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Haptoglobin-Hemoglobin Receptor Independent Killing of African Trypanosomes by Human Serum and Trypanosome Lytic Factors

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ABSTRACT

The haptoglobin-hemoglobin receptor (HpHbR) of African trypanosomes plays a critical role in human innate immunity against these parasites. Localized to the flagellar pocket of the veterinary pathogen *Trypanosoma brucei brucei* this receptor binds Trypanosome Lytic Factor-1 (TLF-1), a subclass of human high-density lipoprotein (HDL) facilitating endocytosis, lysosomal trafficking and subsequent killing. Recently, we found that group 1 *Trypanosoma brucei gambiense*, does not express a functional HpHbR. We now show that loss of the *Tbb*HpHbR reduces the susceptibility of *T. b. brucei* to human serum and TLF-1 by 100 and 10,000 fold respectively. The relatively high concentrations of human serum and TLF-1 needed to kill trypanosomes lacking the HpHbR indicates that high affinity *Tbb*HpHbR binding enhances the cytotoxicity, however in the absence of *Tbb*HpHbR other receptors or fluid phase endocytosis are sufficient to provide some level of susceptibility. Human serum contains a second innate immune factor, TLF-2, that has been suggested to kill trypanosomes independently of the *Tbb*HpHbR. We found that *T. b. brucei* killing by TLF-2 was reduced in *Tbb*HpHbR deficient cells but to a lesser extent than TLF-1. This suggests that both TLF-1 and TLF-2 can be taken up via the *Tbb*HpHbR but that alternative pathways exist for the uptake of these toxins. Together the findings reported here extend our previously published studies (Kieft *et al.*, 2010) and suggest that group 1 *T. b. gambiense* has evolved multiple mechanisms to avoid killing by trypanolytic human serum factors.
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. 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. 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). In addition to these apolipoproteins, Hpr binds free hemoglobin (Hb) in the circulation, which is likely released from erythrocytes during early infection. The heterodimeric Hpr/Hb complex is proposed to be bifunctional, serving both as the ligand for the T. b. brucei HpHbR and directly contributing to high specific activity killing by catalyzing the peroxidation of lysosomal membrane lipids. The other primate specific apolipoprotein in TLF-1, apoL-1, is also directly involved in T. b. brucei killing. 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.
trypanolytic serum complex is called TLF-2 and, while largely devoid of lipids it contains Hpr and apoA-1\textsuperscript{17} and apoL-1 (this paper) suggesting that these complexes share a common origin and perhaps have a similar mechanism of trypanosome killing\textsuperscript{18}.

The two subspecies of human sleeping sickness trypanosomes have evolved distinct mechanisms to survive in the human host. \textit{Trypanosoma brucei rhodesiense} produces the serum resistance associated (SRA) protein that binds and inhibits TLF-1\textsuperscript{19,20,21}. SRA, an intracellular protein largely found in endosomes, co-localizes with TLF-1 in early endosomes and trafficks to the lysosome\textsuperscript{22}. Thus, \textit{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 \textit{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 \textsuperscript{23}. The underlying basis for reduced TLF-1 uptake is two fold. First, \textit{TbgHpHpR} is expressed at very low levels by group 1 \textit{T. b. gambiense} and second, \textit{TbgHpHbR} contains a number of point mutations within the coding sequence, that render the receptor non-functional\textsuperscript{23}. The combination of mutations to the \textit{TbgHpHbR} and reduced expression abolished TLF-1 binding and uptake resulting in resistance to TLF-1. Thus, in contrast to \textit{T. b. rhodesiense}, it appears the mechanism of group 1 \textit{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 \textit{T. b. gambiense}. In this short addendum to the Kieft \textit{et al.} (2010) paper
we now show that while the \( TbbHpHbR \) 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.

**MATERIAL & METHODS**

*TLF-1 and TLF-2 Purification.* Total serum was obtained from a healthy human donor. As previously described, two sequential flotations on sodium bromide gradients (\( \rho=1.063 \) 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)\(^4\). 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 Na\(_2\)HPO\(_4\), 10 mM KH\(_2\)PO\(_4\), 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) and immediately added to an anti-IgM 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 aliquotted and stored at -80\(^\circ\)C.
Size Exclusion Chromatography and Western Blot Analysis. Size exclusion chromatography was performed on a 1 X PBSE equilibrated Superose 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 Superose 6 column at a flow rate of 0.5 ml/min. Fractions were collected (0.5 ml), proteins concentrated six fold with microspin S-300HR columns (GE, 27513001) and the distribution of Hpr, apoL-1 and IgM determined by SDS-PAGE, silver staining and western 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 manufacturers recommendation (Sigma, I0759).

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 VSG800 or VSG060. The T. b. brucei 427-800R and T. b. brucei 427-060R lines showed reduced uptake of TLF-1 relative to the TLF-1 susceptible parental T. b. brucei 427-221S cells and TLF-1 susceptible cells expressing either VSG800 or VSG060. In addition, we showed that the expression of TbbHbHbR 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 $TbgHpHbR$ mRNA reduced but that mutations to the gene abolished function $^{23}$. 

Purification and characterization of TLF-1 and TLF-2. In order to determine whether loss of $TbbHpHbR$ 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 (Figure 1). Freshly collected human plasma was initially separated by density gradient ultracentrifugation to produce HDL-enriched ($\rho \ 1.063 - 1.26 \text{ g/ml}$) and lipoprotein-deficient fractions ($\rho < 1.063 \text{ g/ml}$) 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 Superose 6 and western blot analysis with anti-Hpr, apoL-1 and IgM (Figure 1A and B). Based on size exclusion chromatography TLF-1 and TLF-2 have estimated relative sizes of 576KDa and 1.6MDa respectively$^{17}$. 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 280nM (Figure 1A). Western blot analysis revealed no contaminating TLF-2 in our purified TLF-1
preparations based on the lack of anti-IgM reactive material on western blots (Figure 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 (~28 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\(^2\). Here we have reexamined the susceptibility of these *T. b. brucei* lines using a long-term growth assay (Figure 2). Consistent with previous studies, the parental *T. b. brucei* 427-221\(^s\), *T. b. brucei* 427-800\(^s\), and *T. b. brucei* 427-060\(^s\) were highly susceptible to TLF-1 with a calculated LG\(_{50}\) of 0.8-6 ng/ml. *T. b. brucei* 427-800\(^R\) and *T. b. brucei* 427-060\(^R\) were >10,000 fold more resistant to TLF-1 than wild type *T. b. brucei* suggesting the *TbbHbHbR* is important in TLF-1 susceptibility. However, concentrations of >10 µg/ml overcame the *TbbHpHbR* deficiency leading to reduced survival (Figure 2A). Since the concentration of TLF-1 needed to kill *T. b. brucei* 427-800\(^R\) and *T. b. brucei* 427-060\(^R\) is similar to that found in human serum it is likely that, *TbbHpHbR* 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 *TbgHpHbR* played a critical
role in human infection by African trypanosomes\textsuperscript{23}. The dramatic reduction in susceptibility to TLF-1 in the \textit{TbbHpHbR} deficient cell lines supports this prediction (Figure 2A). However, the possibility remained that human serum contained additional innate immune factors, such as TLF-2, that might not require the \textit{TbbHpHbR}. To test this possibility, we treated TLF-1 resistant and susceptible \textit{T. b. brucei} lines with human serum (Figure 2B). We found that \textit{T. b. brucei} 427-800\textsuperscript{R} and \textit{T. b. brucei} 427-060\textsuperscript{R} were approximately 100 fold more resistant to human serum killing than either \textit{T. b. brucei} 427-800\textsuperscript{S} or \textit{T. b. brucei} 427-060\textsuperscript{S} (Figure 2B). Based on these results, we conclude that loss of \textit{TbbHpHbR} 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 \textit{TbbHpHbR} binding than TLF-1.

It has been proposed that TLF-2 can bind to \textit{T. b. brucei} in the absence of the \textit{TbbHpHbR}\textsuperscript{18}. We tested whether highly purified TLF-2 was able to kill \textit{T. b. brucei} 427-800\textsuperscript{R} and \textit{T. b. brucei} 427-060\textsuperscript{R} (Figure 2C). Similar to our findings with complete human serum, these \textit{TbbHpHbR} deficient cells were more resistant to TLF-2 relative to the wildtype, \textit{TbbHpHbR} expressing, cell lines. Thus, reduced expression of \textit{TbbHpHbR} expression caused a reduced susceptibility of TLF-2 killing suggesting that TLF-2 can bind to the \textit{TbbHpHbR}. However, the toxic concentration of TLF-2 is $>10$ fold less than TLF-1 indicating that \textit{TbbHpHbR} 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 reduced approximately 100 fold in cell lines expressing reduced levels of *Tbb*HpHbR. However, significant killing was still observed at human serum concentrations above 100 µg/ml. Since the only difference in the susceptible and resistance cell lines is the levels of expression of *Tbb*HpHbR it seems likely that human serum contains a second trypanolytic activity that interacts with *T. b. brucei* independently of the *Tbb*HpHbR. Based on this interpretation of the human serum killing results we decided to investigate whether TLF-2 killing of *T. b. brucei* was independent on the level of expression of *Tbb*HpHbR. We found that TLF-2 killing was reduced 500-1000 fold in cell lines with reduced levels of *Tbb*HpHbR suggesting that TLF-2 also binds *Tbb*HpHbR. These results are in apparent contrast to previous studies on TLF-2 showing that TLF-2 killing was not inhibited by the addition of haptoglobin, an inhibitor of HpHb binding to the TbbHpHbR. These results have been used subsequently to argue that TLF-2 does not bind the TbbHpHbR. 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

\textit{Tbb}\textsubscript{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 \textit{Tbb}\textsubscript{HpHbR} independent uptake of TLF-1 is either by fluid phase endocytosis or the trypanosome lipoprotein scavenger receptor\textsuperscript{25,18}. The findings presented here further support the findings of others that TLF-2 killing is less dependent on the \textit{Tbb}\textsubscript{HpHbR} than is TLF-1\textsuperscript{17,10}. Finally, we propose that group 1 \textit{T. b. gambiense} have evolved multiple mechanisms, including but not limited to the loss of a functional HpHbR, to avoid the cytotoxicity of the trypanosome lytic factors. We are currently exploring these mechanisms.

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\textbf{REFERENCES}


FIGURE LEGENDS

**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 ran marker proteins (1 = thyroglobulin (660kDa), 2 = apoferritin (480kDa), 3 = conalbumin (67kDa), 4 = ovalbumin (45kDa). (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, apoL1 and IgM were detected by western blot. (NA = indicates samples that were not analyzed)

**Figure 2.** In vitro activity of human serum, TLF-1 and TLF-2. TLF-1 resistant (R) and susceptible (S) clonal cell lines of bloodstream form T. b. brucei Lister 427 expressing VSG221, 800 and 060 were prepared as previously described. The percentage surviving cells was determined, using phase contrast microscopy, 14 hours following the addition of TLF-1, TLF-2 or complete human serum to exponentially growing cultures at 37°C. (A) TLF-1 susceptibility of T. b. brucei 427-221S (black), T. b. brucei 427-800S (blue), T. b. brucei 427-800R (red), T. b. brucei 427-060S (yellow) and 427-060R (green). (B) Normal human serum (NHS) susceptibility of T. b. brucei 427-221S (black), T. b. brucei 427-800S (blue), T. b. brucei
427-800R (red), *T. b. brucei* 427-060S (yellow) and 427-060R (green). (C) TLF-2 susceptibility of *T. b. brucei* 427-221S (black), *T. b. brucei* 427-800S (blue), *T. b. brucei* 427-800R (red), *T. b. brucei* 427-060S (yellow) and 427-060R (green).
Figure 1

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Figure 2

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