 16:551–557. doi:10.1038/nm.2141, 1p following 557

Robinson NP, Burman N, Melville SE, Barry JD (1999) Predominance of duplicative VSG gene conversion in antigenic variation in African trypanosomes. Mol Cell Biol 19:5839–5846

Rogers MB, Hilley JD, Dickens NJ et al (2011) Chromosome and gene copy number variation allow major structural change between species and strains of Leishmania. Genome Res 21: 2129–2142. doi:10.1101/gr.122945.111

Roth CW, Bringaud F, Layden RE et al (1989) Active late-appearing variable surface antigen genes in Trypanosoma equiperdum are constructed entirely from pseudogenes. Proc Natl Acad Sci USA 86:9375–9379

Salmon D, Vanwalleghem G, Morias Y et al (2012) Adenylate cyclases of Trypanosoma brucei inhibit the innate immune response of the host. Science 337:463–466. doi:10.1126/science.1222753

Scherf A, Lopez-Rubio JJ, Riviere L (2008) Antigenic variation in Plasmodium falciparum. Annu Rev Microbiol 62:445–470. doi:10.1146/annurev.micro.61.080706.093134

Schwede A, Jones N, Engstler M, Carrington M (2011) The VSG C-terminal domain is inaccessible to antibodies on live trypanosomes. Mol Biochem Parasitol 175:201–204. doi:10.1016/j.molbiopara.2010.11.004

Seed JR, Sechelski J (1988) Growth of pleomorphic Trypanosoma brucei rhodesiense in irradiated inbred mice. J Parasitol 74:781–789

Seed JR, Seed JR, Wenck MA, Wenck MA (2003) Role of the long slender to short stumpy transition in the life cycle of the African trypanosomes. Kinetoplastid Biol Dis 2:3. doi:10.1186/1475-9292-2-3

Seyfang A, Mecke D, Duszenko M (1990) Degradation, recycling, and shedding of Trypanosoma brucei variant surface glycoprotein. J Protozool 37:546–552

Smith TK, Vasileva N, Gluenz E et al (2009) Blocking variant surface glycoprotein synthesis in Trypanosoma brucei triggers a general arrest in translation initiation. PLoS One 4:e7532. doi:10.1371/journal.pone.0007532

Stanne TM, Rudenko G (2010) Active VSG expression sites in Trypanosoma brucei are depleted of nucleosomes. Eukaryot Cell 9:136–147. doi:10.1128/EC.00281-09

Stringer JR, Keely SP (2001) Genetics of surface antigen expression in Pneumocystis carinii. Infect Immun 69:627–639. doi:10.1128/IAI.69.2.627-639.2001

Taylor JE, Rudenko G (2006) Switching trypanosome coats: what's in the wardrobe? Trends Genet 22:614–620. doi:10.1016/j.tig.2006.08.003

Thon G, Baltz T, Giroud C, Eisen H (1990) Trypanosome variable surface glycoproteins: composite genes and order of expression. Genes Dev 4:1374–1383

Tiengwe C, Marcello L, Farr H et al (2012) Genome-wide analysis reveals extensive functional interaction between DNA replication initiation and transcription in the genome of Trypanosoma brucei. Cell Rep 2:185–197. doi:10.1016/j.celrep.2012.06.007

Timmers HT, De Lange T, Kooter JM, Borst P (1987) Coincident multiple activations of the same surface antigen gene in Trypanosoma brucei. J Mol Biol 194:81–90

Turner CMR (1997) The rate of antigenic variation in fly-transmitted and syringe-passaged infections of Trypanosoma brucei. FEMS Microbiol Lett 153:227–231

Turner CMR (1999) Antigenic variation in Trypanosoma brucei infections: an holistic view. J Cell Sci 112(Pt 19):3187–3192

Turner C, Barry J (1989) High frequency of antigenic variation in Trypanosoma brucei rhodesiense infections. Parasitology 99:67–75

Turner CM, Barry JD, Maudlin I, Vickerman K (1988) An estimate of the size of the metacyclic variable antigen repertoire of Trypanosoma brucei rhodesiense. Parasitology 97(Pt 2):269–276

Turner CM, Aslam N, Dye C (1995) Replication, differentiation, growth and the virulence of Trypanosoma brucei infections. Parasitology 111(Pt 3):289–300

Ulbert S, Chaves I, Borst P (2002) Expression site activation in Trypanosoma brucei with three marked variant surface glycoprotein gene expression sites. Mol Biochem Parasitol 120: 225–235

Van Den Abbeele J, Claes Y, van Bockstaele D et al (1999) Trypanosoma brucei spp. development in the tsetse fly: characterization of the post-mesocyclic stages in the foregut and proboscis. Parasitology 118(Pt 5):469–478

Van der Ploeg LH, Cornelissen AW, Barry JD, Borst P (1984) Chromosomes of kinetoplastida. EMBO J 3:3109–3115

Van Meirvenne N, Magnus E, Buscher P (1995) Evaluation of variant specific trypanolysis tests for serodiagnosis of human infections with Trypanosoma brucei gambiense. Acta Trop 60: 189–199

Vassella E, Reuner B, Yutzy B, Boshart M (1997) Differentiation of African trypanosomes is controlled by a density sensing mechanism which signals cell cycle arrest via the cAMP pathway. J Cell Sci 110(Pt 21):2661–2671

Vickerman K (1969) On the surface coat and flagellar adhesion in trypanosomes. J Cell Sci 5: 163–193

Wang Q-P, Kawahara T, Horn D (2010) Histone deacetylases play distinct roles in telomeric VSG expression site silencing in African trypanosomes. Mol Microbiol 77:1237–1245. doi:10.1111/j.1365-2958.2010.07284.x

Weiden M, Osheim YN, Beyer AL, Van der Ploeg LH (1991) Chromosome structure: DNA nucleotide sequence elements of a subset of the minichromosomes of the protozoan Trypanosoma brucei. Mol Cell Biol 11:3823–3834

West SA, Griffin AS, Gardner A, Diggle SP (2006) Social evolution theory for microorganisms. Nat Rev Microbiol 4:597–607. doi:10.1038/nrmicro1461

Wickstead B, Ersfeld K, Gull K (2004) The small chromosomes of Trypanosoma brucei involved in antigenic variation are constructed around repetitive palindromes. Genome Res 14: 1014–1024. doi:10.1101/gr.2227704

Young R, Taylor JE, Kurioka A et al (2008) Isolation and analysis of the genetic diversity of repertoires of VSG expression site containing telomeres from Trypanosoma brucei gambiense, T. b. brucei and T. equiperdum. BMC Genomics 9:385

Zhuang Y, Futse JE, Brown WC et al (2007) Maintenance of antibody to pathogen epitopes generated by segmental gene conversion is highly dynamic during long-term persistent infection. Infect Immun 75:5185–5190. doi:10.1128/IAI.00913-07

Bridging Innate and Adaptive Immunity in African Trypanosomiasis

4

John M. Mansfield, Donna M. Paulnock, and Gina M. Hedberg

Abstract

Infection of man and domestic animals with Brucei group African trypanosomes results in a fatal disease. The immunobiological events that underlie temporal host resistance and susceptibility during an infection are complex but informative, because the parasites activate and engage nearly every element of the host innate and adaptive immune system. The ability of trypanosomes to undergo extensive antigenic variation provides a means to escape adaptive immunity, but these protozoan pathogens also activate and regulate many elements of the host immune response to their own end. This chapter highlights the trypanosome elements that bridge innate and adaptive immune responses in the infected mammalian host: the pathogen-associated molecular patterns (PAMPs) that trigger the innate immune response; the associated pattern recognition receptors (PRRs) on innate immune cells and subcellular signaling events that are activated; the resulting pattern of pro-inflammatory gene expression that shapes the nascent adaptive immune response; and, the downstream elements that ultimately cause host resistance to fail. The chapter concludes with promising new approaches, informed by recent studies of immunological memory, aimed at protecting trypanosome infected hosts against a broad range of antigenic variants.

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4.1 Introduction

The immunobiology of infection with African trypanosomes presents a complex and extremely interesting landscape for the contemporary immunologist. The parasites expose the host innate immune system to PAMPs that engage PRRs on myeloid cells such as macrophages and dendritic cells; these results in the early activation of genes and the release of factors that initially are protective and that shape the nascent adaptive immune response to trypanosome antigens. However, early protective events are superseded by negative regulatory events that, in conjunction with antigenic variation by the parasite, ultimately inhibit adaptive immunity, immunological memory, and lead to death of the infected host. It is the aim of this chapter to dissect and evaluate the significant immunological events that are triggered and regulated during trypanosomiasis.

Historical Perspective. First, it is important to shed some light on current knowledge regarding immunity to Brucei group trypanosomes (e.g., *Trypanosoma brucei, T. brucei rhodesiense, and T. b. gambiense*). In many medical texts and research reviews, it is not uncommon to see the immunology of trypanosomiasis presented as a simple cartoon in which numbers of trypanosomes in the bloodstream are shown to fluctuate asynchronously with the production of host antibody to the variant surface glycoprotein (VSG) coat of trypanosomes and with the emergence and immune escape of variant antigenic types (VATs) that express new VSG surface coats. This simplistic view of immunity to African trypanosomes has evolved and persisted in the literature for more than a century. It is, however, hopelessly incomplete and conveys no insight into the biologically more relevant host immune responses and parasite immune evasion mechanisms that we now know to be important in shaping host immunity during infection.

The view that VSG-specific B-cell responses are the sole basis for host resistance began to change over 20 years ago [see recent overviews in (Mansfield and Paulnock 2005; Paulnock et al. 2010)]. The paradigm first began to unravel with publication of studies on trypanosome resistant and susceptible semi-allogeneic bone marrow chimera mice, in which VSG-specific antibody was shown not to be functionally linked to overall host resistance (Fig. 4.1a) (DeGee and Mansfield 1984). Subsequently, classical genetic studies that employed crosses between resistant and susceptible animal strains, as well as results from recombinant inbred strains derived from resistant and susceptible mice, were used to demonstrate that antibody-mediated control of parasitemia by itself was not genetically linked to the host resistance phenotype (Fig. 4.1b) (De Gee et al. 1988). Thus, although VSG-specific antibodies provide an important mechanism for controlling parasite burden in the blood (and also provide an unerringly specific mechanism to select for new VATs in the process), this event alone is not linked functionally or genetically to overall host resistance during infection.

These seminal findings laid the foundation for exploratory studies in which resistant and susceptible animals were examined for comparative T cell-mediated immune responses to trypanosome antigens. The first direct evidence for

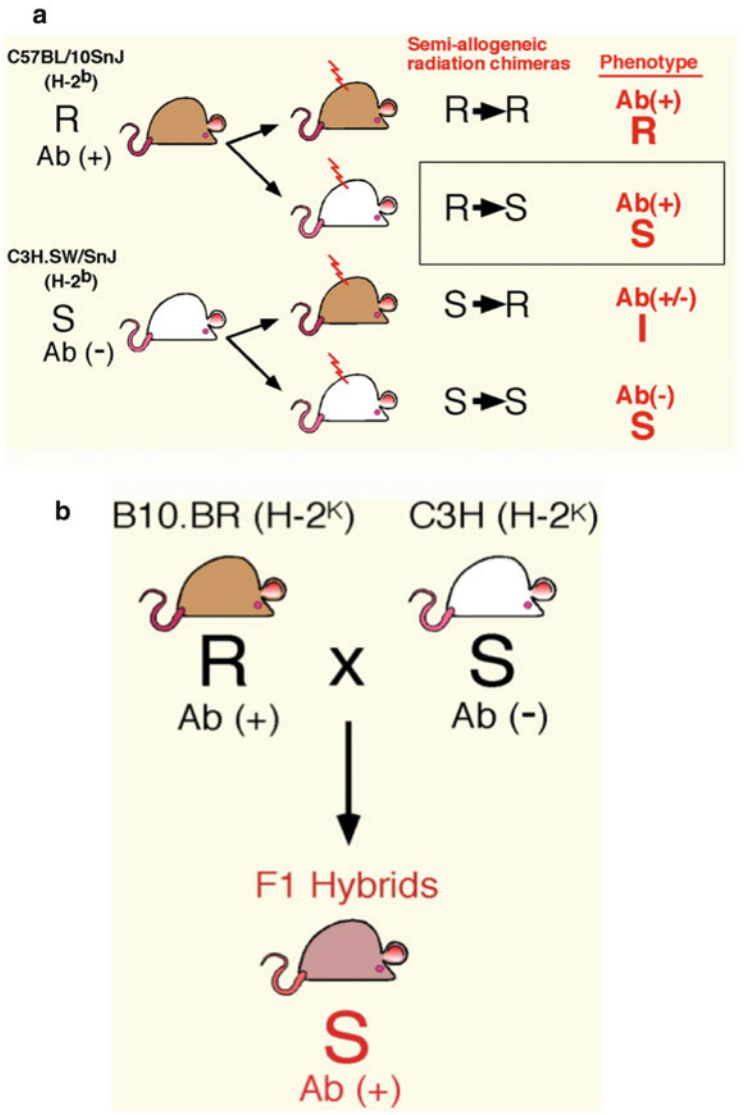


Fig. 4.1 VSG-specific B-cell responses and control of parasitemia, alone, are not functionally (**a**) or genetically (**b**) linked to the overall host resistance phenotype in trypanosomiasis. (**a**) Semi-allogeneic bone marrow cell (BMC) radiation chimeras were constructed between relatively resistant and susceptible mice (R and S mice) in which BMC donors were either positive (Ab+) or negative (Ab−) for VSG-specific antibody responses. The key finding was that R > S chimeras (lethally irradiated S mice that were reconstituted with BMC from R mice) made antibody to VSG and controlled parasitemia like the R donor mice, were but as susceptible as the S mice. (**b**) Classical genetics studies were performed by making crosses between Ab+ R mice and Ab− S mice and examining the F1, F2 and backcross generations. The key finding was that all F1 hybrids made VSG-specific Ab and controlled parasitemia like the R parent strain, but were all susceptible like the S parent strain. Adapted from: DeGee and Mansfield (1984) and De Gee et al. (1988)

VSG-specific T helper (Th)-cell responses came from experimental studies with *T. brucei rhodesiense* infections of mice (Schleifer et al. 1993; Schleifer and Mansfield 1993): VSG-activated T cells were shown to be $CD4^+$ lymphocytes that expressed the CD3+ α/β+ TCR membrane complex, were antigen processing cell (APC)-dependent and MHC II restricted in their responses. The cytokine profiles, both transcriptional and protein secretory, were those associated with highly polarized Th1-cell responses characterized by Type I cytokine production without detectable Type 2 cytokine responses (Schleifer et al. 1993; Hertz et al. 1998; Schopf et al. 1998; Drennan et al. 2005; Dagenais et al. 2009a, b). Thus, VSG preferentially stimulates a highly polarized Th 1 cell cytokine response during trypanosome infection.

An important outcome of VSG-specific Th1-cell activation is the secretion of interferon-gamma (IFN-γ) (Schleifer et al. 1993); this cytokine was formally linked to relative host resistance by using IFN-γ knockout mice (Hertz et al. 1998) as well as by evidence from earlier comparative approaches (De Gee et al. 1985). In fact, infection of mice lacking the IFN-γ gene was shown to result in a susceptibility phenotype similar to that of *scid* mice, which have no adaptive immune system, despite the fact that IFN-γ knockout mice made VSG-specific antibody responses that controlled trypanosomes in the bloodstream like *wt* mice (Hertz et al. 1998; Schopf et al. 1998). The current presumption is that IFN-γ activation of macrophages, along with the concomitant production of microbicidal factors such as TNF-α, RNI, and ROS (Vincendeau et al. 1992; Magez et al. 1993, 1997; Lucas et al. 1994; Drennan et al. 2005), is responsible for control of parasite burden within extravascular tissues where antibody is relatively ineffective in killing trypanosomes. Thus, the current paradigm is that VSG-specific Th1 cells and IFN-γ regulate the core component of host resistance to African trypanosomes. In the sections below we discuss the role of the innate immune system in shaping this Th1-cell response.

4.2 Innate Immunity to Trypanosomes

The innate immune system is alerted to trypanosome infection within hours of infection, leading to predictable patterns of gene expression and downstream resistance mechanisms being activated (De Gee et al. 1985; Magez et al. 1998; Coller and Paulnock 2001; Coller et al. 2003; Drennan et al. 2005; Harris et al. 2006; Leppert et al. 2007; Lopez et al. 2008). Here we review the trypanosome PAMPs and the consequences of their recognition by PRRs expressed on a subset of cells in the innate immune system.

4.2.1 The Glycosylphosphatidylinositol (GPI) Anchor of VSG Molecules

African trypanosomes have evolved a structured monomolecular surface coat that covers the entire plasma membrane of the parasite, including the flagellum (Vickerman and Luckins 1969; Cross 1975). This surface coat consists of a highly ordered and densely packed array of ~10^7 identical GPI-anchored VSG homodimers that both determine the antigenic phenotype of the VAT and prevent antibody and innate immune elements such as complement from binding to subsurface invariant epitopes of the coat or to membrane determinants beneath. Despite extensive primary sequence variation among VSGs that comprise different surface coats, secondary and tertiary structural features of these molecules (and the amino acids residues that determine such features) are highly conserved within the ordered coat structure (Metcalf et al. 1987; Freymann et al. 1990; Carrington et al. 1991a, b; Reinitz et al. 1992; Blum et al. 1993; Chattopadhyay et al. 2005). These structural features are explored in more detail below.

Activation of the Innate Immune System. During infection, GPI anchors are cleaved by activation of an endogenous trypanosome GPI-phospholipase C that appears to be localized to an external linear array along the flagellum (Mensa Wilmot et al. 1990; Field et al. 1991; Carrington et al. 1998; Hanrahan et al. 2009). When GPI-anchored VSG flows across the membrane into this site, the activated enzyme cleaves the anchor and releases VSG from the membrane with residual glycosylinositolphosphate (GIP) residues attached to the shed VSG (GIP-sVSG) (Fig. 4.2) (Webb et al. 1994; Webb et al. 1997a, b; Hanrahan et al. 2009). The release of GIP-sVSG occurs during episodes of high parasite burden in the presence or absence of functional adaptive immunity and is readily detectable in host tissues and blood (Fig. 4.3a).

During early stages of infection macrophages and dendritic cells display the hallmarks of a "classical" or pro-inflammatory activation profile; this includes production of trypanocidal molecules (ROS, RNI, and TNF-α), pro-inflammatory cytokines (including IL-6, IL-12, and IFN-α), and changes in functional capacity (including increased expression of MHC II and co-stimulatory molecules that are relevant for APC activity) (Magez et al. 1998, 2002; Paulnock and Coller 2001; Coller et al. 2003; Harris et al. 2006; Leppert et al. 2007; Barkhuizen et al. 2008; Lopez et al. 2008; Dagenais et al. 2009a, b). It is the GIP residues of shed VSG that activate cells of the innate immune system early in infection (Tachado et al. 1997; Magez et al. 1998, 2002; Paulnock and Coller 2001; Mansfield and Paulnock 2005); in resistant animals this may be supplemented by IFN-γ released by NK cells that have been stimulated by IL-12, below.

GIP-sVSG binds to the Type A scavenger receptor (SR-A) on the macrophage membrane (Leppert et al. 2007). This triggers internalization and delivery of the complex to an endolysosomal compartment, initiating a cascade of subcellular signaling events including activation of NFkB and MAPK pathways and the expression of a subset of pro-inflammatory genes (Fig. 4.3b) (Coller and Paulnock

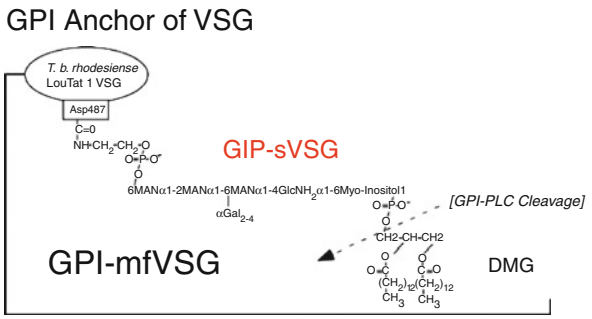


Fig. 4.2 A GPI anchor molecule tethers VSG to the plasma membrane. During infection an endogenous glycosyl-phosphatidylinositol-phospholipase C (GPI-PLC) cleaves the anchor of the membrane form of VSG (GPI-mfVSG) to release the soluble glycosylinositolphosphate-VSG (GIP-sVSG) molecule from the membrane into blood and tissues. Adapted from: Magez et al. (1998) and Coller and Paulnock (2001)

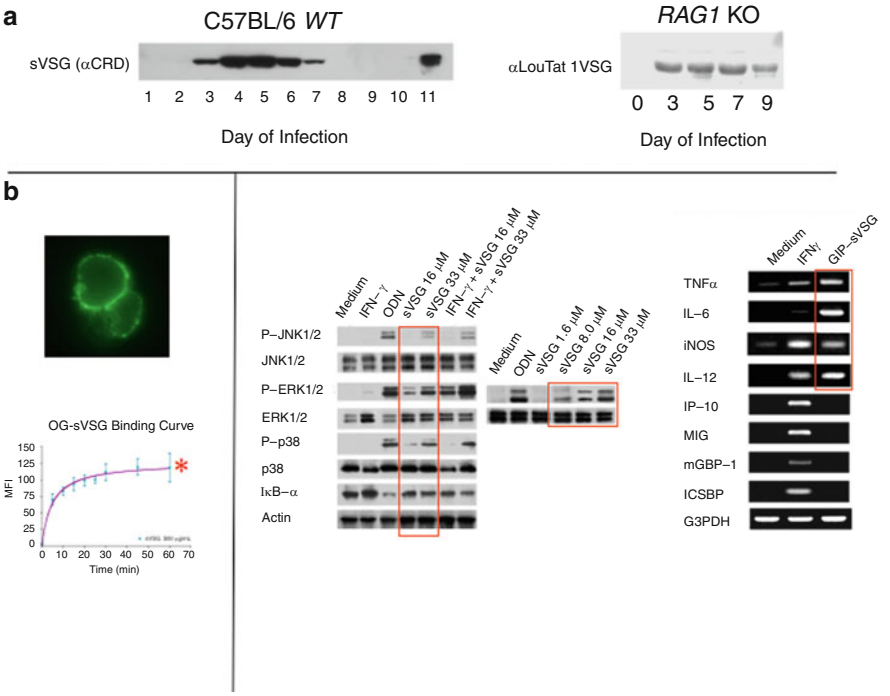


Fig. 4.3 GIP-sVSG triggers a pro-inflammatory innate immune response. (**a**) GIP-sVSG is detectable in the blood of trypanosome infected *wt* mice as well as adaptive immune-deficient *RAG1* knockout mice. (**b**) Binding of GIP-sVSG to macrophage membranes, induction of NFkB- and MAPK-signaling pathways, and upregulation of pro-inflammatory gene expression. Adapted from: Leppert et al. (2007) Coller et al. (2003)

2001; Paulnock and Coller 2001; Coller et al. 2003; Leppert et al. 2007; Lopez et al. 2008). These activation events are modulated by additional factors, including (a) Traf6-, MyD88-, and Toll-Like Receptor (TLR)-dependent *down*regulation of the GIP-sVSG-induced NFkB responses (Leppert et al. 2007); (b) augmentation of the response in the presence of IFN-γ (Coller et al. 2003; Leppert et al. 2007; Lopez et al. 2008); and (c) CpG DNA released from senescent or damaged trypanosomes (see below) (Harris et al. 2006, 2007).

An additional key component of macrophage and dendritic cell activation during infection is engagement of IFN-γ with the IFN-γR that generates the formation and phosphorylation of STAT1 homodimers that activate downstream pro-inflammatory genes. The relative timing of macrophage and innate immune cell exposure to GPI substituents and to IFN-γ is critical for the ability of cells to become activated for APC functions and, for macrophages, to kill the parasites. If there is coincident exposure to significant levels of both IFN-γ and GIP-sVSG, augmentation of cellular activation and transcription of pro-inflammatory genes occurs (Coller et al. 2003; Lopez et al. 2008; Freeman et al. 2013). This event may predominate in early infection with activated NK cells releasing IFN-γ in response to IL-12, (Demick et al. 2013), as well as later with IFN-γ derived from T cells during activation of the adaptive immune response. This augmentation may help to control the early rapid growth of trypanosomes within host tissues. However, if cells are exposed to GIP-sVSG substituents prior to, or in the absence of, sufficient levels of IFN-γ, STAT1 is not phosphorylated in response to IFN-γ and the production of trypanocidal factors as well as APC functions are inhibited (Coller et al. 2003; Dagenais et al. 2009a, b; Freeman et al. 2013). These events likely occur as the infection progresses, when increasing numbers of parasites release GIP-sVSG prior to sufficient activation of new Th1-cell (and NK-cell) responses for the production of IFN-γ.

Thus the interplay and timing of parasite and host factors are critical for control of the parasite burden in host tissues; the ability of GIP-sVSG substituents to block additional parasite killing by interfering with IFN-γ-dependent activation of macrophages and by inhibiting APC function (see below) may be central to downstream parasite evasion of host adaptive immunity. And, there are additional elements of GPI-induced cellular activation that impact on host resistance. For example, GIP-sVSG induces a wide range of interferon response genes including the IRF-7 transcription factor, discussed below (Lopez et al. 2008); the subsequent production of IFN-α/β (De Gee et al. 1985; Lopez et al. 2008) may set in motion the IRF-7- and IFN-α/β-dependent modulation of host resistance during late stage infection that has been shown to inhibit IFN-γ production by Th1 cells (Lopez et al. 2008).

Alteration of GPI-Induced SR-A Signaling. As noted above, GIP-sVSG is delivered to an intracellular endolysosomal compartment of macrophages and dendritic cells by SR-A-mediated uptake (Leppert et al. 2007). SR-As are expressed primarily but not exclusively on cells of the monocyte/macrophage lineage, some DCs, as well as on other cells; they are trimeric integral membrane glycoproteins

that demonstrate broad ligand-binding properties including altered self proteins and many different types of microbial PAMPs. Scavenger receptors were once thought to serve simply as non-opsonic phagocytic receptors for many ligands; however, it is now clear that there are direct signaling events generated by ligand engagement of these receptors, including SR-A, as seen in several parasite-unrelated studies in which tyrosine phosphorylation and activation of MAPK and NFkB pathways appear to be hallmarks of SR-A-mediated signaling (Coller et al. 2003; Todt et al. 2008; DeWitte-Orr et al. 2010). Further, SR-A and other scavenger receptors have now been shown to interact functionally and synergistically with other PRRs of the innate immune system, including TLRs (Bojarova et al. 2008; Bowdish and Gordon 2009; Bowdish et al. 2009; DeWitte-Orr et al. 2010; Stewart et al. 2010; Yew et al. 2010), to generate functional innate immune responses to different microbial pathogens.

PAMP recognition that leads to combinatorial activation of multiple PRRs appears to be an evolutionarily conserved but poorly understood and complex phenomenon (Amit et al. 2009). To date it is clear that TLR-*in*dependent signaling is triggered by GIP-sVSG binding to SR-A, and that NFkB and MAPK signaling pathways are activated after internalization of the complex (Leppert et al. 2007; Paulnock et al. 2010; Mansfield and Paulnock, unpublished observations). However, the GIP-sVSG/SR-A interaction also activates subsequent TLR-, MyD88-, and Traf6-*dependent* signaling events that downregulate the initial response (Leppert et al. 2007; Mansfield and Paulnock, unpublished observations). While scavenger receptor interactions in other studies have been shown to promote positive TLR-mediated signaling effects by catalyzing activation, dimerization, or multimerization of TLRs, the current evidence in trypanosomiasis is that GIP-sVSG/SR-A-induced signaling triggers a coincident negative regulatory TLR-signaling cascade that modifies the initial SR-A-signaling events (Leppert et al. 2007; Paulnock et al. 2010; Mansfield and Paulnock, unpublished observations). Preliminary studies have suggested that TLR9 is the intracellular receptor to which GIP-sVSG is delivered and that mediates the TLR- and Traf6-dependent negative regulatory effect on GIP-sVSG-induced signaling (Mansfield and Paulnock, unpublished observations). It is not yet known which substituent of the GIP moiety serves as the TLR9 ligand. An early concern was that contamination of GIP-sVSG preparations with trypanosome CpG DNA was a contributory factor, since unmethylated CpG DNA released during infection also induces macrophage activation (see below) (Harris et al. 2006, 2007); however, HPLC purification of GIP-sVSG, the use of DNase treatments, and the knowledge that trypanosome CpG DNA is stimulatory only in significantly high concentrations (Harris et al. 2006; Leppert et al. 2007) revealed this was not the case. The current hypothesis is that a phosphate residue of GIP, exposed as part of the GPI anchor residuum after GPI-PLC cleavage of the membrane anchored form of GPI (GPI-mfVSG), is the most likely candidate for TLR9 interaction based on recent studies elaborating similar interactions in other systems (Gangloff and Gay 2008). Thus, the overall evidence is that TLRs are not required for SR-A-induced signaling because the absence, inhibition, or ablation of TLR signaling results in enhanced NFkB and MAPK

signaling by GIP-sVSG (Leppert et al. 2007; Mansfield and Paulnock, unpublished observations).

The overall biological impact of the GIP-sVSG/SR-A-mediated innate immune activation response on trypanosome infection is not entirely clear. The current presumption is that it results in an early protective effect during infection due to the release of trypanocidal effector molecules, but that counter-protective effects supersede as infection progresses (see discussion below) (Coller and Paulnock 2001; Coller et al. 2003; Mansfield and Paulnock 2005; Mansfield and Olivier 2010; Paulnock et al. 2010).

4.2.2 Trypanosome CpG DNA Activation of Innate Immunity

The GPI anchor of VSG is not the only demonstrable trypanosome PAMP that is recognized by the host innate immune system. There is clear evidence that unmethylated CpG DNA, released from dead or dying trypanosomes, is detectable in the tissues of infected mice; this TLR9 ligand is capable of activating macrophages in vitro and in vivo (Harris et al. 2006). Macrophage activation by exposure to trypanosome DNA results in the induction of signal transduction, pro-inflammatory gene expression, and the production of cytokines and factors that may play an integral role in the immune response to this parasite. The presence of cell-free *T. brucei rhodesiense* DNA in the blood of both infected *wt* and *scid* mice suggests that the innate immune system is likely to encounter this PAMP during disease and that macrophages specifically may be activated by this ligand. The patterns of trypanosome DNA appearance in the blood (Harris et al. 2006) and, by inference, in the tissues of infected mice suggests that *T. brucei rhodesiense* DNA may play an accessory role in the response of the innate immune system to the parasite within the first week infection. An early innate response may be a crucial component of relative resistance in trypanosomiasis that, similar to the early activation effects of GIP-sVSG, would upregulate pro-inflammatory gene expression so that products of activated macrophages would contribute to early tissue defense against trypanosomes. Additionally, cells of the innate immune system may be exposed to trypanosome DNA following the phagocytosis of parasites. During antibody-mediated clearance of parasitemia most of the trypanosomes are cleared from blood by Kupffer cells of the liver (Dempsey and Mansfield 1983). Potentially, therefore, Kupffer cells as well as macrophages in extravascular tissues, where products of activated cells destroy trypanosomes, would be exposed to significant levels of DNA. When present, DNA has been found to be cleared from the blood very rapidly with a half-life of less than 30 min; following clearance from the blood, DNA has been found to accumulate in the liver, spleen, and kidneys (Harris et al. 2006). This suggests that there may be constant clearance of trypanosome DNA from the blood, leading to continual exposure of macrophages to this parasite PAMP.

It is worth speculation that trypanosome DNA may actually have another effect, one that alters the magnitude of GIP-sVSG-induced signaling events in innate

immune cells. As noted above, GIP-sVSG/SR-A-mediated activation of innate immune cells encompasses a TLR9-dependent component that downregulates signaling. The treatment of mice with CpG ODN has been shown to promote host resistance and elevate host adaptive immune responses (Harris et al. 2007). Therefore it may be that exposure to DNA released from trypanosomes that have been killed (presumably after the initial protective activation events and as a result of adaptive B- and T-cell immune responses, below) serves as an additional downregulatory signal to counterbalance the sequential and significant innate immune system activation by GIP-sVSG molecules that are released throughout infection. The selective pressure for this may have arisen as a means to reduce immunopathology occurring during infection.

4.2.3 Regulation of Innate Immunity

Downregulation of IFN-γ-Mediated Macrophage Activation. As mentioned above, the interplay and timing of parasite and host factors are critical for control of the parasite burden in host tissues and for regulation of host immune responses that provide such protection. There is clear evidence that GIP-sVSG substituents are capable of blocking production of trypanocidal factors and parasite killing by interference with IFN-γ-dependent activation of macrophages and also by inhibiting APC function; these inhibitory events may be central to down-stream parasite evasion of host adaptive immunity and are discussed here.

Exposure of macrophages to GIP-sVSG induces the rapid production of pro-inflammatory and protective factors including TNF-α, RNI, and ROS and this response is enhanced by initial or prior exposure of the cells to IFN-γ (Magez et al. 1998, 2002; Paulnock and Coller 2001; Coller et al. 2003; Harris et al. 2006; Leppert et al. 2007; Lopez et al. 2008; Dagenais et al. 2009a, b). However, the level and timing of exposure of macrophages to GIP-sVSG and to IFN-γ ultimately determine the macrophage response at the level of induced gene expression. Treatment of macrophages with GIP-sVSG *prior* to IFN-γ exposure results in a marked reduction of IFN-induced responses, including transcription of inducible nitric oxide synthase and production of NO; this effect is mediated through the GIP-sVSG dose-dependent inhibition of STAT1 phosphorylation, which is required for signaling by the IFN-γ receptor (Coller et al. 2003). Thus, in this manner, regulation of the ability of macrophages to respond to IFN-γ during infection is a central mechanism by which the parasites overcome host resistance, and this regulation is mediated by the trypanosome PAMP GIP-sVSG.

These events suggest that cyclical exposure of innate immune cells to varying levels of GIP-sVSG as the infection progresses can result in changes in the macrophage response to IFN-γ during the course of infection. Given that infection with *Trypanosoma brucei* spp. can result in a chronic course of infection in relatively resistant animals and in humans, this may be one mechanism not only for permitting parasites to escape from destruction in the tissues but also for

controlling host tissue pathology. However, a key question is when these changes in macrophage responses, and the accompanying changes in functional activity, occur and at what level they continue to fluctuate during the course of infection. Studies in a *Trypanosoma congolense* experimental model system as well as in some human infections with *Trypanosoma brucei* spp. have suggested that during the later stages of infection there is a permanent shift in the macrophage activation state from a "classical" to an "alternative" activation phenotype, with the latter state associated with production of anti-inflammatory cytokines including IL-10 (Uzonna et al. 1999; Kaushik et al. 2000; MacLean et al. 2001; Sternberg et al. 2005). A similar trend was observed in a *T. brucei brucei* model system, but only when GPI-PLC$^{-/-}$ trypanosomes, which do not shed GIP-sVSG from their VSG coat, were used for infection (Webb et al. 1997a, b), demonstrating both that GIP-sVSG release is a critical component of the pro-inflammatory profile of infected animals infected by *wt* trypanosomes and that such release may inhibit host resistance. Further, other lines of evidence suggest that it is unlikely a full "alternatively activated" macrophage response is induced in Brucei group trypanosome infection (Stijlemans et al. 2007), as a Th2-cell cytokine response, associated with alternatively activated macrophages and capable of IL-4 production, has not been detected during chronic infection, even in IL-12 knockout mice that lack the ability to induce early Th1 cell polarization (Dagenais et al. 2009a, b).

Thus, IL-10 production may have a non-Th2 cell origin in trypanosomiasis, and infected host macrophages are known to be one such source (Harris et al. 2006, 2007). Other elements, as discussed above, may more directly affect the activation profile of macrophages. Macrophages from chronically infected animals are impaired in the ability to release IFN-γ-induced pro-inflammatory cytokines and trypanocidal factors when they encounter PAMPs such as trypanosome GIP-sVSG, CpG DNA, or LPS, *prior* to IFN-γ stimulation (Coller et al. 2003; Harris et al. 2006), yet IL-10 production is expressed in infected animals and humans in both impaired and unimpaired macrophages (Kaushik et al. 2000; Namangala et al. 2000a, b; MacLean et al. 2001; Sternberg et al. 2005; Harris et al. 2006; Lopez et al. 2008; Mansfield and Paulnock, unpublished observations). These alterations are seen in experimental animals as early as the second week of infection and in association with emergence of new waves of parasitemia (Mansfield and Paulnock, unpublished observations). A recent additional example in which trypanosomes modify innate immune resistance of the host is shown in a study where adenylate cyclases released from phagocytosed or damaged parasites exhibited a downregulatory effect on the release of TNF-α from myeloid cells in the liver and, potentially, other tissues (Salmon et al. 2012). The hypothesis from this work is that by selectively reducing TNF-α, the parasites may escape from destruction and gain an advantage following early infection, permitting the organisms to become quickly established in host tissues.

These collective altered macrophage response phenotypes may be similar to those observed during induced tolerance to TLR ligands, and such alterations may provide a significant advantage to the parasite in terms of survival (Medvedev et al. 2000). Overall, the response capability of innate immune cells clearly is diminished

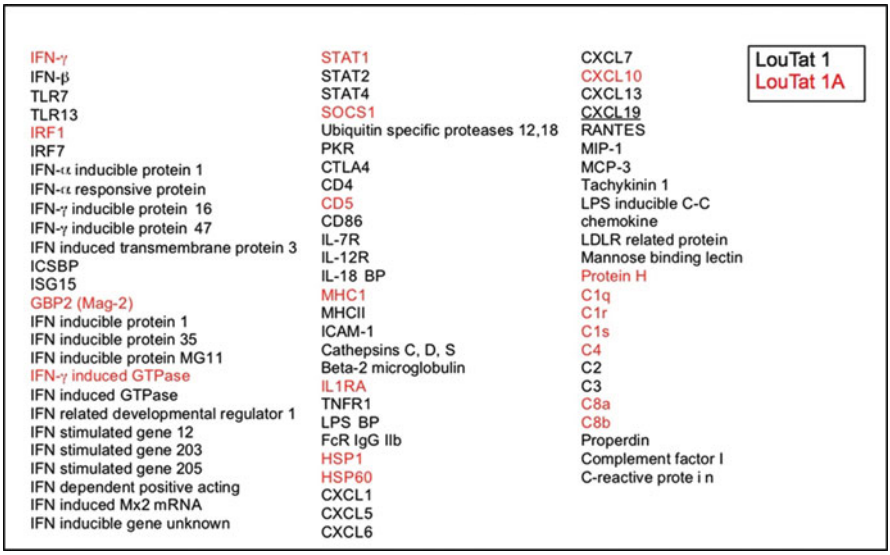
as infection progresses and this may be the partial basis for subsequent alterations in APC function described below. Thus it is clear that macrophage plasticity during infection results in a wide range of activation responses at various times in trypanosomiasis. The ability of GIP-sVSG exposure to dramatically reduce the ability of macrophages to respond to subsequent activation by IFN-γ, above, may be the most important of these changes and has obvious implications for host resistance, since IFN-γ activation of macrophages for the production of trypanocidal or trypanostatic factors in the extravascular environment is a key element in host protection.

Negative Regulation of IFN-γ Production by Type I Interferons. An additional outcome of trypanosome-induced innate immune activation that impacts on IFN-γ-mediated host resistance is the production of Type I interferons (IFN-α/β) during infection (De Gee et al. 1985; Lopez et al. 2008). Microarray analysis of naïve macrophages exposed to GIP-sVSG in vitro reveal that a prominent subset of the genes activated are associated with Type I interferon (IFN-α/β) responses, including IRF-7 and IFN-α expression (Table 4.1) (Lopez et al. 2008), suggesting that GIP-sVSG recognition by macrophages results in activation of the IRF family of transcription factors. The subsequent production of IFN-α/β may set in motion the IRF-7- and IFNAR1-dependent modulation of host resistance observed during late stage infection that inhibits IFN-γ production by Th1 cells (Lopez et al. 2008). The patterns of gene expression observed in macrophages treated with GIP-sVSG in vitro were similar to those seen in macrophages taken directly from trypanosome-infected mice within 72 h postinfection (Lopez et al. 2008), and suggests a role for IFN-α/β in GIP-sVSG-mediated macrophage activation events. Results of in vivo studies with trypanosome infection in IFNAR1$^{-/-}$ mice, which lack the receptor for Type I interferons, and UBP43$^{-/-}$ mice, which are hyper-responsive to Type I interferons, suggest that IFN-α/β may play a pivotal role in overall host resistance (Lopez et al. 2008). The results obtained using UBP43$^{-/-}$ mice are particularly informative, as the mice were more susceptible to African trypanosome infection than their *wt* counterparts; these animals initially displaying macrophage activation profiles that promoted control of parasite burden during early infection but, as the disease progressed, promoted downregulation of host IFN-γ production and a decrease in host resistance (Lopez et al. 2008). These results correlate well with observations in a variety of other non-virus microbial systems that suggest a role for IFN-α/β in modulating the course of infection, although a majority of those bacterial, fungal, and protozoan pathogen systems involve intracellular pathogens.

4.3 Adaptive Immunity to Trypanosomes

Activation of the innate immune system by trypanosome PAMPs, above, triggers a sequence of events that impact on the development of protective Th1-cell cytokine responses to parasite antigens. As discussed here, the production of pro-inflammatory

Table 4.1 Pro-inflammatory genes expressed within 72 h of trypanosome infection with low virulence *T. b. rhodesiense* LT1 (all genes) or the high virulence clone *T. b. rhodesiense* LT1A (subset). Adapted from: Lopez et al. (2008)



cytokines and expression of co-stimulatory molecules on dendritic cells and macrophages early in infection favor both development of Th1-cell responses and the apparent suppression of Th2-cell responses.

4.3.1 The "Bridge" to Polarized Th1-Cell Responses Against Parasite Antigens

African trypanosomiasis is characterized by extreme polarization of Th1-cell responses early in infection, and these T-cell responses and IFN-γ production are functionally and genetically linked to overall host resistance. The "bridge" between infection and development of VSG-specific T-cell responses is activation of the innate immune system as outlined above. As illustrated above, exposure of macrophages and dendritic cells to GIP-sVSG or trypanosome CpG DNA results in the release of antimicrobial factors such as TNF-α, RNI, and ROS plus pro-inflammatory cytokines including IL-6, TNF-α, and IL-12 (Harris et al. 2006; Barkhuizen et al. 2007; Leppert et al. 2007; Lopez et al. 2008). TNF-α plus reactive nitrogen and oxygen species exhibit antimicrobial effects that may provide very early protection against infecting trypanosomes. Subsequently, while IL-6 most likely promotes the rapid expansion of VSG-specific B cells and the production of antibody that controls parasitemia, it is IL-12 that underlies early Th1 polarization

and host tissue protection by IFN-γ secretion during infection: when *wt* and IL-12 knockout mice were infected with *T. brucei rhodesiense*, early development of Type 1 cytokine (e.g., IFN-γ and IL-2)-secreting VSG-specific Th cells was markedly inhibited (Dagenais et al. 2009a, b). Also, as seen in previous studies in which Type 1 cytokines were functionally and genetically linked to relative resistance against trypanosomes (Hertz et al. 1998; Schopf et al. 1998; Drennan et al. 2005), there was no compensatory production of Type 2 cytokines by T cells (e.g., IL-4, IL-5) observed nor the development of a detectable Th2-cell response to trypanosome VSG in the absence of Th1-cell IFN-γ production. Interleukin-12 and IFN-γ both seem to induce and maintain the polarized T-cell phenotype, however there are other as yet unknown elements that contribute to polarization, as seen in the gradual emergence of polarized VSG-specific Th1-cell responses in the absence of IL-12 (Dagenais et al. 2009a, b).

Early T-cell polarization is promoted by upregulation of co-stimulatory molecules on macrophages and dendritic cells that include MHC II, CD80, and CD86 expression, as shown by the comparative downregulation of key co-stimulatory molecules on macrophages and dendritic cells, and loss of $CD8\alpha^+$ dendritic cells, from susceptible mice (absence of VSG-specific Th-cell responses) compared to resistant animals (significant Th1-cell responses) (Hertz et al. 1998; Dagenais et al. 2009a, b). Thus, infection stimulates the outgrowth of a highly polarized Th1-cell response to trypanosome antigens in an environment favoring a rapid Type I cytokine response that is linked to control of parasites by macrophage activation events in relatively resistant hosts.

4.3.2 T-Cell Receptor Specificity for VSG Peptides

VSG molecules are separated into different families based on N-terminal and C-terminal proteolytic domains, sequence homologies, and the number and distribution of cysteine residues (Carrington et al. 1991a, b; Blum et al. 1993). Alignment of different *Trypanosoma brucei spp*. VSGs within Class and Type subgroups has demonstrated that the primary amino acid sequences of VSG N-terminal domains are extremely diverse. However, VSGs share secondary and tertiary structural elements and fold in a similar manner (Carrington et al. 1991a, b; Reinitz et al. 1992; Blum et al. 1993; Chattopadhyay et al. 2005; Marcello et al. 2007). This occurs because critical amino acid residues are the same or similar at key structural sites within VSG molecules and likely reflect the need to maintain integrity of the VSG coat structure during the process of antigenic switching.

Previous genomic and protein sequencing studies revealed the presence of predicted "hypervariable" subregions within the N-terminal domain of VSG molecules (Reinitz et al. 1992; Blum et al. 1993; Field and Boothroyd 1996); these hypervariable sites were predicted to represent evolutionarily selected B- and T-cell recognition sites (Field and Boothroyd 1996; Dagenais et al. 2009a, b). However, Th1 cells appear to recognize peptides in overlapping microvariable sites distributed throughout the N-terminal domain, both within and outside of

hypervariable subregions (Dagenais et al. 2009a, b). Peptide mapping of T-cell reactive sites using VSG-specific T-cell lines, T-cell hybridomas and Th1 cells from infected mice revealed that such sites were restricted to the N-terminal domain of VSG and that no, or very weak, responses were directed against the conserved C-terminal domain (Dagenais et al. 2009a, b). Thus the C-terminal domain appears to be "immunosilent," and the absence of T-cell responses to this domain is likely the basis for a lack of immunological cross-reactivity among different VSG molecules that share C-terminal domain homology (Dagenais et al. 2009a, b).

The absence of T-cell responses to the relatively invariant C-terminal residues of VSGs appears to be a key feature of trypanosome infection, but one that is mirrored by studies of other microbial pathogens including pilin structure and immunogenicity in *Neisseria* (Hansen et al. 2007). The mechanism(s) that prevent potential T-cell recognition of this domain are unknown at present, but recent studies on modulation of APC function during trypanosomiasis reveal a progressive inability to process and present new VSG peptides to Th cells (Dagenais et al. 2009a, b; Paulnock et al. 2010; Freeman et al. 2013; Hedberg et al. 2013a, b). There is also the possibility that the C-terminal domain of VSGs, due to structural or sequence constraints, may not be accessible to cathepsins and other enzymes that degrade the molecule to appropriate peptides during APC processing; these potential features may predispose APCs to process and present only a subset of more readily processed N-terminal domain VSG peptides during infection. Further analysis of Th1-cell specificity and the identities of VSG-derived peptides presented during ongoing infection are necessary to fully understand how VSG is processed and presented by APCs and to identify the spectrum of immunodominant T-cell epitopes recognized in different Classes and Types of VSGs. However, the discovery that T cells fail to recognize peptides within the conserved C-terminal domain of VSGs opens the door to experimental approaches that could stimulate T cells to provide cross-variant protection in an infected host. This is theoretically possible because, although antibodies cannot access buried residues of the C-terminal domain, T cells could be generated that are specific for peptides derived from this domain to provide variant cross-specific protection during African trypanosomiasis. This possibility depends on the ability to generate C-terminal specific T-cell responses by immunization as well as the ability to maintain an intact pool of memory T cells in host animals upon infection. This is explored below in which recent studies of T-cell memory in African trypanosomiasis are discussed.

4.3.3 VSG-Specific T-Cell Memory

The generation of immunological memory is crucial for rapid protection against microbial pathogens upon subsequent exposure. Following clearance of infections that require a robust T-cell response, the vast majority of effector T cells undergo apoptosis (Foulds et al. 2006); however, memory T-cell populations are generated that may be long-lived (Sprent 1994). Subsequently, memory T cells may be rapidly and effectively activated when encountering antigens displayed by the same

pathogen upon reinfection. Thus the term "memory" generally refers to those cells that remain after clearance of an infection and persist in lymphoid tissues to confer lasting protection. Populations of memory $CD4^+$ Th cells play a critical role in preventing secondary microbial infections by recognizing peptides presented by MHC II molecules on APCs and by secreting cytokines that execute protective biological functions, depending on the nature of the target cell and type of infection.

Although primary VSG-specific Th1-cell responses in trypanosomiasis have been characterized, the memory cells derived from them and the persistence of VSG-specific memory $CD4^+$ Th-cell populations have only recently been studied. The presence of over 1,000 different trypanosome VSG genes and VSG pseudogenes (see also Chap. 3) (Van der Ploeg et al. 1992; Berriman et al. 2005) would imply that trypanosomes have been pressured by the immune system to avoid re-expression of the same VSG protein sequences during the course of an infection, since to do so would invite a rapid secondary immune responses to the VAT. This indirectly suggests that memory VSG- and VAT-specific $CD4^+$ Th cells are effective in suppressing reemerging VATs. However, there is evidence that VATs may reemerge during a chronic trypanosome infection (Barry and Turner 1991). Further, there is new evidence for segmental conversion among different VSG genes leading to the expression of "new" mosaic VSG coats in which VSG molecules display large variant sequences derived from other (including previously expressed) VSG genes and VSG pseudo-genes (see Chap. 3) (Barry and Turner 1991; Hall et al. 2013). These collective observations suggest that VSG peptide-specific memory T-cell responses, if generated, are perhaps ineffective or lost as infection progresses. Thus the absence of effective VSG-specific T-cell memory may permit both previous expressed and emerging mosaic trypanosome VATs to survive in chronically infected animals. Clearly this would provide an obstacle to successful vaccination of trypanosome-infected hosts.

Recent studies demonstrate that while *T. brucei rhodesiense* LT1 VSG-specific Th memory cells are generated, they become immunologically unresponsive (Hedberg et al. 2013a, b). The central finding was that when T cells from infected animals were isolated and stimulated with LT 1 VSG ex vivo, they were able to mount a robust primary immune response during the first week of infection but rapidly lost the ability to respond as infection progressed into the chronic stage (Fig. 4.4). However, despite the demonstrable absence of responsiveness, flow cytometric analysis of $CD4^+$ Th cells revealed the presence of a canonical memory T-cell pool: $CD4^+$ Th cells transitioned from a naïve $CD44^{int}CD62L^{hi}$ phenotype to a canonical $CD44^{hi}CD62L^{lo}$ T memory cell phenotype, and this phenotype persisted throughout infection (Hedberg et al. 2013a, b). Thus, although functional VSG-specific Th1-cell memory was lost, phenotypically identifiable memory T cells persisted in lymphoid tissues.

It was important to eliminate the possibility that these T cells exclusively represented memory T cells specific for *different* VATs that had appeared following exposure to the infecting LT 1 VAT. Therefore, animals were drug-cured after the first parasitemia peak; this time frame encompassed a period sufficient for the generation of an effective LT 1 VSG-specific $CD4^+$ Th1-cell response, by 8–10

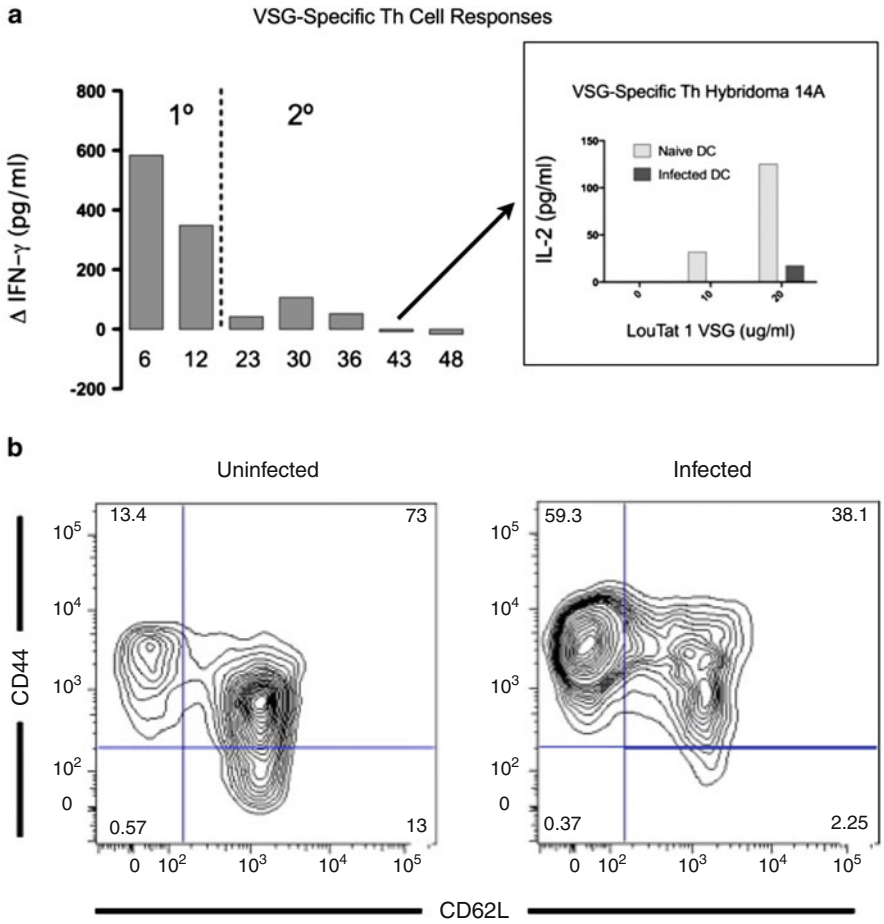


Fig. 4.4 Loss of VSG-specific Th memory cell responses during trypanosome infection. (**a**) Interferon-gamma responses of VSG-stimulated Th cells during the primary immune response (1°) and the subsequent loss of Th memory cell responses (2°), along with loss of dendritic cell (DC) APC function (*above*). (**b**) Maintenance of a Th memory cell $CD44^{hi}CD62L^{lo}$ phenotype throughout infection (*below*). Adapted from: Hedberg et al. (2013a, b)

days postinfection, but a time before new VATs emerged (Levine and Mansfield 1981, 1984; Inverso and Mansfield 1983; Dagenais et al. 2009a, b). Thus, the $CD4^+$ Th cells activated during initial infection would only be specific for antigenic determinants displayed by the infecting LT 1 VAT (De Gee et al. 1988). Drug cure approaches revealed that in the absence of an active infection, $CD4^+$ Th memory cells isolated from these animals at all time points were capable of specific and robust IFN-γ responses following ex vivo stimulation with LT 1 VSG (Hedberg et al. 2013a, b). These results supported a conclusion that memory $CD4^+$ Th cells were indeed generated as part of a large phenotypically identifiable memory T-cell

pool during the course of an infection, but that the protective function of these T cells was suppressed by active infection. Further evidence that T-memory cells in trypanosomiasis were viable and potentially functional came from studies in which infected/drug-cured $CD4^+$ Thy 1.1 congenic donor mouse T cells were transferred into naïve recipient Thy 1.2 recipient mice. The donor Thy 1.1+ cells were detectable and persisted in recipient animal lymphoid tissues for several months and, upon recall infection with the LT 1 VAT, rapidly and differentially expanded in the infected animals (Hedberg et al. 2013a, b).

The reason why viable resident T memory cells normally fail to respond during infection was informed by prior studies in which trypanosome infection resulted in the loss of APC function within 2 weeks of infection (Paulnock et al. 1989; Namangala et al. 2000a, b; Dagenais et al. 2009a, b), a time when these APCs were unable to support T-cell responses to new VATs or to parasite unrelated antigens (Dagenais et al. 2009a, b). This model system used various antigen-specific $CD4^+$ Th-cell hybridomas, in which it was demonstrated that T hybridoma cells incubated with infected mouse APCs and cognate antigen, HEL or VSG, failed to respond to cognate antigen compared to naïve mouse APCs (Dagenais et al. 2009a, b). This approach clearly showed that a defect in the ability of APCs to process and present antigen to $CD4^+$ Th cells occurred within a relatively short period of time after infection. Because animals that were drug cured of infection regained $CD4^+$ Th memory cell responsiveness (Hedberg et al. 2013a, b), this observation was taken a step further to determine if APC function was also rescued. Again, the use of T-cell hybridomas which were specific for HEL peptide or immunodominant LT 1 VSG peptides showed that infected mouse APCs failed to process and present cognate antigens. However, when animals were drug cured, APCs from those animals stimulated antigen-specific T hybridoma cells to the same degree as those incubated with uninfected mouse APCs (Hedberg et al. 2013a, b). Thus the defect observed in antigen presentation is associated with persistent infection and coincides with the loss of memory T cells, but this defect is rapidly reversible and coincides with the recovery of memory T-cell responses. This was an important demonstration, as immune suppression in trypanosomiasis has been shown to be multifaceted, involving both APCs and $CD4^+$ Th cells as well as B cells and macrophages (Mansfield and Paulnock 2005; Mansfield and Olivier 2010; Paulnock et al. 2010; Bockstal et al. 2011a, b). In summary, it was demonstrated that trypanocidal drug treatment results both in the rescue of VSG-specific $CD4^+$ Th memory cell responses and the rescue of innate macrophage and dendritic cell APC functions.

4.3.4 VAT Cross-Protective Immunity

The study discussed above was the first to definitively show that a population of VSG-specific $CD4^+$ Th memory cells is generated following trypanosome infection, that the cells are functionally suppressed during chronic infection, but that they regain the ability to respond to ex vivo or in vivo stimulation if the

trypanosome infection is drug cured concomitant with restoration of APC function. It is of potential importance that the timing of loss of T-cell memory and APC functions within 2–3 weeks of infection coincides precisely with the timing of appearance of mosaic VATs in infected animals (see Chap. 3) (Barry et al. 2012; Hall et al. 2013); the key feature is that mosaic surface coats are encoded by VSG genes that have undergone segmental recombination, permitting the mixing, ultimately, of peptide sequences derived from multiple VSG N-terminal domains into the new mosaic coat VSGs. The biological success of such a recombinant strategy would clearly be dependent on the loss of existing VSG peptide-specific memory T-cell responses, since expression of any previously expressed VSG peptide sequence as part of a mosaic coat would lead to Th cell recognition and immune destruction. The study above demonstrates that the appearance of such mosaic VSGs is precisely coincident with the loss of T cell memory during infection and suggests indirectly that the two events are of potential survival value to the parasite.

However, the further significance of findings regarding VSG-specific Th1 memory cell responses lies in exploiting this information for immunization studies. As mentioned above in Sect. 4.4.3, immunological memory is critical for the generation of effective vaccines, and so far none has been successful in preventing or controlling trypanosomiasis. Many investigators have deemed this a futile attempt for several reasons; namely, that B-cell responses are complicated by factors such as the relative inability of antibodies to access VSG coat subsurface peptides, as well as the destruction of memory B-cell populations in trypanosomiasis (see Chap. 5) (Radwanska et al. 2008; Magez and Radwanska 2009; Bockstal et al. 2011a, b). Because the ability to produce IFN-γ is a critical determinant of relative overall host resistance during infection (Hertz et al. 1998) and the recovery of $CD4^+$ Th1 cell memory have been demonstrated (Hedberg et al. 2013a, b), this permits revisiting the idea of a potential trypanosome vaccine. It has been suggested that the nature of antigenic variation and parasite VSG gene switching make the task impossible, since one can neither accurately predict what VSG will be expressed when parasites infect the host nor which VATs will arise subsequently (Van der Ploeg et al. 1992). However, there is now considerable evidence noted above that, in contrast to the highly variable N-terminal domain of the VSG molecule, the C-terminal domain is highly conserved among parasites of the same type (Carrington et al. 1991a, b; Chattopadhyay et al. 2005). Although the host mounts a robust $CD4^+$ Th-cell response to multiple peptides within the variable N-terminal domain, the response to the C terminus is low or undetectable (Dagenais et al. 2009a, b). It was reasoned that this domain of the molecule remains conserved due to the facts that it is inaccessible to antibody (Schwede et al. 2011) and that it appears to be immunosilent with respect to the protective T-cell response; thus there is no immunological pressure for the parasite to alter amino acid sequences within the C-terminal domain like the immunologically more accessible N-terminal domain.

Theoretically there is no evidence that VSG C terminus region peptides cannot be processed and presented to $CD4^+$ Th cells if animals are immunized in the absence of infection, since peptides from the C-terminal subregion contain sequence and structural motifs predicted to bind to MHC II molecules and to

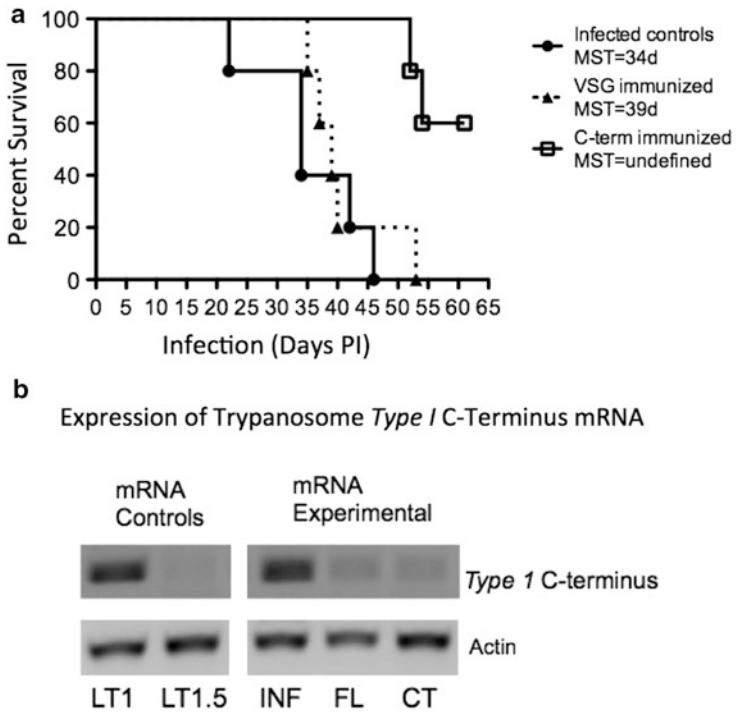


Fig. 4.5 Immunization with a conserved C-terminal VSG peptide provides protection following trypanosome infection. Survival times of control infected mice, mice immunized with full length VSG, and mice immunized with the C-terminal VSG peptide (**a**). Selection against trypanosomes expressing the Type I C-terminal VSG peptide sequence used for immunization (**b**). Adapted from: Hedberg et al. (2013a, b)

T-cell receptors (Mansfield and Paulnock, unpublished observations; Hedberg et al. 2013a, b). Thus, one may be able to induce or augment T-cell responses to this subdominant but highly conserved subregion of the VSG molecule by immunization, since memory $CD4^+$ Th cells specific for the C-terminal domain would recognize multiple parasite VATs of the same Type and could rapidly proliferate and respond soon after an infection (e.g., well in advance of loss of APC function). In fact, current studies have now demonstrated that naive animals can be immunized with VSG C terminus peptides, that Th1-cell responses can be generated by such immunization and are enhanced following infection, and that immunized animals survive significantly longer than nonimmunized infected controls (Fig. 4.5) (Hedberg et al. 2013a, b). Of interest is the observation that surviving animals in this study exhibited parasitemia but that the trypanosomes present no longer expressed the targeted C-terminal domain Type of the infecting VAT (Fig. 4.5); future approaches will include immunization with a peptide cocktail representing all VSG C-terminal domain Types (~4). Thus, the present study lays the groundwork for generating a variant cross-protective memory $CD4^+$

Th-cell population specific for invariant residues of the parasite VSG. Such approaches may be capable of thwarting the parasite's main mechanism of immune evasion by antigenic variation of the immunodominant VSG N terminus.

4.4 Going Forward

This chapter has examined immunity to trypanosomes in the light of recent contemporary immunobiological research on African trypanosomiasis. A centrally important role for VSG-specific Th1-cell responses and attendant macrophage activation in providing relative host resistance has been presented, along with evidence of a critical "bridge" role for trypanosome PAMPs, GIP-sVSG, and CpG DNA, in providing both early protection against infection and in activating and then regulating downstream adaptive immune responses. New information on T-cell memory in trypanosomiasis has been evaluated and exploited to provide new approaches to immunization against this disease by targeting conserved VSG epitopes. The prospect of inducing, prior to a natural infection, VAT cross-protective Th-cell responses is quite exciting and certainly worth pursuing. Also, the prospect of rescuing protective Th cell and APC responses by drug treatment of animals followed by immunization may be another avenue worth exploration. Finally, the observations that GIP-sVSG residues both promote and regulate activation of the infected host immune system may open avenues for the modification of these responses not only during trypanosome infection but also in other types of hypo- or hyperinflammatory diseases. Hopefully the aggregate information and ideas presented here will serve as a springboard to discovering new means to control African trypanosomiasis in man and animals, as well as new basic information on regulation of the innate immune system.

References

Amit I, Garber M, Chevrier N, Leite A, Donner Y, Eisenhaure T, Guttman M, Grenier J, Li W, Zuk O, Schubert L, Birditt B, Shay T, Goren A, Zhang X, Smith Z, Deering R, McDonald R, Cabili M, Bernstein B, Rinn J, Meissner A, Root D, Hacohen N, Regev A (2009) Unbiased reconstruction of a mammalian transcriptional network mediating pathogen responses. Science 326(5950):257–263

Barkhuizen M, Magez S, Atkinson RA, Brombacher F (2007) Interleukin-12p70-dependent interferon- gamma production is crucial for resistance in African trypanosomiasis. J Infect Dis 196(8):1253–1260

Barkhuizen M, Magez S, Ryffel B, Brombacher F (2008) Interleukin-12p70 deficiency increases survival and diminishes pathology in Trypanosoma congolense infection. J Infect Dis 198 (9):1284–1291

Barry JD, Turner CM (1991) The dynamics of antigenic variation and growth of African trypanosomes. Parasitol Today 7(8):207–211

Barry JD, Hall JP, Plenderleith L (2012) Genome hyperevolution and the success of a parasite. Ann N Y Acad Sci 1267:11–17

Berriman M, Ghedin E, Hertz-Fowler C, Blandin G, Renauld H, Bartholomeu DC, Lennard NJ, Caler E, Hamlin NE, Haas B, Bohme U, Hannick L, Aslett MA, Shallom J, Marcello L, Hou L, Wickstead B, Alsmark UC, Arrowsmith C, Atkin RJ, Barron AJ, Bringaud F, Brooks K, Carrington M, Cherevach I, Chillingworth TJ, Churcher C, Clark LN, Corton CH, Cronin A, Davies RM, Doggett J, Djikeng A, Feldblyum T, Field MC, Fraser A, Goodhead I, Hance Z, Harper D, Harris BR, Hauser H, Hostetler J, Ivens A, Jagels K, Johnson D, Johnson J, Jones K, Kerhornou AX, Koo H, Larke N, Landfear S, Larkin C, Leech V, Line A, Lord A, Macleod A, Mooney PJ, Moule S, Martin DM, Morgan GW, Mungall K, Norbertczak H, Ormond D, Pai G, Peacock CS, Peterson J, Quail MA, Rabbinowitsch E, Rajandream MA, Reitter C, Salzberg SL, Sanders M, Schobel S, Sharp S, Simmonds M, Simpson AJ, Tallon L, Turner CM, Tait A, Tivey AR, Van Aken S, Walker D, Wanless D, Wang S, White B, White O, Whitehead S, Woodward J, Wortman J, Adams MD, Embley TM, Gull K, Ullu E, Barry JD, Fairlamb AH, Opperdoes F, Barrell BG, Donelson JE, Hall N, Fraser CM, Melville SE, El-Sayed NM (2005) The genome of the African trypanosome Trypanosoma brucei. Science 309(5733):416–422

Blum ML, Down JA, Gurnett AM, Carrington M, Turner MJ, Wiley DC (1993) A structural motif in the variant surface glycoproteins of Trypanosoma brucei. Nature 362(6421):603–609

Bockstal V, Geurts N, Magez S (2011a) Acute disruption of bone marrow B lymphopoiesis and apoptosis of transitional and marginal zone B cells in the spleen following a blood-stage Plasmodium chabaudi infection in mice. J Parasitol Res 2011:534697

Bockstal V, Guirnalda P, Caljon G, Goenka R, Telfer JC, Frenkel D, Radwanska M, Magez S, Black SJ (2011b) T. brucei infection reduces B lymphopoiesis in bone marrow and truncates compensatory splenic lymphopoiesis through transitional B-cell apoptosis. PLoS Pathog 7(6): e1002089

Bojarova P, Denehy E, Walker I, Loft K, De Souza DP, Woo LW, Potter BV, McConville MJ, Williams SJ (2008) Direct evidence for ArO-S bond cleavage upon inactivation of Pseudomonas aeruginosa arylsulfatase by aryl sulfamates. Chembiochem 9(4):613–623

Bowdish DM, Gordon S (2009) Conserved domains of the class A scavenger receptors: evolution and function. Immunol Rev 227(1):19–31

Bowdish DM, Sakamoto K, Kim MJ, Kroos M, Mukhopadhyay S, Leifer CA, Tryggvason K, Gordon S, Russell DG (2009) MARCO, TLR2, and CD14 are required for macrophage cytokine responses to mycobacterial trehalose dimycolate and Mycobacterium tuberculosis. PLoS Pathog 5(6):e1000474

Carrington M, Miller N, Blum M, Roditi I, Wiley D, Turner M (1991a) Variant specific glycoprotein of Trypanosoma brucei consists of two domains each having an independently conserved pattern of cysteine residues. J Mol Biol 221:823–835

Carrington M, Walters D, Webb H (1991b) The biology of the glycosylphosphatidylinositol-specific phospholipase C of Trypanosoma brucei. Cell Biol Int Rep 15:1101–1114

Carrington M, Carnall N, Crow MS, Gaud A, Redpath MB, Wasunna CL, Webb H (1998) The properties and function of the glycosylphosphatidylinositol-phospholipase C in Trypanosoma brucei. Mol Biochem Parasitol 91(1):153–164

Chattopadhyay A, Jones NG, Nietlispach D, Nielsen PR, Voorheis HP, Mott HR, Carrington M (2005) Structure of the C-terminal domain from Trypanosoma brucei variant surface glycoprotein MITat1.2. J Biol Chem 280(8):7228–7235

Coller SP, Paulnock DM (2001) Signaling pathways initiated in macrophages after engagement of type A scavenger receptors. J Leukoc Biol 70(1):142–148

Coller SP, Mansfield JM, Paulnock DM (2003) Glycosylinositolphosphate soluble variant surface glycoprotein inhibits IFN-gamma-induced nitric oxide production via reduction in STAT1 phosphorylation in African trypanosomiasis. J Immunol 171(3):1466–1472

Cross GA (1975) Identification, purification and properties of clone-specific glycoprotein antigens constituting the surface coat of Trypanosoma brucei. Parasitology 71:393–417

Dagenais TR, Demick KP, Bangs JD, Forest KT, Paulnock DM, Mansfield JM (2009a) T-cell responses to the trypanosome variant surface glycoprotein are not limited to hypervariable subregions. Infect Immun 77(1):141–151

Dagenais TR, Freeman BE, Demick KP, Paulnock DM, Mansfield JM (2009b) Processing and presentation of variant surface glycoprotein molecules to T cells in African trypanosomiasis. J Immunol 183(5):3344–3355

De Gee AL, Sonnenfeld G, Mansfield JM (1985) Genetics of resistance to the African trypanosomes. V. Qualitative and quantitative differences in interferon production among susceptible and resistant mouse strains. J Immunol 134:2723–2726

De Gee AL, Levine RF, Mansfield JM (1988) Genetics of resistance to the African trypanosomes. VI. Heredity of resistance and variable surface glycoprotein-specific immune responses. J Immunol 140(1):283–288

DeGee AL, Mansfield JM (1984) Genetics of resistance to the African trypanosomes. IV. Resistance of radiation chimeras to Trypanosoma rhodesiense infection. Cell Immunol 87(1):85–91

Demick K, Paulnock DM, Mansfield JM (2013) Evaluation of extravascular parasite burden in infected animals by use of African trypanosomes constitutively and stably expressing a cytoplasmic eGFP+ marker (submitted for publication)

Dempsey WL, Mansfield JM (1983) Lymphocyte function in experimental African trypanosomiasis. V. Role of antibody and the mononuclear phagocyte system in variant-specific immunity. J Immunol 130(1):405–411

DeWitte-Orr SJ, Collins SE, Bauer CM, Bowdish DM, Mossman KL (2010) An accessory to the 'Trinity': SR-As are essential pathogen sensors of extracellular dsRNA, mediating entry and leading to subsequent type I IFN responses. PLoS Pathog 6(3):e1000829

Drennan MB, Stijlemans B, Van den Abbeele J, Quesniaux VJ, Barkhuizen M, Brombacher F, De Baetselier P, Ryffel B, Magez S (2005) The induction of a type 1 immune response following a Trypanosoma brucei infection is MyD88 dependent. J Immunol 175(4):2501–2509

Field MC, Boothroyd JC (1996) Sequence divergence in a family of variant surface glycoprotein genes from trypanosomes: coding region hypervariability and downstream recombinogenic repeats. J Mol Evol 42(5):500–511

Field MC, Menon AK, Cross GA (1991) Developmental variation of glycosylphosphatidylinositol membrane anchors in Trypanosoma brucei. Identification of a candidate biosynthetic precursor of the glycosylphosphatidylinositol anchor of the major procyclic stage surface glycoprotein. J Biol Chem 266(13):8392–8400

Foulds KE, Wu CY, Seder RA (2006) Th1 memory: implications for vaccine development. Immunol Rev 211:58–66

Freeman B, Mansfield JM, Paulnock DM (2013) Altered antigen processing and presentation by dendritic cells in chronic African trypanosomiasis (in preparation)

Freymann D, Down J, Carrington M, Roditi I, Turner M, Wiley D (1990) 2.9 Å resolution structure of the N-terminal domain of a variant surface glycoprotein from Trypanosoma brucei. J Mol Biol 216:141–160

Gangloff M, Gay NJ (2008) Baseless assumptions: activation of TLR9 by DNA. Immunity 28 (3):293–294

Hall JP, Wang H, Barry JD (2013) Mosaic VSGs and the scale of Trypanosoma brucei antigenic variation. PLoS Pathog (in press)

Hanrahan O, Webb H, O'Byrne R, Brabazon E, Treumann A, Sunter JD, Carrington M, Voorheis HP (2009) The glycosylphosphatidylinositol-PLC in Trypanosoma brucei forms a linear array on the exterior of the flagellar membrane before and after activation. PLoS Pathog 5(6): e1000468

Hansen JK, Demick KP, Mansfield JM, Forest KT (2007) Conserved regions from Neisseria gonorrhoeae pilin are immunosilent and not immunosuppressive. Infect Immun 75 (8):4138–4147

Harris TH, Cooney NM, Mansfield JM, Paulnock DM (2006) Signal transduction, gene transcription, and cytokine production triggered in macrophages by exposure to trypanosome DNA. Infect Immun 74(8):4530–4537

Harris TH, Mansfield JM, Paulnock DM (2007) CpG oligodeoxynucleotide treatment enhances innate resistance and acquired immunity to African trypanosomes. Infect Immun 75 (5):2366–2373

Hedberg G, Paulnock D, Mansfield J (2013) CD4+ Th memory cell responses to the variant surface glycoprotein during African trypanosomiasis (submitted for publication)

Hedberg G, Paulnock D, Mansfield J (2013) Induction of cross-variant protective immunity to the African trypanosomes following immunization with conserved C-terminal VSG sequences. (in preparation)

Hertz CJ, Filutowicz H, Mansfield JM (1998) Resistance to the African trypanosomes is IFN-gamma dependent. J Immunol 161(12):6775–6783

Inverso JA, Mansfield JM (1983) Genetics of resistance to the African trypanosomes. II. Differences in virulence associated with VSSA expression among clones of Trypanosoma rhodesiense. J Immunol 130(1):412–417

Kaushik RS, Uzonna JE, Zhang Y, Gordon JR, Tabel H (2000) Innate resistance to experimental African trypanosomiasis: differences in cytokine (TNF-alpha, IL-6, IL-10 and IL-12) production by bone marrow-derived macrophages from resistant and susceptible mice. Cytokine 12 (7):1024–1034

Leppert BJ, Mansfield JM, Paulnock DM (2007) The soluble variant surface glycoprotein of African trypanosomes is recognized by a macrophage scavenger receptor and induces I kappa B alpha degradation independently of TRAF6-mediated TLR signaling. J Immunol 179 (1):548–556

Levine RF, Mansfield JM (1981) Genetics of resistance to African trypanosomes: role of the H-2 locus in determining resistance to infection with Trypanosoma rhodesiense. Infect Immun 34(2):513–518

Levine RF, Mansfield JM (1984) Genetics of resistance to the African trypanosomes. III. Variant-specific antibody responses of H-2-compatible resistant and susceptible mice. J Immunol 133 (3):1564–1569

Lopez R, Demick KP, Mansfield JM, Paulnock DM (2008) Type I IFNs play a role in early resistance, but subsequent susceptibility, to the African trypanosomes. J Immunol 181 (7):4908–4917

Lucas R, Magez S, De-Leys R, Fransen L, Scheerlinck JP, Rampelberg M, Sablon E, De-Baetselier P (1994) Mapping the lectin-like activity of tumor necrosis factor. Science 263(5148):814–817

MacLean L, Odiit M, Sternberg JM (2001) Nitric oxide and cytokine synthesis in human African trypanosomiasis. J Infect Dis 184(8):1086–1090

Magez S, Radwanska M (2009) African trypanosomiasis and antibodies: implications for vaccination, therapy and diagnosis. Future Microbiol 4:1075–1087

Magez S, Lucas R, Darji A, Songa EB, Hamers R, De Baetselier P (1993) Murine tumour necrosis factor plays a protective role during the initial phase of the experimental infection with Trypanosoma brucei brucei. Parasite Immunol 15(11):635–641

Magez S, Geuskens M, Beschin A, del Favero H, Verschueren H, Lucas R, Pays E, de Baetselier P (1997) Specific uptake of tumor necrosis factor-alpha is involved in growth control of Trypanosoma brucei. J Cell Biol 137(3):715–727

Magez S, Stijlemans B, Radwanska M, Pays E, Ferguson MA, De Baetselier P (1998) The glycosyl-inositol-phosphate and dimyristoylglycerol moieties of the glycosylphosphatidylinositol anchor of the trypanosome variant-specific surface glycoprotein are distinct macrophage-activating factors. J Immunol 160(4):1949–1956

Magez S, Stijlemans B, Baral T, De Baetselier P (2002) VSG-GPI anchors of African trypanosomes: their role in macrophage activation and induction of infection-associated immunopathology. Microbes Infect 4(9):999–1006

Mansfield JM, Olivier M (2010) Immune evasion by parasites. In: Kaufmann SHE, Sher A, Ahmed R, Sacks D (eds) Immunology of infectious diseases, vol 2. ASM, Washington, DC, pp 379–392

Mansfield JM, Paulnock DM (2005) Regulation of innate and acquired immunity in African trypanosomiasis. Parasite Immunol 27(10–11):361–371

Marcello L, Menon S, Ward P, Wilkes JM, Jones NG, Carrington M, Barry JD (2007) VSGdb: a database for trypanosome variant surface glycoproteins, a large and diverse family of coiled coil proteins. BMC Bioinformatics 8:143

Medvedev AE, Kopydlowski KM, Vogel SN (2000) Inhibition of lipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: dysregulation of cytokine, chemokine, and toll-like receptor 2 and 4 gene expression. J Immunol 164(11):5564–5574

Mensa Wilmot K, Hereld D, Englund PT (1990) Genomic organization, chromosomal localization, and developmentally regulated expression of the glycosyl-phosphatidylinositol-specific phospholipase C of Trypanosoma brucei. Mol Cell Biol 10:720–726

Metcalf P, Blum M, Freymann D, Turner M, Wiley DC (1987) Two variant surface glycoproteins of Trypanosoma brucei of different sequence classes have similar 6 A resolution X-ray structures. Nature 325:84–86

Namangala B, Brys L, Magez S, De Baetselier P, Beschin A (2000a) Trypanosoma brucei brucei infection impairs MHC class II antigen presentation capacity of macrophages. Parasite Immunol 22(7):361–370

Namangala B, de Baetselier P, Brijs L, Stijlemans B, Noel W, Pays E, Carrington M, Beschin A (2000b) Attenuation of Trypanosoma brucei is associated with reduced immunosuppression and concomitant production of Th2 lymphokines. J Infect Dis 181(3):1110–1120

Paulnock DM, Coller SP (2001) Analysis of macrophage activation in African trypanosomiasis. J Leukoc Biol 69(5):685–690

Paulnock DM, Smith C, Mansfield JM (1989) Antigen presenting cell function in African trypanosomiasis. Alan R Liss, New York, pp 135–144

Paulnock DM, Freeman BE, Mansfield JM (2010) Modulation of innate immunity by African trypanosomes. Parasitology 137(14):2051–2063

Radwanska M, Guirnalda P, De Trez C, Ryffel B, Black S, Magez S (2008) Trypanosomiasis-induced B cell apoptosis results in loss of protective anti-parasite antibody responses and abolishment of vaccine-induced memory responses. PLoS Pathog 4(5):e1000078

Reinitz DM, Aizenstein BD, Mansfield JM (1992) Variable and conserved structural elements of trypanosome variant surface glycoproteins. Mol Biochem Parasitol 51(1):119–132

Salmon D, Vanwalleghem G, Morias Y, Denoeud J, Krumbholz C, Lhomme F, Bachmaier S, Kador M, Gossmann J, Dias FB, De Muylder G, Uzureau P, Magez S, Moser M, De Baetselier P, Van Den Abbeele J, Beschin A, Boshart M, Pays E (2012) Adenylate cyclases of Trypanosoma brucei inhibit the innate immune response of the host. Science 337 (6093):463–466

Schleifer KW, Mansfield JM (1993) Suppressor macrophages in African trypanosomiasis inhibit T cell proliferative responses by nitric oxide and prostaglandins. J Immunol 151(10):5492–5503

Schleifer KW, Filutowicz H, Schopf LR, Mansfield JM (1993) Characterization of T helper cell responses to the trypanosome variant surface glycoprotein. J Immunol 150(7):2910–2919

Schopf LR, Filutowicz H, Bi XJ, Mansfield JM (1998) Interleukin-4-dependent immunoglobulin G1 isotype switch in the presence of a polarized antigen-specific Th1-cell response to the trypanosome variant surface glycoprotein. Infect Immun 66(2):451–461

Schwede A, Jones N, Engstler M, Carrington M (2011) The VSG C-terminal domain is inaccessible to antibodies on live trypanosomes. Mol Biochem Parasitol 175(2):201–204

Sprent J (1994) T and B memory cells. Cell 76(2):315–322

Sternberg JM, Rodgers J, Bradley B, Maclean L, Murray M, Kennedy PG (2005) Meningoencephalitic African trypanosomiasis: brain IL-10 and IL-6 are associated with protection from neuro-inflammatory pathology. J Neuroimmunol 167(1–2):81–89

Stewart CR, Stuart LM, Wilkinson K, van Gils JM, Deng J, Halle A, Rayner KJ, Boyer L, Zhong R, Frazier WA, Lacy-Hulbert A, Khoury JE, Golenbock DT, Moore KJ (2010) CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. Nat Immunol 11(2):155–161

Stijlemans B, Baral TN, Guilliams M, Brys L, Korf J, Drennan M, Van Den Abbeele J, De Baetselier P, Magez S (2007) A glycosylphosphatidylinositol-based treatment alleviates trypanosomiasis-associated immunopathology. J Immunol 179(6):4003–4014

Tachado SD, Gerold P, Schwarz R, Novakovic S, McConville M, Schofield L (1997) Signal transduction in macrophages by glycosylphosphatidylinositols of Plasmodium, Trypanosoma, and Leishmania: activation of protein tyrosine kinases and protein kinase C by inositolglycan and diacylglycerol moieties. Proc Natl Acad Sci USA 94(8):4022–4027

Todt JC, Hu B, Curtis JL (2008) The scavenger receptor SR-A I/II (CD204) signals via the receptor tyrosine kinase Mertk during apoptotic cell uptake by murine macrophages. J Leukoc Biol 84 (2):510–518

Uzonna JE, Kaushik RS, Gordon JR, Tabel H (1999) Cytokines and antibody responses during Trypanosoma congolense infections in two inbred mouse strains that differ in resistance. Parasite Immunol 21(2):57–71

Van der Ploeg LH, Gottesdiener K, Lee MG (1992) Antigenic variation in African trypanosomes. Trends Genet 8:452–457

Vickerman K, Luckins AG (1969) Localization of variable antigens in the surface coat of Trypanosoma brucei using ferritin conjugated antibody. Nature 224:1125–1126

Vincendeau P, Daulouede S, Veyret B, Darde ML, Bouteille B, Lemesre JL (1992) Nitric oxide-mediated cytostatic activity on Trypanosoma brucei gambiense and Trypanosoma brucei brucei. Exp Parasitol 75:353–360

Webb H, Carnall N, Carrington M (1994) The role of GPI-PLC in Trypanosoma brucei. Braz J Med Biol Res 27(2):349–356

Webb H, Carnall N, Vanhamme L, Rolin S, Van Den Abbeele J, Welburn S, Pays E, Carrington M (1997a) The GPI-phospholipase C of Trypanosoma brucei is nonessential but influences parasitemia in mice. J Cell Biol 139(1):103–114

Webb H, Carnall N, Vanhamme L, Rolin S, Vandenabbeele J, Welburn S, Pays E, Carrington M (1997b) The Gpi-phospholipase C of Trypanosoma brucei is nonessential but influences parasitemia in mice. J Cell Biol 139(1):103–114

Yew KH, Carsten B, Harrison C (2010) Scavenger receptor A1 is required for sensing HCMV by endosomal TLR-3/-9 in monocytic THP-1 cells. Mol Immunol 47(4):883–893

Adaptive Immunity and Trypanosomiasis-Driven B-Cell Destruction

5

Stefan Magez and Magdalena Radwanska

Abstract

African Trypanosomiasis is an excellent model system to study immune escape by invading extracellular pathogens. Being under continuous attack by the host humoral response, trypanosomes developed a system of antigenic variation of their surface coat in order to evade antibody-mediated immune destruction. In-depth studies on the mechanisms of antigenic variation have resulted in the understanding of both structural and genetic aspects of the surface coat organization of trypanosomes, and the variant-specific glycoproteins (VSG) itself, i.e., the protein that provides the interface between the parasite and the host immune system (see Chaps. 1 and 3). To date, the current hypothesis of VSG-mediated antibody escape implies that during infection the host is capable of mounting an ever changing antibody repertoire, which allows to target in a specific manner each new trypanosome wave. In this chapter, a number of recent and new insights will be discussed that highlight the complexity of this system. Indeed, experimental data obtained in rodent *T. brucei* models suggest that anti-VSG responses are very short lived, and no effective memory is mounted during infection against the successive waves of occurring VATs. In addition, active destruction of the host B-cell compartment occurs during infection, affecting both trypanosome specific and non-specific B-cell memory. These finding will be discussed in the context of the long string of vaccine failure results that have hampered the initiation of an effective vaccine program for trypanosomiasis. Finally, this chapter will also provide new insights into antibody engineering

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that allow interfering with trypanosome biology in ways that are not part of the natural evolutionary pressure. Hence, possible new tools can be developed that can help in a sustainable long-term battle against both human and animal trypanosomiasis.

5.1 Introduction

Ever since the discovery of trypanosomes being the causative agent of Nagana by Sir Bruce in the late nineteenth century, efforts have been undertaken to irradicate both the animal and human form of the disease. Two crucial problems have hampered these efforts so far and will continue to pose major difficulties in the future: (a) the vast wildlife reservoir that can serve as host for the parasite makes total irradication impossible (see Chap. 10) and (b) the parasite is tsetse fly transmitted, hence vector irradication is a prerequisite for disease control (see Chap. 2). Together, these factors make that re-emergence of trypanosomiasis as a human and livestock disease will always remain a threat, as long as total irradication has not been achieved.

In a setting where active irradication appears impossible, vaccination should provide for a feasible alternative. However, one century of vaccine trials have failed to provide any hopeful results. Early vaccine experiments did however result in the acquisition of the fundamental knowledge that to date provides the base for our understanding of the mechanisms of antigenic variation. Indeed, systematic vaccine approaches were started using well-characterized laboratory *T. brucei* clones more than four decades ago, trying to raise protective immune responses against specific variants (Van Meirvenne et al. 1975a, b). The approach taken was to infect laboratory mice with a specific VAT, allow the onset of infection, treat subsequently the mice during the first peak of infection and finally rechallenge these mice with the same variant as previously encountered. Failure to mount a protective response against infection through this strategy, lead investigators to isolate immune serum and realize that the antibodies present were capable of inducing complement-mediated lysis of the vast majority of the targeted population, however, without killing all trypanosomes present. So, antibodies against the first VAT would kill first-peak parasite but would not be able to kill a newly arising VAT that would give rise to a new peak of parasitemia. In the same setting, antiserum raised against the second-peak variant would be effective in providing complement-mediated lysis of the targeted second VAT, but would be unable to prevent a third peak of infection to occur. A combination of parasite cloning, specific in vitro antibody–complement lysis, and characterization of the antigenic type gave rise to (a) the laboratory model in place to date using specific VATs (i.e., for example, *T. b. brucei* AnTat 1, MITat 1, or LOUTat 1) and (b) provided the first insights into the fact that trypanosomes are coated with a dense layer of glycoproteins—the variant surface glycoprotein (VSG)—that is offering a protective coat against immune destruction thanks to the continuous variation of immunodominant epitopes. To date, it has been generally

accepted that vaccination against the main surface glycoprotein of trypanosomes will never result in the build-up of sterile immunity and protection against trypanosomiasis. Indeed, as described elsewhere (Chap. 3) trypanosomes have invested in the development of multiple mechanisms that ensure the ongoing of antigenic variation in such way that it effectively can beat the immune system.

One important remark to be made with respect to the research into VSG biology and its potential target as a vaccine candidate, is the fact that the vast majority of VSG switching research has been executed using bloodstream form trypanosomes, passaged in mice by needle transmission. Very few laboratories to date have access to a functional tsetse fly colony that can safely be used for natural transmission experiments. However it is interesting to observe that the VSG repertoire of metacyclic trypanosomes, i.e., the infective parasite form that populates the salivary glands of the vector has a very limited antigenic reservoir. Indeed, it appeared that here the VAT repertoire is limited to no more than a dozen different VSGs (Le Ray et al. 1978; Barry et al. 1979, 1983; Esser et al. 1982; Crowe et al. 1983). These discoveries initially lead several authors to propose that vaccination against metacyclic trypanosomes could be feasible. In particular, the use of attenuated irradiated trypanosomes for vaccination resulted in preliminary positive results (Esser et al. 1982). Also a combination approach in which mice were infected through tsetse fly bites and subsequently treated with Berenil resulted only in short-term protection against a homologous challenge (Nantulya et al. 1980). Hence, it was suggested in 1985 by Cornelissen and colleagues that due an overlap between the M-VSG and B-VSG repertoires, and the fact that antigenic variation in both expression sites relies on telomeric exchange, vaccination prospects were "not good" (Cornelissen et al. 1985). Later, as the mechanisms of VSG switching were further elucidated, it became apparent that while the M-VSG expression sites (M-ES) had particular unique features (Barry et al. 1998), the VSGs expressed in these expression sites were not any different from the VSGs present in the B-ES (bloodstream form expression site). Hence, so far Cornelissen's prediction appears to hold, as not a single successful vaccine strategy has been made available for effective use since then.

5.2 IgM Responses and Their Crucial Role in the Control of Trypanosomiasis

During the initial vaccination experiments that were reported to provide some VSG-specific protection, it was observed that the antibodies involved were short-lived IgMs (Nantulya et al. 1980; Crowe et al. 1983). In this context, several other reports have indicated the superior anti-trypanosome activity of IgM as compared to IgG antibodies (MacAskill et al. 1983; Mitchell and Pearson 1983). This IgM activity has been proposed to be linked to the capacity of anti-VSG IgM to capture C3 complement fragments (Shi et al. 2005; Pan et al. 2006), which in vivo could contribute to liver-mediated parasite clearance (MacAskill et al. 1980). In recent years the in-depth study of the role of IgM in trypanosomiasis control has been

possible by using genetically modified "knock out" mice lacking either all immunoglobulins (μMT mice) or only the IgM ($IgM^{-/-}$) (Phillips et al. 1996; Lutz et al. 1998; Magez et al. 2008). Combined with the analysis of infection-induced serum antibodies titers (Radwanska et al. 2000a, b), four main conclusions have surfaced: (a) VSG-specific IgM titers reach maximum levels 3–4 days after peak parasitemia, (b) the generation of anti-VSG IgM antibodies does not require T cell help and most likely does not involve MHC class II antigen presentation, (c) infection-induced anti-trypanosome IgMs are crucial for parasitemia clearance, albeit they do not have a major impact on actual peak parasitemia height, and (d) protective anti-VSG IgM titers are short lived and do not provide memory that protect against the re-emergence of VATs that have already been present earlier in infection.

5.2.1 Anti-VSG IgM Peak Titers Occur Subsequent to Peak Parasitemia Control

Even before $IgM^{-/-}$ mice became available, various research laboratories addressed the issue of isoytpe-specific anti-VSG antibody activity during experimental infections. Using the experimental *T. b. brucei* AnTat 1.1 model system in mice, it was shown that IgM antibodies are the major dominant antibody type and that maximum serum IgM titers are reached by day 10 postinfection (Radwanska et al. 2000b). Interestingly, these maximum titers are reached 3 days after the first VAT has completely disappeared from the circulation. These findings enforce the notion already mentioned before (see Chap. 1) that trypanosomes posses very well-controlled mechanisms of growth control and differentiation and that parasitemia control does not only rely on host immunity. Indeed, the occurrence of successive parasitemia peaks coincides in the first place with a transformation stage where actively dividing parasites (known as long slenders) undergo growth arrest and become nondividing (short stumpy) parasites (Vickerman 1985). When the latter fail to be picked up during tsetse fly blood feeding, they disintegrate into the circulation of the infected host. This can help to induce a VSG-specific antibody response (Sendashonga and Black 1982). Hence, it could be argued that during the first week of infection, the increasing presence of disintegrating stumpy form parasites is actually the antibody-inducing driving force, rather than that the occurring antibodies are the driving force behind parasitemia control.

5.2.2 Anti-VSG Antibodies Are Induced in the Absence of T Cells

In order to unravel the immune activation mechanisms involved in trypanosomiasis control, early studies focused not only on antibodies but also on T-cell function during infection. Using various batteries of inbred mouse strains, it was concluded first that VSG-specific antibody control of parasitemia was independent of MHC (H-2 in mice) usage (Levine and Mansfield 1984; De Gee et al. 1988). Subsequent

reports pointed to the fact that T-cell suppression is one of the pathological side effects of trypanosome infections, resulting in the downregulation of expression of both IL-2 and the IL-2 receptor (Sileghem et al. 1989; Darji et al. 1996). Finally, it was shown that *nu*/*nu* mice, which are deprived from thymus-derived matured T-cell subsets, do not suffer from impaired parasitemia control when challenged with cloned *T. b. brucei* parasites (Reinitz and Mansfield 1990; Radwanska et al. 2000a, b). Interestingly, nu/nu mice actually have less overall pathology than WT control animals, as they exhibit a reduced inflammatory reaction resulting in less anemia, less cachexia, and an increased survival time (Magez et al. 2008). These observations link back to data provided above (see Chap. 4) showing that T-cell-derived cytokines such as IFNγ and IL-10 are the major regulators of infection-associated pathology. Together, these data indicate that during infection, T-cell-independent IgM responses are to be considered the first line of host defense against proliferating parasites (Magez et al. 2008), at least in relatively virulent experimental mouse infections.

5.2.3 The Importance of Anti-VSG IgM Antibodies for Parasite Clearance and Initial Peak Parasitemia Control Depends on Parasite Virulence

Having discovered that peak parasitemia clearance coincides with the presence of rising titers of T cell independent anti-VSG IgM serum titers, a function correlation between these events could only be made through the combined use of μMT and $IgM^{-/-}$ mice, i.e., mice that lack either all mature B cells or only the IgM-producing subpopulation. This study showed once again that antibodies per se do not have a major impact on experimental *T. brucei* parasitemia peak height control, but do have major impact on post-peak parasitemia clearance. Indeed, in the absence of proper IgM-mediated VAT-specific immune attack, the overall parasite load quickly increases in the host circulation due to accumulation of an array of different variants. Hence, mice lacking B cells or IgM antibodies succumb early to infection. This is particularly the case in relatively high virulent laboratory models for *T. b. brucei*, a finding confirmed in relatively high virulent *T. evansi* infections (Baral et al. 2007). Important to note is however that when a low virulent *T. brucei* field isolate was grown in $IgM^{-/-}$ mice, the same study showed little or no effect of the lack of infection-induced IgMs on parasitemia progression. While one could be tempted to conclude that this indicated that the remaining IgG was sufficient to control parasitemia, it also could be argued that the innate control of this low virulent infection was the major regulator of parasitemia, as even in the absence of B cells (in μMT mice) this parasite exhibits a prolonged infection. Interestingly, also when low virulent *T. congolense* infections were studied in the same context, the role of IgM seemed to be less pronounced, with parasitemia control mainly relying on control by IgG2a/IgG3 in combination with a pronounced Type I immune response (Uzonna et al. 1999; Magez et al. 2006). These data indicate that in natural infections where trypanosomes are regularly transmitted through the

tsetse vector, (a) autoregulated differentiation signals could actually exert a big impact on parasitemia control, (b) the relative role of IgM/IgG and innate immune reliatory mechanisms can be different, and (c) that the relative virulence of the parasite will determine the need for IgM mediated parasitemia control, with high virulent infection apparently needing a bigger participation of the IgM component than low virulent infections.

5.2.4 Anti-VSG IgM Antibodies Only Offer Short-Term Protection

From the data reviewed above it is clear that in mice, T-cell-independent IgM antibodies have to be considered as the major component of the host humoral immune system in the defense against virulent extracellular trypanosomes. At which time point during infection these responses are actually active, and for how long they remain present during infection, was addressed by a double-infection scheme outlined in Fig. 5.1 (Magez et al. 2008). Here, a primary infection was initiated with *T. b. brucei* AnTat 1.1, a variant that results in an infection that will last for 35–40 days. Subsequently, mice were confronted during infection with a secondary parasite challenge, this time using a hyper virulent *T. b. brucei* variant—killing mice within 4 days of infection. By using either a homologous or heterologous rechallenge (i.e., a second VAT1 challenge or an irrelevant VAT'X' challenge) and repeating the experiment on different time point throughout the primary infection, antibody-mediated VAT-specific effector function was addressed. The conclusion from these experiments was that VAT-specific protection arises between days 7 and 10 post first-challenge, last for about 1 week, and is IgM dependent. Indeed, VAT-specific protection seen on day 10 post primary infection in immune competent hosts, but disappeared by day 17. In $IgM^{-/-}$ mice, no VAT-specific protective responses were obtained at any point of infection, confirming the crucial role of IgMs in control of high virulent infections.

5.2.5 Is There a Role of IgGs in the Anti-Trypanosome Defense in the Natural Host?

The data presented above, all obtained in experimental mouse models for trypanosomiasis, clearly suggest that while IgM is crucial for the control of high virulent trypanosome infections, the role of IgG in vivo remains to be elucidated. Interestingly, to date the contribution of antibodies in trypanosomiasis control in relevant host species and in particular the relative contributions of IgMs versus IgGs remains to be clarified as well. Comparative studies between trypanotolerant cattle breeds such as the N'Dama's and trypanosusceptible breeds such as Boran's have provided a body of data that however remains open for discussion. First, several authors have indicated that no differences in anti-trypanosome antibody titers were found between those breeds (Pinder et al. 1984; Kamanga-Sollo et al. 1991). Others have reported N'Ddama cattle to be characterized by the capacity to generate

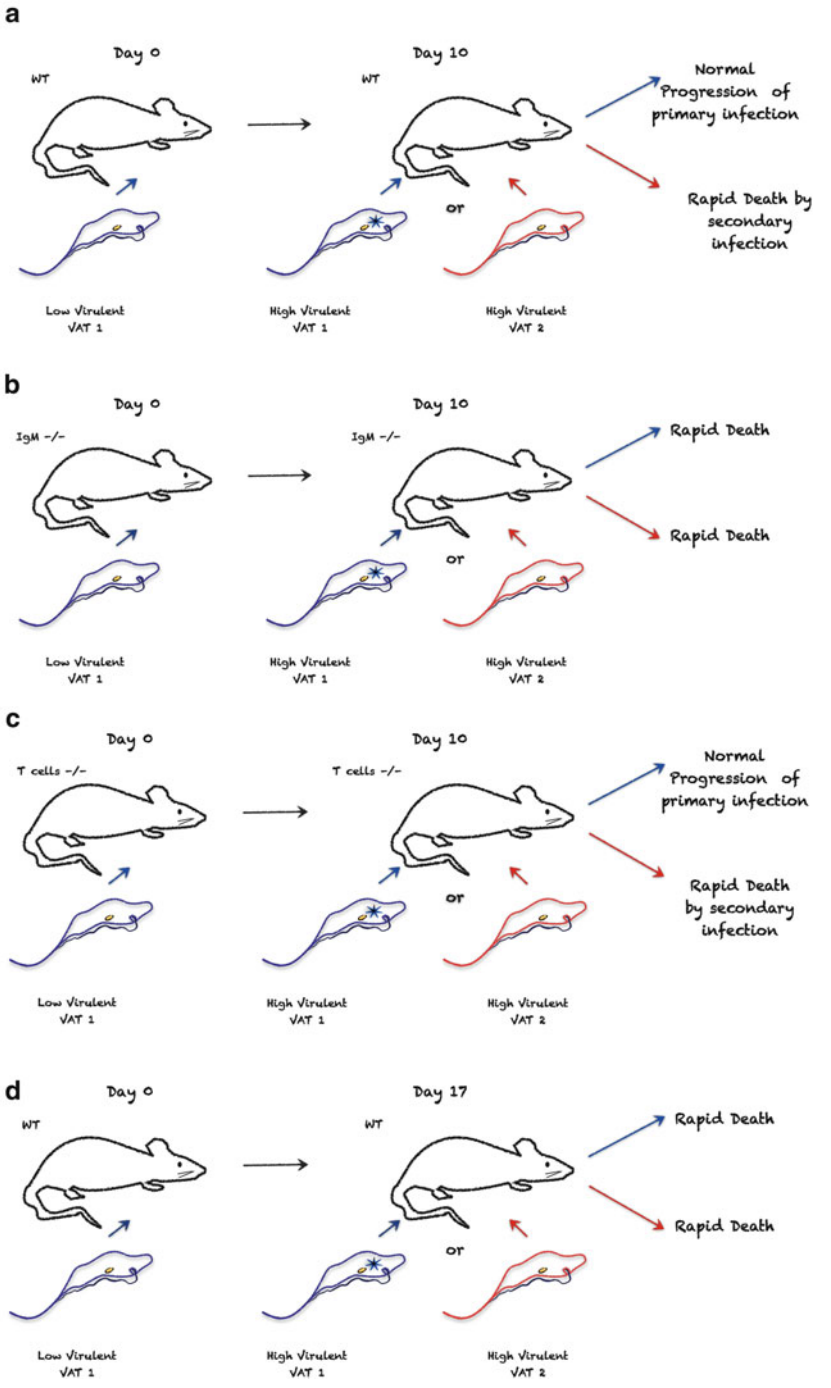

Fig. 5.1 Variant-specific IgM-dependent adaptive immunity is induced during *T. brucei* infections in mice but is short lived. (**a**) When mice infected with a relatively low virulent VSG1

higher IgG1 titers against cryptic VSG epitopes, combined with reduced levels of irrelevant IgM production (Williams et al. 1996). These findings are paralleled by the fact that tolerant cattle mounts a better IgG1 response against congopain (believed to be involved in the induction of trypanosomiasis-pathology) and shows the presence of higher amounts of IgG1-secreting cells in the spleen (Authié et al. 1993a, b; Taylor et al. 1996). However, it should also been noted that the susceptibility differences between the same cattle breeds have been attributed to differences in innate host responses in which it is believed that the presence or absence of inflammation driven pathology determines the susceptibility level. Hence, the quality of the observed antibody responses, including the response against inaccessible cryptic epitopes, could be a consequence of the tolerance status, rather than the result (Lutje et al. 1996; Taylor 1998; O'Gorman et al. 2006; Naessens 2006).

5.3 B-Cell Depletion as a Part of Trypanosomiasis-Associated Immunopathology

As outlined above, all data based on experimental mouse models do suggest that anti-VAT IgM antibodies can cause a major threat to trypanosomes. In order to understand how trypanosomes deal with the potential elimination by the host immune system, it is important to understand how anti-trypanosome antibody responses are generated. During a primary immune response, rapid T-cell-independent IgM responses arise in large from the spleen, where mariganal zone B cells are specialized in providing this specific function. In the recent past, the faith of MZ B cells has been studied in detail in the laboratory *T. b. brucei* AnTat 1.1 model. To address this issue, an in-depth study was performed following all cell subsets that are part of the B-cell development pathway both in bone marrow and in spleen (Radwanska et al. 2008; Bockstal et al. 2011). As indicated in Fig. 5.2, every stage of B-cell development is characterized by the acquisition of specific surface markers, and the expression of surface IgM is considered the hallmark of the immature B cell stage in the bone marrow. Interestingly, in mice B-cell poiesis can occur in the spleen, allowing to rescue infection- and inflammation-associated bone marrow defects that could undermine antibody production. During

Fig. 5.1 (Continued) variant (VAT1) are rechallenged on day 10 of the primary infection with high virulent parasite expressing a homologous VAT1 VSG surface coat, infection control of the secondary challenge prevent early death of the mice. Rechallenge with a high virulent nonrelated VAT2 will lead to early death evoked by an uncontrolled secondary infection. (**b**) Repetition of the same experimental setting in $IgM^{-/-}$ mice shows that the specific immune response involved in day 10 protection is IgM dependent. (**c**) Repetition of the same experimental setting in T $cell^{-/-}$ mice shows that the specific immune response involved in day 10 protection is T cell independent. (**d**) Repetition of the same experimental setting in WT mice shows that the specific immune response involved in day 10 protection is lost by day 17 and indicated the lack of immunological memory induction [adapted from Magez et al. (2008) by Joar Pinto]

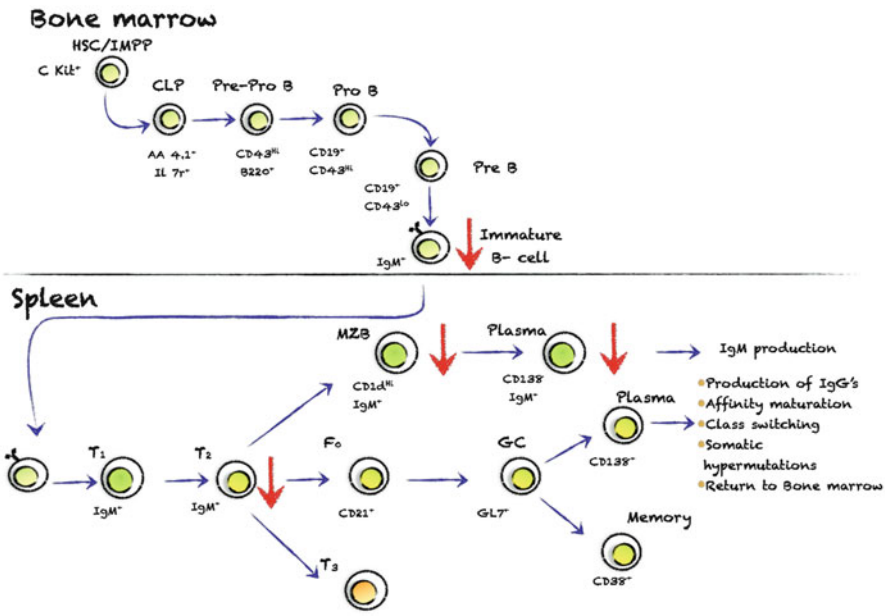

Fig. 5.2 B cell development is severely affected during experimental *T. brucei* infections at all stages, both in bone marrow and the spleen. Striking is that IgM expressing B cells are lost very early on in infection in all experimental models tested so far [adapted from Radwanska et al. (2008) and Bockstal et al. (2011) by Joar Pinto]

trypanosome infections it is clear that both at the level of the bone marrow and the spleen, B-cell poiesis is severely affecting all stages of development. What is striking however is the fact that in all models studied so far, trypanosomiasis results in the rapid and permanent depletion of the IgM producing B cell compartment, i.e., the transitional T1 B cells, the MZ B cells, and following the first peak of infection also the IgM plasma cells. When comparing WT and $IgM^{-/-}$ mice, it became obvious that the main difference between the two is the very strong IgM antibody response and plasma cell differentiation caused by the first wave of parasitemia in WT mice. Important here is that in these mice, rapid MZ differentiation into plasma cells, combined with MZ apoptosis results in a deregulation of spleen compartmentalization. Together with the fact that also the T1 B cells are rapidly depleted, this leaves the spleen incapable of mounting an efficient IgM B cell regeneration process that could offer a defense against the newly arising subsequent VATs. Together, these findings can explain why from the second parasitemia wave onwards, both WT and $IgM^{-/-}$ mice show a very similar progression of infection. Indeed, by day 10 of infection, WT mice are gradually becoming functional $IgM^{-/-}$ mice, and the ongoing trypanosome infections appears to knock down the most dangerous immune component that the host has access to, i.e., the capacity to continuously mount a VAT-specific IgM response.

To date, the underlying mechanism of this B-cell memory destruction remains to be fully elucidated. While trypanosome/B cell cell–cell contact appears to be one of the factors that drive rapid IgM B-cell apoptosis, it is unlikely that the vast scale of the problem can be explained by this mechanism only. Interesting to mention is that recently B-cell development was analyzed in trypanosome-infected MD4 mice. These mice are genetically engineered animals that have a B-cell receptor repertoire that is restricted to the recognition of hen egg lysozyme (HEL). In these mice, B-cell destruction progresses with the same kinetics as in fully immune-competent mice, indicating that the parasite-driven destruction of the IgM compartment is governed by a system that is intrinsically VAT independent. Hence, it results in a gradual abrogation of all IgM responses preventing the host to mount VAT-specific IgMs later-on in infection and hence increasing the success of long-lasting infection needed for efficient transmission. Finally, one important remark that needs to be repeated is that all the detailed observations outlined above are the result of laboratory rodent infection models. Hence, in the future the relevance for human infections as well as "real-life" livestock has now to become the focus of a new investigation.

5.4 Anti-trypanosome Vaccination and Failures Encountered so Far

As African trypanosomes are obligate parasitic organisms, they must rely on the permanent uptake of host factors for survival and multiplication. In order to bind and take up these exogenous macromolecules, parasites must express a number of non- or less-variable surface antigens. In return, parasite must secrete/excrete specific molecules and metabolites throughout infection that will be recognized by the host's immune system and hence can contribute to reactions that include inflammation, ultimately driving immune pathology. Hence, both invariant compartments of this host–parasite interactome could be considered targets for immune intervention or vaccination.

5.4.1 Vaccine Failures Using Invariant Surface Proteins as Targets

To date, a number of invariant trypanosome surface proteins are known. They include the transferrin receptor, the product of expression site-associated gene ESAG6/7 (Steverding et al. 1994), the invariant surface glycoproteins ISG64 (Jackson et al. 1993), ISG65 (Ziegelbauer et al. 1992), ISG75 (Ziegelbauer and Overath 1992), ISG100 (Nolan et al. 1997), and the haptoglobin–hemoglobin receptor (Vanhollebeke et al. 2008; Higgins et al. 2013). The recent description of the molecular structure of the latter indicates that besides the VSG, other molecules must be exposed to the environment, unshielded by the VSG coat.

In trypanosomes, the organelle specialized in binding and endocytosis of host molecules is the flagellar pocket. This membrane invagination at the base of the

parasites' flagella has indeed been described to contain an array of surface-exposed membrane proteins that are relatively conserved among different trypanosome species (Gull 2003) and as such present a potential vaccine target. Unfortunately, so far FP vaccination has only resulted in partial protection in cattle (Mkunza et al. 1995) or mice (Radwanska et al. 2000a, b). In the latter case it was determined that the vaccine efficacy was broken once mice were challenged with 10^3 or more parasites. This point is important to stress, as the infection dose is an important parameter for evaluation of the effectiveness of vaccine candidates. It is well documented that salivary gland of an infected tsetse fly can contain up to 10^4 metacyclic parasites, while several thousand of parasites can be injected during a single blood feed (Gingrich et al. 1981). Hence, vaccine/challenge studies in which only 10^3 or less bloodstream-form parasites are used for infection might be biased toward a positive outcome and do most likely not reflect the situation that is encountered under field conditions. In addition, throughout the anti-trypanosome vaccine literature "partial" protection is being used to describe two different concepts (Magez and Radwanska 2009), i.e., (a) the percentage of mice showing sterile immunity towards infection and (b) the reduction in parasite burden. In case of the latter it can be questioned whether results are positive at all, as all vaccinated animals acquired the infection and finally suffered from infection-associated pathology and succumbed to the disease.

A major pitfall hampering FP immunization could be that the FP contains vast amounts of the highly immunogenic VSG. Therefore, failure of FP vaccination does not rule out the possibility of obtaining an effective vaccine strategy when VSG could be excluded. In view of this, recent efforts have been focused on vaccination with recombinant trypanosome invariant surface-exposed proteins such as ISG75. ISG75 is evenly distributed on the surface of the bloodstream-stage parasites and is conserved among all taxa of the *Trypanozoon* subgenus (Tran et al. 2006). The *E. coli* expressed extracellular domain of ISG75 (Tran et al. 2008) was recently used to immunize mice, resulting in high titers of ISG75-specific antibodies (Magez et al., unpublished results). However, upon challenge with 5×10^3 *T. b. brucei* parasites, no protection was observed, despite the fact that the antigen itself was accessible for antibody binding. In addition, vaccine-induced anti-ISG75 antibody titers decreased rapidly to a level similar to that found in nonvaccinated infected mice. These results indicate that contact of the vaccine-primed immune system with living parasites failed to trigger an effective B-cell memory response, despite the continuous challenge of the immune system with ISG75 during infection. It suggests that an active infection with *T. b. brucei*, or in other words the presence of living and dividing trypanosomes, either suppresses or abolishes the specific antibody response, and possibly rapidly destroys the vaccine memory response. Hence, in order for an anti-trypanosome vaccine to be effective, it should have the ability to eliminate all circulating parasites prior to B-cell memory suppression/destruction. A successful vaccine strategy should therefore rely on the permanent presence of high effective/protective anti-trypanosome titers that can prevent the onset of infection. The antibody titers must be maintained in the absence of continuously circulating trypanosome antigens. This apparent contradiction seems

not only to suggest that anti-trypanosome vaccination might never be feasible, but as discussed below, could have major implications for other vaccination programs in trypanosome-exposed regions. In addition, it could mean that anti-disease vaccination, proposed as an alternative to anti-pathogen vaccination (Authie et al. 2001) would be prone to failure as well.

5.4.2 Failure of Anti-disease Vaccine Strategies as an Alternative Method to Combat Trypanosomiasis

The idea behind anti-disease vaccination is the fact that many indigenous African mammals can harbor natural trypanosome infections without developing severe disease symptoms (Hanotte et al. 2003). In susceptible animals most deaths occur through disease-associated complications and not excessive parasite loads itself (Naessens et al. 2003). This suggests that the negative outcome of trypanosomiasis in case of HAT (Human African Trypanosomiasis) as well as livestock infections is due to the nature of the host immune reaction. Hence, anti-disease vaccination could offer a solution. So far, two main anti-disease vaccination strategies have been reported.

Firstly, the correlation between the capacity to mount an antibody response against the *T. congolense* proteases congopain (CP) and the relative tolerance of bovines towards the trypanosomiasis-associated pathology suggests that CP is a putative anti-disease vaccine candidate (Lalmanach et al. 2002). Interestingly, when trypanosusceptible cattle were vaccinated with CP, the induced IgG titers were much lower as compared to those in trypanotolerant cattle. This suggests that that susceptibility of cattle breeds to trypanosomiasis pathology could correlate with the intrinsic incapacity to mount an immune response against the trypanosome protease. However, when vaccinated with baculovirus expressed central domain of CP, even the susceptible breed showed reduced levels of infection-associated anemia and leucopenia, during the chronic stage of infection (Authie et al. 2001). Despite these early promising results, unfortunately no follow data was generated that suggested the existence of long-term memory induction in this vaccination strategy.

Secondly, based on experimental mouse infections and data obtained in cattle, it was suggested that the inflammatory cytokine TNF plays a major role in the development of trypanosomiasis-associated disease complications (Sileghem et al. 1994; Magez et al. 1999). With the identification of the VSG-GPI anchor as the main TNF-inducing trypanosome moiety (Magez et al. 1998), a liposome-based GPI vaccination strategy was developed in order to prevent excessive immune activation upon infection (Stijlemans et al. 2007). The proposed strategy resulted in a positive outcome for the host in terms of (a) parasitemia control, (b) prolongation of survival, and (c) limitation of infection-associated complications such as anemia, weight loss, and impairment of locomotor activity. However, a detailed analysis of the underlying protective mechanisms elucidated the lack of B-cell and memory involvement. Indeed, GPI vaccination was shown to

modulate host macrophages to become biased to anti-inflammatory alternative activation rather than pro-inflammatory classical activation (Stijlemans et al. 2007). This response was found to be short lived and could even be evoked in B-cell deficient μMT mice.

5.4.3 Trypanosomiasis-Associated Destruction of the B-Cell Memory Compartment Undermines Vaccine Efficacy

In order to explain the continuous failure of anti-trypanosome vaccination attempts, it should be taken into account that since the very early days of the analysis of host–trypanosome interactions and immune modulations, infection-induced immune suppression has been recognized as a hallmark of trypanosomiasis (Murray et al. 1974a, b; Hudson et al. 1976; Corsini et al. 1977; Askonas et al. 1979; Clayton et al. 1979). This suppression could in part explain the trypanosomiasis-associated reduction of the vaccine efficacy against louping-ill virus (Whitelaw et al. 1979), foot and mouth disease (Sharpe et al. 1982), *Brucella abortus* (Rurangirwa et al. 1983), anthrax (Mwangi et al. 1990), and swine fever (Holland et al. 2003). However, as outlined above, in addition to this suppression several host B-cell compartments are rapidly and permanently destroyed during the onset of a trypanosome infection (Radwanska et al. 2008). In order to show the general implication of the latter, a vaccination experiment was performed in which mice were exposed to the commercially available DTPa vaccine Boostrix®. Figure 5.3 outlines how this vaccine protects mice form challenge with *B. pertussis* but is rendered inactive in the presence of trypanosomes (Radwanska et al. 2008). While this could be explained by active parasite-driven immune suppression, treatment of mice with Berenil® did not restore vaccine efficacy, despite the curative effect of the treatment on trypanosomiasis. Based on these results it is concluded that the presence of living and dividing trypanosomes results in the destruction of the host B-cell memory compartment, which is not restricted to antiparasite responses alone. In the near future, it will be crucial to validate these results in more natural infection settings. Indeed, while infection-induced immunosuppression has been reported to occur in humans (Greenwood et al. 1973), no data is available to date with respect to potential permanent immune memory destruction. If this memory B-cell destruction were to occur in human trypanosomiasis, it could suggest that a number of additional immune problems will occur in disease endemic regions. Memory destruction caused by HAT would have a detrimental impact on non-trypanosomiasis vaccination programs that are currently ongoing in sub-Sahara Africa such as the WHO Meningitis Vaccine Project (MVP) and the Pediatric Dengue Vaccine Initiative (PDVI), as well as on future anti-HIV/AIDS and antimalaria vaccine programs. In this case it would be necessary to not only treat HAT victims for trypanosomiasis but subsequently also revaccinate these patients with all previously administered vaccines in order to restore their B-cell memory compartment.

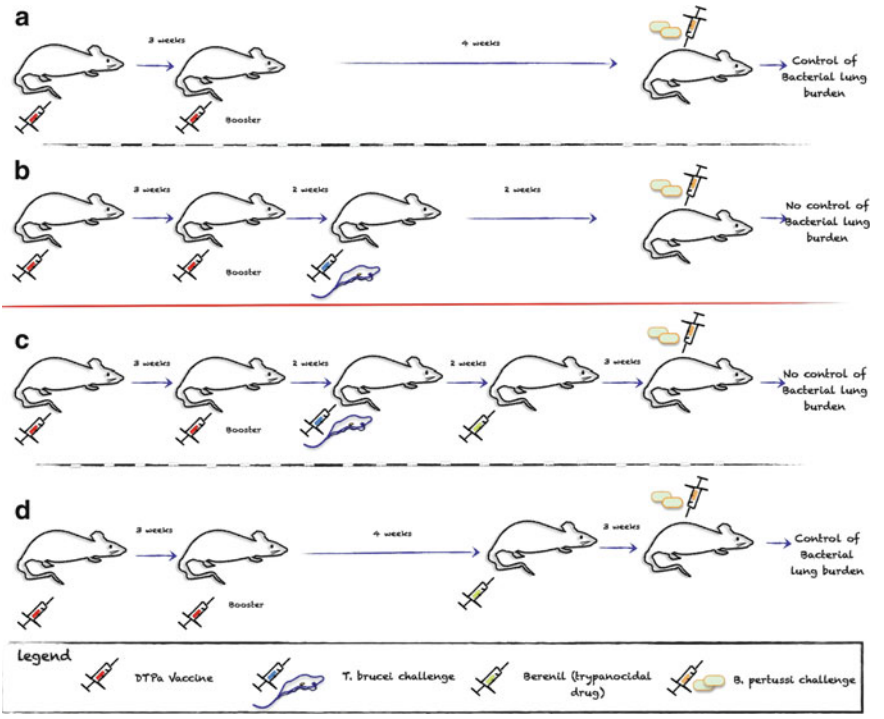

Fig. 5.3 *T. brucei* infections destroy immunological memory evoked by effective nonrelated vaccines such as Boostrix®/DTPa. (**a**) Mice vaccinated and boosted with the DTPa vaccine control a subsequent challenge with *B. pertussis*. (**b**) When DTPa vaccinated mice encounter a *T. brucei* infection, they subsequently loose the protection against a *B. pertussis* challenge. (**c**) Berenil® treatment of trypanosomiasis does not allow the DTPa-induced memory to return. (**d**) Berenil® by itself has no effect on DTPa vaccine efficacy [adapted from Radwanska et al. (2008) by Joar Pinto]

5.5 Mouse Models for Trypanosomiasis and Questions About the Validity of Experimental Trypanosomiasis Data

To date, trypanosome research is mainly executed under strict laboratory settings in which mice and rats are used as the main host model. With respect to the parasite choice, most immunological studies are done with well characterized and most often cloned laboratory strains that are pleomorphic. These strains have the capacity to naturally differentiate from the fast-dividing long slender (LS) bloodstream form, into the nondividing short stumpy (SS) bloodstream form, that obligatory needs to continue its life cycle into the insect vector. Besides the use of pleomorphic parasites, a vast body of trypanosome literature available to date is based upon the use of so-called monomorphic trypanosomes. These parasites have been obtained through cyclic passage in laboratory rodents, in the absence of the natural vector transmission providing for the regular generation of saliva-stage metacyclic trypomastigotes. Monomorphic infections are characterized by the absence of the

natural differentiation of LS forms into SS forms and are hypervirulent often resulting in the death of their host within days after the initiation of infection. Monomorphic trypanosomes are useful for experimental immunological research in rechallenge settings and are preferred tools in settings where gene regulation is studied. However, due to the virulent nature of the infection, they are totally unsuitable to perform basic immunological studies. Indeed, the major early immune stimulation provided by SS degradation is missing. In addition, the extreme fast division time of the parasite leads to excessive numbers of parasites within days of infection outrunning the capacity of the host to induce any effective response.

But even when mouse models using pleomorphic trypanosomes are considered, caution should be given to the general interpretation of result validity and questions could be asked as to the level of confidence that can be given to observations with respect to the possibility to translate them to a field situation with a biological relevant host. Indeed, while HAT (Human African Trypanosomiasis) is characterized by late stage encephalitic complications leading to coma and death, mice suffer either from extreme final parasite loads or multiorgan failure, even when pleomorphic infections are studied. When considering Nagana and other forms of animal trypanosomiasis, here infection-associated anemia and major secondary infections are often the cause of death. In mice, trypanosomiasis-associated anemia does occur, but is by no means to be considered as the main cause of death (Magez et al. 1999). On the other hand, taken up-to-date laboratory conditions where current research is being conducted, death by secondary infection in experimental rodents is a nonexisting factor. Hence, taken these issues together one should dare to ask the question whether or not laboratory rodent models will ever deliver a suitable answer when it comes to the search for immune intervention in trypanosomiasis. To date, only few laboratories have undertaken efforts to study trypanosomiasis in bovine models that reflect Nagana (Mkunza et al. 1995; Taylor et al. 1996; Authie et al. 2001; Kateregga et al. 2012). Even fewer studies are available that have tempted to study relevant models for HAT at these imply the use of nonhuman primates (Ngotho et al. 2011; Gaithuma et al. 2012). While the later have all been subjected to extreme ethical review procedures, holding to the highest standards, emotional issues regarding such studies that affect public opinions in donor countries make it hard to secure sustainable funding, notwithstanding the thousands of HAT victims that yearly succumb to the disease in endemic countries.

5.6 Antibody Engineering and New Ways of Dealing with Trypanosomes

The occurrence of antigenic variation of the trypanosome VSG surface coat, discussed above, appears to be the main reason why antibody-based anti-trypanosome intervention strategies are doomed to fail. With respect to the presence of invariant surface (glyco) proteins (ISGs), it seems that most of these molecules are embedded into the VSG coat and are only poorly accessible for antibodies. Invariant proteins that are exposed in the flagellar pocket might be

directly targeted by antibodies, but antibody binding per se will not harm trypanosomes in general. Here, antibodies bound to more conserved molecules in the flagellar pocket are inaccessible for FcR-mediated binding of immune phagocytosing cells or toxic granulocytes. Even in case of anti-VSG antibody binding, it is well established that trypanosomes posses several mechanisms that provide defenses against antibody-mediated lysis. Indeed, efficient transport of the bound VSG molecule towards the flagellar pocket involving the active process of flagellar beating results in rapidly internalization through the endocytotis pathway, followed by degradation in the lysosomal digestive pathway (Engstler et al. 2007). With respect to complement-mediated killing of trypanosomes, some important issues remain to be solved. While complement-mediated lysis can be used in vitro in a VSG-specific manner to cause killing of trypanosomes, and C3-mediated phagocytosis of trypanosomes has been described to occur in the liver of infected mice, the true contribution of the complement cascade in an in vivo setting can be questioned. While C5-deficient AKR mice have long been known as one of the most resistant mouse strains in the context of experimental trypanosomiasis (Morrison et al. 1978), similar experiments in C3-deficient mice have so far not been reported to date for experimental *T. brucei* infection. However, it is interesting to note that early experimental data obtained with the mouse pathogen *Trypanosoma musculi* indicated no major role for C3 or C5 in parasitemia control (Jarvinen and Dalmasso 1977). In addition, while studies using serum of the trypanotolerant Cape Buffalo have shown that early on in infection complement-dependent antibody-mediated lysis could explain killing of trypanosomes in a VSG-specific manner, the later cryptic phase of the infection was controlled here independent of complement activity (Guirnalda et al. 2007). Together, these data suggest that complement-mediated lysis of trypanosomes is most likely not required for prolonged parasitemia control in a natural trypanotolerant host.

Given the fact that trypanosomes have been selected over the course of history to populate the blood of their mammalian host, it is hardly surprising that they have acquired multiple ways to prevent antibody/complement-mediated elimination. However, with modern antibody engineering techniques available, the possibility offers itself to circumvent these defense mechanism. Indeed, the use of Nanobody (Nb) technology could offer future perspectives for both antibody-mediated drug targeting, as well as the development of antibody-based lytic compounds. Nanobodies are small 15 kDa antibody fragments with unique protein or antigen-recognizing properties (Muyldermans et al. 2009). They are derived from conventional camelid heavy-chain antibodies through recombinant gene technology. Important to stress here is that while almost all antibodies from vertebrate animals are constitutively composed of two identical heavy and two identical light chains, bona fide antibodies devoid of light chains occur in *Camelidae*. These heavy-chain antibodies (HCAbs) bind antigen solely with one single-variable domain with high affinity.

An Nb is the smallest intact antigen-binding entity that can be obtained from any antibody. Methods were developed to clone the Nb repertoire of an immunized camel, dromedary, or llama in phage display vectors and to select the

antigen-specific Nbs from these "immune" Nb libraries. To date, Nbs are economically produced by bacterial, fungal, or plant expression systems to a high yield and possess excellent biophysical properties (e.g., specificity, solubility, stability, long shelf-life). As nanobodies are produced as recombinant molecules, it is also relatively easy to produce larger nanobody complexes, generating, for example, bivalent or bivalent-Fc coupled nanobodies or nanobodies that are directly coupled to other protein carriers (Els Conrath et al. 2001). In addition, in order to reduce possible immunogenicity of nanobodies when injected into non-camelids, they can be customized by altering specific amino acid sequences. For example, for use in man, nanobodies are "humanized" to reduce the risk of the induction of anti-nanobody antibodies in the circulation (Vincke et al. 2009).

Nanobodies generated against trypanosomes have now been used successfully is several settings. First, a Nanobody against a conserved VSG carbohydrate epitope was used to target the enzyme beta-lactamase toward the parasite membrane, this enzyme can convert to pro-drug CCM (7-(4-carboxybutanamido)cephalosporin mustard) into highly toxic PDM (phenylenediamine mustard), resulting in the generation of a local trypanotoxic environment (Stijlemans et al. 2004). Experimental settings in vitro as well as in vivo showed Nanobodies indeed could be used to target larger molecules in a specific manner towards the parasite surface. Next, the same principle was used to target a truncated version of the human trypanolytic molecule ApoL1 towards the parasite membrane (Baral et al. 2006). This resulted in rapid and specific lysis, both in vitro and in vivo. Important to mention here is that human ApoL1 is a natural defense molecule with trypanolytic properties, that is capable of killing all nonhuman infective trypanosome. Human infective trypanosome have developed defense mechanisms against ApoL1 killing, involving in case of *T. rhodesiense* the *sra* gene product that has been proposed to bind to ApoL1. Although the exact mode of action of SRA on ApoL1 remains a matter of debate, it was suggested that removal of the SRA/ApoL1 interaction domain would result in an omnipotent trypanocidal molecule. Unfortunately, the further development of this chemeric construct for use as an anti-trypanosome agent is limited due to the immunogenicity of the ApoL1 moiety in nonhuman mammals. However, a proof-of-principle was delivered that intelligent engineering of antibodies can result in circumventing the conventional defense systems that trypanosomes use against host antibodies. Nb-based targeting of small non-immunogenic molecules is now on trial. Aiming to reduce the toxicity problems that generally accompany anti-trypanosome treatment, targeting of active compounds could indeed allow a reduction of the actual quantity of drug compounds administered to patients or trypanosomiasis-infected animals.

A second example of the use of Nanobodies in the fight against trypanosomiasis was more recently given by the development of Nbs with a direct trypanolytic potential (Stijlemans et al. 2011; Caljon et al. 2012). Here it was observed that high-affinity binding of Nbs to the trypanosome surface coat result in the rapid arrest of the endocytosis pathway, accompanied by immediate glucose and ATP starvation, and followed by lysis of the parasites. While the exact cellular mechanisms at work here are still under investigation, it appears that the defense mechanism that

trypanosomes have developed throughout their evolution to provide for conventional antibody clearance are unable to rescue the parasite from a Nanobody attack.

So far, two different strategies have been used to obtain anti-trypanosome Nbs. While the conventional approach involves vaccination of a camelid with a given antigen, followed by the in vitro selection of Nbs against that same antigen, an alternative method is to derive Nbs from a trypanosome-infected animal. Using the latter technique, a collection of pan-reactive Nbs was obtained that recognize a wide variety of different trypanosomes (Stijlemans et al. 2004). These results suggest that at least in case of animal trypanosomiasis, the experimental infection followed by drug treatment is a very valuable way to force the immune response into generating a wide range of antibodies, which can be used as a starting point for the generation of an Nb library.

5.7 Going Forward

African trypanosomes are extracellular parasites that are continuously exposed to the host antibody environment. Due to natural selection and evolution, these parasites have acquired adaptations that allow perfect survival under conditions of host immune pressure. Besides mechanisms of antigenic variation of the parasites' surface coat (Chaps. 1 and 3), immune modulation appears to be a major parasite defense system. The latter results in destruction of the immune memory—both at the level of the T cell compartment (see Chap. 4) and the B cell compartment as outlined in this chapter—protecting the parasite from the build-up of long-term antibody recall responses. This is important for parasite survival, as besides VSGs, the trypanosome needs to express an array of invariant surface receptors mediating nutrient uptake. The recent elucidation of the structure of the haptoglobin–hemoglobin receptor needed for heme acquisition is just one example where it was shown that an invariant receptor is accessible to the outside and is not shielded by surrounding VSG molecules (Higgins et al. 2013). Yet, continuous exposure of the immune system to this molecule does not lead to the problematic build-up of inhibitory antibodies. It is this need for breakdown of immunological memory responses and the induction of non-specific polyclonal B cell responses combined with the elimination of specific T cell help to remaining B cells that make the prospect for the development of an effective anti-trypanosome vaccine extremely bleak, even when nonvariant exposed parasite immunogens are considered as targets. Indeed, it appears that trypanosomes have not only invested in antigenic variation but also in the strategy to make the host "forget" which variants were present in the past. Hence, it can use different VATs to progress during infection, but can in the long run reuse old VATs as the host's immune system can't remember what it has "seen" already. As such, most recent results obtained in a mouse model for trypanosomiasis suggests that while immune suppression certainly contributes to inefficacy of parasite control by the host immune system, immune destruction results in the absence of any protective antiparasite immune response in the long term. This means that vaccination trails in mice are

doomed to fail, even when nonvariant surface proteins are targeted, and the parasite defense mechanisms of antigenic variation can be circumvented. Hence, while mouse trypanosomiasis has provided us over the last three decades with very valuable infectious immunology data, it remains to be seen whether or not this fundamental knowledge will in the end provide us with practical answers that can help bring forward an immunological intervention strategy that will help to bring both HAT and animal trypanosomiasis under control. Indeed, recent research into the mechanisms of trypanosomiasis-associated B-cell destruction, as well as into the mechanisms of antibody-mediated trypanosome destruction has uncovered a problematic situation, which will be very hard to tackle. Indeed, results obtained in IgM-deficient mice suggest that virulent trypanosomes have developed mechanisms to avoid variant-specific IgG-mediated destruction in vivo. Hence, if future vaccination attempts are to be successful, they should most likely have to be capable of (a) inducing long-lasting IgM memory and (b) trigger complete parasite elimination rapidly upon infection, preventing parasite driven memory destruction. Both of these appear extremely hard targets to obtain.

With respect to diagnoses, also here the dysfunction of the host B-cell compartment remains a problem. To date, only *T. b. gambiense* infections can be detected using the antibody screening CATT assay. For all other African trypanosome infections, including all livestock infections, microscope screening of suspected blood samples remains the only field applicable method available as antibody detection against nonvariant antigens is unfeasible. Hence, besides improvements in antiparasite vaccination, also new developments in both parasite detection and drug therapy are needed in order to win the battle against African trypanosomiasis. Recent developments in biotechnology offer however renewed hopes for the design of novel antibody-based tools that will be cheap, easy-to-use, and reliable under field conditions encountered in trypanosome endemic areas. As microscopic detection/confirmation of parasites in suspected blood samples will always remain the most convincing diagnostic tool, new methods are being designed to overcome the problems of detection limits encounter by conventional microscopy analysis. Here, the use of fluorescent antibody-based probes, such as pan-reactive flouorchrome labeled Nanobodies, in combination with new LED microscopes will be a huge improvement. While this technique was shown to be successful in various laboratory settings, transfer of this technology into a realistic field setting has now to be pursuit.

With respect to Nanobodies it is important to realize that these recombinantly expressed single-chain antibody fragments have a number of unique properties that make them extremely well suited for the development into diagnostic and treatment tools for neglected diseases. While having most properties of conventional antibodies, these molecules have the advantage that, for example, trypanosomes have never been exposed to them in their natural environment. Hence, there has been no evolutionary pressure that has allowed trypanosomes to develop defense systems to protect themselves against these antibody fragments. From a practical point of view, once generated, Nbs can be easily produced and purified using bacterial expression systems, making their production relatively cheap and feasible

even in a nonspecialized African laboratory setting. The future development of Nanobodies as direct anti-trypanosome molecules, drug targeting molecules and components of new diagnostic applications, will have to show that novel immune intervention techniques can be engineered to overcome the various trypanosome defense strategies. If so, this technology can provide a breakthrough not only in the fight against trypanosomiasis but also should be adapted for the detection and cure of other parasitic neglected diseases.

References

Askonas BA, Corsini AC, Clayton CE et al (1979) Functional depletion of T- and B-memory cells and other lymphoid cell subpopulations-during trypanosomiasis. Immunology 36:313–321

Authié E, Duvallet G, Robertson C et al (1993a) Antibody responses to a 33 kDa cysteine protease of *Trypanosoma congolense*: relationship to 'trypanotolerance' in cattle. Parasite Immunol 15 (8):465–474

Authié E, Muteti DK, Williams DJ (1993b) Antibody responses to invariant antigens of *Trypanosoma congolense* in cattle of differing susceptibility to trypanosomiasis. Parasite Immunol 15(2):101–111, Erratum in: Parasite Immunol. 15(3):185

Authie E, Boulange A, Muteti D et al (2001) Immunisation of cattle with cysteine proteinases of *Trypanosoma congolense*: targeting the disease rather than the parasite. Int J Parasitol 31:1429–1433

Baral TN, Magez S, Stijlemans B et al (2006) Experimental therapy of African trypanosomiasis with a nanobody-conjugated human trypanolytic factor. Med Sci 22:914–916

Baral TN, De Baetselier P, Brombacher F, Magez S (2007) Control of Trypanosoma evansi infection is IgM mediated and does not require a type I inflammatory response. J Infect Dis 195:1513–1520

Barry JD, Hajduk SL, Vickerman K et al (1979) Detection of multiple variable antigen types in metacyclic populations of Trypanosoma brucei. Trans R Soc Trop Med Hyg 73:205–208

Barry JD, Crowe JS, Vickerman K (1983) Instability of the *Trypanosoma brucei rhodesiense* metacyclic variable antigen repertoire. Nature 306:699–701

Barry JD, Graham SV, Fotheringham M et al (1998) VSG gene control and infectivity strategy of metacyclic stage Trypanosoma brucei. Mol Biochem Parasitol 91:93–105

Bockstal V, Guirnalda P, Caljon G et al (2011) *T. brucei* infection reduces B lymphopoiesis in bone marrow and truncates compensatory splenic lymphopoiesis through transitional B-cell apoptosis. PLoS Pathog 7:e1002089

Caljon G, Stijlemans B, Saerens D et al (2012) Affinity is an important determinant of the anti-trypanosome activity of nanobodies. PLoS Negl Trop Dis 6:e1902

Clayton CE, Ogilvie BM, Askonas BA (1979) *Trypanosoma brucei* infection in nude mice: B lymphocyte function is suppressed in the absence of T lymphocytes. Parasite Immunol 1:39–48

Cornelissen AW, Bakkeren GA, Barry JD et al (1985) Characteristics of trypanosome variant antigen genes active in the tsetse fly. Nucleic Acids Res 13:4661–4676

Corsini AC, Clayton C, Askonas BA et al (1977) Suppressor cells and loss of B-cell potential in mice infected with *Trypanosoma brucei*. Clin Exp Immunol 29:122–131

Crowe JS, Barry JD, Luckins AG et al (1983) All metacyclic variable antigen types of *Trypanosoma congolense* identified using monoclonal antibodies. Nature 306:389–391

Darji A, Beschin A, Sileghem M et al (1996) In vitro simulation of immunosuppression caused by Trypanosoma brucei: active involvement of gamma interferon and tumor necrosis factor in the pathway of suppression. Infect Immun 64:1937–1943

De Gee AL, Levine RF, Mansfield JM (1988) Genetics of resistance to the African trypanosomes. VI. Heredity of resistance and variable surface glycoprotein-specific immune responses. J Immunol 140:283–288

Engstler M, Pfohl T, Herminghaus S et al (2007) Hydrodynamic flow-mediated protein sorting on the cell surface of trypanosomes. Cell 131:505–515

Els Conrath K, Lauwereys M, Wyns L, Muyldermans S (2001) Camel single-domain antibodies as modular building units in bispecific and bivalent antibody constructs. J Biol Chem 276 (10):7346–7350

Esser KM, Schoenbechler MJ, Gingrich JB (1982) *Trypanosoma rhodesiense* blood forms express all antigen specificities relevant to protection against metacyclic (insect form) challenge. J Immunol 129(4):1715–1718

Gaithuma AK, Karanja SM, Ngotho M et al (2012) Lipid metabolism and other metabolic changes in vervet monkeys experimentally infected with *Trypanosoma brucei rhodesiense*. J Med Primatol 41(2):75–81

Gingrich JB, Ward RA, Macken LM et al (1981) Some phenomena associated with the development of Trypanosoma brucei rhodesiense infections in the tsetse fly, *Glossina morsitans*. Am J Trop Med Hyg 30:570–574

Greenwood BM, Whittle HC, Molyneux DH (1973) Immunosuppression in Gambian trypanosomiasis. Trans R Soc Trop Med Hyg 67:846–850

Guirnalda P, Murphy NB, Nolan D et al (2007) Anti-Trypanosoma brucei activity in Cape buffalo serum during the cryptic phase of parasitemia is mediated by antibodies. Int J Parasitol 37:1391–1399

Gull K (2003) Host-parasite interactions and trypanosome morphogenesis: a flagellar pocketful of goodies. Curr Opin Microbiol 6:365–370

Hanotte O, Ronin Y, Agaba M et al (2003) Mapping of quantitative trait loci controlling trypanotolerance in a cross of tolerant West African N'Dama and susceptible East African Boran cattle. Proc Natl Acad Sci USA 100:7443–7448

Higgins MK, Tkachenko O, Brown A, Reed J, Raper J, Carrington M (2013) Structure of the trypanosome haptoglobin-hemoglobin receptor and implications for nutrient uptake and innate immunity. Proc Natl Acad Sci USA 110(5):1905–1910. doi:10.1073/pnas.1214943110

Holland WG, Do TT, Huong NT et al (2003) The effect of *Trypanosoma evansi* infection on pig performance and vaccination against classical swine fever. Vet Parasitol 111:115–123

Hudson KM, Byner C, Freeman J et al (1976) Immunodepression, high IgM levels and evasion of the immune response in murine trypanosomiasis. Nature 264:256–258

Jackson DG, Windle HJ, Voorheis HP (1993) The identification, purification, and characterization of two invariant surface glycoproteins located beneath the surface coat barrier of bloodstream forms of Trypanosoma brucei. J Biol Chem 268:8085–8095

Jarvinen JA, Dalmasso A (1977) *Trypanosoma musculi* infections in normocomplementemic, C5-deficient, and C3-depleted mice. Infect Immun 16:557–563

Kamanga-Sollo EI, Musoke AJ, Nantulya VM et al (1991) Differences between N'Dama and Boran cattle in the ability of their peripheral blood leucocytes to bind antibody-coated trypanosomes. Acta Trop 49(2):109–117

Kateregga J, Lubega GW, Lindblad EB et al (2012) Effect of adjuvants on the humoral immune response to congopain in mice and cattle. BMC Vet Res 8:63

Lalmanach G, Boulange A, Serveau C et al (2002) Congopain from *Trypanosoma congolense*: drug target and vaccine candidate. Biol Chem 383:739–749

Le Ray D, Barry JD, Vickerman K (1978) Antigenic heterogeneity of metacyclic forms of *Trypanosoma brucei*. Nature 273(5660):300–302

Levine RF, Mansfield JM (1984) Genetics of resistance to the african trypanosomes III variant-specific antibody responses of H-2-compatible resistant and susceptible mice. J Immunol 133:1564–1569

Lutje V, Taylor KA, Kennedy D et al (1996) *Trypanosoma congolense*: a comparison of T-cell-mediated responses in lymph nodes of trypanotolerant and trypanosusceptible cattle during primary infection. Exp Parasitol 84(3):320–329

Lutz C, Ledermann B, Kosco-Vilbois MH et al (1998) IgD can largely substitute for loss of IgM function in B cells. Nature 393:797–801

MacAskill JA, Holmes PH, Whitelaw DD et al (1980) Immunological clearance of 75Se-labelled Trypanosoma brucei in mice.II. Mechanisms in immune animals. Immunology 40:629–635

MacAskill JA, Holmes PH, Whitelaw DD et al (1983) Immune mechanisms in C57B1 mice genetically resistant to *Trypanosoma congolense* infection. II. Aspects of the humoral response. Parasite Immunol 5:577–586

Magez S, Radwanska M (2009) African trypanosomiasis and antibodies: implications for vaccination, therapy and diagnosis. Future Microbiol 4:1075–1087

Magez S, Stijlemans B, Radwanska M et al (1998) The glycosyl-inositol-phosphate and dimyristoylglycerol moieties of the glycosylphosphatidylinositol anchor of the trypanosome variant-specific surface glycoprotein are distinct macrophage-activating factors. J Immunol 160:1949–1956

Magez S, Radwanska M, Beschin A et al (1999) Tumor necrosis factor alpha is a key mediator in the regulation of experimental *Trypanosoma brucei* infections. Infect Immun 67:3128–3132

Magez S, Radwanska M, Drennan M et al (2006) Interferon-gamma and nitric oxide in combination with antibodies are key protective host immune factors during trypanosoma congolense Tc13 Infections. J Infect Dis 193:1575–1583

Magez S, Schwegmann A, Atkinson R et al (2008) The role of B-cells and IgM antibodies in parasitemia, anemia, and VSG switching in *Trypanosoma brucei*-infected mice. PLoS Pathog 4:e1000122

Mitchell LA, Pearson TW (1983) Antibody responses induced by immunization of inbred mice susceptible and resistant to African trypanosomes. Infect Immun 40:894–902

Mkunza F, Olaho WM, Powell CN (1995) Partial protection against natural trypanosomiasis after vaccination with a flagellar pocket antigen from *Trypanosoma brucei rhodesiense*. Vaccine 13:151–154

Morrison WI, Roelants GE, Mayor-Withey KS et al (1978) Susceptibility of inbred strains of mice to Trypanosoma congolense: correlation with changes in spleen lymphocyte populations. Clin Exp Immunol 32:25–40

Murray PK, Jennings FW, Murray M et al (1974a) The nature of immunosuppression in *Trypanosoma brucei* infections in mice. I. The role of the macrophage. Immunology 27:815–824

Murray PK, Jennings FW, Murray M et al (1974b) The nature of immunosuppression in *Trypanosoma brucei* infections in mice.II. The role of the T and B lymphocytes. Immunology 27:825–840

Muyldermans S, Baral TN, Retamozzo VC et al (2009) Camelid immunoglobulins and nanobody technology. Vet Immunol Immunopathol 128:178–183

Mwangi DM, Munyua WK, Nyaga PN (1990) Immunosuppression in caprine trypanosomiasis: effects of acute Trypanosoma congolense infection on antibody response to anthrax spore vaccine. Trop Anim Health Prod 22:95–100

Naessens J (2006) Bovine trypanotolerance: a natural ability to prevent severe anaemia and haemophagocytic syndrome? Int J Parasitol 36(5):521–528

Naessens J, Leak SG, Kennedy DJ et al (2003) Responses of bovine chimaeras combining trypanosomosis resistant and susceptible genotypes to experimental infection with *Trypanosoma congolense*. Vet Parasitol 111:125–142

Nantulya VM, Doyle JJ, Jenni L (1980) Studies on Trypanosoma (nannomonas) congolense III. Antigenic variation in three cyclically transmitted stocks. Parasitology 80:123–131

Ngotho M, Kagira JM, Kariuki C et al (2011) Influence of trypanocidal therapy on the haematology of vervet monkeys experimentally infected with *Trypanosoma brucei rhodesiense*. Acta Trop 19(1):14–18

Nolan DP, Jackson DG, Windle HJ et al (1997) Characterization of a novel, stage-specific, invariant surface protein in *Trypanosoma brucei* containing an internal, serine-rich, repetitive motif. J Biol Chem 272:29212–29221

O'Gorman GM, Park SD, Hill EW et al (2006) Cytokine mRNA profiling of peripheral blood mononuclear cells from trypanotolerant and trypanosusceptible cattle infected with *Trypanosoma congolense*. Physiol Genomics 28(1):53–61

Pan W, Ogunremi O, Wei G et al (2006) CR3 (CD11b/CD18) is the major macrophage receptor for IgM antibody-mediated phagocytosis of African trypanosomes: diverse effect on subsequent synthesis of tumor necrosis factor alpha and nitric oxide. Microbes Infect 8:1209–1218

Phillips JA, Romball CG, Hobbs M et al (1996) CD4+ T cell activation and tolerance induction in B cell knockout mice. J Exp Med 183:1339–1344

Pinder M, Libeau G, Hirsch W et al (1984) Anti-trypanosome specific immune responses in bovids of differing susceptibility to African trypanosomiasis. Immunology 51:247–258

Radwanska M, Magez S, Dumont N et al (2000a) Antibodies raised against the flagellar pocket fraction of *Trypanosoma brucei* preferentially recognize HSP60 in cDNA expression library. Parasite Immunol 22:639–650

Radwanska M, Magez S, Michel A et al (2000b) Comparative analysis of antibody responses against HSP60, invariant surface glycoprotein 70, and variant surface glycoprotein reveals a complex antigen-specific pattern of immunoglobulin isotype switching during infection by Trypanosoma brucei. Infect Immun 68:848–860

Radwanska M, Guirnalda P, De Trez C (2008) Trypanosomiasis-induced B cell apoptosis results in loss of protective anti-parasite antibody responses and abolishment of vaccine-induced memory responses. PLoS Pathog 4:e1000078

Reinitz DM, Mansfield JM (1990) T-cell-independent and T-cell-dependent B-cell responses toexposed variant surface glycoprotein epitopes in trypanosome-infected mice. Infect Immun 58:2337–2342

Rurangirwa FR, Musoke AJ, Nantulya VM (1983) Immune depression in bovine trypanosomiasis: effects of acute and chronic *Trypanosoma congolense* and chronic Trypanosoma vivax infections on antibody response to *Brucella abortus* vaccine. Parasite Immunol 5:267–276

Sendashonga CN, Black SJ (1982) Humoral responses against *Trypanosoma brucei* variable surface antigen is induced by degenerating parasites. Parasite Immunol 4:245–257

Sharpe RT, Langley AM, Mowat GN et al (1982) Immunosuppression in bovine trypanosomiasis: response of cattle infected with *Trypanosoma congolense* to foot-and-mouth disease vaccination and subsequent live virus challenge. Res Vet Sci 32:289–293

Shi M, Wei G, Pan W et al (2005) Impaired Kupffer cells in highly susceptible mice infected with *Trypanosoma congolense*. Infect Immun 73:8393–8396

Sileghem M, Darji A, Hamers R et al (1989) Dual role of macrophages in the suppression of interleukin 2 production and interleukin 2 receptor expression in trypanosome-infected mice. Eur J Immunol 19:829–835

Sileghem M, Flynn JN, Logan-Henfrey L et al (1994) Tumour necrosis factor production by monocytes from cattle infected with *Trypanosoma (Duttonella) vivax* and *Trypanosoma (Nannomonas) congolense*: possible association with severity of anaemia associated with the disease. Parasite Immunol 16:51–54

Steverding D, Stierhof YD, Chaudhri M et al (1994) ESAG 6 and 7 products of Trypanosoma brucei form a transferrin binding protein complex. Eur J Cell Biol 64:78–87

Stijlemans B, Conrath K, Cortez-Retamozo V et al (2004) Efficient targeting of conserved cryptic epitopes of infectious agents by single domain antibodies. African trypanosomes as paradigm. J Biol Chem 279:1256–1261

Stijlemans B, Baral TN, Guilliams M et al (2007) A glycosylphosphatidylinositol-based treatment alleviates trypanosomiasis-associated immunopathology. J Immunol 179:4003–4014

Stijlemans B, Caljon G, Natesan SKA et al (2011) High affinity nanobodies against the Trypanosome brucei VSG are potent trypanolytic agents that block endocytosis. PLoS Pathog 7: e1002072

Taylor KA (1998) Immune responses of cattle to African trypanosomes: protective or pathogenic? Int J Parasitol 28(2):219–240

Taylor KA, Lutje V, Kennedy D et al (1996) Trypanosoma congolense: B-lymphocyte responses differ between trypanotolerant and trypanosusceptible cattle. Exp Parasitol 83:106–116

Tran T, Claes F, Dujardin JC et al (2006) The invariant surface glycoprotein ISG75 gene family consists of two main groups in the Trypanozoon subgenus. Parasitology 133:613–621

Tran T, Buscher P, Vandenbussche G et al (2008) Heterologous expression, purification and characterisation of the extracellular domain of trypanosome invariant surface glycoprotein ISG75. J Biotechnol 135:247–254

Uzonna JE, Kaushik RS, Gordon JR et al (1999) Cytokines and antibody responses during Trypanosoma congolense infections in two inbred mouse strains that differ in resistance. Parasite Immunol 21:57–71

Van Meirvenne N, Janssens PG, Magnus E (1975a) Antigenic variation in syringe passaged populations of *Trypanosoma (Trypanozoon) brucei*. I. Rationalization of the experimental approach. Ann Soc Belg Med Trop 55:1–23

Van Meirvenne N, Janssens PG, Magnus E et al (1975b) Antigenic variation in syringe passaged populations of Trypanosoma (Trypanozoon) brucei. II. Comparative studies on two antigenic-type collections. Ann Soc Belg Med Trop 55:25–30

Vanhollebeke B, De Muylder G, Nielsen MJ et al (2008) A haptoglobin-hemoglobin receptor conveys innate immunity to *Trypanosoma brucei* in humans. Science 320:677–681

Vickerman K (1985) Developmental cycles and biology of pathogenic trypanosomes. Br Med Bull 41:105–114

Vincke C, Loris R, Saerens D et al (2009) General strategy to humanize a camelid single-domain antibody and identification of a universal humanized nanobody scaffold. J Biol Chem 284:3273–3284

Whitelaw DD, Scott JM, Reid HW et al (1979) Immunosuppression in bovine trypanosomiasis: studies with louping-ill vaccine. Res Vet Sci 26:102–107

Williams DJ, Taylor K, Newson J et al (1996) The role of anti-variable surface glycoprotein antibody responses in bovine trypanotolerance. Parasite Immunol 18(4):209–218

Ziegelbauer K, Overath P (1992) Identification of invariant surface glycoproteins in the blood-stream stage of *Trypanosoma brucei*. J Biol Chem 267:10791–10796

Ziegelbauer K, Multhaup G, Overathn P (1992) Molecular characterization of two invariant surface glycoproteins specific for the bloodstream stage of *Trypanosoma brucei*. J Biol Chem 267:10797–10803

Normal Human Serum Lysis of Non-human Trypanosomes and Resistance of *T. b. rhodesiense* and *T. b. gambiense*

6

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Abstract

Trypanosoma brucei can be segregated into three morphologically identical subspecies based on host, geography and pathology. *T. b. brucei* is limited to domestic and wild animals throughout sub-Saharan Africa and is non-infective to humans due to trypanosome lytic factors found in human serum. There are two trypanosome lytic factors in human serum (TLF-1 & 2), both containing the proteins Apolipoprotein L1 (APOL1) and Haptoglobin-related protein (HPR). It has been conclusively demonstrated that the lytic component of TLF is APOL1, although HPR is required for maximal lysis by facilitating uptake of TLF particles via the HpHbR cell surface receptor. *T. b. gambiense* and *T. b. rhodesiense* are able to resist these lytic factors to cause human African sleeping sickness. *T. b. rhodesiense* is able to neutralise APOL1 due to expression of Serum Resistance-Associated gene (*SRA*). *SRA* is not found in the more prevalent human infective sub-species, *T. b. gambiense*, which causes over 97 % of reported human cases. Study of *T. b. gambiense* is complicated in that there are two distinct groups. Group 1 is invariably resistant to lysis and by far the more prevalent group. Group 2 *T. b. gambiense* exhibit a variable resistance phenotype and are only found at a small number of Côte d'Ivoire and Burkina Faso foci. Little is known as to how both groups are able to resist lysis by normal human serum; however, members of group 1 *T. b. gambiense* display both reduced expression and activity of HpHbR that may contribute to the resistance phenotype.

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Fig. 6.1 The distribution of countries reporting *T. b. gambiense* (*black*) and *T. b. rhodesiense* (*grey*) across sub-Saharan Africa. (adapted from WHO 2006)

6.1 Introduction

Trypanosoma brucei can be segregated into three morphologically identical sub-species based on host species, geography and pathology. *T. b. brucei* is limited to non-human animals throughout sub-Saharan Africa and is non-infective to humans due to trypanosome lytic factors (TLF) found in the serum of some old world primates. *T. b. brucei* cells exposed to human serum quickly swell and lyse in a consistent manner (Pays et al. 2006). *T. b. gambiense* and *T. b. rhodesiense* are human infective sub-species due to their ability to resist lysis by human serum. The two sub-species are defined by their relative geographic locations (Fig. 6.1). *T. b. rhodesiense* is found in eastern sub-Saharan Africa and is primarily a disease of animals rather than humans, with little human-to-human transmission (Welburn et al. 2001; Gibson et al. 2002; Radwanska et al. 2002a). It is primarily transmitted by *Glossina morsitans*, a tsetse species that inhabits the dry savannah found in Eastern sub-Saharan Africa (Lane and Crosskey 1993). The human serum resistance phenotype in this sub-species is variable when passaged in laboratory rodents (Willett and Fairbairn 1955). The disease in humans typically progresses rapidly and is always fatal. As it can be readily manipulated in the laboratory, this particular human infective sub-species has been the subject of extensive study.

A serum resistance-associated (*SRA*) gene has been described in *T. b. rhodesiense* and is used as a diagnostic marker to differentiate it from the morphologically identical *T. b. brucei*, which shares the same geographical range (Enyaru et al. 2006; Radwanska et al. 2002a; Gibson 2005). There are, however, several human infective isolates that are defined as *T. b. rhodesiense* on the basis of their geographical location but which do not possess *SRA* (Enyaru et al. 2006; De Greef et al.

1989). The genetic composition of these parasites has not been investigated and it is unknown how they relate to local populations.

T. b. gambiense is transmitted primarily by *G. palpalis*, a riverine tsetse species found along waterways, often close to human settlements. It is by far the most prevalent human infective trypanosome and is responsible for over 97 % of reported sleeping sickness cases (World Health Organization 2006). The disease is typically endemic and although it is believed to be primarily a human disease, domestic pigs have been identified as a potential reservoir (Paindavoine et al. 1986; Simo et al. 2006; Mehlitz 1979; Gibson et al. 1978). However, it is difficult to see how such a reservoir would be maintained as swine clear *T. b. gambiense* infections after a short period of time (Penchenier et al. 2005). Other wild fauna have been shown to carry *T. b. gambiense*, albeit in limited numbers (Njiokou et al. 2006; Mehlitz et al. 1981, 1982; Felgner et al. 1981). The *T. b. gambiense* form of the disease progresses more slowly in humans than that associated with *T. b. rhodesiense* and tends to be either a self-limiting condition or develops into a chronic disease of the lymphatic and nervous systems (Checchi et al. 2008). Research on *T. b. gambiense* is complicated in that there are two discernable groups of *T. b. gambiense*, identifiable by both genetic methods and phenotypic characteristics (Gibson et al. 1980; Mehlitz et al. 1982; Tait et al. 1984; Zillmann et al. 1984; Capewell et al. 2011). Group 1 *T. b. gambiense* parasites cause a chronic infection, are invariably resistant to human serum and are by far the more prevalent of the two groups. Group 2 *T. b. gambiense* are reported to be more virulent and deploy a variable human serum resistance mechanism in a manner similar to *T. b. rhodesiense* (Mehlitz et al. 1982; Zillmann et al. 1984). Most research on *T. b. gambiense* field populations has focused on the more prevalent group 1. Studies with isoenzymes and RFLPs have indicated that group 1 *T. b. gambiense* populations exhibit low genetic variation and appear distinct from *T. b. brucei* populations (Zillmann et al. 1984; Stevens and Tibyrenc 1996; Mathieu-Daude and Bicart-See 1994; Tait et al. 1984; Godfrey and Kilgour 1976; Gibson et al. 1980; Mehlitz et al. 1982; Paindavoine et al. 1989) and recent comprehensive microsatellite genotyping techniques appear to have confirmed this for some populations (Morrison et al. 2008; Koffi et al. 2009). This is in contrast to high genetic variation found in *T. b. brucei* populations (Zillmann et al. 1984; Stevens and Tibyrenc 1996; Mathieu-Daude and Bicart-See 1994; Tait et al. 1984; Godfrey and Kilgour 1976; Gibson et al. 1980; Mehlitz et al. 1982; Balmer et al. 2011). It has also been shown that while group 1 *T. b. gambiense* parasites are clonal within a disease focus, there are substantial genetic differences among isolates from different geographic locations (Morrison et al. 2008). Group 2 isolates show greater genetic variation than group 1 *T. b. gambiense* and appear more similar to local *T. b. brucei* (Zillmann et al. 1984; Stevens and Tibyrenc 1996; Mathieu-Daude and Bicart-See 1994; Tait et al. 1984; Godfrey and Kilgour 1976; Gibson et al. 1980; Mehlitz et al. 1982). The greater variation in genetic markers and similarity to the *T. b. brucei* population suggested that this second group of *T. b. gambiense* may be capable of genetic exchange in the tsetse fly, in contrast to group 1. This has subsequently been confirmed by laboratory crosses between *T. b. gambiense* group 2 and both *T. b. brucei* and *T. b. rhodesiense* strains (Turner et al. 1990;

Jenni et al. 1986; Gibson et al. 1997). The possibility that different mating structures exist in the two groups of *T. b. gambiense* has implications in evaluating the relationships between them and the evolution of several traits in the population, including human infectivity. It is possible that human infectivity has independently evolved in two separate populations of *T. b. gambiense* or, conversely, it has evolved once but due to mating events has become fixed in group 1 and variable in group 2 *T. b. gambiense*.

A recent study of microsatellite multi-locus genotypes of many *T. brucei* isolates from across Africa has found that group 1 *T. b. gambiense* form a clade which is separate from *T. b. brucei*, *T. b. rhodesiense* and group 2 *T. b. gambiense* (Balmer et al. 2011). Group 2 *T. b. gambiense* individuals formed a cluster positioned between *T. b. brucei* and group 1 *T. b. gambiense*, suggesting that group 2 *T. b. gambiense* may be a hybrid of group 1 and *T. b. brucei* and may, perhaps, be expected to share a serum resistance mechanism. However, the *T. b. brucei* used in the Balmer study were from East Africa and are not representative of West African *T. b. brucei* that are sympatric with the two *T. b. gambiense* groups. Consequently, whether group 2 parasites represent a genetically distinct population or are simply host range variants of West African *T. b. brucei* remains unresolved.

6.2 Primate Resistance to Trypanosomes

Due to antigenic variation (see Chaps. 1 and 3) and various mechanisms of immune evasion (see Chaps. 4 and 5) *T. brucei* can effectively evade the mammalian adaptive immune system indefinitely (Barry and McCulloch 2001). However, some species of primate have evolved an innate resistance mechanism in their serum to combat infection. This innate immune mechanism quickly lyses *T. b. brucei*, although *T. b. gambiense* and *T. b rhodesiense* are able to resist lysis and infections can establish in the human host. The lytic effect of human serum on non-human infective trypanosomes was first shown in the early twentieth century (Laveran and Mesnil 1912). When observed by microscopy, trypanosome lysis by human serum proceeds rapidly along a well-defined path (Pays et al. 2006). After a lag phase of approximately 30 min, the parasite cell experiences gross changes to morphology as it swells rapidly until it ruptures within 1–2 h (Rifkin 1978a, 1984). Microscopic evidence indicates that the swelling and lysis of the cell is due to the lysosome swelling (Vanhollebeke and Pays 2006; Pays and Vanhollebeke 2009; Pays et al. 2006). The sera from several catarrhine (old world) primates, including gorillas, baboons and mandrills, were also shown to lyse non-human infective trypanosomes but with differing efficacy (Laveran and Mesnil 1912; Lugli et al. 2004; Poelvoorde et al. 2004; Seed and Sechelski 1990).

The capability to lyse trypanosomes is confined to catarrhine primates and may have originated on two different occasions (Poelvoorde et al. 2004)—once in the *Cercopithecidae* group *(*baboon and mandrills) and once in the *Hominidae* group. It is interesting to note that *Hominidae* of the *Pan* genus (chimpanzees) do not posses trypanocidal serum despite the close evolutionary links with *Gorilla gorilla* and

Homo sapiens. The simplest explanation is that chimpanzees have lost the ability to combat trypanosome infection rather than the lytic effect arising separately in other *Hominidae* lineages. The high similarity of the genes involved in trypanosome lysis make it more likely that the trait evolved prior to divergence of the two groups of primates that occurred approximately 25 million years ago. The serum of *Papio* genus (baboon) primates has a much higher lysis efficiency compared to *Hominidae* and also lyses human infective *T. b. rhodesiense*. In a possible example of convergent evolution, sera from some individuals of West African descent have been shown to affect the growth of *T. b. rhodesiense* and may possess protective alleles of lytic proteins (Lecordier et al. 2009; Genovese et al. 2010). However, this has yet to be confirmed in a case–control study. It is also possible that there exist variant alleles that also protect against *T. b. gambiense*. However, evidence for this is based on a small number of reports of individuals resistant to infection, such as the Bambuti people of the Mbomo region in the Democratic Republic of the Congo (Frezil 1983) or recently described asymptomatic and self-cured cases from Côte d'Ivoire (Ilboudo et al. 2012).

6.3 Trypanosome Lytic Factors, TLF-1 and TLF-2

Early observations of trypanosome lysis indicated that the mechanism was not associated with the classical immune system components of immunoglobin or complement but rather uses an unknown factor in human serum (Laveran and Mesnil 1912). It has also been known for nearly a century that the serum of humans is highly toxic to several trypanosome species (Laveran and Mesnil 1912). While this might have evolved as a specific anti-trypanosome measure, it may also have arisen as a general protozoa defence strategy as the trypanolytic component of human serum also produces measurable physiological effects in *Plasmodium falciparum* (Imrie et al. 2004) and two *Leishmania* species (Samanovic et al. 2009). Nevertheless, most work on elucidating the mechanisms behind the lytic effect has been performed using *T. brucei*. By testing the trypanocidal effect of separated fractions of human serum on *T. b. brucei*, it was shown that the majority of the lytic effect was associated with the densest fraction of human serum, the high density lipoprotein (HDL) component (Rifkin 1978a, b). Purified human HDL from the same fraction size also clears *T. b. brucei* infections in mice (Rifkin 1978b).

HDL bodies are soluble amalgams of phospholipids, cholesterol and apolipoproteins (Pays et al. 2006) and, in addition to a trypanocidal effect, they appear to have an important role in controlling both blood pressure and cholesterol levels (Castelli et al. 1977; Gordon et al. 1977; Miller et al. 1977; Miller 1978). HDLs are a diverse family of particles, containing several different protein families (Blanche et al. 1981). The importance of HDL to trypanosome lysis was confirmed in experiments that showed that the level of HDL in serum correlates with the efficiency of lysis, depletion of HDL reduces lytic activity and that anti-human HDL antibodies can inhibit lysis (Hajduk et al. 1989; Gillett and Owen 1991). Physiological evidence was provided by the characterisation of the human

condition Tangiers disease in which patients have a reduced HDL component in their serum and no corresponding trypanolytic effect (Rifkin 1978a).

Most HDL bodies are not trypanocidal but one particular particle found in separation studies was shown to be highly lytic. This was termed the Trypanosome Lytic Factor (subsequently TLF-1 after the discovery of a second TLF) (Hajduk et al. 1989). Western blots identified two proteins unique to TLF-1 not found in other HDL particles. It was already noted that lysis was inhibited when trypanosomes were incubated below 17 °C, the same temperature at which endocytosis is greatly reduced in eukaryotes (Dunn et al. 1980). This fact, coupled with the initial 30-min lag phase before observable lysis effects, provided compelling evidence that TLF-1 is internalised by endocytosis before it can mediate lysis. Two different receptors for TLF-1 have been suggested to exist based on data from low-temperature-binding assays—a high affinity receptor that has approximately 350 copies per parasite and a low affinity receptor that has 60,000 receptors per cell (Drain et al. 2001). The high affinity receptor has now been acknowledged to be the haptoglobin–haemoglobin receptor HpHbR (Vanhollebeke et al. 2008), although the low affinity receptor is still not described. In addition to TLF-1, a second TLF has since been described in a larger sized fraction of serum (Tomlinson and Raper 1996; Raper et al. 1996; Tomlinson et al. 1995). The larger size of the TLF-2 particle is due to its association with IgM molecules on the particle surface. TLF-2 contains far less lipid than TLF-1 but possesses similar lytic characteristics and is found at a similar concentration in blood (Raper et al. 2001). It does, however, possess a much shorter half-life than TLF-1 and is therefore difficult to study experimentally. The receptor for TLF-2 is less well characterised, although it is known that it has low affinity, high turnover and haptoglobin does not compete for the receptor, unlike TLF-1 (Vanhollebeke et al. 2007). A *T. b. brucei* clone that no longer expresses the HpHbR protein does show increased resistance to lysis by TLF-2 (Bullard et al. 2012), suggesting that this receptor is involved to a degree in uptake of TLF-2, although the particle is primarily internalised via another route. This route has been suggested to be mediated by weak interactions between the VSG coat surrounding the parasite and the IgM bound to TLF-2 (Vanhollebeke and Pays 2010). IgM has been shown to weakly bind VSG on the trypanosome surface with a characteristic low affinity (Vanhollebeke and Pays 2010). TLF-2 would therefore be internalised as part of the normal surface recycling mechanism found in all trypanosome species.

6.4 APOA1, HPR and APOL1

The protein component of TLF-1 and TLF-2 consists of the apolipoproteins L1 (APOL1) and A1 (APOA1) (Tytler et al. 1995) and haptoglobin-related protein (HPR) (Smith et al. 1995; Raper et al. 1996). TLF-2 is also found bound to IgM, contributing to the larger size of the particle (Raper et al. 2001). APOA1 is a ubiquitous apolipoprotein contained within 70 % of all HDL particles and is predominantly involved in providing structure. Both recombinant APOA1 and

transgenic mice expressing human APOA1 are unable to kill *T. b. brucei* indicating that direct involvement in lysis can be discounted (Owen et al. 1992). However, the binding of TLF-1 particles to the low affinity receptor was competed by other HDL particles, including non-lytic bovine particles, suggesting that the ligand could be APOA1 (Vanhamme and Pays 2004). As APOA1 is often bound to lipid and cholesterol, a receptor to scavenge this protein is unsurprising due to the fact trypanosomes have extremely high lipid requirements due to rapid turnover of the VSG coat (Seyfang and Mecke 1990).

Of the remaining two proteins, haptoglobin-related protein appeared to be the most likely candidate to be the lytic factor. HPR is a close paralogue to haptoglobin protein (HP) which has been shown to possess a toxic peroxidase effect when bound to free haemoglobin in low pH conditions, similar to those within the lysosome (Connell and Smithies 1959; Bonkovsky 1991). It was hypothesised that a similar toxicity may be present in HPR and several experiments have implicated HPR in trypanosome lysis. It was shown that the then unknown high affinity TLF-1 receptor is not competed for by other HDLs but by haptoglobin bound to haem (Drain et al. 2001; Smith et al. 1995). Anti-haptoglobin antibodies have been shown to cross-react with HPR and also protect trypanosomes from lysis in the presence of TLF-1 (Smith et al. 1995). It was also noted that non-lytic serum from chimpanzees does not have functional HPR due to a frame-shift mutation in the gene, adding further weight to the argument that HPR is the lytic factor (McEvoy and Maeda 1988).

It was proposed that HPR was the trypanolytic component of HDL with a working hypothesis that haemoglobin-bound HPR enters the parasitic cell via endocytosis. After HPR enters the low pH environment of the lysosome, an iron-based Fenton peroxidase reaction occurs which damages the membrane and initiates lysis (Smith et al. 1995). Adding catalase to the parasite to reduce peroxide concentration also reduced lysis, seemingly supporting the peroxidase hypothesis. It was also shown that the end-products of lipid peroxidation were found in lysed cells and application of Dimethyl-4-phenylenediamine (an inhibitor of the lipid-degrading peroxidase effect) prevented lysis (Bishop et al. 2001). These results suggested that HPR was the lytic component of TLF-1. However, recombinant HPR has also not been shown to be lytic (Vanhollebeke et al. 2007), although this may be because the recombinant protein was constructed without the signal peptide. HPR is one of only a few proteins that possess an un-cleaved signal peptide (Harrington et al. 2012). This signal peptide has been shown to affect membrane fluidity in bloodstream form trypanosomes and has strong trypanocidal properties (Harrington et al. 2010, 2012).

An alternate hypothesis to HPR was proposed, suggesting the trypanocidal protein of TLF was a different common component of both TLF particles, APOL1. APOL1 arose as a lytic factor candidate when it was shown to co-localise with the serum resistance-associated (SRA) protein of *T. b. rhodesiense* (Sect. 6.6) (Vanhamme et al. 2003). APOL1 possesses a pore-forming domain under low pH conditions that is similar to that of bacterial colicins and the mammalian Bcl2 proteins involved in apoptosis (Zhaorigetu et al. 2008). This domain is known to be essential to the lytic function of the protein (Pérez-Morga et al. 2005). APOL1

protein localises with the lysosome membrane marker p67 after endocytosis, suggesting that it embeds in the lysosomal membrane (Vanhamme et al. 2003). Both naturally derived and recombinant APOL1 lyses trypanosomes with a swollen lysosome morphology similar to that seen after exposure to human serum, although unbound APOL1 lyses at a slower rate than normal human serum (Vanhollebeke et al. 2007; Vanhamme and Pays 2004; Vanhamme et al. 2003). It was proposed that APOL1 induces the formation of pores in the lysosome that in turn induce an influx of chloride ions (Vanhamme et al. 2003). The influx of ions upsets the osmotic balance of the lysosome, causing it to swell rapidly and rupture.

Similar to HPR, APOL1 is present in higher primates, but due to the presence of a premature stop codon, it is inactive in chimpanzees, explaining the lack of lytic activity in this species (Poelvoorde et al. 2004). However, HPR has recently been revealed to be ubiquitous in old world primates, even those with non-lytic serum (Vanhollebeke and Pays 2010) suggesting it evolved before APOL1 or innate immunity to trypanosomes. Strong evidence for APOL1 being the lytic factor of TLF came from the discovery of a unique medical case of a human in India found to be infected by the animal trypanosome *T. evansi* (Vanhollebeke et al. 2006). Although closely related to *T. brucei*, this trypanosome species is not normally infective to humans. The evidence indicates that the patient's infection is likely due to two frame-shift mutations, one in each allele of their APOL1 gene, thus rendering the protein non-functional. The patient's HPR gene has no major deviations in sequence from that of normal humans (Vanhollebeke et al. 2006). The addition of natural or recombinant APOL1 to the patient's serum fully rescued the lytic phenotype.

6.5 A Consensus Hypothesis for the Mechanism of Trypanosome Lysis

In light of the mounting evidence that APOL1 and HPR are both vital for efficient trypanosome lysis, a consensus involving both proteins has formed (Fig. 6.2). Reconstituted HDL containing both purified APOL1 and HPR causes 99 % lysis, while those that contain HPR or APOL1 alone are 20 times less effective over the same time-span (Shiflett et al. 2005, 2007; Vanhollebeke et al. 2007). Experiments investigating recombinant APOL1 with serum from the patient with a defective APOL1 gene have come to a similar conclusion that both APOL1 and HPR are necessary for efficient lysis (Vanhollebeke et al. 2006, 2007). Recombinant APOL1 on its own lysed trypanosomes at a slower rate than normal human serum but when added to the serum from the patient with defective APOL1, normal human serum activity was restored (Vanhollebeke et al. 2007). The rate at which APOL1 lysed parasites was also increased when added to recombinant HPR, although they found no evidence that HPR has a direct killing effect on trypanosomes due to the fact that the patient with non-functional APOL1 had perfectly normal HPR. This suggests that HPR has no direct impact on combating trypanosome infection unless paired with APOL1. This is not the case with APOL1, as even transgenic animals

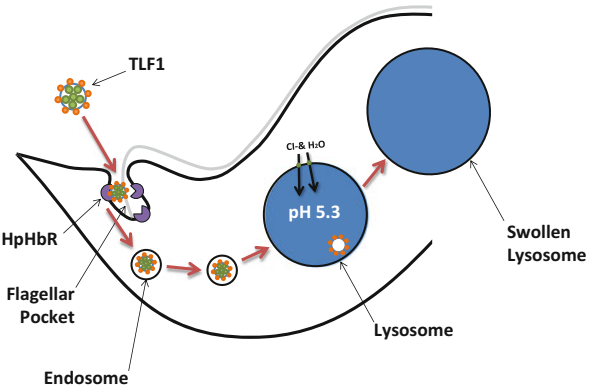

Fig. 6.2 An overview of the current model of TLF-1 mediated lysis in *T. b. brucei* (adapted from Pays et al. 2006; Pays and Vanhollebeke 2009). The TLF particle contains HPR (*Orange*), APOL1 (*Green*) and APOA1. The TLF particle binds to the HpHbR receptor with the HPR protein as the ligand. It is then internalised by endocytosis and transferred into early endosomes (pH 7). As the endosomes mature and eventually integrate with the lysosome, the pH of the organelle falls to 5.3 causing the release of APOL1 (*green*) APOL1 from the TLF particle. APOL1 undergoes a pH-mediated conformational change, exposing the pore-forming and membrane-addressing domains. This new structure allows the integration of APOL1 into the plasma membrane of the lysosome. When integrated within the membrane, APOL1 acts as an ion channel that allows Cl^- to freely enter the organelle and disrupt osmotic balance. Water then enters the lysosome along the new osmotic gradient, causing uncontrolled swelling of the organelle and eventually lysing the trypanosome. TLF-2 acts in a similar manner to TLF-1, although uptake is only partially via HpHbR

expressing human APOL1 are resistant to *T. b. brucei* infection, even without co-expression of HPR (Thomson et al. 2009; Molina-Portela et al. 2008). It appears likely that HPR is the ligand that facilitates the uptake of TLF-1 and APOL1 and a receptor that binds haptoglobin/haemoglobin and HPR/haemoglobin has recently been described, termed HpHbR (Vanhollebeke et al. 2008).

The HpHbR receptor is a GPI-anchored protein of approximately 350 copies found on the cell surface of the parasite. This closely matches the characteristics of the proposed high affinity receptor for TLF-1 (Drain et al. 2001). Down-regulation of the receptor in *T. b. brucei* leads to complete protection against the lytic effects of TLF-1 and an increased resistance to lysis by human serum and TLF-2 (Vanhollebeke et al. 2008; Bullard et al. 2012; Faulkner et al. 2006; Kieft et al. 2010). Therefore, the current hypothesis of innate immunity to trypanosomes in humans is that the TLF particles bind to receptors on the trypanosome surface (Drain et al. 2001; Vanhollebeke et al. 2008) are internalised by endocytosis (Hager et al. 1994) and transported to the lysosome (Shimamura et al. 2001) where they cause disruption of the lysosomal membrane and eventual cell death (Hager et al. 1994; Lorenz et al. 1994). APOL1 is the component of TLF-1 that causes lysosome disruption (Vanhamme et al. 2003). The protein undergoes a conformational change when exposed to the low pH environment of the lysosome, embeds in the

lysosomal membrane and forms channels that allow chloride ions to enter (Pérez-Morga et al. 2005). This perturbs the osmotic balance of the organelle, causing expansion and eventual rupture of the lysosome. HPR facilitates lysis by efficiently binding TLF-1 to the HpHbR receptor so that APOL1 can enter the lysosome. There may also be secondary toxic effects such as peroxidation by HPR (Smith et al. 1995) or membrane fluidity changes caused by the HPR signal peptide (Harrington et al. 2012), making parasites more susceptible to APOL1. The presence of APOL1 in TLF-2 makes it likely that this particle also uses the protein as a lytic mechanism, although uptake of TLF-2 is only partially mediated by HPR binding to HpHbR and may instead involve the weak binding of IgM to VSG (Vanhollebeke and Pays 2010).

6.6 *Trypanosoma brucei rhodesiense* and SRA

It was observed early in human infectivity studies that *T. b. rhodesiense* showed an inconsistent human serum resistance phenotype. Although the serum resistance phenotype was retained during animal passage, individual passages exhibited the phenotype to different degrees (Willett and Fairbairn 1955; Targett and Wilson 1973). Unfortunately, this also invalidated the BIIT somewhat for the original purpose of *T. b. rhodesiense* identification. It was noted that resistance appeared to be related to antigenic variation and changes to the variable antigen type (VAT) of the populations. Isogenic clones of the ETat strain of *T. b. rhodesiense* that differed only in VAT expression showed different serum resistance phenotypes (van Meirvenne et al. 1976). A particular VAT was identified as correlating with human serum resistance, ETat 1.10. This VAT was always present in resistant lines and not in sensitive lines. Gene switching of the *VSG* did not reduce resistance to lysis, implicating an *ESAG* rather than the *VSG* itself (Rifkin et al. 1994). All of these data indicate the presence of a gene in an expression site that was differently expressed depending on the expression site being used. Although the likely location of the serum resistance element was now understood, the actual gene was unknown.

In an attempt to narrow down the options, mRNA was compared between isogenic resistant and susceptible lines. A serum resistance associated transcript (SRA) was identified and predicted to be for a *VSG*-like gene (De Greef et al. 1989, 1992). Both lines possessed the gene for the protein but only resistant lines expressed it. The gene was later confirmed to be an *ESAG* in the ETat 1.10 expression site by Southern blot analysis. Conclusive proof that human serum resistance in *T. b. rhodesiense* was due to a single gene was shown when transgenic *T. b. brucei* expressing *SRA* gained resistance to lysis (Xong et al. 1998). Harking back to the earlier days of trypanosome research, the result was confirmed by inoculation into a human when a *T. b. brucei-SRA* line infected a researcher (Gibson 2005). Unlike the pioneering work of the Tinde experiment, this human inoculation was accidental, but it clearly demonstrated that the *SRA* gene was sufficient to confer human serum resistance to a *T. brucei* line.

SRA has been shown to be present in the vast majority of studied *T. b. rhodesiense* lines but absent in human serum sensitive *T. b. brucei* isolates (Welburn et al. 2001; Radwanska et al. 2002a; Picozzi 2005; Enyaru et al. 2006), although this has become a somewhat circular argument as *SRA* is diagnostic of *T. b. rhodesiense*, so *T. b. rhodesiense* must always possess *SRA*. However, a study in Uganda has identified several human infective *T. brucei* in a *T. b. rhodesiense* focus that did not PCR amplify with *SRA* primers (Enyaru et al. 2006). Additionally, in a study comparing serum sensitive and resistant isogenic lines expressing the same ETAT, the *SRA* transcript was not present in all resistant lines (Rifkin et al. 1994; De Greef et al. 1989). It is unknown as to whether these phenomena are due to mutations in the *SRA* gene, the expression site or an entirely new resistance trait arising in a *T. brucei* population.

Structurally, *SRA* appears to be a truncated *VSG* with a large deletion of a region in the centre of the N-terminal domain (Xong et al. 1998; De Greef et al. 1992; De Greef and Hamers 1994). SRA is present on the parasite cell surface (Milner and Hajduk 1999; Stephens and Hajduk 2011), where it is internalised in the flagellar pocket during cell surface protein turnover, leading to accumulation in the endosomes and lysosome (Shiflett et al. 2007; Vanhamme et al. 2003; Oli et al. 2006; Stephens and Hajduk 2011). This internalisation is essential to the function of SRA and interaction between APOL1 and SRA occurs within the cell rather than the flagellar pocket (Stephens and Hajduk 2011). This agrees with the fact that human TLF localises in the lysosome before lysis.

Genetically, *SRA* is well conserved in *T. b. rhodesiense*, with less than 3 % sequence variation between strains (Gibson et al. 2002). This would make it likely that the gene arose only once (Campillo and Carrington 2003). The slight differences present in *SRA* sequences segregate into northern and southern groups (Gibson et al. 2002; MacLean et al. 2004). This coincides with a previous distinction of *T. b. rhodesiense* into two sub-groups based on RFLP, minisatellite analysis (Godfrey et al. 1990; Gibson et al. 2002; MacLeod et al. 2000) and disease severity (MacLean et al. 2004). However, the differing genotypes and phenotypes may also be due to genetic exchange between strains resulting in the transfer of the *SRA* gene (and other genetic material) to *T. brucei* of different genetic backgrounds. This is supported by the fact that some populations of *T. b. rhodesiense* are more related to neighbouring *T. b. brucei* than other *T. b. rhodesiense* populations, suggesting that *T. b. rhodesiense* is a human-infective variant of *T. b. brucei* (MacLeod et al. 2001). This leads to a working hypothesis that on entry into the human bloodstream, some trypanosomes are expressing *SRA* at an active expression site. Consequently, these parasites are selected and the population within the host becomes resistant. In non-human animals this selective pressure is not applied, explaining the variable nature of serum resistance when *T. b. rhodesiense* is passaged through these species.

Although *SRA* is now well described in the literature, theories of how SRA confers resistance to human serum are somewhat polarised. The prevailing theory holds that SRA inhibits the activity of APOL1 based on the observed co-localisation of the two proteins in parasite cells (Vanhamme et al. 2003). The deletion of 126 amino acids in SRA relative to a non-truncated VSG removes two

surface loops normally present in the VSG protein, exposing the internal α-helices (Campillo and Carrington 2003). Based on modelled tertiary structure, it was proposed that the deletion resulted in human serum resistance as it allows an inter-chelating interaction between the exposed helices and the helical section of APOL1, either in the lysosome or on the cell surface (Vanhamme et al. 2003; Vanhamme and Pays 2004). The alternative hypothesis is that SRA disrupts internal trafficking of TLF to the lysosome and it remains within pre-lysosome vesicles (Shiflett et al. 2007; Oli et al. 2006). These trafficking experiments, however, used the laboratory adapted *T. b. brucei* strain Lister 427 ectopically expressing *SRA* and may not necessarily be indicative of what occurs in *T. b. rhodesiense*.

Regardless of mechanism, the discovery of *SRA* has led to advancements in epidemiology, diagnosis and interventions to combat sleeping sickness caused by *T. b. rhodesiense*. With the advent of specific PCR markers for *SRA*, trypanosomes with the potential to infect humans can be identified from non-human hosts without the need for laborious human serum resistance assays such as the BIIT (Welburn et al. 2001; Gibson et al. 2002; Radwanska et al. 2002a). This has proved useful in understanding the role of wildlife and livestock in the epidemiology of the disease. For example, traditional microscopy techniques and serum resistance assays estimated the number of livestock cattle to be carrying human infective *T. b. rhodesiense* at approximately 1 %. Modern analysis has indicated that instead this value is closer to 18 % (Welburn et al. 2001).

The importance of the cattle reservoir of *T. b. rhodesiense* may become increasingly important in countries such as Uganda that are now emerging from a period of long-term political unrest and where population migration is increasing. In the town of Soroti in Uganda, a recent *T. b. rhodesiense* outbreak has emerged that appears to coincide with a recent movement of cattle from a traditional endemic focus of sleeping sickness in Busoga district (Fèvre et al. 2001, 2005). There is a significant correlation between the presence of the disease and distance from the Soroti cattle market since 1998. Cluster analysis also identified that a patient's history of visiting the market was a statistically significant risk factor in contracting sleeping sickness. Although the evidence is circumstantial, the emergence of a *T. b. rhodesiense* disease focus in Soroti would seem to correlate with the recent movement of cattle into the area (Fèvre et al. 2001; Hutchinson et al. 2003).

The knowledge of how SRA acts to inhibit the lytic component of TLF has also led to an explanation of mechanism by which the serum of baboons can lyse *T. b. rhodesiense*. The APOL1 present in this species possesses two amino acid substitutions in the C-terminal helix of the protein. Normal *T. b. rhodesiense* SRA is unable to bind this protein variant and cannot inactivate the pore-forming domain (Thomson et al. 2009; Pérez-Morga et al. 2005; Lecordier et al. 2009). Demonstrating convergent evolution, APOL1 alleles from humans with sera that can reduce growth of *T. b. rhodesiense* in vitro also show modification to the same C-terminal portion of the gene (Genovese et al. 2010). It has been proposed that semen from transgenic cattle expressing modified baboon or human APOL1 with modified C termini could be utilised for artificially inseminating cattle at foci in Africa to generate herds of cattle resistant to *T. b. brucei* and *T. b. rhodesiense*,

as well as the major livestock pathogenic trypanosome species, *T. vivax* and *T. congolense*. In addition to the cost and logistical issues of this endeavour, the lack of widespread acceptance of transgenic animals by the African public may make the scheme difficult to succeed. The removal of African trypanosomes by the application of these transgenic animals is also likely to promote selection for trypanosomes that do not possess the *SRA* gene as a serum resistance mechanism. Without competition from *T. b. brucei* or *T. b. rhodesiense* it is impossible to predict how other trypanosomes will evolve to exploit this new niche—past experience indicates that it is unlikely to be to the benefit of the human population. An alternative use of such modified APOL1 would be the creation of a stable delivery system to deliver modified APOL1 to people infected with *T. b. rhodesiense*. This method has already been used with limited success in vitro on *T. b. rhodesiense* by conjugating the pore-forming domain of APOL1 with a single domain antibody that enters the cell after binding to the VSG (Baral et al. 2006).

Although the discovery of *SRA* has provided benefits to the understanding of *T. b. rhodesiense* genetics and biology, it is unable to be applied to the dominant human infective trypanosome *T. b. gambiense*. *SRA* is not present in either group of *T. b. gambiense* (De Greef et al. 1992; Radwanska et al. 2002b; Turner et al. 2004). An '*SRA*-like' motif has been located in the intermediate TREU927 strain, but it is not involved in resistance (Vanhamme et al. 2004). It should be reiterated that *T. b. rhodesiense* causes only around 3 % of human sleeping sickness cases (World Health Organization 2006) and elucidating the mechanism of the most prevalent human infective sub-species, *T. b. gambiense*, is vital for future disease control in Africa.

6.7 *Trypanosoma brucei gambiense* and Resistance to Human Serum Lysis

In comparison to *T. b. rhodesiense*, very little is known about the serum resistance mechanism of *T. b. gambiense*. As previously mentioned, there are two groups of *T. b. gambiense* (Zillmann et al. 1984; Stevens and Tibyrenc 1996; Mathieu-Daude and Bicart-See 1994; Tait et al. 1984; Godfrey and Kilgour 1976; Gibson et al. 1980; Mehlitz et al. 1982; Paindavoine et al. 1989). Group 2 has a similar serum resistance phenotype to *T. b. rhodesiense*, with a great deal of variance in the degree of human serum resistance displayed (Mehlitz et al. 1982; Zillmann et al. 1984). It is possible that group 2 *T. b. gambiense* may possess a similar resistance mechanism to *T. b. rhodesiense* in that an *ESAG* has adapted to inhibit TLF. The fact that a *T. b. brucei* strain artificially selected in vitro to be resistant to TLF-1 shows some changes in *ESAG* expression indicates that *ESAG* modification linked to human serum resistance can occur in *T. b. brucei* (Faulkner et al. 2006). However, in this particular line it has been shown that these changes to *VSG* and *ESAG* are probably unrelated to the resistance phenotype and rather it is down-regulation of the expression of the *HpHbR* gene that has conferred TLF resistance (Kieft et al. 2010; Bullard et al. 2012). There have been few studies on the human serum

resistance phenotype among group 2 *T. b. gambiense*. Preliminary in vivo studies with the group 2 *T. b. gambiense* strain TxTat have shown a correlation between the VAT of the parasite and resistant or sensitive phenotypes, suggesting it is linked to *VSG* and ES switching (Ortiz et al. 1994). It was also shown in the same line that resistant TxTat clones depleted the lytic potential of human serum more slowly than the sensitive clones (Ortiz-Ordóñez et al. 1994). It has, however, come to light that there is some confusion as to the exact identity of some TxTat lines, with some derivatives being revealed to be *T. b. rhodesiense* (Grab and Kennedy 2008). Lineage data for the TxTat used in the ex vivo assays is unavailable and it is possible that it has been misidentified as a type 2 *T. b. gambiense*. A recent study comparing the resistance phenotypes of group 1 and 2 *T. b. gambiense* suggest that ESAG switching does not correlate with a switch in resistance phenotypes (Capewell et al. 2011). The parasite transferrin receptor formed by ESAG6 and ESAG7 shows high variability between expression sites in both group 2 *T. b. gambiense* and *T. b. brucei* (Salmon et al. 1994; Bitter et al. 1998; Ligtenberg et al. 1994; Van Luenen et al. 2005). By sequencing the expressed ESAG6 and ESAG7 in both sensitive and resistance isogenic clones of a group 2 *T. b. gambiense* and comparing it to high throughput sequence data identifying all ESAG sequences, it was shown that both sensitive and resistant forms were using the same expression site (Capewell et al. 2011). As it does not appear that expression site switching is related to resistance switching in group 2 *T. b. gambiense*, there could be other mechanisms involved that vary transcription in a similar manner, such as the stochastic expression that emerges from gene network interactions found in some eukaryotes (Kærn et al. 2005). Indeed, in *T. brucei*, procyclic forms are always human serum resistant, while bloodstream forms are always sensitive, even in the same isogenic line (Natesan et al. 2010). This suggests that every *T. b. brucei* has the potential to become human infective merely by modifying expression of pre-existing genes. The potential for isogenic human serum sensitive and resistant forms of group 2 *T. b. gambiense* provides a powerful tool for forward genetics techniques, such as comparative transcriptomics, that will help reveal the genes involved in resistance.

Very little is known about the resistance mechanisms in the most prevalent human infective trypanosome, group 1 *T. b. gambiense*, due to the difficulty of working with it under laboratory conditions. BIIT tests indicate it has a robust and invariant resistance phenotype (Zillmann et al. 1984; Stevens and Tibyrenc 1996; Mathieu-Daude and Bicart-See 1994; Tait et al. 1984; Godfrey and Kilgour 1976; Gibson et al. 1980; Mehlitz et al. 1982; Paindavoine et al. 1989). This would indicate a genetic mechanism of resistance that is being constitutively expressed, completely unlike the expression linked system of *T. b. rhodesiense* or group 2 *T. b. gambiense*. A component of resistance has been revealed in a recent study showing that group 1 *T. b. gambiense* from several studied foci exhibit a marked decrease in HpHbR expression compared to other *T. brucei* sub-species. Decreased expression of this gene is correlated with reduced uptake of TLF-1 and TLF-2 (Kieft et al. 2010). Furthermore, this study also suggested that the HpHbR is also non-functional in group 1 *T. b. gambiense* as ectopic expression of the *T. b. gambiense*

HpHbR allele in a HpHbR null mutant *T. b. brucei* did not restore sensitivity to TLF. Several group 1 *T. b gambiense*-specific polymorphisms in HpHbR were identified and it has now been shown that a single substitution of serine for leucine at position 210 in HpHbR reduces binding affinity of HpHbR 20-fold (Higgins et al. 2013). This polymorphism is conserved across all group 1 *T. b. gambiense* foci that have been investigated so far, suggesting it has potential as a tool for identifying *T. b. gambiense* (Symula et al. 2012). All of this information indicates that group 1 *T. b. gambiense* have evolved to avoid TLF-1 by modification of both expression and function of the HpHbR gene. However, it is unclear how this sub-species group avoids lysis by TLF-2 that is primarily taken up via a route that does not involve HpHbR (Raper et al. 2001) or whether the uncharacterised low affinity receptor is still functional (Drain et al. 2001). Evidence that group 1 *T. b. gambiense* is resistant to lysis by recombinant APOL1 internalised via fluid phase endocytosis suggests that the parasites have mechanisms to directly protect against lysis by APOL1, although they may need to be combined with avoidance (Capewell et al. 2011).

6.8 Going Forward

Understanding the mechanisms of human infectivity in *T. brucei* is both an interesting case study of the co-evolutionary arms race between parasite and host and also provides notable advances in the detection and treatment of disease. It is hoped that by exploiting the natural defence mechanisms that have evolved to combat trypanosome infection, it will limit the opportunities for the parasite to evolve and overcome treatment options. For example, the increasing understanding of APOL1 has allowed the formation of several possible treatments for African trypanosomiasis. One such proposed solution to African sleeping sickness involves the distribution of transgenic cows expressing either recombinant baboon ApoL1 (Thomson et al. 2009) or truncated APOL1 (Lecordier et al. 2009). Both baboon APOL1 and the truncated APOL1 have key mutations at the SRA-binding site that allow them to kill *T. b. rhodesiense* and mice expressing these genes ectopically are unable to be infected by either *T. b. brucei* or *T. b. rhodesiense* (Thomson et al. 2009). There are also APOL1 alleles in some humans of African descent that naturally possess modifications similar to baboon and truncated APOL1, and the sera of these patients reduce growth of *T. b. rhodesiense* in culture. However, patients who were homozygous for the mutant alleles exhibited acute kidney problems during mid-life, suggesting that ectopic expression of such genes in livestock may prove deleterious to their health (Genovese et al. 2010).

Unfortunately, utilising the currently identified APOL1 alleles for intervention will have a limited effect on the human disease across Africa due to the fact that both the baboon and truncated APOL1 used in these experiments are not able to kill either group of *T. b. gambiense* (Lecordier et al. 2009; Thomson et al. 2009). *T. b. rhodesiense* causes less than 3 % of human African sleeping sickness cases across the continent (World Health Organization 2006). Application of these transgenic cows is also likely to open new niches for non-SRA *T. b. rhodesiense* (De Greef

et al. 1989; Enyaru et al. 2006) and both groups of *T. b. gambiense* due to the removal of not only *T. b. brucei* and *T. b. rhodesiense* from the cattle herd, but the more widespread animal trypanosomes *T. congolense* and *T. vivax*. This will need to be considered as the *T. b. gambiense* and *T. b. rhodesiense* foci in Uganda converge (Fèvre et al. 2005; Picozzi 2005). The removal of so many competitors to *T. b. gambiense* as it expands south may result in cattle herds predominantly infected not with human serum sensitive species, but with *T. b. gambiense*. Until a variant of APOL1 can be found that kills group 1 *T. b. gambiense*, it would be prudent to advise caution in the use of treatments across Africa that would greatly upset the natural populations of parasitic species. Such variant alleles may exist in the Bambuti people of the Democratic Republic of the Congo (Frezil 1983) or asymptomatic patients and self-cured cases from Côte d'Ivoire (Ilboudo et al. 2012). There is evidence that the APOL1 gene is under intense selection in African individuals and it is likely that other alleles exist that protect against trypanosomes (Conrad et al. 2010). These APOL1 must also be delivered via particles other than TLF-1 as group 1 *T. b. gambiense* has markedly less uptake of TLF (Higgins et al. 2013; Kieft et al. 2010). These could be either TLF-2 or artificially created TLF particles with novel ligands. An alternative to using lytic particles is the utilisation of APOL1 conjugates that are internalised by interactions with known surface proteins of *T. brucei*, such as transferrin (Ligtenberg et al. 1994) or VSG. This has effectively been demonstrated using an APOL1 nanobody conjugate against *T. b. brucei* and *T. b. rhodesiense*, although not against *T. b. gambiense* (Baral et al. 2006). Although not utilised in *T. b. rhodesiense* to date, an additional intervention strategy that could be utilised are large peptide or drug screens that target SRA. These peptides are unlikely to work on *T. b. gambiense* so this sub-species will require a unique targeted intervention.

Group 1 *T. b. gambiense* are still able to resist lysis by recombinant APOL1 and TLF-2, neither of which are principally internalised via HpHbR. This group must therefore possess further resistance mechanisms to resist lysis. Additionally, group 2 *T. b. gambiense* possess a fully functional HpHbR but still display resistance, albeit variably expressed, so this sub-species group possibly possess a third novel resistance mechanism (Capewell et al. 2011). Finding the mechanisms by which both groups are able to avoid lysis by APOL1 is essential to combating human sleeping sickness across Africa. Now that laboratory adapted strains of both groups of *T. b. gambiense* have been generated (Capewell et al. 2011), several techniques are available to identify such genes. Group 2 *T. b. gambiense* exists as both resistant and sensitive forms and undergoes sexual recombination. This makes it amenable to forward genetics techniques such as quantitative trait analysis and comparative transcriptomics. The lack of sensitive forms of group 1 *T. b. gambiense*, and the apparent lack of mating displayed in the field in this sub-species group, makes such forward genetics difficult in group 1 parasites (Zillmann et al. 1984; Stevens and Tibyrenc 1996; Mathieu-Daude and Bicart-See 1994; Tait et al. 1984; Godfrey and Kilgour 1976; Gibson et al. 1980; Mehlitz et al. 1982; Paindavoine et al. 1989; Koffi et al. 2009; Morrison et al. 2008). A reverse genetics technique, such as a genome-wide RNAi screen, may prove useful (Alsford et al. 2011), especially as a

protocol for genetically modifying *T. b. gambiense* has been developed (Giroud et al. 2009). This RITSeq technique has proven useful in discovering numerous genes involved in drug resistance in *T. brucei* (Alsford et al. 2012).

In conclusion, human infectivity in *T. brucei* has conceivably arisen on at least four occasions. In Eastern Africa the evolution of *SRA* and the high levels of mating have caused the human infectivity trait to spread through the local *T. brucei* population, generating the *T. b. rhodesiense* sub-species (De Greef et al. 1989, 1992; Welburn et al. 2001; Gibson 2005; Xong et al. 1998; Gibson et al. 2002). It is important to also note that there are human infective trypanosomes in this area that do not possess *SRA*, suggesting a second mechanism to resist lysis (De Greef et al. 1989; Enyaru et al. 2006). In West and Central Africa, group 1 *T. b. gambiense* is the dominant cause of human African sleeping sickness. This group of parasites has evolved a constitutively expressed human serum resistance mechanism that does not depend on *SRA*. After evolution of this trait, the sub-species appears to have expanded clonally in the human population. Just as in East Africa, there is also a human infective *T. brucei* population separate from the dominant form that appears to have a novel resistance mechanism, group 2 *T. b. gambiense*. Taken together, all of these data indicate that *T. brucei* has a high zoonotic potential despite specific trypanolytic countermeasures that have been inherited by humans. Understanding the co-evolution of these trypanolytic factors in primates and resistance to them in parasites is of key importance to combating human trypanosomiasis.

References

Alsford S et al (2011) High-throughput phenotyping using parallel sequencing of RNA interference targets in the African trypanosome. Genome Res 21(6):915–924

Alsford S et al (2012) High-throughput decoding of antitrypanosomal drug efficacy and resistance. Nature 482(7384):232–236

Balmer O et al (2011) Phylogeography and taxonomy of *Trypanosoma brucei*. PLoS Negl Trop Dis 5(2):e961

Baral TN et al (2006) Experimental therapy of African trypanosomiasis with a nanobody-conjugated human trypanolytic factor. Nat Med 12(5):580–584

Barry JD, McCulloch R (2001) Antigenic variation in trypanosomes: enhanced phenotypic variation in a eukaryotic parasite. Adv Parasitol 49:1–70

Bishop JR, Shimamura M, Hajduk SL (2001) Insight into the mechanism of trypanosome lytic factor-1 killing of *Trypanosoma brucei brucei*. Mol Biochem Parasitol 118(1):33–40

Bitter W et al (1998) The role of transferrin-receptor variation in the host range of *Trypanosoma brucei*. Nature 391(6666):499–502

Blanche PJ et al (1981) Characterization of human high-density lipoproteins by gradient gel electrophoresis. Biochim Biophys Acta 665(3):408–419

Bonkovsky HL (1991) Iron and the liver. Am J Med Sci 301(1):32

Bullard W et al (2012) Haptoglobin-hemoglobin receptor independent killing of African trypanosomes by human serum. Virulence 3(1):72–76

Campillo N, Carrington M (2003) The origin of the serum resistance associated (SRA) gene and a model of the structure of the SRA polypeptide from Trypanosoma brucei rhodesiense. Mol Biochem Parasitol 127(1):79–84

Capewell P et al (2011) Differences between *Trypanosoma brucei gambiense* groups 1 and 2 in their resistance to killing by trypanolytic factor 1. PLoS Negl Trop Dis 5(9):e1287

Castelli WP et al (1977) HDL cholesterol and other lipids in coronary heart disease. The cooperative lipoprotein phenotyping study. Circulation 55(5):767–772
Checchi F et al (2008) The natural progression of gambiense sleeping sickness: what is the evidence? PLoS Negl Trop Dis 2(12):e303
Connell GE, Smithies O (1959) Human haptoglobins: estimation and purification. Biochem J 72 (1):115–121
Conrad DF et al (2010) Origins and functional impact of copy number variation in the human genome. Nature 464(7289):704–712
De Greef C, Hamers R (1994) The serum resistance-associated (*SRA*) gene of *Trypanosoma brucei rhodesiense* encodes a variant surface glycoprotein-like protein. Mol Biochem Parasitol 68 (2):277–284
De Greef C et al (1989) A gene expressed only in serum-resistant variants of *Trypanosoma brucei rhodesiense*. Mol Biochem Parasitol 36(2):169–176
De Greef C et al (1992) Only the serum-resistant bloodstream forms of *Trypanosoma brucei rhodesiense* express the serum resistance associated (SRA) protein. Ann Soc Belg Med Trop 72 (Suppl 1):13–21
Drain J, Bishop JR, Hajduk SL (2001) Haptoglobin-related protein mediates trypanosome lytic factor binding to trypanosomes. J Biol Chem 276(32):30254–30260
Dunn W, Hubbard A, Aronson N (1980) Low temperature selectively inhibits fusion between pinocytic vesicles and lysosomes during heterophagy of 125I-asialofetuin by the perfused rat liver. J Biol Chem 255(12):5971
Enyaru JCK et al (2006) Detection of *T.b. rhodesiense* trypanosomes in humans and domestic animals in south east Uganda by amplification of serum resistance-associated gene. Ann N Y Acad Sci 1081:311–319
Faulkner SD et al (2006) In vitro generation of human high-density-lipoprotein-resistant *Trypanosoma brucei brucei*. Eukaryot Cell 5(8):1276–1286
Felgner P et al (1981) Epidemiological studies on the animal reservoir of *gambiense* sleeping sickness. Part II. Parasitological and immunodiagnostic examination of the human population. Tropenmed Parasitol 32(3):134–140
Fèvre EM et al (2001) The origins of a new *Trypanosoma brucei rhodesiense* sleeping sickness outbreak in eastern Uganda. Lancet 358(9282):625–628
Fèvre EM et al (2005) A burgeoning epidemic of sleeping sickness in Uganda. Lancet 366 (9487):745–747
Frezil JL (1983) Human trypanosomiasis in the Congo. ORSTOM, Paris
Genovese G et al (2010) Association of Trypanolytic ApoL1 variants with kidney disease in African Americans. Science 329(5993):841–845
Gibson WC (2005) The SRA gene: the key to understanding the nature of *Trypanosoma brucei rhodesiense*. Parasitology 131(2):143
Gibson W, Mehlitz D, Lanham SM (1978) The identification of *Trypanosoma brucei gambiense* in Liberian pigs and dogs by isoenzymes and by resistance to human plasma. Tropenmed Parasitol 29(3):335–345
Gibson WC, Marshall DC, Godfrey DG (1980) Numerical analysis of enzyme polymorphism: a new approach to the epidemiology and taxonomy of trypanosomes of the subgenus *Trypanozoon*. Adv Parasitol 18:175–246
Gibson W et al (1997) Intraclonal mating in *Trypanosoma brucei* is associated with out-crossing. Microbiology 143(3):909–920
Gibson W, Backhouse T, Griffiths A (2002) The human serum resistance associated gene is ubiquitous and conserved in *Trypanosoma brucei rhodesiense* throughout East Africa. Infect Genet Evol 1(3):207–214
Gillett MP, Owen JS (1991) *Trypanosoma brucei brucei*: differences in the trypanocidal activity of human plasma and its relationship to the level of high density lipoproteins. Trans R Soc Trop Med Hyg 85(5):612–616

Giroud C et al (2009) Murine models for *Trypanosoma brucei gambiense* disease progression—from silent to chronic infections and early brain tropism. PLoS Negl Trop Dis 3(9):e509

Godfrey DG, Kilgour V (1976) Enzyme electrophoresis in characterizing the causative organism of Gambian trypanosomiasis. Trans R Soc Trop Med Hyg 70(3):219–224

Godfrey DG et al (1990) The distribution, relationships and identification of enzymic variants within the subgenus *Trypanozoon*. Adv Parasitol 29:1–74

Gordon T et al (1977) High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. Am J Med 62(5):707

Grab DJ, Kennedy PGE (2008) Traversal of human and animal trypanosomes across the blood-brain barrier. J Neurovirol 14(5):344–351

Hager KM et al (1994) Endocytosis of a cytotoxic human high density lipoprotein results in disruption of acidic intracellular vesicles and subsequent killing of African trypanosomes. J Cell Biol 126:155–167

Hajduk SL et al (1989) Lysis of *Trypanosoma brucei* by a toxic subspecies of human high density lipoprotein. J Biol Chem 264(9):5210–5217

Harrington JM et al (2010) The plasma membrane of bloodstream-form African trypanosomes confers susceptibility and specificity to killing by hydrophobic peptides. J Biol Chem 285 (37):28659–28666

Harrington JM et al (2012) Novel African trypanocidal agents: membrane rigidifying peptides. PLoS One 7(9):e44384

Higgins MK, Tkachenko O, Brown A (2013) Structure of the trypanosome haptoglobin–hemoglobin receptor and implications for nutrient uptake and innate immunity. Proc Natl Acad Sci U S A 110(5):1905–1910

Hutchinson OC et al (2003) Lessons learned from the emergence of a new *Trypanosoma brucei rhodesiense* sleeping sickness focus in Uganda. Lancet Infect Dis 3(1):42–45

Ilboudo H et al (2012) APOL1 expression is induced by *Trypanosoma brucei gambiense* infection but is not associated with differential susceptibility to sleeping sickness. Infect Genet Evol 12 (7):1519–1523

Imrie H, Ferguson D, Day KP (2004) Human serum haptoglobin is toxic to *Plasmodium falciparum* in vitro. Mol Biochem Parasitol 133(1):93–98

Jenni L et al (1986) Hybrid formation between African trypanosomes during cyclical transmission. Nature 322(6075):173–175

Kærn M et al (2005) Stochasticity in gene expression: from theories to phenotypes. Nat Rev Genet 6(6):451–464

Kieft R et al (2010) Mechanism of *Trypanosoma brucei gambiense* (group 1) resistance to human trypanosome lytic factor. Proc Natl Acad Sci U S A 107(37):16137–16141

Koffi M et al (2009) Population genetics of *Trypanosoma brucei gambiense*, the agent of sleeping sickness in Western Africa. Proc Natl Acad Sci U S A 106:209–214

Lane R, Crosskey R (1993) Medical insects and arachnids. Chapman & Hall, London

Laveran A, Mesnil F (1912) Trypanosomes et Trypanosomiases. Trypanosomiase humaine ou maladie du sommeil. Masson, Paris

Lecordier L et al (2009) C-terminal mutants of apolipoprotein L-I efficiently kill both *Trypanosoma brucei brucei* and *Trypanosoma brucei rhodesiense*. PLoS Pathog 5(12): e1000685

Ligtenberg MJ et al (1994) Reconstitution of a surface transferrin binding complex in insect form *Trypanosoma brucei*. EMBO J 13(11):2565

Lorenz P et al (1994) Importance of acidic intracellular compartments in the lysis of *Trypanosoma brucei brucei* by normal human serum. Trans R Soc Trop Med Hyg 88(4):487–488

Lugli EB et al (2004) Characterization of primate trypanosome lytic factors. Mol Biochem Parasitol 138(1):9–20

MacLean L et al (2004) Severity of human African trypanosomiasis in East Africa is associated with geographic location, parasite genotype, and host inflammatory cytokine response profile. Infect Immun 72(12):7040–7044

MacLeod A et al (2000) Minisatellite marker analysis of *Trypanosoma brucei*: reconciliation of clonal, panmictic, and epidemic population genetic structures. Proc Natl Acad Sci 97 (24):13442–13447
MacLeod A et al (2001) Evidence for multiple origins of human infectivity in *Trypanosoma brucei* revealed by minisatellite variant repeat mapping. J Mol Evol 52(3):290–301
Mathieu-Daude F, Bicart-See A (1994) Identification of *Trypanosoma brucei gambiense* group I by a specific kinetoplast DNA probe. Am J Trop Med Hyg 50(1):13–19
McEvoy SM, Maeda N (1988) Complex events in the evolution of the haptoglobin gene cluster in primates. J Biol Chem 263(30):15740
Mehlitz D (1979) Trypanosome infections in domestic animals in Liberia. Tropenmed Parasitol 30 (2):212–219
Mehlitz D, Brinkmann U, Haller L (1981) Epidemiological studies on the animal reservoir of Gambiense sleeping sickness. Part I. Review of literature and description of the study areas. Tropenmed Parasitol 32(3):129–133
Mehlitz D, Zillmann U, Scott CM (1982) Epidemiological studies on the animal reservoir of Gambiense sleeping sickness. Part III. Characterization of trypanozoon stocks by isoenzymes and sensitivity to human serum. Tropenmed Parasitol 33(3):113–118
Miller N (1978) The evidence for the antiatherogenicity of high density lipoprotein in man. Lipids 13(12):914–919
Miller NE et al (1977) The Tromsø Heart Study: high density lipoprotein and coronary heart disease: a prospective case control study. Lancet 309(8019):965–968
Milner JD, Hajduk SL (1999) Expression and localization of serum resistance associated protein in *Trypanosoma brucei rhodesiense*. Mol Biochem Parasitol 104(2):271–283
Molina-Portela MP, Samanovic M, Raper J (2008) Distinct roles of apolipoprotein components within the trypanosome lytic factor complex revealed in a novel transgenic mouse model. J Exp Med 205(8):1721–1728
Morrison LJ et al (2008) *Trypanosoma brucei gambiense* Type 1 populations from human patients are clonal and display geographical genetic differentiation. Infect Genet Evol 8(6):847–854
Natesan SK et al (2010) Evidence that low endocytic activity is not directly responsible for human serum resistance in the insect form of African trypanosomes. BMC Res Notes 3(1):63
Njiokou F et al (2006) Wild fauna as a probable animal reservoir for *Trypanosoma brucei gambiense* in Cameroon. Infect Genet Evol 6(2):147–153
Oli MW et al (2006) Serum resistance-associated protein blocks lysosomal targeting of trypanosome lytic factor in *Trypanosoma brucei*. Eukaryot Cell 5(1):132–139
Ortiz JC, Sechelski JB, Seed JR (1994) Characterization of human serum-resistant and serum-sensitive clones from a single *Trypanosoma brucei gambiense* parental clone. J Parasitol 80 (4):550–557
Ortiz-Ordóñez JC, Sechelski JB, Seed JR (1994) Mechanism of lysis of *Trypanosoma brucei gambiense* by human serum. J Parasitol 80(6):924–930
Owen JS, Gillett M, Hughes TE (1992) Transgenic mice expressing human apolipoprotein AI have sera with modest trypanolytic activity in vitro but remain susceptible to infection by *Trypanosoma brucei brucei*. J Lipid Res 33(11):1639–1646
Paindavoine P et al (1986) The use of DNA hybridization and numerical taxonomy in determining relationships between *Trypanosoma brucei* stocks and subspecies. Parasitology 92(Pt 1):31–50
Paindavoine P et al (1989) Different allele frequencies in *Trypanosoma brucei brucei* and *Trypanosoma brucei gambiense* populations. Mol Biochem Parasitol 32(1):61–71
Pays E, Vanhollebeke B (2009) Human innate immunity against African trypanosomes. Curr Opin Immunol 21(5):493–498
Pays E et al (2006) The trypanolytic factor of human serum. Nat Rev Microbiol 4(6):477–486
Penchenier L et al (2005) Spontaneous cure of domestic pigs experimentally infected by *Trypanosoma brucei gambiense*: implications for the control of sleeping sickness. Vet Parasitol 133(1):7–11

Pérez-Morga D et al (2005) Apolipoprotein L-I promotes trypanosome lysis by forming pores in lysosomal membranes. Science 309(5733):469–472
Picozzi K (2005) Sleeping sickness in Uganda: a thin line between two fatal diseases. BMJ 331 (7527):1238–1241
Poelvoorde P et al (2004) Distribution of apolipoprotein L-I and trypanosome lytic activity among primate sera. Mol Biochem Parasitol 134(1):155–157
Radwanska M, Chamekh M et al (2002a) The serum resistance-associated gene as a diagnostic tool for the detection of *Trypanosoma brucei rhodesiense*. Am J Trop Med Hyg 67(6):684–690
Radwanska M, Claes F et al (2002b) Novel primer sequences for polymerase chain reaction-based detection of *Trypanosoma brucei gambiense*. Am J Trop Med Hyg 67(3):289–295
Raper J, Nussenzweig V, Tomlinson S (1996) The main lytic factor of *Trypanosoma brucei brucei* in normal human serum is not high density lipoprotein. J Exp Med 183(3):1023
Raper J et al (2001) Trypanosome lytic factors: novel mediators of human innate immunity. Curr Opin Microbiol 4(4):402–408
Rifkin MR (1978a) Identification of the trypanocidal factor in normal human serum: high density lipoprotein. Proc Natl Acad Sci 75(7):3450–3454
Rifkin MR (1978b) *Trypanosoma brucei*: some properties of the cytotoxic reaction induced by normal human serum. Exp Parasitol 46(2):189–206
Rifkin M (1984) *Trypanosoma brucei*: biochemical and morphological studies of cytotoxicity caused by normal human serum. Exp Parasitol 58(1):81–93
Rifkin MR et al (1994) Human serum-sensitive *Trypanosoma brucei rhodesiense*: a comparison with serologically identical human serum-resistant clones. Mol Biochem Parasitol 66 (2):211–220
Salmon D et al (1994) A novel heterodimeric transferrin receptor encoded by a pair of VSG expression site-associated genes in *T. brucei*. Cell 78(1):75–86
Samanovic M, Molina-Portela MP, Chessler A (2009) Trypanosome lytic factor, an antimicrobial high-density lipoprotein, ameliorates *Leishmania* infection. PLoS Pathog 5(1)
Seed JR, Sechelski O (1990) A survey for a trypanocidal factor in primate sera. J Protozool 37 (5):393–400
Seyfang A, Mecke D (1990) Degradation, recycling, and shedding of *Trypanosoma brucei* variant surface glycoprotein. J Protozool 37(6):546–552
Shiflett AM et al (2005) Human high density lipoproteins are platforms for the assembly of multi-component innate immune complexes. J Biol Chem 280(38):32578–32585
Shiflett A et al (2007) African trypanosomes: intracellular trafficking of host defense molecules. J Eukaryot Microbiol 54(1):18–21
Shimamura M, Hager KM, Hajduk SL (2001) The lysosomal targeting and intracellular metabolism of trypanosome lytic factor by *Trypanosoma brucei brucei*. Mol Biochem Parasitol 115 (2):227–237
Simo G et al (2006) High prevalence of *Trypanosoma brucei gambiense group* 1 in pigs from the Fontem sleeping sickness focus in Cameroon. Vet Parasitol 139(1–3):57–66
Smith AB, Esko JD, Hajduk SL (1995) Killing of trypanosomes by the human haptoglobin-related protein. Science 268(5208):284–286
Stephens NA, Hajduk SL (2011) Endosomal localization of the serum resistance-associated protein in African trypanosomes confers human infectivity. Eukaryot Cell 10(8):1023–1033
Stevens JR, Tibyrenc M (1996) *Trypanosoma brucei* sl: evolution, linkage and the clonality debate. Parasitology 112(5):481–488
Symula RE, Beadell JS, Sistrom M (2012) *Trypanosoma brucei gambiense* group 1 is distinguished by a unique amino acid substitution in the HpHb receptor implicated in human serum resistance. PLoS Negl Trop Dis 6(7):e1728
Tait A, Babiker EA, Le Ray D (1984) Enzyme variation in *Trypanosoma brucei* ssp. I. Evidence for the sub-speciation of Trypanosoma brucei gambiense. Parasitology 89(2):311–326
Targett GA, Wilson VC (1973) The blood incubation infectivity test as a means of distinguishing between *Trypanosoma brucei brucei* and *T. brucei rhodesiense*. Int J Parasitol 3(1):5–11

Thomson R et al (2009) Hydrodynamic gene delivery of baboon trypanosome lytic factor eliminates both animal and human-infective African trypanosomes. Proc Natl Acad Sci U S A 106(46):19509–19514
Tomlinson S, Raper J (1996) The lysis of *Trypanosoma brucei brucei* by human serum. Nat Biotechnol 14(6):717–721
Tomlinson S et al (1995) High-density-lipoprotein-independent killing of *Trypanosoma brucei* by human serum. Mol Biochem Parasitol 70(1–2):131–138
Turner CMR et al (1990) Evidence that the mechanism of gene exchange in *Trypanosoma brucei* involves meiosis and syngamy. Parasitology 106(1):209–214
Turner C et al (2004) Human infectivity trait in *Trypanosoma brucei*: stability, heritability and relationship to sra expression. Parasitology 129(4):445–454
Tytler EM et al (1995) Reconstitution of the trypanolytic factor from components of a subspecies of human high-density lipoproteins. Mol Biochem Parasitol 69(1):9–17
Van Luenen HGAM et al (2005) Trypanosomes change their transferrin receptor expression to allow effective uptake of host transferrin. Mol Microbiol 58(1):151–165
van Meirvenne N, Maginus E, Janssens PG (1976) The effect of normal human serum on trypanosomes of distinct antigenic type (ETat 1 to 12) isolated from a strain of *Trypanosoma brucei rhodesiense*. Ann Soc Belg Med Trop 56(1):55–63
Vanhamme L, Pays E (2004) The trypanosome lytic factor of human serum and the molecular basis of sleeping sickness. Int J Parasitol 34(8):887–898
Vanhamme L et al (2003) Apolipoprotein L-I is the trypanosome lytic factor of human serum. Nature 422(6927):83–87
Vanhamme L et al (2004) The *Trypanosoma brucei* reference strain TREU927/4 contains *T. brucei rhodesiense*-specific *SRA* sequences, but displays a distinct phenotype of relative resistance to human serum. Mol Biochem Parasitol 135(1):39–47
Vanhollebeke B, Pays E (2006) The function of apolipoproteins L. Cell Mol Life Sci 63 (17):1937–1944
Vanhollebeke B, Pays E (2010) The trypanolytic factor of human serum: many ways to enter the parasite, a single way to kill. Mol Microbiol 76(4):806–814
Vanhollebeke B et al (2006) Human *Trypanosoma evansi* infection linked to a lack of apolipoprotein L-I. N Engl J Med 355(26):2752–2756
Vanhollebeke B et al (2007) Distinct roles of haptoglobin-related protein and apolipoprotein LI in trypanolysis by human serum. Proc Natl Acad Sci U S A 104(10):4118
Vanhollebeke B et al (2008) A haptoglobin-hemoglobin receptor conveys innate immunity to *Trypanosoma brucei* in humans. Science 320(5876):677–681
Welburn S et al (2001) Identification of human-infective trypanosomes in animal reservoir of sleeping sickness in Uganda by means of serum-resistance-associated (*SRA*) gene. Lancet 358 (9298):2017–2019
Willett KC, Fairbairn H (1955) The Tinde experiment: a study of *Trypanosoma rhodesiense* during eighteen years of cyclical transmission. Ann Trop Med Parasitol 49(3):278–292
World Health Organization (2006) Human African Trypanosomiasis (sleeping sickness): epidemiological update. Weekly epidemiological record 81:69–80
Xong HV et al (1998) A VSG expression site-associated gene confers resistance to human serum in *Trypanosoma rhodesiense*. Cell 95(6):839–846
Zhaorigetu S et al (2008) ApoL1, a BH3-only lipid-binding protein, induces autophagic cell death. Autophagy 4(8):1079–1082
Zillmann U, Mehlitz D, Sachs R (1984) Identity of *Trypanozoon* stocks isolated from man and a domestic dog in Liberia. Tropenmed Parasitol 35(2):105–108

Proteomics and African Trypanosomes: Shedding New Light on Host–Vector–Parasite Interactions and Impact on Control Methods

7

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Abstract

Most mammalian and vector host species have acquired strategies by selective pressure to mislead the trypanosome and to win the fight during their molecular dialogue. Due to the same evolutionary pressure, trypanosomes have acquired strategies to bypass the host defences and to ensure the completion of their complex life cycles. Elucidation of these complex molecular crosstalks will improve the understanding of trypanosomes' variability with respect to virulence and pathogenicity, will help to define trypansome-specific host biomarkers and will help to refine control strategies for African trypanosomoses. Advances in proteomics applications have provided new insights on African trypanosomes and on the biochemical interactions with their tsetse vectors and mammalian hosts. In this chapter, we present the interest of proteomics to characterise trypanosomes–hosts interactions, a synthetic review of proteomics studies performed on the parasite and its respective hosts, a discussion on the contributions and pitfalls of using diverse proteomics tools, a view for future prospects on proteomics dedicated to African trypanosomes and a projection of new conceptual approaches (i.e. metabolomics, interactomics, population proteomics) to accurately decipher insect vector–trypanosome–mammalian host interactions, with the idea of further developing new tools to improve trypanosomoses control.

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7.1 Introduction

7.1.1 Relevance of Proteomics in Studying African Trypanosomosis

Advances in proteomics have offered new tools to better understand host–vector–-parasite crosstalks and have opened new ways to improve detection and control of neglected tropical diseases (NTDs) caused by trypanosomatids (Cuervo et al. 2010). African trypanosomosis is a promising model to study host–vector–parasite interactions, as trypanosomes can be considered as specialists in escaping and diverting the immune system of their mammalian hosts (e.g. humans and bovines) and in the behavioural manipulation of their main vector (i.e. tsetse fly) to increase their transmission. One striking biological strategy developed by trypanosome species is the ability of changing their cellular surface coat, composed by variant surface glycoproteins (VSG), during their life cycle in their intermediate hosts (tsetse flies) and their final hosts (human and livestock) (Roditi and Lehane 2008). Moreover, trypanosomes remain extracellular all along their life cycle, which makes them as rare cellular model to study parasite strategy through cell differentiation and maturation. *Trypanosoma brucei* has thus emerged as a model for the study of evolutionary cell biology, with regard to molecular dialogue or conflict tightly regulated with its hosts. During trypanosome–host–vector interactions, all analytical levels (genome, transcriptome, proteome: whole cell content, secretome: naturally excreted-secreted molecules and metabolome) interact with each other within one actor, then between two and at the protein level between the three actors. Proteomics applications on molecular crosstalks between a trypanosome and its hosts during the infection process are recent but have already led to new insights (Holzmuller et al. 2010), as they offer an excellent way to examine the host and parasite genomes in action, through the qualitative/quantitative characterisation of both hosts and parasite proteomes. Moreover, as a cellular eukaryotic model, trypanosome exhibits a highly organised cellular structure with specialised organelles, which may give the impression of a relatively small whole proteome when analysed directly in a global approach because the different protein contents are extremely compartmentalised and can be under-represented after purification processes [illustrated in Bridges et al. (2008)].

7.1.2 Pathogenoproteomics: An Analytical Concept to Integrate the Diversity of Host–Vector–Trypanosome Crosstalks

Integrative "omics" approaches have been proposed to study molecular crosstalks, reflecting the adaptative processes and determining the outcome of the infection (Holzmuller et al. 2008b; Biron et al. 2006). Nevertheless, significant technical challenges used to hamper initially the full exploitation of this organism, but advances in proteomics provide novel high throughput approaches to acquiring rapid functional data. Proteomics tools have evolved at an exponential speed these

last 10 years, from mono- or bi-dimensional electrophoresis (1-DE, 2-DE) to protein chips, concomitantly to progresses in mass spectrometry technology [reviewed in Holzmuller et al. (2010)].

The complexity of the potential molecular interactions occurring all along the trypanosome's transmission and infection makes it quite unreasonable to try to elucidate these coevolutionary processes in a global manner. By contrast, through the characterization of either specific sub-proteomes, from both the trypanosome and its hosts, or modulation of the level of expression of particular molecules correlated with the infectious process, pathogenoproteomics brings data to characterise specific molecular interactions to rebuild the puzzle of crosstalks occurring during the parasitic developmental cycle. Moreover, integrating data from different analytical levels increase knowledge in cell biology processes established by trypanosomes to ensure their differentiation (e.g. from bloodstream to procyclic to metacyclic forms), maturation (e.g. from long slender to short stumpy forms in *T. brucei*) and finally transmission to complete the infectious cycle [trypanosome developmental cell biology reviewed in Matthews (2005)]. Pathogenoproteomics promotes integrative approaches based on a holistic view of host–vector–parasite interactions to decipher the molecular dialogues occurring and governing the parasite life cycle (Holzmuller et al. 2008b), which may further lead to definition of more efficient diagnostic and therapeutic tools or improved control strategies to fight against African trypanosomoses.

7.1.3 Retrospective of Proteomics Studies on African Trypanosomoses

Pioneer studies on the proteome of African trypanosome have evidenced protein spots quantitatively different between cultured trypanosomes and tsetse midgut forms, originating from both the parasite and the vector (Anderson et al. 1985; Pearson et al. 1987). Then, Van Deursen et al. proposed a reproducible protocol to analyse the proteome of *T. brucei* during its life cycle (i.e. tsetse midgut procyclics and bloodstream forms) (Van Deursen et al. 2003). They highlighted the need of standard procedures to allow comparison of the results obtained in different laboratories working on trypanosomes proteomics. They also demonstrated that the analysis of the whole proteome of trypanosomes would generate data concerning thousands of protein spots, among which it would be difficult to evidence low variation of expression of proteins of interest. Nevertheless, improved proteomics techniques based on stable isotope labelling by amino acids in culture (SILAC) allowed the large-scale comparison of procyclic and bloodstream forms of *T. brucei*, showing that around 10 % of the proteins exhibited a fivefold differential regulation between the two lifestages and that fold changes were larger at the protein level than at the transcript level. This high throughput proteomics technology also evidenced previously not known stage-specifically expressed proteins in more than 4,000 protein groups quantified (Urbaniak et al. 2012; Butter et al. 2013). The identification of stage-specific proteins helps to understand how trypanosomes

adapt to different hosts and provides new insights into differences in metabolism, gene regulation and cell architecture.

Integrating the risk of pitfalls and the standardisation needs, most researchers have done their proteomics studies on specific compartments (i.e. sub-proteomes) of trypanosomes to increase the chance to find candidate proteins, which differential expression could be associated with the infection process: glycosome (Colasante et al. 2006), plasma membrane (Bridges et al. 2008), nucleus (Degrasse et al. 2008), secretome (Holzmuller et al. 2008a; Grébaut et al. 2009), mitochondria (Panigrahi et al. 2009), flagellum (Hart et al. 2009) and cytoskeleton (Portman and Gull 2012). Some studies demonstrated differential expression of the components of sub-proteomes depending on the parasite stage considered, virulence (capacity to multiply inside a host)/pathogenicity (ability to produce mortality) properties or drug resistance acquirement (Holzmuller et al. 2008a; Grébaut et al. 2009; Parsons and Nielsen 1990; Foucher et al. 2006; Vertommen et al. 2008). Moreover, as protein translation and turnover are key elements of genome expression regulation, some research groups focused their work on the analysis of phosphoproteome, since phosphorylation is a key regulatory mechanism for cellular function in trypanosomatids (Nett et al. 2009).

From the hosts' point of view, impact of trypanosome development on life traits was also investigated by proteomics. For instance, to better understand tsetse's feeding behaviour, which is crucial for trypanosomes transmission, analysis of the tsetse head proteome was performed to evaluate the potential vector manipulation by trypanosomes (Lefèvre et al. 2007). In the same way, the extracellular location of trypanosomes in the bloodstream of their mammalian hosts and the deregulations of the host immune system strongly suggest that there must be serological markers, other than antibodies or parasite VSG, which sign the molecular dialogues and conflicts of the host–parasite interaction. Using different high-throughput proteomics technologies, human biological fluids as serum and cerebrospinal fluid (CSF) were analysed to find out biomarkers of both infection and disease progression in order to improve diagnosis and treatment management of HAT. This first objective has been partially reached with the characterisation of promising candidates for staging HAT (Hainard et al. 2009, 2011; Tiberti et al. 2010, 2012, 2013b; Courtioux et al. 2009). The urgent goal is now to quickly include these candidates, alone or in association, to accomplish the yet unfulfilled pledge of improved accurate, robust and affordable HAT diagnostic kits as expected for long and to make them available for field surveys of sleeping sickness.

Until now, what did proteomics tell us about the mammal–arthropod vector–trypanosome crosstalk? This chapter is divided into four parts to synthesise the contributions of proteomics in better deciphering the molecular interactions of the trypanosome with its hosts: (1) trypanosome–tsetse fly molecular crosstalk and consequences on vector control strategies, (2) biochemical characterisation of the trypanosome and impact on infection control and (3) characterisation of trypanosome-induced metabolic and biochemical changes in the mammalian host and consequences on African trypanosomoses management. The contribution of proteomics will be discussed and projected including advances and pitfalls of

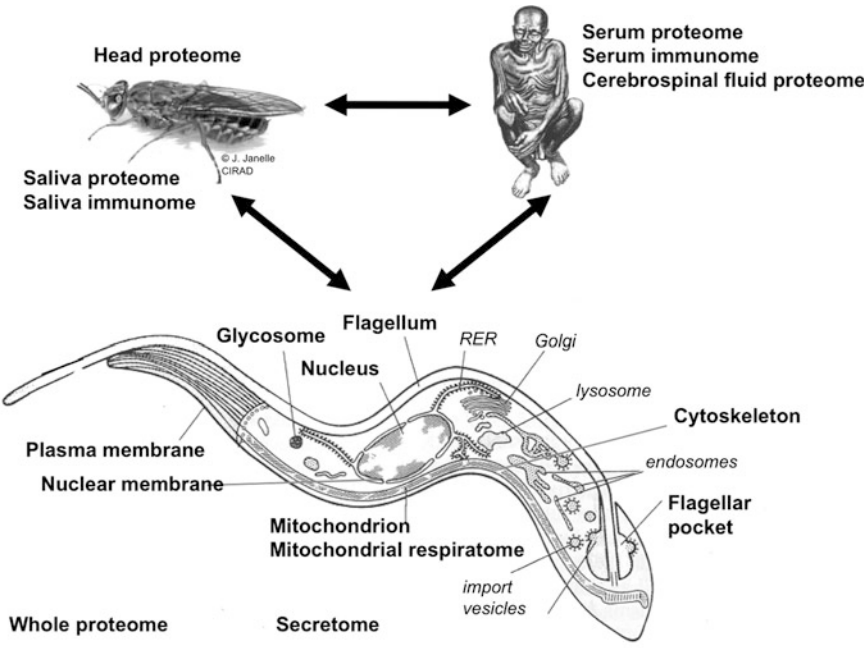

Fig. 7.1 Synthetic view of proteomics investigations on crosstalks occurring within African trypanosomoses triptych: mammalian host–arthropod vector–protozoan parasite. In *bold*: biological compartments already studied in proteomics. In *italic*: trypanosome compartments of further interest. *RER* rough endoplasmic reticulum

current approaches and the expectations of integrated approaches such as metabolomics and interactomics (Fig. 7.1).

7.2 Trypanosome–Tsetse Fly Molecular Crosstalk and Consequences on Vector Control Strategies

An intriguing and little known molecular dialogue between trypanosome species and their tsetse fly vectors begins when an uninfected tsetse fly takes a blood meal on an infected mammalian host. Additionally, the developmental cycles inside the tsetse fly are different according the trypanosome species. For instance, *T. brucei* and *T. congolense* both initiate their infection process in the fly midgut and subsequent stages occur exclusively in the salivary glands for *T. brucei* and in the mouthparts for *T. congolense*, where they transform into metacyclic forms, acquiring VSG coat and becoming infective, ready to be inoculated during the next blood meal. Molecular studies on tsetse–trypanosome interactions have highlighted the trypanosome difficulty to initial establishment in the vector (Aksoy et al. 2003).

7.2.1 Establishment of Trypanosomes Species in Their Tsetse Fly Vectors

During the evolutionary "arm race" between tsetse flies and its parasites, both actors have acquired strategies by selective pressure to mislead the other interlocutor and to win the fight during the molecular dialogue. For instance, the tsetse midgut epithelium is protected by the peritrophic matrix (PM), a glycosaminoglycan-rich layer strengthened with chitin, to prevent the infection by parasites but actually it is unclear if the PM is an efficient strategy to disrupt the trypanosome development (Lehane and Msangi 1991). Many key molecules are produced and used by tsetse flies to prevent the trypanosome infection in midgut: proteases, reactive oxygen species (ROS), antimicrobial peptides (e.g. cecropin, attacin, diptericin and defensin) and tsetse EP protein (Hao et al. 2001; Hu and Aksoy 2006; Chandra et al. 2004). These molecular strategies are very efficient since most of tsetse flies of natural populations are refractory to infection by trypanosomes. However, trypanosomes developed an astonishing strategy to bypass the insect vector defences by switching from the blood stream VSG surface coat to a new stage-specific procyclin coat, limiting the efficiency of the tsetse innate immune response (Pearson 2001; Güther et al. 2006). A recent mass spectrometry analysis performed on a mutant of *T. brucei* unable to express cell surface procyclins evidenced multispanning membrane proteins, among which are the THT2A glucose transporter, the TbN10 nucleoside transporter and the TbPAD2 putative carboxylate transporter of the PAD (proteins associated with differentiation) family (Güther et al. 2009). The use of available antibodies allowed the identification of two other surface proteins: P-type ATPase and VHPPase. Moreover, the analysis of the lipid mass of the procyclin coat by electrospray ionisation–mass spectrometry (ES–MS) demonstrated inositolphosphoceramide (IPC) ions as constitutive and specific elements of the membrane (Güther et al. 2006), and potential drug target (Mina et al. 2009).

When procyclic forms of *T. brucei* succeed in surviving in the midgut then migrate up to the salivary glands, several molecules were thought to be intimately associated to the maturation and development of the parasite. A protein chemical approach using high-resolution 2D gel electrophoresis and mass spectrometry was used to identify the salivary proteins of teneral *Glossina morsitans morsitans*. The first protein was a tsetse salivary gland growth factor-1 (TSGF-1). Two proteins with no known function were identified as tsetse salivary gland protein 1 (Tsal 1) and tsetse salivary gland protein 2 (Tsal 2). The fourth protein was identified as tsetse antigen-5 (TAg-5), which is a member of a large family of anti-haemostatic proteins (Haddow et al. 2002), showing also allergen properties in humans (Caljon et al. 2009). Moreover, immunogenic profiles of saliva proteins seemed to be not only specific to the *Glossina* species but also to the infectious status of exposed individuals (Poinsignon et al. 2007), and an immunoproteomics study further defined four tsetse salivary proteins, including TAg-5 and TSGF-1, as relevant biomarkers of human–tsetse contacts (Dama et al. 2013).

7.2.2 Alteration of Tsetse-Feeding Behaviour by Metacyclic Stages of the Trypanosome

Parasites as a whole can modify the behaviour of their vector hosts in ways that seem to increase the parasite's chances of completing its life cycle (Lefèvre et al. 2009), including a change in preferred substrate, temperature preferences, locomotor activity, visual cycle, circadian rhythms, geo- or phototropism, cessation of food consumption and feeding rate (Schaub 2006; Lefèvre et al. 2008). Moreover, it is known for long that arthropod vector–pathogen associations alter the behaviour of their vectors, notably feeding behaviour, in a way that increases the contact with the vertebrate host to enhance the pathogen's transmission (Schaub 2006; Moore 1993). In the tsetse fly–trypanosome associations, infected flies showed increased probing rate and more voracious feeding than uninfected flies (Jenni et al. 1980), but as often the molecular crosstalks underlying these behavioural changes are by no means well characterised (Biron et al. 2005; Libersat et al. 2009). One of the main characteristics of the central nervous system (CNS) of insects is to convert sensory receptors' activity into patterns of muscle activity that will define different behaviours, which support the hypothesis that any changes in host behaviour should have a molecular basis in its CNS. In that way, one proteomics study was performed on tsetse fly–trypanosome associations to decipher the parasite influence on the tsetse head proteome expression and to improve the understanding of the proximate causes impacting the tsetse vector behaviour. This study provides evidence that *T. b. brucei*, at metacyclic stage, alter the head proteome of *G. palpalis gambiensis* through modifications in protein families from sugar metabolism, signal transduction and heat shock response (Lefèvre et al. 2007). It was also suggested that *T. b. brucei* can alter serotonin and/or dopamine in the brain of metacyclic-infected flies, as well as induce apoptosis in neurones, which contributes to explain the behaviour modification of infected tsetses. On the other hand, modifications in the saliva composition in *T. brucei*-infected flies were associated with prolonged feeding time, enhancing the potential in transmitting trypanosomes. Actually, trypanosome colonisation of salivary glands induce significant decrease in anti-platelet aggregation and anti-thrombin activities, plus inhibition of thrombin-induced blood coagulation, hampering tsetse's feeding performance and increasing the vector/host contact and parasite transmission in field conditions (Van Den Abbeele et al. 2010).

Better characterising the molecular interactions between tsetse flies and trypanosomes influencing the vector behaviour (and thus the contact with the mammalian host) will first increase the knowledge of tsetse immune responses to different trypanosome species but also bring new epidemiological data on tsetse fly population dynamics. This new knowledge will help to set up new ways to interfere with the transmission processes of trypanosomes.

7.3 Biochemical Characterisation of Trypanosome's Anatomy and Cellular Metabolism and Impact on Infection Control

The host–parasite interactions have been analysed mainly on the side of the trypanosome in order to find its Achille's heel to fight against infection. From this point of view, the complex highly structured cell organisation of the trypanosome may reflect the evolution of parasitic strategies developed to interact with the environment "host".

7.3.1 The Nucleus: A Simplified Genome Expression and Regulation Organelle

Analysis of the nuclear proteome of procyclic and bloodstream forms of *T. brucei* demonstrated that the protein composition of trypanosome nuclei does not vary greatly between life stages, with only a minor fraction of the protein bands analysed in 1-D gel (Rout and Field 2001). By contrast, the overall shapes of the nuclei were different for the two life stages, with procyclic forms nuclei being spherical and nuclei from bloodstream trypanosomes being significantly more elongated, and nucleolus being compact in procyclics whereas more irregular in bloodstream forms. The overall similarity in the nuclei protein profiles of procyclic and bloodstream trypanosomes reflected the majority of nuclear proteins being structural and involved in chromatin/matrix assembly and maintenance with only a very small fraction responsible for control of differential gene expression and other stage-specific functions (Rout and Field 2001). The nuclear pore complex is the only mean to transfer information from the nucleus to the cell and is composed of about 30 distinct nucleoporins. Using a subcellular proteomic approach complemented with complementary computational strategies, Degrasse and Devos (2010) identified five nucleoporins by sequence similarity alone, plus 17 by bioinformatics, among the 865 proteins identified in the nuclear envelope. Their molecular and functional results confirmed the evolutionary originality in trypanosomes subcellular organisation.

7.3.2 The Plasma Membrane: The Physical Interface with the Insect Vector and Mammalian Host

Because exposed to the extracellular environment, plasma membrane of the trypanosome is a key element of its defence against the aggression resulting from the immune responses of both invertebrate and vertebrate hosts and chemical compounds. Plasma membrane constitutes a selective interface to import from, export to and more generally communicate with the extracellular environment. Because of their hydrophobic properties and their low abundance, plasma membrane proteins are difficult to study by conventional proteomic approaches. Nevertheless, Bridges et al. recently isolated plasma membranes from bloodstream forms

of *T. brucei* and applied a battery of complementary protein separation and identification techniques to identify a large number of proteins in this fraction (Bridges et al. 2008). Considering plasma membrane as a two-dimensional sheet, the number of integral membrane proteins to be inserted into the membrane is subjected to a very finite spatial restriction. This is further exacerbated by the large amount of the GPI-anchored VSG (~10^7 copies/cell), which is the key element of the trypanosome's plasma membrane and a specific drug target (Barry and McCulloch 2001; Ferguson et al. 1999). Therefore the modest fraction of plasma membrane identified proteins (16 % of all the proteins in *T. brucei* with five or more transmembrane domains) represents a large proportion of the integral membrane proteins expressed in bloodstream form trypanosomes (Bridges et al. 2008). Among the identified proteins there was a large number annotated as hypothetical, but for which a homologue was identified in the closely related trypanosomatids *Leishmania* or *Trypanosoma cruzi*. This may indicate that the majority (particularly in the case of plasma membrane) are encoded by genes that are functionally unique and features of the trypanosomatids. Moreover, the larger number of proteins in the plasma membrane of bloodstream forms of *T. brucei* compared to yeast may reflect the greater complexity of trypanosome plasma membrane, particularly because of their necessity to interact with and defend against the host (Bridges et al. 2008). The identified proteins were essentially membrane transporters, with a large number belonging to the equilibrative nucleoside transporter family, of which several were demonstrated to be involved in purine nucleoside/nucleobase transport. This protein family was highly represented, reflecting the absolute requirement of trypanosomes to scavenge purines from their external environment, which can be considered as a trypanosome's dependency and thus represent a potential therapeutic target (De Koning et al. 2005). By contrast, few amino acid transporters have been detected, despite a large number of genes annotated to encode these proteins in the genome of *T. brucei*. One explanation is that these transporters operate at high turnover rate and, consequently, can be expressed at low copy number, as suggested for glucose, for which maximum rate of uptake could be supported by 500 receptors per cell (Bridges et al. 2008). Finally, a large number of adenylate cyclases were identified in the *T. brucei* plasma membrane, confirming that multiple adenylate cyclases are expressed at any one time in trypanosomes, where they may be acting as extracellular receptors (Seebeck et al. 2004). These two last protein families are evidence of trypanosome strategies to interact with his host, by using the host resources in a discrete manner and by developing sensors to analyse the parasitic environment.

7.3.3 The Glycosome and Mitochondria: The Trypanosome's Energetic Metabolism Specifically Adapted to Different Hosts

Trypanosomes exhibit two particular cellular organelles: they contain an unusual peroxisome-like microbody named glycosome, which contains the first 7–9

enzymes of glycolysis (Parsons 2004) and a large, single, mitochondrion (Van Hellemond et al. 2005). Contrary to procyclic trypanosomes, bloodstream forms mitochondrial metabolism is severely suppressed. In their study, Vertommen et al. evidenced only a few mitochondrial enzymes, such as pyruvate dehydrogenase E1 subunit and the α and β subunits of succinyl-CoA ligase. They found no evidence for the presence of either a functional tricarboxylic acid cycle or respiratory chain. In bloodstream trypanosomes, pyruvate was secreted as the sole end-product of glycolysis and they expressed specifically the alternative oxidase, the enzyme responsible for the transfer of the reducing equivalents generated in this pathway (Vertommen et al. 2008). By contrast, the mitochondrial respiratome of *T. brucei* procyclics demonstrated functional molecular respiratory complexes I–V with many proteins unique to trypanosomes (Acestor et al. 2011).

In addition to glycolysis, other pathways have been postulated to be present in the glycosome, like ether lipid biosynthesis, β-oxidation of fatty acids, sterol and isoprenoid biosynthesis, purine salvage, pyrimidine biosynthesis, the pentose phosphate pathway and gluconeogenesis (Parsons 2004). Interestingly, compartmentation of microbody enzymes is essential for survival of both bloodstream and procyclic *T. brucei*, probably by protecting trypanosomes from the negative side effects of the "turbo" structure of glycolysis (Guerra-Giraldez et al. 2002). Nevertheless, glycosomes of procyclic trypanosomes appeared to be more complex than those of bloodstream trypanosomes and contain in addition enzymes of the oxidative branch of the pentose phosphate pathway, the Calvin–Benson cycle and two different pathways involved in the detoxification of oxygen radicals and peroxides (Colasante et al. 2006). This compartmentation appears to be associated to the own metabolism of the parasite but reflects in fact an adaptative process to complete the life cycle of the parasite and its adaptation to different hosts. An example with aldose-1-epimerase, an enzyme repressed at high glucose concentration concentrations and de-repressed during deficiency of glucose, which was detected in cultured procyclic (0.1–0.3 mM glucose in culture medium) but not in animal-purified bloodstream (3–5 mM glucose in blood) trypanosomes (Colasante et al. 2006). By contrast, several enzymes of the glycerol–ether lipid biosynthesis pathway were absent in both life forms of the parasite; suggesting that part of this pathway is down-regulated. Such a regulation indicates that trypanosomes emphasise the uptake of glycerol–ether lipids from their host, rather than a de novo biosynthesis. This argues in favour of indirect information on adaptation of trypanosomes to the environment "host" can be given by proteomic analysis of biological compartments not directly interacting with the host. This was also illustrated by the presence of arginine kinases in bloodstream form glycosomes, suggesting the existence of an additional temporal energy buffer (arginine-phosphate) in these organelles, which could stabilise the ATP/ADP ratio and could provide energy under stress conditions (Colasante et al. 2006; Vertommen et al. 2008).

Another characteristic of trypanosomes' glycosomes is the presence of a pyruvate phosphate dikinase, which converted all inorganic pyrophosphate (PPi) to ATP at the expense of phosphoenolpyruvate and AMP. Moreover, what the proteomic

analysis revealed clearly is that none of the enzymes involved in β-oxydation, the hallmark of peroxysomes, are present in glycosomes of *T. brucei* (Colasante et al. 2006). This could be informative of the evolution of peroxysomal functions, if we consider the glycosome as an ancestor of the peroxysome.

Operational mitochondrion in procyclic trypanosomes could be considered as an adaptation to the insect vector or being a result of trypanosome–tsetse fly interactions. In the mitochondrion of procyclic trypanosomes, most of the enzymes of the tricarboxylic acid cycle were detected (Vertommen et al. 2008). Moreover, mitochondrion contains complex machinery for energy metabolism through oxidative phosphorylation, iron-sulfur clusters and network of metabolic processes, and proteomic data evidenced also many of the core components of respiratory complexes I–V (Panigrahi et al. 2009). More recently, the same authors published the protein composition of *T. brucei* mitochondrial membrane (Acestor et al. 2009), which both completes the list of previously identified proteins and provides the subcellular assignment of the mitochondrial proteins. Importantly, a large proportion of proteins assigned to the mitochondrion have no known function, which confirms that a large fraction of the components in mitochondrial complexes are unique to trypanosomes (Panigrahi et al. 2008, 2009), and constitute therefore preferred targets to counter the development of the trypanosome.

7.3.4 The Flagellum and Flagellar Pocket: The Trypanosome's Motility and Understanding of Its Environment

Whereas the flagellum-mediated migration between the gut and salivary glands in the tsetse fly vector is essential for progression of the trypanosome life cycle, the necessity for motility of bloodstream trypanosomes remains unclear. In both forms, a single attached flagellum emerges from the cytoplasm in a specialised membrane domain termed flagellar pocket. Flagellum is surrounded by its own membrane and attached along most of its length to the cell body by a network of cytoskeletal and membranous connections: the flagellum attachment zone (FAZ). Within the flagellum, the microtubule axoneme drives the flagellar movement. A large lattice-like structure, the paraflagellar rod (PFR) is physically attached to both FAZ and axoneme. Broadhead et al. identified 331 proteins of *T. brucei* flagellum proteome, among which 208 were trypanosomatid specific and probably represent organism-specific flagellar structures and functions. Functional analysis of a set of proteins from *T. brucei* flagellum proteome by RNA interference resulted in bloodstream trypanosomes that did not complete cytokinesis, yielding monstrous cells with an inability to proliferate (Broadhead et al. 2006). These parasites ceased dividing but continued to progress through cell cycle, leading to large contorted cells containing multiple kinetoplasts and nuclei, with a rapid lethality of these monstrous cells. This integrated work with identification of *T. brucei* flagellum proteome showed that flagellum function cannot be compromised and is essential in the bloodstream trypanosome, probably by playing a key role in sensing the host environment. Moreover, the severity of the phenotype induced by ablation of proteins of diverse

function and location in either the axoneme or the trypanosome-specific paraflagellar rod, its rapid onset and its lethality, demonstrated the significance of identifying key active molecules in trypanosome development (Broadhead et al. 2006). Proteomics of extracted flagellar complexes also evidenced a new bi-lobe component, TbLRRP1, the expression of which was demonstrated to be essential for Golgi apparatus and flagellar pocket collar duplication as well as new FAZ biogenesis (Zhou et al. 2010). Overall data suggest that the flagellum could represent the Achille's heel of the trypanosome, and among the trypanosomatid-specific proteins identified, some promising candidates, such as PFR, TAX-1 or TbLRRP1, could now be use to screen natural or chemical compounds able to block their assembly and that may be further evaluated for disease control in HAT and AAT.

7.3.5 The Secretome: A Trypanosome Language?

Excreted/secreted components (secretome) of microorganisms have been intensively studied over these last years. By developing a standard methodology to access complex mixture of proteins, carbohydrates and lipids excreted from the surface or secreted via exocytosis vesicles and the flagellar pocket of the parasite, analyses were conducted on the secretome of African trypanosomes: *T. congolense*, *T. evansi* and *T. b. gambiense* (Holzmuller et al. 2008a, c; Grébaut et al. 2009). These studies had the originality to develop a comparative approach of the proteome and the secretome with particular attention to common and specific molecules between strains of differing virulence and pathogenicity. Proteomics analysis of *T. congolense* by nano liquid chromatography/tandem mass spectrometry (LC/MS–MS) of 1D gel protein bands led to identification of 108 characterised proteins. Among these characterised proteins, 75 % were predicted to have known features of secreted proteins, but only 25 % were triggered by a signal peptide, the other secretory pathways were non-classical, bacteria-like or Gram-negative specific bacteria-like ones (Grébaut et al. 2009). Moreover, vesicles resembling exosomes were evidenced by electron microscopy in *T. b. gambiense*, concomitantly with proteins belonging to the general exosome molecular signature (Geiger et al. 2010). Five major types of potential functions were associated to the identified proteins: protein binding, oxidoreductase activity, hydrolase activity, ion binding and nucleotide binding. These predicted functions argue in favour of an active compartment dedicated to interactions with the immune system of the host. Moreover, an unusual aspect of trypanosomes secretome was the identification of proteins related to translation that could suggest, as it was proposed by Silverman et al. for *Leishmania* (Silverman et al. 2008), an elimination of proteins issued from the extremely productive translational machinery of trypanosomes. Another particular aspect stands in some multifunctional proteins, as elongation factor 1a (Nandan et al. 2002) or calreticulin, which could play ancillary role in the pathogenesis or pathogen survival. Calreticulin has been demonstrated to be involved in host immunosuppression, inhibiting the classical pathway of complement activation

by interaction with host C1q (Ferreira et al. 2004). In the same way, tubulin was reported to be an important T-cell stimulating antigen during *Leishmania* infection (Probst et al. 2001), and inhibition of tubulin was demonstrated to prevent the development of trypanosomes in vitro (Lubega et al. 2002). The last point enhanced by analysis of differential secretome/proteome expression of the two studied *T. congolense* strains, which expressed opposite levels of virulence and pathogenicity in infected hosts, was that identified proteins with the same identification (e.g. cysteine protease) were expressed as different isoforms (different levels of phosphorylation or/and glycosylation). Cysteine proteases are known to play an indispensable role in the biology of parasitic organisms (Sajid and McKerrow 2002) and suspected to act as a major pathogenic factor in mammalian hosts (Lalmanach et al. 2002).

Finally, proteomics studies of the secretome of trypanosomes brought precious information about the first elements of the molecular dialogue with the host that both intervenes before the first peak of parasitemia and may be considered as paracrine immunomodulators (Holzmuller et al. 2008a). The characterization of these elements offers new ways for the definition of both tools for precocious diagnosis of trypanosome infection and targets for the development of improved trypanosomosis treatment.

7.4 Characterisation of Trypanosome-Induced Metabolic and Immune Biochemical Changes in the Mammalian Host and Consequences on African Trypanosomosis Management

Apart from specific and nonspecific serologic responses, immunological and physiological disorders have been described for long and are keys to the outcome of trypanosome infection [reviewed in Bucheton et al. (2011)]. All these pathophysiological disturbances have molecular signatures in biological fluids of the infected host, which could be used for diagnosis purposes; the only limitation will be given by specificity. Only one study has been published so far, dealing with animal trypanosomosis: the clinical proteome of *T. evansi* camel isolates during experimental infection in mice, which revealed expression of known drug targets such as cysteine proteases, oligopeptidases or kinases (Roy et al. 2010). By contrast, the fast advances in proteomics development offered new high-throughput analytical tools to investigate human–trypanosome molecular crosstalks and could open new ways to improve detection and control of a NTD such as HAT.

7.4.1 Diagnostic Biomarkers

In the last decade, even if there were few studies really dedicated to using proteomics tools to characterise diagnosis biomarkers for HAT, these studies were of high relevance to renew the available toolbox of candidate molecules (Table 7.1). Surface-Enhanced Laser Desorption–Ionisation Time-Of-Flight (SELDI-TOF)

Table 7.1 Dicovery of biomarkers for Sleeping Sickness diagnosis and staging

Analysed biomarkers	Techniques	Host biological compartments	Patients cohorts	Diagnostic and staging biomarkers	References
Protein profiles	SELDI-TOF	Serum		Not available	Papadopoulos et al. (2004)
CXCL-13	ELISA	Paired serum and CSF from *T. b. gambiense*-infected patients	42 individuals; 26 HAT patients and 16 controls	CXCL-13	Courtioux et al. (2009)
H-FABP, GSTP-1, S100β	ELISA	CSF from *T. b. gambiense* patients	100 individuals; 21 stage 1 and 79 stage 2 patients	CXCL-10, CXCL-8, H-FABP	Hainard et al. (2009)
Cytokines and chemokines: IL- 1ra, IL-1β, IL-6, IL-9, IL-10, G-CSF, VEGF, IFN-γ,TNF-α, CCL2, CCL4, CXCL8 and CXCL10	Bioplex bead suspension assays				
T. b. brucei sub-proteomes	1-DE and 2-DE Western Blot	Serum from *T. b. rhodesiense*-infected patients	60 individuals; 30 HAT patients and 30 controls	TbHSP70	Manful et al. (2010)
Acetone precipitated CSF proteins	2-DE, MALDI TOF-TOF MS, LTQ-QT MS	CSF from *T. b. gambiense*-infected patients	9 individuals; 5 stage 1 and 4 stage 2 patients (+ pool of 5 stage 2 patients)	β2MG, osteopontin	Tiberti et al. (2010)
CSF proteins depleted with MARS Hu-14 column	TMT® MS/MS		18 individuals; 9 stage 1 and 9 stage 2 patients		
MMP-2, MMP-9, ICAM-1, VCAM-1,	ELISA	CSF from *T. b. gambiense*-infected patients	63 individuals; 15 stage 1	ICAM-1, MMP-9, H-FABP	Hainard et al. (2011)

(continued)

Table 7.1 (continued)

Analysed biomarkers	Techniques	Host biological compartments	Patients cohorts	Diagnostic and staging biomarkers	References
E-selection, CXCL-10, CXCL-8, H-FABP			and 48 stage 2 patients		
β2MG, CXCL-13, Neopterin, IgM	ELISA	CSF from *T. b. gambiense*-infected patients	512 individuals training cohort: 44 stage 1 and 56 stage 2 patients validation cohort: 412 HAT patients	Neopterin, IgM	Tiberti et al. (2012)
IgM, β2MG, Neopterin, CXCL-13	ELISA	CSF from *T. b. rhodesiense*-infected patients	85 individuals; 14 stage 1 and 71 stage 2 patients	CXCL-13, CXCL-10, MMP-9, IgM	Tiberti et al. (2013b)
CXCL-10, VCAM-1, ICAM-1, MMP-9	Multiplex bead suspension arrays				
T. b gambiense soluble proteome	Immunoprecipitation 1-DE, LC-MS/MS	Serum from *T. b. gambiense*-infected patients	163 individuals; 154 HAT patients and 9 controls	ISG64, ISG65	Sullivan et al. (2013)

Mass Spectrometry (MS) is a proteomics technique that generates high-resolution mass spectra of biologic samples on chemically active chip arrays (De Bock et al. 2010) in particular when associated with pre-fractionation methods (Roche et al. 2006). Papadopoulos et al. (2004), using this "naïve" global approach (SELDI-TOF-MS technology), were the first who successfully associated the different stages of HAT to some specific circulating protein profiles in the serum of HAT patients. This approach brought a new hope for its interest in providing new candidates for the development of improved diagnostic tests, disease staging and identification of potential novel drug targets in HAT (Agranoff et al. 2005), but until now authors did not achieve the molecular characterisation of the biomarkers. Nevertheless, it opened the way the proteomics screening of biological fluids to evidence molecular biomarkers associated the kinetics of HAT. Using Dot enzyme-linked immunosorbent assay (Dot ELISA), which can be considered as the non-miniaturised ancestor of protein chip arrays currently available, Courtioux et al.

demonstrated that antineurofilament (anti-NF) and antigalactocerebrosides (anti-GalC) antibodies were promising markers of CSF invasion by trypanosomes (Courtioux et al. 2005). Compared to the staging criteria they used (CSF cell count $\geq$ 20 cells/μl, CSF IgM titer $\geq$100 mg/l and/or trypanosomes in the CSF), authors obtained 83.2 % sensitivity and 100.0 % specificity by combining anti-NF and anti-GalC detection in CSF.

In the last 5 years, the use of proteomic tools for the identification of biomarkers for staging Sleeping Sickness has advanced at a vertiginous pace (illustrated in Table 7.1). Proteomics studies were performed using CSF as biological sample, to refine the characterisation of elevated CSF protein levels observed in late-stage *T. b. gambiense* HAT. Hainard et al. initiated in 2009 the first molecular profiling of CSF of patients suffering HAT versus controls and proposed that individual or combination of biomarkers signing the invasion of CNS by trypanosomes could be defined among brain damage markers evidenced by proteomics and inflammation-related proteins, as previously shown for CXCL-13 (Courtioux et al. 2009). The concentrations of S100β protein, heart-fatty acid binding protein (H-FABP) and Glutathione S-transferase P1 (GSTP-1) were determined by ELISA, and the levels of 13 cytokines and chemokines (IL-1ra, IL-1β, IL-6, IL-9, IL-10, G-CSF, VEGF, IFN-γ, TNF-α, CCL2, CCL4, CXCL8 and CXCL10) were determined using the Bioplex bead suspension arrays (Bio-Rad, Hercules, CA). The results showed that CXCL10, a 10 kDa interferon γ-induced protein, most accurately distinguished stage 1 and stage 2 patients, with a sensitivity of 84 % and specificity of 100 %. Multiplex data analysis using modified iterative permutation-response calculation named Rule Induction Like (RIL) allowed to demonstrate that combination of CXCL10, CXCL8 and H-FABP biomarkers increased sensitivity and specificity to 97 % and 100 %, respectively, which could dramatically improve the detection of Stage 2 patients (Hainard et al. 2009). Using a similar approach (multiplex bead suspension assays and RIL) with cell adhesion molecules [intercellular adhesion molecule (ICAM) 1, vascular cell adhesion molecule (VCAM) 1 and E-selectin (endothelial leucocyte adhesion molecule 1)] and matrix metalloproteinases (MMP-2 and -9), the same research group showed that ICAM-1 and MMP-9 could be new powerful CSF biomarkers for staging HAT (Hainard et al. 2011). The same team also performed a more conventional proteomics approach on CSF of patients suffering Sleeping Sickness by 2-DE and protein spot identification by mass spectrometry LC-MS/MS, compared to quantitative MS analysis by sixplex tandem mass tag (TMT) isobaric labelling quantitative MS/MS. Eighty-five differentially expressed proteins were identified by the two complementary strategies between CSF from HAT patients; especially 73 were overexpressed in Stage 2 CSF samples, among three candidate proteins [complement factor H, osteopontin (OPN) and β-2-microglobulin (B2MG)] were further evaluated by immunoassays and RIL (Western Blot and ELISA) on a larger number of patients. Association of B2MG and OPN was able to discriminate between Stage 1 and Stage 2 with a sensitivity of 91 % for 100 % specificity (Tiberti et al. 2010). Very recently, the same group of researchers analysed on a multi-centre cohort the CSF levels of their six most promising staging markers: B2MG, CXCL10, CXCL13, ICAM-1, VCAM-1 and

MMP-9 (Tiberti et al. 2012, 2013b), and they added neopterin that was already indicated as a staging biomarker of *T. b. rhodesiense* infection (Maclean et al. 2006). The seven markers, as well as IgM showed high ability to discriminate between early and late Stage 2 patients, but only IgM and neopterin were >80 % specific at 100 % sensitivity, leading to the selection of neopterin as a promising new staging marker for *T. b. gambiense* Sleeping Sickness (Tiberti et al. 2012). Moreover, neopterin was demonstrated to be a CSF biomarker of interest to predict the treatment outcome for *T. b. gambiense* Sleeping Sickness (Tiberti et al. 2013a). For *T. b. rhodesiense* Sleeping Sickness, the best biomarkers were different, as IgM, MMP-9 and CXCL-13 were shown to be the most discriminating between early and late Stage 2 patients, which could also be important to better explain the differences in immunopathogenesis and clinical presentations of the two forms of HAT (Tiberti et al. 2013a).

An alternative approach has been published 2 years ago on the antigenic profiles of *T. b. brucei* sub-proteomes given by sera of acute Sleeping Sickness patients (Manful et al. 2010). The proteomics strategy was to combine 1D or 2D electrophoresis of trypanosome proteins with Western Blot recognition by sera from *T. b. rhodesiense* patients, but the originality was to perform a cell fractionation step prior to analysis in order to remove VSGs (which in abundance could mask minority proteins of interest) and to purify glycosomes and cytoskeletons. Unfortunately, in bloodstream-form sub-proteomes no specific signal seemed to be differentially recognised by control and infection sera. Using procyclic forms, two targets were identified in the soluble fraction of cytoskeletons; a 70 kDa band was identified as Heat Shock Protein (HSP70) and a 35 kDa band that was not identified by MS (no technical precision). Precision was brought by 2D profiling: few spots exhibited potential specificity for infection sera, but confirmed HSP70 and evidenced mitochondrial proteins as strong targets. Unfortunately, these proteins are highly conserved during evolution and preferentially expressed by the trypanosome's insect stage, which made the authors conclude that they are unlikely to be useful for diagnostic purpose. Integrating technical limitations, the most important message delivered in the article was that "none of the *T. brucei* proteins that were detected by Coomassie staining was likely to be useful as a diagnostic antigen" for *T. b. rhodesiense* Sleeping Sickness (Manful et al. 2010). By contrast, the same type of immunoproteomics approach with purified IgG from *T. b. gambiense*-infected patients allowed the identification of 24 immunodiagnostic antigens, among which four were selected for further functional analysis: ISG64, ISG65, ISG75 and GRESAG4. ISG64 and ISG65 demonstrated promising power to detect *T. b. gambiense* infections, whereas they were less powerful for *T. b. rhodesiene* ones (Sullivan et al. 2013).

7.4.2 Therapeutic Markers

Relapses in the treatment of sleeping sickness have increased in number because of limited therapeutic armamentarium, and acquirement of chemoresistance by

trypanosomes is becoming a serious public health and veterinary problem. Again, proteomics can help to determine the biochemical changes allowing trypanosomes to resist the effect of the chemicals used. In the case of drug resistance, despite analysis of procyclic forms (i.e. insect stage) of trypanosomes, an interesting study compared total proteome from drug-sensitive and drug-resistant isogenic lines of *T. brucei*. Authors evidenced a protein spot corresponding to a putative nascent polypeptide-associated complex subunit, whose absence was associated with resistance to arsenical drug Cymelarsan and which also occurred as isoforms (same molecular weight, different p*I*) in both resistant and sensitive lines. MS matched this protein to an identical pair of tandem genes exhibiting identical sequence, which strongly suggested that the missing protein isoform arose due to the lack of a post-translational modification (Foucher et al. 2006). This illustrates the importance of protein regulation systems in trypanosomes to adapt the host environment and the need of having a sophisticated sensing system.

Other studies were recently published on new drug targeting using a similar approach: affinity chromatography followed by mass spectrometry characterisation of the eluted proteins. Using a chemical proteomics approach validated by functional RNA interference experiments, the specific antitrypanosomal activity of 4-[5-(4-phenoxyphenyl)-2*H*-pyrazol-3-yl]morpholine on *T. b. rhodesiense* was associated with disabling adenosine kinase, a key enzyme of the parasite purine salvage pathway (Kuettel et al. 2009). A naphtoquinone active against *T. b. rhodesiense* was also demonstrated to target and inhibit glycosomal glycerol kinase and glycosomal glyceraldehyde-3-phosphate dehydrogenase at micromolar concentrations and to concomitantly generate oxygen radicals to exert its trypanocidal activity (Pieretti et al. 2013). In the same way, 4-[4-amino-5-(2-methoxy-benzoyl)-pyrimidin-2-ylamino]-piperidine-1-carboxylic acid phenylamide, a compound able to cure mice from *T. b. brucei* infection after oral administration, was demonstrated to bound specifically to mitogen-activated protein kinases (MAPKs) and cdc2-related kinases (CRKs). This latter result could explain the mode of action and the deleterious pharmacological effects of 2,4-diaminopyrimidines on trypanosomatids (Mercer et al. 2011). Using the *T. brucei* model, two other studies characterised the trypanocidal activities and molecular targets of existing clinical inhibitors of cysteine protease and lipases, respectively (Yang et al. 2012a, b). This highlights the power of proteomics not only in improving diagnosis African trypanosomoses but also in evidencing new future drugs to treat infected humans and animals.

7.5 Going Forward

During the parasite life cycle Trypanosome–hosts interactions occur through evolutionary distant molecular "languages". Subcellular proteomic identification of the constituents of highly enriched organelles provides manageably sized proteomes for detailed *in silico* functional assignment of the molecular dialogues and conflicts occurring between the trypanosome and its insect vector or mammalian host in the

time course of the infection. The refined analysis of trypanosome sub-proteomes may thus highlight strategies developed by the parasite to survive and replicate within the host and thus brought new avenues to better understand the infectious process and interfere with it in the frame of elimination of African trypanosomoses. The precise molecular characterization of trypanosome's sub-proteomes deserve further investigations to complete the understanding of the complex trypanosome infectious strategies and to define parasite-specific molecular components that are evolutionary divergent from the mammalian hosts.

Pitfalls of the Current Proteomics Approaches. As illustrated all along this chapter, one classic pitfall in studies of the molecular crosstalk in host–parasite associations is the use of a single proteomic technique to survey complex molecular systems. This was particularly true before the advent of high throughput proteomics technologies associated with advanced MS.

Despite the fact that specific immune responses to salivary proteins of *Glossina* were detected in humans, most studies on molecular crosstalks in host–trypanosome associations are performed either by following the expression of the parasite proteome during infection, by the reaction of the host proteome following an invasion by a parasite species or by the injection of immune elicitors (Zhang et al. 2005). Moreover, two questions must be kept in mind when analysing proteomics data according to the sampling strategy: do the host genomic expressions during the infection by a parasite represent a nonspecific response that might be induced by any pathogen? Is the parasite proteome/secretome used during the infection process specific to a host or not?

This is well illustrated by the promising candidates MMP-9, ICAM-1, OP, B2MG, H-FABP, NP, CXCL-8, -10 and -13 that were demonstrated to be overexpressed in CSF of patients suffering Stage 2 HAT, but which are not specific of a trypanosome infection and their elevated expression level can also be the signature of other neurodegenerative or infectious diseases. For example, the study, which proposed neopterin as the best one for staging HAT, was based on sick-versus-control analysis of patients through the prism of Sleeping Sickness (Tiberti et al. 2012). But neopterin was also described as an excellent marker for viral infections such as herpetic encephalitis (Bociąga-Jasik et al. 2011), HIV (Edén et al. 2007) and also for patients suffering psychiatric diseases such as schizophrenia (Bechter et al. 2010) or in paediatric neurology as a marker of active central nervous system inflammation (Dale et al. 2009). Other biomarkers that were promising for staging HAT, as S100β protein and neurofilament or CXCL-10, have been shown to be overexpressed in CSF of patients infected by HIV (Du Pasquier et al. 2012; Angel et al. 2012; Valcour et al. 2012), a significant limitation because of the HIV epidemics and its evolution in the African continent (De Cock et al. 2012). Moreover, especially in the case of neopterin, high levels of this protein might persist for long in the CSF of patients that follows an effective highly active antiretroviral therapy against HIV (Edén et al. 2007), and this should pose ethical issues because of the risks associated with the treatment of HAT Stage 2, but above all it must raise questions about the real diagnostic value of such biomarkers in the

context of multiple infections. Additionally, despite the huge interest of these recent findings in CSF markers for a better staging and treatment management of people suffering HAT, the search for non-invasive tests for diagnosis and treatment follow-up appears to be also a priority, because of the problem of acceptability of multiple lumbar punctures by HAT patients.

Future in Proteomics for African Trypanosomes. "Omics" studies have contributed to the discovery of candidate genes and biochemical pathways potentially involved for the molecular crosstalk in host–vector–trypanosome associations. Proteomics datasets still offered a panel of protein candidates to develop new diagnostic tools or to investigate new therapeutic targets, which must be evaluated and validated in the field to answer current needs of screening and control of African trypanosomoses. Further proteomics approaches, because they relate to functional proteins, should be designed with regards both to increase of knowledge on trypanosome's specific virulence and pathogenic factors and especially to concrete applications in diagnosis or drug design fields. For instance, multiplex combination of highly specific biomarkers would help simultaneously to integrate inter-individual variability (from both trypanosome and its hosts) and to maintain high efficiency in detecting infections. In that way, fine screening of serum and plasma of patients suffering HAT, in comparison with serum and plasma of non-HAT people suffering other infections, will be the most important challenge in the coming years. It will be closely related not only to the advances of proteomics and mass spectrometry technology but also to bioinformatics development and molecular databases updates. One encouraging point is that methodologies to fraction human serum or plasma have been widely developed these last years because circulating low molecular weight proteome (LMWP) could easily be associated with a clinical pathological status in a given patients. One new fast, efficient and reliable multi-fractionation system is based on mesoporous silica chips MPS that specifically target and enrich LMWP, can be applied at the nanoscale depending on both the biological queries and biological sample constraints: various pore size distributions, pore structures, connectivity and surface properties can be applied for selective recovery of low mass proteins (Fan et al. 2012). Additionaly, a 4D protein profiling method is based on abundant protein depletion coupled with microscale 2D electrophoresis followed by reversed-phase separation of tryptic peptides and LC-MS/MS. This profiling strategy can routinely identify low abundant proteins from very low volumes of biological sample and distinguish variations a large number of proteins over nine orders of magnitude, including proteins at the ng/ml level or below (Tang et al. 2011). Moreover, the improvement of protein chip arrays will make this technology a future gold standard for biomarkers discovery, as it will allow both antigen capture and antibody detection on different chemical or biochemical matrix (Hause et al. 2011). For example, in the case of sleeping sickness, it could be used to capture low abundant circulating antigens from the secretome or proteome of trypanosomes by antibodies from serum, and CSF in the case of HAT, of an infected host.

Despite sustained efforts in proteomics application to research on African trypanosomes, there are still many black boxes in the characterisation of the molecular dialogues established between a trypanosome and its hosts. Among the studies to be developed, some particular points that proteomics could shed light on are: the host, the vector and the trypanosome LMWP and peptidome expression during their molecular crosstalk's; the insoluble/hydrophobic proteome linked to host–vector–trypanosome systems; the molecular changes in host, vector, trypanosome secretomes/proteomes within and between populations based on the population proteomics approach (Nedelkov 2005); and biochemical signatures linked to a particular habitat and/or environmental conditions.

Metabolomics a Key "Omics" Tool for the Pathogenoproteomics. As extracellular parasites, African trypanosomes must constantly monitor and respond to environmental changes they induce in their insect vector and human and/or animal hosts. How trypanosomes detect these changes is a black box, but they must have the ability to sample changes in nutrients and other small molecules in order to reprogram their gene expression profile. Metabolomics quantify modifications of low molecular weight chemicals within a given system and will therefore bring new information on the discrete biochemical changes occurring when the trypanosome interacts with its hosts (Creek et al. 2012). This is illustrated by the recent evidencing of a novel sterol metabolic network of *T. brucei*, which operates through the acetate-mevalonate pathway using leucine as an alternative precursor of ergosterol. Fluctuations of the acetyl-CoA and the cytosolic sterol metabolic signatures between procyclic and bloodstream forms strongly suggested that modulation of synthetic pathways involved in ergosterol biosynthesis lead to sterol profiles specifically adapted to parasite growth needs (Nes et al. 2012). Another metabolomics pioneering study on *T. b. brucei* infections revealed that co-infections, which are a common problem in the field, did not show marked alteration of urinary biochemical composition compared to single strain infection, but that differences were related to the trypanosome strain considered (Li et al. 2011). Other applications of metabolomics are to bring information on the mode of action of drugs and drug targets as proven by a LC-MS based non-targeted metabolomics approach of eflornithine and nifurtimox. Metabolomics confirmed changes in the polyamines pathway induced by eflornithine or nifurtimox metabolic activation. But more interestingly, it highlighted eflornithine-modified metabolic pathways not previously described (e.g. lack of arginase activity, *N*-acetylated ornithine and putrescine) or nifurtimox-modified carbohydrate and nucleotide metabolism (Vincent et al. 2012).

Bridging metabolomics with other "omics" data makes enter trypanosomes knowledge in a new era with the ability to dissect the vector–parasite–host interactions in real time, by shifting from protein–protein interaction to complex metabolic changes, with the constant objective of diagnostic or even prognostic metabolic signatures of the infection (Holmes 2010). Considering the complexity of biomolecular events regulating metabolic patterns, network biology approaches as interactome mapping have been recently developed.

Interactomics: Toward in Silico Deciphering of Host–Vector–Trypanosome Molecular Interactions. Functional analysis of "omics" molecular data and interactome bioassays will be necessary to confirm the involvement of candidate peptides and proteins in the host–vector–trypanosome molecular dialogue. The last few years have witnessed the birth of new biological entities named "interactomes", corresponding to an "ideal world" of the complete set of protein–protein interactions existing between all the proteins of an organism (Lievens et al. 2010). One study on *T. brucei* dissected the structural organisation of paraflagellar rod proteins, demonstrating two clusters of interactions (Lacomble et al. 2009). Integrating high throughput "omics" datasets (genomics, transcriptomics, proteomics, metabolomics, glycomics, lipidomics) allows the systems biology of organisms to be explored (Biron et al. 2007). Several computational techniques as the mirror tree method based on protein co-evolution, co-adaptation and interactions have emerged to complement these experimental approaches (Juan et al. 2008). These bioinformatics tools are promising to create the first comprehensive, experiment-based, multi-scale mathematical model of trypanosome physiology: the "Silicon Trypanosome" (Bakker et al. 2010). This makes one believe that in the near future, as initiated by Uetz et al. (2006), the docking of the interactomes of trypanosomes onto those of their hosts will soon be possible and will be a significant step to both understand and interfere with the infectious process in African trypanosomoses.

References

Acestor N, Panigrahi AK, Ogata Y et al (2009) Protein composition of *Trypanosoma brucei* mitochondrial membranes. Proteomics 9:5497–5508

Acestor N, Zíková A, Dalley RA et al (2011) *Trypanosoma brucei* mitochondrial respiratome: composition and organization in procyclic form. Mol Cell Proteomics 10:M110.006908

Agranoff D, Stich A, Abel P et al (2005) Proteomic fingerprinting for the diagnosis of human African trypanosomiasis. Trends Parasitol 21:154–157

Aksoy S, Gibson WC, Lehane MJ et al (2003) Perspectives on the interactions between tsetse and trypanosomes with implications for the control of trypanosomiasis. Adv Parasitol 62:1–83

Anderson NL, Parish NM, Richardson JP et al (1985) Comparison of African trypanosomes of different antigenic phenotypes, subspecies and life cycle stages by two-dimensional gel electrophoresis. Mol Biochem Parasitol 16:299–314

Angel TE, Jacobs JM, Spudich SS et al (2012) The cerebrospinal fluid proteome in HIV infection: change associated with disease severity. Clin Proteomics 9:3

Bakker BM, Krauth-Siegel RL, Clayton C et al (2010) The silicon trypanosome. Parasitology 137:1333–1341

Barry JD, McCulloch R (2001) Antigenic variation in trypanosomes: enhanced phenotypic variation in a eukaryotic parasite. Adv Parasitol 49:1–70

Bechter K, Reiber H, Herzog S et al (2010) Cerebrospinal fluid analysis in affective and schizophrenic spectrum disorders: identification of subgroups with immune responses and blood-CSF barrier dysfunction. J Psychiatr Res 44:321–330

Biron DG, Joly C, Galeotti N et al (2005) The proteomics: a new prospect for studying parasitic manipulation. Behav Processes 68:249–253

Biron DG, Loxdale HD, Ponton F et al (2006) Population proteomics: an emerging discipline to study metapopulation ecology. Proteomics 6:1712–1715

Biron DG, Hughes AL, Loxdale HD et al (2007) The need for megatechnologies: massive sequencing, proteomics and bioinformatics. In: Tibayrenc M (ed) Encyclopedia of infectious diseases: modern technologies. Wiley, New York, pp 357–377

Bociąga-Jasik M, Cieśla A, Kalinowska-Nowak A et al (2011) Role of IL-6 and neopterin in the pathogenesis of herpetic encephalitis. Pharmacol Rep 63:1203–1209

Bridges DJ, Pitt AR, Hanrahan O et al (2008) Characterisation of the plasma membrane subproteome of bloodstream form *Trypanosoma brucei*. Proteomics 8:83–99

Broadhead R, Dawe HR, Farr H et al (2006) Flagellar motility is required for the viability of the bloodstream trypanosome. Nature 440:224–227

Bucheton B, MacLeod A, Jamonneau V (2011) Human host determinants influencing the outcome of *Trypanosoma brucei gambiense* infections. Parasite Immunol 33:438–447

Butter F, Bucerius F, Michel M et al (2013) Comparative proteomics of two life cycle stages of stable isotope-labeled *Trypanosoma brucei* reveals novel components of the parasite's host adaptation machinery. Mol Cell Proteomics 12:172–179

Caljon G, Broos K, De Goeyse I et al (2009) Identification of a functional Antigen5-related allergen in the saliva of a blood feeding insect, the tsetse fly. Insect Biochem Mol Biol 39:332–341

Chandra M, Liniger M, Tetley L et al (2004) TsetseEP, a gut protein from the tsetse *Glossina morsitans*, is related to a major surface glycoprotein of trypanosomes transmitted by the fly and to the products of a *Drosophila* gene family. Insect Biochem Mol Biol 3:1163–1173

Colasante C, Ellis M, Ruppert T, Voncken F (2006) Comparative proteomics of glycosomes from bloodstream form and procyclic culture form *Trypanosoma brucei brucei*. Proteomics 6:3275–3293

Courtioux B, Bisser S, M'belesso P et al (2005) Dot enzyme-linked immunosorbent assay for more reliable staging of patients with Human African trypanosomiasis. J Clin Microbiol 43:4789–4795

Courtioux B, Pervieux L, Vatunga G et al (2009) Increased CXCL-13 levels in human African trypanosomiasis meningo-encephalitis. Trop Med Int Health 14:529–534

Creek DJ, Anderson J, McConville MJ et al (2012) Metabolomic analysis of trypanosomatid protozoa. Mol Biochem Parasitol 181:73–84

Cuervo P, Domont GB, De Jesus JB (2010) Proteomics of trypanosomatids of human medical importance. J Proteomics 73:845–867

Dale RC, Brilot F, Fagan E et al (2009) Cerebrospinal fluid neopterin in paediatric neurology: a marker of active central nervous system inflammation. Dev Med Child Neurol 51:317–323

Dama E, Cornelie S, Bienvenu Somda M et al (2013) Identification of *Glossina palpalis gambiensis* specific salivary antigens: towards the development of a serologic biomarker of human exposure to tsetse flies in West Africa. Microbes Infect 15(5):416–427

De Bock M, de Seny D, Meuwis MA et al (2010) Challenges for biomarker discovery in body fluids using SELDI-TOF-MS. J Biomed Biotechnol 2010:906082

De Cock KM, Jaffe HW, Curran JW (2012) The evolving epidemiology of HIV/AIDS. AIDS 26:1205–1213

De Koning HP, Bridges DJ, Burchmore RJ (2005) Purine and pyrimidine transport in pathogenic protozoa: from biology to therapy. FEMS Microbiol Rev 29:987–1020

Degrasse JA, Devos D (2010) A functional proteomic study of the Trypanosoma brucei nuclear pore complex: an informatic strategy. Methods Mol Biol 673:231–238

Degrasse JA, Chait BT, Field MC et al (2008) High-yield isolation and subcellular proteomic characterization of nuclear and subnuclear structures from trypanosomes. Methods Mol Biol 463:77–92

Du Pasquier RA, Jilek S, Kalubi M et al (2012) Marked Increase of the Astrocytic Marker S100B in the Cerebrospinal Fluid of HIV-infected Patients on LPV/r-Monotherapy. AIDS 27:203–210

Edén A, Price RW, Spudich S et al (2007) Immune activation of the central nervous system is still present after >4 years of effective highly active antiretroviral therapy. J Infect Dis 196:1779–1783

Fan J, Gallagher JW, Wu HJ et al (2012) Low molecular weight protein enrichment on mesoporous silica thin films for biomarker discovery. J Vis Exp 62. doi:10.3791/3876

Ferguson MA, Brimacombe JS, Brown JR et al (1999) The GPI biosynthetic pathway as a therapeutic target for African sleeping sickness. Biochim Biophys Acta 1455:327–340

Ferreira V, Molina MC, Valck C et al (2004) Role of calreticulin from parasites in its interaction with vertebrate hosts. Mol Immunol 40:1279–1291

Foucher AL, McIntosh A, Douce G et al (2006) A proteomic analysis of arsenical drug resistance in *Trypanosoma brucei*. Proteomics 6:2726–2732

Geiger A, Hirtz C, Bécue T et al (2010) Exocytosis and protein secretion in *Trypanosoma*. BMC Microbiol 10:20

Grébaut P, Chuchana P, Brizard JP et al (2009) Identification of total and differentially expressed excreted-secreted proteins from *Trypanosoma congolense* strains exhibiting different virulence and pathogenicity. Int J Parasitol 39:1137–1150

Guerra-Giraldez C, Quijada L, Clayton CE (2002) Compartmentation of enzymes in a microbody, the glycosome, is essential in *Trypanosoma brucei*. J Cell Sci 115:2651–2658

Güther ML, Lee S, Tetley L et al (2006) GPI-anchored proteins and free GPI glycolipids of procyclic form *Trypanosoma brucei* are nonessential for growth, are required for colonization of the tsetse fly, and are not the only components of the surface coat. Mol Biol Cell 17:5265–5274

Güther ML, Beattie K, Lamont DJ et al (2009) Fate of glycosylphosphatidylinositol (GPI)-less procyclin and characterization of sialylated non-GPI-anchored surface coat molecules of procyclic-form *Trypanosoma brucei*. Eukaryot Cell 8:1407–1417

Haddow JD, Poulis B, Haines LR et al (2002) Identification of major soluble salivary gland proteins in teneral *Glossina morsitans morsitans*. Insect Biochem Mol Biol 32:1045–1053

Hainard A, Tiberti N, Robin X et al (2009) A combined CXCL10, CXCL8 and H-FABP panel for the staging of human African trypanosomiasis patients. PLoS Negl Trop Dis 3:e459

Hainard A, Tiberti N, Robin X et al (2011) Matrix metalloproteinase-9 and intercellular adhesion molecule 1 are powerful staging markers for human African trypanosomiasis. Trop Med Int Health 16:119–126

Hao Z, Kasumba I, Lehane MJ et al (2001) Tsetse immune responses and trypanosome transmission: implications for the development of tsetse-based strategies to reduce trypanosomiasis. Proc Natl Acad Sci USA 98:12648–12653

Hart SR, Lau KW, Hao Z et al (2009) Analysis of the trypanosome flagellar proteome using a combined electron transfer/collisionally activated dissociation strategy. J Am Soc Mass Spectrom 20:167–175

Hause RJ, Kim HD, Leung KK et al (2011) Targeted protein-omic methods are bridging the gap between proteomic and hypothesis-driven protein analysis approaches. Expert Rev Proteomics 8:565–575

Holmes E (2010) The evolution of metabolic profiling in parasitology. Parasitology 137:1437–1449

Holzmuller P, Biron DG, Courtois P et al (2008a) Virulence and pathogenicity patterns of *Trypanosoma brucei gambiense* field isolates in experimentally infected mouse: differences in host immune response modulation by secretome and proteomics. Microbes Infect 10:79–86

Holzmuller P, Grébaut P, Brizard JP et al (2008b) "Pathogeno-Proteomics": toward a new approach of host–vector–pathogen interactions. Ann N Y Acad Sci 1149:66–70

Holzmuller P, Grébaut P, Peltier JB et al (2008c) Secretome of animal trypanosomes. Ann N Y Acad Sci 1149:337–342

Holzmuller P, Grébaut P, Cuny G et al (2010) Tsetse flies, trypanosomes, humans and animals: what is proteomics revealing about their crosstalks? Expert Rev Proteomics 7:113–126

Hu CY, Aksoy S (2006) Innate immune responses regulate trypanosome parasite infection of the tsetse fly *Glossina morsitans morsitans*. Mol Microbiol 60:1194–1204

Jenni L, Molyneux DH, Livesey JL et al (1980) Feeding behavior of tsetse flies infected with salivarian trypanosomes. Nature 283:383–385

Juan D, Pazos F, Valencia A (2008) High confidence prediction of global interactomes based on genome-wide coevolutionary networks. Proc Natl Acad Sci USA 105:934–939

Kuettel S, Mosimann M, Mäser P et al (2009) Adenosine Kinase of *T. b. Rhodesiense* identified as the putative target of 4-[5-(4-phenoxyphenyl)-2H-pyrazol-3-yl]morpholine using chemical proteomics. PLoS Negl Trop Dis 3:e506

Lacomble S, Portman N, Gull K (2009) A protein-protein interaction map of the Trypanosoma brucei paraflagellar rod. PLoS One 4:e7685

Lalmanach G, Boulangé A, Serveau C et al (2002) Congopain from *Trypanosoma congolense*: drug target and vaccine candidate. Biol Chem 383:739–749

Lefèvre T, Thomas F, Ravel S et al (2007) *Trypanosoma brucei brucei* induces alteration in the head proteome of the tsetse fly vector *Glossina palpalis gambiensis*. Insect Mol Biol 16:651–660

Lefèvre T, Roche B, Poulin R et al (2008) Exploiting host compensatory responses: the 'must' of manipulation? Trends Parasitol 24:435–439

Lefèvre T, Adamo S, Biron DG et al (2009) How to make someone do something: the diversity and evolution of manipulative strategies in host-parasite interactions. Adv Parasitol 68:45–83

Lehane MJ, Msangi AR (1991) Lectin and peritrophic membrane development in the gut of *Glossina m. morsitans* and a discussion of their role in protecting the fly against trypanosome infection. Med Vet Entomol 5:495–501

Li JV, Saric J, Wang Y et al (2011) Metabonomic investigation of single and multiple strain *Trypanosoma brucei brucei* infections. Am J Trop Med Hyg 84:91–98

Libersat F, Delago A, Gal R (2009) Manipulation of host behavior by parasitic insects and insect parasites. Annu Rev Entomol 54:189–207

Lievens S, Eyckerman S, Lemmens I et al (2010) Large-scale protein interactome mapping: strategies and opportunities. Expert Rev Proteomics 7:679–690

Lubega GW, Ochola DO, Prichard RK (2002) *Trypanosoma brucei*: anti-tubulin antibodies specifically inhibit trypanosome growth in culture. Exp Parasitol 102:134–142

Maclean L, Odiit M, Sternberg JM (2006) Intrathecal cytokine responses in *Trypanosoma brucei rhodesiense* sleeping sickness patients. Trans R Soc Trop Med Hyg 100:270–275

Manful T, Mulindwa J, Frank FM et al (2010) A search for Trypanosoma brucei rhodesiense diagnostic antigens by proteomic screening and targeted cloning. PLoS One 5:e9630

Matthews KR (2005) The developmental cell biology of *Trypanosoma brucei*. J Cell Sci 118:283–290

Mercer L, Bowling T, Perales J et al (2011) 2,4-Diaminopyrimidines as potent inhibitors of *Trypanosoma brucei* and identification of molecular targets by a chemical proteomics approach. PLoS Negl Trop Dis 5:e956

Mina JG, Pan SY, Wansadhipathi NK et al (2009) The *Trypanosoma brucei* sphingolipid synthase, an essential enzyme and drug target. Mol Biochem Parasitol 168:16–23

Moore J (1993) Parasites and the behaviour of biting flies. J Parasitol 79:1–16

Nandan D, Yi T, Lopez M et al (2002) *Leishmania* EF-1alpha activates the Src homology 2 domain containing tyrosine phosphatase SHP-1 leading to macrophage deactivation. J Biol Chem 277:50190–50197

Nedelkov D (2005) Population proteomics: addressing protein diversity in humans. Expert Rev Proteomics 2:315–324

Nes CR, Singha UK, Liu J et al (2012) Novel sterol metabolic network of Trypanosoma brucei procyclic and bloodstream forms. Biochem J 443:267–277

Nett IR, Martin DM, Miranda-Saavedra D et al (2009) The phosphoproteome of bloodstream form *Trypanonosoma brucei*, causative agent of African sleeping sickness. Mol Cell Proteomics 8:1527–1538

Panigrahi AK, Zíková A, Dalley RA et al (2008) Mitochondrial complexes in *Trypanosoma brucei*: a novel complex and a unique oxidoreductase complex. Mol Cell Proteomics 7:534–545

Panigrahi AK, Ogata Y, Zíková A et al (2009) A comprehensive analysis of *Trypanosoma brucei* mitochondrial proteome. Proteomics 9:434–450

Papadopoulos MC, Abel PM, Agranoff D et al (2004) A novel and accurate diagnostic test for human African trypanosomiasis. Lancet 363:1358–1363

Parsons M (2004) Glycosomes: parasites and the divergence of peroxisomal purpose. Mol Microbiol 53:717–724

Parsons M, Nielsen B (1990) *Trypanosoma brucei*: two-dimensional gel analysis of the major glycosomal proteins during the life cycle. Exp Parasitol 70:276–285

Pearson TW (2001) Procyclins, proteases and proteomics: dissecting trypanosomes in the tsetse fly. Trends Microbiol 9:299–301

Pearson TW, Moloo SK, Jenni L (1987) Culture form and tsetse fly midgut form procyclic *Trypanosoma brucei* express common proteins. Mol Biochem Parasitol 25:273–278

Pieretti S, Haanstra JR, Mazet M et al (2013) Naphthoquinone derivatives exert their antitrypanosomal activity via a multi-target mechanism. PLoS Negl Trop Dis 7:e2012

Poinsignon A, Cornelie S, Remoue F et al (2007) Human/vector relationships during human African trypanosomiasis: initial screening of immunogenic salivary proteins of *Glossina* species. Am J Trop Med Hyg 76:327–333

Portman N, Gull K (2012) Proteomics and the *Trypanosoma brucei* cytoskeleton: advances and opportunities. Parasitology 139:1168–1177

Probst P, Stromberg E, Ghalib HW et al (2001) Identification and characterization of T cell-stimulating antigens from *Leishmania* by CD4 T cell expression cloning. J Immunol 166:498–505

Roche S, Tiers L, Provansal M et al (2006) Interest of major serum protein removal for Surface-Enhanced Laser Desorption/Ionization – Time Of Flight (SELDI-TOF) proteomic blood profiling. Proteome Sci 4:20

Roditi I, Lehane M (2008) Interactions between trypanosomes and tsetse flies. Curr Opin Microbiol 11:345–351

Rout MP, Field MC (2001) Isolation and characterization of subnuclear compartments from *Trypanosoma brucei*. Identification of a major repetitive nuclear lamina component. J Biol Chem 276:38261–38271

Roy N, Nageshan RK, Pallavi R et al (2010) Proteomics of *Trypanosoma evansi* infection in rodents. PLoS One 5:e9796

Sajid M, McKerrow JH (2002) Cysteine proteases of parasitic organisms. Mol Biochem Parasitol 120:1–21

Schaub GA (2006) Parasitogenic alterations of vector behaviour. Int J Med Microbiol 296:37–40

Seebeck T, Schaub R, Johner A (2004) cAMP signalling in the kinetoplastid protozoa. Curr Mol Med 4:585–599

Silverman JM, Chan SK, Robinson DP et al (2008) Proteomic analysis of the secretome of *Leishmania donovani*. Genome Biol 9:R35

Sullivan L, Wall SJ, Carrington M et al (2013) Proteomic selection of immunodiagnostic antigens for human african trypanosomiasis and generation of a prototype lateral flow immunodiagnostic device. PLoS Negl Trop Dis 7:e2087

Tang HY, Beer LA, Speicher DW (2011) In-depth analysis of a plasma or serum proteome using a 4D protein profiling method. Methods Mol Biol 728:47–67

Tiberti N, Hainard A, Lejon V et al (2010) Discovery and verification of osteopontin and Beta-2-microglobulin as promising markers for staging human African trypanosomiasis. Mol Cell Proteomics 9:2783–2795

Tiberti N, Hainard A, Lejon V et al (2012) Cerebrospinal fluid neopterin as marker of the meningo-encephalitic stage of *Trypanosoma brucei gambiense* sleeping sickness. PLoS One 7:e40909

Tiberti N, Lejon V, Hainard A et al (2013a) Neopterin Is a Cerebrospinal Fluid Marker for Treatment Outcome Evaluation in Patients Affected by *Trypanosoma brucei gambiense* Sleeping Sickness. PLoS Negl Trop Dis 7:e2088

Tiberti N, Matovu E, Hainard A et al (2013b) New biomarkers for stage determination in *Trypanosoma brucei rhodesiense* sleeping sickness patients. Clin Transl Med 2:1

Uetz P, Dong YA, Zeretzke C et al (2006) Herpesviral protein networks and their interaction with the human proteome. Science 311:239–242

Urbaniak MD, Guther ML, Ferguson MA (2012) Comparative SILAC proteomic analysis of *Trypanosoma brucei* bloodstream and procyclic lifecycle stages. PLoS One 7:e36619

Valcour V, Chalermchai T, Sailasuta N et al (2012) Central nervous system viral invasion and inflammation during acute HIV infection. J Infect Dis 206:275–282

Van Den Abbeele J, Caljon G, De Ridder K et al (2010) *Trypanosoma brucei* modifies the tsetse salivary composition, altering the fly feeding behavior that favors parasite transmission. PLoS Pathog 6:e1000926

Van Deursen FJ, Thornton DJ, Matthews KR (2003) A reproducible protocol for analysis of the proteome of *Trypanosoma brucei* by 2-dimensional gel electrophoresis. Mol Biochem Parasitol 128:107–110

van Hellemond JJ, Opperdoes FR, Tielens AG (2005) The extraordinary mitochondrion and unusual citric acid cycle in *Trypanosoma brucei*. Biochem Soc Trans 33:967–971

Vertommen D, Van Roy J, Szikora JP et al (2008) Differential expression of glycosomal and mitochondrial proteins in the two major life-cycle stages of *Trypanosoma brucei*. Mol Biochem Parasitol 158:189–201

Vincent IM, Creek DJ, Burgess K et al (2012) Untargeted metabolomics reveals a lack of synergy between nifurtimox and eflornithine against *Trypanosoma brucei*. PLoS Negl Trop Dis 6: e1618

Yang PY, Wang M, He CY et al (2012a) Proteomic profiling and potential cellular target identification of K11777, a clinical cysteine protease inhibitor, in *Trypanosoma brucei*. Chem Commun (Camb) 48:835–837

Yang PY, Wang M, Liu K et al (2012b) Parasite-based screening and proteome profiling reveal orlistat, an FDA-approved drug, as a potential anti Trypanosoma brucei agent. Chemistry 18:8403–8413

Zhang CG, Chromy BA, McCutchen-Maloney SL (2005) Host-pathogen interactions: a proteomic view. Expert Rev Proteomics 2:187–202

Zhou Q, Gheiratmand L, Chen Y et al (2010) A comparative proteomic analysis reveals a new bi-lobe protein required for bi-lobe duplication and cell division in *Trypanosoma brucei*. PLoS One 5:e9660

Diagnosis of African Trypanosomiasis

8

Philippe Büscher

Abstract

In African trypanosomiasis, diagnosis is complex since several diseases are caused by different parasites and occur in different host species giving rise to a variety of parasite–host relationships. At one end of the spectrum we find acute or chronic but fatal diseases, while at the other end long-lasting subclinical and almost commensal infections are observed. The result is that all diagnostic methods, whether clinical or molecular, have their limitations that will define how, where and for what particular trypanosomiasis they will eventually be applied, alone or in combination. Research on diagnosis of African trypanosomiasis perfectly reflects the technological and socio-economical environment wherein it is conducted. After the discovery of African trypanosomes causing sleeping sickness and nagana in the early twentieth century, refinement of clinical diagnosis was soon followed by the development of improved parasitological methods. Later, serological and molecular diagnostics appeared but hardly found their way to the non-academic end user with one exception, a direct agglutination test for *gambiense* sleeping sickness. Only in the last decade, African trypanosomiases were freed from their status of neglected tropical diseases and received much more attention from public and private financial donors and from researchers. This evolution has led to major breakthroughs in diagnostics development that may have a huge impact on control of human and animal African trypanosomiases.

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8.1 Introduction

African trypanosomiasis is a complex of parasitic infections caused by various trypanosome species (*Trypanosoma brucei*, *T. congolense*, *T. evansi*, *T. equiperdum* and *T. vivax*) and subspecies in many different hosts, including mammals, reptiles and insects (tsetse flies). Among the mammals, we count man and his domestic animals (bovine, buffalo, camel, dog, goat, horse, pig, sheep. . .) as well as a range of wild animals. In mammals, infections can lead not only to acute or chronic but fatal diseases, like sleeping sickness in man, but also to long-lasting subclinical and almost commensal infections, particularly in animals that have evolved over millions of years together with their trypanosome parasites. Related to the diversity of African trypanosomiases is the diversity of the methods to diagnose these infections and their applicability. All diagnostic methods, whether clinical or molecular, have their limitations that will define how, where and for what particular trypanosomiasis they will eventually be applied, alone or combined in a diagnostic algorithm. For instance, the specifications of a diagnostic test used in epidemiological surveys of animal trypanosomiasis are quite different from those of a test to confirm sleeping sickness in an individual patient. For the first situation, a high throughput format that needs a laboratory environment, e.g. ELISA, and that allows adjusting specificity and sensitivity depending on the expected prevalence, will perfectly do. When it comes to individual diagnosis in rural settings where an immediate result is required to decide on treatment with rather toxic drugs, a rapid diagnostic test (RDT) format, e.g. an immunochromatography test (ICT), with high specificity is far more appropriate.

With this respect, some performance characteristics of diagnostic tests may be worth clarifying. Each diagnostic test has its intrinsic ***sensitivity*** and ***specificity***, usually assessed on a collection of specimens that are classified as positive (infected) or negative (non-infected) based on results obtained in a single or a combination of reference tests. The formulas are then as follows: **sensitivity = true positives/(true positives + false negatives)** and **specificity = true negatives/(true negatives + false positives)** (Brady 1995). In African trypanosomiasis, the generally accepted reference test is based on microscopic detection of the parasite in a biological specimen of the infected host. In many instances the parasite load is very low and under the detection limit of the reference test. Therefore, within the positives (infected) there may be a bias towards specimens from individuals with high parasite loads that may not be fully representative for the whole infected population. In addition, the collection of negatives (non-infected) may contain specimens from actually infected individuals. In that case, no-gold standard mathematics should be used to calculate test sensitivity and specificity (Joseph et al. 1995; Rutjes et al. 2007; Enøe et al. 2000; de Clare Bronsvoort et al. 2010). Apart from their intrinsic characteristics, the performance of diagnostic tests can be described in terms of ***positive*** and ***negative predictive value*** (PPV and NPV) that take into account the prevalence of the infection or the disease within a given population. The formulas are as follows: **PPV = (sensitivity)(prevalence)/[(sensitivity)(prevalence)+(1−specificity)(1−prevalence)]** and **NPV = (specificity)**

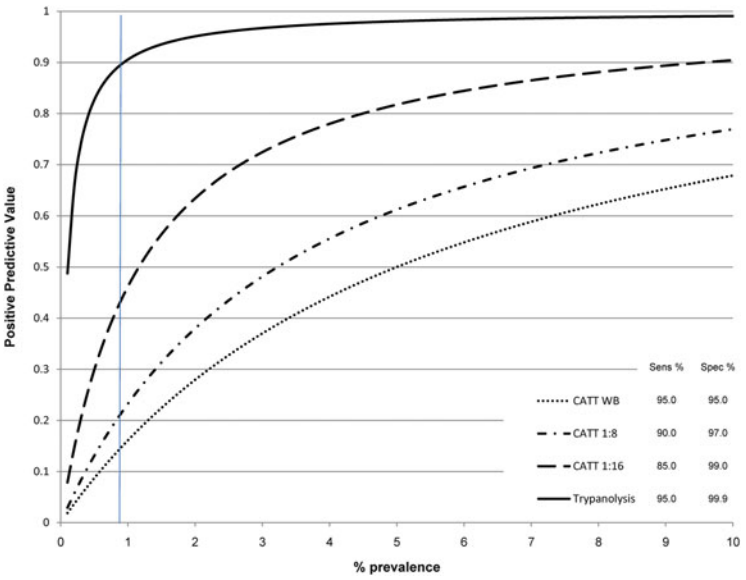

Fig. 8.1 Positive predictive value of four antibody detection tests in function of the prevalence in the target population and of the intrinsic specificity and sensitivity of the tests

(1−prevalence)/[(specificity)(1−prevalence) + (1−sensitivity)(prevalence)]. The prevalence of an infection will thus define which type of diagnostic test will perform best in a given population. For instance, when the prevalence is low, a highly specific test is required to avoid a high number of false positives as illustrated in Fig. 8.1 representing the PPV calculated in function of the prevalence with the sensitivity (Se) and specificity (Sp) data for three versions of the Card Agglutination Test for Trypanosomiasis (CATT) and for the Immune Trypanolysis test (TL).

Research on diagnosis of African trypanosomiases perfectly reflects the technological and socio-economical environment wherein it is conducted. After the discovery of African trypanosomes causing sleeping sickness and nagana, refinement of clinical diagnosis was soon followed by the development of improved parasitological methods. Later, serological and molecular diagnostics appeared but hardly found their way to the non-academic end user. In 2001 when human and animal African trypanosomiases were still seriously neglected tropical diseases, we wrote "*Despite decennia of research into the development of reliable diagnostic tests for African trypanosomiasis, little has changed in current practice, particularly at the level of farmers, veterinarians and health personnel in developing countries where African trypanosomiasis prevails*" (Büscher 2001). In the meantime, African trypanosomiases were freed from their status of neglected tropical diseases and received much more attention from researchers and from public and private financial donors. Thanks to this evolution some major breakthroughs in diagnostics development have occurred and may have a huge impact on control of

African trypanosomiases in the near future. Still, we shouldn't be too optimistic since not only the performance of diagnostic tests but also their price, particularly in comparison with the costs of treatment, their applicability and their availability to the end user will have an influence on their deployment. For example, in the absence of a rapid diagnostic test that is cheaper than the currently used drugs, the practice of treating nagana in cattle or surra in camel on clinical suspicion only will continue. Another problem may arise from the "pressure to deliver", imposed on diagnostics developers by the funding agencies that tend to shift from public to private, introducing the risk that quantity and visibility become more important than quality. This may result in the large-scale application of new diagnostics that have not been fully evaluated or validated and thus may compromise the outcome of efforts to control human and animal African trypanosomiases. International organisations like the World Health Organization (WHO), the Food and Agriculture Organization (FAO) and the World Animal Health Organization (OIE) might have a role to play in encouraging the proper validation.

In the following paragraphs, an overview of currently available diagnostic tools for human and animal African trypanosomiasis is given with special attention to recent achievements.

8.2 Clinical Signs and Symptoms

In African trypanosomiasis, clinical signs and symptoms are rather unspecific or may even be obscure, particularly in trypanotolerant hosts. No single symptom is pathognomonic and African trypanosomiasis may simulate many other infections. Therefore, clinical diagnosis remains only presumptive and relies upon the combination of several clinical signs occurring in a susceptible host in a given epidemiological situation.

In domestic animals, the acute phase of trypanosomiasis is often associated with fever and with anaemia, reflected by low packed cell volumes (PCV) in the blood. Some *T. vivax* strains can cause a haemorrhagic syndrome in bovine (Assoku and Gardiner 1989). Neurological symptoms such as paralysis of the hindquarters occur in chronic stages of *Trypanozoon* infections, particularly in camel and horses. Also in horses, ventral oedema and local skin indurations (plaques) are more or less pathognomonic for *T. evansi* or *T. equiperdum* infection (OIE 2012).

In human, irregular fever that does not resolve with anti-malaria treatment is an important feature of the sleeping sickness syndrome. All too often, sleeping sickness patients are first treated for malaria and remain undiagnosed for several weeks to months with a substantial risk to die before confirmation of trypanosomiasis (Hasker et al. 2011; Bukachi et al. 2009). Other important early symptoms, although they can be caused by other infections, are general fatigue, joint pain (Kerandal sign) and swollen cervical lymph nodes (Winterbottom sign). In the neurological stage of sleeping sickness, symptoms become more specific but do not occur in all patients. Archaic reflexes, behaviour changes, irregular sleep and impairment of motor function are just some of the symptoms that can be observed

and that strongly suggest human African trypanosomiasis when present in a patient living in an endemic area (for a review see World Health Organization (1986) that will be updated during 2013). Particularly challenging however, and described in a recent review, is recognising human African trypanosomiasis in patients with a history of travel or residence in a sleeping sickness focus, e.g. immigrants or tourists, that seek medical care in a non-endemic health service where knowledge on sleeping sickness is poor (Lejon et al. 2003b).

8.3 Parasite Detection

In the mammal host, trypanosomes invade different compartments, depending on the species. *T. congolense* and *T. vivax* are mainly blood-dwelling parasites. Species belonging to the *Trypanozoon* subgenus, on the other hand, are notorious tissue invading parasites that can be found in the extravascular spaces of all kind of organs, including the central nervous system. Only a fraction of the total parasite population circulates in body fluids that are accessible for diagnostic examinations, in casu the blood, lymph and cerebrospinal fluid.

In many instances, particularly in the chronic phase of the infection, the parasite load in these body fluids remains below the detection limit of common microscopic techniques thus making parasite detection cumbersome and poorly sensitive. For example, even when combining different methods, it is estimated that less than 80 % of *gambiense* sleeping sickness patients can be confirmed parasitologically (Büscher et al. 2011). Similar low confirmation rates are obtained in buffalo and camel infected with *T. evansi* or in cattle infected with *T. vivax*, *T. congolense* or *T. brucei*, often occurring as mixed infections (Fikru et al. 2012). In dourine (*T. equiperdum*), detecting the parasite is so difficult that in a recent outbreak in Italy, the definition of a confirmed case was based on indirect diagnostic evidence and reads as follows: an animal giving a positive result in a serological antibody detection test or in PCR and showing clinical signs compatible with dourine or showing an increase of serological titre in two consecutive testing or epidemiologically linked with a confirmed case of dourine (Calistri et al. 2013). Typically, only in 2 of the 10 cases confirmed according to the above definition, a living parasite was seen under the microscope in the mammary secretion and in the joint fluid (Pascucci et al. 2013). For obvious reason concentration or separation techniques have thus become the standard for parasite detection in human and animal African trypanosomiasis. Several techniques exist (Büscher and Lejon 2004).

Blood can be taken up in capillary tubes that are coated with an anticoagulant and closed at one end with sealant. After high speed centrifugation in an haematocrit centrifuge, the blood is separated in three phases: erythrocytes at the bottom, plasma on top and a layer (buffy coat) of white blood cells (WBC) in the middle. The tubes are mounted under the microscope in a specially designed or custom-made capillary holder so that they are surrounded by water to minimise light diffraction and covered with a cover slip. Since trypanosomes have the same density as the WBCs, they will be concentrated in the buffy coat thus facilitating

their detection under the microscope. This method is called Capillary Tube Centrifugation (CTC), Haematocrit Centrifugation Technique (HTC) or Woo technique (Woo 1970). With this method, identification of the trypanosome species is not obvious although the morphology and particularly the movement of the parasites may be indicative. When examining animal blood, the microscopist should be aware that bovine and sheep may carry non-pathogenic trypanosomes from the subgenus *Megatrypanum*, respectively, *Trypanosoma theileri* and *Trypanosoma melophagium*. Also, when the blood contains microfilaria that are much bigger than trypanosomes and that disturb the buffy coat by their movement, it becomes almost impossible to detect the smaller trypanosomes. In case differential diagnosis is desired, the capillary tubes can be broken and the buffy coat spread on a microscope slide for examination according to Murray (Murray et al. 1977). Given the risk of infection when manipulating human samples, the Murray technique is not recommended in sleeping sickness diagnosis.

The mini Anion Exchange Centrifugation Technique (mAECT), developed by Lumsden et al. (1979), separates trypanosomes from blood cells on an anion exchange chromatography gel based on their differential surface charge in function of pH and ionic strength of the gel equilibration buffer. Trypanosomes that are eluted from the gel are taken up in a clear glass or plastic collector tube that is centrifuged where after the parasites concentrate at the bottom of the tube. Based on the work of Sheila Lanham and David Godfrey, the mAECT buffer composition can be adapted in function of the host and the trypanosome species (Lanham and Godfrey 1970). The same buffer composition can be used to separate *T. brucei*, *T. evansi* and *T. equiperdum* from mouse, rat, human, horse and camel blood but not for goat blood (Gutierrez et al. 2004). Separating *T. congolense*, *T. vivax* or *Trypanozoon* trypanosomes from bovine blood is less straightforward. Recently, an improved model of mAECT column and collector tube has been designed and is available as a 20-test kit from the Institut National de Recherche Biomédicale (INRB) in Kinshasa (Büscher et al. 2009). mAECT is mainly used for diagnosis of *gambiense* sleeping sickness where it has a lower detection limit of <30 trypanosomes/ml of blood due to the large volume of blood (up to 0.5 ml) that is examined. This detection limit can even be lowered by prior centrifugation of a larger volume of blood (up to 5 ml) and loading the buffy coat on the mAECT column (Camara et al. 2010). mAECT is without doubt the most sensitive parasite detection technique with an analytical sensitivity that is similar to most molecular diagnostics for African trypanosomiasis. Unfortunately, also the price is similar (7 €/test).

The Quantitative Buffy Coat (QBC) was originally developed for diagnosis of malaria but is useful for detection of other parasites, including trypanosomes (Levine et al. 1989; Bailey and Smith 1992; Chatel et al. 1999). In QBC, a capillary tube containing acridine orange, a DNA intercalating fluorescent dye, is filled with some 50 µl of blood and a small float with the same density as WBC is inserted in the tube. By high speed centrifugation, the buffy coat settles between the inner wall of the capillary tube and the outer wall of the float thus facilitating recognition of nucleated cells in epifluorescence microscopy. If trypanosomes are present, they

can be seen as cells with green fluorescent nuclei and kinetoplasts, moving between the white blood cells in the expanded buffy coat. In the new version of the QBC test, the halogen bulb has been replaced by a light emitting diode (LED) (Kuhn et al. 2010). The QBC test is available from QBC Diagnostics (http://www.qbcdiagnostics.com).

Recently, another application of acridine orange for diagnosis of African trypanosomiasis has been described where trypanosomes are stained after lysis of the red blood cells (Bieler et al. 2012). Although the technique seems promising, it still suffers from the fact that not only the nuclei and kinetoplast of the trypanosomes but also the nuclei of the white blood cells are fluorescent. It is possible to specifically stain trypanosomes in blood with peptide nucleic acid probes hybridising to ribosomal RNA or with RNA aptamers or antibodies binding to cell surface epitopes (Radwanska et al. 2002c; Lorger et al. 2003; Lejon et al. 2003a). However, these probes and antibodies have not yet been applied in combination with concentration techniques neither with the new generation of LED-powered fluorescence microscopes such as the Primo Star iLED (Carl Zeiss Microimaging, Gottingen, Germany) nor with the FLUOLED™ 2CSL reflected light illuminator (Fraen corporation, MA, USA). The fact that epifluorescence microscopy is now possible with relatively simple and portable microscopes is a major breakthrough in the microscopic diagnosis of infectious diseases and opens perspectives for the further development of parasitological diagnostics for African trypanosomiasis.

In human African trypanosomiasis, the trypanosomes are not only to be found in blood but also in inoculation chancres, lymph nodes or cerebrospinal fluid. Particularly in the early stage of *gambiense* sleeping sickness, patients may present with swollen cervical lymph nodes that can be punctured with a dry needle to aspirate some microlitres of lymph for examination as fresh preparation under the microscope. The technique is very interesting since it is simple and cheap, and, in a considerable fraction of patients, the parasites are only detectable in the lymph and not in the blood.

It also happens that patients with strong neurological and/or serological suspicion of late stage HAT cannot be parasitologically confirmed in blood or lymph. In such cases, lumbar puncture and examination of the cerebrospinal fluid may reveal the parasites that have entered the central nervous system (see below under Sect. 8.7). In some *rhodesiense* HAT patients, a chancre may develop at the inoculation site and trypanosomes may be detected in an aspirate of the chancre juice before they are detectable in the blood (Apted 1970). However, examination of chancre aspirate is seldom applied, since in most cases, patients are diagnosed when the chancre has already disappeared.

8.4 Antibody Detection

Because of the limited sensitivity of microscopic parasite detection techniques in African trypanosomiasis, much interest has gone in the development of indirect diagnostic tests. All trypanosome infections induce a rapid and strong humoral

immune response that can be exploited for diagnosis. For more than 60 years now, researchers have been developing antibody detection tests for African trypanosomiasis in all kinds of formats, including direct and indirect agglutination tests, complement fixation tests (CFT), enzyme linked immunosorbent assay (ELISA), immunofluorescence tests (IFAT), Western blot, immune trypanolysis, etc. Many of these tests have been developed for serum of plasma but some of them are also applicable on whole blood, on blood dried on filter paper, on cerebrospinal fluid and even on saliva (Balozet 1946; Binz 1972; Woo and Soltys 1972; Luckins and Mehlitz 1978; Rae and Luckins 1984; Rebeski et al. 2001; Van Meirvenne et al. 1995; Bailey et al. 1967; Lejon et al. 1997, 2006).

One important feature shared by all antibody detection tests is that they will remain positive after curative treatment by the mere fact that trypanosome-specific antibodies can remain detectable in the circulation for months or even years. On the other hand, antibodies against some particular antigens may become detectable only after a couple of weeks of infection. This means that the result in an antibody detection test should always be interpreted in the light of the history of an animal or a person, and particularly for sleeping sickness, suspicion of infection by a positive serological result should be confirmed by microscopic detection of the parasite. In animal African trypanosomiasis on the other hand, the combination of a serological positive result with a low PCV is often considered sufficient to confirm infection.

Another common feature is that the specificity of an antibody detection test is highly dependent on the purity and specificity of the antigen. Since most antigen preparations used in CFT, ELISA or Western blot consist of crude lysates of a given trypanosome strain cultured in vivo or in vitro, it is not surprising that in most instances cross reactions occur with antibodies due to infections with other trypanosome species and even with other parasites. A recent example is given by Desquesnes et al. (2007) who describe the cross-reactivity of *T. evansi* antigens with anti-*T. cruzi* antibodies and therefore show how crude antigens can compromise a diagnostic test. Also, the reader should be aware that most antibody detection tests described in the literature are using antigens prepared from poorly documented trypanosome strains of which the antigenic composition is not necessarily relevant for infections by other strains of the same trypanosome species. All too often, also the preparation of the antigen itself and the whole diagnostic protocol are not standardised. For *T. congolense* and *T. vivax* in bovine, the International Atomic Energy Agency (IAEA) in Vienna has invested successfully in the delivery of standardised and properly validated ELISA tests including precoated plates and control specimen (Rebeski et al. 1999, 2000). Unfortunately, the production of the test kits has proven not to be sustainable and the test is not available anymore.

Several ways exist to overcome problematic cross-reactions in antibody detection tests. One way is to develop an inhibition test based on a monospecific antibody that reacts very specifically with one particular epitope on a complex antigen (Bossard et al. 2010). Another way is to use highly purified native, recombinant or synthetic antigens. Recombinant and synthetic antigens also overcome problems with the standardisation of antigen production from in vitro- or in vivo-cultured

trypanosomes and with risk for infection of persons that manipulate human infective *T.b. gambiense* and *T.b. rhodesiense* strains (Herwaldt 2001).

The hitherto most successful antibody detection tests for African trypanosomiasis are based on some particular Variant Surface Glycoproteins (VSG) of *T.b. gambiense* and *T. evansi*. Studies on the phenomenon of antigenic variation in *Trypanozoon* parasites revealed that almost all infected individuals carry antibodies against some, so called, predominant Variable Antigen Types (VATs). The VAT is defined by very specific epitopes of the VSGs exposed at the surface of a living trypanosome and that are recognised by VAT-specific antibodies. Based on this principle, a direct card agglutination test for trypanosomiasis (CATT/*T.b. gambiense*) was developed for sleeping sickness (Magnus et al. 1978). Till today, this is the only serological test that is used at a large scale to actively screen inhabitants of endemic villages for *gambiense*-specific antibodies. A similar test has been developed for *T. evansi* and is now recommended by the World Animal Health Organisation (OIE) for testing for surra (Bajyana Songa and Hamers 1988; OIE 2012). CATT/*T.b. gambiense* uses VAT LiTat 1.3 as major antigen, while CATT/*T. evansi* uses VAT RoTat 1.2. Although very useful, both test will not be 100 % sensitive since in some endemic regions, e.g. in Nigeria, the LiTat 1.3 is not recognised by all sleeping sickness patients and in Kenya, a *T. evansi* circulates in camel that does not express the RoTat 1.2 VAT (Van Meirvenne et al. 1995; Ngaira et al. 2005). For *T.b. gambiense*, the combination of LiTat 1.3 with LiTat 1.5 allows picking up the patients not reacting with LiTat 1.3 alone, but this combination is not possible in a direct agglutination format. Fortunately, during the production of the CATT tests, other, non-VAT specific but still trypanosome-specific antigens become available for the agglutination reaction thus limiting the loss in sensitivity of the tests. On the other hand, CATT/*T.b. gambiense* and CATT/*T. evansi* are rapid diagnostic tests intended for screening of whole blood or diluted plasma thus inevitably inducing the risk of false positive results. Therefore, their performance is best in situations with relatively high disease prevalence (high negative predictive value). Other test formats using VSGs as antigens are indirect latex agglutination, ELISA, immune trypanolysis and recently also immunochromatography.

Immune trypanolysis is used as the reference test for antibody detection for *gambiense* sleeping sickness and for surra (*T. evansi*) based on their very high specificity and thus positive predictive value (Jamonneau et al. 2010; Holland et al. 2002; Verloo et al. 1998). The test format makes use of living cloned populations of trypanosomes, all expressing the same VAT. When incubated with a specimen that contains VAT-specific antibodies and with guinea pig serum as complement source, the trypanosomes will be destroyed by antibody-dependent complement-mediated lysis (ADCML) (Van Meirvenne et al. 1995). It goes without saying that the test can only be performed in reference laboratories with facilities to cryopreserve the cloned populations and to inoculate rodents with human infective trypanosomes. We therefore are developing an immune trypanolysis test based on *T.b. brucei* expressing constitutively the *T.b. gambiense*-specific VSGs LiTat 1.3 and LiTat 1.5 in vitro (Rogé et al. 2011b). The trypanolysis test can also be performed with blood dried on filter paper which allows to implement it for large-scale epidemiological

surveys, e.g. to monitor the effect of trypanosomiasis control activities. As stated above, VSGs have also been incorporated in ELISA for antibody detection in serum, plasma, filter paper eluates, cerebrospinal fluid and saliva (Lejon et al. 1998, 2003d, 2005, 2006; Holland et al. 2002). However, since ELISA is restricted to fully equipped laboratories, a more field applicable format was developed as a latex agglutination test with good diagnostic performance but that could not replace CATT since it is not applicable on whole blood but only on diluted blood or plasma (Büscher et al. 1999).

Very recently, the first lateral flow ICTs for *gambiense* sleeping sickness using native trypanosome antigens have been introduced (Büscher et al. 2013; Ebeja 2012) (HAT Sero-*K*-Set, Coris BioConcept, Belgium and SD-Bioline HAT, Standard Diagnostics, South Korea). Lateral flow tests have some major advantages over other serological tests formats that make them genuine Rapid Diagnostic Tests (RDT) applicable in field conditions without any laboratory facility. RDTs are supposed to fulfil the ASSURED criteria: Affordable (typically around 1 USD/test), Sensitive, Specific, User-friendly (minimal manipulations, easy readout), Rapid and Robust (readout within <30 min, stable at ambient temperature) and Equipment free and Deliverable to the end user (Peeling et al. 2006). Another advantage of lateral flow tests that is of particular importance in African trypanosomiasis, is their design that allows to detect antibodies without the need of host species-specific or Ig class-specific conjugates. Thus, as for direct agglutination and inhibition test formats, the same device can be use for testing bovine, camel, horse, human, etc.

Given the difficulties in preparing native antigens in a standardised way and in sufficient amounts to produce diagnostic tests, much effort has gone in identification of putative diagnostic antigens and their expression as recombinant antigens. Recombinant expression of the *T. evansi*-specific VSG RoTat 1.2 has been achieved in *Spodoptera frugiperdis* but large-scale production was not successful (Lejon et al. 2005). In contrast, expression of RoTat 1.2 VSG and the *T.b. gambiense*-specific VSGs LiTat 1.3 and LiTat 1.5 in *Pichia pastoris* has been proven efficient and the recombinant antigens have clear diagnostic potential (Rogé et al. 2011a, 2012a, b, c, 2013). This research has led to the development of the first lateral flow ICT for surra (Surra Sero-*K*-Set, Coris BioConcept, Belgium). *Pichia pastoris* has also been used as expression system for other trypanosomal proteins with diagnostic potential, e.g. congopain and cathepsin B (Mendoza-Palomares et al. 2008; Boulangé et al. 2011). More common is the recombinant expression of potentially diagnostic trypanosomal proteins in bacteria such as *Escherichia coli*. Some examples are VSG RoTat 1.2, Invariant Surface Glycoprotein 65 (ISG65) and ISG75, GM6, MARP1 and HSP70/BiP (Tran et al. 2009; Bossard et al. 2010; Sengupta et al. 2013; Thuy et al. 2012; Müller et al. 1992; Imboden et al. 1995). Most of these antigens have been tested in ELISA against sera from experimentally or naturally infected animals or human. With recombinant ISG65, a prototype ICT for sleeping sickness has been developed (Sullivan et al. 2013).

Going one step further, research has been conducted in order to replace native or recombinant antigens by mimotopes, i.e. short synthetic peptides that mimic linear

or conformational epitopes of antigens. Screening of random phage display libraries with anti-VSG LiTat 1.3 and anti-VSG LiTat 1.5 mouse and human IgGs resulted in the selection of phages expressing putative mimotopes among which some had clear diagnostic potential (Van Nieuwenhove et al. 2011, 2012, 2013).

Regarding *T. vivax*, *T. congolense* and *T. equiperdum*, all current antibody detection test still rely on crude native antigens, with one exception, the HSP70/BiP inhibition test (Bossard et al. 2010). Moreover, they all come in the format of ELISA, except for *T. equiperdum* where only the ancient CFT format, which cannot distinguish between *T. evansi* and *T. equiperdum* infection in horses, is recommended by OIE (OIE 2012). Fortunately and very recently, the non-for-profit organisation GALVmed has taken up the development of RDTs for *T. congolense* and *T. vivax* in their activity portfolio (http://www.galvmed.org/activities/animal-african-trypanosomosis/).

8.5 Antigen Detection

Attempts to develop antigen detection tests for African trypanosomiasis have been undertaken by many research groups but hitherto without success (Liu and Pearson 1987; Nantulya and Lindqvist 1989; Olaho-Mukani et al. 1993; Kashiwazaki et al. 1994). Although it is possible to detect particular antigens in controlled experimental infections, no single test performs acceptably well on specimens from naturally infected individuals to warrant implementation in clinical practice. Most probably, concentrations of circulating parasite antigen are just too low to be detectable with the current diagnostic test formats. Moreover, such antigens may be hidden in immune complexes. Still, targeting high copy number structural or secreted trypanosome antigens against which the host does not mount an antibody response and combining this strategy with novel biosensor technologies, may eventually lead to reliable antigen detection tests.

8.6 Molecular Diagnosis

A large variety of molecular diagnostic tests, based on detection of trypanosome DNA or RNA , have been developed for human and animal African trypanosomiasis [for a review on sleeping sickness, see Deborggraeve and Büscher (2012)]. Molecular diagnostics can be applied on all biological specimens that may contain the parasite's nucleic acids such as blood, CSF, lymph node and chancre aspirate, biopsy and histological material. Even from stained microscopic slides, DNA can be extracted for subsequent PCR (Deborggraeve et al. 2008).

Detection of RNA has the advantage that it is the best surrogate for detection of living parasites, while DNA of dead trypanosomes can remain in the circulation for a couple of days. On the other hand, RNA rapidly degrades thus making proper specimen processing more delicate. Till today, all RNA detection tests target ribosomal RNA that is also the target in the PNA hybridisation test for fluorescent

parasite detection mentioned above (Radwanska et al. 2002c). As an alternative to parasite detection, Shiraishi and co-workers described the amplification-free detection of 18S rRNA, making use of a pair of biotinylated PNA probes hybridising with two sequences in *Trypanozoon* 18S rRNA at a distance of ca. 60 nm from each other. The PNA probes are individually bound to (strept)avidin-coated fluorescent beads, differing in size. When the probes simultaneously hybridise with their corresponding sequence on rRNA, the PNA-coated beads are co-localised and as such are detectable by fluorescence microscopy (Shiraishi et al. 2011). Amplification of rRNA can be achieved by isothermal nucleic acid sequence-based amplification (NASBA) technology making use of simultaneous activity of T7 RNA polymerase and RNase (Mugasa et al. 2009). In African trypanosomiasis, NASBA has only been developed for detection of *Trypanozoon*-specific sequences where amplicon visualisation is performed with fluorescent molecular beacons for real-time recording or by gold-labelled probes for oligochromatography (Mugasa et al. 2008, 2009).

DNA detection tests are based on the amplification of a variety of coding and non-coding sequences of genomic or kinetoplast DNA, including highly and medium repetitive sequences as well as single copy sequences. Examples of single copy targeting PCRs are those that allow distinction of *T.b. gambiense* type I and *T.b. rhodesiense* from the other pathogenic trypanosomes based on the presence of respectively *tgsgp* and *sra* genes (Radwanska et al. 2002a, b; Njiru et al. 2004, 2011; Welburn et al. 2001; Picozzi et al. 2008). Other targeted genes are *esag6* and *esag7*, 18*s*, *pfra*, *RoTat 1.2 vsg*, and *cathepsin L–like genes* (Kabiri et al. 1999; Holland et al. 2001; Deborggraeve et al. 2006; Kuboki et al. 2003; Njiru 2011; Claes et al. 2004; Sengupta et al. 2010; Bromidge et al. 1993; Cortez et al. 2009). Non-coding sequences for which PCRs were developed are: satellite DNA, internal transcribed sequences (ITS1 and ITS2) and repetitive insertion mobile elements (RIME) (Moser et al. 1989; Desquesnes et al. 2001; Njiru et al. 2008b; Thekisoe et al. 2007). The interest of these non-coding sequences lies in their usually high copy number thus conferring high analytical sensitivity but with the consequence that they are more prone to contamination during specimen collection or processing. Of special interest in animal trypanosomiasis are PCRs that allow discrimination of the trypanosome species in one single run and even in the same specimen in case of mixed infection. One example is the 18S-PCR-RFLP where cleaving of the amplicon with two restriction enzymes generates fragment profiles that are characteristic for *T. congolense*, *T. vivax*, *Trypanozoon* and *T. theileri* (Geysen et al. 2003). Similar differential diagnosis can be obtained with the less complex ITS1 PCR that generates amplicons of taxon-specific lengths (Desquesnes et al. 2001; Fikru et al. 2012; Njiru et al. 2005). Species-specific PCRs may suffer from unexpected genomic variation within the target taxon. A notorious example is the RoTat 1.2 VSG PCR that has been proven to detect specifically *T. evansi* type A occurring in Africa, Latin America and South East Asia but that will not detect the rare *T. evansi* type B strains that circulate in camels in Kenya (Claes et al. 2004; Ngaira et al. 2005). Also for *T. vivax*, unpublished evidence exists that the current PCRs may not be able to detect all circulating strains, particularly those in East

Africa. Despite all the efforts to develop taxon-specific PCRs, important gaps remain. At present, no *T. equiperdum*-specific primers exist that can discriminate them unequivocally from *T. brucei* and the same applies for *T.b. gambiense* type II.

To amplify DNA, PCR is the most commonly used technique, although sometimes real-time PCR is applied (Becker et al. 2004; Mumba Ngoyi et al. 2011; Sharma et al. 2012). In view of bringing molecular diagnostics closer to the patient, visualisation of PCR amplicons by hybridisation with specific probes on a nitrocellulose membrane (oligochromatography) has been achieved, yielding a highly sensitive and specific diagnostic test for sleeping sickness (Deborggraeve et al. 2006; Matovu et al. 2010b). Still the technique relies on high-quality purified DNA and on sophisticated equipment like a thermocycler that can hardly be implemented in rural health centres where even electricity supply is not fully secured. Loop-mediated isothermal amplification (LAMP) of DNA is a less demanding technology that may prove a real breakthrough in molecular diagnostics of African trypanosomiasis (Njiru 2012). In LAMP, DNA is amplified without prior heat denaturation by a *Bst* DNA polymerase and the amplicons are visualised by turbidimetry, colorimetric reactions or fluorescence (Mori et al. 2001; Notomi et al. 2000; Tomita et al. 2008; Wastling et al. 2010). Over the last 10 years, multiple LAMP tests for diagnosis of animal and human African trypanosomiasis have been developed targeting more or less the same coding and non-coding DNA sequences as in conventional PCR (Njiru et al. 2008a, b; Njiru 2011, 2012; Matovu et al. 2010a; Thekisoe et al. 2007; Kuboki et al. 2003). Of particular interest for the diagnosis of sleeping sickness is the development of the Loopamp™ *Trypanosoma brucei* Detection Kit and the LF-160 incubator (Eiken Chemical, Tochigi, Japan). The kit consists of ready-to-use reaction tubes containing lyophilised reagents and control specimens (http://www.finddiagnostics.org). The test is run in the LF-160 incubator that contains two heating blocks, one for the DNA extraction and one for the LAMP reaction. Attached to this incubator is an UV illuminator to visualise the fluorescent reaction products. With RIME as target, the Loopamp™ *Trypanosoma brucei* Detection Kit reaches high analytical sensitivities and diagnostic performance that are comparable to conventional 18S PCR (unpublished results). Importantly, the LAMP reaction is much less sensitive to inhibitory factors than PCR, making specimen preparation much easier (Namangala et al. 2012).

Techniques for specimen collection, storage and subsequent nucleic acid extraction critically influence the eventual results obtained in molecular diagnostics. Since RNA is more prone to degradation by nucleases and hydrolysis, collection and long-term storage under field conditions is not evident. In a recent study, Basiye et al. (2011) compared different storage media and temperatures on their performance to protect DNA and RNA from degradation during 10 weeks prior to extraction. Their findings suggest that the guanidinium-based L3™ buffer is suitable for preservation of parasite nucleic acid material at −20 °C or 4 °C and is compatible with field work. Some studies report on the use of filter paper for easy collection and preservation of biological specimens at ambient temperature (Vitouley et al. 2011). Several methods are in use to extract trypanosome DNA from such filters (Ahmed et al. 2011; Carducci et al. 1992; Pereira de Almeida et al.

1998). In our hands, it is not necessary to use special filters such as the FTA or 903 Protein Saver cards (Whatman, Maidstone, UK). Cheap plain cellulose filters such as the Whatman N°4 are fine and have the advantage that they are also perfect for storage of specimens for serological testing. However, given the usually low parasitaemia in African trypanosomiasis, we recommend collecting larger volumes of specimens, between 0.2 and 0.8 ml, in stabilisation buffers such as the Qiagen AS-1 storage buffer (Westburg, Leusden, the Netherlands) or home-made guanidinium EDTA buffers (Deborggraeve et al. 2011a; Duffy et al. 2013). These buffers allow to store the specimens for months at ambient temperature and are compatible with most of the commercial DNA extraction kits. For diagnostic purposes, we further recommend to run a mammalian cytochrome B-specific PCR along with the trypanosome-specific PCRs to ensure that a negative result is not due to the presence of inhibitors or to the degradation of DNA during storage or extraction (Kocher et al. 1989). In some particular situations, it is useful to first apply a broad-spectrum *Trypanosomatidae*-specific 18S PCR that, when positive, is followed by taxon-specific PCRs. In this way, atypical human infections, e.g. with the stercorarian rat pathogen *T. lewisi*, can be diagnosed (Verma et al. 2011; Desquesnes et al. 2011).

In principle, molecular diagnostics have a high analytical sensitivity and, depending on fine tuning of reaction conditions, primers and/or probes, can be very specific. Interpretation of the analytical sensitivity reported in the literature is however not straightforward. In a recent review of molecular diagnostics for sleeping sickness, we calculated the lower detection limit for more than 20 tests, taking into account the volume of template in the reaction mixture. We found reported lower detection limits varying between 0.001 and 10,000 parasites per reaction (Deborggraeve and Büscher 2010, 2012). The sometimes extreme analytical sensitivity of molecular tests looks promising but can only be reached with pure DNA or RNA or when a larger specimen volume is processed for nucleic acid extraction prior to the amplification reaction. To reflect real-life situations, analytical sensitivity is best assessed on serial dilutions of living trypanosomes in blood from which a volume is processed that corresponds with common diagnostic practice. Thus, we have unpublished data showing that the lower detection limit of the Loopamp™ *Trypanosoma brucei* Detection Kit for *T.b. gambiense* is only 100 trypanosomes/ml of blood and that the lower detection limit of ITS1 PCR is 10 trypanosomes/ml for *T. congolense* and *T. brucei* but only 100 trypanosomes/ml for *T. vivax*. In addition, diagnostic accuracy in terms of diagnostic sensitivity and specificity are not only defined by the analytical sensitivity and specificity of a test. Based on an extensive literature review, Mugasa et al. (2012) presented an overview of the diagnostic accuracies of 12 molecular tests that have been evaluated in clinical studies on human African trypanosomiasis patients and endemic controls. Sensitivities ranged from 82 % to 100 %, while specificities ranged from 59 % to 100 %. With these characteristics, the molecular tests do not perform much better than the best parasitological tests for sleeping sickness. While the latter are robust, relatively cheap and applicable in field conditions, molecular diagnostics in their current format are expensive and not adapted for field work and therefore their

direct benefit for the patient is questionable (Deborggraeve and Büscher 2010). For animal trypanosomiases, many studies provide evidence that molecular tests are much more sensitive than the conventional haematocrit centrifugation technique. However, except in special conditions such as diagnosis of import cases in non-endemic countries, they are only applied for research purposes such as epidemiological studies, monitoring drug resistance and control interventions, animal reservoir of human infective trypanosomes, etc.

8.7 Stage Determination and Follow-Up in Sleeping Sickness

In sleeping sickness, primary diagnosis has to be followed by stage determination to assess whether the trypanosomes have already reached the brain (late or second or neurological stage of the disease). In the brain, the parasites are protected against the trypanocidal action of drugs that are used to treat the early or first disease stage, since these drugs do not pass the blood–brain barrier in sufficient amounts to kill the trypanosomes. Stage determination is performed by examining the cerebrospinal fluid (CSF), obtained via lumbar puncture, for parameters reflecting an ongoing infection in the brain [for a review see Lejon and Büscher (2005)]. Since none of the drugs are fully effective, patients can relapse after treatment. Therefore, it is mandatory to follow up the patients for up to 24 months to assess the treatment outcome. In the vast majority of relapsing patients, the parasite cannot be detected anymore in blood or lymph node aspirate but only in CSF. Therefore, follow-up consists of lumbar punctures and CSF examination at 6, 12, 18 and 24 months after treatment (World Health Organization 1986). For accurate staging and follow-up, it is important to collect a larger volume of CSF (4–8 ml) which does not harm the patient more than taking 1 ml (Kjeldsberg and Knight 1993). Further, the CSF should be examined as soon as possible since trypanosomes do not survive for a long time in this fluid (Pentreath et al. 1992). According to the WHO, a patient is in second stage when the white blood cell count >5 cells/μl or when the presence of the trypanosome is confirmed or if the protein concentration is abnormally high (World Health Organization 1986). Strict adherence to these cut-off values is rather contested. Particularly the protein quantification has been proven unreliable under the conditions prevailing in rural laboratories where sleeping sickness is endemic and also has no added value compared to parasite detection and cell counting (Bisser et al. 2002; Lejon et al. 2003e). The cut-off cell count of 5 cells/μl is near the detection limit of most cell counting cells which makes the accuracy of the counting problematic. Furthermore, all too often, the glass cell-counting chambers like the Fuchs Rosenthal or the Neubauer chambers used in rural health centres in Africa are broken or worn and devoid of the original cover slips thus resulting in erroneous counts. Therefore, we strongly recommend to use the single-use plastic cell counting chambers like the Kova Glasstic (Hycor) or the Uriglass (Menarini) (Mumba Ngoyi et al. 2013). It should be noted that in some endemic countries like Angola and Côte d'Ivoire, the cut-off for normal cell count has been set at 20 cell/μl. However a study in Uganda has shown that almost half of the patients with cells

between 5 and 20/μl relapsed after treatment with pentamidine, the drug of choice for first stage *gambiense* HAT (Lejon et al. 2003c). Parasites can be detected in the CSF during the cell counting only when they are present in high numbers. Better is to use concentration techniques such as the Modified Single Centrifugation as described by Miézan et al. and recently improved by the use of the same collector tube as for mAECT (Miézan et al. 2000; Mumba Ngoyi et al. 2013). For examination of CSF during the follow-up after treatment, only cell count and parasite detection are performed but literature learns that the criteria to define relapse or cure are not standardised (Mumba Ngoyi et al. 2009). Only very recently, one prospective and one retrospective study proved that by using objective criteria for cell count (the so-called 5-50-20 algorithm) about 90 % of *gambiense* second-stage patients can be classified as cured already after 12 months thus drastically reducing the number of follow-up lumbar punctures (Mumba Ngoyi et al. 2010; Priotto et al. 2012).

Alternative CSF markers for the neurological stage of sleeping sickness have been proposed. Most of them are markers related to inflammation and neurodegeneration like total IgM, neurofilament, glial fibrillary acidic protein, anti-galactocerebroside antibodies and cytokines (Bisser et al. 2000; Lejon et al. 1999, 2002; MacLean et al. 1999). For semi-quantification of IgM in CSF, a latex agglutination test has been developed but eventually showed no added value to the existing parameters (Lejon and Büscher 2002). The same applies for another marker that was found up-regulated in second stage sleeping sickness, CXCL 10 (Hainard et al. 2009; Amin et al. 2009). The recent discovery that neopterin in CSF can replace cell count for stage determination as well as for follow-up deserves attention since the development of an RDT for neopterin detection in CSF seems feasible (Tiberti et al. 2013a, b).

As surrogate for parasite detection in CSF, molecular diagnostics such as PCR, LAMP and NASBA have been used in some rare occasions (Jamonneau et al. 2007; Truc et al. 1999, 2012; Kirchhoff 1998; Kyambadde et al. 2000; Mugasa et al. 2009). Only one large-scale prospective study on 360 *gambiense* HAT patients has been conducted to investigate the role of an 18S PCR in stage determination and follow-up. The 18S PCR showed 88 % sensitivity and 83 % specificity for stage determination but most surprisingly trypanosome DNA was still detectable 2 years after treatment in about 20 % of patients that were considered cured according to their clinical presentation and normal CSF values (Deborggraeve et al. 2011b). A similar phenomenon was already observed in a patient and in experimentally infected rats (Pereira de Almeida 1999; Kirchhoff 1998). These observations learn that PCR has no value as test of cure. At the same time it challenges the dogma that clinical cure after treatment equals parasitological cure. With this respect, the finding that some *T.b. gambiense* strains induce silent infections in the brains of mice that survived without clinical signs and without detectable parasites for more than 1 year is highly relevant (Giroud et al. 2009).

Investigations in non-invasive diagnostics for stage determination and follow-up by measuring the Sudden Onset Rapid Eye Movement (SOREM) sleep episodes with polysomnography or by recording the patient's movements with actigraphy have not yet resulted in reliable and field applicable diagnostic techniques (Buguet et al. 2005; Mpandzou et al. 2011; Njamnshi et al. 2012).

8.8 Going Forward

For diagnosis of human and animal African trypanosomiases, clinical signs and symptoms may be suggestive but are not sufficient for confirmation of the infection that still has to be obtained via microscopic parasite detection techniques. Because of the often low number of circulating parasites, parasite concentration techniques such as the HCT and the mAECT for blood and the MSC for cerebrospinal fluid are recommended. Although molecular diagnostic techniques have evolved tremendously over the last decades and bear unprecedented potential for sensitive and specific diagnosis, none can yet replace microscopy as point-of-care test in most situations. Therefore, the application of molecular diagnostics and particularly those that can simultaneously detect different trypanosome species is mainly indicated for research purposes, e.g. epidemiological and clinical investigations. Serological antibody detection has been proven reliable for screening in *gambiense* sleeping sickness and in surra (*T. evansi*). The development of a field applicable test in the format of a card agglutination test (CATT/*T.b.gambiense*) has largely contributed to the control of *gambiense* sleeping sickness through active case detection. Today, the test has become victim of its own success since, with sharply decreasing prevalences, the limited specificity of the CATT/*T.b.gambiense* becomes problematic. For passive case detection in low endemic foci or for surveillance in "controlled" foci, tests with higher specificity are needed. The recently developed immunochromatography tests may fulfil this requirement of high specificity and in addition are designed as RDTs. Yet, their diagnostic performance remains to be evaluated at a larger scale. With respect to stage determination and follow-up after treatment in sleeping sickness, lumbar puncture and examination of CSF on white blood cell count and on presence of the parasite remain compulsory.

On the level of parasite detection, no major improvements are to be expected in the near future unless someone takes up the challenge to develop imaging software for digital recognition of trypanosomes in a larger volume of blood through the microscope. Technically, this should be feasible but probably is too sophisticated for application in African trypanosomiasis. Whether LED-powered epifluorescence will eventually finds its way to the field will depend on the compatibility of specific fluorescent markers with parasite concentration techniques.

The new serological rapid diagnostic tests (RDTs), on the other hand, will certainly contribute to WHO's aim of eliminating *gambiense* sleeping sickness by the year 2020. The native antigens in these RDTs will soon be replaced by recombinant and/or synthetic peptides as is already the case in the Surra Sero-K-SeT for *T. evansi*. Thanks to the GALVmed initiative, also RDTs for *T. vivax* and *T. congolense* will become available within a couple of years. Only *T.b. brucei* and *T. equiperdum* remain largely under investigated and deserve more attention. Following the development and proper validation of these RDTs, the challenge will be to define their rational use at point of care, in combination with high throughput tests, like ELISA, deployed in reference laboratories.

Among the existing molecular test formats, only the LAMP has reasonable perspectives to find its way to district hospitals or veterinary centres with limited technical resources. Yet, the current price and low added value compared to microscopy are prohibitive for large-scale use of LAMP in routine practice. The same constraint may be expected for new generation lab-on-chips tests for which the market value of African trypanosomiasis is probably too low. Notwithstanding the tremendous output of research on African trypanosomes, for some trypanosome taxa, specific molecular markers are lacking (*T. equiperdum*, *T.b. gambiense* type II). For others (*T.b. gambiense* and *T.b. rhodesiense*) diagnosis as well as animal reservoir studies would greatly benefit from the discovery of subspecies specific multi-copy sequences.

With respect to stage determination and follow-up after treatment of sleeping sickness, recent investigations have not yet delivered new diagnostic tests but have greatly improved the application of some existing tests, in casu white blood cell count and parasite detection in CSF, particularly for the assessment of treatment outcome. It is however not unrealistic to hope that the newly identified biomarkers in CSF will soon be translated into field applicable RDTs. Furthermore, with the rapidly evolving proteomic analytical technologies, one may expect that sooner or later some second stage-specific biomarkers may be detectable in the blood so that lumbar punctures are not needed anymore. On the other hand, blood biomarker discovery may be overtaken by the venue of new, safe drugs that are effective in both disease stages thus avoiding the need for lumbar punctures.

8.9 Reference Laboratories

Expert advice and technical assistance in African trypanosomiasis diagnosis can be obtained from several reference laboratories endorsed by the World Health Organization, the World Animal Health Organization and the Food and Agriculture Organization.

Human African Trypanosomiasis

WHO Collaborating Center for Research and Training on human African trypanosomiasis diagnostics (BEL-43)
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References

Ahmed HA, Macleod ET, Hide G et al (2011) The best practice for preparation of samples from FTA cards for diagnosis of blood borne infections using African trypanosomes as a model system. Parasit Vectors 4:e68

Amin DN, Rottenberg ME, Thomson AR et al (2009) Expression and role of CXCL10 during the encephalitic stage of experimental and clinical African trypanosomiasis. J Infect Dis 200:1556–1565

Apted FIC (1970) Clinical manifestations and diagnosis of sleeping sickness. In: Mulligan HW (ed) The African trypanosomiases. George Allen and Unwin, London, pp 661–683

Assoku RKG, Gardiner PR (1989) Detection of antibodies to platelets and erythrocytes during infection with haemorrhage-causing *Trypanosoma vivax* in Ayrshire cattle. Vet Parasitol 31:199–216

Bailey JW, Smith DH (1992) The use of the acridine orange QBC technique in the diagnosis of African trypanosomiasis. Trans R Soc Trop Med Hyg 86:630

Bailey NM, Cunningham MP, Kimber CD (1967) The indirect fluorescent antibody technique applied to dried blood, for use as a screening test in the diagnosis of human trypanosomiasis in Africa. Trans R Soc Trop Med Hyg 61:696–700

Bajyana Songa E, Hamers R (1988) A card agglutination test (CATT) for veterinary use based on an early VAT RoTat 1/2 of *Trypanosoma evansi*. Ann Soc Belg Med Trop 68:233–240

Balozet L (1946) La réaction de déviation du complément après le traitement de la dourine par la méthode de Ciuca. Bull Acad Vét 19:240–241

Basiye FL, Schoone GJ, Beld M et al (2011) Comparison of short-term and long-term protocols for stabilization and preservation of RNA and DNA of *Leishmania*, *Trypanosoma*, and *Plasmodium*. Diagn Microbiol Infect Dis 69:66–73

Becker S, Franco JR, Simarro PP et al (2004) Real-time PCR for detection of *Trypanosoma brucei* in human blood samples. Diagn Microbiol Infect Dis 50:193–199

Bieler S, Matovu E, Mitashi P et al (2012) Improved detection of *Trypanosoma brucei* by lysis of red blood cells, concentration and LED fluorescence microscopy. Acta Trop 121:135–140

Binz G (1972) An evaluation of the capillary and latex aggluntination and heterophile antibody tests for the detection of *Trypanosoma rhodesiense* infections. Bull World Health Organ 47:773–778

Bisser S, Ayed Z, Bouteille B et al (2000) Central nervous system involvement in African trypanosomiasis: presence of anti-galactocerebroside antibodies in patients' cerebrospinal fluid. Trans R Soc Trop Med Hyg 94:225–226

Bisser S, Lejon V, Preux PM et al (2002) Blood-cerebrospinal fluid barrier and intrathecal immunoglobulins compared to field diagnosis of central nervous system involvement in sleeping sickness. J Neurol Sci 193:127–135

Bossard G, Boulange A, Holzmuller P et al (2010) Serodiagnosis of bovine trypanosomosis based on HSP70/BiP inhibition ELISA. Vet Parasitol 173:39–47

Boulangé AF, Khamadi SA, Pillay D et al (2011) Production of congopaiin, the major cysteine protease of *Trypanosoma* (*Nannomonas*) *congolense*, in *Pichia pastoris* reveals unexpected dimerisation at physiological pH. Protein Express Purif 75:95–103

Brady JF (1995) Interpretation of immunoassay data. Immunoanalysis of agrochemicals. Ciba Crop Protection, Greensboro, pp 266–287

Bromidge T, Gibson W, Hudson K et al (1993) Identification of *Trypanosoma brucei gambiense* by PCR amplification of variant surface glycoprotein genes. Acta Trop 53:107–119

Buguet A, Bisser S, Josenando T et al (2005) Sleep structure: a new diagnostic tool for stage determination in sleeping sickness. Acta Trop 93:107–117

Bukachi SA, Wandibba S, Nyamongo IK (2009) The treatment pathways followed by cases of human African trypanosomiasis in western Kenya and eastern Uganda. Ann Trop Med Parasitol 103:211–220

Büscher P (2001) Diagnosis of human and animal African trypanosomiasis. In: Black SJ, Seed JR (eds) The African trypanosomes. Kluwer, Boston, pp 51–63

Büscher P, Lejon V (2004) Diagnosis of human African trypanosomiasis. In: Maudlin I, Holmes P, Miles MA (eds) The trypanosomiases. CABI, Oxfordshire, pp 203–218

Büscher P, Lejon V, Magnus E et al (1999) Improved latex agglutination test for detection of antibodies in serum and cerebrospinal fluid of *Trypanosoma brucei gambiense* infected patients. Acta Trop 73:11–20

Büscher P, Mumba Ngoyi D, Kaboré J et al (2009) Improved models of mini anion exchange centrifugation technique (mAECT) and modified single centrifugation (MSC) for sleeping sickness diagnosis and staging. PLoS Negl Trop Dis 3:e471

Büscher P, Mumba Ngoyi D, Balharbi F, Kande Betu V, Van der Veken W, Sese C, Lejon V (2011) Improved parasitological and molecular techniques for the diagnosis and surveillance of sleeping sicknes. In: Abstracts of the 31st meeting of the international scientific council for trypanosomiasis research and control, Bamako, Mali, 12–16 Sept 2011

Büscher P, Gilleman Q, Lejon V (2013) Novel rapid diagnostic tests for sleeping sickness. N Engl J Med 368:1069–1070

Calistri P, Narcisi V, Atzeni M et al (2013) Dourine reemergence in Italy. J Eq Vet Sci 33:83–89

Camara M, Camara O, Ilboudo H et al (2010) Sleeping sickness diagnosis: use of buffy coats improves the sensitivity of the mini anion exchange centrifugation test. Trop Med Int Health 15:796–799

Carducci C, Ellul L, Antonozzi I et al (1992) DNA elution and amplification by polymerase chain reaction from dried blood spots. Biotechniques 13:735–737

Chatel G, Gulletta M, Matteelli A et al (1999) Diagnosis of tick-borne relapsing fever by the quantitative buffy coat fluorescence method. Am J Trop Med Hyg 60:738–739

Claes F, Radwanska M, Urakawa T et al (2004) Variable surface glycoprotein RoTat 1.2 PCR as a specific diagnostic tool for the detection of *Trypanosoma evansi* infections. Kinetoplastid Biol Dis 3:1–6

Cortez AP, Rodrigues AC, Garcia HA et al (2009) Cathepsin L-like genes of Trypanosoma vivax from Africa and South America–characterization, relationships and diagnostic implications. Mol Cell Probes 23:44–51

Pereira de Almeida PJL (1999) Contributions to the diagnostic evaluation of the Polymerase Chain Reaction for the detection of Salivarian trypanosomes. PhD Thesis, Université Libre de Bruxelles, Brussels, Belgium

de Clare Bronsvoort BM, von Wissmann B, Fèvre EM et al (2010) No gold standard estimation of the sensitivity and specificity of two molecular diagnostic protocols for *Trypanosoma brucei spp*. in Western Kenya. PLoS One 5:e8628

Deborggraeve S, Büscher P (2010) Molecular diagnostics for sleeping sickness: where's the benefit for the patient? Lancet Infect Dis 10:433–439

Deborggraeve S, Büscher P (2012) Recent progress in molecular diagnosis of sleeping sickness. Expert Rev Mol Diagn 12:719–730

Deborggraeve S, Claes F, Laurent T et al (2006) Molecular dipstick test for diagnosis of sleeping sickness. J Clin Microbiol 44:2884–2889

Deborggraeve S, Koffi M, Jamonneau V et al (2008) Molecular analysis of archived blood slides reveals an atypical human *Trypanosoma* infection. Diagn Microbiol Infect Dis 61:428–433

Deborggraeve S, Lejon V, Ali Ekangu R et al (2011a) Diagnostic accuracy of PCR in *gambiense* sleeping sickness diagnosis, staging and post-treatment follow-up: a 2-year longitudinal study. PLoS Negl Trop Dis 5:e972

Deborggraeve S, Lejon V, Ali Ekangu R et al (2011b) How reliable is PCR for diagnosis, staging and follow-up of *gambiense* sleeping sickness? Trop Med Int Health 16(S1):168

Desquesnes M, McLaughlin G, Zoungrana A et al (2001) Detection and identification of *Trypanosoma* of African livestock through a single PCR based on internal transcribed spacer 1 of rDNA. Int J Parasitol 31:610–614

Desquesnes M, Bosseno M-F, Brenière SF (2007) Detection of Chagas infections using Trypanosoma evansi crude antigen demonstrates high cross-reactions with Trypanosoma cruzi. Infect Genet Evol 7:457–462

Desquesnes M, Kamyingkird K, Yangtara S et al (2011) Specific primers for PCR amplification of the ITS1 (ribosomal DNA) of *Trypanosoma lewisi*. Infect Genet Evol 11:1361–1367

Duffy T, Cura CI, Ramirez JC et al (2013) Analytical performance of a multiplex Real-Time PCR assay using TaqMan probes for quantification of Trypanosoma cruzi satellite DNA in blood samples. PLoS Negl Trop Dis 7:e2000

Ebeja AK (2012) Journée Scientifique THA à Kinshasa. Bull HAT Platform 12:9–10

Enøe C, Georgiadis MP, Johnson WO (2000) Estimation of sensitivity and specificity of diagnostic tests and disease prevalence when the true disease state is unknown. Prev Vet Med 45:61–81

Fikru R, Goddeeris BM, Delespaux V et al (2012) Widespread occurrence of *Trypanosoma vivax* in bovines of tsetse- as well as non-tsetse-infested regions of Ethiopia: a reason for concern? Vet Parasitol 190:355–361

Geysen D, Delespaux V, Geerts S (2003) PCR-RFLP using Ssu-rDNA amplification as an easy method for species-specific diagnosis of *Trypanosoma* species in cattle. Vet Parasitol 110:171–180

Giroud C, Ottones F, Coustou V et al (2009) Murine models for *Trypanosoma brucei gambiense* disease progression-from silent to chronic infections and early brain tropism. PLoS Negl Trop Dis 3:e509

Gutierrez C, Corbera JA, Doreste F et al (2004) Use of the miniature anion exchange centrifugation technique to isolate *Trypanosoma evansi* from goats. Ann N Y Acad Sci 1026:149–151

Hainard A, Tiberti N, Robin X et al (2009) A combined CXCL10, CXCL8 and H-FABP panel for the staging of human African trypanosomiasis patients. PLoS Negl Trop Dis 3:e459

Hasker E, Lumbala C, Mbo F et al (2011) Health care-seeking behaviour and diagnostic delays for Human African Trypanosomiasis in the Democratic Republic of the Congo. Trop Med Int Health 16:869–874

Herwaldt BL (2001) Laboratory-acquired parasitic infections from accidental exposures. Clin Microbiol Rev 14:659–688

Holland WG, Claes F, My LN et al (2001) A comparative evaluation of parasitological tests and a PCR for *Trypanosoma evansi* diagnosis in experimentally infected water buffaloes. Vet Parasitol 97:23–33

Holland WG, Thanh NG, My LN et al (2002) Evaluation of whole fresh blood and dried blood on filter paper discs in serological tests for *Trypanosoma evansi* in experimentally infected water buffaloes. Acta Trop 81:159–165

Imboden M, Müller N, Hemphill A et al (1995) Repetitive proteins from the flagellar cytoskeleton of African trypanosomes are diagnostically useful antigens. Parasitology 110:249–258

Jamonneau V, Solano P, Garcia A et al (2007) Application of PCR/CSF for stage determination and therapeutic decision in human African trypanosomiasis in Cöte d'Ivoire. In: Crowther JR (ed) Developing methodologies for the use of polymerase chain reaction in the diagnosis and monitoring of trypanosomosis. International Atomic Energy Agency, Vienna, pp 27–35

Jamonneau V, Bucheton B, Kabore J et al (2010) Revisiting the immune trypanolysis test to optimise epidemiological surveillance and control of sleeping sickness in west Africa. PLoS Negl Trop Dis 4:e917

Joseph L, Gyorkos TW, Coupal L (1995) Bayesian estimation of disease prevalence and the parameters of diagnostic tests in the absence of a gold standard. Am J Epidemiol 141:263–272

Kabiri M, Franco JR, Simarro PP et al (1999) Detection of *Trypanosoma brucei gambiense* in sleeping sickness suspects by PCR amplification of expression-site-associated genes 6 and 7. Trop Med Int Health 4:658–661

Kashiwazaki Y, Snowden K, Smith DH et al (1994) A multiple antigen detection dipstick colloidal dye immunoassay for the field diagnosis of trypanosome infections in cattle. Vet Parasitol 55:57–69

Kirchhoff LV (1998) Use of a *PCR* assay for diagnosing African trypanosomiasis of the CNS: a case report. Cent Afr J Med 44:134–136

Kjeldsberg CR, Knight JA (1993) Cerebrospinal fluid. In: Johnson KD (ed) Body fluids. American Society of Clinical Pathologists (ASCP), Chicago, IL, pp 65–157

Kocher TD, Thomas WK, Meyer A et al (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc Natl Acad Sci USA 86:6196–6200

Kuboki N, Inoue N, Sakurai T et al (2003) Loop-mediated isothermal amplification for detection of African trypanosomes. J Clin Microbiol 41:5517–5524

Kuhn W, Armstrong D, Atteberry S et al (2010) Usefulness of the paralens fluorescent microscope adaptor for the identification of mycobacteria in both field and laboratory settings. Open Microbiol J 4:30–33

Kyambadde JW, Enyaru JCK, Matovu E et al (2000) Detection of trypanosomes in suspected sleeping sickness patients in Uganda using the polymerase chain reaction. Bull World Health Organ 78:119–124

Lanham SM, Godfrey DG (1970) Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. Exp Parasitol 28:521–534

Lejon V, Büscher P (2002) Sleeping sickness: from intrathecal IgM synthesis to card agglutination. Acta Trop 83:S70

Lejon V, Büscher P (2005) Cerebrospinal fluid in human African trypanosomiasis: a key to diagnosis, therapeutic decision and post-treatment follow-up. Trop Med Int Health 10:395–403

Lejon V, Moons A, Büscher P, et al (1997) Trypanosome specific antibody profile in serum and cerebrospinal fluid of *T.b. gambiense* patients. In: Ndung'u JM (ed) Twenty-third meeting of the international scientific council for trypanosomiasis research and control (ISCTRC), Banjul, The Gambia, 11–15 Sept 1995. OAU/STRC, Nairobi, pp 78–91

Lejon V, Büscher P, Magnus E et al (1998) A semi-quantitative ELISA for detection of *Trypanosoma brucei gambiense* specific antibodies in serum and cerebrospinal fluid of sleeping sickness patients. Acta Trop 69:151–164

Lejon V, Rosengren LE, Büscher P et al (1999) Detection of light subunit neurofilament and glial fibrillary acidic protein in cerebrospinal fluid of *Trypanosoma brucei gambiense* -infected patients. Am J Trop Med Hyg 60:94–98

Lejon V, Lardon J, Kenis G et al (2002) Interleukin-6, IL-8 and IL-10 in serum and CSF of *T.b. gambiense* sleeping sickness patients before and after treatment. Trans R Soc Trop Med Hyg 96:329–333

Lejon V, Ameel V, Brandt J, Van den Bossche P, Büscher P (2003a) Development of a direct immunofluorescence test (DIFAT) for parasitological diagnosis of African trypanosomiasis. In: Abstracts of the 27th meeting of the international scientific council for trypanosomiasis research and control (ISCTRC), Nairobi, Kenya, Sept 2003

Lejon V, Boelaert M, Jannin J et al (2003b) The challenge of *Trypanosoma brucei gambiense* sleeping sickness diagnosis outside Africa. Lancet Infect Dis 3:804–808

Lejon V, Legros D, Savignoni A et al (2003c) Neuro-inflammatory risk factors for treatment failure in "early second stage" sleeping sickness patients treated with Pentamidine. J Neuroimmunol 144:132–138

Lejon V, Rebeski DE, Ndao M et al (2003d) Performance of enzyme-linked immunosorbent assays for detection of antibodies against *T. congolense* and *T. vivax* in goats. Vet Parasitol 116:87–95

Lejon V, Reiber H, Legros D et al (2003e) Intrathecal immune response pattern for improved diagnosis of central nervous system involvement in trypanosomiasis. J Infect Dis 187:1475–1483

Lejon V, Claes F, Verloo D et al (2005) Recombinant RoTat 1.2 variable surface glycoprotein for diagnosis of *Trypanosoma evansi* in dromedary camel. Int J Parasitol 35:455–460

Lejon V, Jamonneau V, Solano P et al (2006) Detection of trypanosome-specific antibodies in saliva, towards non-invasive serological diagnosis of sleeping sickness. Trop Med Int Health 11:620–627

Levine RA, Wardlaw SC, Patton CL (1989) Detection of haemoparasites using quantitative buffy coat analysis tubes. Parasitol Today 5:132–134

Liu MK, Pearson TW (1987) Detection of circulating trypanosomal antigens by double antibody ELISA using antibodies to procyclic trypanosomes. Parasitology 95:277–290

Lorger M, Engstler M, Homann M et al (2003) Targeting the variable surface of African trypanosomes with variant surface glycoprotein-specific, serum-stable RNA aptamers. Eukaryot Cell 2:84–94

Luckins AG, Mehlitz D (1978) Evaluation of an indirect fluorescent antibody test, enzyme-linked immunosorbent assay and quantification of immunoglobulins in the diagnosis of bovine trypanosomiasis. Trop Anim Health Prod 10:149–159

Lumsden WHR, Kimber CD, Evans DA et al (1979) *Trypanosoma brucei*: miniature anion-exchange centrifugation technique for detection of low parasitaemias: adaptation for field use. Trans R Soc Trop Med Hyg 73:312–317

MacLean L, Odiit M, Okitoi D et al (1999) Plasma nitrate and interferron-gamma in *Trypanosoma brucei rhodesiense* infections: evidence that nitric oxide production is induced during both early blood-stage and late meningoencephalitic-stage infections. Trans R Soc Trop Med Hyg 93:169–170

Magnus E, Vervoort T, Van Meirvenne N (1978) A card-agglutination test with stained trypanosomes (C.A.T.T.) for the serological diagnosis of *T.b.gambiense* trypanosomiasis. Ann Soc Belg Med Trop 58:169–176

Matovu E, Kuepfer I, Boobo A et al (2010a) Comparative detection of trypanosomal DNA by loop-mediated isothermal amplification and PCR from flinders technology associates cards spotted with patient blood. J Clin Microbiol 48:2087–2090

Matovu E, Mugasa CM, Ali Ekangu R et al (2010b) Phase II evaluation of sensitivity and specificity of PCR and NASBA followed by oligochromatography for diagnosis of human African trypanosomiasis in D.R. Congo and Uganda. PLoS Negl Trop Dis 4:e737

Mendoza-Palomares C, Biteau N, Giroud C et al (2008) Molecular and biochemical characterization of a cathepsin B-like protease family unique to *Trypanosoma congolense*. Eukaryot Cell 7:684–697

Miézan TW, Meda AH, Doua F et al (2000) Single centrifugation of cerebrospinal fluid in a sealed pasteur pipette for simple, rapid and sensitive detection of trypanosomes. Trans R Soc Trop Med Hyg 94:293

Mori Y, Nagamine K, Tomita N et al (2001) Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem Biophys Res Commun 289:150–154

Moser DR, Cook GA, Ochs DE et al (1989) Detection of *Trypanosoma congolense* and *Trypanosoma brucei* subspecies by DNA amplification using the polymerase chain reaction. Parasitology 99:57–66

Mpandzou G, Cespuglio R, Ngampo S et al (2011) Polysomnography as a diagnosis and post-treatment follow-up tool in human African trypanosomiasis: a case study in an infant. J Neurol Sci 305:112–115

Mugasa CM, Schoone GJ, Ekangu RA et al (2008) Detection of *Trypanosoma brucei* parasites in blood samples using real-time nucleic acid sequence-based amplification. Diagn Microbiol Infect Dis 61:440–445

Mugasa CM, Laurent T, Schoone GJ et al (2009) Nucleic acid sequence-based amplification with oligochromatography for detection of *Trypanosoma brucei* in clinical samples. J Clin Microbiol 47:630–635

Mugasa CM, Adams ER, Boer KR et al (2012) Diagnostic accuracy of molecular amplification tests for human African trypanosomiasis-systematic review. PLoS Negl Trop Dis 6:e1438

Müller N, Hemphill A, Imboden M et al (1992) Identification and characterization of two repetitive non-variable antigens from African trypanosomes which are recognized early during infection. Parasitology 104:111–120

Mumba Ngoyi D, Lejon V, N'Siesi FX et al (2009) Comparison of operational criteria for treatment outcome in *gambiense* human African trypanosomiasis. Trop Med Int Health 14:438–444

Mumba Ngoyi D, Lejon V, Pyana P et al (2010) How to shorten patient follow-up after treatment for *Trypanosoma brucei gambiense* sleeping sickness? J Infect Dis 201:453–463

Mumba Ngoyi D, Bohorquez E, Messina J et al (2011) Prevalence of human African trypanosomiasis in the Democratic Republic of the Congo. PLoS Negl Trop Dis 5:e1246

Mumba Ngoyi D, Menten J, Pyana PP et al (2013) Stage determination in sleeping sickness: comparison of two cell counting and two parasite detection techniques. Trop Med Int Health 18 (6):778–782

Murray M, Murray PK, McIntyre WIM (1977) An improved parasitological technique for the diagnosis of African trypanosomiasis. Trans R Soc Trop Med Hyg 71:325–326

Namangala B, Hachaambwa L, Kajino K et al (2012) The use of Loop-mediated Isothermal Amplification (LAMP) to detect the re-emerging Human African Trypanosomiasis (HAT) in the Luangwa and Zambezi valleys. Parasit Vectors 5:e282

Nantulya VM, Lindqvist KJ (1989) Antigen-detection enzyme immunoassays for the diagnosis of *Trypanosoma vivax*, *T. congolense* and *T. brucei* infections in cattle. Trop Med Parasitol 40:267–272

Ngaira JM, Olembo NK, Njagi ENM et al (2005) The detection of non-RoTat 1.2 *Trypanosoma evansi*. Exp Parasitol 110:30–38

Njamnshi AK, Seke Etet PF, Perrig S et al (2012) Actigraphy in human African trypanosomiasis as a tool for objective clinical evaluation and monitoring: a pilot study. PLoS Negl Trop Dis 6: e1525

Njiru ZK (2011) Rapid and sensitive detection of human African trypanosomiasis by loop-mediated isothermal amplification combined with a lateral-flow dipstick. Diagn Microbiol Infect Dis 69:205–209

Njiru ZK (2012) Loop-mediated isothermal amplification technology: towards point of care diagnostics. PLoS Negl Trop Dis 6:e1572

Njiru ZK, Ndung'u K, Matete G et al (2004) Detection of *Trypanosoma brucei rhodesiense* in animals from sleeping sickness foci in East Africa using the serum resistance (*SRA*) gene. Acta Trop 90:249–254

Njiru ZK, Constantine CC, Guya S et al (2005) The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. Parasitol Res 95:186–192

Njiru ZK, Mikosza ASJ, Armstrong T et al (2008a) Loop-mediated isothermal amplification (LAMP) method for rapid detection of *Trypanosoma brucei rhodesiense*. PLoS Negl Trop Dis 2:e147

Njiru ZK, Mikosza ASJ, Matovu E et al (2008b) African trypanosomiasis: Sensitive and rapid detection of the sub-genus *Trypanozoon* by loop-mediated isothermal amplification (LAMP) of parasite DNA. Int J Parasitol 38:589–599

Njiru ZK, Traub R, Ouma JO et al (2011) Detection of group 1 *Trypanosoma brucei gambiense* by Loop-Mediated Isothermal Amplification. J Clin Microbiol 49:1530–1536

Notomi T, Okayama H, Masubuchi H et al (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28:7–9

OIE (2012) Manual of diagnostic tests and vaccines for terrestrial animals. http://www.oie.int/manual-of-diagnostic-tests-and-vaccines-for-terrestrial-animals/

Olaho-Mukani W, Munyua WK, Mutugi MW et al (1993) Comparison of antibody- and antigen-detection enzyme immunoassays for the diagnosis of *Trypanosoma evansi* infections in camels. Vet Parasitol 45:231–240

Pascucci I, Di Provvido A, Camara C et al (2013) Diagnosis of dourine outbreaks in Italy. Vet Parasitol 193:30–38

Peeling RW, Holmes KK, Mabey D et al (2006) Rapid tests for sexually transmitted infections (STIs): the way forward. Sex Transm Dis 82(Suppl V):v1–v6

Pentreath VW, Owolabi AO, Doua F (1992) Survival of *Trypanosoma brucei brucei* in cerebrospinal fluid. Ann Trop Med Parasitol 86:29–34

Pereira de Almeida PJL, Ndao M, Van Meirvenne N et al (1998) Diagnostic evaluation of PCR on dried blood samples from goats experimentally infected with *Trypanosoma brucei brucei*. Acta Trop 70:269–276

Picozzi K, Carrington M, Welburn SC (2008) A multiplex PCR that discriminates between *Trypanosoma brucei brucei* and zoonotic *T. b. rhodesiense*. Exp Parasitol 118:41–46

Priotto G, Chappuis F, Bastard M et al (2012) Early prediction of treatment efficacy in second-stage gambiense human African trypanosomiasis. PLoS Negl Trop Dis 6:e1662

Radwanska M, Chamekh M, Vanhamme L et al (2002a) The serum resistance-associated gene as a diagnostic tool for the detection of *Trypanosoma brucei rhodesiense*. Am J Trop Med Hyg 67:684–690

Radwanska M, Claes F, Magez S et al (2002b) Novel primer sequences for a polymerase chain reaction-based detection of *Trypanosoma brucei gambiense*. Am J Trop Med Hyg 67:289–295

Radwanska M, Magez S, Perry-O'Keefe H et al (2002c) Direct detection and identification of African trypanosomes by fluorescence in situ hybridization using peptide nucleic acid probes. J Clin Microbiol 40:4295–4297

Rae PF, Luckins AG (1984) Detection of circulating trypanosomal antigens by enzyme immunoassay. Ann Trop Med Parasitol 78:587–596

Rebeski DE, Winger EM, Rogovic B et al (1999) Improved methods for the diagnosis of African trypanosomosis. Mem Inst Oswaldo Cruz 94:249–253

Rebeski DE, Winger EM, Okoro H et al (2000) Detection of *Trypanosoma congolense* with indirect ELISAs using antigen-precoated microtitre plates. Vet Parasitol 89:187–198

Rebeski DE, Winger EM, Ouma JO et al (2001) Charting methods to monitor the operational performance of ELISA method for the detection of antibodies against trypanosomes. Vet Parasitol 96:11–50

Rogé S, Heykers A, Brouwer de Koning A, Guisez Y, Büscher P (2011a) Recombinant expression of LiTat 1.3 VSG in *Pichia pastoris* for serodiagnosis of *gambiense* sleeping sickness. In: Abstracts of the 31st meeting of the international scientific council for trypanosomiasis research and control, Bamako, Mali, 12–16 Sept 2011

Rogé S, Van Reet N, Wand N, Guisez Y, Rudenko G, Büscher P (2011b) Immune trypanolysis on non-human infective trypanosomes for diagnosis of *gambiense* sleeping sickness. In: Abstracts of the 31st meeting of the international scientific council for trypanosomiasis research and control, Bamako, Mali, 12–16 Sept 2011

Rogé S, Guisez Y, Büscher P (2012a) A new latex agglutination test for surra based on recombinant *Trypanosoma evansi* RoTat 1.2 variant surface glycoprotein expressed in the *Pichia pastoris* GlycoSwitch™ M5 strain. In: Abstracts of the Pichia 2012, Alpbach, Austria, 29 Feb–3 Mar 2012

Rogé S, Meul M, Guisez Y, Gilleman Q, Simon T, Büscher P (2012b) Development of rapid diagnostic tests for surra and sleeping sickness based on recombinant antigens expressed in *Pichia pastoris*. In: Abstracts of the Pathogens' survival strategies, from fundamentals to field. ITM 54th international colloquium, Antwerp, Belgium, 3–5 Dec 2012

Rogé S, Van Reet N, Odiwuor SO, Schildermans K, Vandamme S, Vandenberghe I, Vervecken W, Gillingwater K, Claes F, Devreese B, Guisez Y, Büscher P (2012c) Recombinant expression of trypanosome surface glycoproteins in *Pichia pastoris* for the diagnosis of *Trypanosoma evansi* infection. In: Abstracts of the Pathogens' survival strategies, from fundamentals to field. ITM 54th international colloquium, Antwerp, Belgium, 3–5 Dec 2012

Rogé S, Van Reet N, Odiwuor S, Tran T, Schildermans K, Vandamme S, Vandenberghe I, Vervecken W, Gillingwater K, Claes F, Devreese B, Guisez Y, Büscher P (2013) Recombinant expression of trypanosome surface glycoprtoteins in *Pichia pastoris* for the diagnosis of *Trypanosoma evansi* infection. Vet Parasitol. doi:10.1016/j.vetpar.2013.05.009

Rutjes AW, Reitsma JB, Coomarasamy A et al (2007) Evaluation of diagnostic tests when there is no gold standard. A review of methods. Health Technol Assess 11:iii, ix-51

Sengupta PP, Balumahendiran M, Suryanaryana VVS et al (2010) PCR-based diagnosis of surra-targeting VSG genen: experimental studies in small laboratory rodents and buffalo. Vet Parasitol 171:22–31

Sengupta PP, Balumahendiran M, Balamurugan V et al (2013) Expressed truncated N-terminal variable surface glycoprotein (VSG) of *Trypanosoma evansi* in *E. coli* exhibits immunoreactivity. Vet Parasitol 187:1–8

Sharma P, Juyal PD, Singla LD et al (2012) Comparative evaluation of real time PCR assay with conventional parasitological techniques for diagnosis of *Trypanosoma evansi* in cattle and buffaloes. Vet Parasitol 190:375–382

Shiraishi T, Deborggraeve S, Büscher P et al (2011) Sensitive detection of nucleic acids by PNA hybridization directed co-localization of fluorescent beads. Artif DNA PNA XNA 2:60–66

Sullivan L, Wall SJ, Carrington M et al (2013) Proteomic selection of immunodiagnostic antigens for human African trypanosomiasis and generation of a protoype lateral flow immunodiagnostic device. PLoS Negl Trop Dis 7:e2087

Thekisoe OMM, Kuboki N, Nambota A et al (2007) Species-specific loop-mediated isothermal amplification (LAMP) for diagnosis of trypanosomosis. Acta Trop 102:182–189

Thuy NT, Goto Y, Lun ZR et al (2012) Tandem repeat protein as potential diagnostic antigen for *Trypanosoma evansi* infection. Parasitol Res 110:733–739

Tiberti N, Hainard A, Lejon V et al (2013a) Cerebrospinal fluid neopterin is a marker of the meningo-encephalitic stage of *Trypanosoma brucei gambiense* sleeping sickness. PLoS One 7: e40909

Tiberti N, Lejon V, Hainard A et al (2013b) Neopterin is a cerebrospinal fluid marker for treatment outcome evaluation in patients affected by *Trypanosoma brucei gambiense* sleeping sickness. PLoS Negl Trop Dis 7:e2088

Tomita N, Mori Y, Kanda H et al (2008) Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat Protoc 3:877–882

Tran T, Claes F, Verloo D et al (2009) Towards a new reference test for surra in camels. Clin Vaccine Immunol 16:999–1002

Truc P, Jamonneau V, Cuny G et al (1999) Use of polymerase chain reaction in human African trypanosomiasis stage determination and follow-up. Bull World Health Organ 77:745–748

Truc P, Lando A, Penchenier L et al (2012) Human African trypanosomiasis in Angola: clinical observations, treatment, and use of PCR for stage determination of early stage of the disease. Trans R Soc Trop Med Hyg 106:10–14

Van Meirvenne N, Magnus E, Büscher P (1995) Evaluation of variant specific trypanolysis tests for serodiagnosis of human infections with *Trypanosoma brucei gambiense*. Acta Trop 60:189–199

Van Nieuwenhove L, Rogé S, Balharbi F et al (2011) Identification of peptide mimotopes of *Trypanosoma brucei gambiense* variant surface glycoproteins. PLoS Negl Trop Dis 5:e1189

Van Nieuwenhove L, Büscher P, Balharbi F et al (2012) Identification of mimotopes with diagnostic potential for *Trypanosoma brucei gambiense* variant surface glycoproteins with human antibody fractions. PLoS Negl Trop Dis 6:e1682

Van Nieuwenhove L, Büscher P, Balharbi F et al (2013) A LiTat 1.5 variant surface glycoprotein-derived peptide with diagnostic potential for *Trypanosoma brucei gambiense*. Trop Med Int Health 18:461–465

Verloo D, Tibayrenc R, Magnus E et al (1998) Performance of serological tests for *Trypanosoma evansi* infections in camels from Niger. J Protozool Res 8:190–193

Verma A, Manchanda S, Kumar N et al (2011) *Trypanosoma lewisi* or *T. lewisi*-like infection in a 37-day-old Indian infant. Am J Trop Med Hyg 85:221–224

Vitouley HS, Mungube EO, Allegye-Cudjoe E et al (2011) Improved PCR-RFLP for the detection of diminazene resistance in *Trypanosoma congolense* under field conditions using filter papers for sample storage. PLoS Negl Trop Dis 5:e1223

Wastling SL, Picozzi K, Kakembo AS et al (2010) LAMP for human African trypanosomiasis: a comparative study of detection formats. PLoS Negl Trop Dis 4:e865
Welburn SC, Picozzi K, Fèvre EM et al (2001) Identification of human-infective trypanosomes in animal reservoir of sleeping sickness in Uganda by means of serum-resistance-associated (*SRA*) gene. Lancet 358:2017–2019
Woo PTK (1970) The haematocrit centrifuge technique for the diagnosis of African trypanosomiasis. Acta Trop 27:384–386
Woo PTK, Soltys MA (1972) The indirect haemagglutination and charcoal-agglutination tests in the diagnosis of African sleeping sickness. Tropenmed Parasitol 23:324–327
World Health Organization (1986) Epidemiology and control of African trypanosomiasis. WHO Technical Report Series 739, World Health Organization, Geneva

Progress Towards New Treatments for Human African Trypanosomiasis

9

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Abstract

The treatment of African trypanosomiasis has essentially remained unchanged for decades. A mountain of excellent work has been produced on many aspects of trypanosome biochemistry, biology, genetics, etc., but this has not translated into new therapies, although the disease burden has steadily increased through the latter half of the twentieth century. The only new drug to be introduced in the last 50 years or so is eflornithine, in the late 1970s, for the treatment of late-stage gambiense sleeping sickness only. However, this was in many ways unsatisfactory and melarsoprol remained the first-line treatment for late-stage sleeping sickness until an alarming increase in treatment failures necessitated change. Since the emerging sleeping sickness epidemic became widely recognised, around the year 2000, needs-driven development of new drugs, and the preservation of the production of old drugs, has been the result of dedicated work by organisations such as the World Health Organisation, the Drugs for Neglected Diseases initiative (DNDi), the Access to Essential Medicines campaign, and the Consortium for Parasitic Drug Development (CPDD) among others, much of it in partnership with academia and the pharmaceutical industry. This has already

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resulted in milestones such as the donations of free treatments by producers; improved drug distribution, case finding and clinical care; an improved 10-day melarsoprol treatment; the first clinical trial for an oral sleeping sickness drug—pafuramidine and the introduction of eflornithine–nifurtimox combination therapy to begin replacing melarsoprol. While these efforts have undoubtedly contributed to reducing the disease burden in central Africa, newer treatments are still very necessary, especially as most current treatments are threatened by drug resistance. Here, we review recent advances in understanding drug resistance mechanisms, progress towards new drugs, and new delivery systems to improve efficacy.

9.1 Introduction

Chemotherapy of human African trypanosomiasis (HAT) started as early as 1905 with the introduction of the arsenic compound atoxyl, the name optimistically meaning "non-toxic", at the time of terrible sleeping sickness epidemics in Eastern and Central Africa (Duggan 1970), estimated to have killed 250,000 people in Uganda alone (Fèvre et al. 2004). Remarkably, this was only a decade after the discovery by David Bruce that trypanosomes were the aetiological agents of what was known as "tsetse fly disease" (Bruce 1895). While atoxyl was found to cause blindness in 2 % of patients through atrophy of the optic nerve, it prompted a systematic programme of drug discovery initiated by Paul Ehrlich (Steverding 2010), leading to a succession of organoarsenic drugs including arsphenamine (compound 606; Salvarsan) in 1907, which was ineffective against trypanosomiasis but, in 1909, was found to have excellent curative properties for syphilis (Bosch and Rosich 2008). For sleeping sickness, however, it was not until 1919 that an effective arsenical drug, tryparsamide, was synthesised (Jacobs and Heidelberger 1919). Tryparsamide was able to cross the blood–brain barrier, becoming the first drug against late-stage sleeping sickness and was used into the 1960s for both human and animal trypanosomiasis (Steverding 2010). However, it was gradually replaced by melarsoprol, first introduced in 1949 (Friedheim 1949), which unlike tryparsamide did not display any ocular toxicity—but causes instead a reactive encephalopathy in up to 10 % of patients that is fatal in about half the cases (Kuepfer et al. 2012).

Paul Ehrlich initiated not only the discovery of the organoarsenical trypanocides but also started a programme developing a series of dyes that were specifically accumulated by trypanosomes, as new drugs. This progressed from nagana red and trypan blue to afridol violet. These compounds were systematically tested and their activity and specificity optimised, leading after his death to the development of suramin (Bayer 205; Germanin®) by Bayer in 1916 (Schlitzer 2009). Suramin is still the only drug available for the treatment of East African HAT, just as melarsoprol is the only drug against the late stage of the disease. Even for West African sleeping sickness melarsoprol is only now beginning to be replaced by a recently introduced combination of eflornithine and nifurtimox (Yun et al. 2010).

The only other sleeping sickness drug, pentamidine, is used against early stage West African HAT, and was developed in the early 1930s from a compound called synthalin, a synthetic analogue of insulin, which was believed to kill trypanosomes, which need glucose as their sole energy source, by inducing hypoglycaemia in the host. However, Lourie and Yorke showed in 1937 that synthalin is 10,000 times more powerful than insulin as a trypanocide in vitro (Lourie and Yorke 1937). Replacement of synthalin's guanidine groups by benzamidines gave rise to the entire class of aromatic diamidines, which includes pentamidine, stilbamidine and diminazene (Berenil®) (Williamson 1970), and while it is clear that at least some of these accumulate in the parasite's mitochondrion (Basselin et al. 2002; Lanteri et al. 2008), their mode of action has never been satisfactorily resolved.

Thus, the current treatments for HAT (and animal African trypanosomiasis; AAT) have been at the very root of the development of chemotherapy and its principles over the last century and more—and unfortunately stayed there. HAT and AAT are still mostly treated with organoarsenicals, aromatic diamidines and suramin. In addition, AAT is treated with the two phenanthridine drugs ethidium bromide [Homidium® (Watkins and Woolfe 1952)] and isometamidium [methidium; Samorin® (Wragg et al. 1958)], introduced in 1952 and 1958, respectively. These are direct derivatives of phenidium chloride, a trypanocide from 1938 (Browning et al. 1938). The phenanthridinium drugs are generally considered too dangerous to use in humans. In fact, most if not all of the current pharmacopoeia against African trypanosomiasis would be considered to have unacceptable side effects if they were now introduced for human use.

If drug toxicity was not enough to make the case that new drugs are urgently needed for this neglected disease, drug resistance threatens the continued usefulness of most of the compounds (Delespaux and De Koning 2007). For instance, only the alarming rate of melarsoprol treatment failures (Legros et al. 1999; Robays et al. 2008) prompted the introduction of eflornithine as first-line treatment, initially as mono-therapy and recently in combination with nifurtimox. Moreover, resistance to the veterinary trypanocides is widespread (Geerts et al. 2001). Fortunately, a considerable effort towards new HAT treatments has been made over the last decade, resulting in a situation where several promising compounds or compound classes are nearing or entering clinical evaluation. In addition, there are new strategies to target (existing) trypanocides much more specifically to the parasite, potentially bypassing resistance mechanisms. Some of these developments will be highlighted below.

9.2 New Insights into Drug Action and Resistance of Current Trypanocides

9.2.1 Role of Transporters in Selective Activity and in Drug Resistance

The issues concerning drug entry into protozoan target cells by the processes of (1) simple diffusion through membranes, (2) diffusion enabled by passive transport proteins (facilitated diffusion) and (3) energy-dependent transporters (active transport) have recently been thoroughly reviewed (Delespaux and De Koning 2013). However, a short reiteration of transporters involved in drug delivery and resistance in trypanosomes is important for a proper understanding of HAT and AAT chemotherapy.

Of current chemotherapy against African trypanosomiasis, it is believed that only nifurtimox enters the cell by simple diffusion, having physico-chemical properties that allow a reasonable diffusion rate. Other trypanocides are too highly charged to have a substantial diffusion rate, e.g. suramin with six negative charges, and diamidines with two unshielded positive charges. While melarsoprol is highly lipophilic, it is not stable in circulation and is rapidly converted to the water-soluble form melarsen oxide (Keiser et al. 2000), and as such needs a transporter to be taken up by trypanosomes (Scott et al. 1997). This transporter was identified as an adenosine/adenine transporter and called P2 (Carter and Fairlamb 1993). The gene encoding this transporter was identified several years later and called Tb*AT1* (Mäser et al. 1999). Meanwhile, it had become clear that this transporter is also able to transport diamidine drugs including pentamidine (Carter et al. 1995; De Koning 2001) and diminazene (De Koning et al. 2004).

However, deletion of the P2/Tb*AT1* transporter created a trypanosome line that, while substantially resistant to diminazene, had only marginally lost sensitivity to pentamidine and the melaminophenyl arsenical drugs (Matovu et al. 2003), indicating the presence of (an) additional transporter(s) for these drugs. It is now clear that pentamidine is additionally taken up by a High Affinity Pentamidine Transporter (HAPT1) and a Low Affinity Pentamidine Transporter (LAPT1) and that HAPT1 is also the secondary transporter for the arsenical drugs (Bridges et al. 2007; De Koning 2008). Eflornithine entry into trypanosomes is also transporter mediated and several groups have recently identified this transporter as one of the *T. b. brucei* amino acid transporters, TbAAT6 and established that loss of the transporter leads to eflornithine resistance (Vincent et al. 2010; Baker et al. 2011; Schumann et al. 2011). Indeed, as two groups independently identified this transporter gene using an RNAi library approach, it could be argued that clinical resistance is also likely to be due to loss of TbAAT6, although this remains to be established. A laboratory strain with induced eflornithine resistance was previously shown to be deficient in the uptake of this drug (Phillips and Wang 1987).

9.2.2 Trypanosoma brucei Aquaporin 2 Is a Drug Transporter with a Unique Selectivity Filter and Substrate Specificity

Aquaporins are members of the major intrinsic protein family (MIP), a superfamily of aquaporins and aquaglyceroporins, which allow the movement of water and small neutral solutes across cell membranes in a wide selection of organisms from bacteria to humans, with most eukaryotic organisms containing at least one Aquaporin. The aquaporins of parasitic protozoa are reviewed in (Beitz 2005). There are three aquaglyceroporins encoded by the *T. brucei* genome, AQP1-3, which have been shown to transport water, glycerol, urea, dihydroacetone (Uzcategui et al. 2004) and ammonia (Zeuthen et al. 2006). AQP1 has been located to the flagellar membrane, AQP3 to the plasma membrane (Bassarak et al. 2011) and AQP2 to the flagellar pocket in bloodstream form parasites and the plasma membrane in procyclic-form parasites (Baker et al. 2012).

Aquaporins 2 and 3 were first identified as potential genes involved in resistance to both pentamidine and melarsoprol by an RNAi library screen, the two genes being in tandem array and sharing 83 % sequence identity (Alsford et al. 2012). Expression of each gene separately in *aqp2–aqp3* double null cells unambiguously identified TbAQP2 as the determinant for pentamidine and arsenical sensitivity/resistance (Baker et al. 2012). TbAQP2 has non-standard motifs in key regions of the protein thought to determine the selectivity of the pore, whereas AQP3 contains standard selectivity region amino acids. AQP2 is the only Major Intrinsic Protein (MIP) family member described to date that has NSA/NPS and IVLL motifs. In contrast, AQP3 and AQP1 both contain the classical NPA/NPA motifs found in most other family members (Baker et al. 2012), and a WGYR motif in their selectivity region that is common to aquaglyceroporin-type pores, being found in 118 of the other family members (Baker et al. 2013). In particular, the lack of the aromatic/arginine (ar/R) motif in AQP2 may lead to an increase in its ability to transport larger and charged molecules (Beitz et al. 2006; Li et al. 2011).

The status of AQP2 was investigated in a well-characterised laboratory-selected strain, B48, which has high levels of resistance to both pentamidine and melarsoprol (Bridges et al. 2007); an *AQP2*/*AQP3* chimeric gene, inactive with respect to pentamidine sensitivity, was found to have replaced *AQP2*, with a 272-bp section towards the 3′ end of *AQP2* replaced with an in-frame section of *AQP3* (Baker et al. 2012). This suggests that it is this section of the gene which is responsible for the drug-sensitivity profile of *AQP2*, which includes the second half of the likely selectivity region (NPS/IVLL is replaced with the classical regions of NPA/IGYR in the chimera). The B48 line has been shown to owe its resistance to pentamidine and melarsoprol to the loss of HAPT1, thus suggesting that *AQP2* may be the long-searched-for gene that encodes this drug transporter.

The role of AQP2 should now be investigated in drug-resistant *T. b. gambiense* field strains. The *T. b. gambiense* genome contains two tandem copies of AQP2 and lacks AQP3, thus it is of particular interest to investigate the presence or mutation status of these genes in any drug-resistant isolates that can be acquired. It is also imperative to determine whether AQP2 is directly transporting the relatively bulky

drugs melarsoprol and pentamidine (molecular masses of 398 Da and 340 Da, respectively), or somehow enabling this indirectly. Known AQP2 substrates are considerably smaller, with masses of 92 Da for glycerol, 60 Da for urea and 90 Da for dihydroxyacetone. However, the *Leishmania* aquaglyceroporin AQP1 has been shown to transport trivalent arsenic and antimony and to be involved in resistance to trivalent antimony (Gorbal et al. 2004; Figarella et al. 2007). Human aquaglyceroporin 9, a homologue of AQP2, has also been reported to transport purines and pyrimidines (Tsukaguchi et al. 1999).

It has thus been shown that AQP2 has a critical role in melarsoprol-pentamidine cross-resistance, and it is likely that AQP2 is in fact the previously reported HAPT1 drug transporter (Munday, De Koning et al., unpublished results). If AQP2 can be shown to be mutated or lost in melarsoprol/pentamidine cross-resistant field isolates it may mean the development of a test for drug sensitivity will be possible, allowing rational choices in clinical treatment.

9.2.3 Drug Uptake in Animal African Trypanosomiasis

The genomes of the main veterinary trypanosomiasis-causing species, *T. congolense* and *T. vivax*, do not contain a syntenic equivalent of *TbAQP2*; only a *TbAQP3* homologue is annotated in their genomes (Jackson et al. 2013). Resistance to veterinary trypanocides, particularly diminazene, is increasing (Geerts et al. 2001; Delespaux et al. 2008) but the resistance mechanisms are currently unknown. Sensitivity to veterinary arsenicals (cymelarsan) and diamidines (diminazene) was believed to be mediated by an equivalent of TbAT1/P2 instead of an aquaporin. However, the proposed homologue of TbAT1 (Delespaux et al. 2006) has now been shown to be a P1-type, rather than P2-type transporter and is not involved in diamidine or melaminophenyl arsenical transport after all (Munday et al. 2013).

9.3 New Targets

The perilously low number of drugs against the various forms and stages of trypanosomiasis, with its variety of parasite and host species, the difficulties of administration in the field, their high toxicity levels and the emergence of resistance in areas with high transmission rates and high levels of drug use, all contribute to the urgency in finding new trypanocidal drugs. A common strategy is the discovery and exploitation of unique targets in the trypanosome, whose absence in the mammalian host will reduce adverse side effects, and allow the development of the proverbial magic bullet. At the same time, however, new advances in the development of effective drug delivery systems, as well as the possibility of targeting these vehicles using immunological tools, may allow an improvement of the sleeping sickness treatment by recycling of the outdated drugs.

9.3.1 *N*-Myristoltransferase

Myristoyl-CoA:protein *N*-myristoyltransferase (NMT; E.C. 2.3.1.97) is a cytosolic monomeric enzyme which catalyses the transfer of a rare 14-carbon saturated fatty acid, myristate, from myristoyl-CoA to the amino group of N-terminal glycine residues in eukaryotic and viral proteins (Bhatnagar et al. 1999). *N*-myristoylation is a co- and post-translational modification required for membrane targeting and functional activation of proteins, thus it is an essential process in a wide range of living organisms (Farazi et al. 2001). In *T. brucei*, RNAi knockdown of NMT has been shown to be lethal in cell culture (Price et al. 2003) and renders the parasites incapable of establishing an infection in a mouse model (Price et al. 2010) making TbNMT a promising therapeutic target (Bowyer et al. 2008). More than 60 potential protein substrates are predicted to be myristoylated by TbNMT in *T. brucei* (Mills et al. 2007). Among them, ADP-ribosylation factor-1 protein (ARF-1) (Price et al. 2007), ADP-ribosylation factor-like protein (ARL-1) (Price et al. 2005) and a calpain-type protease (CAP5.5) (Olego-Fernandez et al. 2009), are essential for bloodstream trypanosome viability. Inhibition of the TbNMT is expected to have pleiotropic effects on multiple pathways. A high-throughput screening of a diversity-based compound library identified a pyrazole sulfonamide scaffold as a promising lead towards NMT inhibitors. Further modifications yielded the compound DDD85646, a potent TbNMT inhibitor (Frearson et al. 2010; Brand et al. 2012). DDD85646 inhibited proliferation of bloodstream forms of *T. brucei* in culture at nM concentrations, being 200-fold more sensitive than a human primary cell line. Moreover, exposure of *T. brucei* bloodstream forms, to DDD85646 resulted in a rapid enlargement of the flagellar pocket; this so-called BigEye phenotype typically indicates disruption of endocytosis (Allen et al. 2003). DDD85646 cured animals from acute infection with *T. b. brucei* and *T. b. rhodesiense* strains after oral administration, being well tolerated at effective doses (Frearson et al. 2010). It is hoped that these encouraging results will be followed up with preclinical development and will eventually lead to clinical trials of TbNMT inhibitors for sleeping sickness.

9.3.2 Proteases

Proteases are ubiquitous enzymes in nature that regulate and coordinate a large number of cellular processes. Protozoan proteases are closely associated with processes that support parasitism, such as the digestion of host components and the cellular remodelling of parasites between life-cycle stages (Williams et al. 2006). Thus, they are considered as potentially effective drug targets for treatment of many parasitic diseases (Vermelho et al. 2007). The major groups of *T. brucei* proteases suitable as drug targets are threonine-, serine- and cysteine proteases.

T. brucei threonine proteases are present in the proteasome, a multicatalytic proteinase complex which plays a critical role in intracellular protein degradation. The trypanosome proteasome differs from the mammalian proteasome in protease

activity, substrate specificity and inhibitor sensitivity. Unlike the mammalian proteasome, the trypanosome proteasome exhibits high trypsin-like and low chymotrypsin-like activities (Hua et al. 1996; Wang et al. 2003). This kindled interest in the proteasome as a potential drug target for sleeping sickness treatment. In this line, trileucine methyl vinyl sulfones, which are selective inhibitors of the trypsin-like activity of the proteasome, showed anti-*T. brucei* activity in vitro (Steverding et al. 2005). Recently, vinyl ester tripeptides, which are potent inhibitors of the chymotrypsin-like activity, also showed a strong trypanocidal activity without displaying cytotoxicity against human cells. This difference in sensitivity between mammalian and trypanosome proteasome may be exploited for rational anti-trypanosomal drug development (Steverding et al. 2011). The vinyl sulfone K777, an inhibitor of *T. cruzi* protease cruzain has been cleared for clinical trials against Chagas' disease (Leslie 2011), confirming the potential of protease inhibitors in kinetoplastid disease.

African trypanosomes also contain Oligopeptidase B (TbOPB) (Morty et al. 1999), a serine protease proposed as target of the trypanocidal drugs pentamidine, suramin and diminazene (Morty et al. 1998), despite the fact that the same authors suggest that this protease, which is not released by live trypanosomes, appears to have a role in pathogenesis once it enters the host circulation (Morty et al. 2001). This would be hard to reconcile with the strong in vitro trypanocidal activity of pentamidine, suramin and diminazene if their target was TbOPB; in addition, the cross-resistance patterns have never suggested a similar mechanism of action for suramin and diamidines (Kaminsky and Mäser 2000; Delespaux and De Koning 2007). It should be obvious that the mere fact that trypanocides are able to inhibit the purified enzyme in an in vitro assay does not prove that this is the primary target of the drugs, and no other supportive (e.g. genetic) evidence has been reported. Whatever the merit of that claim, the authors also showed that peptidyl phosphonate diphenyl esters, which are serine protease inhibitors, inhibited trypanosome growth in vitro and in vivo, although this could not be definitively ascribed to inhibition of TbOPB (Morty et al. 2000).

Bloodstream trypanosomes express two cysteine proteases: rhodesain (brucipain, trypanopain), a cathepsin L-like enzyme and TbCatB, a cathepsin B-like protease. Significantly, a study demonstrated that the specific RNAi knockdown of TbCatB expression, but not rhodesain expression, was lethal in *T. brucei* (Mackey et al. 2004). Moreover, knockdown of TbCatB in vivo was able to rescue mice from a lethal *T. brucei* infection, whereas only a partial cure was observed after knockdown of rhodesain (Abdulla et al. 2008). O'Brien et al. (2008) showed that TbCatB has an essential role in the degradation of host proteins, including transferrin and that this compromises the essential acquisition of iron. These results identified TbCatB as a prime target for developing anti-trypanosome therapies. Treatment of parasites in culture with the cysteine protease inhibitor, benzyloxycarbonyl–phenylalanyl–alanyl diazomethylketone (Z–Phe–Ala–CHN_2) was lethal to *T. brucei* in vitro (Scory et al. 1999). Parasites treated with this inhibitor exhibited altered cell morphology, displaying enlarged lysosomes, appeared to be defective in host protein degradation and seemed to be in cell cycle arrest

(Scory et al. 2007). Thus, Z–Phe–Ala–CHN_2 treatment produced phenotypic defects similar to those seen with the knockdown of TbCatB; however, the Z–Phe–Ala–CHN_2 treatment produced cells that exhibited cell cycle arrest in apparent G1 stage (one nucleus, one kinetoplast) (Scory et al. 2007), whereas the cathepsin B-specific RNAi caused a phenotype with all cells containing at least two nuclei and kinetoplasts after 24 h of induction (Mackey et al. 2004). The likely reason for the discrepancy is the inhibition of multiple cysteine proteases by Z-Phe-Ala-CHN_2.

The screening of a collection of small molecules donated by Celera Genomics identified two ketobenzoxazole analogues as cysteine proteases inhibitors with in vitro potency and selectivity, but these require further optimization to enhance their anti-trypanosomal activity (Ang et al. 2011). A disadvantage of peptide-based drugs is that they are vulnerable to degradation by proteases. For this reason, other strategies have been focused on the synthesis of non-peptidic cysteine protease inhibitors, like thiosemicarbazones and macrocyclic vinyl sulfones, which indeed showed promising inhibitory effects on rhodesain and other parasite cysteine proteases including cruzain (Greenbaum et al. 2004; Chen et al. 2008). Optimization of other TbCatB inhibitors, the purine nitrile compounds, improved selectivity for TbCatB over human cathepsins B and L (Mallari et al. 2008, 2009). Additionally, purine nitrile compounds were metabolically stable, orally available and apparently crossed the blood–brain barrier well (Mallari et al. 2010). However, mice infected with *T. b. brucei* and treated with the most potent lead compound only showed a somewhat longer average survival time compared to untreated, and the same compound was completely ineffective in a murine *T. b. rhodesiense* model, even when treated with 200 mg/kg for 5 days (Mallari et al. 2010). Recently, it has been described that nicotinamide, the amide form of vitamin B3, inhibited TbCatB and induced trypanosome cell death in vitro (Unciti-Broceta et al. 2013). Consistent with the proposed mechanism of action, nicotinamide induced a cell division defect leading to a rapid increase in cells with multiple nuclei and kinetoplasts. These results certainly make nicotinamide a very interesting compound for further investigation although the very high concentrations necessary for its activity in vitro may not be easily achieved in vivo.

9.3.3 Phosphodiesterases

Cyclic nucleotide-specific phosphodiesterases (PDEs) play a central role in cell signalling mechanisms mediated via cyclic nucleotides. PDEs are highly specific hydrolases that convert the signalling molecules 5′,3′-cyclic adenosine and/or guanosine monophosphate (cAMP or cGMP) into the corresponding 5′-monophosphates (5′-AMP or 5′-GMP). Four different PDEs families are presents in *T. brucei* genome (PDEs A–D) (Kunz et al. 2006; Gould and De Koning 2011). Although the catalytic domains of PDEs are highly conserved between *T. brucei* and humans, they are promising drug targets (Seebeck et al. 2011). Two *T. b. brucei* isoforms of the PDEB family, TbPDEB1 and TbPDEB2, have been validated as

drug targets, but only if both are inhibited simultaneously, as they can complement each other (Oberholzer et al. 2007; Shakur et al. 2011). Fortunately, the binding pockets of the isoenzymes are virtually identical, giving them the same pharmacology (De Koning et al. 2012). RNAi experiments targeting both PDEBs led to an increase of intracellular cAMP concentration and cell lysis in vitro and in vivo (Zoraghi and Seebeck 2002; Oberholzer et al. 2007). Moreover, a high-throughput screening of a compound library (owned by Nycomed Pharma) identified a group of molecules with high inhibitory activity against TbPDEB1 (De Koning et al. 2012). The lead compound, the tetrahydrophthalazinone (Cpd A), inhibited TbPDEB1 and TbPDEB2 and stopped *T. brucei* proliferation at nM concentrations in vitro and, importantly, displayed identical activity against cell lines resistant to diamidine and melaminophenyl arsenical drugs currently used in the field. The exposure of cells to Cpd A induced the same phenotype as that observed in the TbPDEB knock-down experiments, confirming Cpd A specificity for its target. Just as important, the same dose dependency (and structure–activity relationship) was observed when assessing trypanocidal activity, TbPDEB inhibition and cellular cAMP increases. Although selectivity for the trypanosomal phosphodiesterase is essential, and was not yet achieved with Cpd A (De Koning et al. 2012), the publication of the TbPDEB1 crystal structure highlights a parasite-specific "P-pocket" at the active site (Jansen et al. 2013), also found in the *Leishmania major* PDEB1 (Wang et al. 2007) and *T. cruzi* PDEC structures (Wang et al. 2012b). These results offer a new strategy to combat HAT through the design or identification of specific TbPDEB inhibitors and have led to several groups actively pursuing these (Orrling et al. 2012; Wang et al. 2012a; Ochiana et al. 2012; Jansen et al. 2013).

9.3.4 Ergosterol Biosynthesis Inhibitors

All eukaryotic organisms require sterols, and as the biosynthesis pathways for these molecules are complex, there is much potential for therapeutic intervention. This is most clear in antifungal therapy, which is to a large degree based on exploiting the fact that fungi rely on ergosterol rather than the cholesterol found in the mammalian hosts (Kathiravan et al. 2012). Kinetoplastid parasites similarly produce ergosterol using a biosynthesis pathway very similar to that operating in fungi, and as such their growth is susceptible to sterol biosynthesis inhibitors (Roberts et al. 2003). *Leishmania* species and *T. cruzi* exclusively produce their sterols endogenously, whereas *T. brucei* can also scavenge some sterols from the host environment (Lepesheva et al. 2011). However, *T. brucei* can only utilise the endogenously made sterols into trypanosome-specific sterols necessary for various regulatory processes (Lepesheva et al. 2010a, b) and this is what makes African trypanosomes sensitive to inhibition of sterol biosyntheis. *T. brucei* cytochrome P450 enzyme, sterol 14α-demethylase (CYP51A1), is an enzyme of the ergosterol biosynthesis pathway. Inhibition of CYP51A1 blocks ergosterol synthesis which is lethal in unicellular organisms (Lepesheva and Waterman 2007). A number of azole-based drugs (anti-fungals such as ketoconazol, posaconazole and ravuconazole) are potent

inhibitors of *T. cruzi* CYP51A1 enzyme; posaconazole and a ravuconazole prodrug have now entered Phase II clinical trials against Chagas' disease (Clayton 2010; Urbina 2013). A recent high-throughput screening of a collection of azole derivatives identified two imidazoles, VNF (SDZ285604) and VNI (SDZ285428), as inhibitors of trypanosomal CYP51 (Lepesheva et al. 2007). Both compounds also inhibited the human enzyme but at a 100-fold excess (Lepesheva et al. 2008). The study of the crystal structure of *T. brucei* and *T. cruzi* CYP51 interacting with VNI has opened an opportunity for rational design of novel and selective inhibitors of these enzymes (Lepesheva et al. 2010a, b).

9.3.5 Protein Kinases

The critical role of protein kinases in signalling and cell division make them potential targets in kinetoplastids but the critical issue for kinase inhibitors is selectivity, as many kinases are extremely well conserved and have important roles in both protozoan and mammalian biochemistry. The *T. b. brucei* genome is estimated by bioinformatic analysis to encode some 180 distinct kinases (Parsons et al. 2005; Urbaniak et al. 2012). Amongst these, *T. brucei* expresses two TOR (Target of Rapamycin) kinases, TbTOR1 and TbTOR2, both of which have essential regulatory functions in cell biology; treatment with rapamycin potently inhibited trypanosome cell growth (Barquilla et al. 2008). TOR kinases are part of the phosphoinositide 3-kinase (PI3K) family of kinases and a set of eight known inhibitors of human PI3K was tested for effects on a panel of kinetoplastid parasites, with several displaying submicromolar activity against *Leishmania* species and/or African trypanosomes in vitro. The most active of these compounds, NVP-BEZ235 (Novartis), also displayed in vivo activity, although this was not fully curative at the highest tolerated dose (Diaz-Gonzalez et al. 2011). Given that this study was performed with only a small set of inhibitors optimised for the human orthologues, this result is encouraging and should lead to structure-based optimisation.

Glycogen synthase kinase-3 (GSK-3) is another potential drug target for African trypanosomiasis therapy, as RNAi knockdown of either of two *T. b. brucei* isoforms led to growth arrest and morphological defects (Ojo et al. 2008). Recently, a set of 16,000 compounds from the Pfizer compound library, selected for inhibition of human GSK-3, was screened for inhibition of TbGSK-3 and for trypanocidal activity, identifying potent and selective compounds for further optimisation (Oduor et al. 2011).

The above shows that a drug development strategy targeting an essential trypanosomal kinase is possible. On the other hand, SCYX-5070, a 2,4-diaminopyrimidine identified by high-throughput screening of SCYNEXIS proprietary libraries against trypanosome viability, was found to bind preferentially to *T. brucei* mitogen-activated protein kinases (MAPKs) and cdc2-related kinases (CRKs), by chemical proteomics (Mercer et al. 2011). While these 2,4-diaminopyrimidines were promising lead compounds, they exhibited poor

in vitro permeability characteristics, making it unlikely they would be effective against the cerebral stage of trypanosomiasis; it was shown that 4-desamino analogues (i.e. 2-aminopyrimidines) had improved in vitro permeability but had lost in vivo trypanocidal activity (Perales et al. 2011). It is not yet clear which kinase(s) these compounds targeted for their trypanocidal activity, but CRK12 (a cdc2-related kinase) and ERK8 (an extracellular signal-related kinase) were identified as essential for *T. brucei* viability through genetic knockdown studies with RNAi (Mackey et al. 2011). Recently, a kinase-focused chemoproteomics strategy, using beads with a covalently attached mixture of kinase inhibitors identified up to 50 kinases in *T. b. brucei* lysates. Co-incubation with different concentrations of the inhibitors titrated the kinases from the beads, allowing the calculation of IC_{50} values, several of which were in the mid-nanomolar range (Urbaniak et al. 2012).

9.4 New Clinical Drug Candidates for HAT

9.4.1 Fexinidazole

Although fexinidazole is being hailed as potentially the first oral treatment for late-stage sleeping sickness, and came out of a screening for new nitroheterocycle trypanocides (Torreele et al. 2010), its curative action was first reported by Frank Jennings in 1983 (Jennings and Urquhart 1983). However, fexinidazole, a 2-substituted 5-nitroimidazole, the first new clinical candidate for treating advanced-stage sleeping sickness to enter clinical development for 30 years, entered Phase I in 2009 (Torreele et al. 2010), and is now ready to enter Phase II/III trials (http://www.dndi.org; Barrett and Croft 2012). Like nifurtimox, currently used in combination with eflornithine (NECT) against late-stage sleeping sickness, fexinidazole is an orally available nitroheterocycle capable of efficiently crossing the blood–brain barrier. However, whereas nifurtimox is too toxic to be used as a standalone drug (Pépin et al. 1992; Delespaux and De Koning 2007), fexinidazole appears to be safe and curative in acute and chronic (late stage) murine models and in Phase I clinical trials (Torreele et al. 2010; Kaiser et al. 2011). Indeed, even the perennial worry with nitrocompounds, about genotoxicity, has been allayed for fexinidazole (Tweats et al. 2012). Importantly, the drug also showed no cross-resistance with pentamidine, eflornithine or melarsoprol (Kaiser et al. 2011), although reciprocal cross-resistance with nifurtimox, with which it shares the same mechanism of action, is a potential concern, as is the ease by which stable resistance to the nitroheterocycles can be induced in the laboratory (Sokolova et al. 2010). It seems therefore imperative that fexinidazole, like nifurtimox, is introduced as a combination in order to improve efficacy and delay the onset of otherwise rapid resistance development which would also fatally undermine NECT. Combinations with suramin (Jennings and Urquhart 1983) and melarsoprol have previously been reported to be effective in chronic mouse models, in the case of melarsoprol using a single (!) topical administration (Jennings et al. 1996).

9.4.2 Benzoxaboroles

By far the newest chemical class of trypanocides in clinical development, the antiprotozoal activity of benzoxaboroles was only recently discovered in a collaboration of Anacor Pharmaceuticals, SCYNEXIS and DNDi, with the original screening identifying one compound with the promising in vitro activity of 0.12 μg/ml (Ding et al. 2010). Further medicinal chemistry and structure–activity analysis quickly led to the identification of further compounds with even better activity (0.02 μg/ml) (Ding et al. 2010), identifying oxaborole 6-carboxamides as particularly attractive leads based on in vitro and in vivo activity and pharmacokinetic properties (Nare et al. 2010). Most importantly, selected compounds including SCYX-6759 and AN3520 were curative in murine models of cerebral trypanosomiasis, without any overt toxicity in mice or non-human primates. The high level of penetration into the central nervous system was ascribed to a lack of interaction with the P-glycoprotein efflux transporters that are prevalent on the blood–brain barrier (Nare et al. 2010). Like fexinidazole, the oxaboroles are effective after oral administration, with doses of SCYX-7158 as low as 25 mg/kg once daily for 7 days giving 100 % efficacy in the late-stage model (Jacobs et al. 2011). SCYX-7158 toxicity was minimal in preclinical evaluation and entered First-in-Human studies (Phase I trial) in early 2012 (http://www.dndi.org; Barrett and Croft 2012). Meanwhile, the benzoxaboroles scaffold is being used to search for further antitrypanosomal strategies, leading to the development of chalcone–benzoxaborole hybrid molecules (Qiao et al. 2012), prompted by earlier reports of trypanocidal activity by chalcone compounds (Troeberg et al. 2000).

9.4.3 Aromatic Diamidines and Their Prodrugs

Pentamidine is the drug of choice to treat the first stage of *T. b. gambiense* infection. It is a water-soluble aromatic dicationic diamidine that is administered by intramuscular injection due to its poor absorption when administered orally. New approaches have focussed on modifying its structure to improve oral bioavailability. The Consortium for Parasitic Drug Development (CPDD; http://www.thecpdd.org) has screened in excess of 2,000 compounds, of which 517 displayed in vitro EC_{50} values below 200 nM against *T. b. brucei* as well as >1,000 selectivity compared to human cell lines (Mäser et al. 2012), showing the enormous promise this old scaffold still holds. There was much hope for an aromatic dicationic diamidine derivate, Pafuramidine (DB289), which is the oral methoxyamidine prodrug of furamidine (DB75, an aromatic diamidine and pentamidine analogue). DB289 displayed oral activity, curing early stage but not cerebral-stage trypanosomiasis models in rodents (Wenzler et al. 2009) and in nonhuman primates (Mdachi et al. 2009).

Pafuramidine was duly entered into clinical development by the CPDD, with funding from the Bill and Melinda Gates Foundation, as an oral treatment for early-stage gambiense sleeping sickness. Although it achieved the target cure rates in

phase III clinical trials, it ultimately failed due to the delayed manifestation of liver toxicity and renal insufficiency in a number of recipients from a retrospective phase I safety trial (Paine et al. 2010). It is likely that the methoxy prodrug strategy was at least in part responsible for the liver and renal toxicity, as the lipophilic compound would be trapped in these cells after conversion to the active compound, furamidine. The newer generation of aromatic diamidines has thus dropped the prodrug approach. An aza analogue of furamidine, CPD0802 (DB829), was curative in murine and vervet monkey models of late-stage HAT (Wenzler et al. 2009; Mdachi et al. 2009; Paine et al. 2010). Importantly, the accumulation of CPD0802 in kidney cells is tenfold lower than previously found with furamidine (Barrett and Croft 2012). The high potency of these compounds supports their further pre-clinical evaluation and development.

9.5 New Drug Delivery Systems

New therapeutic approaches for chemotherapy treatment have also been focussed on the design of different drug delivery systems, such as polymeric nanoparticles, liposomes, micelles or cyclodextrins. Cyclodextrins are cyclic oligosaccharide molecules composed of six or more glycopyranose units linked by α-1,4 glycosidic bonds that have been used to improve the solubility and to reduce toxicity of a wide variety of drugs (Loftsson and Duchene 2007). A recently proposed new strategy for HAT treatment was to occlude melarsoprol into cyclodextrins to improve its water solubility. The melarsoprol–cyclodextrin complex, given orally, was able to cure the late-stage mouse model of trypanosomiasis without any signs of toxicity (Rodgers et al. 2011). The normal route for melarsoprol administration is intravenously suspended in propylene glycol, which leads to destruction of the veins and tissue necrosis at the inject site and severe reactive encephalopathy in some 10 % of patients (Atougia and Kennedy 2000).

Another drug-targeting strategy that was recently developed, and even much more specific, is to conjugate the active substance onto a nanobody raised against a specific pathogen (Stijlemans et al. 2004). Nanobodies are small antibody fragments derived from heavy-chain camelid antibodies that can be used to target biologically active components (Nguyen et al. 2001; Muyldermans 2001). For instance, nanobody NbAn33, which binds specifically to a conserved epitope of the VSG of *T. brucei*, is an excellent candidate to target trypanosomes (Stijlemans et al. 2004; Baral et al. 2006). The proof of this concept was the specific targeting of the active principle of the human serum trypanolytic factor, apolipoprotein L1 to trypanosomes in vivo, resulting in curative effects with both human serum-sensitive and -resistant strains (Baral et al. 2006). Considerable efforts are ongoing to exploit this principle for specific drug delivery to trypanosomes, which is expected to drastically reduce toxicity and may bypass some drug resistance mechanisms, particularly those associated with drug uptake (Unciti-Broceta, Garcia Salcedo et al. unpublished results).

Polymeric nanoparticles are another drug delivery system frequently used for targeting therapeutic agents. They are biodegradable and biocompatible colloidal particles in the range of 10–1,000 nm in size, where the drug can be entrapped, dissolved, attached or encapsulated. These effective and flexible targeted drug delivery systems represent alternative therapeutic approaches to the current ways of administrating anti-trypanosome therapies, even when using the same active principle. One example in antiprotozoal chemotherapy is the use of liposomal (AmBisome®) and colloidal dispersion (Amphocil®) formulations of amphotericin B, which has vastly improved the utility, and decreased the toxicity, of amphotericin B (Singh et al. 2012).

9.6 Going Forward

The above has sought to illustrate that the progress towards new treatment options for African trypanosomiasis is now more promising than ever, with some half dozen good leads being actively developed in late preclinical or early clinical development, backed by organisations dedicated to new drugs for neglected diseases. This is encouraging indeed but it is most important that this does not lead to complacency: the problem has *not* been solved. The most obvious consideration here is that none of the new chemical entities or formulations have as yet passed rigorous clinical trials. The example of pafuramidine serves to remind us that even very promising new compounds can fall at the last hurdle. Furthermore, African trypanosomiasis is a complex of diseases, in many different hosts, involving multiple species of trypanosome and different disease stages—all with distinct pharmacological requirements. "African trypanosomiasis" will therefore not be cured with one new drug. There is a need for an oral early stage treatment that can safely be prescribed locally, without the need for hospitalisation, and for paediatric formulations; there is an urgent need for a more effective and safer treatment of late stage HAT that can be used against both *T. b. gambiense* and *T. b. rhodesiense* infections and finally an urgent need for new curative and prophylactic veterinary trypanosomiasis medicines. With the current decline in patient numbers across much of Africa there is a risk of disengagement by researchers and funders alike, before the battle is won. It is hoped that this time around the international community will not let this happen and that new treatments will help in a push towards the eradication of this neglected disease.

References

Abdulla MH, O'Brien T, Mackey ZB et al (2008) RNA interference of *Trypanosoma brucei* cathepsin B and L affects disease progression in a mouse model. PLoS Negl Trop Dis 2:e298

Allen CL, Goulding D, Field MC (2003) Clathrin-mediated endocytosis is essential in Trypanosoma brucei. EMBO J 22:4991–5002

Alsford S, Eckert S, Baker N, Glover L, Sanchez-Flores A, Leung K-F, Turner DJ, Field MC, Berriman M, Horn D (2012) High-throughput decoding of anti-trypanosomal drug efficacy and resistance. Nature 482:232–236

Ang KKH, Ratnam J, Gut J et al (2011) Mining a cathepsin inhibitor library for new antiparasitic drug leads. PLoS Negl Trop Dis 5:e1023

Atougia JLM, Kennedy PGE (2000) Neurological aspects of human trypanosomiasis. In: Le D, Kennedy PGE (eds) Infectious diseases of the nervous system. Butterowrth-Heinemann, Oxford, pp 321–372

Baker N, Alsford S, Horn D (2011) Genome-wide RNAi screens in African trypanosomes identify the nifurtimox activator NTR and the eflornithine transporter AAT6. Mol Biochem Parasitol 176:55–57

Baker N, Glover L, Munday JC et al (2012) Aquaglyceroporin 2 controls susceptibility to melarsoprol and pentamidine in African trypanosomes. Proc Natl Acad Sci USA 109:10996–11001

Baker N, de Koning HP, Mäser P, Horn D (2013) Drug resistance in African trypanosomiasis: the melarsoprol and pentamidine story. Trends Parasitol 29:110–118

Baral TN, Magez S, Stijlemans B et al (2006) Experimental therapy of African trypanosomiasis with a nanobody-conjugated human trypanolytic factor. Nat Med 12:580–584

Barquilla A, Crespo JL, Navarro M (2008) Rapamycin inhibits trypanosome cell growth by preventing TOR complex 2 formation. Proc Natl Acad Sci USA 105:14579–14584

Barrett MP, Croft SL (2012) Management of trypanosomiasis and leishmaniasis. Br Med Bull 104:175–196

Bassarak B, Uzcátegui NL, Schönfeld C, Duszenko M (2011) Functional characterization of three aquaglyceroporins from *Trypanosoma brucei* in osmoregulation and glycerol transport. Cell Physiol Biochem 27:411–420

Basselin M, Denise H, Coombs GH, Barrett MP (2002) Resistance to pentamidine in *Leishmania mexicana* involves exclusion of the drug from the mitochondrion. Antimicrob Agents Chemother 46:3731–3738

Beitz E (2005) Aquaporins from pathogenic protozoan parasites: structure, function and potential for chemotherapy. Biol Cell 97:373–383

Beitz E, Wu B, Holm LM, Schultz JE, Zeuthen T (2006) Point mutations in the aromatic/arginine region in aquaporin 1 allow passage of urea, glycerol, ammonia, and protons. Proc Natl Acad Sci USA 103:269–274

Bhatnagar RS, Futterer K, Waksman G, Gordon JI (1999) The structure of myristoyl-CoA:protein N-myristoyltransferase. Biochim Biophys Acta 1441:162–172

Bosch F, Rosich L (2008) The contributions of Paul Ehrlich to Pharmacology: a tribute on the occasion of the centenary of his Nobel prize. Pharmacology 82:171–179

Bowyer PW, Tate EW, Leatherbarrow RJ et al (2008) N-Myristoyltransferase: a prospective drug target for protozoan parasites. ChemMedChem 3:402–408

Brand S, Cleghorn LAT, McElroy SP et al (2012) Discovery of a novel class of orally active trypanocidal N-myristoyltransferase inhibitors. J Med Chem 55:140–152

Bridges D, Gould MK, Nerima B et al (2007) Loss of the High Affinity Pentamidine Transporter is responsible for high levels of cross-resistance between arsenical and diamidine drugs in African trypanosomes. Mol Pharmacol 71:1098–1108

Browning CH, Morgan GT, Robb JVM, Walls LP (1938) The trypanocidal action of certain phenanthridinium compounds. J Pathol Bacteriol 46:203

Bruce D (1895) Preliminary report on the tsetse fly disease or nagana, in Zululand. Harrison & Sons, London

Carter NS, Fairlamb AH (1993) Arsenical-resistant trypanosomes lack an unusual adenosine transporter. Nature 361:173–176

Carter NS, Berger BJ, Fairlamb AH (1995) Uptake of diamidine drugs by the P2 nucleoside transporter in melarsen-sensitive and -resistant *Trypanosoma brucei brucei*. J Biol Chem 270:28153–28157

Chen YT, Lira R, Hansell E et al (2008) Synthesis of macrocyclic trypanosomal cysteine protease inhibitors. Bioorg Med Chem Lett 18:5860–5863

Clayton J (2010) Chagas disease: pushing through the pipeline. Nature 465:S12–S15

De Koning HP (2001) Uptake of pentamidine in *Trypanosoma brucei brucei* is mediated by three distinct transporters. Implications for crossresistance with arsenicals. Mol Pharmacol 59:586–592

De Koning HP (2008) The ever-increasing complexities of arsenical-diamidine cross-resistance in African trypanosomes. Trends Parasitol 24:345–349

De Koning HP, Stewart M, Anderson L et al (2004) The trypanocide diminazene aceturate is accumulated predominantly through the TbAT1 purine transporter; additional insights in diamidine resistance in African trypanosomes. Antimicrob Agents Chemother 48:1515–1519

De Koning HP, Gould MK, Sterk GJ et al (2012) Pharmacological validation of *Trypanosoma brucei* phosphodiesterases as novel drug targets. J Infect Dis 206:229–237

Delespaux V, De Koning HP (2007) Drugs and drug resistance in African trypanosomiasis. Drug Resist Updat 10:30–50

Delespaux V, De Koning HP (2013) Transporters in antiparasitic drug development and resistance. In: Jäger T, Koch O, Flohé L (eds) Trypanosomatid diseases: molecular routes to drug discovery. Wiley-VCH, Weinheim, Germany, pp 335–350

Delespaux V, Chitanga S, Geysen D et al (2006) SSCP analysis of the P2 purine transporter *TcoAT1* gene of *Trypanosoma congolense* leads to a simple PCR-RFLP test allowing the rapid identification of diminazene resistant stocks. Acta Trop 100:96–102

Delespaux V, Geysen D, Van den Bossche P, Geerts S (2008) Molecular tools for the rapid detection of drug resistance in animal trypanosomes. Trends Parasitol 245:236–242

Diaz-Gonzalez R, Kuhlmann FM, Galan-Rodriguez C et al (2011) The susceptibility of trypanosomatid pathogens to PI3/mTOR kinase inhibitors affords a new opportunity for drug repurposing. PLoS Negl Trop Dis 5:e1297

Ding D, Zhao Y, Meng Q et al (2010) Discovery of novel benzoxaborole-based potent antitrypanosomal agents. ACS Med Chem Lett 1:165–169

Duggan AJ (1970) An historical perspective. In: Mulligan HW (ed) The African trypanosomiases. George Allen & Unwin, London, pp 41–88

Farazi TA, Waksman G, Gordon JI (2001) The biology and enzymology of protein N-myristoylation. J Biol Chem 276:39501–39504

Fèvre EM, Coleman OG, Welburn SC, Maudlin I (2004) Reanalysing the 1900–1920 sleeping sickness epidemic in Uganda. Emerg Infect Dis 10:567573

Figarella K, Uzcategui NL, Zhou Y et al (2007) Biochemical characterization of *Leishmania major* aquaglyceroporin LmAQP1: possible role in volume regulation and osmotaxis. Mol Microbiol 65:1006–1017

Frearson JA, Brand S, McElroy SP et al (2010) N-myristoyltransferase inhibitors as new leads to treat sleeping sickness. Nature 464:728–732

Friedheim EA (1949) Mel B in the treatment of human trypanosomiasis. Am J Trop Med Hyg 29:173–180

Geerts S, Holmes PH, Eisler MC, Diall O (2001) African bovine trypanosomiasis: the problem of drug resistance. Trends Parasitol 17:25–28

Gorbal B, Sonuc N, Bhattacharjee H et al (2004) Drug uptake and modulation of drug resistance in *Leishmania* by an aquaglyceroporin. J Biol Chem 279:31010–31017

Gould MK, De Koning HP (2011) Cyclic nucleotide signalling in protozoa. FEMS Microbiol Rev 35:515–541

Greenbaum DC, Mackey Z, Hansell E et al (2004) Synthesis and structure-activity relationships of parasiticidal thiosemicarbazone cysteine protease inhibitors against *Plasmodium falciparum*, *Trypanosoma brucei*, and *Trypanosoma cruzi*. J Med Chem 47:3212–3219

Hua S, To WY, Nguyen TT et al (1996) Purification and characterization of proteasomes from *Trypanosoma brucei*. Mol Biochem Parasitol 78:33–46

Jackson AP, Allison HC, Barry JD et al (2013) A cell-surface phylome for African trypanosomes. PLoS Negl Trop Dis 7:e2121

Jacobs WA, Heidelberger M (1919) Aromatic arsenic compounds V. N-substituted glycylarsanilic acids. J Am Chem Soc 41:1809–1821

Jacobs RT, Nare B, Wring SA et al (2011) SCYX-7158, an Orally-Active Benzoxaborole for the Treatment of Stage 2 Human African Trypanosomiasis. PLoS Negl Trop Dis 5:e1151

Jansen C, Wang H, Kooistra AJ et al (2013) Discovery of Novel *Trypanosoma brucei* Phosphodiesterase B1 Inhibitors by Virtual Screening against the Unliganded TbrPDEB1 Crystal Structure. J Med Chem 56:2087–2096

Jennings FW, Urquhart GM (1983) The use of the 2 substituted 5-nitroimidazole, Fexinidazole (Hoe 239) in the treatment of chronic *T. brucei* infections in mice. Z Parasitenkd 69:577–581

Jennings FW, Atouguia JM, Murray M (1996) Topical chemotherapy for experimental murine African CNS-trypanosomiasis: the successful use of the arsenical, melarsoprol, combined with the 5-nitroimidazoles, fexinidazole or MK-436. Trop Med Int Health 1:590–598

Kaiser M, Bray MA, Cal M et al (2011) Antitrypanosomal activity of fexinidazole, a new oral nitroimidazole drug candidate for treatment of sleeping sickness. Antimicrob Agents Chemother 55:5602–5608

Kaminsky R, Mäser P (2000) Drug resistance in African trypanosomes. Curr Opin Anti-infect Investig Drugs 2:76–82

Kathiravan MK, Salake AB, Chothe AS et al (2012) The biology and chemistry of antifungal agents: a review. Bioorg Med Chem 20:5678–5698

Keiser J, Ericsson O, Burri C (2000) Investigations of the metabolites of the trypanocidal drug melarsoprol. Clin Pharmacol Ther 67:478–488

Kuepfer I, Schmid C, Allan M et al (2012) Safety and efficacy of the 10-day melarsoprol schedule for the treatment of second stage rhodesiense sleeping sickness. PLoS Negl Trop Dis 6:e1695

Kunz S, Beavo JA, D'Angelo MA et al (2006) Cyclic nucleotide specific phosphodiesterases of the kinetoplastida: a unified nomenclature. Mol Biochem Parasitol 145:133–135

Lanteri CA, Tidwell RR, Meshnick SR (2008) The mitochondrion is a site of trypanocidal action of the aromatic diamidine DB75 in bloodstream forms of *Trypanosoma brucei*. Antimicrob Agents Chemother 52:875–882

Legros D, Evans S, Maiso F et al (1999) Risk factors for treatment failure after melarsoprol for Trypanosoma brucei gambiense trypanosomiasis in Uganda. Trans R Soc Trop Med Hyg 93:439–442

Lepesheva GI, Waterman MR (2007) Sterol 14α-demethylase cytochrome P450 (CYP51), a P450 in all biological kingdoms. Biochim Biophys Acta 1770:467–477

Lepesheva GI, Ott RD, Hargrove TY et al (2007) Sterol 14α-demethylase as a potential target for antitrypanosomal therapy: enzyme inhibition and parasite cell growth. Chem Biol 14:1283–1293

Lepesheva GI, Hargrove TY, Kleshchenko Y, Nes WD, Villalta F, Waterman MR (2008) CYP51: a major drug target in the cytochrome P450 superfamily. Lipids 43:1117–1125

Lepesheva GI, Hargrove TY, Anderson S et al (2010a) Structural insights into inhibition of sterol 14alpha-demethylase in the human pathogen *Trypanosoma cruzi*. J Biol Chem 285:25582–25590

Lepesheva GI, Park HW, Hargrove TY et al (2010b) Crystal structures of *Trypanosoma brucei* sterol 14α-demethylase and implications for selective treatment of human infections. J Biol Chem 285:1773–1780

Lepesheva GI, Villalta F, Waterman MR (2011) Targeting *Trypanosoma cruzi* sterol 14α-demethylase (CYP51). Adv Parasitol 75:65–87

Leslie M (2011) Drug developers finally take aim at a neglected disease. Science 333:933–935

Li H, Chen H, Steinbronn C et al (2011) Enhancement of proton conductance by mutations of the selectivity filter of aquaporin-1. J Mol Biol 407:607–620

Loftsson T, Duchene D (2007) Cyclodextrins and their pharmaceutical applications. Int J Pharm 329:1–11

Lourie EM, Yorke W (1937) Studies in Chemotherapy XVI: the trypanocidal action of synthalin. Ann Trop Med Parasitol 31:435–445
Mackey ZB, O'Brien TC, Greenbaum DC et al (2004) A cathepsin B-like protease is required for host protein degradation in *Trypanosoma brucei*. J Biol Chem 279:48426–48433
Mackey ZB, Koupparis K, Nishino M, McKerrow JH (2011) High-throughput analysis of an RNAi library identifies novel kinase targets in *Trypanosoma brucei*. Chem Biol Drug Des 78:454–463
Mallari JP, Shelat AA, Obrien T et al (2008) Development of potent purine-derived nitrile inhibitors of the trypanosomal protease TbcatB. J Med Chem 51:545–552
Mallari JP, Shelat AA, Kosinski A et al (2009) Structure-guided development of selective TbcatB inhibitors. J Med Chem 52:6489–6493
Mallari JP, Zhu F, Lemoff A et al (2010) Optimization of purine-nitrile TbcatB inhibitors for use in vivo and evaluation of efficacy in murine models. Bioorg Med Chem 18:8302–8309
Mäser P, Sütterlin C, Kralli A, Kaminsky R (1999) A nucleoside transporter from *Trypanosoma brucei* involved in drug resistance. Science 285:242–244
Mäser P, Wittlin S, Rottman M et al (2012) Antiparasitic agents: new drugs on the horizon. Curr Opin Pharmacol 12:562–566
Matovu E, Stewart M, Geiser F et al (2003) The mechanisms of arsenical and diamidine uptake and resistance in *Trypanosoma brucei*. Eukaryot Cell 2:1003–1008
Mdachi RE, Thuita JK, Kagira JM et al (2009) Efficacy of the novel diamidine compound 2,5-Bis (4-amidinophenyl)- furan-bis-O-Methlylamidoxime (Pafuramidine, DB289) against Trypanosoma brucei rhodesiense infection in vervet monkeys after oral administration. Antimicrob Agents Chemother 53:953–957
Mercer L, Bowling T, Perales J et al (2011) 2,4-Diaminopyrimidines as potent inhibitors of *Trypanosoma brucei* and identification of molecular targets by a chemical proteomics approach. PLoS Negl Trop Dis 5:e956
Mills E, Price HP, Johner A et al (2007) Kinetoplastid PPEF phosphatases: dual acylated proteins expressed in the endomembrane system of *Leishmania*. Mol Biochem Parasitol 152:22–34
Morty RE, Troeberg L, Pike RN et al (1998) A trypanosome oligopeptidase as a target for the trypanocidal agents pentamidine, diminazene and suramin. FEBS Lett 433:251–256
Morty RE, Lonsdale-Eccles JD, Morehead J et al (1999) Oligopeptidase B from *Trypanosoma brucei*, a new member of an emerging subgroup of serine oligopeptidases. J Biol Chem 274:26149–26156
Morty RE, Troeberg L, Powers JC et al (2000) Characterisation of the antitrypanosomal activity of peptidyl alpha-aminoalkyl phosphonate diphenyl esters. Biochem Pharmacol 60:1497–1504
Morty RE, Lonsdale-Eccles JD, Mentele R et al (2001) Trypanosome-derived oligopeptidase B is released into the plasma of infected rodents, where it persists and retains full catalytic activity. Infect Immun 69:2757–2761
Munday JC, Rojas López KE, Eze AA et al (2013) Functional expression of *TcoAT1* reveals it to be a P1-type nucleoside transporter with no capacity for diminazene uptake. Int J Parasitol Drugs Drug Resist 3:69–76
Muyldermans S (2001) Single domain camel antibodies: current status. J Biotechnol 74:277–302
Nare B, Wring S, Bacchi C et al (2010) Discovery of novel orally bioavailable oxaborole 6-carboxamides that demonstrate cure in a murine model of late-stage central nervous system African trypanosomiasis. Antimicrob Agents Chemother 54:4379–4388
Nguyen VK, Desmyter A, Muyldermans S (2001) Functional heavy-chain antibodies in Camelidae. Adv Immunol 79:261–296
O'Brien TC, Mackey ZB, Fetter RD et al (2008) A parasite cysteine protease is key to host protein degradation and iron acquisition. J Biol Chem 283:28934–28943
Oberholzer M, Marti G, Baresic M et al (2007) The *Trypanosoma brucei* cAMP phosphodiesterases TbrPDEB1 and TbrPDEB2: flagellar enzymes that are essential for parasite virulence. FASEB J 21:720–731

Ochiana SO, Gustafson A, Bland ND et al (2012) Synthesis and evaluation of human phosphodiesterases (PDE) 5 inhibitor analogs as trypanosomal PDE inhibitors. Part 2. Tadalafil analogs. Bioorg Med Chem Lett 22:2582–2584

Oduor RO, Ojo KK, Williams GP et al (2011) *Trypanosoma brucei* glycogen synthase kinase-3, a target for anti-trypanosomal drug development: a public-private partnership to identify novel leads. PLoS Negl Trop Dis 5:e1017

Ojo KK, Gillespie JR, Riechers AJ et al (2008) Glycogen synthase kinase 3 is a potential drug target for African trypanosomiasis therapy. Antimicrob Agents Chemother 52:3710–3717

Olego-Fernandez S, Vaughan S, Shaw MK et al (2009) Cell morphogenesis of Trypanosoma brucei requires the paralogous, differentially expressed calpain-related proteins CAP5.5 and CAP5.5V. Protist 160:576–590

Orrling KM, Jansen C, Vu XL et al (2012) Catechol pyrazolinones as trypanocidals: fragment-based design, synthesis, and pharmacological evaluation of nanomolar inhibitors of trypanosomal phosphodiesterase B1. J Med Chem 55:8745–8756

Paine MF, Wang M, Boykin D et al (2010) Diamidines for human African trypanosomiasis. Curr Opin Investig Drugs 11:876–883

Parsons M, Worthey EA, Ward PN, Mottram JC (2005) Comparative analysis of the kinomes of three pathogenic trypanosomatids: *Leishmania major*, *Trypanosoma brucei* and *Trypanosoma cruzi*. BMC Genomics 6:127

Pépin J, Milord F, Meurice F et al (1992) High-dose nifurtimox for arseno-resistant *Trypanosoma brucei gambiense* sleeping sickness: an open trial in central Zaire. Trans R Soc Trop Med Hyg 86:254–256

Perales JB, Freeman J, Bacchi CJ et al (2011) SAR of 2-amino and 2,4-diamino pyrimidines with in vivo efficacy against *Trypanosoma brucei*. Bioorg Med Chem Lett 21:2816–2819

Phillips MA, Wang CC (1987) A *Trypanosoma brucei* mutant resistant to alpha-difluoromethylornithine. Mol Biochem Parasitol 22:9–17

Price HP, Menon MR, Panethymitaki C et al (2003) Myristoyl-CoA:protein N-myristoyltransferase, an essential enzyme and potential drug target in kinetoplastid parasites. J Biol Chem 278:7206–7214

Price HP, Panethymitaki C, Goulding D, Smith DF (2005) Functional analysis of TbARL1, an *N*-myristoylated Golgi protein essential for viability in bloodstream trypanosomes. J Cell Sci 118:831–841

Price HP, Stark M, Smith DF (2007) *Trypanosoma brucei* ARF1 plays a central role in endocytosis and Golgi-lysosome trafficking. Mol Biol Cell 18:864–873

Price HP, Guther ML, Ferguson MA, Smith DF (2010) Myristoyl-CoA:protein N-myristoyltransferase depletion in trypanosomes causes avirulence and endocytic defects. Mol Biochem Parasitol 169:55–58

Qiao Z, Wang Q, Zhang F et al (2012) Chalcone-benzoxaborole hybrid molecules as potent antitrypanosomal agents. J Med Chem 12:3553–3557

Robays J, Nyamowala G, Sese C et al (2008) High failure rates of melarsoprol for sleeping sickness, Democratic Republic of Congo. Emerg Infect Dis 14:966–967

Roberts CW, McLeod R, Rice DW et al (2003) Fatty acid and sterol metabolism: potential antimicrobial targets in apicomplexan and trypanosomatid parasitic protozoa. Mol Biochem Parasitol 126:129–142

Rodgers J, Jones A, Gibaud S et al (2011) Melarsoprol cyclodextrin inclusion complexes as promising oral candidates for the treatment of human African trypanosomiasis. PLoS Negl Trop Dis 5:e1308

Schlitzer M (2009) Wirkstoffe zur Behandlung der Afrikanischen Schlafkrankheit. Im letzten Jahrhundert entwickelt. Pharm Unserer Zeit 6:552–558

Schumann Burkard G, Jutzi P, Roditi I (2011) Genome-wide RNAi screens in bloodstream form trypanosomes identify drug transporters. Mol Biochem Parasitol 175:91–94

Scory S, Caffrey CR, Stierhof YD et al (1999) *Trypanosoma brucei*: killing of bloodstream forms in vitro and in vivo by the cysteine proteinase inhibitor Z-phe-ala-CHN2. Exp Parasitol 91:327–333

Scory S, Stierhof YD, Caffrey CR, Steverding D (2007) The cysteine proteinase inhibitor Z-Phe-Ala-CHN2 alters cell morphology and cell division activity of *Trypanosoma brucei* bloodstream forms in vivo. Kinetoplastid Biol Dis 6:2

Scott AG, Tait A, Turner CM (1997) Trypanosoma brucei: lack of cross-resistance to melarsoprol in vitro by cymelarsan-resistant parasites. Exp Parasitol 86:181–190

Seebeck T, Sterk GJ, Ke H (2011) Phosphodiesterase inhibitors as a new generation of antiprotozoan drugs: exploiting the benefit of enzymes that are highly conserved between host and parasite. Future Med Chem 3:1289–1306

Shakur Y, De Koning HP, Ke H et al (2011) Therapeutic potential of phosphodiesterase inhibitors in parasitic diseases. Handb Exp Pharmacol 204:487–510

Singh N, Kumar M, Singh RK (2012) Leishmaniasis: current status of available drugs and new potential drug targets. Asian Pac J Trop Med 5:485–497

Sokolova AY, Wyllie S, Patterson S et al (2010) Cross-resistance to nitro drugs and implications for treatment of human African trypanosomiasis. Antimicrob Agents Chemother 54:2893–2900

Steverding D (2010) The development of drugs for treatment of sleeping sickness: a historical review. Parasit Vectors 3:15

Steverding D, Spackman RW, Royle HJ, Glenn RJ (2005) Trypanocidal activities of trileucine methyl vinyl sulfone proteasome inhibitors. Parasitol Res 95:73–76

Steverding D, Baldisserotto A, Wang X, Marastoni M (2011) Trypanocidal activity of peptidyl vinyl ester derivatives selective for inhibition of mammalian proteasome trypsin-like activity. Exp Parasitol 128:444–447

Stijlemans B, Conrath K, Cortez-Retamozo V et al (2004) Efficient targeting of conserved cryptic epitopes of infectious agents by single domain antibodies. African trypanosomes as paradigm. J Biol Chem 279:1256–1261

Torreele E, Bourdin Trunz B, Tweats D et al (2010) Fexinidazole – a new oral nitroimidazole drug candidate entering clinical development for the treatment of sleeping sickness. PLoS Negl Trop Dis 4:e923

Troeberg L, Chen X, Flaherty TM et al (2000) Chalcone, acyl hydrazide, and related amides kill cultured *Trypanosoma brucei brucei*. Mol Med 6:660–669

Tsukaguchi H, Meremowicz S, Morton CC, Hediger MA (1999) Fuctional and molecular characterization of the human neutral solute channel aquaporin-9. Am J Physiol 277:F685–F696

Tweats D, Bourdin Trunz B, Torreele E (2012) Genotoxicity profile of fexinidazole–^{4}/a drug candidate in clinical development for human African trypanomiasis (sleeping sickness). Mutagenesis 27:523–532

Unciti-Broceta JD, Maceira J, Morales S et al (2013) Nicotinamide inhibits the lysosomal cathepsin b-like protease and kills African trypanosomes. J Biol Chem 288:10548–10557

Urbaniak MD, Mathieson T, Bantscheff M et al (2012) Chemical proteomic analysis reveals the drugability of the kinome of *Trypanosoma brucei*. ACS Chem Biol 7:1858–1865

Urbina JA (2013) Ergosterol biosynthesis for the specific treatment of Chagas disease: from basic science to clinical trials. In: Jäger T, Koch O, Flohé L (eds) Trypanosomatid diseases: molecular routes to drug discovery. Wiley-VCH, Weinheim, Germany, pp 489–514

Uzcategui NL, Szallies A, Pavlovic-Djuranovic S et al (2004) Cloning, heterologous expression, and characterization of three aquaglyceroporins from *Trypanosoma brucei*. J Biol Chem 279:42669–42676

Vermelho AB, Giovanni-de-Simone S, d'Avila-Levy CM et al (2007) Trypanosomatidae peptidases: a target for drugs development. Curr Enzym Inhib 3:19–48

Vincent IM, Creek D, Watson DG et al (2010) A molecular mechanism for eflornithine resistance in African trypanosomes. PLoS Pathog 6:e1001204

Wang CC, Bozdech Z, Liu CL et al (2003) Biochemical analysis of the 20 S proteasome of *Trypanosoma brucei*. J Biol Chem 278:15800–15808

Wang H, Yan Z, Geng J et al (2007) Crystal structure of the *Leishmania major* phosphodiesterase LmjPDEB1 and insight into the design of the parasite-selective inhibitors. Mol Microbiol 66:1029–1038

Wang C, Ashton TD, Gustafson A et al (2012a) Synthesis and evaluation of human phosphodiesterases (PDE) 5 inhibitor analogs as trypanosomal PDE inhibitors. Part 1. Sildenafil analogs. Bioorg Med Chem Lett 22:2579–2581

Wang H, Kunz S, Chen G et al (2012b) Biological and structural characterization of *Trypanosoma cruzi* phosphodiesterase C and Implications for design of parasite selective inhibitors. J Biol Chem 287:11788–11797

Watkins TI, Woolfe G (1952) Effect of changing the quaternizing group on the trypanocidal activity of dimidium bromide. Nature 169:506

Wenzler T, Boykin DW, Ismail MA et al (2009) New treatment option for second-stage African sleeping sickness: in vitro and in vivo efficacy of aza analogs of DB289. Antimicrob Agents Chemother 53:4185–4192

Williams RA, Tetley L, Mottram JC, Coombs GH (2006) Cysteine peptidases CPA and CPB are vital for autophagy and differentiation in *Leishmania mexicana*. Mol Microbiol 61:655–674

Williamson J (1970) Review of chemotherapeutic and chemoprophylactic agents. In: Mulligan HW (ed) The African trypanosomiases. George Allen & Unwin, London, pp 125–221

Wragg WR, Washbourne K, Brown KN, Hill J (1958) Metamidium: a new trypanocidal drug. Nature 182:1005–1006

Yun O, Priotto GG, Tong J et al (2010) NECT is next: implementing the new drug combination therapy for *Trypanosoma brucei gambiense* sleeping sickness. PLoS Negl Trop Dis 4:e720

Zeuthen T, Wu B, Pavlovic-Djuranovic S et al (2006) Ammonia permeability of the aquaglyceroporins from *Plasmodium falciparum, Toxoplasma gondii* and *Trypansoma brucei*. Mol Microbiol 61:1598–1608

Zoraghi R, Seebeck T (2002) The cAMP-specific phosphodiesterase TbPDE2C is an essential enzyme in bloodstream form *Trypanosoma brucei*. Proc Natl Acad Sci USA 99:4343–4348

Animal African Trypanosomosis in Sub-Saharan Africa and Beyond African Borders

10

Boniface Namangala and Steven Odongo

Abstract

This chapter mainly focuses attention on trypanosome infections occurring in domestic animal species, with particular emphasis on those in the African continent. Specific trypanosome species cause three major disease syndromes within Africa namely *nagana*, surra and Dourine. *Nagana*, mainly affecting domestic ruminants, pigs and pets in sub-Saharan Africa, is caused by *Trypanosoma congolense*, *Trypanosoma vivax*, *Trypanosoma brucei* subspecies as well as *Trypanosoma simiae*, primarily transmitted by tsetse flies (*Glossina* species). Surra, caused by *Trypanosoma evansi*, mainly affects camels, horses, ruminants, pigs and dogs in North and East Africa. Beyond the African borders, surra occurs in the Middle East, Asia and Latin America. Unlike other trypanosome syndromes, Dourine is non-vector borne, but rather transmitted through coitus from stallions to mares and vice versa. It occurs worldwide wherever horses are reared in large numbers. Thus non-vector-borne trypanosomes have a wider geographical distribution beyond the African continent as they may also be spread through international trade.

Although some trypanosome species strictly cause disease to animals, others such as *T. b. rhodesiense* and *T. b. gambiense* cause disease to both animals and humans. In aggregate, trypanosome infections have serious socio-economic implications and significantly contribute to poverty and underdevelopment experienced in the affected regions where livestock production is the main

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livelihood of the local communities. As such, efforts towards effective control of the disease are justified.

10.1 Introduction

Agriculture and particularly livestock production are the main drivers of most of the sub-Saharan African economies. Indeed, the agricultural sector contributes significantly to the gross domestic products and employs the largest part of the populations in the region (Mukhebi and Perry 1992; Swallow 1999). The majority of the livestock is owned by small-scale pastoralists whose livelihoods revolve around their livestock (Mukhebi and Perry 1992; Simuunza et al. 2011). The livestock kept by such traditional farmers include cattle, goats, sheep, pigs, chickens, ducks, guinea fowls, camels and donkeys. Cattle, and to a lesser extent small ruminants, are the principal livestock reared by most of the small-scale pastoralists. Livestock production is critical as a source of the much needed protein (meat, eggs milk and dairy products), income, draught power as well as manure for enhanced crop production. Animal hide and fur are important in manufacturing industries. Livestock is also used as a medium of exchange and may further be kept just for prestige. As such, any level of impediment to livestock production has serious consequences on the livelihoods of local communities in sub-Saharan Africa. Unfortunately, however, livestock production faces several challenges, most of which are as a result of animal diseases caused by viruses, bacteria, protozoa, fungi, ecto- and endo-parasites. Vector-borne diseases, including trypanosomosis, theileriosis, babesiosis and anaplasmosis are among the major livestock diseases in sub-Saharan Africa (Mukhebi and Perry 1992; Simuunza et al. 2011; Kristjanson et al. 1999; Stich et al. 2002). This chapter will address animal African trypanosomosis (AAT), its distribution and the possible impacts on livestock productivity.

10.2 Animal African Trypanosomosis

Despite several decades of research, AAT remains one of the major vector-borne diseases with serious impediment to agricultural and economic advancement in sub-Saharan Africa (Coustou et al. 2012; Morrison and MacLeod 2011). It is caused by various trypanosome species mainly transmitted through the saliva of tsetse flies during a blood meal. Trypanosomes are unusual among the haemoprotozoan parasites in that in the mammalian host, they are completely covered by a thick immunogenic coat called variant surface glycoprotein (VSG) (Borst and Rudenko 1994). Moreover, these parasites constantly modify their VSG by the process of antigenic variation that enables them to evade immune destruction but which is also responsible for the current challenge to design an effective vaccine against trypanosomosis. Infection with trypanosomes is also associated with suppression

of the host lymphocyte blastogenesis in response to parasite-related and -unrelated antigens. Such trypanosome-induced immunosuppression has been amply documented in livestock (Flynn and Sileghem 1991) and mice (De Baetselier 1996). This has been linked with increased susceptibility to secondary infections and decreased responsiveness to vaccination (Sileghem et al. 1994; Namangala et al. 2000; Radwanska et al. 2008). There is cumulative evidence that macrophages of a suppressive phenotype elicited during AAT are the central effector cells in the inhibition of lymphoproliferative responses to antigens and mitogens (De Baetselier 1996; Namangala 2011).

Tsetse flies, only found in Africa, belong to the genus *Glossina* within which three groups are recognised on the basis of their preference for habitat: (1) the riverine (*palpalis*) group (2) the forest (*fusca*) group and (3) the savannah (*morsitans*) group (Manful et al. 2010). AAT is mainly transmitted by *G. morsitans* (savannah species), *G. palpalis* (riverine species) and *G. fusca* (forest species). Other tsetse species that may transmit AAT include *G. pallidepes*, *G. austeni*, *G. swynnertoni* (savannah species); *G. longipennis*, *G. brevipalpalis* (forest species) and *G. tachnoides* (riverine species). Tsetse flies mainly inhabit thickets, particularly in national parks and game management areas where they primarily feed on wildlife habouring a number of different trypanosome species but do not suffer pathogenic consequences of AAT (Anderson et al. 2011). As such, they are referred to as "trypanotolerant" and serve as the major reservoirs of trypanosomes for domestic animals and humans (Namangala 2011). In the same vein, some West African cattle breeds such as the N'Dama, are also trypanotolerant and thus remain productive and gain weight even when they are infected with African trypanosomes (Taylor 1998). Chronically and sub clinically infected domestic animals may also serve as trypanosome reservoirs. During epidemics, tsetse flies and other haematophagous arthropods may also pick trypanosomes from acutely infected individuals with massive parasitosis to susceptible ones within the same vicinity.

Domestic animals get infected following encroachment of humans and their animals near or into tsetse-infested areas. Tsetse flies may thus pick bloodstream trypomastigotes from wildlife and transmit them as metacyclics to such domestic animal, usually with fatal consequences. Larger animals such as cattle (mainly adult cows and oxen), possibly due to the larger odour plumes they produce, and indeed their greater availability as compared to others livestock, are more attractive to tsetse flies than smaller animals that are usually kept in fewer numbers (Torr and Mangwiro 2000; Torr et al. 2006; Simukoko et al. 2007). Thus, in a given pasture where several domestic animals graze, their susceptibility to AAT is influenced by their size and age. As such, cattle tend to shield smaller and fewer livestock from AAT in most African communities. AAT is usually debilitating to affected animals. Although there are more reports on the impact of AAT in cattle, being the main livestock reared in sub-Saharan Africa, AAT is also a major constraint to the productivity of several other domestic animals including small ruminants, pigs, horses, camels and dogs (Snow et al. 1996; Matete 2003; Dhollander et al. 2006; Simukoko et al. 2007; Salim et al. 2011).

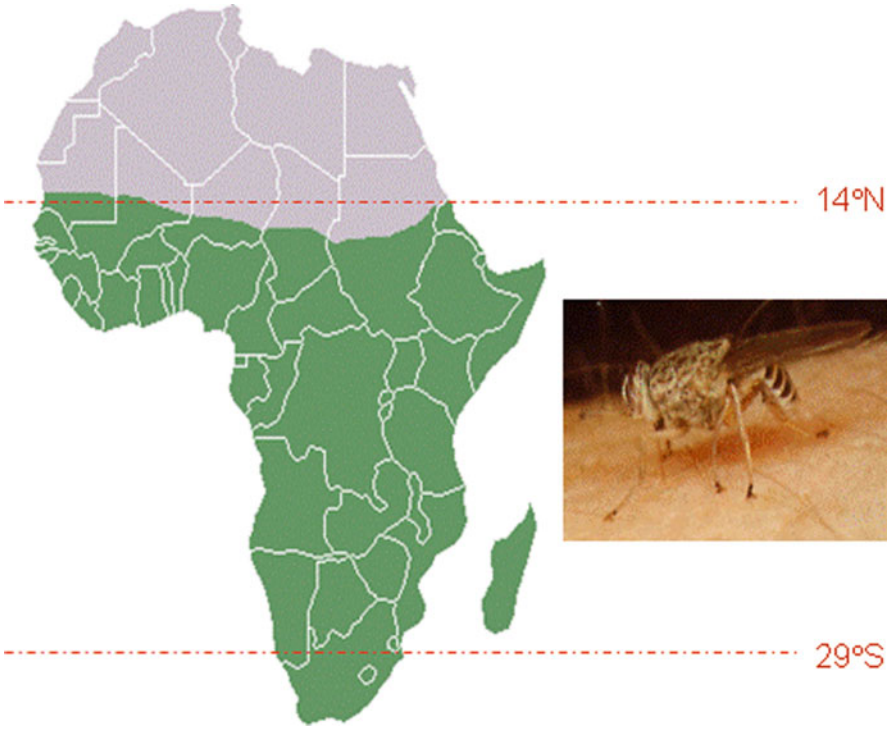

Fig. 10.1 Tsetse-infested sub-Saharan Africa extending between latitude 14° North and 29° South of the Equator (except for Madagascar and most of the Republic of South Africa below Latitude 29° South)

10.3 Aetiology, Transmission and Geographical Distribution

Unlike human African trypanosomiasis that occurs in specific foci within the 20 countries in sub-Saharan Africa, AAT is much more widespread in distribution. AAT occurs literally wherever there is livestock being reared in sub-Saharan Africa within a tsetse-infested region stretching 14° North and 29° South of the equator, affecting about 40 countries (Cattand 2001; Fig. 10.1). Depending on the trypanosome species, there are three major syndromes of AAT: (1) *Nagana*, (2) Surra and (3) Dourine (Table 10.1). Chagas disease, the fourth trypanosomosis syndrome, occurs outside the African continent, in Latin America.

10.3.1 Nagana

Nagana, mainly caused by the tsetse-transmitted *Trypanosoma congolense* (subgenus *Nannomonas*) followed by *Trypanosoma vivax* (subgenus *Duttonella*) and to a lesser extent *Trypanosoma brucei* subspecies (subgenus *Trypanozoon*), is the major

Table 10.1 Major characteristic features of animal trypanosomosis syndromes

	Trypanosomosis syndrome			
Parameter	*Nagana*	Surra	Dourine	Chagas disease
Aetiology	*T. congolense*, *T. vivax*, *T. brucei subspecies*, *T. simiae*	*T. evansi*	*T. equiperdum*	*T. cruzi*
Transmission	Mainly by tsetse flies; other biting flies may play a role	Biting flies	By coitus, non-vector-borne,	Blood-sucking triatomine bugs
Main susceptible domestic animals	Cattle, small ruminants, pigs, horses, dogs	Camels, horses, mules, donkeys, domestic ruminants, Indian elephants, dogs, cats	Horses, donkeys	Dogs, cats, pigs, rodents
Main clinical signs	Intermittent fever, emaciation, anaemia, abortion, lymphadenopathy, oedema of the throat and ventrum, ocular discharge	As for *nagana*	Mucopurulent discharge from genitalia, oedema of the genitalia, surrounding tissues and ventrum, anaemia, hyperaemia, skin plaques, nervous symptoms	Sudden death, lethargy, tachycardia, exercise intolerance, fever, anorexia, lymphadenopathy, diarrhoea
Geographical distribution	Sub-Saharan Africa	North and North-East Africa, Middle and Far East, Mexico, Central and South America	In all the continents wherever large numbers of horses are reared	Mexico, Central and South America

AAT syndrome affecting cattle, small ruminants, horses, pigs and dogs, in sub-Saharan Africa (Table 10.1). Although AAT affects several domestic animals, it is of major importance in cattle, being the dominant animals reared in sub-Saharan Africa. *Nagana* is a Zulu word that means "to be in low or depressed spirit", describing the debilitation or wasting associated with AAT (Stephen 1986). *Trypanosoma congolense* accounts for more than 80 % of AAT in domesticated animals (cattle, goats, sheep, horses, pigs, dogs) in Southern, East and Central Africa and the losses associated with the disease (Van den Bossche et al. 2006; Simukoko et al. 2007; Laohasinnarong et al. 2011; Tafese et al. 2012). Even in West Africa, *T. congolense* remains one of the major causes of AAT in livestock (Nakayima et al. 2012; Takeet et al. 2012). Clinical signs of *T. congolense*-infected animals are aspecific and include intermittent fever, abortion, cachexia, anaemia, lympadenopathy, lethargy, anorexia, oedema of the throat, ventrum and forelimbs, ocular discharge and death (Taylor 1998).

Trypanosoma congolense is further classified into three different types: Savannah, Forest and Kilifi (Young and Godfrey 1983; Knowles et al. 1988). *Trypanosoma congolense* strains within the Savannah type are regarded to be the most pathogenic/virulent and widespread throughout the savannah ecosystem of sub-Saharan Africa (Bengaly et al. 2002). Whereas *T. congolense* Forest occurs in humid forest ecosystems of West, Central and East Africa, *T. congolense* Kilifi seems to be more restricted to East Africa and to a lesser extent in Southern Africa (Mamabolo et al. 2009). Although *T. congolense* is cyclically transmitted by members of the *Glossina* species, mechanical transmission by other haematophagous arthropods has also been reported (Desquesnes and Dia 2003). The latter is important in that *T. congelense* may still cause AAT in non-tsetse-infested regions inhabited by other biting flies.

In endemic areas where livestock constitute the main reservoir of infection, low pathogenic *T. congolense* parasites circulate, resulting in more chronic AAT and generally low impact of the disease (Van den Bossche and Rowlands 2001; Chitanga et al. 2013). However, epidemics of *T. congolense* AAT may occur with severe impacts (increased animal mortalities and reduced calving rates) especially following recent introduction of livestock to tsetse-infested areas occurring at the livestock/wildlife interface areas. In such situations, tsetse flies or other haematophagous flies transmit high virulent *T. congolense* strains to domestic animals from wildlife reservoirs (Van den Bossche and Rowlands 2001; Chitanga et al. 2013).

Trypanosoma vivax is the second most important cause of *nagana* to ruminants, pigs and horses in sub-Saharan Africa (see also Chap. 11). AAT due to *T. vivax* is generally milder than the one caused by *T. congolense*. In West Africa, *T. vivax* accounts for the majority of AAT cases (Adam et al. 2012; Sow et al. 2012). *Trypanosoma vivax* may be transmitted mechanically by biting *Diptera*. *Trypanosoma vivax* is also a major cause of trypanosomosis (with occasional reports of epidemics), mainly in ruminants, in Latin America (Galiza et al. 2011).

Trypanosoma brucei brucei, cyclically transmitted by tsetse flies, is the most widely distributed African trypanosome, affecting virtually every domestic mammal including ruminants, horses and dogs. Horses and dogs are highly susceptible to *T. brucei* infection, as well as the human-infective *T. b. rhodesiense*, resulting in acute fulminant disease (Stephen 1986; Matete 2003; Dhollander et al. 2006). However, the pathogenicity of *T. b. brucei* and *T. b. rhodesiense* in ruminants is considered to be relatively low. Rather, ruminants are considered to be domestic reservoirs of these trypanosome subspecies (Fevre et al. 2001, 2005). On the other hand, humans are thought to be the main reservoirs of *T. b. gambiense*, although pigs, and to a lesser extent, sheep and goats, are implicated to be domestic animal reservoirs in West and Central Africa (Simo et al. 2006; Cordon-Obras et al. 2009).

Trypanosoma simiae (subgenus *Nannomonas*) causes an acute fatal disease mainly in pigs and to a lesser extent in camels and sheep in the tsetse-infested sub-Saharan Africa. Although the above trypanosome species and subspecies may cause monolytic infections in specific domestic animals, co-infections of a

combination of two or more different trypanosome species and/or subspecies are frequently reported in the field.

10.3.2 Surra

Surra is the trypanosome syndrome caused by *Trypanosoma evansi* (subgenus *Trypanozoon*). *Trypanosoma evansi*, mechanically transmitted by haematophagous *Diptera*, particularly those belonging to the *Tabanidae* family, was first discovered as the causative agent of surra in a horse in India by Griffith Evans in 1880. The local people recognised the disease as an occasional scourge which they termed "surra", denoting anything rotten (Stephen 1986). Surra, with similar clinical manifestations to *nagana*, is now known to mainly affect camels, horses and water buffalos. Donkeys, mules, goats, sheep, cattle, Indian elephants, dogs and cats are also susceptible to surra (Stephen 1986; Delafosse and Doutoum 2004; Dobson et al. 2009) (Table 10.1). The disease tends to be of much more economic importance in camels than in any other animal species. In equine species, horses are more susceptible to surra, followed by mules and donkeys. In dogs, the disease is usually acute with fatal consequences within a few days in the absence of treatment. In Africa, surra is confined to the northern and north-eastern part of the continent. Surra is also endemic in the Middle and Far East, Asia, Mexico, Central and South America (Stephen 1986; Delafosse and Doutoum 2004; Dobson et al. 2009).

10.3.3 Dourine

Dourine, a trypanosome syndrome of horses and donkeys, is a unique trypanosome syndrome in that it is non-vector borne. Instead, it is naturally transmitted by coitus from stallions to mares and vice versa (Stephen 1986). In stallions, clinical signs include oedema of the penis, scrotum, prepuce and surrounding skin up to the chest; anaemia; severe loss of weight; inguinal lymph nodes may be swollen; and moderate mucopurulent urethral discharge (Table 10.1). In the case of mares, clinical signs include vulval oedema with profuse fluid discharge; ulceration of vulva mucosa; oedema of the perineum, udder and abdominal floor; hyperemia; anaemia and emaciation. Nervous signs usually follow genital involvement and generalised loss of condition. Unlike in the case of *nagana* and surra, these clinical signs are pathognomonic.

Dourine is also referred to as "Covering Disease" or "Horse Syphilis", denoting its sexual transmission (Stephen 1986). The causative agent for dourine is *Trypanosoma equiperdum* (subgenus *Trypanozoon*). This parasite requires only the vertebrate host to complete its developmental cycle. Because of non-requirement of specific vectors for its transmission, it is widespread in its distribution, occurring in all regions of the world where large population of horses are found, including Europe, USA, Latin America, Africa and Asia (Alemu et al. 1997; Clausen et al. 2003) (Table 10.1).

10.3.4 Animal *T. cruzi* Trypanosomosis

T. cruzi infections or "American trypanosomiasis", referred to in humans as Chagas' disease, is a tropical parasitic zoonosis caused by a *Stercorarian* trypanosome. It is mainly found in the rural areas of Mexico, Central America and South America, where it is mostly transmitted to humans and domestic animals (mainly dogs, cats, pigs and rodents) by the infected faeces of triatomine bugs ("kissing bugs") during blood meal (Table 10.1). Clinical signs of Chagas disease are mainly seen in young animals. In these animals, the disease may be acute or chronic, with symptoms such as sudden death, tachycardia, exercise intolerance, lethargy, fever, anorexia, lymphadenopathy and diarrhoea. The chronic form may be completely asymptomatic. Wild and domestic animals, as well as subclinically and/or chronically infected humans, are natural reservoirs of *T. cruzi* (Bern et al. 2007). Originally, *T. cruzi* only affected wild animals. It later spread to domestic animals and people (Dias et al. 2002). Over 25 million people in Latin America are at risk of contracting Chagas disease (Dias et al. 2002; Bern et al. 2007). The resurgence of Chagas disease and its increasing geographic distribution is mainly due to large-scale population movements from rural to urban areas of Latin America and to other regions of the world. It may also be spread through blood transfusion, vertical transmission or organ donation.

10.4 Wild Animals as Reservoir of African Trypanosomosis

Wild animals are important reservoir of human and animal trypanosomes (Anderson et al. 2011). Infection of wild animals with animal trypanosomes occurs under experimental condition (Olubayo et al. 1991) and in nature (Anderson et al. 2011; Van den Bossche et al. 2011). Natural infections are encountered in African buffalos, duikers, bushbucks, impalas, greater kudus, warthogs, giraffes, elephants, zebras, lions and leopards (Anderson et al. 2011) supporting the occurrence of sylvatic–domestic transmission cycle (Swai and Kaaya 2012). The frequency of transmission is high where buffer zones surrounding game reserves have been turned into grazing ground (Anderson et al. 2011). So far no effective strategy for controlling trypanosomiasis in wild animals exists. Tsetse control is a strategy most applicable in this situation. However, the use of fly traps and insecticides for controlling tsetse vector is not feasible on large area.

10.5 Diagnosis of Anila Trypanosomosis

Unlike Dourine, field diagnosis of *nagana* and surra is difficult mainly because clinical and post-mortem signs of these AAT syndromes are not pathognomonic. Therefore diagnosis must rely on laboratory techniques that confirm the presence in blood of trypanosomes or the presence of anti-trypanosomal antibodies.

10.5.1 Clinical Signs and Post-mortem Findings

The course of trypanosomosis is variable depending on host and parasite factors. Generally clinical signs associated with *nagana* and surra in domestic animals include intermittent fever, wasting, lymphadenopathy, loss of condition, pallor, abortion in pregnant animals and lacrimation. Infected animals are usually weak and lethargic and hence lag behind the herd. The course of the infection may be acute (particularly when animals have just been introduced to a tsetse-infested area), subacute or chronic (mainly in endemic areas), with possible fatal consequences in the absence of intervention. Anaemia is thought to be the most pathogenic consequences of infection with African trypanosomes (Van den Bossche and Rowlands 2001; Taylor 1998). Furthermore, post-mortem examination of an animal that died of AAT reveal generalised carcass emaciation, enlarged lymph node, enlarged liver and petechial haemorrhages of the serosal membranes, especially in the peritoneal cavity. Although these findings could help veterinarians suspect AAT in a herd, they are not fully diagnostic as several other endemic disease conditions including malnutrition, tick-borne diseases, tuberculosis and intestinal helminths exhibit similar clinical signs and post-mortem findings to trypanosomosis.

10.5.2 Microscopy

The detection of circulating trypanosomes in host blood, cerebral spinal fluid and lymph biopsy by light microscopy is specific, definitive and is the most reliable applied method for diagnosis of AAT (Chappius et al. 2005). Body fluids may be directly examined as wet smears for trypanosome presence by light microscopy. The trypanosomes are detected by their movement among the blood cells. In addition, trypanosomes may be examined in Giemsa-stained thin or thick smears. Thin blood films preserve the morphology of trypanosomes and are useful in morphological differentiation of species (Büscher et al. 2009). Thick smears are useful for the detection of scanty trypanosomes.

However, the disadvantage of the above techniques is their low sensitivity, mainly in view of the fact that in endemic areas, parasitaemia in domestic animals naturally infected with trypanosomes tends to be low. Various concentration techniques, including body fluid centrifugation, aimed at improving the sensitivity of microscopy, are currently available. Animal blood in a heparinised (or EDTA) capillary tube may be centrifuged and the resultant buffy coat examined for the presence of trypanosomes (Waiswa and Katunguka-Rwakishaya 2004). Centrifugation has further advantages in that packed cell volume (PCV), used for anaemia determination, is simultaneously determined.

10.5.3 Xenodiagnosis

Xenodiagnosis is the inoculation of trypanosome-infected blood into laboratory animals such as mice, rats and rabbits. This technique can be used to detect some *T. congolense* and *T. brucei* (but not the non-rodent-adapted *T. vivax*) infections. The rodent inoculation technique may be more sensitive than microscopy, especially when the parasitaemia is scanty. However, its disadvantage is that not all trypanosome species, including some strains of *T. congolense* and *T. brucei*, become established in the rodents (Duleu et al. 2004).

10.5.4 Immunodiagnosis

Commonly used techniques in AAT serodiagnosis include the enzyme-linked immunosorbent assay (ELISA), complement fixation test (CFT), indirect fluorescent antibody test (IFAT) and card agglutination test (CATT) (Monzon et al. 2003). Serological tests are particularly useful in the diagnosis of Dourine and surra. However, the disadvantages of serodiagnosis are (1) strong cross-reactions among antibodies to various pathogenic trypanosome species (Desquesnes et al. 2001) and (2) no distinction can be made between the past (recovered animals) and present (active infection) infections, restricting their usefulness to measuring exposure. Antigen ELISA for detection of *T. congolense* was developed but later abandoned because of low specificity and sensitivity (Rebeski et al. 1999). The low sensitivity is caused by existence of circulating antigen in complex with host antibodies leaving few epitopes for attachment of monoclonal antibody used in the test (Rebeski et al. 1999). Therefore no commercial antigen ELISA for specific detection of *T. congolense* is available.

10.5.5 Molecular Diagnosis

Molecular techniques such as polymerase chain reaction (PCR) have significantly improved the sensitivity and accuracy of trypanosome diagnosis compared to the traditional parasitological methods (Chappius et al. 2005). For regular diagnosis, the sensitivity of PCR has increased to reach a level of 1 trypanosome/ml of blood, which may be up to thrice the sensitivity of microscopic observation of the buffy coat. PCR differentiates between trypanosome species and subspecies using specific primers (Desquesnes and Dávila 2002). However, the cost implications and requirement for highly skilled manpower are obstacles to their wide application in clinical settings in resource-limited sub-Saharan Africa. Advances in molecular methods of diagnosis have led to the development of a multispecies PCR capable of distinguishing all the major pathogenic trypanosomes of domestic animals in a single test, hence reducing the overall cost (Desquesnes and Dávila 2002). The internal transcribed spacer (ITS) region of rDNA is a preferred target for such a universal trypanosome test because of its highly conserved flanking regions and

size variability among trypanosome species and subspecies (Desquesnes et al. 2001; Desquesnes and Dávila 2002). Thus the ITS1-CF/ ITS1-BR and the KIN1/ KIN2 primers, both targeting the ITS1 of rDNA, offer promise in the routine diagnosis of pathogenic trypanosomes in clinical specimens from infected animals (Njiru et al. 2005).

Loop-mediated isothermal amplification (LAMP) is a novel strategy which amplifies DNA with high sensitivity and rapidity under isothermal conditions (60–65 °C), producing large quantities of DNA within 30–60 min (Notomi et al. 2000). LAMP has the advantage over PCR of being cheaper and simpler/easier to perform as it only requires a heating block or water bath and may hence be performed even in the field. Compared to PCR, the LAMP assay is also rapid; the whole process, including DNA extraction, can be done within an hour and the LAMP products can be visualised by naked eyes or through measurement of turbidity or fluorescence (Notomi et al. 2000; Thekisoe et al. 2007; Namangala et al. 2012) and may thus be more practical for resource-limited communities in sub-Saharan Africa where AAT is endemic.

10.6 Anaemia During Animal African Trypanosomosis

Anaemia is reportedly the most pathogenic consequence of infection with animal-infective trypanosomes (Logan-Henfrey et al. 1992). Thus all the three trypanosomosis syndromes (*nagana*, surra and Dourine) are associated with the development of anaemia in the affected animals. In particular, intravascular haematic trypanosomes such as *T. congolense*, *T. vivax* and *T. simiae* are associated with induction of severe anaemia (Losos and Ikede 1972). Although anaemia also occurs during infection with extravascular humoral trypanosomes (*T. brucei* complex and *T. evansi*), tissue degeneration and inflammation are the main pathologies associated with these species.

Although the exact mechanisms of anaemia during trypanosomosis largely remain unknown, they may involve interactions between the parasite molecules and host immune system, resulting in immunopathology. During trypanosome infection, a number of trypanosome stimuli and host immune mediators synergize to produce the haemophagocytic syndrome and the resultant anaemia (Naessens 2006). The aggressive parasitaemic waves occurring during early-stage trypanosomosis result in massive quantities of trypanosome molecules including soluble and membrane-bound VSG molecules in circulation, hyperactivation of macrophages (and concomitant production of tumour necrosis factor and interferon gamma) and other phagocytes which in turn lead to massive erythrophagocytosis in the spleen, liver and lymph nodes (Taylor 1998; Namangala 2011; Stijlemans et al. 2007). During chronic trypanosomosis, anaemia may be the result of insufficient haemopoiesis. Current evidence suggests that both non-specific erythrophagocytosis by hyperactivated phagocytic system and specific phagocytosis of damaged host cells contribute to anaemia (Taylor 1998). Erythrophagocytosis may

occur following deposition of immune complexes, together with C3 molecules, on erythrocytes or following generation of erythrocyte autoantibodies.

What could lead to the induction of auto-reactive antibodies during trypanosomosis? Firstly, this may be possible if trypanosomes share certain antigens with host erythrocytes. Members of the *Trypanosoma brucei* complex possess proteins that are similar to spectrin, a highly conserved structural protein occurring on erythrocyte membranes (Schneider et al. 1988). Evidence also suggests the existence of autoreactive antibodies that do not only recognise VSG antigens, but also human, murine and bovine epitopes (Müller et al. 1996). Furthermore, an increase in the levels of antibodies that bind to sheep and chicken erythrocytes has been observed in *T. vivax*- and *T. congolense*-infected cattle as well as *T. vivax*-infected mice (Musoke et al. 1981; Tabel et al. 1981; Mahan et al. 1986).

Secondly, current evidence suggests that *T. vivax* and *T. brucei* complex secrete enzymes including neuraminidases, proteases and phospholipases that may damage red blood cell membranes, exposing epitopes that are normally hidden (Taylor 1998). The latter will be recognised as "foreign" by the host immune system, resulting in the production of autoantibodies to the erythrocytes.

10.7 Impacts of Animal African Trypanosomosis

AAT is a major constraint to livestock productivity in about 40 sub-Saharan African countries, adversely affecting millions of people in rural communities who depend on livestock for their livelihoods (Coustou et al. 2012). Up to 11 million km^2 of potential grazing land in sub-Saharan Africa (nearly 50 % of total land in the region) inhabited by up to 300 million people is rendered unsuitable for livestock rearing (Mcdermott and Coleman 2001). AAT also has direct impacts on the number of livestock kept by farmers, the breed and species composition of the livestock herd and the grazing pattern of livestock (Swallow 1999). The potential benefits of improved AAT control on the continent, in terms of meat and milk productivity alone, are $700 million annually (Kristjanson et al. 1999). In aggregate, AAT significantly contribute to poverty and under-development in sub-Saharan Africa. The exact impact of AAT may not be measured accurately because of the many indirect effects the disease has to the affected communities and their livestock and the difficulty in carrying out such assessment. The following are some of the impacts AAT has on agricultural productivity mainly in rural sub-Saharan communities:

Livestock Mortality. AAT is detrimental to livestock productivity as it puts up to 50 million cattle and 70 million goats and sheep at risk, costing livestock producers and consumers up to $5 billion annually (Coustou et al. 2012). However, this is an under-estimation considering the numerous indirect losses encountered. Such loss does not only contribute to the continent's economic under-development but also deprive the affected communities of the much needed proteins from meat and milk.

Thus AAT reduces the availability of the animal draught power required for general transportation and particularly for crop cultivation, and further reduces the availability of cattle manure, both of which result in reduction in crop yield (Swallow 1999).

Reduction of Calving Rate. Recent data suggest that the largest and most consistent impacts of AAT are on birth rates and mortality of young animals (Swallow 1999). Compared to animals kept in AAT free areas, animals kept in areas of moderate risk of AAT have lower calving rates, lower milk yields and higher rates of calf mortality. Because of the fever associated with AAT, affected pregnant animal usually abort. In susceptible cattle breeds, AAT may reduce calving by up to 20 %, and causes the deaths of another 20 % of calves that are born alive (Swallow 1999). Current evidence suggests that AAT induces a reduction in the calving rates of even the so-called trypanotolerant cattle, sheep and goats in West Africa (Trail et al. 1993; Swallow 1999). Accordingly, AAT reduces calving rates by up to 12 % and increases calf mortality by up to 10 % in trypanotolerant cattle. Furthermore, AAT has been shown to reduce milk offtake by up to 26 % and lambing and kidding rates by as much as 37 % in trypanotolerant sheep and goats in West Africa (Swallow 1999).

Debilitation and Reduced Productivity. AAT is usually chronic and debilitating. As such affected animals are weak and lethargic, further contributing to the reduction in the availability and work efficiency of draught animals (oxen and donkeys) used for preparing land for crops. Thus the most important indirect impact of AAT on crop production is through compromising the availability and health of animals that provide animal traction (Swallow 1999). Additional traction capacity can allow farmers to expand the area that they cultivate, increase yields of existing crops, grow a different mix of crops or allocate labour, land and fertiliser more efficiently. Furthermore, in tsetse-infested sub-Saharan Africa, AAT reduces the offtake of meat and milk by at least 50 %, resulting in lower income from milk and meat sales and less access to liquid capital (Swallow 1999). Moreover, farmers are unable to successfully keep high-yielding exotic livestock breeds in such AAT-endemic areas, which have great economic consequences (Stich et al. 2002).

Compared to animals kept in AAT-free areas, animals kept in areas of moderate risk of AAT also require more frequent treatment with prophylactic and curative doses of trypanocidal drugs. African farmers and governments bear the increasing cost of treating cattle exposed to AAT. As such, at least $30 million is spent on administration of up to 30 million trypanocidal drugs and payments for veterinary services (Swallow 1999).

10.8 Treatment and Control

Because of the lack of an effective vaccine against AAT, current control measure against the disease is achieved by targeting either the parasite or the tsetse vector.

10.8.1 Targeting the Trypanosome

Early and accurate diagnosis is essential for successful treatment of AAT. Continuous surveillance of AAT is an important ingredient for successful treatment and ultimate control of the disease. Effective treatment contributes to disease control by reducing the number of infected individuals that may otherwise act as a source of infection to susceptible livestock within their vicinity. The main drugs used for AAT treatment are diminazene aceturate (berenil) and isometamidium (samorin) (Chitanga et al. 2011). Berenil, with only curative properties, has high activity against *T. congolense* and *T. vivax*, particularly those strains that are resistant to other trypanocides, and is hence effective in treating *nagana*. Furthermore, berenil has low toxic side effects. *Nagana* may also be treated curatively or prophylactically with isometamidium chloride (samorin). Berenil, samorin and suramin are also effective against surra (Tuntasuvan et al. 2003). However, although suramin and berenil may be used for treatment of Dourine, these drugs do not completely eliminate the parasites such the treated animals become carriers (Gillingwater et al. 2007). Thus if the aim is to eradicate Dourine, treatment should not be recommended.

Drug Resistance. Currently, single or multiple trypanocidal drug resistant has been reported in at least 18 African countries where the problem is increasing and rapidly spreading (Delespaux et al. 2008). Mechanism of resistance varies with drugs; resistance to diminazene aceturate is caused by low drug uptake resulting from of mutation P2-type purine transporter (Delespaux et al. 2008), whereas isometamidium chloride resistance is attributed to active drug efflux (Sutherland and Holmes 1993) or reduced uptake (Delespaux et al. 2005). Emergence of strains that are resistance to drug is attributed to inappropriate drug use (Delespaux et al. 2002; Clausen et al. 2010).

Detection of Drug Resistance. Without carrying explicit experiment, it is difficult to differentiate parasitaemia due to drug resistance from re-infection (Glover 1948) and drug under dose. Direct and indirect approaches are being used for detection of drug resistant strains. Direct approach involves incubation of trypanosomes in various concentration of trypanocidal for a defined time period followed by establishment of treatment effect (Delespaux et al. 2008). Such direct tests are akinetoplastic induction (Chitambo et al. 1992), drug incubation infectivity test (Kaminsky et al. 1990; Kaminsky and Brun 1993) and drug incubation *Glossina* infectivity test (P. H. Clausen et al. 1999). An approach close to this is where mice or cattle is inoculated with field isolates and the patent animals are treated with specified curative dosage of trypanocidal (Whiteside 1963; Chitambo and Arakawa 1991; Eisler et al. 2001). Use of mice limits drug sensitivity test only to those isolates that able to grow in them.

Indirectly bio-markers can be used to detect drug-resistant strains and where there is doubt the test is performed in combination with a suitable direct in vitro method. Tests relying on detection of marker are PCR (Gall et al. 2004) and PCR-RFLP

(Vitouley et al. 2011; Moti et al. 2012). *Mbo*II-PCR-RFLP is used for detection of resistance to isometamidium chloride in *T. congolense*. With this technique resistant strains are distinguishable from sensitive strains based on size polymorphism of a putative gene. The gene in resistant and sensitive strains is 384 bp and 381 bp, respectively (Delespaux et al. 2008). It is also possible to differentiate diminazene aceturate-resistant strains with a variant of PCR-RFLP which uses *Bcl*I enzyme for digestion of amplified gene coding for P2-type purine transporter *Tco*AT1. In this case restriction patterns of resistant and sensitive strains differ because there is a mutation on the gene where guanine is substituted with adenine (Delespaux et al. 2008).

Wide-scale longitudinal survey for presence of drug-resistant trypanosomes in endemic is done (Wilson et al. 1976; Gall et al. 2004). In this survey trypanosome-free herd is administered chemoprophylactic treatment and continuously followed for development of parasitaemia for a given time period. Concurrently, level of isometamidium chloride in circulation is monitored by ELISA (Whitelaw et al. 1991). This approach distinguishes parasitaemia due to drug resistance from re-infection or under dose.

Targeting the Tsetse Vector. Elimination of the tsetse vector in the transmission cycle is critical and more sustainable in the reduction of the prevalence/incidence of tsetse-transmitted AAT. Current methods for tsetse population control methods include aerial application of ultra-low volume insecticide (such as pyrethroids), the use of insecticide-laced targets and traps, pour-on application on livestock and sterile insect technique (Torr et al. 2006). Chemical control depends upon sufficient contact between the tsetse fly and the insecticide for the fly to pick up lethal dose. Preference for vector control is now given to elaborate combination of techniques (Krafsur 2009). These combinations may include selective aerial and/or ground spraying to obtain an initial knock down of the flies, followed by deployment of targets, traps and screens and the use of insecticide (pyrethroid)-treated livestock and possibly achieving localised eradication through the use of the sterile insect technique (Krafsur 2009). Most of the earlier control methods including ground spraying of insecticides using dichlorodiphenyl-trichloroethane (DDT), the clearing of bush and extermination of native mammals that provide tsetse fly blood meals and act as trypanosome reservoirs, are no longer extensively applied due to environmental considerations (Krafsur 2009). The limitation of vector control in the control of AAT is its heavy reliance on the use of insecticides which are liable to inducing resistance to the targeted insects as well as the high cost of insecticides.

10.9 Going Forward

While progress has been made on fighting AAT, there are challenges which continue to hinder complete eradication of the disease. For instance, when compared to diseases of livestock such as Foot-and-Mouth Disease, Rinderpest and Anthrax, AAT is unknown by majority of people in communities where the disease

occurs (Machila et al. 2007). Lack of public awareness campaign and community involvement is an obstacle undermining AAT control programmes. For effective control of AAT control, more attention should be paid to community education. Where stakeholders are fully aware of the disease, instituting and implementation of control programme will become easy. Another important challenge is the increase in human population which has led to encroachment of tsetse-infested areas by livestock keepers in search for pastures. The dilemma can be prevented by promoting rangeland management to ensure availability of pastures. Another crucial factor which has contributed to the spread of AAT beyond its known boundaries is climate change caused by global warming. The first impact of global warming on livestock keeping is inadequate pastures and water for animals. This has lead to migration of livestock keepers in search for pastures and water, resulting in the spread of AAT. Secondly, some regions in Africa were too cold but with the global warming the climate became conducive for survival of tsetse vector and biting flies. The two situations have aggravated the spread of infection.

Much as our own failure is impeding AAT control, African trypanosomes are continuously evolving by means of genetic exchange (Tait and Turner 1990; Morrison et al. 2009). Genetic exchange could have serious negative impact on the control of African trypanosomes because it underlies emergence of strains with undesirable phenotypes such as drug resistance, high host diversity, high virulence, absence of usual diagnostic markers and unknown mode of transmission. Therefore to keep pace with ever-evolving trypanosomes, where feasible, research should be tailored to field isolates of trypanosomes than the usual laboratory strains.

Besides challenges being experienced, hope for stumping out AAT is in sight. Novel technologies have solved some hurdles which had delayed progress on vaccines, trypanocidal drugs and diagnostic tools research. In order to design effective tools for controlling African trypanosomes, an in-depth knowledge of the parasites biology is a requirement. To-date unravelling the biology of African trypanosomes is being aided by technologies such as RNA interference, microarray, mass spectrometer, in vitro culture system of trypanosomes and availability of full genome sequences of most pathogenic animal trypanosomes. Application of these technologies synergistically has proven useful for identification of virulent factors of trypanosomes and drug transporters boosting pursuit for anti-disease vaccine and trypanocidal drug development.

Regarding advance on development of screening test tools for African trypanosomes, there is a current shift from the conventional laboratory based methods to rapid test kits such as immunodiagnostic lateral flow test (Sullivan et al. 2013). This is a simple test which is suitable for field situation and interpretation of result does not require expertise. Cost of producing antibody is high and in the end this cost will be met by the poor farmers making the test kit unaffordable. Once fully established, Nanobody® (Nb) will be a suitable substitute for antibody because it is cheap and easy to produce. This thermo-stable recombinant protein is derived from cloning a gene which codes for the antigen-binding fragment of camelid IgG2 and IgG3 (Hamers-Casterman et al. 1993; Lauwereys et al. 1998). Moreover, Nb has robust finger-like complementary determining region three

(CDR3) domain used for antibody–antigen interaction and has got the ability to reach hidden epitopes usually inaccessible to the conventional IgG CDR 3 (De Genst et al. 2006). Recognition of unique epitopes by Nb is advantageous to circumvent low sensitivity encountered with monoclonal antibody-based tests where hosts' IgGs form immune complex with antigen, thereby depriving monoclonal antibody of binding to the shared exposed epitopes (Rebeski et al. 1999). Thus availability of a user-friendly and affordable on-farm diagnostic test kits will strengthen disease surveillance.

While the search for good vaccine candidate against AAT is on-going, breeding of livestock with natural resistance to trypanosomiasis is encouraged. The short comings of these indigenous trypanotolerant breeds include their low quality in terms of meat and milk yield. With genes responding to trypanosome infection under investigation (Noyes et al. 2011), in future, a more precise selection of breeding parents for desirable traits will be made possible.

African Union, through its technical arm, Pan Africa Tsetse and Trypanosomiasis Eradication Campaign (PATTEC), in partnership with Foundation for Innovative New Diagnostics (FIND), is dedicated to eradicating African trypanosomiasis and tsetse vectors (Kabayo 2002). A strategic plan for action was drawn by the member states and progress is evaluated yearly. The programme success has been reported in Botswana and Namibia. The success story of these two countries is indeed an indication of tremendous achievement towards eradication of African trypanosomiasis worth emulation by other African states.

Finally, climate change, industrialization, population pressure and inherent parasite characteristics have rendered obsolete some strategies that were previously effective in controlling African trypanosomiasis. Thus current control methods against AAT should be re-evaluated and updated in order to achieve the desired goal. Furthermore, AAT is trans-boundary disease whose effective control requires a multi-disciplinary approach, targeting both the insect vectors and the trypanosomes; and active involvement by nations where the disease is prevalent. To design and effectively implement tsetse flies control; agro-foresters, wildlife scientists, entomologists and ecologists should be involved because they are conversant with the flies' habitat. Countries where trypanosomiasis is endemic should be at the frontline to fight the infection through continuous surveillance of the disease. In the end successful control of the disease will ultimately improve the livelihood of the communities where AAT is endemic.

References

Adam Y, Marcotty T, Cecchi G et al (2012) Bovine trypanosomosis in the Upper West Region of Ghana: entomological, parasitological and serological cross-sectional surveys. Res Vet Sci 92:462–468

Alemu T, Luckins AG, Phipps LP et al (1997) The use of enzyme linked immunosorbent assays to investigate the prevalence of Trypanosoma equiperdum in Ethiopian horses. Vet Parasitol 71:239–250

Anderson NE, Mubanga J, Fevre EM et al (2011) Characterisation of the wildlife reservoir community for human and animal trypanosomiasis in the Luangwa Valley, Zambia. PLoS Negl Trop Dis 5(6):e1211

Bengaly Z, Sidibe I, Ganaba R et al (2002) Comparative pathogenicity of three genetically distinct types of Trypanosoma congolense in cattle: clinical observations and haematological changes. Vet Parasitol 108:1–19

Bern C, Montgomery SP, Herwaldt BL et al (2007) Evaluation and treatment of chagas disease in the United States: a systematic review. JAMA 298:2171–2181

Borst P, Rudenko G (1994) Antigenic variation in African trypanosomes. Science 264:1872–1873

Büscher P, Mumba Ngoyi D, Kaboré J et al (2009) Improved models of mini anion exchange centrifugation technique (mAECT) and modified single centrifugation (MSC) for sleeping sickness diagnosis and staging. PLoS Negl Trop Dis 3:e471

Cattand P (2001) African Trypanosomiasis, human. In: Service MW (ed) Encyclopedia of arthropod–transmitted infections of man and domestic animals. CABI, Oxon, pp 13–23

Chappius F, Loutan L, Simarro P et al (2005) Options for field diagnosis of human African Trypanosomiasis. Clin Microbiol Rev 18:133–146

Chitambo H, Arakawa A (1991) Therapeutic effect of Berenil and Samorin in mice infected with four trypanosome populations isolated from Zambian cattle. Vet Parasitol 39:43–52

Chitambo H, Arakawa A, Ono T (1992) In vivo assessment of drug sensitivity of African trypanosomes using the akinetoplastic induction test. Res Vet Sci 52:243–249

Chitanga S, Marcotty T, Namangala B et al (2011) High prevalence of drug resistance in animal trypanosomes without a history of drug exposure. PLoS Negl Trop Dis 5(12):e1454

Chitanga S, Namangala B, De Deken R et al (2013) Shifting from wild to domestic hosts: the effect on the transmission of Trypanosoma congolense to tsetse flies. Acta Trop 125:32–36

Clausen PH, Leendertz FH, Blankenburg A et al (1999) A drug incubation Glossina infectivity test (DIGIT) to assess the susceptibility of Trypanosoma congolense bloodstream forms to trypanocidal drugs (Xenodiagnosis). Acta Trop 72:111–117

Clausen PH, Chuluun S, Sodnomdarjaa R et al (2003) A field study to estimate the prevalence of Trypanosoma equiperdum in Mongolian horses. Vet Parasitol 115:9–18

Clausen PH, Bauer B, Zessin KH et al (2010) Preventing and containing trypanocide resistance in the cotton zone of West Africa. Transbound Emerg Dis 57:28–32

Cordon-Obras C, Berzosa P, Ndong-Mabale N et al (2009) Trypanosoma brucei gambiense in domestic livestock of Kogo and Mbini foci (Equatorial Guinea). Trop Med Int Health 14:535–541

Coustou V, Plazolles N, Guegan F et al (2012) Sialidases play a key role in infection and anaemia in Trypanosoma congolense animal trypanosomiasis. Cell Microbiol 14:431–445

De Baetselier P (1996) T cell subsets and cytokine interplay in infectious diseases. In: Mustafa AS, Al-Attiyah RJ, Nath I, Chugh TD (eds) Mechanisms underlying trypanosome-induced T-cell immunosuppression. Karger, Basel

De Genst E, Silence K, Decanniere K et al (2006) Molecular basis for the preferential cleft recognition by dromedary heavy-chain antibodies. Proc Natl Acad Sci USA 103:4586–4591. doi:10.1073/pnas.0505379103

Delafosse A, Doutoum AA (2004) Prevalence of *Trypanosoma evansi* infection and associated risk factors in camels in eastern Chad. Vet Parasitol 119:155–164

Delespaux V, Geerts S, Brandt J et al (2002) Monitoring the correct use of isometamidium by farmers and veterinary assistants in Eastern Province of Zambia using the isometamidium-ELISA. Vet Parasitol 110:117–122

Delespaux V, Geysen D, Majiwa PAO et al (2005) Identification of a genetic marker for isometamidium chloride resistance in Trypanosoma congolense. Int J Parasitol 35:235–243

Delespaux V, Dinka H, Masumu J et al (2008) Five-fold increase in Trypanosoma congolense isolates resistant to diminazene aceturate over a seven-year period in Eastern Zambia. Drug Resist Updat 11:205–209

Desquesnes M, Dávila AM (2002) Applications of PCR-based tools for detection and identification of animal trypanosomes: a review and perspectives. Vet Parasitol 109:213–231
Desquesnes M, Dia ML (2003) Mechanical transmission of Trypanosoma congolense in cattle by the African tabanid Atylotus agrestis. Exp Parasitol 105:226–2231
Desquesnes M, McLaughlin G, Zoungrana A et al (2001) Detection and identification of Trypanosoma of African livestock through a single PCR based on internal transcribed spacer 1 of rDNA. Int J Parasitol 31:610–614
Dhollander S, Jallow A, Mbodge K et al (2006) Equine trypanosomosis in the Central River Division of The Gambia: a study of veterinary gate-clinic consultation records. Prevent Vet Med 75:152–162
Dias JC, Prata A, Schofield CJ (2002) Chagas' disease in the Amazon: an overview of the current situation and perspectives for prevention. Rev Soc Bras Med Trop 35:669–678
Dobson RJ, Dargantes AP, Mercado RT et al (2009) Models for Trypanosoma evansi (surra), its control and economic impact on small-hold livestock owners in the Philippines. Int J Parasitol 39:1115–1123
Duleu S, Vincendeau P, Courtois P et al (2004) Mouse strain susceptibility to trypanosome infection: an arginase-dependent effect. J Immunol 172:6298–6303
Eisler MC, Brandt J, Bauer B et al (2001) Standardised tests in mice and cattle for the detection of drug resistance in tsetse-transmitted trypanosomes of African domestic cattle. Vet Parasitol 97:171–182
Fevre EM, Coleman PG, Odiit M et al (2001) The origins of new *Trypanosoma brucei rhodesiense* sleeping sickness outbreaks in eastern Uganda. Lancet 358:625–628
Fevre EM, Picozzi K, Fyfe J et al (2005) A burgeoning epidemic of sleeping sickness in Uganda. Lancet 366:745–747
Flynn JN, Sileghem M (1991) The role of the macrophage in induction of immunosuppression in Trypanosoma congolense-infected cattle. Immunology 74:310–316
Galiza GJ, Garcia HA, Assis AC et al (2011) High mortality and lesions of the central nervous system in trypanosomosis by *Trypanosoma vivax* in Brazilian hair sheep. Vet Parasitol 182:359–363
Gall Y, Woitag T, Bauer B et al (2004) Trypanocidal failure suggested by PCR results in cattle field samples. Acta Trop 92:7–16
Gillingwater K, Büscher P, Brun R (2007) Establishment of a panel of reference Trypanosoma evansi and Trypanosoma equiperdum strains for drug screening. Vet Parasitol 148:114–121
Glover P (1948) The epidemiology of trypanosomiasis in man and animals. Proc R Soc Med XLI (551–558):23
Hamers-Casterman C, Atarhouch T, Muyldermans S et al (1993) Naturally occurring antibodies devoid of light chains. Nature 363:446–448
Kabayo JP (2002) Aiming to eliminate tsetse from Africa. Trends Parasitol 18:473–475
Kaminsky R, Brun R (1993) In vitro assays to determine drug sensitivities of African trypanosomes: a review. Acta Trop 54:279–289
Kaminsky R, Gumm ID, Zweygarth E et al (1990) A drug incubation infectivity test (DIIT) for assessing resistance in trypanosomes. Vet Parasitol 34:335–343
Knowles G, Betschart B, Kukla BA et al (1988) Genetically discrete populations of Trypanosoma congolense from livestock on the Kenyan coast. Parasitology 96(Pt 3):461–474
Krafsur ES (2009) Tsetse flies: genetics, evolution, and role as vectors. Infect Genet Evol 9:124–141
Kristjanson PM, Swallow BM, Rowlands GJ et al (1999) Measuring the costs of African animal trypanosomosis, the potential benefits of control and returns to research. Agr Syst 59:79–98
Laohasinnarong D, Thekisoe OM, Malele I et al (2011) Prevalence of Trypanosoma sp. in cattle from Tanzania estimated by conventional PCR and loop-mediated isothermal amplification (LAMP). Parasitol Res 109:1735–1739

Lauwereys M, Arbabi Ghahroudi M, Desmyter A et al (1998) Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. EMBO J 17:3512–3520. doi:10.1093/emboj/17.13.3512

Logan-Henfrey LL, Gardiner PR, Mahmoud MM (1992) Animal trypanosomiasis in sub-Saharan Africa. In: Kreier JP, Baker JR (eds) Parasitic protozoa, vol 2. Academic, San Diego, CA, pp 157–275

Losos GJ, Ikede BO (1972) Review of pathology of diseases in domestic and laboratory animals caused by *Trypanosoma congolense*, *T. vivax*, *T. brucei*, *T. rhodesiense* and *T. gambiense*. Vet Pathol Suppl 9:1–71

Machila N, Emongor R, Shaw AP et al (2007) A community education intervention to improve bovine trypanosomiasis knowledge and appropriate use of trypanocidal drugs on smallholder farms in Kenya. Agr Syst 94:261–272

Mahan SM, Hendershot L, Black SJ (1986) Control of trypanodestructive antibody responses and parasitemia in mice infected with *Trypanosoma* (*Duttonella*) *vivax*. Infect Immun 54:213–221

Mamabolo MV, Ntantiso L, Latif A et al (2009) Natural infection of cattle and tsetse flies in South Africa with two genotypic groups of *Trypanosoma congolense*. Parasitology 136:425–431. doi:10.1017/S0031182009005587

Manful T, Mulindwa J, Frank FM et al (2010) A search for *Trypanosoma brucei rhodesiense* diagnostic antigens by proteomic screening and targeted cloning. PLoS One 5:e9630

Matete GO (2003) Occurrence, clinical manifestation and the epidemiological implications of naturally occurring canine trypanosomosis in western Kenya. Onderstepoort J Vet Res 70:317–323

McDermott JJ, Coleman PG (2001) Comparing apples and oranges–model-based assessment of different tsetse-transmitted trypanosomosis control strategies. Int J Parasitol 31:603–609

Monzon CM, Mancebo OA, Russo AM (2003) Antibody levels by indirect ELISA test in Trypanosoma evansi infected horses following treatment with quinapyramine sulphate. Vet Parasitol 111:59–63

Morrison LJ, MacLeod A (2011) African trypanosomiasis. Parasite Immunol 33:421–422

Morrison LJ, Tweedie A, Black A et al (2009) Discovery of mating in the major African livestock pathogen Trypanosoma congolense. PLoS One 4:e5564

Moti Y, Fikru R, Van Den Abbeele J et al (2012) Ghibe river basin in Ethiopia: present situation of trypanocidal drug resistance in Trypanosoma congolense using tests in mice and PCR-RFLP. Vet Parasitol 189:197–203

Mukhebi AW, Perry BD (1992) Economic implications of the control of East Coast fever in eastern, central and southern Africa. International Laboratory for Research on Animal Diseases (ILRAD, Nairobi

Müller N, Mansfield JM, Seebeck T (1996) Trypanosome variant surface glycoproteins are recognized by self-reactive antibodies in uninfected hosts. Infect Immun 64:4593–4597

Musoke AJ, Nantulya VM, Barbet AF et al (1981) Bovine immune response to African; trypanosomes: specific antibodies to variable surface glycoproteins of Trypanosoma brucei. Parasite Immunol 3:97–106

Naessens J (2006) Bovine trypanotolerance: a natural ability to prevent severe anaemia and haemophagocytic syndrome? Int J Parasitol 36:521–528

Nakayima J, Nakao R, Alhassan A et al (2012) Molecular epidemiological studies on animal trypanosomiases in Ghana. Parasit Vectors 5:217. doi:10.1186/1756-3305-5-217

Namangala B (2011) How the African trypanosomes evade host immune killing. Parasite Immunol 33:430–437

Namangala B, De Baetselier P, Brijs L et al (2000) Attenuation of Trypanosoma brucei is associated with reduced immunosuppression and concomitant production of Th2 lymphokines. J Infect Dis 181:1110–1120

Namangala B, Hachaambwa L, Kajino K et al (2012) The use of loop-mediated isothermal amplification (LAMP) to detect the re-emerging human African Trypanosomiasis (HAT) in the Luangwa and Zambezi valleys. Parasit Vectors 5:282

Njiru ZK, Constantine CC, Guya S et al (2005) The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. Parasitol Res 95:186–192

Notomi T, Okayama H, Masubuchi H et al (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28:E63

Noyes H, Brass A, Obara I et al (2011) Genetic and expression analysis of cattle identifies candidate genes in pathways responding to Trypanosoma congolense infection. Proc Natl Acad Sci USA 108(22):9304–9309. doi:10.1073/pnas.1013486108

Olubayo RO, Mihok S, Wesonga DF et al (1991) Pathogenicity of tsetse-transmitted Trypanosoma congolense for waterbuck (Kobus defassa) and Boran cattle (Bos indicus). Acta Trop 49:173–183

Radwanska M, Guirnalda P, De Trez C et al (2008) Trypanosomiasis-induced B cell apoptosis results in loss of protective anti-parasite antibody responses and abolishment of vaccine-induced memory responses. PLoS Pathog 4(5):e1000078

Rebeski DE, Winger EM, Van Rooij EM et al (1999) Pitfalls in the application of enzyme-linked immunoassays for the detection of circulating trypanosomal antigens in serum samples. Parasitol Res 85:550–556

Salim B, Bakheit MA, Kamau J et al (2011) Molecular epidemiology of camel trypanosomiasis based on ITS1 rDNA and RoTat 1.2 VSG gene in the Sudan. Parasit Vectors 4:31

Schneider A, Lutz HU, Marugg R et al (1988) Spectrin-like proteins in the paraflagellar rod structure of Trypanosoma brucei. J Cell Sci 90(Pt 2):307–315

Sileghem M, Flynn JN, Logan-Henfrey L et al (1994) Tumour necrosis factor production by monocytes from cattle infected with Trypanosoma (Duttonella) vivax and Trypanosoma (Nannomonas) congolense: possible association with severity of anaemia associated with the disease. Parasite Immunol 16:51–54

Simo G, Asonganyi T, Nkinin SW et al (2006) High prevalence of Trypanosoma brucei gambiense group 1 in pigs from the Fontem sleeping sickness focus in Cameroon. Vet Parasitol 139:57–66

Simukoko H, Marcotty T, Phiri I et al (2007) The comparative role of cattle, goats and pigs in the epidemiology of livestock trypanosomiasis on the plateau of eastern Zambia. Vet Parasitol 147:231–238

Simuunza M, William W, Emily C et al (2011) Epidemiological analysis of tick-borne disease in Zambia. Vet Parasitol 175:331–342

Snow WF, Wacher TJ, Rawlings P (1996) Observations on the prevalence of trypanosomosis in small ruminants, equines and cattle, in relation to tsetse challenge, in The Gambia. Vet Parasitol 66:1–11

Sow A, Sidibé I, Bengaly Z et al (2012) Field detection of resistance to isometamidium chloride and diminazene aceturate in Trypanosoma vivax from the region of the Boucle du Mouhoun in Burkina Faso. Vet Parasitol 187:105–111

Stephen LE (1986) Trypanosomiasis: a veterinary perspective. Pergamon/A. Wheaton & Co, Exter/London, pp 1–487

Stich A, Abel PM, Krishna S (2002) Human African trypanosomiasis. BMJ 325:203–206

Stijlemans B, Guilliams M, Raes G et al (2007) African trypanosomosis: from immune escape and immunopathology to immune intervention. Vet Parasitol 148:3–13

Sullivan L, Wall SJ, Carrington M et al (2013) Proteomic selection of immunodiagnostic antigens for human African trypanosomiasis and generation of a prototype lateral flow immunodiagnostic device. PLoS Negl Trop Dis 7:e2087. doi:10.1371/journal.pntd.0002087

Sutherland IA, Holmes PH (1993) Alterations in drug transport in resistant Trypanosoma congolense. Acta Trop 54:271–278

Swai E, Kaaya J (2012) A parasitological survey for bovine trypanosomosis in the livestock-wildlife ecozone of Northern Tanzania. Vet World 5:459

Swallow BM (1999) Impacts of African animal trypanosomosis on migration, livestock and crop production. In: International Scientific Council for Trypanosomiasis Research and Control. International Livestock Research Institute, Nairobi

Tabel H, Losos GJ, Maxie MG et al (1981) Experimental bovine trypanosomiasis (Trypanosoma vivax and T. congolense). III. Serum levels of immunoglobulins, heterophile antibodies, and antibodies to T. vivax. Tropenmed Parasitol 32:149–153

Tait A, Turner CM (1990) Genetic exchange in Trypanosoma brucei. Parasitol Today 6:70–75

Tafese W, Melaku A, Fentahun T (2012) Prevalence of bovine trypanosomosis and its vectors in two districts of East Wollega Zone, Ethiopia. Onderstepoort J Vet Res 79:E1–E4

Takeet MI, Fagbemi BO, Donato MD et al (2012) Molecular survey of pathogenic trypanosomes in naturally infected Nigerian cattle. Res Vet Sci 94(3):555–561, pii: S0034-5288(12)00321-9

Taylor KA (1998) Immune responses of cattle to African trypanosomes: protective or pathogenic? Int J Parasitol 28:219–240

Thekisoe OM, Kuboki N, Nambota A et al (2007) Species-specific loop-mediated isothermal amplification (LAMP) for diagnosis of trypanosomosis. Acta Trop 102:182–189

Torr SJ, Mangwiro TN (2000) Interactions between cattle and biting flies: effects on the feeding rate of tsetse. Med Vet Entomol 14:400–409

Torr SJ, Mangwiro TN, Hall DR (2006) The effects of host physiology on the attraction of tsetse (Diptera: Glossinidae) and Stomoxys (Diptera: Muscidae) to cattle. Bull Entomol Res 96:71–84

Trail JC, D'Ieteren GD, Murray M et al (1993) Measurement of trypanotolerance criteria and their effect on reproductive performance of N'Dama cattle. Vet Parasitol 45:241–255

Tuntasuvan D, Jarabrum W, Viseshakul N et al (2003) Chemotherapy of surra in horses and mules with diminazene aceturate. Vet Parasitol 110:227–233

Van den Bossche P, Rowlands GJ (2001) The relationship between the parasitological prevalence of trypanosomal infections in cattle and herd average packed cell volume. Acta Trop 78:163–170

Van den Bossche P, Akoda K, Kubi C et al (2006) The transmissibility of Trypanosoma congolense seems to be associated with its level of resistance to isometamidium chloride. Vet Parasitol 135:365–367

Van den Bossche P, Chitanga S, Masumu J et al (2011) Virulence in Trypanosoma congolense Savannah subgroup. A comparison between strains and transmission cycles. Parasite Immunol 33:456–460

Vitouley HS, Mungube EO, Allegye-Cudjoe E et al (2011) Improved PCR-RFLP for the detection of diminazene resistance in Trypanosoma congolense under field conditions using filter papers for sample storage. PLoS Negl Trop Dis 5:e1223

Waiswa C, Katunguka-Rwakishaya E (2004) Bovine trypanosomiasis in south-western Uganda: packed-cell volumes and prevalence of infection in the cattle. Ann Trop Med Parasitol 98:21–27

Whitelaw DD, Gault EA, Holmes PH et al (1991) Development of an enzyme-linked immunosorbent assay for the detection and measurement of the trypanocidal drug isometamidium chloride in cattle. Res Vet Sci 50:185–189

Whiteside EF (1963) A strain of Trypanosoma congolense directly resistant to Berenil. J Comp Pathol Ther 73:167–175

Wilson AJ, Paris J, Luckins AG et al (1976) Observations on a herd of beef cattle maintained in a tsetse area. II. Assessment of the development of immunity in association with trypanocidal drug treatment. Trop Anim Health Prod 8:1–11

Young CJ, Godfrey DG (1983) Enzyme polymorphism and the distribution of *Trypanosoma congolense* isolates. Ann Trop Med Parasitol 77:467–481

Trypanosoma (*Duttonella*) *vivax* and Typanosomosis in Latin America: Secadera/Huequera/Cacho Hueco

11

Mary Isabel Gonzatti, Bernardo González-Baradat, Pedro M. Aso, and Armando Reyna-Bello

Abstract

The disease caused by *T. vivax* is commonly called *Nagana* in Africa and "*secadera*/*cachera*/*cacho hueco*/*huequera*" in parts of South America. This chapter will focus on the disease and its causative agent, reviewing new diagnostic methods, economic impact, chemotherapy, phylogenetic analysis of *T. vivax* isolates from Africa and South America, epidemiological studies in Latin America, and the analysis of recent genomic and transcriptomic data. *T. vivax* has a significant economic impact on livestock production in sub-Saharan Africa, where it is transmitted by the tsetse fly, and elsewhere in the African continent and in Central and South America, where it is transmitted mechanically. *T. vivax* is enzootic in most Latin American countries, and recurrent epizootic outbreaks causing significant morbidity and mortality have been reported over the past decades. Several significant landmarks in *T. vivax* research have been achieved in the last 2 years, including the publication of high-quality draft genome sequences and partial RNA-seq data for the Y486 strain, as well as the complete transcriptome of the LIEM-176 strain. Comparative analysis of the *T. vivax*, *T. brucei*, and *T. congolense* genomes revealed important differences in

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the surface proteins responsible for host immune response evasion in these species, and data from the *T. vivax* LIEM-76 transcriptome support the participation of other surface proteins, in addition to the VSG, in immune evasion. Proteins of the trans-sialidase family have been identified as important virulence factors that catalyze the desialylation of the host red blood cell, which in turn triggers the erythrophagocytosis that results in anemia. These findings will provide novel tools to tackle the challenge of controlling animal trypanosomosis caused by *T. vivax* in the developing world.

11.1 Introduction

Animal trypanosomoses (AT) are a group of diseases caused by various species of the Salivarian section of the *Trypanosoma* genus that affect cattle, as well as domestic and wild mammals causing serious socioeconomic losses throughout Africa, South and Central America, and large parts of Asia. Salivarian trypanosomes are characterized by an inoculative type of transmission and constitute a monophyletic clade with four different subgenera, Nannomonas, Trypanozoon, Duttonella, and the less characterized Pycnomonas. (Hoare 1972; Haag et al. 1998; Stevens and Brisse 2004). *T.* (*Nannomonas*) *congolense*, *Trypanosoma* (*Trypanozoon*) *brucei brucei*, and *Trypanosoma* (*Duttonella*) *vivax* are transmitted by tsetse flies (Diptera: Glossnidae) throughout sub-Saharan Africa. A tsetse-independent, mechanical mode of transmission by horse flies (tabanids) and stable flies (*Stomoxys sp.*) has allowed *T. evansi*, the etiological agent of the disease known as surra, *derrengadera* or *mal de cadeiras*, to expand worldwide. Two other African Trypanosome species are found beyond the African tsetse belt, *T. equiperdum*, the causative agent of Dourine, a sexually transmitted, cosmopolitan, but often unreported equine disease and *T. vivax*, the focus of this chapter (OIE Terrestrial Manual 2008a, b; Gibson 2007).

Trypanosoma vivax is generally considered a vascular parasite that causes mild to severe disease in various domestic and wild ungulates (Taylor and Authié 2004). However, *T. vivax* has also been found in nervous tissues, cerebrospinal fluid, and aqueous humor, causing neurological signs in bovine, goats, and sheep (Batista et al. 2007, 2011; Galiza et al. 2011). *T. vivax* is the only species of this group of African trypanosomes that has a dual mode of transmission: biologically by the tsetse fly in sub-Saharan Africa and mechanically by blood-sucking insects in Central and South America and parts of Africa. The livestock diseases caused by *T. vivax*, *T. brucei brucei*, and *T. congolense* are known in Africa by the common name of *Nagana* or tsetse disease. In some South American countries, notably Venezuela and Colombia, bovine trypanosomosis caused by *T. vivax* is often accompanied by other hemoparasitic or bacterial infections and is known by the

local names of "*secadera*/*cachera*/*cacho hueco*/*huequera*" (Rivera 1996; Desquesnes 2004; Benavides et al. 2011).[1,2]

In spite of their wide distribution and socioeconomic impact, the non-tsetse transmitted animal trypanosomoses (NTTAT) caused by *T. evansi*, *T. equiperdum*, and *T. vivax* have been less well studied than their TTAT counterparts. About 30 years ago, however, an ad hoc working Group on *T. evansi* was set up at the *Office International des Épizooties* (OIE), with the agreement of the United Nations (FAO) and the World Health Organization (WHO).[3] In February 1991, based on the achievements of the *T. evansi* working Group, the OIE extended its scope to include all NTTAT around the world. From its creation, the goal of the NTTAT Group has been to involve investigators from affected, member countries in Africa, Asia, and the Americas in the study, discussion, and information sharing on such critical topics as differential diagnosis of the aforementioned trypanosomes, epidemiology, characterization, and comparison of strains isolated in different geographical regions, chemotherapy, and chemoresistance to trypanocidal drugs (Touratier 1993, 1999, 2000).

Some trypanosomes, previously thought to be restricted to animals, have been linked to infections and disease in humans. In Ghana in 1917, the morphological identification of *T. vivax* led to the first report of atypical human trypanosomosis (a-HT). Eighteen other cases have been linked to *T. congolense*, *T.b. brucei*, *T. evansi*, *T. lewisi*, and *T. lewisi-like* parasites (Truc et al. 2013). One of the a-HT infections due to *T. evansi* was explained by the lack of apolipoprotein L-I, a human serum factor involved in trypanosome lysis. All of the recent cases of a-HT have been confirmed by molecular diagnosis (PCR) and two of them were fatal. The evaluation of the actual incidence of a-HT by *T. vivax* and other trypanosomes in endemic regions will require new integrated approaches to human and animal health, including the development and implementation of specific diagnostic tools and the study of the factors that influence infections, transmission, and susceptibility.

11.2 Characteristics and Morphology of *T. vivax*

T. vivax has been considered essentially monomorphic although recent results suggest that it could be pleomorphic (Gómez-Piñeres et al (2013). It presents two distinctive features that are useful for direct diagnosis with a light microscopy: a

[1] "Secadera" (from *seco* = dried) refers to the emaciated/dehydrated physical condition of the animals suffering from a *T. vivax* infection often accompanied by other diseases such as anaplasmosis, babesiosis, theileriosis, as well as vitamin and mineral deficiencies that trigger weight loss and skin that appears attached to the underlying muscles and bones.

[2] The terms *cachera*/*cacho hueco*/*huequera* (from *cacho* = horn; *hueco* = hollow) relates to the paranasal sinuses bone resorption, including the base of the horn, associated with this disease and due to mineral deficiencies and/or bacterial opportunistic infections.

[3] On May 26th, 1983 at the OIE Headquarters.

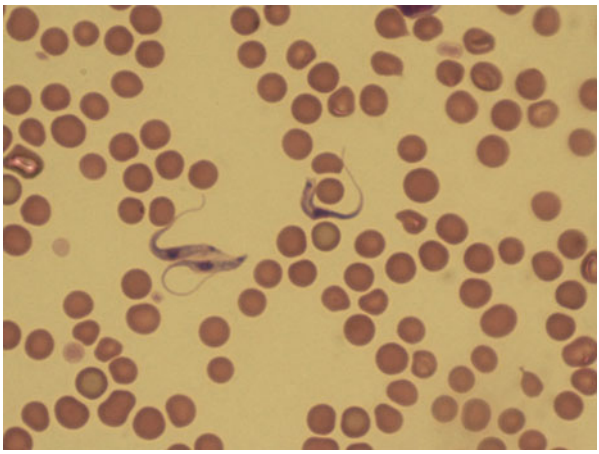

Fig. 11.1 Venezuelan *T. vivax* TvMT1 in a thin blood smear from an ovine experimental infection. A thin-peripheral blood smear was prepared from an ovine experimentally infected with *T. vivax* TvMT1 at the first peak of parasitemia. The blood smear was stained with Hemacolor® (×400)

large kinetoplast and a rapid crossing of the field of vision in fresh blood (Fig. 11.1). Its free and terminal flagellum is attached to the parasite by an undulating membrane that is characteristically less developed than that in *T. brucei* and *T. evansi*. The posterior end of *T. vivax* is rounded, broad, and wide, with the prominent kinetoplast ($d = 1.1$ μm) usually positioned near or at the terminal end. The nucleus, either rounded or elongated, depending on the isolate, is located in the mid portion of the parasite. Biometric studies have shown significant variation in the total length of both African and South American *T. vivax* isolates, with the length from the tip of the flagellum to the end terminal ranging from 18 to 31 μm, with mean values between 21 and 25.4 μm [reviewed in Hoare (1972), Shaw and Lainson (1972), Desquesnes (2004) and Osório et al. (2008)].

Morphological studies and morphometric characterization have been performed on five Venezuelan *T. vivax* isolates of various degrees of virulence (Gómez-Piñeres et al. 2013), including the LIEM-176 isolate whose transcriptome has been recently reported (Greif et al. 2013). Using scanning electron microscopy, their extremity appeared flat or pointed depending on the angle, and the extended flagellum showed pronounced contortions along the axis of the parasite body. The dimension of the flagellar pocket was 2 μm, and it was positioned 3–4 μm from the posterior terminal end. The study showed statistical differences in the total length between the five *T. vivax* isolates, with an average length of 20.99 μm. The LIEM 176 isolate was the smallest, at 18.12 ± 2.42 μm, and the TvGZ1 isolate was the largest, with a total length of 24.35 ± 5.55 μm (Fig. 11.2). Thus, the average length of the Venezuelan *T. vivax* isolates varied from 16 to 30 μm, within the range determined for other isolates in previous biometric studies.

In spite of the distinctive character of the *T. vivax* kinetoplast, there have been fewer molecular studies on the structure in this species compared to other Salivarian trypanosomes. The kinetoplast contains the mitochondrial DNA (K-DNA) and is located within the mitochondrial matrix of all kinetoplastid protozoa, close to the basal body of the flagellum. The K-DNA contains two types of concatenated

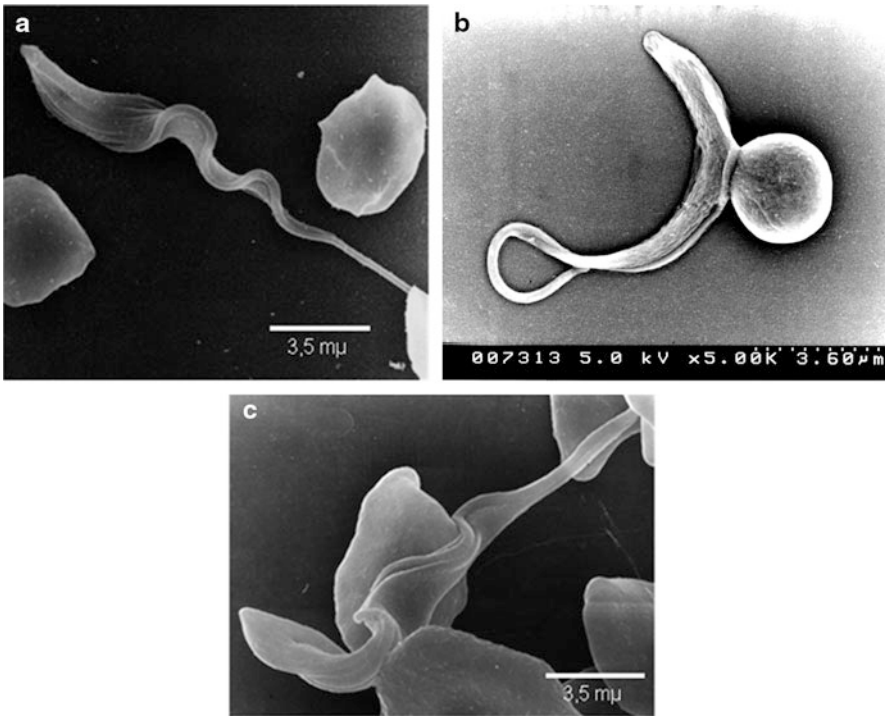

Fig. 11.2 Scanning electron micrographs of *T. vivax* Liem-176. (**a**) Scanning electron micrograph of *T. vivax* LIEM-176 in peripheral blood in close pràximity to two red blood cells from an experimentally infected ovine (×6,000). Courtesy of Dr. Alpidio A. Boada Soucre, IDECYT-Universidad Nacional Experimental Simón Rodríguez, Caracas, Venezuela. (**b**) Scanning electron micrograph showing a *T. vivax* LIEM-176 parasite adhered to a peripheral blood ovine red blood cell from that of an experimental infection (×10,000). Courtesy of Dr. Alpidio A. Boada Sucre, IDECYT-Universidad Nacional Experimental Simón Rodríguez, Caracas, Venezuela. (**c**) Scanning electron micrograph of a peripheral blood *T. vivax* LIEM-176 trypomastigote from an ovine experimental infection (×6,000). Courtesy of Dr. Alpidio A. Boada Sucre, IDECYT-Universidad Nacional Experimental Simón Rodríguez, Caracas, Venezuela

circular structures, known as minicircles and maxicircles. Depending on the species, the K-DNA contains 5–50 × 10^3 minicircles and 20–50 maxicircles. The *T. vivax* K-DNA contains the largest maxicircles (40 kpb), as well as the smallest minicircles (465 pb) yet characterized (Borst et al. 1985; Masiga and Gibson 1990; Yurchenko and Kolesnikov 2001).

11.3 Genome, Transcriptome, and Immune Evasion

Significant advances on *T. vivax* have been reported in the last few years including the deposition and publication of high-quality draft genome sequences as well as partial RNA-seq data for the rodent adapted Y486 strain (Jackson et al. 2012) and

the transcriptome analysis of the LIEM-176 strain (Greif et al. 2013). Both *T. vivax* strains were initially isolated from bovines, the Y486 from zebu cattle in Zaria, Nigeria in 1976 (Leeflang et al. 1976; Gibson 2012), and the LIEM-176 in Trujillo state, Venezuela, in 2002 (Gómez-Piñeres et al. 2013). The comparison of the *T. brucei* 927, *T. vivax* Y486, and *T. congolense* IL3000 genomes revealed similar GC contents (46–52 %), comparable genome sizes and putative protein-coding genes, 29,273 kbp and 9,266 protein-coding genes in *T. brucei* and 41,775 kbp with 11,870 genes in *T. vivax* (Jackson et al. 2012).

The most significant differences between the three genomes appear to be related to their surface proteins and immune evasion mechanisms (Jackson et al. 2012, 2013). Salivarian Trypanosomes share a largely extracellular lifestyle and have evolved antigenic variation to escape host innate and acquired immunity. The variable surface glycoprotein (VSG) is the predominant component of the dense surface coat of *T. brucei* and blocks the access of antibodies to the invariant antigens. Antigenic variation results from the spontaneous and continuous switching and expression of VSG gene variants from a large repertoire of silent and antigenically different genes, during the course of individual infections and upon entry into new hosts within the same population (Turner et al. 1995; Barry 1997; Turner 1999; Barry et al. 2012). Early biochemical studies showed that the *T. vivax* VSG, differed from the *T. brucei* and *T. congolense* counterparts in several features, including its size, ~40 kDa, pattern of glycosylation, and C-terminal end (Gardiner et al. 1996).

The three-way genome comparative analysis and the estimated phylogenies based on the cell surface proteins of *T. congolense*, *T. brucei*, and *T. vivax* shed some light on the evolution of immune evasion mechanisms in these trypanosomes (Jackson et al. 2012, 2013). They reported 79 gene families that potentially encode cell-surface proteins based on the presence of a signal peptide, a Glycosyl-Phosphatidyl-Inositol (GPI) anchor, or a transmembrane helix. *T. vivax*, the earliest branch in the African trypanosome evolution, contains a highly diverse VSG repertoire including genes belonging to two VSG-like protein families (Fams 25 and 26) and two of the ancestral families, a-VSG (Fam 23) and b-VSG (Fam 24). In addition, the entire transferrin-receptor lineage is absent from *T. vivax*, suggesting that this protein family appeared later, after the separation of *T. vivax* from *T. congolense* and *T. brucei*. Nineteen out of the seventy-nine were *T. vivax*-specific gene families, including several coding for as yet-unidentified putative membrane proteins. The loss and expansion of surface-protein gene families in *T. vivax* are best illustrated by the absence of the major-surface protease *MSP-B* lineage (No 46) and the appearance of 11 *MSP-C genes* (gp63). Thirty-three additional shared cell-surface families were described, including the trans-sialidase, invariant surface glycoprotein, amino acid transporter, glucose transporter, peptidyl-prolyl cis-trans isomerase, and several hypothetical proteins. The emerging picture is that of a complex surface architecture that might well explain the observed biological differences and specific mechanisms of immune evasion that characterize *T. vivax* and distinguish it from other African trypanosomes (Jackson et al. 2012, 2013).

The *T. vivax* LIEM-76 transcriptome data illustrates that other cell surface proteins, in addition to the VSG, are likely to play a strategic role in immune evasion (Greif et al. 2013, http://www.bioinformatica.fcien.edu.uy/Tvivax/). The VSG represent 55 % of the externally disposed cell-surface proteins in *T. vivax*, a considerably lower figure than the 95–98 % reported for the *T. brucei* VSG. Greif et al. (2013) identified a candidate VSG mRNA with a 90.4 % sequence identity to the VSG from the West African *T. vivax* ILDat 2.1 isolate (Gardiner et al. 1996). VSG expression levels in *T. vivax* LIEM-176 were found to be high, but considerably lower than in *T. brucei*. The authors proposed that other cell-surface proteins, including the GP63 peptidase, might be relevant. In this Transcriptome analysis, a total of 6,500 proteins and RNA-coding sequences were identified, including over 1,000 that were species specific and 50 that were found only in the genome of the Venezuelan strain. Contrary to the accepted view that trypanosomatids regulate their gene expression principally at a posttrancriptional level, the *T. vivax* LIEM-176 RNA-seq analysis showed that some protein-coding regions of the genome were not transcribed. The finding of these silent genes raises the possibility that transcription initiation may be regulated in the bloodstream stage of *T. vivax* LIEM-176, and perhaps this transcriptional regulation could also occur in other trypanosomatids. The trans-splicing sites for 5959 *T. vivax* genes were mapped to locations immediately before or after the AUG start codons. Thus, the pattern of trans-splicing appears to be conserved in all trypanosomatids, including the distant Stercoraria relative, *T. cruzi*.

11.4 Molecular Phylogeny of *T. vivax*

Several molecular markers have been used to study the genetic diversity of *T. vivax* and to better understand its phylogenetic relationship to other African trypanosomes (Duffy et al. 2009; Cortez et al. 2009; Adams et al. 2010; Tait et al. 2011; Hamilton et al. 2012; Auty et al. 2012). The genetic diversity of *T. vivax* was analyzed using species-specific microsatellite markers on 31 isolates from horses, donkeys, and cattle from The Gambia (Duffy et al. 2009). The results showed that *Trypanosoma vivax* presents a clonal population structure, limited genetic diversity, and asexual replication. Based on these analysis, Tait et al. (2011) suggested that *T. vivax* presents limited genetic variability, as would be expected from the lack of genetic exchange in this species. However, other molecular studies, using isoenzymes (Fasogbon et al. 1990), microsatellites (Morlais et al. 2001; Biryomumaisho et al. 2011), and sequence analysis of various markers with *T. vivax* isolates from West and East Africa and South America showed greater complexity and genetic diversity (Dirie et al. 1993; Malele et al. 2003; Cortez et al. 2006; Rodrigues et al. 2008; Adams et al. 2010).

Initial phylogenetic analysis of isolates from South American (Brazil and Venezuela) and Eastern (Kenya and Mozambique) and Western Africa (Nigeria) were reported by Cortez et al. (2006) and Rodrigues et al. (2008). They used SSUrDNA and ITS sequences and showed that South American and West Africa

T. vivax are closely related, presenting a small 0.4 % divergence, while the genetic distance between the South American isolates and those from East Africa (Kenya) was estimated at ~3.2 %.

Highly heterogeneous *T. vivax* strains were isolated in Mozambique, including some that were closely related to South American and West Africa isolates and one highly divergent *T. vivax*-like isolate from a Nyala antelope (*Tragelaphus angasi*), designated TviMzNy (Rodrigues et al. 2008). The analysis of the 5.8SrDNA sequences yielded similar results. This led the authors to propose two *T. vivax* genotypes, named A and B. All the molecular phylogeny studies support the view that *T. vivax* was introduced to the New World by infected bovines that originated in Western Africa (Hoare 1972).

Another molecular marker that has been successfully used to study genetic diversity and to compare African and South American *T. vivax* isolates is the gene that encodes the papain-like Cathepsin L (CatL) peptidase (Cortez et al. 2009). The CatL is encoded by a multicopy gene family that is present as tandem copies in various trypanosome species which makes them good polymorphic markers to study variability among geographical isolates.

The phylogenetic analysis (NJ) of the CatL sequences confirmed that *T. vivax* belongs to a marginal clade within the Salivaria section, as had been inferred from SSU rDNA and gGAPDH sequences (Stevens and Rambaut 2001; Cortez et al. 2006; Hamilton et al. 2007). All the *T. vivax* and *T. vivax*-like isolates grouped within the same cluster, with a genetic distance between ~1.1 and 10.6 %. The CatL sequences were distributed amongst nine subclades, named TviCatL1–9. TviCatL1 was found only in the West Africa isolates (WA), while TviCatL 3–4 were found in both WA and South American isolates. TviCatL 2 and TviCatL3 are closely related to each other (~1.7 % divergence) compared to TviCatL2 and TviCatL4 (~8 % divergence). The TviCatL8 and TviCatL9 sequences corresponded to the Kenyan genotypes (Eastern Africa) separated from each other by ~12 % divergence. Most of the genotypes from Mozambique were grouped in TviCatL6–7 (divergence ~1.1 %). The remaining Mozambique isolates were grouped with TviCatL5, which showed a ~3.5 % divergence with TviCatL6–7. The TviCatL5–9 sequences did not show significant homology to those reported in the draft *T. vivax* genome from the Y486 strain (Nigeria, West Africa). In agreement with previous reports, the CatL sequences from East Africa showed a large degree of sequence polymorphism (Cortez et al. 2006; Rodrigues et al. 2008).

Genotyping of *T. vivax* isolates from infected tsetse flies caught in Tanzania was performed using the method of fluorescent fragment length barcoding (FFLB), which discriminates based on size polymorphisms of various rRNA regions and sequence analysis of the 18SrRNA, combined with the glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) (Adams et al. 2010). Based on the results, the authors proposed the existence of three *T. vivax* lineages, A, B, and C within the *Dutonella* subgenus. The lineage initially referred as C contained isolates from Africa and South America, whereas the A and B lineages contained the isolates obtained from tsetse flies in Tanzania (Adams et al. 2010). Subsequent studies showed a close relationship between the *T. vivax* C lineage and the TviMzNy nyala

isolate (Rodrigues et al. 2008; Adams et al. 2010). The *T. vivax* lineages from Tanzania, A and B, could correspond to completely new *Trypanosoma sp*., for which no mammalian host has yet been identified. Alternatively, they could be related to *T.v. uniforme* or *T.v. ellipsiprymni*, two subspecies of the Dutonella subgenus that have been poorly characterized (Adams et al. 2010).

The genetic characterization of additional geographical *T. vivax* isolates revealed a much greater degree of complexity than originally proposed. This genetic diversity should be further analyzed with regard to affected vertebrate hosts, degrees of virulence and pathogenicity, as well as drug sensitivity and response to therapeutic drug regimens.

11.5 Clinical Signs and Pathogenicity of Bovine Trypanosomosis

Bovine trypanosomosis caused by *T. vivax* is mostly a chronic disease in Latin America (Osório et al. 2008), but a hyperacute "haemorrhagic syndrome," characterized by widespread internal bleeding, has been described in cattle in East Africa (Uilenberg 1998).

According to Anosa (1988), three stages can be recognized in bovine trypanosomosis:

Prepatent Period. No clinical manifestations of the disease are present during this period, which begins with the transmission of the parasite by the vector and ends at the time the parasites are detected in the animal blood. In ovine and bovine experimental infections in our laboratory, with Venezuelan isolates of *T. vivax*, we have observed a variable prepatent period ranging from 2 to 10 days (Reyna-Bello and Gonzatti, unpublished results). The variation in the length of the prepatent period correlated with the parasite load in the inoculum and the virulence of the isolate, as has been reported elsewhere (Desquesnes 2004).

Acute Period. This begins with the appearance of parasites in the blood and may last up to 2–3 months. In the initial phase, one of the first signs of colonization of the host by the parasite is the temperature increase. The recurrence of the fever is characteristic and coincides with the parasitemia waves that are often observed every 8–15 days. A dramatic descent of the erythrocytes in circulation is observed during this period, which engenders a moderate or severe anemia. Other signs that are characteristic of this period include lachrymation, sub-mandibullar edema, nasal discharges, progressive weight loss, lethargy, and decreased milk production. Clinical examination of the animal would reveal pallor of mucous membranes and in some cases, an enlargement of the lymph nodes. A variable percentage of the affected animals will die during this period. The mortality rate varies significantly, 3–50 %, depending on the isolate, the degree of stress of the host, the breed, and species [Hoare (1972), Desquesnes (2004), Osório et al. (2008) and personal observations of the authors]. Severe reproductive disorders often appear during

this period in both males and females. In males infected with *T. vivax*, there is a total inhibition of spermatogenesis, with testicular and epididimal degeneration that leads to infertility or even sterility. The motility and quality of the sperm decrease and percentage of atypias increase. (De Stefano et al. 1999; Adamu et al. 2006). In experimentally infected sheep, structural alterations were observed in Sertoli and Leydig cells, as well as spermatozoids, and a lymphocytic infiltrate was seen in the testicular cellular interstitium. Ultrastructural observation revealed significant alterations of the mitochondria, rough and smooth endoplasmic reticulum, Golgi, nucleus, and plasma membrane (Boada-Sucre 2010). In female animals, *T. vivax* induces severe genital lesions, transient or permanent anestrus, abnormal estrus cycles, and a higher incidence of abortions (Sekoni 1994).

Chronic Period. Two to three months after infection, the animals that have survived the acute phase will maintain cryptic parasitemia, but start to recover normal blood parameters and appear asymptomatic. However, different types of stress (scarce food, drought, etc.) will likely trigger the reappearance of the parasitemia and concomitantly a return of the clinical signs of the acute period (Desquesnes 2004). It would be valuable to evaluate the degree of reversibility, if any, of the damage and alterations induced by *T. vivax* in the host reproductive systems and capacity.

Anemia is a clinical sign that characterizes the trypanosomosis caused by African trypanosomes, as well as other parasitic diseases and bacterial infections (babesiosis, anaplasmosis, gastrointestinal helminthiasis). An important contribution to the fundamental understanding of the pathogenic factors and mechanisms involved in the progressive anemia during *T. vivax* infection has been recently reported (Guegan et al. 2013). The authors used a murine model of *T. vivax* infection (Y486 strain) to show that trans-sialidases are directly involved in the desialylation of the host red blood cell (RBC) that precedes the erythrophagocytosis phenomenon responsible for anemia. The desialylation of major RBC glycoproteins, principally the glycophorins, by the parasite trans-sialidases resembles the changes in the erythrocytes membranes that precede their removal from the blood during the aging process.

11.6 Epidemiological Aspects

The generally accepted view is that *T. vivax* was introduced into French Guyana and the French West Indies around 1830 with Zebu cattle originating in Senegal (Leger and Vienne 1919; Carougeau 1929), and the first report of bovine infection by *T. vivax* Ziemann, 1905 in South America was in French Guyana (Leger and Vienne 1919). However, archival and historical evidence suggests that the arrival of this trypanosome might have occurred as early as 1733, through the importation of cattle from Senegal, the Gulf of Guinea, and Spanish colonies to Martinique and Guadalupe (Maillard and Maillard 1998). Leger and Vienne named the parasite *T. guyanense* and proposed that it originated in Venezuela, where Tejera described a

morphologically identical isolate a year later, calling it *T. cazalboui* Laveran, 1906 (Kubes 1944). However, the name *T. guyanense* was already in use to describe a different parasite, so the South American cattle isolate was renamed *T. viennei* Lavier, 1921. Eventually, *T. vivax* Ziemann, 1905 became the prevailing designation and is currently used for both *T. vivax vivax* [4] and *T.* (*D*) *vivax viennei* Lavier, 1921 (Desquesnes 2004).

Following this initial work, there were several reports of the presence of trypanosomoses caused by *T. vivax* in Central and South America, Guadeloupe and Martinique (1926), Colombia (1931), Suriname and Guyana (1938), Panama (1941), and Brazil (1944) (Shaw and Lainson 1972; Jones and Davila 2001; Desquesnes 2004). In 1995, the disease was diagnosed in Pantanal, Matto Grosso, Brazil, at the border with Bolivia and Paraguay, and soon after this, *T. vivax* outbreaks were reported in the Bolivian lowlands (Silva et al. 1996) and in Paraguay (Silva et al. 1998). Some of the factors that might have contributed to the geographic extension of severe epizootic waves include the high prevalence of *T. vivax* in the Pantanal region, the development of new roads, the increase in animal trade, and possibly iatrogenic transmission in the course of a vaccination program against foot and mouth disease [Jones and Davila (2001) and Desquesnes (2004), quote by Dr. Touratier]. A comprehensive seroepidemiological study in six areas of the Pantanal region of Brazil, using indirect ELISA, in the state of Mato Grosso (Madruga et al. 2006) reported seroprevalence rates ranging from 28.1 % in Rio Negro to 71.9 % in Curumbá, with an average of 56 %. One year later, a prevalence of 27 % was reported in the neighbor Pantanal region in Bolivia (Gonzales et al. 2007) and the disease spread across to Paraguay (Silva et al. 1998).

Low to medium seroprevalences have been reported for *T. vivax* in other Latin American countries, with 14.3 % in Peru, 22.5 % in Ecuador, and 40 % in Paraguay, but the indirect immunofluorescence (IFI) used in these studies cannot discriminate between *T. vivax* or *T. evansi*. Serological surveys suggested that *T. vivax* had also spread to Costa Rica in Central America (Wells et al. 1977), and *T. vivax* was identified by microscopic examination of blood smears in Costa Rica several years later, in the course of an outbreak of typanosomosis, babesiosis, and anaplasmosis between 2007 and 2008 (Oliveira et al. 2009). *T. vivax* infections were detected in 32.1 % of Brown Swiss bovines, and 10.7 % of the animals had coinfections with *T. vivax* and *A. marginale*. Unlike previous outbreaks in the Pantanal regions of Brazil, Paraguay, and Bolivia (Silva et al. 1996, 1998), and also French Guyana (Desquesnes 2004), mortality was not observed in Costa Rica.

In Venezuela, local and nationwide studies have been carried out to determine trypanosome seroprevalence in bovines. Toro M (1990) reported prevalences ranging from 21 % in Zulia and the Andean region to 24 % in Guayana, 28 % in the plains states, and 33.5 % in the Central-Western region. Two years later, Duno

[4] *T. vivax vivax*, corresponds to the previous denomination for the African parasite, whereas, *T. (D.) vivax viennei* Lavier, 1921 was used for the non-tsetse transmitted closely related parasite in South and Central America.

(1992) presented a higher, 57.80 %, seroprevalence in Falcón, one of the Northwestern states. The same year, Perrone et al. (1992) showed a direct relationship between age and trypanosome seroprevalence in Guárico state. The values rose from 10 % for the younger animals to 80 % for those over 36 months. Later studies reported 30 % and 38.5 % values in bovines from Bolívar and Guárico states, respectively (Espinoza et al. 1999; García et al. 2001) and a similar 29.5 % seroprevalence in buffaloes in Guárico state (Tamasaukas et al. 2006). The most recent survey sampled 49 farms in various regions of Venezuela and reported an overall seropositivity of 33.1 % by IFI with crude *T. vivax* antigens and using indirect ELISA with a *T. evansi* crude extract (Suárez et al. 2009). A *T. vivax* outbreak in September–October 2004 was documented in a double-purpose farm in Zulia state, just 12 days after the introduction of zebu cattle from Colombia without the corresponding sanitary controls or quarantine (Simoes et al. 2009). In this Zulia outbreak, in the extreme west of Venezuela, the high level of parasitological prevalence, 77 %, was accompanied by a mortality rate of 15.15 %. A second *Trypanosoma vivax* outbreak occurred in May 2007 in El Temblador, Monagas state, in the eastern region of Venezuela (García et al. 2008). Clinical signs and symptoms were present that are common in trypanosomosis/cachera, but are also typical of other hemotropic pathogens: fever, pale mucosas, lethargy, lymphadenopathy, emaciation, anorexia, severe anemia, jaundice, and lachrymation (Fig. 11.3). The outbreak occurred shortly after the double-purpose herd returned to the Monagas farm from the high Orinoco river area, where they had been kept during the dry season. *T. vivax* infection was confirmed by PCR diagnosis; 27 % of the animals were positive for *Trypanosoma* by Woo and 50 % were serologically positive by iELISA (Garcia et al. 2008). As coinfection with *A. marginale* was demonstrated, the animals were treated with isometamidium chloride and oxytetracicline hydrochloride, and no deaths were reported.

In Colombia, *T. vivax* is enzootic, especially in areas below 1,000 m above sea level. Otte et al. (1988) reported an increase in the incidence of infection at the end of the rainy season in the Northern coastal regions, which coincides with the increase in the tabanid population. The seroprevalence varied from 0 to 61 % depending on the farm. Mateus and González (1991) reported an outbreak with 30 % morbidity, 6–7 % combined mortality, and an abortion rate of l.3 %.

The seroprevalence by indirect ELISA in Guyana, Suriname, and French Guyana was estimated at 42 ± 3 %, 31 ± 2 %, and 22.2 ± 1.5 %, respectively (Desquesnes 2004). Thus, the current picture reflects the same pattern described almost a decade ago. *T. vivax* represents a limiting factor for cattle trade in almost all of South and Central America, because it appears to be enzootic from Paraguay to Central America, with sporadic epizootic outbreaks that cause significant morbidity, mortality, and economic losses.

Fig. 11.3 Trypanosoma vivax outbreak in the Eastern region of Venezuela, Monagas state, El Temblador, May 2007. (**a**) *T. vivax* was confirmed in 27 % of the animals by Woo and in 50 % by iELISA (García et al. 2008). No deaths were reported. (**b**) Clinical signs and symptoms most frequently observed for the affected animals included lethargy, lymphadenopathy, emaciation, pallor of mucous membranes, low haematocrit, jaundice, and lachrymation

11.6.1 Economic Impact of *Nagana/Cachera*

In Africa, bovine trypanosomosis causes close to three million deaths in cattle herds, and 35 million doses of trypanocides are administered per year. In Sub-Saharan Africa, total livestock economic losses reach 1.2 billion per annum (FAO 2004). *T. vivax* is considered one of the principal pathogens associated with a decrease in livestock production in Venezuela (Rey 2004) and other South American countries (Desquesnes 2004), but there is a relative dearth of current estimates on the economic impact of this parasitosis in South and Central America.

Over 30 years ago, economic losses due to trypanosomosis caused by *T. vivax* were estimated at 56.6 US$ per animal in Colombia. This ranked trypanosomosis third in livestock economic impact, after tick-transmitted diseases and fasciolosis (Betancourt and Wells 1979; Wells 1982). The cost of this disease was estimated at 64 US$ per animal, equivalent to 17 % of its total value for the Pantanal region in Brazil and the lowlands of Bolivia (Seidl et al. 1999). If this estimate were to be extended to all of the 11 million cattle in the area, the total loss would reach $160 million (Dávila and Silva 2000). The first report of *T. vivax* in Minas Gerais,

another region of Brazil, calculated a 27 % decrease in milk production and a 45 % decrease in pregnancy rates (Abrão et al. 2009).

In French Guyana, the economic cost of the bovine trypanosomosis outbreak was estimated at 3.3 % of the weight of the live animal. Extrapolating to the market value of the animal, this would be equivalent to a 30 € loss per animal, plus an additional 3 € per animal for the therapeutic treatment (Desquesnes 2004).

A complete economic assessment must take into account the complex epidemiologic situation in the region, where bovines, goats, sheep, and horses can all be infected, and in which recurrent and sporadic *T. vivax* outbreaks have been reported. In addition, *T. vivax* bovine infections are often accompanied by other hemotropic pathogens that may contribute to the cattle morbidity, mortality, and infertility, with the consequent impact on productivity. Quantitation of economic losses due to *T. vivax* must include other costs, such as curative or prophylactic treatments, personnel and veterinarian assistance, and the indirect impact on crops and farming systems. Facilities for a timely and accurate laboratory diagnosis are not always readily available, and preventive or curative treatments are sometimes applied based only on general clinical signs, so the parasite may spread throughout much of the herd before appropriate therapeutic measures are implemented. Reversal of this situation will require the implementation of good husbandry practices to monitor livestock for anemia, weight gain and reproductive health and the implementation of parasitological, clinical, molecular and serological diagnosis to apply appropriate treatment programs.

11.7 Transmission and Vectors

Trypanosomosis transmission depends upon the geographical location and the pathogenic species. In Africa, *T. vivax* transmission is essentially cyclical due to the presence of various species of the tsetse fly Glossina genus, including *G. m. centralis*, *G. m. morsitans*, *G. pallidipes*, *G. austeni*, *G. brevipalpis*, *G. tachinoides*, *G. palpalis palpalis*, *G. p. gambiensis*, and *G. fuscipes fuscipes*. During the cyclical transmission, the parasite transforms from the trypomastigote stage to epimastigotes that multiply in the insect proboscis, pharynx and esophagus and finally into infective metacyclics. *T. vivax* can also be transmitted mechanically both in Africa and in Central and South America, but in this case both the differentiation and multiplication processes occur in the vertebrate host rather than in the insect host (Hoare 1972; Gardiner 1989).

This second mode of transmission has allowed *T. vivax* propagation beyond the tsetse belt, but has also important implications for the animal trypanosomosis control programs in Africa. Two species of tabanids, *Atylotus agriestes* and *Atylotus fuscipes*, were shown to transmit *T. vivax* mechanically to cattle in an experimental infection (Desquesnes 2004; Desquesnes and Dia 2003, 2004). The relative importance of mechanical transmission in Africa is still poorly understood. The capacity of *T. vivax* to be transmitted both cyclically and mechanically might allow the coexistence of both forms of transmission in the same area, the same host, and

perhaps, in the same vector (Desquesnes et al. 2009). *T. vivax* seroprevalence and molecular incidence (by PCR) were compared between tsetse-infested and tsetse-free areas in Northwestern Ethiopia, over an 8 months period (Cherenet et al. 2006). At the end of this period, the seroprevalence of *T. vivax* was high in both regions. The average monthly incidence was 20.9 % in the tsetse-free area and 25.7 % in the vector-infested zone. The seroprevalence of *T congolense* and *T. vivax* in the tsetse-infested area was the same (36.1 %), whereas *T. vivax* was responsible for 90.9 % of the trypanosome infections in the tsetse-free area. A recent report confirmed the importance of mechanical transmission of *T. vivax* in various regions of Ethiopia by studying the prevalence of *T. vivax* in tsetse-infested and tsetse-free areas using parasitological methods (Woo), as well as PCR (ITS-1 primers) (Fikru et al. 2012). Similar prevalence rates, 22.6 % and 25.7 %, were reported for the tsetse-infested and the vector-free areas, respectively.

T. vivax mechanical transmission is affected by the level of parasitemia of the vertebrate host and the propagation efficiency of the vector (Roberts et al. 1989). A tabanid vector may keep up to 1–12 nL of infected blood in its biting mouthparts (Foil et al. 1987). The vector then approaches a second host almost immediately and inoculates the infected blood, favoring the mechanical transmission of the parasites that have a limited survival time in the insect mouthparts. In the field, a vector may bite the same host several times, which would lower the efficiency of the mechanical transmission. Foil (1983) has estimated that only 2–3 % of the tabanids will reach a second host within 10 min, and the percentage decreases to 0, after 50 min, probably due to the travelled distance between animals in the herd. Desquesnes et al. (2009) have proposed a predictive mathematical model for mechanic transmission to cattle that is applicable not only to *T. vivax* but also to other pathogens transmitted by tabanids. The model simulates the evolution of the incidence and prevalence rates of a pathogen depending on a number of variable parameters such as parasitemia, size of the herd, initial prevalence of infection, and insect number and burden.

11.8 Diagnosis

Bovine trypanosomosis caused by *T. vivax* is currently diagnosed by a combination of clinical, parasitological, molecular, and serological techniques.

Clinical diagnosis is based on several signs including: recurrent fever; anemia evidenced by pallor of mucous membranes (vulvar, ocular, or gingival); lachrymation; emaciation; overall loss of physical condition; cachexia; and an evident decrease in productivity, measured by milk production and/or rate of weight gain. Even though these clinical signs may be used to diagnose the acute phase of the disease, they can be confused with other hemotropic agents such as babesiosis and anaplasmosis (Hoare 1972; Desquesnes 2004; Osório et al. 2008).

Parasitological diagnosis is the most applied method to identify *T. vivax* in the field. It uses the direct microscopic observation of a fresh drop of blood and the Woo's test, whose detection limit (Woo 1969) is 10^3 parasites/mL, which is

adequate to diagnose the acute phase of the disease (Masake et al. 1997; Ramírez-Iglesias et al. 2011).

Molecular diagnosis includes the use of molecular hybridization techniques allowing the initial identification of *T. vivax* in tsetse flies (Kukla et al. 1987) and the characterization of South American (Colombia) and African isolates (Dickin and Gibson 1989; Dirie et al. 1993). That DNA probe, however, hybridized to the Colombian and West African isolates but did not recognize the Kenyan (Eastern Africa) isolate (Dickin and Gibson 1989). The PCR and the development of species-specific and sensitive primers constituted an important advance in the diagnosis of many pathogens, including *T. vivax*. Several molecular markers have been utilized, including: a specific antigen recognized by the Tv27 monoclonal antibody (Masake et al. 1994); satellites or microsatellite sequences (Masiga 1992; Morlais et al. 2001); spliced-leader sequences; (Ventura et al. 2001) and the internal transcribed spacers of ribosomal DNA (Desquesnes et al. 2001; Adams et al. 2008).

At least eight different bovine trypanosome genotypes have been identified by PCR: *T. brucei sp.*; *T. congolense savannah*, *forest and kilifi*; *T. simiae*; *T. simiae tsavo*; *T. godfreyi and T. vivax* (Gibson 2009). The molecular identification of several *T. vivax* isolates from different geographical regions has been particularly difficult due to genetic variability, particularly in African isolates (Simpson et al. 2006; Stevens 2008; Cortez et al. 2009). For example, the use of spliced-leader sequences did not allow the identification of *T. vivax* isolates from East Africa. In addition, some primers (Masake et al. 1997) that were initially considered "universal" for all the *T. vivax* African isolates were later shown to fail to recognize some genetic variants (Malele et al. 2003).

A significant improvement in the PCR sensitivity was achieved by targeting a microsatellite repeat for the detection of *T. vivax* in the mouthparts of field-collected tsetse flies (Masiga et al. 1992; Masake et al. 1994; Morlais et al. 2001). The design of species-specific and universal molecular markers will require comprehensive genomic studies on *T. vivax* variants from distinct geographical regions.

The PCR based on the inter-ribosomal spacer 1 (ITS1) of the rDNA) amplifies fragments of different sizes in some of the African trypanosome species, including the following three groups: *T. vivax* (307 bp); *T. evansi*, *T. brucei*, and *T. equiperdum* (540 bp); and *T. congolense* (780 bp). The ITS1 PCR is an ideally suited diagnostic test to discriminate *T. vivax* and *T. evansi* infections or coinfections in a single agarose gel (Desquesnes et al. 2001; Njiru et al. 2005). In spite of the discriminating power of the single ITS1 PCR, the comparative analysis of several sets of primers to detect *T. evansi* infections showed that they had a lower sensitivity than the TBR1/2 primers (Fernández et al. 2008). Additional *T. vivax*-specific primers have been used to identify isolates from various geographical regions including South America, East, and West Africa (Cortez et al. 2009) or to diagnose *T. vivax* prevalence in the Pantanal outbreak in Bolivia (Gonzales et al. 2006).

A new technique that employs the *Bst* DNA Polymerasa (from *Bacillus stearothermophilus*) to isothermically amplify target DNA was developed over a decade ago (Notomi et al. 2000). The Loop-mediated Isothermal Amplification

(LAMP) allows the efficient and rapid amplification, of up to 10^9 copies, of specific segments of a DNA sequence, under isothermal conditions, in less than an hour. The product can either be visualized directly as a precipitate using magnesium pyrophosphate or monitored in real time using a turbidimeter (Morlais et al. 2001). LAMP assays to detect *T cruzi*, *T. brucei brucei*, *T. brucei gambiense*, *T. brucei rhodesiense*, *T. rangeli*, *T. congolense*, and *T. evansi* have a higher sensitivity than PCR by one or two orders of magnitude (Kuboki et al. 2003; Thekisoe et al. 2005, 2007, 2010; Njiru et al. 2008, 2010; Matovu et al. 2010; Njiru et al. 2011a; Njiru 2011). The *T. vivax* LAMP test, based on satellite DNA sequences, also evidenced a higher sensitivity (1 tryp/mL) than PCR assays ($10–10^3$ tryp/mL) (Njiru et al. 2011b). Thus, the LAMP technique constitutes a promising tool for the molecular diagnosis of *T. vivax*.

Serological diagnosis of animal trypanosomosis is often performed by the indirect immunofluorescence assay (IFI) and ELISA. The indirect ELISA is a simple, effective assay that can be used to quantitate the presence of antibodies in a large number of samples (Reyna-Bello et al. 1998; Osório et al. 2008). This technique has been used with crude extracts of either *T. vivax* or *T. evansi* to diagnose animals in both the acute and chronic phases of the disease (Ramírez-Iglesias et al. 2011) and to determine trypanosomosis seroprevalence (Desquesnes 2004; Madruga et al. 2006; Suárez et al. 2009; Cadioli et al. 2012). Soluble *T. evansi* crude antigens have been used to detect *T. vivax* because of the high antigenic cross-reactivity and the ease of propagating *T. evansi* in rodents to obtain the extracts (Reyna-Bello et al. 1998; Suárez et al. 2009). Bossard et al. (2010) developed an anti- HSP70/BiP monoclonal antibody inhibition ELISA assay that can identify cattle infected with *T. congolense* or *T. vivax*, the two most pathogenic African animal trypanosomes. However, it would be highly desirable to use purified or recombinant antigens to perform *T. vivax*-specific serodiagnosis as the severity and treatment for the disease depends upon the species (Desquesnes 2004; Mekata et al. 2009; Desquesnes et al. 2011; Eleizalde et al. 2012; Mekata et al. 2013). *T. vivax* appears to be much more pathogenic than *T. evansi* for sheep, cattle, and goats and is treated with diminazene aceturate or isometamidium chloride, whereas quinapyramine, suramin, and melarsomine are recommended for surra or derrengadera, the disease caused by *T. evansi*.

11.9 Treatment

Due to the high endemicity and sporadic outbreaks of *T. vivax* bovine trypanosomosis in Latin America, many high-productivity farms maintain constant control and supervision and use preventive, regular trypanocidal treatments and other anti-trypanosomal control measurements that vary according to the geographical area, livestock condition, and overall epidemiological situation, among others. The two most common drugs for the treatment of bovine trypanosomosis caused by *T. vivax* are diminazene aceturate (Babenil®, Diminazene-A) and isometamidium chloride (Trypamidium®), available since 1955 and 1961, respectively. No new

drugs have been developed for the treatment of trypanosomosis due to *T. vivax* in the last 50 years (Rivera 1996; Desquesnes 2004; Holmes et al. 2004).

Diminazene aceturate 4,4′ (*azoamino*) *dibenzamidine* treatment is applied intramuscular at dosis that vary from 3.3 to 7 mg/kg and is also effective against bovine babesiosis (Mosqueda et al. 2012; Gohil et al. 2012). The mode of action of diminazene aceturate is not totally clear; it appears to block K-DNA replication and polyamine synthesis. Drug resistance has been found in *T. vivax* isolates due to its indiscriminate use and the long periods of treatment. We have used diminazene aceturate at doses ranging from 7 to 12 mg/kg for the treatment of *T. vivax* experimentally infected ovines. An apparent recovery and cure is evidenced for 1–1½ months, after which new waves of parasitemia are evidenced in the blood and the clinical signs of the disease reappear. Some veterinarians recommend the use of a second dose of diminazene aceturate 7 days after the first treatment to minimize relapses (Manuel Rivera, unpublished results).

Isometamidium chloride is used as a sterilizing, prophylactic treatment at a dose of 1 mg/kg that normally confers 5 months of protection. The kinetoplast is the main organelle where the drug is accumulated and its mechanism of action involves the cleavage of K-DNA-topoisomerase complex required for its replication (Holmes et al. 2004). Thus, isometamidium chloride is the preferred treatment in enzootic areas with a high prevalence of *T. vivax*.

In our experience, the use of isometamidium chloride rapidly eliminates *T. vivax* from the peripheral blood, and the Venezuelan strains appear to respond well to this treatment. However, there are reports of *T. vivax* isolates resistant to this drug in other regions of South America and Africa (Desquesnes 2004; Sow et al. 2012). The evaluation of risk factors for bovine trypanosomosis in farms in various states of Venezuela (Suárez et al. 2009) showed statistically significant lower seroprevalence rates in farms that regularly used isometamidium chloride treatments, compared to those that used diminazene aceturate. In addition, 2 % of the animals from the farms where isometamidium chloride treatment was applied presented active infections. These results confirmed the efficacy of isometamidium chloride prophylactic treatment to control *T. vivax* in endemic regions.

11.10 Going Forward

Epidemiological studies of *T. vivax* show that it is a major pathogen that causes significant morbidity and mortality of livestock in developing countries within and beyond the tsetse belt in Africa and in Central and South America. In spite of the fragmented information on its real economic impact, recurrent epizootic outbreaks of *T. vivax*, associated with seasonal factors and trade of infected livestock, continue to be reported in Latin America. *T. vivax* was introduced into the New World through the importation of Senegalese cattle to French Guyana and the French West Indies. Phylogenetic analyses support this origin, as South American *T. vivax* isolates are closely related to West African strains. Contrary to the earlier proposal that *T. vivax* presents limited genetic divergence, new clades within the

Duttonella subgenus have been identified in Africa. Comparative analysis between the *T. vivax*Y486, *T. brucei* 927, and *T. congolense* IL3000 genomes shows distinct cell surface proteins and immune evasion mechanisms. The *T. vivax* LIEM-76 transcriptome provides further insight into the participation of additional surface proteins, besides the VSG, in immune evasion. In a recent major breakthrough, trans-sialidases have been identified as *T. vivax* virulence factors that promote erythrophagocytosis and cause anemia in the acute phase of the trypanosomosis. No species-specific serodiagnostic test is available at the moment for *T. vivax*; however, several PCR assays based on species-specific primers and a highly sensitivity *T. vivax* LAMP test, based on satellite DNA sequences, have been developed. The LAMP technique constitutes a promising tool for the molecular diagnosis of *T. vivax* under field conditions due to its high sensitivity, low cost, and efficient and rapid amplification of the target DNA.

Going forward, we envision that the analysis of the *T. vivax* Y486 genome and the LIEM-76 transcriptome will bring about significant progress into the development of new therapeutic targets to overcome the problems of drug resistance. However, the comparative analysis of additional genomes and transcriptomes might be compulsory to address the genetic diversity of this parasite and the transcriptional regulation of gene expression. It is foreseeable that in the "omics" era, the analysis of *T. vivax* strains, of various degrees of pathogenicity and/or virulence, will contribute to the discovery of additional factors that play a role in the pathology of this complex disease. Further exploitation of recent advances on *T. vivax* genetic engineering, in vitro culture systems, and the use of mouse models (D'Archivio et al. 2011; Chamond et al. 2010) will generate additional information on pathological mechanisms, disease progression, CNS involvement, drug candidates, antigenic variation, humoral immune response, and anti-inflammatory mediators.

There is an urgent need to develop both simple and sophisticated species-specific tests for field and laboratory diagnosis in wide geographical areas to conduct epidemiological surveys in endemic countries throughout the developing world. The aim would be to use additional molecular markers, monoclonal antibodies, recombinant protein(s), and nanobodies to diagnose active *T. vivax* infections.

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References

Abrão D, de Carvalho A, Facury E et al (2009) Impacto económico causado por *Trypanosoma vivax* em rebanho boviono leiteiro no estado de Minas Gerais Ciência Animal Brasileira – Suplemento 1, – Anais do VIII Congresso Brasileiro de Buiatria

Adams ER, Hamilton PB, Malele II et al (2008) The identification, diversity and prevalence of trypanosomes in field caught tsetse in Tanzania using ITS-1 primers and fluorescent fragment length barcoding. Infect Genet Evol 8:439–444

Adams E, Hamilton P, Rodrigues A et al (2010) New *Trypanosoma* (*Duttonella*) *vivax* genotypes from tsetse flies in East Africa. Parasitology 137:641–645

Adamu S, Fatihu MY, Useh NM et al (2006) Sequential testicular and epididymal damage in Zebu bulls experimentally infected with *Trypanosoma vivax*. Vet Parasitol 143:29–34

Anosa VO (1988) Haematological and biochemical changes in human and animal Trypanosomiasis. Part II. Rev Elev Med Vet Pays Trop 41:151–164

Auty H, Anderson NE, Picozzi K et al (2012) Trypanosome diversity in wildlife species from the serengeti and Luangwa Valley ecosystems. PLoS Negl Trop Dis 6:e1828

Barry JD (1997) The relative significance of mechanism of antigenic variation in African Trypanosomes. Parasitol Today 13:212–218

Barry JD, Hall JP, Plenderleith L (2012) Genome hyperevolution and the success of a parasite. Ann NY Acad Sci 1267:11–17

Batista JS, Riet-Correa F, Teixeira MM, Madruga CR, Simões SD, Maia TF (2007) Trypanosomiasis by *Trypanosoma vivax* in cattle in the Brazilian semiarid: description of an outbreak and lesions in the nervous system. Vet Parasitol 143:174–181

Batista JS, Rodrigues CM, García HA et al (2011) Association of *Trypanosoma vivax* in extracellular sites with central nervous system lesions and changes in cerebrospinal fluid in experimentally infected goats. Vet Res 42:63–69

Benavides E, López M, Alayón L (2011) Enfermedades del ganado en la región de La Macarena (Meta). Un ejercicio de epidemiología participativa. Rev Med Vet 21:41–62

Betancourt A, Wells E (1979) Pérdidas económicas en un brote de tripanosomosis bovina causada por *Trypanosoma vivax*. Rev Acovez 3: 6–9

Biryomumaisho S, Katunguka-Rwakishaya E, Lubega G et al (2011) Identification of *Trypanosoma vivax* subtypes isolated from cattle and goats using microsatellite markers. Vet Arhiv 81:13–24

Boada-Sucre A (2010) Histopatología y Patología Ultraestructural de la Glándula Adrenal, el Hígado y el Testículo de Ovinos Infectados Experimentalmente con *Trypanosoma vivax*. Tesis Doctorado, Universidad Central de Venezuela. Facultad de Ciencias

Borst P, Fase-Fowler F, Weijers PJ, Barry JD, Tetley L, Vickerman K (1985) Kinetoplast DNA from *Trypanosoma viva*x and *T. congolense*. Mol Biochem Parasitol 15:129–142

Bossard G, Boulangé A, Holzmuller P et al (2010) Serodiagnosis of bovine trypanosomosis based on HSP70/BiP inhibition ELISA. Vet Parasitol 173:39–47

Cadioli F, de Barnabé PA, Machado R et al (2012) First report of *Trypanosoma vivax* outbreak in dairy cattle in São Paulo state, Brazil. Rev Bras Parasitol Vet 21:118–124

Carougeau M (1929) Trypanosomiase bovine à la Martinique. Bull Soc Pathol Exot 22:246–247

Chamond N, Cosson A, Blom-Potar MC, Jouvion G, D'Archivio S, Medina M, Droin-Bergère S, Huerre M, Goyard S, Minoprio P (2010) Trypanosoma vivax infections: pushing ahead with mouse models for the study of Nagana. I. Parasitological, hematological and pathological parameters. PLoS Negl Trop Dis 4:e792

Cherenet T, Sani R, Speybroeck N et al (2006) A comparative longitudinal study of bovine trypanosomiasis in tsetse-free and tsetse-infested zones of the Amhara Region, northwest Ethiopia. Vet Parasitol 140:251–258

Cortez AP, Ventura R, Rodrigues A et al (2006) The taxonomic and phylogenetic relationships of *Trypanosoma vivax* from South America and Africa. Parasitology 133:159–169

Cortez AP, Rodrigues A, Garcia H et al (2009) Cathepsin L-like genes of *Trypanosoma vivax* from Africa and South America – characterization, relationships and diagnostic implications. Mol Cell Probes 23:44–51

D'Archivio S, Medina M, Cosson A, Chamond N, Rotureau B, Minoprio P, Goyard S (2011) Genetic engineering of *Trypanosoma* (*Dutonella*) *vivax* and in vitro differentiation under axenic conditions. PLoS Negl Trop Dis 5:e1461

Dávila AM, Silva RA (2000) Animal trypanosomiasis in South America. Current status, partnership, and information technology. Ann NY Acad Sci 916:199–212

De Stefano H, González B, Boada-Sucre A et al (1999) Efecto de la infección con *Trypanosoma vivax* sobre la calidad espermática de toros Siboney. Rev Cient FCV-LUZ IX:411–417

Desquesnes M (2004) Livestock trypanosomes and their vectors in Latin America. OIE. World Organization for Animal Health, Paris

Desquesnes M, Dia ML (2003) Mechanical transmission of *Trypanosoma congolense* in cattle by the African tabanid *Atylotus agrestis*. Exp Parasitol 105:226–231

Desquesnes M, Dia ML (2004) Mechanical transmission of *Trypanosoma vivax* in cattle by the African tabanid *Atylotus fuscipes*. Vet Parasitol 119(1):9–19

Desquesnes M, McLaughlin G, Zoungrana A et al (2001) Detection and identification of *Trypanosoma* of African livestock through a single PCR based on internal transcribed spacer 1 of rDNA. Int J Parasitol 31:610–614

Desquesnes M, Biteau-Coroller F, Bouyer J et al (2009) Development of a mathematical model for mechanical transmission of trypanosomes and other pathogens of cattle transmitted by tabanids. Int J Parasitol 39:333–346

Desquesnes M, Kamyingkird K, Vergne T et al (2011) An evaluation of melarsomine hydrochloride efficacy for parasitological cure in experimental infection of dairy cattle with *Trypanosoma evansi* in Thailand. Parasitology 138:1134–1142

Dickin SK, Gibson WC (1989) Hybridisation with a repetitive DNA probe reveals the presence of small chromosomes in *Trypanosoma vivax*. Mol Biochem Parasitol 33:135–142

Dirie MF, Otte MJ, Thatthi R et al (1993) Comparative studies of *Trypanosoma* (*Duttonella*) *vivax* isolates from Colombia. Parasitology 106(Pt 1):21–29

Duffy C, Morrison L, Black A et al (2009) *Trypanosoma vivax* displays a clonal population structure. Int J Parasitol 39:1475–1483

Duno F, Garcia F, Rivera M (1992) Epidemiological aspects of bovine tripanosomosis in Falcón State, Venezuela. In: Premier Séminaire International sur les Trypanosomoses Animales Non Transmises par les Glossines, Annecy, 14–16 Oct 1992, 184 pp

Eleizalde MC, Gómez-Piñeres E, Tavares-Márquez LM et al (2012) Determination by PCR of the animal trypanosomiasis caused by *T. evansi* in samples from cattle in the San Casimiro Municipality Aragua State, Venezuela. Congreso Panamericano de Ciencias Veterinaria. Cartagena, Colombia

Espinoza E, González N, Aso P, Perrone T (1999) Incidencia serológica de Trypanosoma vivax en becerros a pastoreo en sabanas del Estado Guárico. Vet Trop 24:5–15

FAO (2004). The state of food and agriculture 2003–2004. Food and Agriculture Organisation of the United Nations (FAO), Rome, Italy. Accessed 5 Mar 2013

Fasogbon A, Knowles G, Gardiner P (1990) A comparison of the isoenzymes of *Trypanosoma* (*Duttonella*) *vivax* isolates from East and West Africa. Int J Parasitol 20:389–394

Fernández D, González-Baradat B, Eleizalde M et al (2008) *Trypanosoma evansi*: a comparison of PCR and parasitological diagnostic tests in experimentally infected mice. Exp Parasitol 121:1–7

Fikru R, Goddeeris B, Delespaux V et al (2012) Widespread occurrence of *Trypanosoma vivax* in bovines of tsetse- as well as non-tsetse-infested regions of Ethiopia: a reason for concern? Vet Parasitol 190:355–361

Foil L (1983) A mark-recapture method for measuring effects of spatial separation of horses on tabanid (Diptera) movement between hosts. J Med Entomol 20:301–305

Foil L, Adams W, Mcmanus J et al (1987) Bloodmeal residues on mouthpart of *Tabanus fuscicostatus* and the potential for mechanical transmission of pathogens. J Med Entomol 24:613–616

Galiza GJ, Garcia HA, Assis AC et al (2011) High mortality and lesions of the central nervous system in Trypanosomosis by Trypanosoma vivax in Brazilian hair sheep. Vet Parasitol 182:359–363

García H, Aguirre A, Pérez G et al (2001) Diagnóstico parasitológico y serológico de infecciones por *Trypanosoma sp* en dos rebaños bufalinos (*Bubalus bubalis*) del estado Guárico. Rev Fac Cien Vet UCV 42:15–28

García FA, Alfaro EC, Reyna A et al (2008) Evaluación de la eficacia de un producto a base de cloruro de isometamidium y clorhidrato de oxitetraciclina en un rebaño de ceba del Edo. Monagas naturalmente infectado con *Trypanosoma vivax* y *Anaplasma marginale*. Venezuela Bovina 23:34–43

Gardiner PR (1989) Recent studies of the biology of *Trypanosoma vivax*. Adv Parasitol 28:229–317

Gardiner PR, Nene V, Barry MM et al (1996) Characterization of a small variable surface glycoprotein from *Trypanosoma vivax*. Mol Biochem Parasitol 82:1–11

Gibson W (2007) Resolution of the species problem in African trypanosomes. Int J Parasitol 37:829–838

Gibson W (2009) Species-specific probes for the identification of the African tsetse-transmitted trypanosomes. Parasitology 136:1501–1507

Gibson W (2012) The origins of the trypanosome genome strains *Trypanosoma brucei brucei* TREU 927, *T. b. gambiense* DAL 972, *T. vivax* Y486 and *T. congolense* IL3000. Parasit Vectors 5:71–76

Gohil S, Herrmann S, Günther S, Cooke BM (2012) Bovine babesiosis in the 21st century: advances in biology and functional genomics. Int J Parasitol 43:125–132

Gómez-Piñeres E, Boada-Sucre A, Bretaña A et al (2013) Morphometric characterization of *Trypanosoma vivax* isolates from Venezuela. Rev Fac Cien Vet (UCV) (in press)

Gonzales JL, Loza A, Chacon E et al (2006) Sensitivity of different *Trypanosoma vivax* specific primers for the diagnosis of livestock trypanosomosis using different DNA extraction methods. Vet Parasitol 136:119–126

Gonzales JL, Chacon E, Miranda M et al (2007) Bovine trypanosomosis in the Bolivian Pantanal. Vet Parasitol 146:9–16

Greif G, de Leon MP, Lamolle G et al (2013) Transcriptome analysis of the bloodstream stage from the parasite Trypanosoma vivax. BMC Genomics 14(1):149

Guegan F, Plazolles N, Baltz T et al (2013) Erythrophagocytosis of desialylated red blood cells is responsible for anaemia during *Trypanosoma vivax* infection. Cell Microbiol. doi:10.1111/cmi.12123

Haag J, O'hUigin C, Overath P (1998) The molecular phylogeny of trypanosomes: evidence for an early divergence of the Salivaria. Mol Biochem Parasitol 91:37–49

Hamilton P, Gibson W, Stevens J (2007) Patterns of co-evolution between trypanosomes and their hosts deduced from ribosomal RNA and protein-coding gene phylogenies. Mol Phylogenet Evol 44:15–25

Hoare C (1972) The trypanosomes of mammals. A zoological monograph. Blackwell Scientific Publications, Oxford, 749 pp

Holmes PH, Eisler MC, Geerts S (2004) Current chemotherapy of animal trypanosomiasis. In: Maudlin I, Holmes P, Miles M (eds) The Tripanosomiases, 1st edn. CABI Publishing, Boston, MA, pp 431–464

Jackson AP, Berry A, Aslett M et al (2012) Antigenic diversity is generated by distinct evolutionary mechanisms in African trypamosome species. Proc Natl Acad Sci USA 109:3416–3421

Jackson AP, Allison H, Barry JD et al (2013) A cell-surface phylome for African Trypanosomes. PLoS Negl Trop Dis 7:e2121

Jones TW, Davila AM (2001) *Trypanosoma vivax*-out of Africa. Trends Parasitol 17:99–101

Kubes V (1944) El *Trypanosoma vivax* Americano. Agente de la tripanosomiasis bovina en Venezuela. Su comparación con el del Africa, Instituto de Investigaciones Veterinarias. Ed, Grafolit, Caracas, Venezuela

Kuboki N, Inoue N, Sakurai T et al (2003) Loop-mediated isothermal amplification for detection of African trypanosomes. J Clin Microbiol 41:5517–5524

Kukla B, Majiwa P, Young J et al (1987) Use of species-specific DNA probes for detection and identification of trypanosome infection in tsetse flies. Parasitology 95:1–16

Leeflang P, Buys J, Blotkamp C (1976) Studies on *Trypanosoma vivax*: infectivity and serial maintenance of natural bovine isolates in mice. Int J Parasitol 6:413–417

Leger L, Vienne M (1919) Epizootie à trypanosomes chez les Bovidés de la Guyane Française. Bull Soc Pathol Exot 12:258–266

Madruga C, Araújo F, Cavalcante-Goes G et al (2006) The development of an enzyme-linked immunosorbent assay for *Trypanosoma vivax* antibodies and its use in epidemiological surveys. Mem Inst Oswaldo Cruz 101:801–807

Maillard N, Maillard J (1998) Historique du peuplement bovin et de l'introduction de la tique *Amblyomma variegatum* dans les îles françaises des Antilles: synthèse bibliographique. Ethnozootech 61:19–36

Malele I, Craske L, Knight C et al (2003) Identification of new trypanosome species from wild tsetse flies in Tanzania. infection. Infect Genet Evol 3:271–279

Masake RA, Nantulya VM, Pellé R, Makau JM, Gathuo H, ole-MoiYoi OK (1994) A species-specific antigen of Trypanosoma (Duttonella) vivax detectable in the course of infection is encoded by a differentially expressed tandemly reiterated gene. Mol Biochem Parasitol 64:207–218

Masake RA, Majiwa PA, Moloo SK et al (1997) Sensitive and specific detection of *Trypanosoma vivax* using the polymerase chain reaction. Exp Parasitol 85:193–205

Masiga DK, Gibson WC (1990) Specific probes for *Trypanosoma* (*Trypanozoon*) *evansi* based on kinetoplast DNA minicircles. Mol Biochem Parasitol 40:279–283

Masiga D, Smyth A, Hayes P et al (1992) Sensitive detection of trypanosomes in tsetse flies by DNA amplification. Int J Parasitol 22:909–918

Mateus G, González M (1991) Características de un brote de *Trypanosoma vivax* en Colombia. Rev Cuba Cien Vet 22:167–172

Matovu E, Kuepfer I, Boobo A, Kibona S, Burri C (2010) Comparativte detection of trypanosomal DNA by loop-mediated isothermal amplification and PCR from flinders technology associates cards spotted with patient blood. J Clin Microbiol 48(6):2087–2090

Mekata H, Konnai S, Witola WH et al (2009) Molecular detection of trypanosomes in cattle in South America and genetic diversity of *Trypanosoma evansi* based on expression-site-associated gene 6. Infect Genet Evol 9:1301–1305

Mekata H, Konnai S, Mingala CN, Abes NS, Gutierrez CA, Dargantes AP, Witola WH, Inoue N, Onuma M, Murata S, Ohashi K (2013) Isolation, cloning and pathologic analysis of Trypanosoma evansi field isolates. Parasitol Res 112:1513–1521

Morlais I, Ravel S, Grébaut P et al (2001) New molecular marker for *Trypanosoma* (*Duttonella*) *vivax* identification. Acta Trop 80:207–213

Mosqueda J, Olvera-Ramírez A, Aguilar-Tipacamú G et al (2012) Current advances in detection and treatment of babesiosis. Curr Med Chem 19:1504–1518

Njiru Z (2011) Rapid and sensitive detection of human African trypanosomiasis by loop-mediated isothermal amplification combined with a lateral-flow dipstick. Diagn Microbiol Infect Dis 69:205–209

Njiru Z, Constantine C, Guya S et al (2005) The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. Parasitol Res 95:186–192

Njiru Z, Mikosza A, Armstrong T et al (2008) Loop-mediated isothermal amplification (LAMP) method for rapid detection of *Trypanosoma brucei rhodesiense*. PLoS Negl Trop Dis 2:e147. doi:10.1371/journal.pntd.0000147

Njiru Z, Ouma J, Enyaru J et al (2010) Loop-mediated isothermal amplification (LAMP) test for detection of *Trypanosoma evansi* strain B. Exp Parasitol 125:196–201

Njiru Z, Traub R, Ouma J, Enyaru JC et al (2011a) Detection of group 1 *Trypanosoma brucei gambiense* by loop-mediated isothermal amplification. J Clin Microbiol 49:1530–1536

Njiru Z, Ouma J, Bateta R et al (2011b) Loop-mediated isothermal amplification test for *Trypanosoma vivax* based on satellite repeat DNA. Vet Parasitol 180:358–362

Notomi T, Okayama H, Masubuchi H et al (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28:E63

OIE (2008) Terrestrial manual. Chapter 2. 4.1 8. Trypanosomosis (tsetse-transmitted). http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.18_TRYPANOSOMOSIS.pdf. Accessed 25 Sep 2012

OIE (2008a) Terrestrial manual. Chapter 2.5.3. Dourine. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.05.03_DOURINE.pdfdf. Accessed 11 Mar 2013

Oliveira J, Hernández-Gamboa J, Jiménez-Alfaro C et al (2009) First report of *Trypanosoma vivax* infection in dairy cattle from Costa Rica. Vet Parasitol 163:136–139

Osório A, Madruga C, Desquesnes M et al (2008) *Trypanosoma* (*Duttonella*) *vivax*: its biology, epidemiology, pathogenesis, and introduction in the new world—a review. Mem Inst Oswaldo Cruz 103:1–13

Otte M, Abuabara J, Nieto MI et al (1988) Incidence of *Trypanosoma vivax* infection on three cattle farms on the north coast of Colombia. Acta Vet Scand Suppl 84:104–106

Perrone T, Lesseur M, Reveron I et al (1992) Seroepidemiology of bovine trypanosomosis in the area of Santa Maria de Ipire, Venezuela. In: Premier séminaire international sur les trypanosomoses animales non transmises par les Glossines, Annecy, 14–16 Oct 1992, p 196

Ramírez-Iglesias JR, Eleizalde MC, Gómez-Piñeres E et al (2011) *Trypanosoma evansi*: a comparative study of four diagnostic techniques for trypanosomosis using rabbit as an experimental model. Exp Parasitol 128:91–96

Rey C (2004) Hemoparasitosis en América Latina: El Caso Venezuela. Red Electrónica de Garrapatas y Enfermedades Transmitidas por Garrapatas para América Latina y el Caribe, RedEctopar. Cuarta Conferencia Electrónica

Reyna-Bello A, García FA, Rivera M et al (1998) Enzyme-linked immunosorbent assay (ELISA) for detection of anti-*Trypanosoma evansi* equine antibodies. Vet Parasitol 80:149–157

Rivera M (1996) Hemoparasitosis Bovinas. Consejo de Desarrollo Científico y Humanístico UCV. Colección Estudios. Caracas, Venezuela, 238 pp

Roberts W, Wellde B, Reardon M et al (1989) Mechanical transmission of *Trypanosoma brucei rhodesiense* by *Glossina morsitans morsitans* (Diptera:Glossinidae). Ann Trop Med Parasitol 83(Suppl 1):127–131

Rodrigues A, Neves L, Garcia H et al (2008) Phylogenetic analysis of Trypanosoma vivax supports the separation of South American/West African from East African isolates and a new *T. vivax*-like genotype infecting a nyala antelope from Mozambique. Parasitology 135:1317–1328

Seidl A, Dávila AMR, Silva RA (1999) Estimated financial impact of *Trypanosoma vivax* on the Brazilian Pantanal and Bolivian lowlands. Mem Inst Oswaldo Cruz 94:269–272

Sekoni VO (1994) Reproductive disorders caused by animal trypanosomiases: a review. Theriogenology 42:557–570

Shaw J, Lainson R (1972) *Trypanosoma vivax* in Brazil. Ann Trop Med Parasitol 66:25–32

Silva RA, da Silva JA, Schneider RC et al (1996) Outbreak of Trypanosomiasis due to *Trypanosoma vivax* Ziemann, 1905. in bovines of the Pantanal, Brazil. Mem Inst Oswaldo Cruz 52:561–562

Silva RA, Equez A, Morales G et al (1998) Bovine trypanosomosis in Bolivian and Brazilian lowlands. Mem Inst Oswaldo Cruz 93:29–32

Simoes D, Sánchez M, González Y, Rivera F, Parra R, Gil M, García ME, Quijada J y García F (2009) Brote de tripanosomosis en un rebaño doble propósito del municipio Mara del estado Zulia, Venezuela. CIENCIA 17:124–132

Simpson AG, Stevens JR, Lukes J (2006) The evolution and diversity of kinetoplastid flagellates. Trends Parasitol 22:168–174

Sow A, Sidibé I, Bengaly Z et al (2012) Field detection of resistance to isometamidium chloride and diminazene aceturate in *Trypanosoma vivax* from the region of the Boucle du Mouhoun in Burkina Faso. Vet Parasitol 187:105–111

Stevens JR (2008) Kinetoplastid phylogenetics, with special reference to the evolution of parasitic trypanosomes. Parasite 15:226–232

Stevens J, Rambaut A (2001) Evolutionary rate differences in trypanosomes. Infect Genet Evol 1:143–150

Stevens J, Brisse S (2004) Systematics of Trypanosomes of medical and veterinary importance. In: Maudlin I, Holmes P, Miles M (eds) The Tripanosomiases, 1st edn. CABI Publishing, Boston, MA, pp 1–24

Suárez C, García F, Román D et al (2009) Factores de riesgo asociados a la tripanosomosis bovina en explotaciones ganaderas de Venezuela. Zootecnia Trop 27:363–372

Tait A, Morrison L, Duffy C et al (2011) Trypanosome genetics: populations, phenotypes and diversity. Vet Parasitol 181:61–68

Tamasaukas R, Roa N, Cobo M (2006) Trypanosomosis por *Trypanosoma vivax* en búfalos (*Bubalis bubalis*), en dos fincas del estado Guárico, Venezuela. Rev Cient FCV-LUZ XVI:575–578

Taylor K, Authié EM-L (2004) Pathogenesis of animal Trypanosomiasis. In: Maudlin I, Holmes P, Miles M (eds) The Tripanosomiases, 1st edn. CABI Publishing, Boston, MA, pp 331–353

Thekisoe O, Inoue N, Kuboki N et al (2005) Evaluation of loop-mediated isothermal amplification (LAMP), PCR and parasitological tests for detection of Trypanosoma evansi in experimentally infected pigs. Vet Parasitol 130:327–330

Thekisoe OM, Kuboki N, Nambota A et al (2007) Species-specific loop-mediated isothermal amplification (LAMP) for diagnosis of trypanosomosis. Acta Trop 102:182–189

Thekisoe O, Rodriguez C, Rivas F et al (2010) Detection of *Trypanosoma cruz*i and *T. rangeli* infections from *Rhodnius pallescens* bugs by loop-mediated isothermal amplification (LAMP). Am J Trop Med Hyg 82:855–860

Toro M (1990) Seroepidemiología de las hemoparasitosis en Venezuela. In: Giardina S, and, Garcia F (eds.) Hemoparásitos: Biología y Diagnóstico. Cuadernos Serie Biología No. 1. Universidad Simón Bolívar, Caracas, Venezuela

Touratier L (1993) First International Seminar on Non Tsetse-Transmitted Animal Trypanosomoses:conclusions and recommendations. Rev Sci Tech 12:273–281

Touratier L (1999) The Office International des epizooties ad hoc group on non tsetse animal Trypanosomoses: its origin, scope and perspectives. Mem Inst Oswaldo Cruz 94:191–194

Touratier L (2000) Challenges of non-tsetse transmitted animal trypanosomoses (NTTAT). An outline and some perspectives. Ann NY Acad Sci 916:237–239

Truc P, Buscher P, Cuny G et al (2013) Atypical human infections by animal trypanosomes: new emerging diseases? PLoS Negl Trop Dis (in press)

Turner CM (1999) Antigenic variation in *Trypanosoma brucei* infections: a holistic view. J Cell Sci 112:3187–3192

Turner CMR, Aslam N, Dye C (1995) Replication, diferentiation, growth and virulence of *Trypanosoma brucei* infections. Parasitology 111:289–300

Uilenberg G (1998) A field guide for the diagnosis, treatment and prevention of African animal Trypanosomosis. Food and Agriculture Organization of the United Nations. Rome -FAO 1998. http://www.fao.org/DOCREP/006/X0413E/X0413E00.HTM. Accessed 17 Mar 2013

Ventura R, Paiva F, Silva R et al (2001) *Trypanosoma vivax*: characterization of the spliced-leader gene of a Brazilian stock and species-specific detection by PCR amplification of an intergenic spacer sequence. Exp Parasitol 99:37–48

Wells E, Betancourt A, Ramirez L (1977) Serological evidence for the geographical distribution of *Trypanosoma vivax* in the New World. Trans R Soc Trop Med Hyg 71:448–449

Wells E, Ramirez L, Betancourt A (1982) *Trypanosoma vivax* in Colombia: interpretation of field results. Trop Anim Health Prod 14:141–150

Woo PTK (1969) The haematocrit centrifuge for detection of trypanosomes in blood. Can J Zool 47:921–923

Yurchenko VY, Kolesnikov AA (2001) Minicircular kinetoplast DNA of Trypanosomatidae. Mol Biol 35:1–10

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