Haptoglobin-hemoglobin receptor independent killing of African trypanosomes by human serum and trypanosome lytic factors

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Introduction

African trypanosomes are eukaryotic pathogens that cause important human and animal diseases. These parasites have evolved a variety of mechanisms to escape innate and acquired immunity, including the use of the variant surface glycoprotein (VSG) coat to cover the plasma membrane of the parasite providing a barrier against attack by complement.1 The VSG coat also serves as the molecular decoy during antigenic variation, presenting an ever-changing target to the adaptive immune system of the mammal, thus allowing the parasites to evade antibody-mediated killing.1 The subspecies of trypanosomes that infect humans face the additional challenge of encountering a unique innate defense mechanism mediated by two related serum proteins complexes. In the circulation of humans, TLF-1 is a minor subclass of HDL containing apolipoprotein A-1 (apoA-1), the defining protein of all HDLs, and two primate specific proteins, apolipoprotein L-1 (apoL-1) and haptoglobin-related protein (Hpr).3-7 In addition to these apolipoproteins, Hpr binds free hemoglobin (Hb) in the circulation, which is likely released from erythrocytes during early infection.3 The heterodimeric Hpr/Hb complex is proposed to be bifunctional, serving both as the ligand for the T. b. brucei HpHbR3,9 and directly contributing to high specific activity killing by catalyzing the peroxidation of lysosomal membrane lipids.6,7 The other primate specific apolipoprotein in TLF-1, apoL-1, is also directly involved in T. b. brucei HpHbR3,9 and directly contributing to high specific activity killing by catalyzing the peroxidation of lysosomal membrane lipids.6,7 An ion channel forming protein, apoL-1 undergoes conformation...
changes at lysosomal pH and can integrate into membranes. The combined action of Hpr/Hb and apoL-1 results in the osmotic lysis of the parasite. The other members of Hpr/Hb and apoL-1 results in the binding and inhibiting TLF-1. SRA, an serum resistance associated (SRA) protein Trypanosoma brucei rhodesiense produces the serum resistance associated (SRA) protein that binds and inhibits TLF-1. SRA, an intracellular protein largely found in endosomes, co-localizes with TLF-1 in early endosomes and traffics to the lysosome. Thus, T. b. rhodesiense survives in humans largely because it is able to produce an antidote to TLF-1. While untested, it is likely that TLF-2 is inhibited by SRA by the same mechanism since both serum complexes contain apoL-1. In contrast, we recently showed that group 1 T. b. gambiense does not bind or take up TLF-1, suggesting that these cells have evolved a different mechanism to avoid the cytotoxicity of TLF-1. The underlying basis for reduced TLF-1 uptake is 2-fold. First, TbgHprR is expressed at very low levels by group 1 T. b. gambiense and second, TbgHprR contains a number of point mutations within the coding sequence that render the receptor non-functional. The combination of mutations to the TbgHprR and reduced expression aboliished TLF-1 binding and uptake resulting in resistance to TLF-1. Thus, in contrast to T. b. rhodesiense, it appears the mechanism of group 1 T. b. gambiense resistance to TLF-1 involves reduced uptake and avoidance of the toxin. To date no evidence for an inhibitory protein with SRA-like characteristics has been described in T. b. gambiense. In this short addendum to the Kieft et al. paper we now show that while the TbgHprR enhances susceptibility to human serum, TLF-2 and TLF-1 other receptors or fluid phase endocytosis also contribute to trypanosome killing. Further, our results suggest that the resistance of group 1 T. b. gambiense to human serum and TLF involves other mechanisms beyond the simple loss of a single receptor.

Materials and Methods

TLF-1 and TLF-2 purification. Total serum was obtained from a healthy human donor. As previously described, two sequential flotation on sodium bromide gradients (ρ = 1.063 and 1.26 g/ml) resulted in an HDL-rich fraction (TLF-1; top third of the gradient) and a lipoprotein-deficient fraction (TLF-2; bottom third of the gradient). The TLF-1 fraction was passed over an anti-IgM column (Sigma, A9935). The unbound material was then passed over an anti-Hpr column, washed with PBSE (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO 4 , 10 mM KH2PO 4 , 3 mM EDTA) and bound protein was eluted in 100 mM glycine (pH 2.5) and neutralized with 1 M Tris (pH 7.5). The TLF-2 fraction was passed over an anti-Hpr column and washed with PBSE. Bound protein was eluted in 100 mM glycine (pH 2.5), neutralized with 1 M Tris (pH 7.5). All protein samples were aliquoted and stored at -80°C.

Size exclusion chromatography and protein gel blot analysis. Size exclusion chromatography was performed on a 1 X PBSE equilibrated Supersose 6 10/300 GL column (GE Healthcare). Individual protein standards were used to estimate the molecular weights of TLF-1 and TLF-2. Samples of TLF-1 and TLF-2 from immuno-affinity purification (70 μg) were run on the Supersose 6 column at a flow rate of 0.5 ml/min. Fractions were collected (0.5 ml), proteins concentrated 6-fold with microspin S-300HR columns (GE, 27513001) and the distribution of Hpr, apoL-1 and IgM determined by SDS-PAGE, silver staining and protein gel blot analysis. Characterization of antibodies against Hpr and apoL-1 has previously been described. Anti-IgM was purchased from Sigma and used according to the manufacturer's recommendation (Sigma, I0799).

Results

TLF-1 resistant T. b. brucei. During the course of our studies on the mechanism of TLF-1 resistance in group 1 T. b. gambiense we developed a laboratory model for TLF-1 resistance using well-characterized clonal cell lines of T. b. brucei that had been selected for resistance to human HDLs. We isolated TLF-1 resistant (R) or susceptible (S) T. b. brucei lines expressing either the VSG080 or VSG060. The T. b. brucei 427-800 R and T. b. brucei 427-600 R lines showed reduced uptake of TLF-1 relative to the TLF-1 susceptible parental T. b. brucei 427-221 cells and TLF-1 susceptible cells expressing either VSG080 or VSG060. In addition, we showed that the expression of TbgHprR mRNA was reduced approximately 20-fold in resistant cells. These findings led us to examine group 1 T. b. gambiense where we found that not only was expression of the TbgHprR mRNA reduced but that mutations to the gene abolishes function.

Purification and characterization of TLF-1 and TLF-2. In order to determine whether loss of TbgHprR was sufficient to provide complete protection from human serum, TLF-1 and TLF-2 activity we developed a purification protocol exploiting physical and compositional differences in these human serum innate immune complexes (Fig. 1). Freshly collected human plasma was initially separated by density gradient ultracentrifugation to produce HDL-enriched (ρ = 1.063-1.26 g/ml) and lipoprotein-deficient fractions (ρ < 1.063 g/ml) that were used as the starting materials for TLF-1 and TLF-2 purification respectively. During the purification of TLF-1 small amounts of contaminating TLF-2 were removed from the HDL-containing fraction by absorption with anti-IgM.

TLF-1 was subsequently bound to anti-Hpr resin, washed extensively at neutral pH to remove human HDLs lacking Hpr and eluted at low pH. TLF-2 was purified from the lipoprotein-deficient serum by sequential affinity chromatography with anti-Hpr followed by binding and elution from an anti-IgM column. The purity of TLF-1 and TLF-2 was evaluated by size exclusion chromatography on Superox 6 and protein gel blot analysis with anti-Hpr, apoL-1 and IgM (Fig. 1A and B). Based on size exclusion chromatography, TLF-1 and TLF-2 have estimated relative sizes of 576 kDa and 1.6 MDa.
respectively. Superose 6 chromatography of purified TLF-1 and TLF-2 revealed somewhat dispersed distributions consistent with particle heterogeneity but there was minimal overlap of the TLF-1 and TLF-2 absorbance peaks at 280 nM (Fig. 1A). Protein gel blot analysis revealed no contaminating TLF-2 in our purified TLF-1 preparations based on the lack of anti-IgM reactive material on protein gel blots (Fig. 1B, data not shown). TLF-2 preparations were highly enriched in particles containing Hpr, apoL-1 and IgM; however, these preparations also contained small amounts of IgM deficient complexes with an elution time (~38 min) from the Superose 6 column consistent with TLF-1. Based on the distribution of the Hpr dimer and IgM across the size exclusion fractions we estimate the amount of contaminating TLF-1 in these preparations to be ~10%.

Susceptibility of *T. b. brucei* to human serum, TLF-1 and TLF-2. Our previous studies compared the short-term killing of trypanosomes to TLF-1. Here we have re-examined the susceptibility of these *T. b. brucei* lines using a long-term growth assay (Fig. 2). Consistent with previous studies, the parental *T. b. brucei* 427-221.

![Figure 1. Characterization of purified TLF-1 and TLF-2. (A) Superose 6 size exclusion chromatography of TLF-1 and TLF-2. Absorbance profiles (280 nM) of TLF-1 and TLF-2, superimposed on individually run marker proteins (1, thyroglobulin (660 kDa); 2, apoferritin (480 kDa); 3, conalbumin (67 kDa); 4, ovalbumin (45 kDa)). (B) Analysis of individual Superose 6 column fractions of TLF-1 and TLF-2 separated on non-denaturing 12% SDS-PAGE and silver stained (top panel). Hpr, apoL-1 and IgM were detected by protein gel blot. NA, not analyzed.](image-url)
T. b. brucei 427-800, and T. b. brucei 427-060 were highly susceptible to TLF-1 with a calculated LD₅₀ of 0.8–6 ng/ml. T. b. brucei 427-800 and T. b. brucei 427-060 were >10,000-fold more resistant to TLF-1 than wild-type T. b. brucei, suggesting the TbbHpHbR is important in TLF-1 susceptibility. However, concentrations of >10 µg/ml overcome the Tbb HpHbR deficiency leading to reduced survival (Fig. 2A). Since the concentration of TLF-1 needed to kill T. b. brucei 427-800 and T. b. brucei 427-060 is similar to that found in human serum it is likely that Tbb HpHbR-independent mechanisms of TLF-1 uptake play a significant role in trypanosome killing.

Based on our studies with both the group 1 T. b. gambiense and the TLF-1 resistant T. b. brucei lines we predicted that loss of a functional Tbb HpHbR played a critical role in human infection by African trypanosomes. However, concentrations of TLF-1 are approximately 100-fold more resistant to human serum killing than either T. b. brucei 427-800 or T. b. brucei 427-060 (Fig. 2B). Based on these results, we conclude that loss of Tbb HpHbR expression contributes to the overall resistance of these cells to human serum; however the level of resistance is much less than the high level of resistance seen for TLF-1 (10,000-fold). A possible interpretation of these findings is that other human serum components, such as TLF-2, are less dependent on Tbb HpHbR binding than TLF-1.

It has been proposed that TLF-2 can bind to, T. b. brucei, in the absence of the Tbb HpHbR. We tested whether highly purified TLF-2 was able to kill T. b. brucei 427-800 and T. b. brucei 427-060 (Fig. 2C). Similar to our findings with complete human serum, these Tbb HpHbR-deficient cell lines were more resistant to TLF-2 relative to the wild-type Tbb HpHbR-expressing cell lines. Thus, reduced expression of Tbb HpHbR expression caused a reduced susceptibility of TLF-2 killing suggesting that TLF-2 can bind to the Tbb HpHbR. However, the toxic concentration of TLF-2 is >10-fold less than TLF-1, indicating that Tbb HpHbR-independent mechanisms may play a greater role in TLF-2 binding, uptake and killing.

**Discussion**

In the studies presented here human serum, TLF-1 and TLF-2 susceptibility was examined in isogenic lines of T. b. brucei differing in Tbb HpHbR expression. Cells deficient in Tbb HpHbR expression were 10,000-fold more resistant to TLF-1 relative to wild-type susceptible cells. However, at concentrations of TLF-1 typically found in serum (>10 µg/ml), both resistant and susceptible cell lines were killed. Human serum killing was also observed approximately 100-fold in cell lines expressing reduced levels of Tbb HpHbR. However, significant killing was still observed at human serum concentrations.
results have been used subsequently to argue that TLF-2 does not bind the Tbb HpHbR. It is possible that our results are influenced by the small amount of contaminating TLF-1 in our TLF-2 preparations. Clearly, a detailed characterization of the TLF-2 binding, uptake and cellular location is needed.

Our results are consistent with previous findings indicating the importance of the Tbb HpHbR in TLF-1 killing but also suggest that other mechanisms of TLF-1 binding and uptake may contribute to trypanosome killing. The most likely pathways for HpHbR-independent uptake of TLF-1 is either by fluid phase endocytosis or the trypanosome lipoprotein scavenger receptor. The findings presented here further support the findings of others that TLF-2 killing is less dependent on the HpHbR.

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