In Vitro Generation of Human High-Density-Lipoprotein-Resistant Trypanosoma brucei brucei

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The host range of African trypanosomes is influenced by innate protective molecules in the blood of primates. A subfraction of human high-density lipoprotein (HDL) containing apolipoprotein A-I, apolipoprotein L-I, and haptoglobin-related protein is toxic to Trypanosoma brucei brucei but not the human sleeping sickness parasite Trypanosoma brucei rhodesiense. It is thought that T. b. rhodesiense evolved from a T. b. brucei-like ancestor and expresses a defense protein that ablates the antitrypanosomal activity of human HDL. To directly investigate this possibility, we developed an in vitro selection to generate human HDL-resistant T. b. brucei. Here we show that conversion of T. b. brucei from human HDL sensitive to resistant correlates with changes in the expression of the variant surface glycoprotein (VSG) and abolished uptake of the cytotoxic human HDLs. Complete transcriptome analysis of the HDL-susceptible and-resistant trypanosomes confirmed that VSG switching had occurred but failed to reveal the expression of other genes specifically associated with human HDL resistance, including the serum resistance-associated gene (SRA) of T. b. rhodesiense. In addition, we found that when the original active expression site was still utilized, expression of three expression site-associated genes (ESAG) was altered in the HDL-resistant trypanosomes. These findings demonstrate that resistance to human HDLs can be acquired by T. b. brucei.

Three subspecies of Trypanosoma brucei have been described previously based on host range and course of infection (17, 18, 19, 26). Trypanosoma brucei brucei causes nagana in cattle and livestock in sub-Saharan Africa, Trypanosoma brucei rhodesiense causes acute human African sleeping sickness in East Africa, and Trypanosoma brucei gambiense causes chronic human African sleeping sickness in West Africa. The phylogenetic relationship of these three organisms has been difficult to resolve, particularly for T. b. brucei and T. b. rhodesiense, which are morphologically indistinguishable and infect the same wild-animal hosts (17).

A distinguishing feature of T. b. brucei is its inability to infect humans. This innate protection is provided by trypanolytic activity found in normal human blood and also in the blood of most apes and Old World monkeys (24, 31, 46, 56). The trypanosome lytic factor (TLF) is a minor subfraction of heterogeneous human high-density lipoprotein (HDL) particles containing apolipoprotein A-I (apoA-I), apolipoprotein A-II (apoA-II), apolipoprotein L-I (apoL-I), and haptoglobin-related protein (Hpr) (23, 29, 38, 48, 49, 50, 51, 52, 57, 59, 60, 64, 65, 69). The mechanism of TLF killing of T. b. brucei has been controversial. Initially, Hpr was suggested to be the active component in TLF, based on the evolution of the Hpr gene during primate evolution, the correlation of the trypanolytic activity in primate sera, and the presence of the Hpr gene in T. b. brucei-resistant animals (33, 56, 59). More recently, highly purified, recombinant apoL-I has been shown to have trypanosome-killing activity (45, 69). It now appears that Hpr and apoL-I both have trypanosome-killing activity and that the assembly of these proteins into the same HDL particle is synergistic, resulting in a naturally occurring supercomplex with enhanced activity compared with that of HDLs lacking either Hpr or apoL-I (57). The biochemical mechanism of TLF killing of T. b. brucei also continues to be debated (4, 36, 37, 45). However, there is good agreement that trypanosome killing by TLF follows a pathway beginning with high-affinity binding to a receptor in the flagellar pocket of the trypanosome, followed by endocytosis and localization to the lysosome. Within the acidic lysosome, TLF is activated and causes cell death. Inhibition of TLF trafficking to the lysosome or neutralization of the lysosomal pH abolishes TLF-mediated killing of T. b. brucei (13, 20, 21, 28, 30, 41, 49, 58, 70).

The discovery of a human serum resistance-associated gene (SRA) in T. b. rhodesiense, but not in T. b. brucei, provided a molecular explanation for human infectivity by these parasites (9, 10, 11). Several experiments have shown a correlation between the expression of the SRA gene and resistance to human serum (11, 35), and transfection of this gene into T. b. brucei conferred resistance to human serum and TLF (41, 74). In addition, the SRA gene has proven to be a reliable marker for T. b. rhodesiense in epidemiological studies throughout East Africa (16, 18, 39, 47, 72, 73).

The expression of the SRA gene is unstable when T. b.
rhodesiense is maintained outside the human host. Repeated passage of human serum-resistant T. b. rhodesiense in mice results in a loss of SRA expression and a corresponding loss of serum resistance. When these human serum-susceptible lines are placed under TLF selection, some cells revert to the TLF resistance phenotype by reactivation of SRA gene expression (22, 35, 74).

Although differing in susceptibility to TLF, all three subspecies of T. brucei evade the host adaptive immune system by periodically changing the expressed variant surface glycoprotein (VSG) (7, 12, 32, 68). The expressed VSG gene lies at the end of a telomere in one of 20 polycistronically transcribed expression sites (ES). These expression sites also contain genes known as expression site-associated genes (ESAGs) that code for bloodstream-specific proteins, including the transferrin receptor (Tf-R). It has been postulated that genes in ES may be grouped and arranged to help the trypanosome survive in different hosts and environments (1, 44). In T. b. rhodesiense, SRA is an expression site-associated gene and shares some features with VSGs (74). Because of the similarities between T. b. rhodesiense and T. b. brucei, the origins of human serum resistance have been debated. However, it is generally thought that the SRA gene originated from a VSG gene in an ancestral, human-serum-sensitive trypanosome (8, 11).

We wanted to determine whether repeated exposure of T. b. brucei to human HDLs would generate resistance to human HDL killing without the presence of the SRA gene. Several HDL-resistant populations emerged during the selection period, and the molecular and cellular characteristics of one of these trypanosome populations are reported here. Endocytosis of TLF did not occur in these trypanosomes, and they could be cultivated in media containing high concentrations of human HDL or normal human serum. Surprisingly, the selection for human HDL resistance correlated with a change in the expressed VSG and recombination events in the 221 expression site (221ES), leading to changes in the expression of ESAG1, -2, and -3. These in vitro-generated lines may provide important insight into the mechanism and evolution of human infectivity by African trypanosomes.

MATERIALS AND METHODS

Trypanosome strains and in vitro selection. Bloodstream stages of the T. b. brucei Lister strain 427 (MiTat 1.2), expressing VSG221, were used in these studies. Cells were cultured in HMI-9 medium with the addition of heat-inactivated fetal bovine serum (FBS) (10%) and Serum Plus (10%) (IRH Biosciences, Kansas). T. b. brucei 427-221 is an antigenically stable line and contains a single gene within the 221ES (3). To verify that the observed resistance to human HDL was specific to TLF, several cell lines (427-221, 427-12, 427-20, 427-60, and 427-800) were retested for susceptibility to both highly purified TLF and intact human serum. TLF was purified from total human HDLs by immunoaffinity chromatography using an Hpr-specific monoclonal antibody (13). The anti-Hpr affinity column was prepared with Affi-gel 10 (Bio-Rad) according to the manufacturer's recommendations. Total human HDL (density, 1.063 to 1.26 g/ml) was purified by flotation on sodium bromide, dialyzed against phosphate-buffered saline (PBS) containing 3 mM EDTA, pH 7.5, and concentrated to 0.4 mg/ml with an Amicon filtration concentrator (Amicon no. 4306). Hpr-containing HDLs were bound to the anti-Hpr column, washed with PBS containing 0.4 mg/ml of Amicon filtration concentrator, and eluted using 100 mM glycine at pH 2.0. Following elution, this highly purified preparation of TLF was neutralized by the addition of 1 M Tris, pH 7.5. The Hpr-specific monoclonal antibody reacts with human Hpr, whether free or in Hpr-containing HDLs, but shows no cross-reactivity with other human serum proteins (57). This TLF was used for all standard lysis assays presented.

SDS-polyacrylamide gel electrophoresis and Western blotting. For the analysis of total cell protein, trypanosomes were incubated in lysis buffer (100 mM Tris, pH 8.0, 10 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), and an equivalent of 3 × 107 cells per lane was run on 8% or 10% SDS-polyacrylamide gels. Following electrophoresis, proteins were transferred to a nitrocellulose filter membrane (0.45 µm; Protran, Schleicher and Schuell, Dassel, Germany) for 1.5 h at 60 V and probed for VSG221 by standard Western blotting methods.

Alexa labeling of TLF and immunofluorescence microscopy. Cultured trypanosomes were centrifuged at 2,500 × g for 10 min at room temperature. Cells were then resuspended in HMI-9 medium at a density of 3 × 107 cells/ml. TLF (~100 µg) was fluorescently labeled using an Alexa Fluor 488 protein labeling kit (Molecular Probes, California). Alexa-labeled TLF (6 µg) was added to 3 × 107 trypanosomes and then incubated at 37°C for 1.5 h. Cells were then collected and washed three times with ice-cold PBS containing 1% glucose. After the last wash, the cell pellet was resuspended in ice-cold PBS containing 1% glucose at one-fourth the original volume of media. The cells were immobilized to a glass slide and stained with the VSG-specific antibody M chloroquine for 30 min at 37°C prior to TLF incubation. This blocked TLF-mediated lysis and allowed maximal accumulation of TLF in the lysosome (58).

Identification of the expressed VSGs and transferrin receptor. The expressed VSGs and transferrin receptor (ESAG6 and ESAG7) were identified by cDNA cloning of reverse transcriptase PCR (RT-PCR) products. A universal 3′ VSG primer (5′-GGGTAACCTACGGTTAATAATATACG-3′) and a 5′ spliced leader RNA primer (5′-CGCTATTATTAGAACAGTTTCTGTAC-3′) were used to amplify and clone the expressed VSG cDNA. ESAG6/7 primers (5′-CGAAATCTCCTTAAACTTGGAGATCTC-3′ and 5′-GCTTCAGACATCAGCGATTTGCTTC-3′) were used to amplify and clone the expressed ESAG6/7 cDNA. Following reverse transcription and PCR, amplified bands were excised from the gel, purified, and cloned into the TA vector for DNA sequencing.
TABLE 1. In vitro selection for human HDL-resistant T. b. brucei.

<table>
<thead>
<tr>
<th>Amt(µ) human HDL (µl)/well</th>
<th>Population designation</th>
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</tr>
<tr>
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</tr>
<tr>
<td>0, 300, 500, 700, 800, 1,000</td>
<td>427-800</td>
</tr>
</tbody>
</table>

a Shown are the amounts of human HDL added to each well in a 3-ml culture of T. b. brucei 427-221 for 24 h. The highest concentration of HDL that yielded viable cells during a round of selection is indicated in bold. These cells were allowed to recover for 5 to 14 days and then were used in the next round of selection.

RNA isolation, RT-PCR, probe labeling, and Northern blot analysis. Total trypanosomina RNA was isolated using Tripure isolation reagent (Roche, Mannheim, Germany), and RT-PCR was performed on 1 to 3 µg by use of a SuperScript III first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). Primers for ESAG1 (5'-AAGGACGATGTTGGAGGATG-3' and 5'-GAGAGGGTGGTGAGTGAA-3'), ESAG2 (5'-CCCGATGGGACACATTACC-3' and 5'-CATTGTGAAATCCGCAGATGAT-3'), ESAG3 (5'-TCTGTCGACACACCAAGATGGT-3' and 5'-ATTAGGCTGTCGCGGAAAAT-3'), and ESAG4 (5'-CCTTGAGGCTTGTGTCCGCTCTG-3' and 5'-CAATAATCAGCAATTTCCCGAGA-3') were used to amplify the expressed ESAG from 427-221 and 427-800 trypanosomes. After RT-PCR, products were gel purified and cloned into the TA vector for sequencing. For Northern blot analysis, 2.5 µg total RNA was separated on a 1% agarose formaldelyde gel and electroblotted at 15 V for 1 h and then for an additional 2 h at 50 V onto a charged nylon membrane. The blot was hybridized overnight with a digoxigenin-labeled probe at 40°C in Easy Hyb buffer (Roche, Mannheim, Germany). Immunological detection of digoxigenin-labeled probe by chemiluminescence was performed according to the manufacturer's instructions.

Radiolabeled probes were generated using a random primer labeling kit (Stratagene, La Jolla, CA). The SR4 probe was generated from the cloned T. b. rhodesiense SR4 gene (KETRI 2482) by using SR4-specific primers (5'-CCA CTCCTAAGATACCTAAAT-3' and 5'-AATTCATGAAAATGTGTTAAA G-3'). VSG probes were generated from cloned VSG221 and VSG800 by using primers specific for VSG221 (5'-CGAGGAGGCTAGCGACCAAC-3' and 5'-T CCGTGTTGCAAGATGCTCTG-3') and VSG800 (5'-ATGCCGCTAACTG TGAACATCT-3' and 5'-TATCTTGTGACGCCGCTGCT-3').

Pulsed-field gel electrophoresis and Southern blot analysis. Chromosome separation was performed by contour-clamped homogeneous electric field (CHEF) electrophoresis with a Bio-Rad CHEF-DR II system (Bio-Rad, Hercules, CA). Genomic plasmids were prepared by immobilizing trypanosomes at a concentration of 2 x 10^8 cells/ml in 0.7% low-melting-temperature agarose. The plugs were poured, allowed to solidify at 4°C, and then treated with proteinase K (1 mg/ml) for 48 h before use. Agarose gels were prepared in 1 x TAE (0.01 M Tris, 0.5 mM Trisphosphate, 0.025% acetic acid) and were electrophoresed at 12°C with buffer recirculation. Hansenula wingei (Bio-Rad) chromosomes were run concurrently as a standard. The switch time was fixed at 900 s. The overall running time was 72 h at 2.0 V/cm, and the included angle was 120° in 1 x TAE. Radiolabeled probes for VSG221 and VSG800 for Southern blotting were prepared as described above. The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was used as a control probe for trypanosome chromosome Vla (34).

SAGE. Serial analysis of gene expression (SAGE) libraries were constructed using ~30 µg total RNA, purified as described above and DNase treated, from T. b. brucei 427-221 and 427-800 populations following the I-SAGE Long kit protocol (Invitrogen, Carlsbad, CA). Recombiant p2ER1 clones produced by SAGE were purified using GeneMachines RevPrcp Orbit (Genomic Solutions, Ann Arbor, MI) and were sequenced on an ABI 3730xl DNA sequencer (Applied Biosystems, Foster City, CA). Sequences collected were analyzed with software created at the MLB specifically for T. b. brucei SAGE analysis. The SAGE software extracts ditag sequences from the ABI 3730xl results according to the SAGE sequence grammar, parses out individual SAGE tags, excludes tags with sequence ambiguities, and reduces all SAGE tags to a look-up table of unique SAGE tag sequences and their observed frequencies among all of the T. b. brucei SAGE libraries. SAGE tags not found more than once in at least one SAGE library were excluded from analyses as putative sequencing error, unless the tag sequence had a perfect match to available T. b. brucei genomic data. The unique tag sequences were mapped to all available T. b. brucei DNA sequences to determine the identities of expressed genes. For this, draft assembly contigs and open reading frame (ORF) calls from the TIGR and Sanger sequencing centers were downloaded from their respective FTP sites and supplemented with nonredundant T. b. brucei genome sequences available at NCBI (113 total contigs summing to 25,496,703 bp with 9,737 total predicted ORFs). The 21-bp SAGE tags were mapped to assembly contigs based on exact sequence matches. Tags were then visualized in a generic model organism database using the Gbrowse interface (61) in the context of genome sequences and predicted open reading frames. As SAGE tags are generated from the most 3' NlaIII restriction site of transcripts, tags with significant differential expression among the SAGE libraries were manually assigned to predicted genes based on their position relative to predicted ORFs. These genes were annotated by BLAST and other homology detection methods. SAGE tags were scored for differential expression among the 427-221 and 427-800 libraries by use of the Stekel et al. (62) R statistic, a log likelihood ratio statistic which scores tags by their deviation from the null hypothesis of equal frequencies given the tag sampling depth for each SAGE library. Higher scores represent a greater deviation from the null hypothesis, while scores close to zero represent near-constitutive expression. To reduce the effects of sampling error in highlighting differential expression, only tags with an R value of 4 or greater were considered for RT-PCR and other post-SAGE analysis.

RESULTS

In vitro generation of HDL-resistant T. b. brucei. In the initial round of selection for HDL-resistant T. b. brucei, cells were treated with low concentrations of human HDL (Table 1). Cultures exposed to concentrations of HDL above 0.03% (0.5, 1.0, 1.5, and 2.0 µl) failed to yield viable cells. Trypanosomes that survived the initial round of selection (427-0.1) were expanded and a second round of human HDL selection started. During each round of selection, the population of cells able to grow at the highest HDL concentration was expanded and

FIG. 1. T. b. brucei 427-800 cells are resistant to human HDLs. The percentages of trypanosomes lysed following incubation for 4 h at 37°C with designated trypanolytic units of human HDLs are shown. A unit of HDL is defined as the amount of HDL needed to lyse 50% of 1 x 10^7 trypanosomes during a 2-h incubation at 37°C. T. b. brucei 427-221 (blue circles), 427-12 (black inverted triangles), 427-20 (black squares) and 427-800 (red triangles) and T. b. rhodesiense (KETRI 2482) (green diamonds) were analyzed.
treated with increasing amounts of human HDL. Following eight rounds of selection over a 9-month period of time, a population of *T. b. brucei* resistant to 800 μl of human HDL added to growth media was obtained. The 427-800 cell line is highly resistant not only to human HDL but also to purified TLF and is able to grow in the presence of 25% normal human serum (data not shown).

To determine whether the observed HDL resistance was a stable trait, trypanosome populations and clonal lines of each were expanded and tested for susceptibility to lysis by human HDLs (Fig. 1). Trypanosome populations selected with increasing concentrations of human HDLs were resistant to human HDLs at concentrations corresponding to those used in the selection protocols. Both the 427-12 and 427-20 populations were intermediate in susceptibility to human HDL killing compared to the *T. b. brucei* 427-221 population and the highly resistant *T. b. brucei* 427-800 and *T. b. rhodesiense* populations (Fig. 1).

Uptake and localization of TLF: The uptake and localization of TLF may contribute to the resistance of *T. b. rhodesiense* to normal human serum killing (22). To investigate TLF uptake by *T. b. brucei* 427-800, TLF was conjugated with Alexa-488 and incubated with both 427-221 and 427-800 cells (Fig. 2). *T. b. brucei* 427-221 rapidly endocytosed the labeled TLF. Intracellular, fluorescent granules were initially visible within 30 min and accumulated during the 1.5-h incubation at 37°C (Fig. 2A, C, D, and E). In contrast, *T. b. brucei* 427-800 cells failed to take up TLF (Fig. 2B, F, G, and H). We had shown previously that treatment of *T. b. brucei* with low concentrations of chloroquine (50 μM) inhibited acidification of the lysosome, ablated TLF killing, and resulted in the accumulation of TLF in the lysosome (21, 58). Therefore, even low levels of TLF uptake would be detectable under these conditions, since it would accumulate in the lysosome of the chloroquine-treated cells. When *T. b. brucei* 427-221 cells were preincubated with chloroquine, Alexa-conjugated TLF levels were elevated relative to cells not treated with chloroquine. Consistent with previous results, the Alexa-conjugated TLF levels colocalized with the lysosomal protein p67 in the *T. b. brucei* 427-221 cells (Fig. 3) (21, 58). No TLF uptake or accumulation was detected in the *T. b. brucei* 427-800 cells. These results contrast our findings for *T. b. rhodesiense*, where TLF was taken up by human HDL-resistant trypanosomes but failed to localize to the lysosome (22).

Identification of expressed VSGs: Since *T. b. brucei* 427 lacks the 5R4 gene, we took an unbiased approach to look for differences in the 427-221 and 427-800 trypanosomes that might explain the HDL resistance trait. When total cell protein
from the 427-221, 427-1, 427-3, 427-12, 427-20, and 427-800 populations was fractionated on SDS-polyacrylamide gels, changes in the overall protein banding patterns were observed (Fig. 4A). A major protein migrating at approximately 50 kDa (Fig. 4A) in the 427-221, 427-1, and 427-3 cells was absent in the 427-12, 427-20, and 427-800 cells. A new protein migrating at approximately 62 kDa was present in the 427-12 and 427-20 cells, while a protein migrating at approximately 55 kDa was present in the 427-800 cells (Fig. 4A). As we will show later, these differences correspond to changes in VSG expression.

Western blotting and immunofluorescence microscopy confirmed that the 427-221, 427-1, and 427-3 trypanosomes express VSG221 (Fig. 4B and D). Low levels of VSG switching were expected to occur during the course of the in vitro selection experiment, but it was unexpected that the trypanosomes that had switched VSGs would become the predominant cells in the resistant populations in the absence of immune selection. This suggested that expression of a different VSG provided a selective survival advantage in the presence of human HDL.

In order to determine the VSG expressed by the *T. b. brucei* cell line highly resistant to human HDL, we cloned and sequenced the expressed VSG cDNA from 427-800 cells. The cDNAs for the VSG expressed by the 427-221 and 427-800 cells were amplified from total cell RNA by reverse transcriptase PCR with primers specific to the conserved sequence within the 3' untranslated region of all VSG genes and the spliced leader RNA that is trans-spliced to the 5' end of all trypanosome mRNAs. These cDNAs were cloned and sequenced. The 427-221 cDNA corresponded to the expected VSG221, while the 427-800 cDNA was unique. To verify that the 427-800 VSG corresponded to the expressed protein, the VSG was purified using conditions specific for the release of glycosylphosphatidylinositol (GPI)-anchored proteins (43). The 55-kDa protein was trypsin treated and analyzed by mass spectroscopy. We were able to unambiguously identify the VSG800 based on the determined mass of 20 peptides covering roughly half of the predicted VSG800 sequence (data not shown).

The in vitro selection of human HDL-resistant 427-800 cells raised the possibility that VSG800 might be functionally or structurally similar to the SRA protein expressed by *T. b. rhodesiense* (9). To examine this, the predicted protein sequences for VSG221, VSG800, and SRA were aligned using the computer program MUSCLE (14) (Fig. 5). As reported by
others, SRA contains a large internal deletion relative to other known VSG genes (8). Overall, VSG and SRA sequences show limited sequence similarity outside of the conserved C-terminal portion of the proteins. However, SRA and VSGs do share several structural and functional features, including an N-terminal signal sequence, a C-terminal GPI anchor site, and extended regions of predicted α-helices (Fig. 5). Comparison of SRA with VSG221 and VSG800 did not suggest that VSG800 had greater homology to SRA than other VSGs.

Based on the cDNA sequences of SRA, VSG221, and VSG800, specific primers were designed to determine whether these genes were present in the 427-221 and 427-800 cell lines as well as the KETRI 2482 line of T. b. rhodesiense (Fig. 6A). The VSG221 and VSG800 genes were detected in T. b. brucei 427-221 genomic DNA, but the VSG221 gene was absent from T. b. brucei 427-800. This is consistent with studies from other labs that have reported that the VSG221 gene is single copy and that gene conversion within the 221ES could lead to loss of the VSG221 gene (53). PCR amplification of genomic DNA failed to detect a copy of the SRA gene in either T. b. brucei 427-221 or T. b. brucei 427-800. However, the SRA4 gene was readily amplified from total genomic DNA from T. b. rhodesiense (KETRI 2482) (Fig. 6A).

The expression of VSG221 and VSG800 was confirmed by Northern blot hybridization with cDNA probes and total RNA from T. b. brucei 427-221 and 427-800, respectively (Fig. 6B). The SRA4 mRNA was detected in the T. b. rhodesiense total mRNA but not in RNA samples from either of the T. b. brucei cell lines (Fig. 6B). These findings show that our in vitro-generated, human HDL-resistant T. b. brucei line does not express SRA and that acquisition of HDL resistance correlates with VSG switching.

**Transcriptome analysis by SAGE.** SAGE offers a powerful, unbiased approach for the analysis of genome-wide mRNA abundance differences in different cell populations. After the removal of putative sequencing error, a total of 38,545 and 38,334 SAGE tags (21 bp each) were sampled from 427-221 and 427-800, respectively. The sampled tags represented 26,385 unique 21-bp sequences, of which roughly 85% mapped to a single location in the genome. The SAGE analysis revealed that a major difference between the 427-221 and 427-800 transcriptomes was the presence of VSG221 and VSG800 transcripts in the respective cell lines (Table 2). We also found that SAGE tags specific for ESAG1, ESAG2, the terminal ESAG3, and ESAG8b encoded in the 221ES were absent in 427-800. Other, differentially abundant tags corresponded to transcripts for housekeeping proteins including tubulin, translation elongation factors, ribosomal proteins, and enolase. These were present in both the 427-221 and 427-800 libraries.

![FIG. 6. Analysis of the VSG221, VSG800, and SRA genes and transcripts in T. b. brucei and T. b. rhodesiense.](image)

**TABLE 2. Differences in mRNA abundance between T. b. brucei 427-221 and 427-800, as detected by SAGE**

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<th>Tag ID no.</th>
<th>Tag sequence</th>
<th>Annotation</th>
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<th>Fold change</th>
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<td>43126</td>
<td>CATGTATGGGATGGATTGCGG</td>
<td>Beta tubulin</td>
<td>142</td>
<td>266</td>
</tr>
</tbody>
</table>

*Tags are sorted by change (n-fold), which has been calculated with correction for total sampling effort. Data presented in bold represent tags from the 221ES.

*Primary tags are those from the most 3'-proximal NlaIII site of transcripts, whereas secondary tags are produced for highly abundant transcripts by incomplete NlaIII digestion during SAGE library construction.

*SAGE is performed without replicates upon a single RNA extract, making traditional statistics unworkable. As such, SAGE is best viewed as a measure of relative change. To reduce the effects of sampling and sequencing error in highlighting differential expression, only tags with log likelihood (R) values of 4 or greater are shown.

*Higher log likelihood scores represent a greater deviation from the null hypothesis of equal abundance among the two SAGE libraries.

*Two copies of esag3 exist in the 221ES. SAGE tag no. 177 maps to the terminal copy only.
reflect differences in cell density at the time when RNA was extracted for SAGE library construction. These results showed that selection for HDL resistance accompanied VSG switching. We sequenced three ESAG1, seven ESAG2, and 11 ESAG3 cDNA clones from the 427-800 cells. No sequences identical to that of the 221ES ESAG1 were identified in the 427-800 cDNA clones. However, we did identify one ESAG1-like sequence with 97% nucleotide similarity to the 221ES ESAG1 sequence. A second cDNA had extensive similarity to small regions of the 221ES ESAG1 but only 7% overall similarity (Fig. 8C). We also did not find cDNAs with sequences identical to that of the 221ES ESAG2 in the 427-800 cell line. However, we did identify two ESAG2-like sequences with 97% and 93% nucleotide similarity to the 221ES ESAG2 sequence (Fig. 8C). The analysis of ESAG3 expression is complicated by the presence of three copies of ESAG3 in the

### TABLE 3. Mapping of SAGE tags to the 221ES

<table>
<thead>
<tr>
<th>Tag ID no.</th>
<th>Tag sequence</th>
<th>Annotation</th>
<th>Frequency in:</th>
</tr>
</thead>
<tbody>
<tr>
<td>41524</td>
<td>CATGTGGTATGTACAAGCTAC</td>
<td>esag6/7r</td>
<td>44 44</td>
</tr>
<tr>
<td>41878</td>
<td>CATGGGAACACTGCTGCTGCT</td>
<td>esag6</td>
<td>32 32</td>
</tr>
<tr>
<td>43692</td>
<td>CATGTAAGGAAACGGCAACAG</td>
<td>Downstream of esag6</td>
<td>58 58</td>
</tr>
<tr>
<td>63</td>
<td>CATGATATAAAACGTGGATGC</td>
<td>esag5r</td>
<td>40 40</td>
</tr>
<tr>
<td>87</td>
<td>CATGAAATCTCTGGGGCAAGCA</td>
<td>esag3/3sr</td>
<td>34 34</td>
</tr>
<tr>
<td>42811</td>
<td>CATGGGGAGGGGTTGACCTTT</td>
<td>esag4</td>
<td>46 46</td>
</tr>
<tr>
<td>42492</td>
<td>CATGACTTCTTCCTTCATT</td>
<td>Hypothetical protein H25N7.21</td>
<td>38 38</td>
</tr>
<tr>
<td>501</td>
<td>CATGGAAATGGATGTTTCC</td>
<td>esag8/8r</td>
<td>10 10</td>
</tr>
<tr>
<td>41933</td>
<td>CATGTGCCTGCTGCTTCTTTC</td>
<td>Hypothetical protein H25N7.25</td>
<td>15 15</td>
</tr>
<tr>
<td>263</td>
<td>CATGTCGACTGGACTATGAGC</td>
<td>esag8b</td>
<td>15 15</td>
</tr>
<tr>
<td>177</td>
<td>CATGAAAATCTTGCAGGCAAGA</td>
<td>esag3</td>
<td>20 20</td>
</tr>
<tr>
<td>56</td>
<td>CATGTCGAGGGAAGACTAAAAA</td>
<td>esag1</td>
<td>44 44</td>
</tr>
</tbody>
</table>

---

*Tags are approximately sorted by position on the 221ES (GenBank accession no. AL671259) (Fig. 8). Data presented in bold represent tags with sequences entirely unique to the 221ES. All other tag sequences can be mapped to other known T. b. brucex expression sites but not non-ES locations.

b: Tag ID, identification.

c: Tag no. 41524 cannot discriminate between esag6 and esag7 transcripts.

d: Tag no. 87 maps to both the esag3 pseudogene and the functional esag3 copy in the middle of the ES. It does not map to the terminal copy of esag3.

e: Tag no. 501 maps to both copies of esag8 as well as to esag8b. As tag no. 263 independently suggests an absence of esag8b transcripts in 427-800, we assume esag8b does not contribute to the frequency of tag no. 501 in 427-800.
Within the 221ES is an upstream ESAG3 pseudogene (ESAG3p) and two nearly identical copies of ESAG3. We designated these copies ESAG3 (for the upstream copy of the gene) and ESAG3b (for the downstream copy) (Fig. 8A). As expected from the SAGE analysis, we identified cDNAs for ESAG3, -3b and -3p in 421-221 cells (Fig. 8C). While SAGE detected the possible presence of ESAG3 transcripts in 427-800 cells, we could detect transcripts only from ESAG3 and a non-221ES copy of an ESAG3-like transcript by RT-PCR. While RT-PCR analysis of ESAG8 revealed the two products expected for ESAG8 and -8b, sequencing failed to resolve whether one or both copies of ESAG8 were expressed (data not shown).
Based on the combination of SAGE analysis and cDNA sequence analysis, we conclude that the duplication and transposition of VSG800 into the 221ES resulted in deletion of coding sequences for ESAG1, -2, and -3b. The transcripts in the 427-800 cells are the result of transcription of related genes from other copies in the 221ES, other expression sites, or internal chromosomal copies. We are currently preparing telomere clones of the 221ES from 427-800 cells to precisely define the ESAGs deleted during the transposition of VSG800 into the 221ES.

DISCUSSION

The resistance of humans to infection by *T. b. brucei* is a consequence of an innate, protective class of human HDLs containing apoL-1, Hpr, apoA-1, and apoA-II (23, 50, 57, 59, 60, 65, 69). African trypanosomes that cause human sleeping sickness circumbent the cytotoxic activity of this HDL subclass. The infectivity of *T. b. rhodesiense* for humans requires the expression of the VSG-like protein, SRA, which localizes to intracellular organelles of the endocytic pathway and the lysosome (9, 35, 41, 69, 74). While the mechanism of TLF inhibition by SRA is not completely understood, recent studies suggest that binding of SRA to apoL-1 neutralizes the trypanosome-killing activity of TLF (69). Both the SRA gene and its telomeric ES are highly conserved in *T. b. rhodesiense* (16). This has led to the suggestion that the SRA gene arose only once during evolution and subsequently spread through genetic exchange (17, 18).

Although compelling evidence supports the role of SRA in human infectivity by *T. b. rhodesiense*, several investigators have reported the occurrence of human serum-resistant lines of African trypanosomes that lack the SRA gene (2, 47, 66, 67). To investigate the mechanism of SRA-independent resistance, we generated human serum-resistant lines of *T. b. brucei* 427 (MiTat 1.2) by repeated in vitro exposure to human HDLs. We extensively characterized one human HDL-resistant line, *T. b. brucei* 427-800, which was also highly resistant to purified TLF and could grow in media containing 25% normal human serum. The resistance phenotype correlated with the absence of TLF binding and accumulation in *T. b. brucei* 427-800 cells (Fig. 2 and 3). Analysis of both protein and RNA profiles indicated that *T. b. brucei* 427-800 continued to transcribe the 221ES, but ESAG1, -2, -3, and -8b mRNAs (from the 221ES) were not detected and VSG800 was transcribed (Fig. 8; Table 3). Duplication and transposition of VSG800 into the 221ES were confirmed by CHEF blotting (Fig. 7). These studies show that human HDL-resistant variants of *T. b. brucei* 427 can be generated in vitro and that the resistance phenotype is directly linked to antigenic variation. Based on these findings, we propose that resistance to human HDLs is due to either the expression of VSG800, the deletion of ESAG1, -2, -3, or -8b from the 221ES, or some other unknown mechanism.

The potential for antigenic variation to directly influence human serum susceptibility or resistance was previously proposed based on studies of *T. b. rhodesiense* and *T. b. gambiense* isolates that had lost human serum resistance following VSG switching (25, 42, 74). Based on the genomic organization of the SRA gene within bloodstream expression sites, it is possible that antigenic switching by in situ activation of a new, SRA-lacking ES is the mechanism of loss of human serum resistance in *T. b. rhodesiense* (74).

By analogy to the role that SRA acts as an inhibitor of human HDL killing of *T. b. rhodesiense*, one might expect human HDL-resistant *T. b. brucei* 427-800 to express a protein that specifically interacts with TLF and ablates its activity. Since SRA is a member of the VSG family of proteins, it is possible that in vitro exposure of *T. b. brucei* to human HDL may have selected for the expression of a VSG that conferred resistance. The *T. b. brucei* genome contains a large number of silent VSG genes, many of which are clustered at internal locations on large chromosomes, with a minority of these genes distributed at telomeres of minichromosomes, intermediate chromosomes, and megabase chromosomes. The expression of VSG genes is tightly regulated to ensure that a single telomeric VSG is transcribed at a time. This leaves hundreds of silent VSG genes free of functional constraints and susceptible to random sequence drift contributing to the variability of VSGs. The high multiplicity of silent VSG genes provides a potential pool for selection of a gene that could confer resistance to human HDLs. Based on limited sequence comparison, we have been unable to identify features of VSG800 that might contribute to inhibition of human HDL killing of trypanosomes. However, the role of VSG800 in the resistance trait can be evaluated directly by replacement of the VSG221 gene in the human HDL-susceptible *T. b. brucei* 427-221 cell line. This experiment is under way.

In addition to SRA, there is at least one other example of a VSG that has evolved to take on a different function. The trypanosome Tf-Rs are encoded by ESAG6 and ESAG7 and assemble as a heterodimeric receptor anchored to the flagellar pocket by a single GPI on the ESAG6-encoded subunit (6, 27, 54, 63). The ESAG6 and ESAG7 subunits contain VSG-like N-terminal domains that facilitate packing within the VSG surface coat in the flagellar pocket and variable C-terminal sequences with differing affinities for mammalian transferrins (55). Growth of trypanosomes in different sera can select for VES ES switching and results in the expression of different Tf-Rs (5, 15). A correlation to the relative transferrin binding affinity of the expressed trypanosome Tf-R and the host transferrin has been made previously (15, 71). *T. b. brucei* 427-800 and *T. b. rhodesiense* clearly share the human HDL resistance phenotype in a standard lysis assay. Despite this superficial similarity, analysis of TLF uptake and localization revealed differences. Two reports show that TLF (22) or purified apoL-1 (69) is taken up by *T. b. rhodesiense*. In a study with native purified TLF we have found that, although TLF accumulation was reduced in *T. b. rhodesiense*, flagellar pocket binding and perhaps endocytosis occurred. However, TLF did not traffic to the lysosome (22). Vanhamme and co-workers also found that *T. b. rhodesiense* bound recombinant apoL-1 but, in contrast to our initial studies, found that apoL-1 was efficiently taken up and colocalized to the trypanosome lysosome with SRA (69). In the studies reported here, we were unable to detect TLF accumulation in *T. b. brucei* 427-800 cells, raising the possibility that human HDL resistance in *T. b. brucei* 427-800 may result from loss of the TLF receptor. Consistent with this possibility, we found that ESAG1, -2, and -3 were potentially deleted during the transposition of VSG800 into the 221ES and that transcripts from these genes were
absent in the SAGE library and also in cDNAs prepared from *T. b. brucei* 427-800 (Table 3; Fig. 8). ESAG1 and -2 are interesting candidates for the TLF receptor in *T. b. brucei*. ESAG2 is a GPI-anchored protein found exclusively in the flagellar pocket of bloodstream trypanosomes (40, 44). Less is known about ESAG1, but it is predicted to be a transmembrane cell surface protein in bloodstream trypanosomes (44). While the function of each of these proteins is unknown, it is interesting to note that another human serum-resistant line of *T. b. brucei* (TREU 927; GuTat 10.1) also lacks these genes in the active expression site (66, 67).

We have shown here that *T. b. brucei* can gain serum resistance after repeated exposure to human HDL. The genetic cause of resistance has not been thoroughly determined; we have shown, however, that the lack of TLF accumulation in the lysosome is the most likely cellular mechanism. This phenotype may be due to the expression of VSG800, which, by some unknown mechanism, inhibits uptake of TLF. Alternatively, TLF resistance might be a consequence of loss of a gene encoding the TLF receptor during recombination of VSG800 into the 221ES. These possibilities are currently being tested.

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