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Invited Review

The trypanosome lytic factor of human serum and the molecular basis of sleeping sickness

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Abstract

Trypanosoma brucei brucei infects a wide range of mammals but is unable to infect humans because this subspecies is lysed by normal human serum (NHS). The trypanosome lytic factor is associated with High Density Lipoproteins (HDLs). Several HDL-associated components have been proposed as candidate lytic factors, and contradictory hypotheses concerning the mechanism of lysis have been suggested. Elucidation of the process by which *Trypanosoma brucei rhodesiense* resists lysis and causes human sleeping sickness has indicated that the HDL-bound apolipoprotein L-I (apoL-I) could be the long-sought after lytic component of NHS. This research also allowed the identification of a specific diagnostic DNA probe for *T. b. rhodesiense*, and may lead to the development of novel anti-trypanosome strategies for use in the field.

Keywords: *Trypanosoma brucei*; Trypanosome lytic factor; Haptoglobin-related protein; Apolipoprotein L-I; Serum resistance-associated protein; Lysosome

1. Introduction

African trypanosomes include several species and subspecies that infect a wide range of game and domestic animals as well as humans. Some of these parasites are the agents of diseases of major veterinary or medical importance. Whereas *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* cause sleeping sickness in humans, *Trypanosoma brucei brucei*, *Trypanosoma congolense* and *Trypanosoma vivax* cause nagana in cattle, and *Trypanosoma evansi* and *Trypanosoma equiperdum* are, respectively, responsible for surra in camels and dura in horses. Sleeping sickness is amongst the worst plagues of Africa. Close to 50,000 cases are now reported annually, but the medical surveillance covers less than 5% of the population at risk, and according to WHO, 500,000 people could actually be infected. For instance, in villages located in epidemical foci in Sudan, Angola or Congo, the prevalence is 20–50%, and sleeping sickness may be a more common cause of mortality than AIDS (www.who.org).

Most trypanosomes are transmitted from host to host by tsetse flies (sp. *Glossina*). During their bloodmeal on a mammal, the tsetse fly ingests trypanosome bloodstream forms. Some of these, termed stumpy forms, are able to quickly differentiate into procyclic forms that will colonise the midgut of the insect. After a journey of four to six weeks in the digestive tract, the parasites reach the salivary glands where they differentiate into non-dividing metacyclic forms. Upon entry into the mammalian blood (following a bite) these cells resume cell division and differentiate into long slender forms. By a mechanism believed to be dependent on cell density (Vassella et al., 1997), most of these forms will eventually differentiate into non-proliferative stumpy forms, ready for differentiation into procyclic forms.

Survival of the trypanosome population in the blood is linked to the spectacular process of antigenic variation. The trypanosome is entirely covered with a dense coat made of a single type of protein, the variant surface glycoprotein (VSG), which is highly immunogenic and therefore elicits an efficient antibody response. Repeated variation of the VSG continuously allows trypanosomes to escape this response. The interplay between parasite antigenic variation and host antibody response shapes

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the typical pattern of chronic infection characterised by successive waves of parasitaemia. Prolonged infection is vital for the survival of trypanosomes, because the parasite transmission to healthy hosts is dependent on the bite by tsetse flies, and this process is both infrequent and inefficient. The molecular basis for antigenic variation is transcriptional switching from one *VSG* gene to another. In order to be transcribed, the *VSG* must be located in one of several dedicated telomeres termed *VSG* Expression Sites (ESs). These harbour long polycistronic transcription units containing not only the *VSG*, but also several other genes called Expression Site Associated Genes (*ESAG*s). At any time only a single ES is active, and therefore only one *VSG* and set of *ESAG*s is transcribed. Antigenic variation occurs either through replacement of the *VSG* in the active ES by DNA recombination, or following transcriptional switching between different ESs, a mechanism referred to as in situ activation (for recent reviews, see Borst and Ulbert, 2001; Pays et al., 2001; Vanhamme et al., 2001; Donelson, 2003).

2. Trypanosome lysis by human serum

African trypanosomes have a large range of mammalian hosts. *T. brucei* and *T. congolense*, for instance, are able to infect most game as well as domestic animals. However, humans are naturally resistant to infection by these parasites. In 1912 Laveran and Mesnil (1912) had already noted that normal human serum (NHS) was able to lyse *T. brucei*, and reported that this was not associated with immunoglobulins or complement activation. Laveran also uncovered trypanolytic activity in the serum of some baboon (*Papio*) and mandrill (*Mandrillus*) species, although considerable differences of activity were noticed, the *Papio papio* serum being much more efficient than human serum, and the activity of mandrill serum being low and inconsistent. Subsequent studies extended the trypanolytic property to gorilla serum, and confirmed the low and irreproducible nature of the activity of mandrill serum, which was also observed for the serum of *Papio hamadryas* (Seed et al., 1990; Kageruka et al., 1991; Poelvoorde et al., 2004). Worth mentioning are differences between humans and gorillas on one side and baboons on the other side, as lytic factors from these two groups showed distinct antigenic and functional properties (Seed et al., 1990; Poelvoorde et al., 2004). Therefore, the ability to lyse trypanosomes was probably acquired by at least two primate species independently. This ability could have been maintained upon selective pressure imposed by trypanosomiasis. Interestingly, in at least two cases the trypanolytic activity seems to have been lost, as the sera from chimpanzees and *P. hamadryas* cannot lyse trypanosomes despite the close evolutionary relatedness of these species to humans, gorillas and other *Papio* species.

3. Trypanosome resistance to lysis

Resistance to NHS is the primary character distinguishing *T. b. rhodesiense* and *T. b. gambiense* from *T. b. brucei*. The two former subspecies differ both in their geographical distribution and the acuteness of the disease they cause. *T. b. gambiense* is found in West and Central Africa while *T. b. rhodesiense* is widespread in East Africa. Infections by either parasite species are fatal if left uncured, but *T. b. rhodesiense* causes an acute disease whereas *T. b. gambiense* is associated with a slowly progressing chronic infection. Until very recently no morphological, biochemical or genetic criteria, including isoenzyme, amplified fragment length polymorphism (AFLP) or restrictions fragment length polymorphism (RFLP) analysis, unequivocally allowed the discrimination between the three subspecies (Hide et al., 1990; MacLeod et al., 2000, 2001; Gibson, 2002a). This has led to the suggestion that *T. b. rhodesiense* and *T. b. gambiense* are only extended host range variants of *T. b. brucei*.

T. b. gambiense, at least the group I as defined by Gibson (1986), is constitutively resistant to lysis by NHS (the so-called *T. b. gambiense* strain TXTaR (Ortiz-Ordonez et al., 1994), which exhibits conditional resistance to NHS, does probably not belong to this group). In contrast, the resistance phenotype of *T. b. rhodesiense* appears to be more complex. Laveran and Mesnil (1912) noticed that *T. b. rhodesiense* becomes sensitive to NHS if passaged in animals. Extensive characterisation of some isolates of this subspecies demonstrated that the ability to become resistant is both conditional and reversible. In the ETaR strain, many clonal antigenic variants exhibited sensitivity to NHS, but upon selective pressure by NHS a resistant variant, ETat 1.10, systematically emerged (Van Meirvenne et al., 1976). In the continuous presence of NHS new antigenic variants, including those previously found to be sensitive, remained resistant. On the contrary, if passaged in the absence of NHS the new variants reverted to NHS-sensitive forms, even if selected for expression of the same VSG as that present in resistant clones (Van Meirvenne et al., 1976; Xong et al., 1998) (Fig. 1). Overall, these analyses suggested that, if transition between resistance and sensitivity is associated with antigenic variation, neither phenotype could be assigned to the expression of a particular VSG.

4. A gene responsible for resistance in *T. b. rhodesiense*

A major step in the characterisation of the mechanism of resistance to NHS in *T. b. rhodesiense* was achieved when Hamers and collaborators performed substractive hybridisation between cDNAs from sensitive (S) and resistant (R) clones of the ETaR strain. This led to the identification of an mRNA specifically expressed in R cells. This mRNA was shown to encode a VSG-like

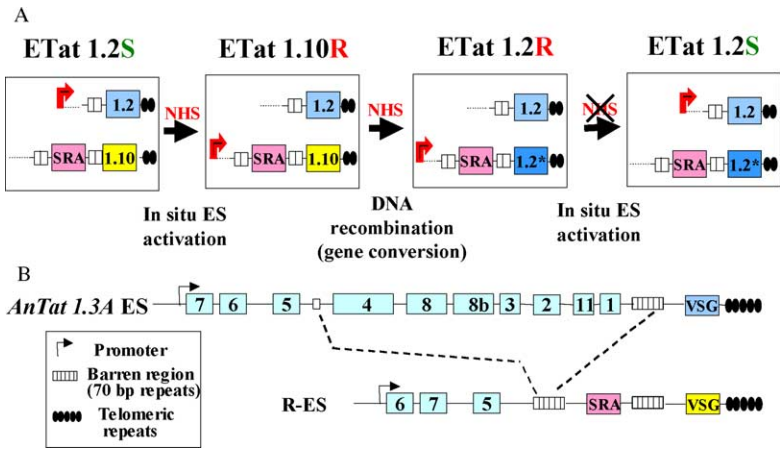

Fig. 1. Antigenic variation-linked expression of both Serum Resistance Associated protein (SRA) and resistance to normal human serum (NHS) in the ETaR strain of *Trypanosoma brucei rhodesiense*. *Panel A* summarises the observations reported by Xong et al. (1998). Different variant clones from the ETaR strain were derived from each other as indicated. In some cases NHS was injected into mice to select for resistance to NHS. These clones express either the ETat 1.2 or ETat 1.10 variant surface glycoprotein (VSG), and exhibit either sensitivity (S) or resistance (R) to NHS. The mechanisms found to trigger these antigenic switches are indicated below, and are illustrated in the highly schematised diagrams of the relevant ESs (VSG expression sites). In the latter only two genes from each polycistronic transcription unit are represented, namely those of the VSG and SRA. The broken arrow symbolises full transcription of the ES, a single ES being active at any time. The ETat 1.2 gene labelled with an asterisk is an identical copy, generated by gene conversion, of the gene active in the first clone of this series. *Panel B* details the actual structure of the R-ES, comparing this structure with that of the *T. b. brucei AnTat 1.3A* ES (Pays et al., 2001). In addition to the presence of *SRA*, the R-ES is also characterised by a large deletion encompassing *ESAG4–ESAG1*, which probably resulted from recombination between 70 bp repeats as indicated by the dotted lines.

protein named Serum Resistance Associated protein, or SRA (De Greef et al., 1989; De Greef and Hamers, 1994). Ten years later PCR-assisted differential display analysis of R and S variants from a different *T. b. rhodesiense* isolate detected the same R-specific transcript (Milner and Hajduk, 1999) (see EMBL Z37159 for updated sequence). Today three independent groups have confirmed that the presence of *SRA* generally correlates with resistance to NHS in this subspecies (Xong et al., 1998; Welburn et al., 2001; Gibson et al., 2002; Radwanska et al., 2002).

Detailed analysis of the antigenic switches associated with the appearance/disappearance of the resistance trait in the ETaR strain provided an explanation for the linkage of this phenotype to both antigenic variation and *SRA* expression. It was found that a given *VSG* ES, termed R-ES, is systematically used in R forms, and that this ES contains *SRA* as an additional *ESAG* (Xong et al., 1998) (Fig. 1). Thus, when this ES is active, trypanosomes both resist NHS and express *SRA* irrespective of the *VSG* present in this site. The involvement of *SRA* in resistance to NHS was evaluated by transfection of this gene into *T. b. brucei*. Transgenic *T. b. brucei* constitutively expressing *SRA* became fully resistant to NHS both in vitro and in mice, proving that *SRA* is necessary and sufficient to confer the R phenotype (Xong et al., 1998). This result indicated that *SRA* is the gene that allowed *T. b. rhodesiense* to infect humans. Interestingly, *SRA* was not found in *T. b. gambiense* (De Greef et al., 1992) suggesting that another gene is responsible for resistance in this subspecies.

5. Expression of SRA and resistance to NHS during the life cycle of *T. b. rhodesiense*

According to the data obtained for the ETaR strain, a population of S cells can generate R forms by antigenic variation, through random in situ switching to the R-ES. As antigenic variation is a stochastic process, with frequencies of 10^{-7} to 10^{-2} depending on the strain, the larger is the population, the higher is the probability that such a cell will be generated. In the presence of NHS selective pressure will favour that cell. Therefore, upon infection of a laboratory animal or seeding of a culture flask by a population of S forms in the presence of NHS, if the size of the population is in the same order of magnitude or higher than the frequency of antigenic switching in this strain, the probability of generating a population of resistant trypanosomes, is high.

Metacyclic forms are the first invaders of mammalian blood. Therefore, when injected in NHS these cells should resist lysis. Brun and Jenni found that in *T. b. rhodesiense* NHS resistance is only exhibited by a minority of metacyclic cells, and is very inconsistent (Brun and Jenni, 1987). In a given experiment only two out of 11 salivary samples from individual flies (between 800 and 8000 metacyclic forms) contained resistant cells, whereas pools of cells from several flies (3000–14,000 metacyclic forms) were infective in all three cases tested. In the same tests metacyclic forms from *T. b. brucei* were always sensitive, and metacyclic forms from *T. b. gambiense* always resistant. In these in vitro experiments, the detection of R metacyclics was based on the reappearance of a trypanosome population

several days after seeding NHS-containing medium with metacyclics. If this clearly indicated that some metacyclics survived the test, it does not necessarily mean that these cells were intrinsically resistant to NHS. Indeed, it is possible that in some cells activation of the bloodstream-specific R-ES occurred rapidly enough to save them from lysis. As metacyclic forms are normally not injected straight in the blood but rather in tissue spaces or lymphatic vessels where they can remain for hours or days, it can be envisaged that the natural environmental conditions are relaxed enough to permit frequent transformation of metacyclics into bloodstream forms that express the R-ES. In any rate, the data of Brun and Jenni (1987) strongly suggest that the infectivity of *T. b. rhodesiense* to humans, at least when transmission occurs by tsetse flies, is very low.

Procyclic forms are fully resistant to lysis by NHS, a character that appears very quickly at differentiation from bloodstream forms (Moore et al., 1995). Resistance of these forms is probably due to their very low level of endocytosis, as suggested by the appearance of a similar phenotype in *T. b. brucei* bloodstream forms rendered defective for endocytosis by conditional knock-down of actin (Garcia-Salcedo et al., 2004).

6. Purification and characterisation of the trypanosome lytic factor

6.1. HDL and TLF1

A first characterisation of the trypanolytic factor was reported by Rifkin, who found this activity associated with high density lipoproteins (HDL) (Rifkin, 1978). The results received further support from several observations (Hajduk et al., 1989,1994; Gillett and Owen, 1991, 1992a,b; Lorenz et al., 1995a,b) specifically: (i) the level of serum HDL correlates positively with the level of trypanolytic activity; (ii) depletion of HDL reduces the lytic activity of NHS; (iii) anti-HDL antiserum inhibits trypanolytic activity. HDLs have a lipid core of triglycerides and cholesterol esters coated by an external layer of phospholipids and cholesterol that contain apolipoproteins. These particles carry water-insoluble molecules to various places in the body and the major function of HDLs is to return cholesterol to the liver. HDLs have been classified in as many as a dozen subclasses dependent on their size, density, electrophoretic mobility and apolipoprotein content. The trypanosome lytic activity (TLF) is present in a sub fraction of HDLs commonly refered to as HDL3 (Lorenz et al., 1994b, 1995b). Further, purification allowed the identification of a minimal complex termed TLF1 (for trypanosome lytic factor 1), a small subfraction with very high density (1.20–1.26 g/ml.), constituted of large particles of 18–22 nm (550 kDa) present at 10 μg/ml in the plasma and containing apoA-I, apoA-II, haptoglobin (Hp), haptoglobin-related protein (Hpr), paraoxonase as well as a few unidentified proteins (Smith et al., 1995; Hajduk et al., 1989; Raper et al., 1999; Drain et al., 2001).

6.2. TLF2

Further analysis of NHS by the group of Raper detected a second TLF with higher molecular weight (1000 kDa) and a much higher density (1.28 g/ml.) related to its very low lipid content (<1%) (Raper et al., 1996b; Tomlinson et al., 1997). This fraction was found to contain apoA-I, Hpr and IgM as well as some unidentified proteins.

6.3. TLF1 or TLF2?

Which TLF is active in vivo has been a matter of debate. In particular, much attention has been paid to Hp, since this protein was reported to be a natural inhibitor of TLF (Smith and Hajduk, 1995). Hp levels were found to inversely correlate with TLF activity. Moreover, immunodepletion with anti-Hp antibodies led to increased trypanolysis, whereas the addition of purified Hp inhibited the process. There is some evidence indicating that the inhibitory effect of Hp targets TLF1 (Tomlinson et al., 1995; Raper et al., 1996a,b, 1999; Barker et al., 2001): (i) TLF1 activity increases when it is purified from the serum; (ii) excess Hp inhibits TLF1, but not TLF2, in a dose-dependent and reversible manner; (iii) excess Hp does not inhibit NHS activity; (iv) patients with vascular haemolysis or paroxysmal nocturnal haemoglobinurea, where Hp is titrated out by a large excess of haemoglobin and cleared by the liver, have a 10 times higher trypanolytic activity. According to these observations, TLF2 would be the physiological lytic factor (Raper et al., 2001).

TLF1 and TLF2 are present at a similar concentration (respectively 10 and 12 μg/ml in plasma, Raper et al., 2001), contain some common proteins and seem to act on trypanosomes with the same time- and temperature-dependence, as well as the same sensitivity to the endosome-alkalising agents chloroquine and NH_4Cl. Therefore, it seems reasonable to assume that the same active component is present in the two different serum particles, and would operate following the same mechanism once inside the trypanosome. These two particles would differ in their mechanism of binding and/or entry into trypanosomes, the binding of TLF1 being possibly inhibited by Hp (Drain et al., 2001).

7. TLF binding to trypanosomes

For trypanosomes, the only site of entry for external macromolecules is the flagellar pocket (for a recent review, see Gull, 2003). The binding and uptake of known ligands (transferrin, LDL) is highly polarised and extremely rapid. In the case of HDLs several studies concluded that there are 16,000–65,000 binding sites per cell, with a moderate

affinity (mid-micromolar range) (Gillett and Owen, 1992a; Hager et al., 1994). Work with purified TLF1 found 65,000 low affinity and 350 high affinity-binding sites (Drain et al., 2001). These studies were consistent with the existence of high capacity/low affinity receptors that would bind all HDL particles, as well as a few specific and saturable receptors for TLF. Based on (i) binding of purified Hpr, (ii) inhibition of this binding by TLF1 but not by nonlytic HDL, (iii) inhibition of TLF1 binding by purified Hpr, and (iv) inhibition of TLF binding by anti-Hpr antibodies, it was concluded that the TLF receptors recognise Hpr as a specific ligand (Drain et al., 2001).

Little is known about the molecular nature of these receptors. However, a recent report proposed that a lipoprotein scavenger receptor would be responsible for the binding of low/moderate affinity. The main arguments were the following: (i) the estimated numbers of LDL and HDL binding sites are roughly the same (16,000–65,000 HDL receptors, versus 30,000–52,000 LDL receptors); (ii) HDL, LDL and TLF1 compete for binding to the same sites; (iii) lipids of lipoprotein particles are taken up preferentially compared with proteins (Green et al., 2003).

Whatever the nature of the receptor, an obvious question immediately arises: is there a difference between resistant and sensitive trypanosomes regarding the binding and uptake of TLF? Answers to this question were contradictory. While Hajduk and collaborators reported binding but no uptake of TLF in resistant trypanosomes (Hager and Hajduk, 1997), other studies did not find any difference between resistant and sensitive trypanosomes at both binding and uptake levels when HDL3 (Lorenz et al., 1995b) or NHS reconstituted with fluorescent apoL-I (Vanhamme et al., 2003) were used as ligands.

8. The mechanism of lysis

8.1. Endocytosis of TLF

The lysis of trypanosomes is very fast, occurring in a matter of minutes and is irreversible. While a lag phase of 20–40 min precedes the observable effects, incubation with NHS for only 6 min at 37 °C is enough to achieve a 50% loss of infectivity in mice (Rifkin, 1984). After the lag phase trypanosomes start to swell, round up with a free flagellum and look like tadpoles. This process is osmotically driven and paralleled by an increase in ion permeability of the plasma membrane (Rifkin, 1984). This phenomenon could be ascribed to an immediate and non-specific transfer of lipids or proteins from the HDL particles to the trypanosome membrane, which would result in membrane destabilisation and subsequent disruption. However, all observations rather indicate that TLF is taken up by receptor-mediated endocytosis followed by intracellular delivery into the lysosome (Hager et al., 1994; Hajduk et al., 1994; Lorenz et al., 1994a; Ortiz-Ordonez et al., 1994; Hager and Hajduk, 1997; Shimamura et al., 2001). The following evidence indicate this:

(i) TLF binds to saturable and specific high affinity sites.
(ii) Lysis involves at least two temperature-sensitive steps: it is inhibited at 4 °C where there is binding but no endocytosis, but is also inhibited at 17 °C, which allows endocytosis but not fusion between endosomes and the lysosome.
(iii) Lysis is sensitive to agents such as chloroquine and NH_4Cl, which prevent acidification of internal compartments.
(iv) Immunohistochemical methods directly demonstrated the internalisation of TLF or its components, as well as their progression towards the lysosome.
(v) Finally, trypanosomes whose components of the endocytic machinery such as actin were abrogated by conditional RNA interference, displayed a phenotype of resistance to NHS (Garcia-Salcedo et al., 2004).

8.2. TLF action in the lysosome

While there seems to be a general consensus concerning the intracellular routing of TLF, the mechanism by which TLF triggers lysis from the lysosome is disputed. The arguments can be differentiated on the basis of their relative support for a model of membrane peroxidation.

8.2.1. Arguments for the peroxidative mechanism

Hajduk and collaborators have proposed that TLF triggers acidic pH-dependent oxidative activity that would generate free radicals, peroxidation of lipids in the lysosomal membrane and subsequent membrane disruption resulting in the release of lysosomal enzymes in the cytoplasm and auto digestion of the cell. This model is based on the following observations.

(i) TLF1 possesses peroxidase activity at pH 4 (Smith et al., 1995). Such an activity was also demonstrated for the Haptoglobin-Hemoglobin complex (Hp–Hb). Since traces of Hb had been detected in the purified TLF, the peroxidase activity was first ascribed to the Hpr–Hb dimer (Smith et al., 1995). However, the pH of lysosomes is close to 5.5, thus higher than that used for the detection of the peroxidase activity of the Hpr–Hb complex. Moreover, TLF was later shown not to contain any Hb (Muranjan et al., 1998; Raper et al., 1999), and this conclusion was further supported by the observations that anti-Hb antibodies do not precipitate TLF, that Hpr does not bind Hb, and that Hpr is not cleared from patients with intravascular haemolysis, which generates a large amount of free haemoglobin in the blood (Muranjan et al., 1998). In a second version of the initial model,

the lipid peroxidation was ascribed to the Fenton reaction between H_2O_2 and iron, since trypanolysis was found to be inhibited by iron chelators (Bishop et al., 2001). This reaction would generate free hydroxyl radicals, which would trigger the chain reaction of lipid peroxidation. This alternative hypothesis is still not fully satisfactory, because the intriguing question of the origin of iron remains.

(ii) Catalase, an enzyme with antioxidative activity, is not present in trypanosomes. When supplied to trypanosomes, this enzyme was able to inhibit TLF1-mediated lysis (Smith et al., 1995).

(iii) DPPD, an antioxidant known to inhibit lipid peroxidation, inhibited trypanolysis (Bishop et al., 2001). However, this compound is also a weak base, which could therefore act on the pH of intracellular vesicles.

(iv) Lysis was associated with the detection of end products of lipid peroxidation (Bishop et al., 2001). However, the latter could have been due to a contamination by Hp–Hb in these experiments.

(v) Trypanolysis was inhibited by protease inhibitors (Hager et al., 1994; Bishop et al., 2001).

8.2.2. Arguments against a peroxidative mechanism

In a set of experiments with contrasting results (Molina Portela et al., 2000), Raper and colleagues reported that:

(i) Peroxide-specific intracellular probes did not detect any TLF-induced reactive oxygen intermediates.

(ii) There was no evidence of lipid peroxidation upon exposure to TLF, although in control experiments membrane lipids could indeed be peroxidated when trypanosomes were exposed to low peroxide concentrations.

(iii) TLF activity was not blocked by antioxidants or metal chelating agents able to break the free radical chain reaction of lipid peroxidation. Antioxidants enhanced trypanolysis marginally.

(iv) TLF activity was not blocked by catalase or superoxide dismutase.

(v) There was no inhibition of trypanolysis by protease inhibitors.

The clear contradiction between these two sets of results leaves the field open for alternative explanations. Molina Portela et al. (2000) suggested that Hpr could be membranolytic. However, recent observations (Vanhamme et al., 2003) argue against a possible fusogenic effect of TLF on the lysosomal membrane. Alternatively, the change in ion permeability that seems to be associated with lysis (Rifkin, 1984) supports the idea of pore formation in cellular membranes. According to this hypothesis, pore formation would be triggered by acidic pH-induced conformational change of a TLF component.

9. The TLF toxin candidates

9.1. ApoA-I

Apolipoprotein A-I is the most abundant protein of HDLs. This protein serves as a specific cofactor for the enzyme that esterifies cholesterol in the plasma. It is a single chain polypeptide of 28 kDa. Because of its abundance, the trypanolytic activity of this protein was the first to be tested. ApoA-I purified from human serum, but not that from bovine or sheep serum, was found to lyse trypanosomes (Gillett and Owen, 1992b). Moreover, serum deficient in apoA-I had no measurable TLF activity. In these experiments, sheep or bovine HDLs reconstituted with human apoA-I acquired trypanolytic activity. Because these experiments were performed with native apoA-I, which is present in amounts up to a thousand times higher than other components it is likely that this protein was contaminated, possibly by the genuine TLF. In order to clarify this question the trypanolytic activity of recombinant apoA-I should be tested, but this has not been reported yet. However, different observations cast serious doubts on the putative lytic activity of this protein.

(i) Other studies did not confirm the trypanolytic activity of apoA-I (Rifkin, 1991).

(ii) As mentioned previously, only a subfraction of the human HDL possesses trypanolytic activity, despite the overwhelming presence of apoA-I in all HDL subfractions.

(iii) Transgenic mice expressing physiological levels of apoA-I were not resistant to trypanosome infection although their serum showed a slight trypanolytic activity. This phenotype was tentatively ascribed to the presence of some TLF inhibitory activity in mouse HDL (Owen et al., 1992).

(iv) The serum from patients with Tangier disease, associated with undetectable levels of HDL and apoA-I, still shows trypanolytic activity (Tomlinson et al., 1995).

9.2. Hpr

The haptoglobin-related protein is present in the human plasma at concentrations ranging from 25 to 50 μg/ml, and is associated with apoA-I in HDLs. This protein is organised as a heterodimer of peptides of 13 and 36 kDa, both processed from the same precursor. Hpr shares 94% identity with Hp, an abundant (2 mg/ml) protein that strongly binds free Hb and transports it back to the liver. The function of Hpr is unknown.

Hpr has been identified as a common component of TLF1 and TLF2 (Tomlinson et al., 1997). Because anti-Hp antibodies, which strongly cross-react with Hpr, inhibited TLF activity, it has been claimed that Hpr is the active component of TLF (Smith et al., 1995). This view was

supported by the distribution of Hpr in primate sera, since both Hpr and TLF were missing from chimpanzee sera. The absence of Hpr was found to be due to a frameshift mutation in the Hpr gene of chimpanzees (McEvoy and Maeda, 1988). However, the potential lytic activity of recombinant Hpr was never assessed, and, more importantly, trypanolytic activity could not be recorded in transgenic mice producing human Hpr (Hatada et al., 2002). Finally, arguments against the involvement of Hpr in trypanosome lysis were obtained when analysing the role of apoL-I (see next paragraph).

An alternative hypothesis involving Hpr in TLF is that Hpr is the ligand responsible for binding of the lytic particles to the trypanosome (Drain et al., 2001). This function would explain the observation that TLF activity is neutralised by an excess of Hp, which would act as competitor for the same surface receptors. However, some observations argue against this new hypothesis (see next paragraph).

9.3. ApoL-I

This candidate was not identified after purification of TLF, but following a completely different approach based on the study of SRA, the natural TLF antidote of *T. b. rhodesiense* (Xong et al., 1998; Vanhamme et al., 2003). A detailed mutagenesis study of the VSG-like protein SRA was undertaken to identify the determinants responsible for its activity. The results pointed to an essential role of the N-terminal α-helix A, whose function in VSGs is to organise coiled-coil protein interactions. This observation suggested that SRA neutralises TLF through coiled-coiling interaction. A doublet of proteins (42 and 39 kDa) specifically bound to SRA via an affinity column based on the N-terminal domain of recombinant SRA loaded with NHS. These proteins were identified as being the two secreted forms of apoL-I, a human-specific apolipoprotein mainly associated with the HDL3 fraction (Duchateau et al., 1997). That apoL-I is the active lytic component of TLF was supported by the following results (Vanhamme et al., 2003, and unpublished data).

(i) Mutated SRA without the capacity to confer resistance to NHS were not able either to bind apoL-I.
(ii) NHS depleted of apoL-I following chromatography on SRA totally lost trypanolytic activity.
(iii) NHS depleted of apoL-I following chromatography on anti-apoL-I antibodies also lost trypanolytic activity.
(iv) The lytic activity of these depleted sera was fully recovered when supplemented with physiological concentrations of native apoL-I, although the role of possible contaminants cannot be excluded.
(v) More importantly, physiological concentrations of recombinant apoL-I produced either by in vitro translation, expression in yeast or expression in CHO cells was also able to restore trypanolytic activity of apoL-I depleted sera.
(vi) In the reconstitution experiments, the kinetics of trypanosome lysis by recombinant apoL-I was similar to that observed with NHS.
(vii) Physiological concentrations of recombinant apoL-I lysed trypanosomes in fetal calf serum, which lacks Hpr, suggesting that apoL-I does not require Hpr to be internalised and lyse the trypanosomes. Again, in these experiments the kinetics of lysis resembled that observed with NHS.
(viii) The trypanolytic activity of apoL-I was only observed on NHS-sensitive trypanosomes, and never on NHS-resistant parasites such as R clones of *T. b. rhodesiense* or SRA-expressing transgenic *T. b. brucei*. This result demonstrated that recombinant apoL-I exhibits the same biological activity as NHS.

Finally, one of the main arguments invoked to support the identification of Hpr as TLF, namely its absence from chimpanzees, was recently challenged by the demonstration that chimpanzees lack apoL-I too (Poelvoorde et al., 2004).

In humans the apoL-I gene is part of a multigene family that contains six members (apoL-I to apoL-VI) organised as a cluster on chromosome 22 (Duchateau et al., 2001; Page et al., 2001). Related genes exist in zebrafish, mouse and rat, but *apoL-I* appears to be specific to only gorilla and man (Poelvoorde et al., 2004). It has been proposed that a tandem duplication of one of the apoL family members resulted in the generation of an additional copy linked to the sequence of a N-terminal signal peptide, enabling the secretion of its product into serum (Page et al., 2001). All apoL isoforms appear to have a broad tissue distribution, suggesting a basic function for these proteins. More precisely, apoL-I is expressed in the liver, spleen, kidney, placenta, lungs and small intestine as well as in endothelial cells and macrophages. A role for apoL-I in lipolysis of triglyceride-rich particles has been suggested due to its specific association with HDLs and its overexpression associated with hypercholesterolemia and hypertriglyceridemia (Duchateau et al., 2000). This hypothesis correlates with the expression of apoL-I in endothelial cells of the vascular tissue and its induction by TNF-α, a cytokine involved in inflammation (Horrevoets et al., 1999; Monajemi et al., 2002). Therefore, apoL-I could play a role in the modulation of the lipid content during inflammation. A protective role has indeed been attributed to HDLs against the development of atherosclerotic diseases, which themselves result from inflammatory processes initiated by endothelial injury. In addition, although apoL-I expression in the brain appears to be low, elevated levels of mRNAs were detected in neurones of the prefrontal cortex from schizophrenic patients (Mimmack et al., 2002). Here again, inflammatory causes have been invoked for the development of schizophrenia (Naudin et al., 1997; Marx et al., 2001).

10. The molecular basis for resistance

The demonstration that SRA is necessary and sufficient to confer resistance to NHS raised the question of its molecular action. To answer this question the SRA gene was subjected to heavy mutational and deletional analysis, and chimeras between various parts of the SRA and a VSG were also constructed. More than 40 altered versions of the SRA gene were examined for their capacity to confer resistance to NHS in transgenic *T. b. brucei*. These experiments allowed the following conclusions (Vanhamme et al., 2003): (i) SRA molecules devoid of signal peptide, GPI anchor, C-terminal domain or all N-glycosylation sites are still able to confer resistance; (ii) the loss of either surface-exposed loops or the C-terminal cysteines presumably involved in disulphide bridging does not affect resistance; (iii) the N-terminal α-helix A is indispensable to confer resistance. These findings indicated that the phenotype conferred by SRA is extremely robust, since important deletions such as those of the N- and C-terminal signal peptides did not prevent activity. Presumably the intracellular targeting of SRA does not require these signals. As mentioned above, the main conclusion from these studies was that SRA neutralises TLF by direct coiled-coiling interaction, a hypothesis that led to the identification of apoL-I as the lytic component of TLF. Thus, the N-terminal α-helix of SRA is able to interact with apoL-I. Deletional and mutational analysis of apoL-I allowed the delineation of the interacting segment of this protein to its C-terminal α-helix (Vanhamme et al., 2003). Computational modelling suggested an antiparallel interaction, which would be as stable as the interaction between dimeric VSG molecules, but more stable than those in putative SRA dimers (Vanhamme et al., 2003). Although SRA and apoL-I can interact in vitro, so far apoL-I-SRA complexes have not been detected in vivo. However, two independent lines of evidence support the hypothesis that these exist. First, apoL-I and SRA colocalise in the lysosome. Second, a C-terminal truncated version of apoL-I, which lacks the α-helix interacting with SRA, was found able to lyse resistant clones of *T. b. rhodesiense* (Vanhamme et al., 2003). This last result suggests that this interaction is involved in the resistance of this subspecies to NHS.

Together, these data are best explained by the following model (Fig. 2). The HDL-bound apoL-I is taken up by the parasite by endocytosis, and finally reaches the lysosome. The acidic pH of this environment triggers the lytic activity of apoL-I, by a molecular mechanism that remains to be solved. In resistant *T. b. rhodesiense*, SRA neutralises this activity following physical interaction with the C-terminal helix of the toxin. How the complex is discarded afterwards is unknown. Since recombinant SRA and recombinant apoL-I are necessary and sufficient to mimic in *T. b. brucei* the natural phenotype of *T. b. rhodesiense*, resistance to NHS in this subspecies cannot be considered as a complex trait as previously proposed.

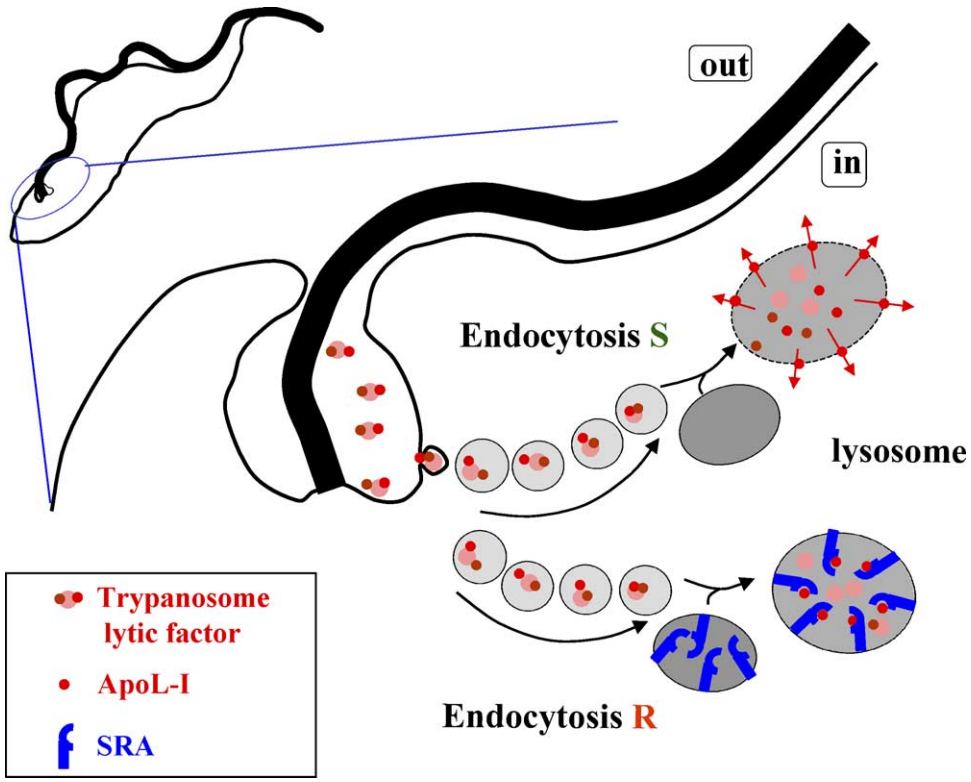

Fig. 2. Model of trypanosome lysis by normal human serum (NHS), and resistance to lysis in *Trypanosoma brucei rhodesiense*.

11. The clonal origin of *T. b. rhodesiense*

As already mentioned, until recently *T. b. brucei*, *T. b. rhodesiense* and *T. b. gambiense* were indistinguishable by any specific biochemical, genetical or morphological marker, although the high genomic stability of the so-called group 1 of *T. b. gambiense* allowed its identification through the use of a variety of probes (Gibson, 1986). Isoenzyme patterns, RFLP or RAPD analyses did not allow the discrimination between *T. b. rhodesiense* and *T. b. brucei*, but revealed genetic polymorphism between foci of the former subspecies (Hide et al., 1990; MacLeod et al., 2000, 2001). For example, genetic differences between Northern and Southern isolates were associated with different clinical manifestations (Gibson, 2002b). Together these analyses suggested that *T. b. rhodesiense* might have multiple origins (MacLeod et al., 2001).

The discovery of *SRA* completely changed the perspective. A simple sequence analysis of *SRA* allowed its classification within the *VSG* family (De Greef and Hamers, 1994). Moreover, the alignment with *VSG* sequences indicated that *SRA* lacks the domain encoding the antigenic surface-exposed loops of the protein (Vanhamme et al., 2004). This conclusion was independently reached by structural modelling of the SRA sequence (Campillo and Carrington, 2003). A careful comparison with the reference sequence database for *T. brucei* (TREU927/4) identified a gene that we termed *SRA-BC*, for basic copy of *SRA* (Vanhamme et al., 2004). Indeed this gene showed all the characteristics of a regular *VSG*, including the presence of a domain encoding surface-exposed loops similar to those of the BoTat1.20 VSG. Thus, it is likely that *SRA* evolved from *SRA-BC* by internal in-frame deletion of this domain. Whether this deletion was required to achieve proper targeting of SRA into the lysosome instead of the plasma membrane, is open to investigation. At any rate, the presence of *SRA* in a given ES strongly suggests that this event occurred during DNA rearrangements associated with antigenic variation. Three independent groups have evaluated the generality and relevance of this event for the *T. b. rhodesiense* subspecies as a whole (Welburn et al., 2001; Gibson, 2002a,b; Gibson et al., 2002; Radwanska et al., 2002; Welburn and Odiit, 2002). According to these studies, *SRA* is ubiquitous in *T. b. rhodesiense* and can be considered as the best marker of this subspecies. Moreover, the genomic location of this gene in the R-ES was found to be highly conserved between isolates (Radwanska et al., 2002; Gibson and Ferris, 2003). These findings strongly suggest a single molecular origin for the appearance of NHS resistance in *T. b. rhodesiense*, confirming that *SRA* probably arose once by DNA recombination within the R-ES. The genetic heterogeneity observed with several markers (MacLeod et al., 2001; Gibson, 2002a) could be explained by further genetic divergence, spreading of *SRA* by genetic exchange and alterations or duplications linked to repeated transcriptional activation of this gene in the R-ES.

12. A new role for apolipoproteins: innate immunity against parasites?

Lipoprotein particles, and in particular HDLs, have long been suspected to have an anti-inflammatory effect. This has been particularly studied in connection with their protective effect against the atherogenic process. Thus, the decrease of the general level of HDL often observed during infection by viruses, bacteria and parasites (Nilsson-Ehle and Nilsson-Ehle, 1990; Van Lenten et al., 2001) could influence the development of the atherosclerotic lesion. The relationship between infection and the level and/or action of HDL components is poorly characterised at the molecular level. A few reported examples show that HDL and in particular apoA-I protects against endotoxins or cell infection by the HIV (Owens et al., 1990; Levine et al., 1993). It is also known that some HDL sub fractions (apoE-containing particles) are specifically enhanced.

Apart from their primary role in lipid transport, HDL particles could have evolved other functions. One of these functions could be to fight against infections, as their location in the blood is ideal to defend the body against invaders. The anchoring of anti-infectious proteins in lipoprotein particles would ensure their sequestration when not needed to prevent cytotoxic effects and preservation of high plasma levels. ApoL-I (Vanhamme et al., 2003) and antibacterial defensins or cathelicidins (Sorensen et al., 1999) are such examples of HDL proteins that participate in these anti infectious functions.

13. Questions to be answered

Although significant progresses have been made in the identification of the active component of TLF and the gene conferring resistance to lysis by NHS in *T. b. rhodesiense*, many questions remain open.

(i) How does TLF enter the trypanosome? Is there a specific receptor? Does this receptor bind apoL-I or another HDL component?
(ii) How does apoL-I lyse trypanosomes?
(iii) How and why is SRA targeted to the lysosome?
(iv) How does SRA neutralise the lytic activity?
(v) What is the mechanism of resistance to apoL-I in *T. b. gambiense*?
(vi) Is it conceivable to generate apoL-I derived peptides that would kill human-infective trypanosomes, and therefore could be used to cure sleeping sickness?
(vii) Is it conceivable to generate transgenic cattle that would express these apoL-I derived peptides, and would thus be trypanoresistant and productive in endemic areas?

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