Endocytosis of a Cytotoxic Human High Density Lipoprotein Results in Disruption of Acidic Intracellular Vesicles and Subsequent Killing of African Trypanosomes

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Abstract. The host range of *Trypanosoma brucei brucei* is restricted by the cytolytic effects of human serum high-density lipoprotein (HDL). The lytic activity is caused by a minor subclass of human serum HDL called trypanosome lytic factor (TLF). TLF binds in the flagellar pocket to specific TLF-binding sites. Internalization and localization of TLF to a population of endocytic vesicles, and ultimately large lysosome-like vesicles, precedes lysis of *T. b. brucei*. The membranes of these large vesicles are disrupted by the accumulation of TLF particles. Inhibitor studies with lysosomotropic amines have shown these large vesicles to be acidic in nature and that prevention of their rupture spares the cells from TLF-mediated lysis. Furthermore, leupeptin inhibition suggests that a thioprotease may be involved in the mechanism of TLF-mediated lysis of *T. b. brucei*. Based on these results, we propose a lytic mechanism involving cell surface binding, endocytosis and lysosomal targeting. This is followed by lysosomal disruption and subsequent autodigestion of the cell.

TRYPANOSOMES are the causative agents of both human and cattle diseases in large regions of sub-Saharan Africa (8). *T. b. gambiense* and *T. b. rhodesiense* cause the chronic and acute forms of human sleeping sickness. *T. b. brucei* causes the cattle disease, nagana, but is unable to infect humans (16, 17, 19). The three subspecies of *T. brucei* are biochemically and morphologically indistinguishable and are defined by human infectivity or sensitivity to normal human serum in vitro (27). Early work by Rifkin (28) identified the cytolytic component of human serum as a high-density lipoprotein (HDL)[1]. A number of recent studies support the role of human HDL in the killing of *T. b. brucei*. HDL is now widely accepted as being a major factor in preventing the infection of humans by *T. b. brucei* (11, 13, 15, 28).

The nature of the cytotoxic HDL particles is more controversial, due to the heterogeneity of human serum HDLs. Human serum HDLs vary in size, apolipoprotein, and lipid content (10). In addition, a number of enzymatic activities have been found to be associated with specific subclasses of HDL (20, 25). In Rifkin's initial study, the lytic activity overlapped but did not coincide with HDL, suggesting that the lytic activity might be a subclass of HDL particles (28). Gillett and Owen (11, 13, 24) have reported that all human serum HDLs are lytic and that the active component is apolipoprotein A-I. On the other hand, we found the toxicity of human HDL for *T. b. brucei* was not a general feature of all human serum HDL but was restricted to a minor subpopulation of HDL particles of very high density (ρ = 1.24 g/ml) and unusually large size (20-nm diam) (15, 17). This trypanolytic subclass of human serum HDL has been named trypanosome lytic factor (TLF). In addition to the typical HDL associated apolipoproteins A-I and A-II, two unique apolipoproteins, L-I (94,500 D) and L-III (49,500 D) (15, 17) are also constituents of TLF (see Materials and Methods). These studies showed trypanosome lytic activity was limited to a subpopulation of human HDLs, supporting the earlier work of Rifkin (28).

Other host macromolecules have also been shown to interact with the trypanosome. The most striking examples are the studies on the binding and uptake of transferrin (2, 33) and low density lipoprotein (LDL) (7) by bloodstream try-

1. *Abbreviations used in this paper*: HDL, high density lipoprotein; HPLC, high pressure liquid chromatography; LDL, low density lipoprotein; TLF, trypanosome lytic factor.

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panosomes. These studies have shown LDL and transferrin are taken up by receptor-mediated endocytosis. It is now clear endocytosis only occurs at the flagellar pocket of trypanosomes. Binding studies indicate that LDL and transferrin bind to the flagellum and the flagellar pocket and are endocytosed via coated pits and vesicles which ultimately fuse with degradative organelles (lysosomes?).

Several mechanisms have been proposed to explain the lysis of *T. b. brucei* by human serum (13, 30, 31). The simplest requires specific binding of TLF to *T. b. brucei*. In this report we show TLF binds specifically to the flagellar pocket membrane of *T. b. brucei*. Electron and fluorescence microscopy reveal that TLF is endocytosed and a series of inhibitor studies with lysosomotropic amines verify the role of endocytosis in the lytic pathway. Finally, we used a variety of techniques showing localization of endocytosed TLF to lysosome-like vesicles and demonstrating the essential nature of vesicle breakdown in the lytic pathway.

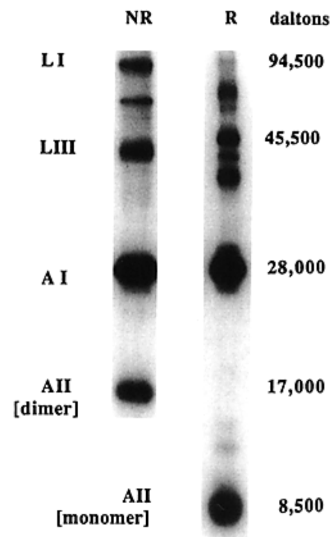


Figure 1. ^{125}I-labeling of TLF apolipoproteins (see Materials and Methods). TLF was purified and ^{125}I labeled as described in Materials and Methods. Autoradiogram of apolipoproteins separated on 12% SDS-polyacrylamide gels containing 4 M urea under reducing (*R*) and nonreducing (*NR*) conditions. The size of the apo L-I (94,500 D), apo L-III (45,500 D), apo A-I (28,000 D), and apo A-II dimer (17,000 D) was estimated relative to protein standards (Sigma Chemical Co.).

Materials and Methods

Cell Culture and Lysis Assay

A clonal line of *T. b. brucei*, ILTat 1.3, was used in all experiments. ILTat 1.3 is an antigenically stable cell line which produces acute, monomorphic infections in laboratory rodents. Trypanosomes were grown to 1-5 $\times$ 10^8 cells/ml in Swiss CD-1 mice and harvested using a protocol modified from Lanham (22). Briefly, cells are harvested either by cardiac puncture or a post vena cava puncture and diluted with an equal volume of 4°C bicine buffer (50 mM bicine at pH 8.0, 70 mM glucose, 5 mM KCl, and 50 mM NaCl) supplemented with 100 U/ml heparin (1). Cells are separated from the blood by passing over a DEAE-cellulose column (22) previously equilibrated in bicine buffer. Trypanosomes are washed and resuspended to a final cell density of 3 $\times$ 10^7/ml in F-12 medium (GIBCO BRL, Gaithersburg, MD), containing 15% (vol/vol) fetal bovine serum (heat-inactivated), 1% (wt/vol) glucose, 30 mM Hepes (pH 7.4). Lysis assays carried out as previously described (15). Inhibitor lysis assays were performed as described (15) with the exception of a pre incubation period of 30 min at 4°C with either ammonium chloride (1 to 32 mM), chloroquine (0.25 to 100 μM), or monensin (0.01 to 0.1 μM). Higher concentrations of these weak basic amines ($\geq$64 mM for NH_4Cl and $\geq$300 μM for chloroquine), were toxic to the trypanosomes. Higher concentrations ($\geq$1.0 μM) of monensin were also lethal to trypanosomes. The cells were then shifted to 37°C and treated with TLF for 2 h. In the case of the protease inhibitor studies, cells were pre-incubated with 0.01 mM to 1.0 mM leupeptin for 1 h at 37°C and subsequently treated for an additional 2 h at 37°C with TLF. All inhibitors were made fresh the day of assay and dissolved in PBS/EDTA phosphate buffered saline. Inhibitors were supplied from Sigma Chemical Co. (St. Louis, MO) except monensin which was obtained from Calbiochem-Behring Corp. (Palo Alto, CA). Monensin was dissolved in DMSO and insoluble particulates were spun out before use.

Serum Lipoprotein and TLF Purification

Normal human blood was obtained from healthy, fasted donors and LDL, non-lytic HDL, and TLF were purified as described previously (15). Typically, ~500 μg of highly purified TLF (10,000-20,000 U) was recovered from 200 ml of human serum. Non-lytic HDL (ρ = 1.063-1.17 g/ml) and LDL (ρ = 1.002-1.063 g/ml) were also prepared by sodium bromide flotation and were dialyzed against PBS-EDTA prior to use.

Characterization of Purified TLF

SDS-PAGE of ^{125}I-TLF and autoradiography (Fig. 1) indicated that each TLF associated protein labeled with ^{125}I in proportion to the relative amount of staining by Coomassie blue (data not shown). Apolipoproteins A-I and A-II were identified based on reactivity with monospecific polyclonal and monoclonal antibodies (data not shown) and apparent molecular weights estimated under nonreducing and reducing conditions. Two additional proteins are enriched in purified TLF preparations. The 94,500 D (apo L-I) and 49,500 D (apo L-III) proteins were previously referred to as "band a" and "band c" (15). To be more consistent with established nomenclature and to avoid confusion with apolipoproteins A-I and A-II we have adopted this new nomenclature (17). The previously reported "band b", seen in earlier preparations, was absent in more highly purified TLF preparations. The protein which runs at ~65,000 D is human serum albumin. For comparison we also radioiodinated non-lytic human HDL (ρ = 1.063-1.17 g/ml). The major structural apolipoproteins A-I (28,016 D) and A-II (8,500 D) were seen under reducing conditions while the A-II dimer (17,000 D) is seen under nonreducing conditions (data not shown). L-I and L-III were not detected in these preparations since TLF represents <0.5% of the total HDL.

Binding Studies

To examine the binding of serum high density lipoproteins to *T. b. brucei*, purified TLF and non-lytic HDL's were radioiodinated. TLF and HDL were radiolabeled according to a modified method of Goldstein et al. (14). The reaction was carried out in a solution of PBS/EDTA and 78 mM Tris base (pH 9.9). The catalyst used was Iodogen (1, 3, 4, 6-tetrachloro- 3 α, 6 α-diphenylglycouril) (Sigma Chemical Co.). Sodium ^{125}I (10 μCi/μg protein) was added to purified TLF or HDL and incubated at 26°C for 15 min. Unincorporated ^{125}I was removed by dialysis against PBS/EDTA or by filtration on a PD10 column (Pharmacia Fine Chemicals, Piscataway, NJ). Less than 5% of the ^{125}I radioactivity was extractable into chloroform-methanol (14). Specific activities of 500-5,000 cpm/ng of TLF or HDL protein were obtained. SDS-polyacrylamide and autoradiography of ^{125}I-TLF and ^{125}I-HDL were used to verify the labeling of the apolipoproteins. For binding studies, *T. b. brucei* was grown to 1-5 $\times$ 10^8 cells/ml in Swiss CD-1 mice and harvested as discussed above. The cells were washed with F-12/BSA buffer (F-12 media, 1% [wt/vol] bovine serum albumin, 1% [wt/vol] glucose, 30 mM Hepes [pH 7.4]). Binding studies were carried out for 1 h at 4°C in the same buffer at a final cell density of 1 $\times$ 10^7/ml. Following binding, cells were washed three times with PBS/EDTA (pH 7.4), centrifuged at 4°C at 6,000 rpm in a microfuge, and counted immediately. The specificity of TLF was established by competition studies. In these studies, *T. brucei* was incubated for 1 h at 4°C with 5 μg/ml ^{125}I-TLF and no competitor or with a 10-fold mass excess of unlabeled TLF, non-lytic HDL, LDL, or ILTat 1.3 VSG. The number of TLF binding sites was based on the half-maximal binding and an estimated molecular weight of 5 $\times$ 10^5 for TLF (15).

Fluorescence Microscopy

Fluorescent studies were carried out in F-12/BSA buffer. The cells were incubated (at a final cell density of 1 $\times$ 10^7/ml) with inhibitors as described in the lysis assay above. The optimal inhibitor concentrations for the fluorescent studies were determined from the inhibitor lysis assays. The concentrations used were 8 mM NH_4Cl, 50 μM chloroquine, 0.6 mM

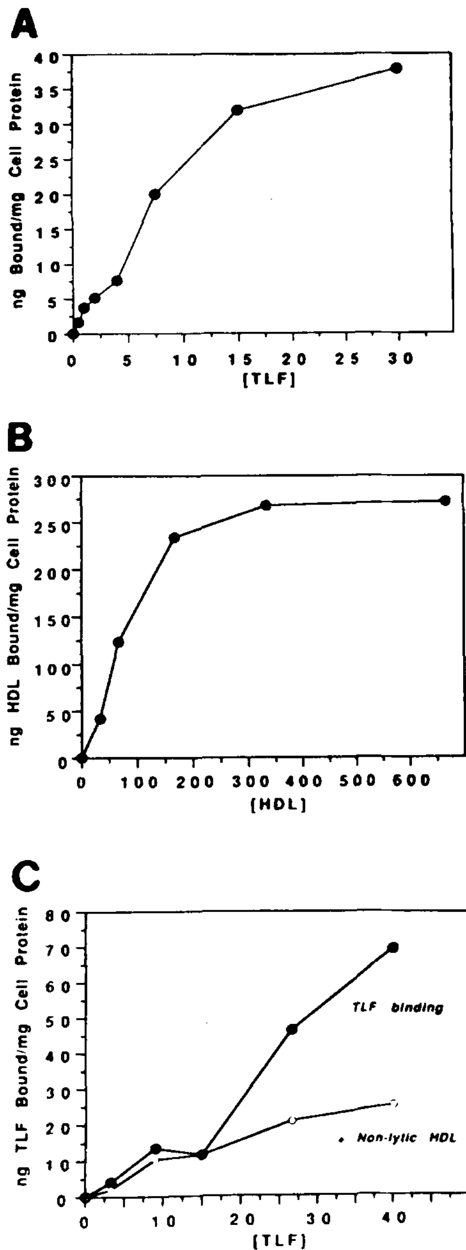


Figure 2. Binding of ^{125}I-TLF and ^{125}I-HDL to *T. b. brucei*. Binding studies were carried-out as described in Materials and Methods. (*A*) Concentration dependence of TLF binding. TLF concentration is expressed as μg/ml. Trypanosomes were incubated at 4°C for 1 h with varying concentrations of ^{125}I-TLF, washed, and counted. Each point is the average of three binding experiments. (*B*) Concentration dependence of HDL binding (expressed as μg/ml). Trypanosomes were incubated at 4°C for 1 h with varying concentrations of non-lytic ^{125}I-HDL (33–700 μg/ml), washed, and counted. (*C*) Competition of high and low affinity TLF binding with non-lytic HDL. The competition for TLF binding was examined at concentrations ranging from 3 to 40 μg/ml in the presence of a constant mass excess (300 μg) of non-lytic HDL. The black filled dots represent the amounts of TLF binding at different TLF concentrations. The white filled dots represent the amount of TLF binding in the presence of competitor (a fixed amount of non-lytic HDL competitor at a concentration of 1,000 μg/ml).

leupeptin, and 0.1 μM monensin. The cells were then incubated with 3 mg/ml of tetramethylrhodamine-labeled dextran (Molecular Probes, Eugene, OR; M_r = 10,000; neutral charge) for 30 min at 37°C. Dextran is a bulk phase endocytic marker used to fluorescently monitor endocytic vesicle integrity. Sample cells were subsequently treated with 4 U of TLF for 45 min at 37°C, control cells were mock treated at same time and temperature. The cells were fixed in EM grade 1% final concentration formaldehyde (Toumisin Res. Corp., Rockville, MD), washed three times with 0.37% EM grade formaldehyde and resuspended in ∼100 μl of 0.37% EM grade formaldehyde. One third of the cell suspension was mounted onto slides (by cytospinning the sample onto the slide), stained with 20 μg/ml Hoescht dye and treated with a modified mountant to inhibit fading. The mountant was in 1:9 PBS/glycerol containing 0.1% phenylene diamine (Fisher Scientific Co., Pittsburgh, PA).

Image Acquisition

The slide specimens were viewed using a Leitz fluorescence microscope equipped with a Vario Orthomat II camera system. The magnification factors include: 100× oil immersion lens, zoom of 9.7, tube length of 1.25×, and camera lens magnification of 0.32×. Cells were photographed using Kodak Ektachrome (EES 135-6) 800/1600 film (pushed to 1600 ASA in the development).

Electron Microscopy

TLF was conjugated to colloidal gold as described by Handley et al. (18). Conjugation reactions were verified by negative staining with 2% (wt/vol) sodium phosphotungstate (pH 5.9) and examination with a Zeiss 10 electron microscope. In order to study the localization of TLF binding, cells were prepared for transmission electron microscopy following incubation with TLF-gold complexes (6.7 μg/ml at 4°C for 1 h). Cells were washed extensively in PBS-EDTA at 4°C to remove unbound TLF gold complexes. Samples were then fixed with 2% (vol/vol) glutaraldehyde and postfixed in 1% (wt/vol) osmium tetroxide in 0.1 M sodium phosphate buffer (pH 7.5) containing 4% (wt/vol) sucrose. The uptake of TLF at higher temperatures was studied by first binding TLF to the cells at 4°C as described above. After removing unbound TLF, the cells were shifted to the higher temperatures. The distribution of TLF-gold complexes was determined by examination of >200 cells from samples incubated at 4°, 17°, and 37°C. The number of TLF-gold particles were counted in sections through the FP, SV and tubules adjacent to the flagellar pocket, and LV or free in the cytoplasm.

Results

TLF Binding to T. B. brucei

Killing of trypanosomes by human serum or HDL involves

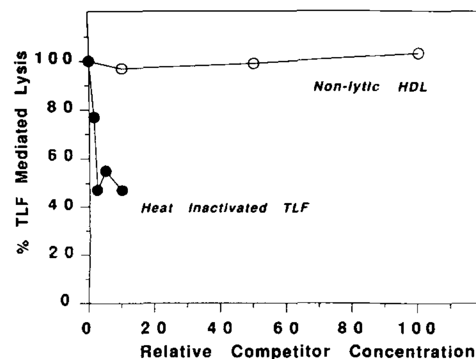


Figure 3. Inhibition of TLF-mediated lysis of *T. b. brucei* by heat-inactivated TLF and non-lytic HDL. Non-lytic human serum HDL (5, 50, and 100 μg) or heat-inactivated TLF (2, 4, 6, and 10 U) was incubated with 2 U of active TLF (∼0.5 μg protein) and the lysis of *T. b. brucei* was determined following standard assay conditions (Materials and Methods). TLF was inactivated by thermal denaturation at 90°C for 15 min.

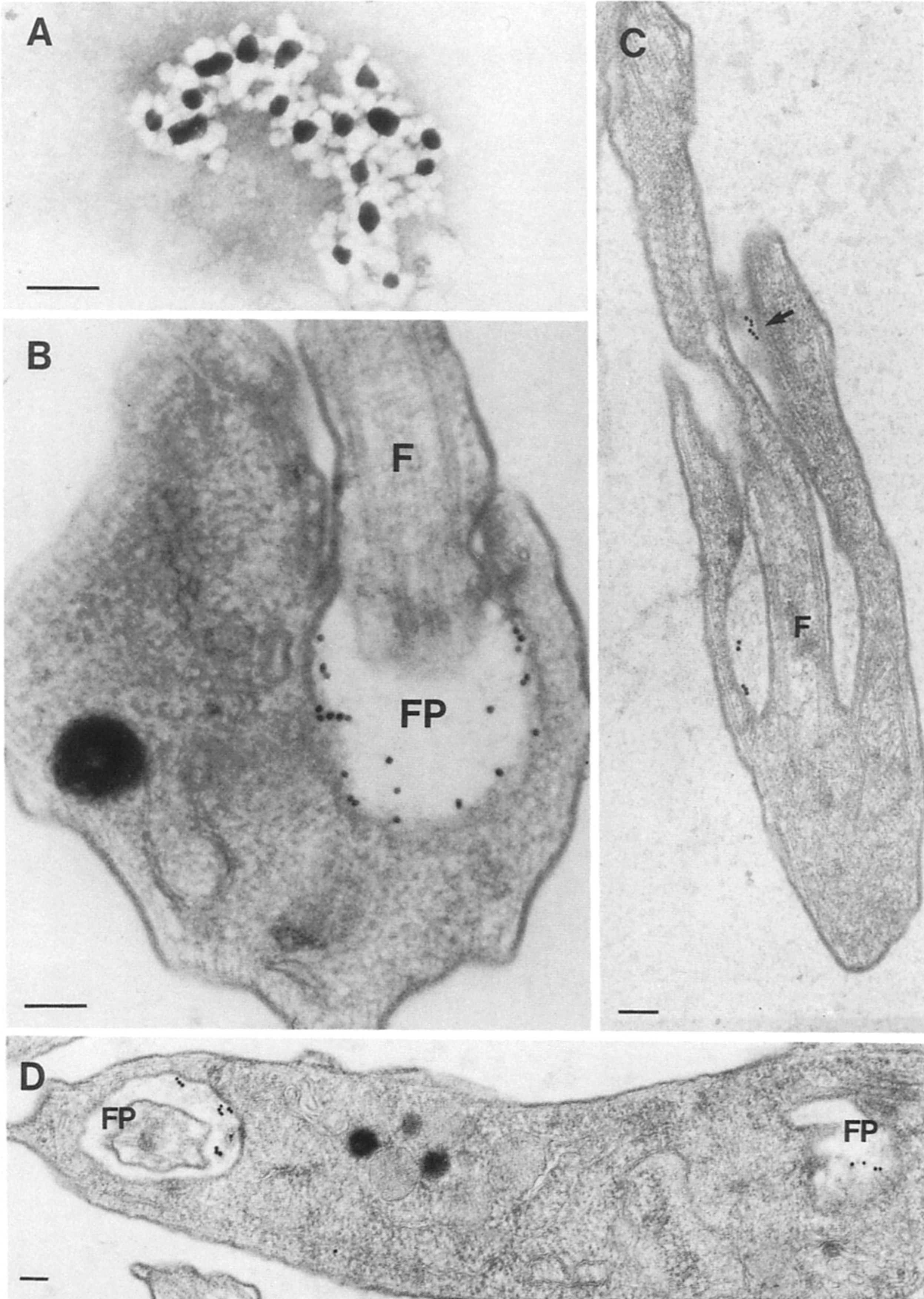


Figure 4.

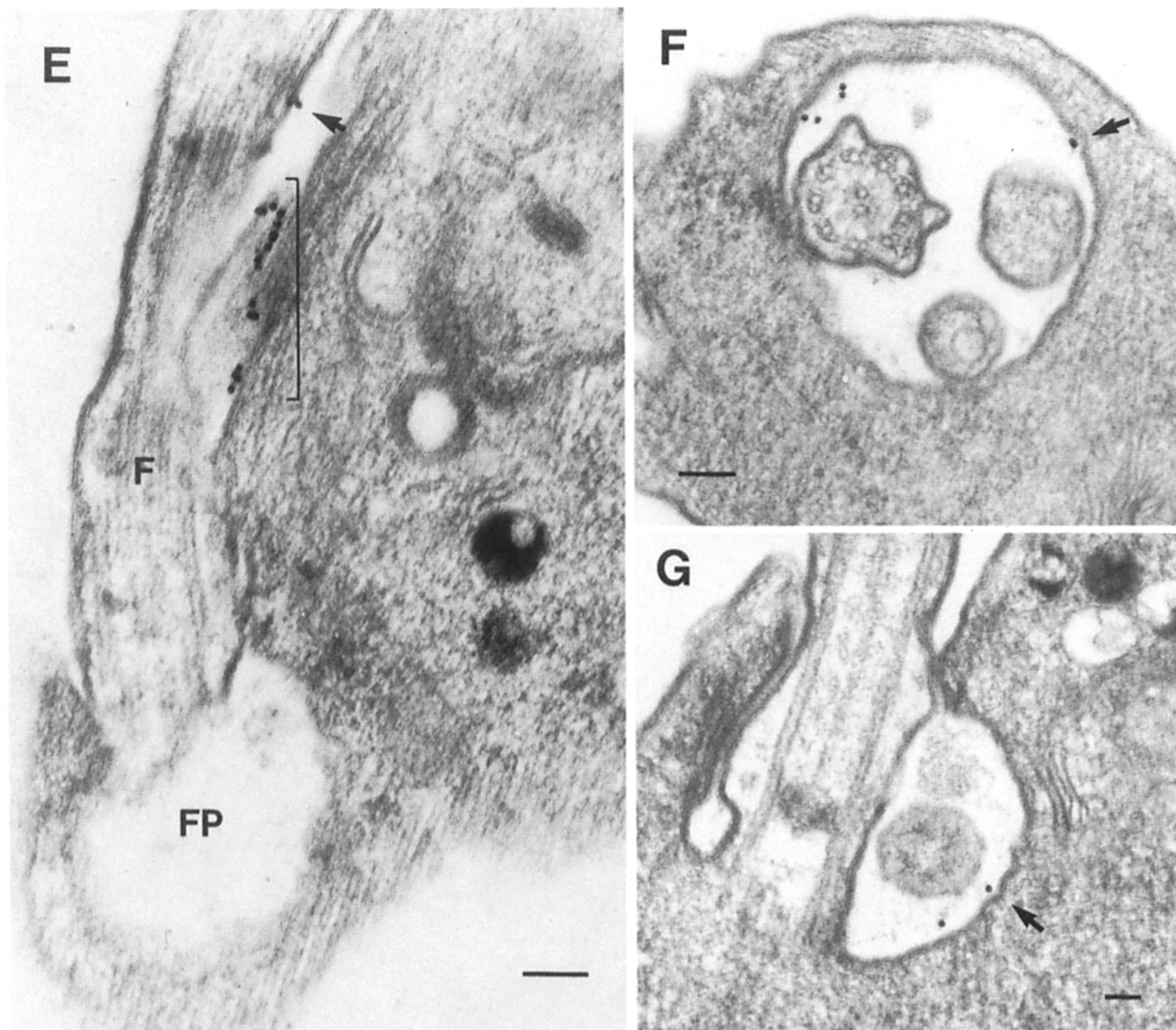


Figure 4. Localization of TLF binding at 4°C to *T. b. brucei*. TLF was conjugated to colloidal gold as described (18). Cells were incubated with the TLF–gold complexes at a concentration of approximately 6.7 μg/ml. (*A*) Negative staining of TLF–gold complexes. Complexes were stained with sodium phosphotungstate, collected on formvar coated grids and examined by transmission electron microscopy. (*B*) Thin section electron micrograph of *T. b. brucei* incubated at 4°C for 1 h with TLF–gold complexes. TLF–gold complexes bound to the flagellar pocket (*FP*) membrane. (*C*) Thin section electron micrograph of *T. b. brucei* incubated at 4°C with TLF–gold complexes. Binding is seen both in the flagellar pocket and to the junction region at the flagellum (*F, arrow*). (*D*) Thin section electron micrograph of a dividing *T. b. brucei* with TLF–gold complexes in both flagellar pockets (*FP*). (*E*) Thin section electron micrograph of TLF–gold complexes bound to the junction region (*bracket*) and to the flagella (*F, arrow*). (*F*) Thin section electron micrograph of TLF–gold complexes bound to the flagella pocket membrane. One of the complexes is associated with a small "pit" in the flagellar pocket membrane (*arrow*). (*G*) Thin section electron micrograph of TLF–gold complexes associated with flagellar pocket "pits" (*arrow*). Bar, 0.1 μm.

disruption of the osmotic stability of the cells (23, 28, 31). *T. b. brucei* lysis may be a direct consequence of TLF binding to the cell surface forming pores in the plasma membrane. Alternatively, lysis may require uptake and processing of the particles. The latter case is supported by a 20–40-min delay observed in the onset of cell lysis following the addition of TLF to cells (15, 29). The availability of highly purified TLF made it possible to study binding and uptake of TLF by *T. b. brucei* and to discriminate between these possibilities. When trypanosomes were incubated with ^{125}I-TLF (2 μg/ml) at 4°C binding was rapid and approached equilibrium within 1 h (data not shown). The extent of binding was a function of the extracellular concentration of TLF (Fig. 2 *A*). The shape of the binding curves suggests multiple binding components; a high affinity/low capacity binding site which shows half-maximal binding at 0.75 μg/ml and a low affinity/high capacity binding site which fails to show saturable binding at concentrations of TLF as high as 30 μg/ml (Fig. 2 *A*). The lower concentration (0.75 μg/ml) is similar to the TLF concentration (0.66 μg/ml) required for in vitro lysis. This correlation suggests that the high-affinity binding site may mediate killing. From this

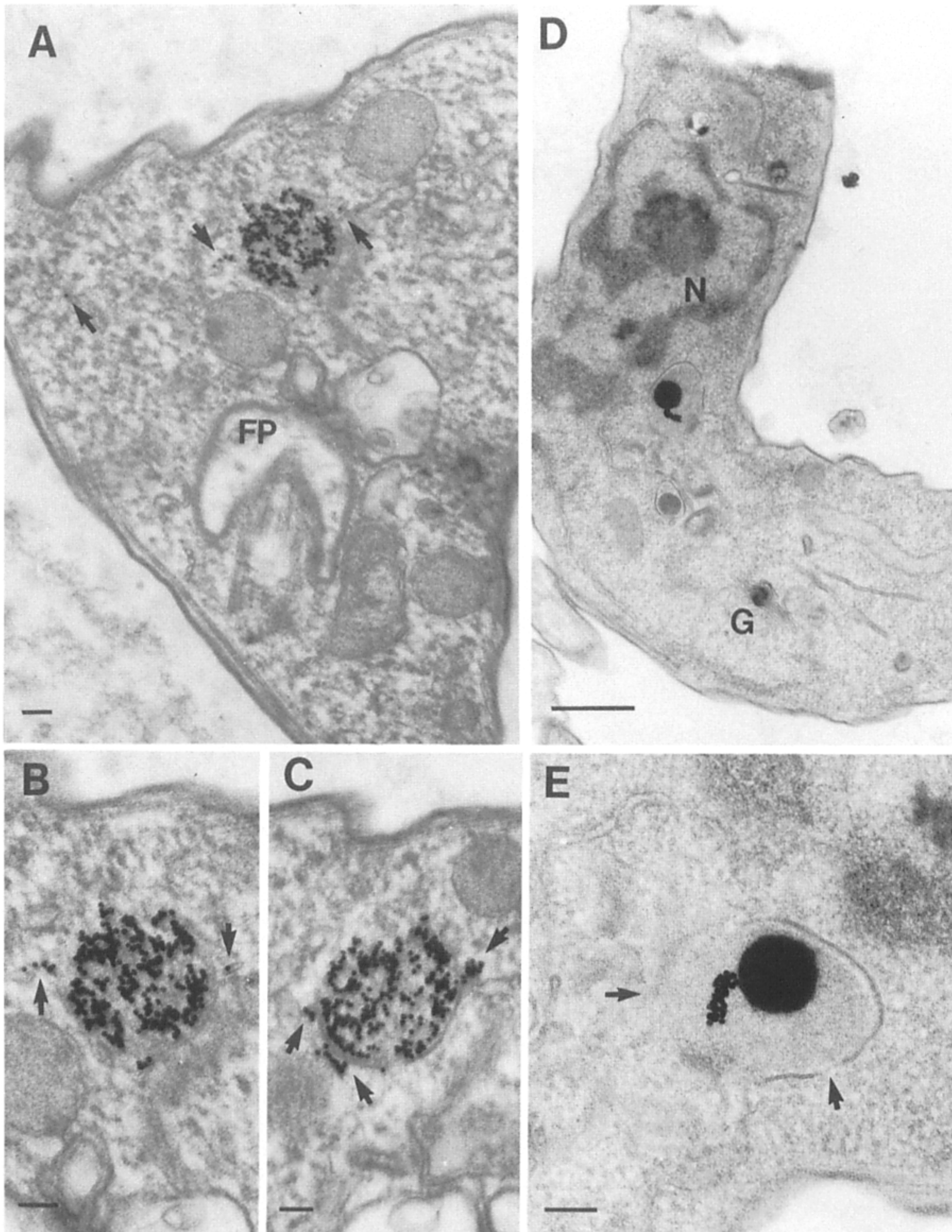


Figure 5. Localization of TLF–gold complexes in *T. b. brucei* at 37°C. Thin section electron microscopy of TLF-gold-treated cells incubated at 4°C for 1 h, washed to remove any unbound TLF, and then shifted to 37°C for 1 h. (*A*) TLF–gold complexes are absent from the flagellar pocket (*FP*) and are now localized within large vesicles and free in the cytoplasm (*arrow*). (*B*, *C*) High magnification electron micrographs of serial sections though a large vesicle containing TLF-gold complexes (*A*). TLF–gold complexes released due to disruption of the vesicle membranes are seen (*arrows*). (*D*) TLF–gold complexes in a large vesicle containing electron dense material. The cell nucleus (*N*), Golgi (*G*), and other membrane bound organelles are intact. (*E*) High magnification electron micrograph of the TLF containing vesicle (*D*) reveals membrane disruption (*arrows*). (*F*) Section through a pair of cells containing TLF–gold complexes. (*G*, *H*) High magnification electron micrograph of the TLF–gold complexes reveals in *F* that the membranes of the vesicles are disrupted and localized cytoplasmic damage is seen. Bars: (*A–C*, *E–H*) 0.1 μm; (*D* and *E*) 0.5 μm.

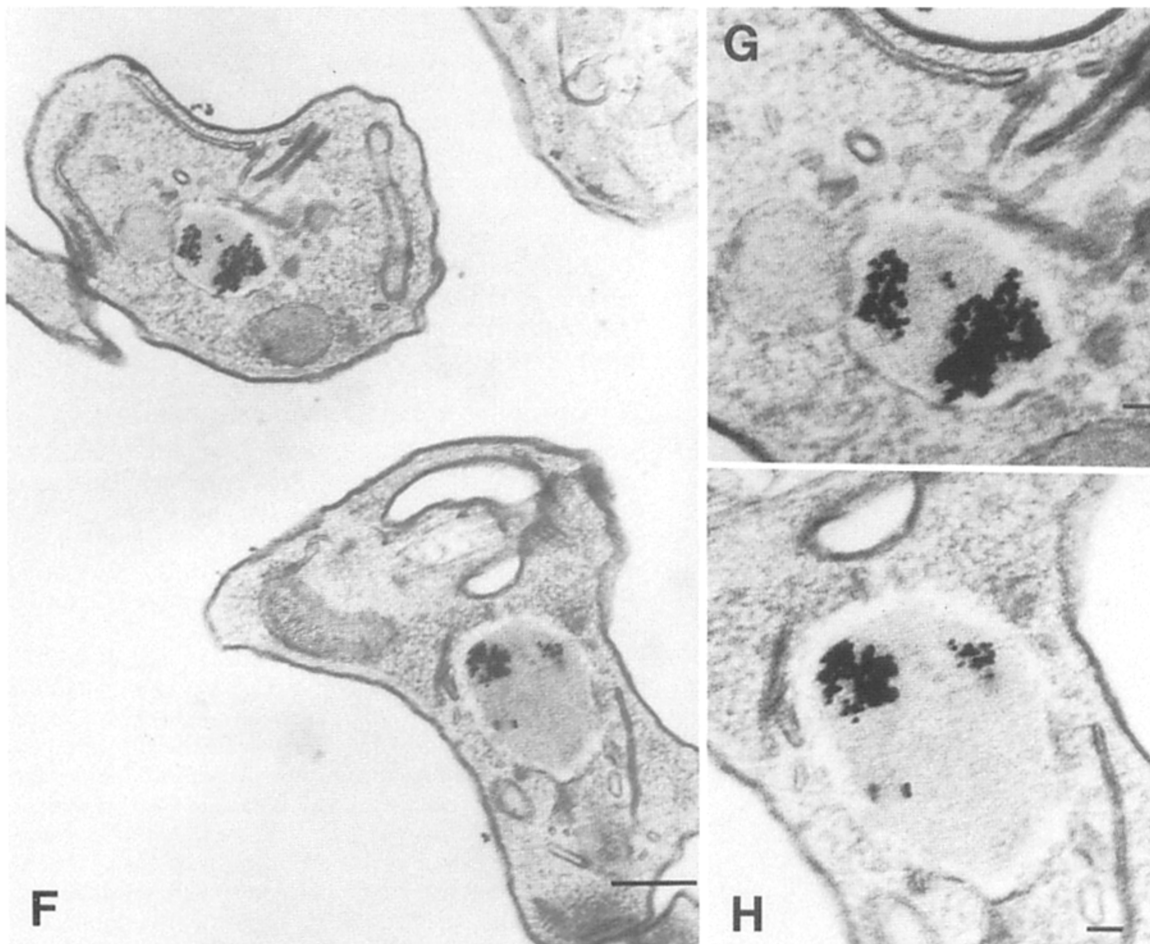


Figure 5.

binding data, we calculate each trypanosome contains ~350 high affinity TLF binding sites. Non-lytic human serum HDL (ρ = 1.063-1.17 g/ml) also binds rapidly and in a saturable fashion to *T. b. brucei* when assayed under identical conditions at 4°C. The half-maximal binding for non-lytic HDL is 80 μg/ml with ~22,000 molecules per trypanosome (Fig. 2 *B*). Taken together, these results suggest the presence of multiple binding sites on the trypanome surface for TLF and the lower binding sites might also bind non-lytic HDL.

Since TLF and non-lytic HDL contain both apo A-I and apo A-II, it seemed likely that the low affinity TLF binding was to a general HDL binding site. To test this possibility, we have carried out competition experiments with various lipoproteins. An excess of unlabeled TLF reduces TLF binding to *T. b. brucei* by >90%. Human LDL does not compete for ^{125}I-TLF binding (<10% inhibition) and incubation with a 50-fold mass excess of purified VSG inhibited binding by <24%. Thus nonspecific binding to VSG was a minor component in the binding studies.

Non-lytic HDL, added at a 100-fold mass excess, reduced overall ^{125}I-TLF binding by ~84% but had no effect on high affinity binding (Fig. 2 *C*). Addition of 100-fold mass excess non-lytic HDL also had no effect on the lytic activity of TLF (Fig. 3). By comparison, heat inactivated TLF (90°C, 15 min) inhibited by the lytic activity of TLF up to 50% (Fig. 3). Adequate amounts of heat-inactivated TLF was not available to test if lysis could be lowered further.

TLF Binding Is Localized to the Flagellar Pocket

To determine the localization of TLF-binding sites on intact trypanosomes, purified TLF was complexed with colloidal gold, and incubated at 4°C with trypanosomes (Fig. 4). The TLF–gold complexes appeared in negatively stained preparations as a single 20-nm gold particle surrounded by 6–10 lipoprotein particles (Fig. 4 *A*). When trypanosomes were incubated with TLF–gold complexes, extensive binding to the flagellar pocket membrane (Fig. 4, *B–D*), and to a lesser extent to the flagellar membrane outside the pocket was seen (Fig. 4, *D* and *E*). TLF–gold complexes tend to associate with invaginations or pits along the flagellar pocket membrane (Fig. 4, *B*, *C*, *F*, and *G*). Association with membrane pits represents the initial stage in the endocytosis of TLF. Since all binding and washing steps were carried out at low temperature, presumably no internalization occurred. To rule out nonspecific trapping of TLF–gold complexes in the flagellar pocket, colloidal gold alone (i.e., lacking TLF) was incubated with trypanosomes under identical conditions. No significant binding to the cell surface or trapping of gold particles within the flagellar pocket was seen (data not shown).

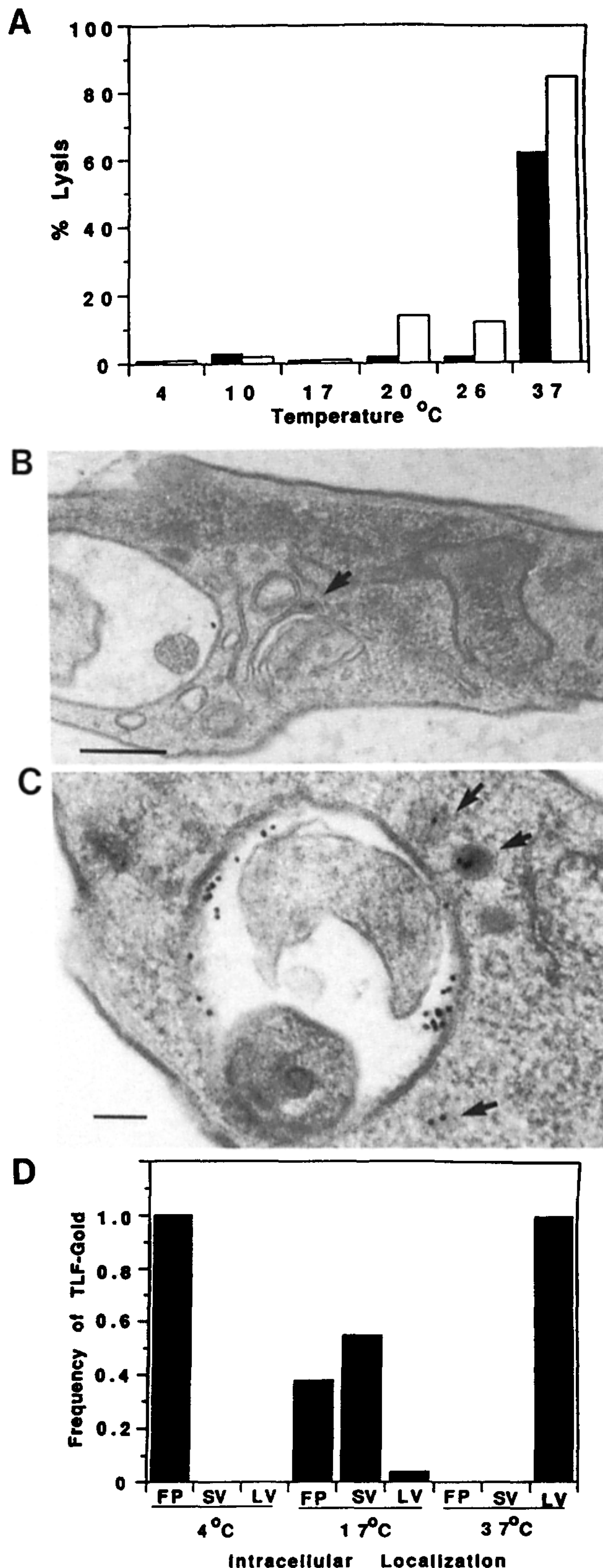


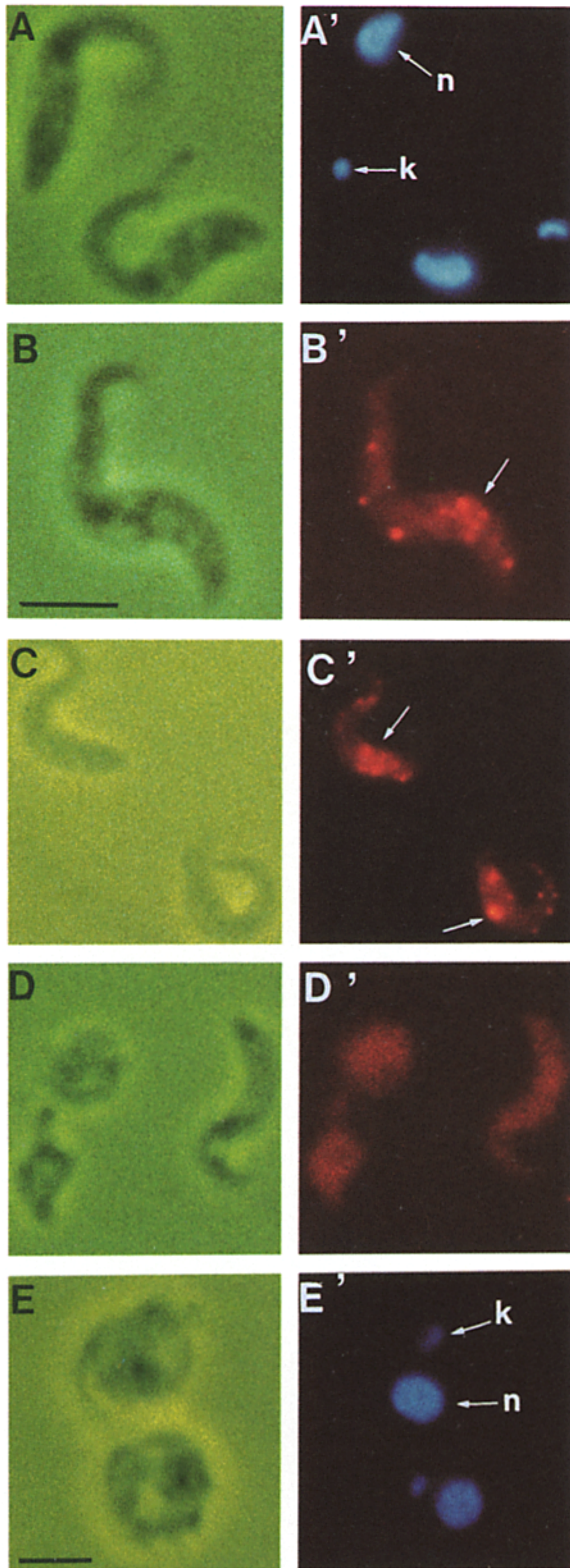
Figure 6. (*A*) Effect of temperature on the lysis of *T. b. brucei* by TLF. *T. b. brucei* was incubated for 2 h at 4, 10, 17, 20, 26, and 37°C with 2 U (*closed bars*) and 8 U (*open bars*) of TLF added. The % lysis was determined as described in Material and Methods. (*B*) Effect of temperature on TLF localization. Thin section electron microscopy of TLF-gold-treated cells incubated at 4°C for 1 h, washed to remove any unbound TLF, and then shifted to 17°C for 1 h. TLF-gold complexes were found within the flagellar pocket and a tubular network system (*arrow*) near the flagella pocket. (*C*) Thin section electron micrograph of TLF-gold complexes in cells incubated at 4°C, washed to remove unbound TLF, and shifted to 17°C for 1 h. TLF-gold complexes are localized in the flagellar pocket (*FP*) and in small vesicles (*arrows*) in the cytoplasm near the flagellar pocket (*FP*). (*D*) Quantitation of thin section electron microscopy results. Histogram of the distribution of TLF-gold complexes at 4°, 17°, and 37°C. *FP*, flagellar pocket; *SV*, small vesicles; *LV*, large vesicles. The relative frequency of TLF-gold distribution is shown for each temperature. Bars: (*B*) 0.1 μm; (*C*) 0.5 μm.

Endocytosis of TLF

The uptake of gold conjugated TLF was examined by electron microscopy. When trypanosomes were incubated with TLF-gold complexes at 4°C, washed, and then shifted to 37°C for 1 h, gold-TLF particles were no longer restricted to the flagellar pocket. TLF-gold particles were seen within the cells, mainly localized in large, lysosome-like vesicles or free in the cytoplasm (Fig. 5, *A–H*). Redistribution of TLF to internal vesicles at 37°C supports vesicle-mediated endocytosis of TLF. Surprisingly, the membranes of the large, TLF containing vesicles were disrupted and TLF-gold complexes were released into the cytoplasm (Fig. 5, *A–H*).

TLF Lysis Involves a Temperature-sensitive Step

Several aspects of TLF mediated lysis suggest a multi-step pathway toward cell death (Figs. 4 and 5). Earlier studies have shown that a lag period of at least 20 min precedes the lysis of *T. b. brucei* by TLF (29). This lag period can not be shortened by raising the concentration of TLF (15). Consistent with these observations, cells treated with native TLF for 15 min appear morphologically normal (reference 29; see also Fig. 7 *C*). It is also observed that lysis does not occur at 17°C regardless of the concentration of TLF (up to a 20-fold increase) or the time of TLF incubation (up to 6 h) (15). At temperatures of 20° and 26°C, increasing the concentration of TLF (Fig. 6 *A*) or incubating the cells with TLF for longer than 2 h gradually increases TLF lysis. The "cold-sensitive" step (17°C and below) in TLF-mediated lysis of trypanosomes might involve a membrane fusion event during the endocytic pathway, analogous to the temperature sensitive step involving inhibition of endosome/lysosome fusion in higher eukaryotic cells (9). When trypanosomes were incubated with TLF-gold at 4°C for 1 h, washed to remove unbound TLF, and then incubated for an additional hour at 17°C (Fig. 6, *B* and *C*), TLF-gold complexes were seen in small vesicles and tubular structures in the cytoplasm near the flagellar pocket. As shown previously, at 37°C the TLF moves to LV which are then disrupted (Fig. 5). The relative occurrence of TLF-gold particles within the different cellular compartments or free in the cytoplasm at 4°, 17°, and 37°C was determined from electron micrographs (Figs. 4, 5, and 6, *B* and *C*) and was expressed as the relative frequency of TLF-gold particle distribution (Fig. 6 *D*). The redistribution of TLF to internal vesicles at elevated temperatures again supports the possibility that TLF binds the trypanosome surface and is taken-up into small vesicles by endocytosis and then transferred to large vesicles (Figs. 4, 5, *A–J*, and 6; see reference 15).



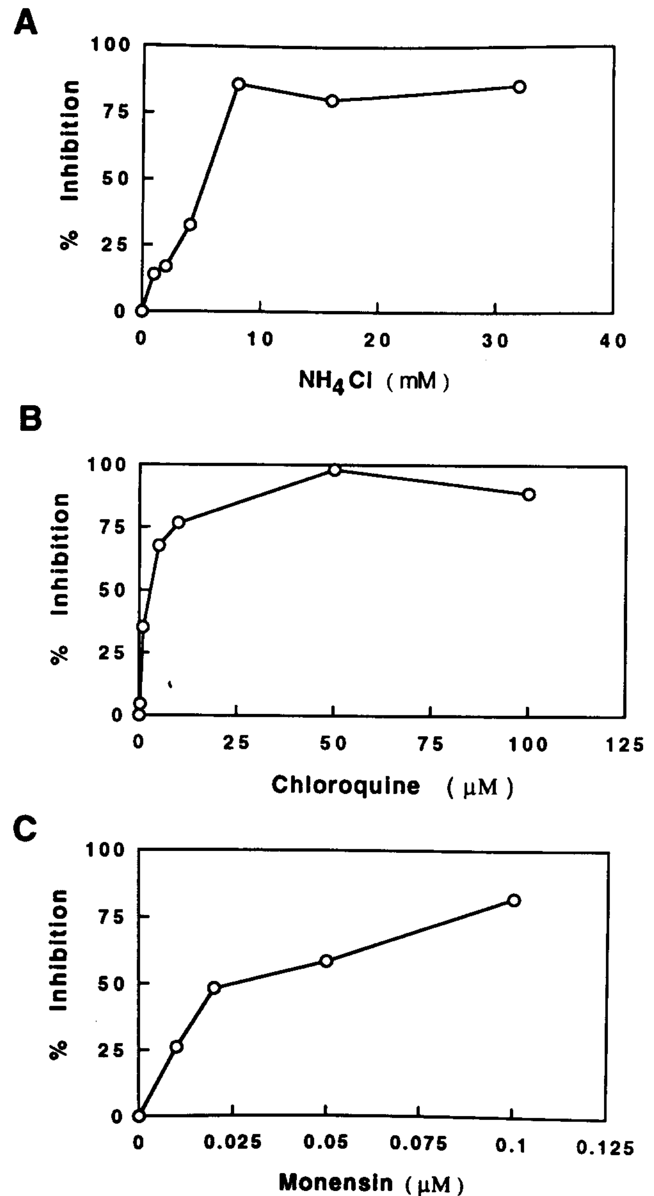


Figure 8. Inhibition of TLF lysis by pH-raising agents. Lysis assays were carried out as previously described in Materials and Methods. (*A*) NH_4Cl effects on lysis of *T. b. brucei*. *T. b. brucei* was pre-treated at 4°C for 30 min with 1 to 32 mM NH_4Cl and then shifted to 37°C and incubated with ∼2 U of TLF for 2 h. (*B*) Chloroquine effects on lysis of *T. b. brucei*. *T. b. brucei* was pre-treated at 4°C for 30 min with 0.25 to 100 μM chloroquine, and then shifted to 37°C and incubated with ∼2 U of TLF for 2 h. (*C*) Monensin effects on lysis of *T. b. brucei*. *T. b. brucei* was pre-treated at 4°C for 30 min with 0.01 to 0.1 μM monensin, and then shifted to 37°C and incubated with ∼2 U of TLF for 2 h.

Figure 7. Loss of dextran staining upon exposure to native TLF at 37°C. Phase contrast (*left*) and fluorescence (*right*) microscopy of bloodstream trypanosomes pre-loaded with 3 mg/ml rhodamine-labeled dextran 37°C 30′ followed by mock or TLF treatment. The same bloodstream trypanosomes were viewed by phase contrast (*A–E*) or fluorescence (*A–E′*). (*A′*) Hoescht staining (of DNA) in mock treated cells. *k*, kinetoplast; *n*, nucleus (*arrows*). (*B′*) The bulk of dextran appears to have accumulated between the kinetoplast and nucleus (*arrow*). Other small vesicles are seen throughout the body. (*C′*) Punctate fluorescence is maintained in cells exposed to TLF for 15 min (*arrows*). (*D′*) Punctate fluorescence is lost in TLF exposed cells (37°C, 45′). (*E′*) Cells exposed to TLF for 45 min maintain Hoescht staining. The kinetoplast and nucleus, however, appear swollen (*arrow*). *k*, kinetoplast; *n*, nucleus. Bars: (*A*, *B*, and *E*) 4 μm; (*C* and *D*) 5 μm.

We were concerned that gold conjugation might affect both the activity and localization of TLF. To examine the uptake and localization of native TLF, a bulk phase endocytic marker, rhodamine-labeled dextran, was incubated with trypanosomes in the presence or absence of native TLF (Fig. 7). Fluorescence microscopy of trypanosomes incubated at 37°C revealed accumulation of labeled dextran in small vesicles at several locations in the cytoplasm (Fig. 7 *B'*). The bulk of the dextran was centrally located between the Hoescht stained kinetoplast mitochondrial DNA (at the base of the flagellum) and the nucleus (Fig. 7 *A'*), consistent with lysosomal localization in trypanosomes (4, 5). Co-incubation of *T. b. brucei* for 45 min with TLF and dextran dramatically altered both the general morphology and distribution of rhodamine containing granules (Fig. 7 *D'*). These cells lacked the crisp, punctate fluorescence seen in the absence of TLF. Loss of vesicle staining coincided with an increase in overall cytoplasmic staining. Vesicle disruption appeared to precede loss of osmoregulation since some cells had lost vesicle staining but retained normal morphology (Fig. 7, *D* and *D'*). Vesicle disruption may be specific to the vesicles containing rhodamine, the bulk phase endocytic marker, since both kinetoplast and nucleus, although swollen, were intact as judged by Hoescht staining (Fig. 7 *E'*) and electron microscopy (Fig. 5, *B* and *F*). To determine if the fluorescence observed above represented internalized material versus externally bound material, cells were incubated with dextran alone at 4°C for 30 min. The cells were then washed and viewed under the microscope. No fluorescence was seen (data not shown).

The Pathway of TLF-mediated Lysis Requires an Acidification Step

Although the nature of the large vesicles can not be determined directly from either electron or fluorescence micros-

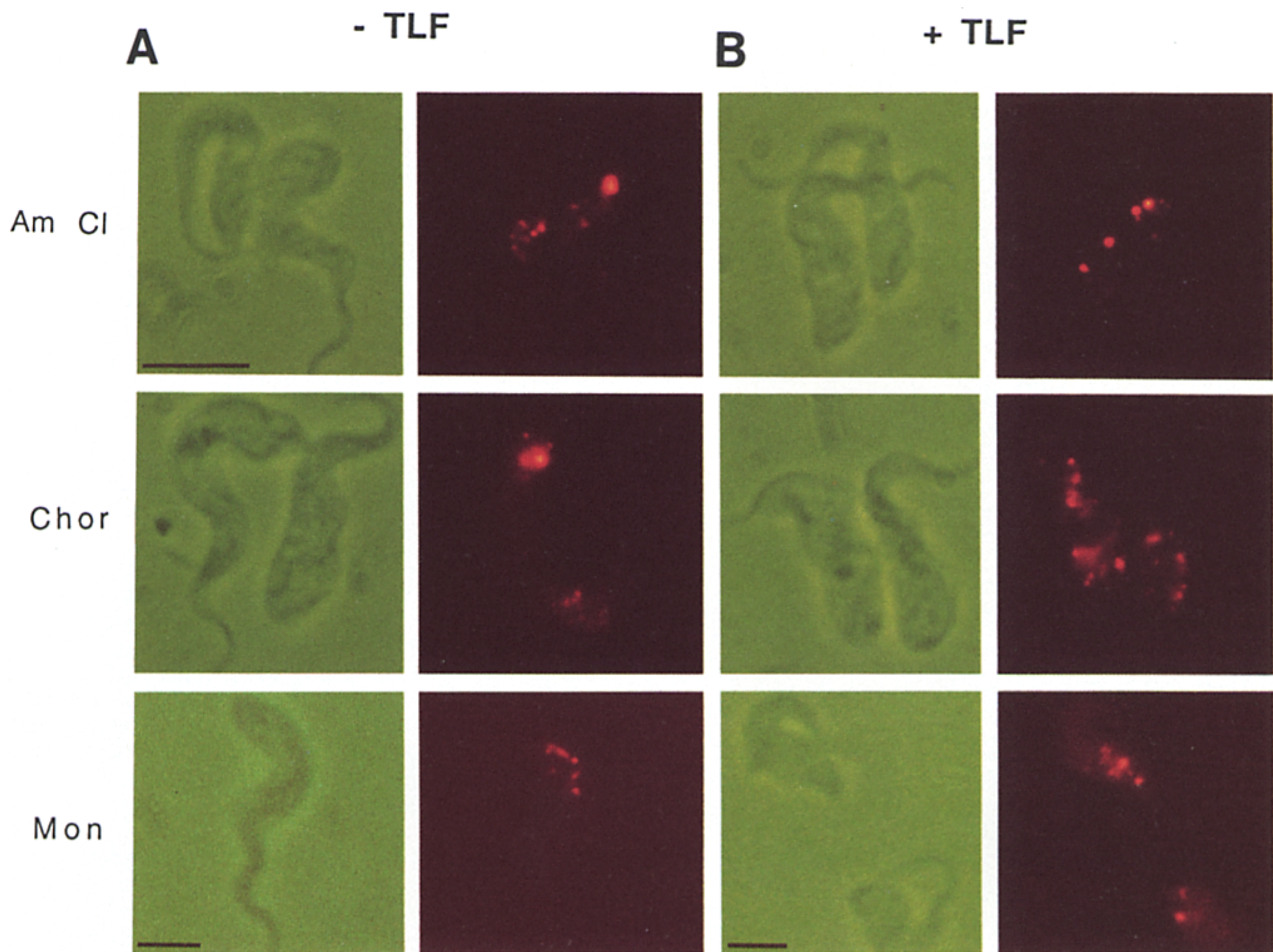
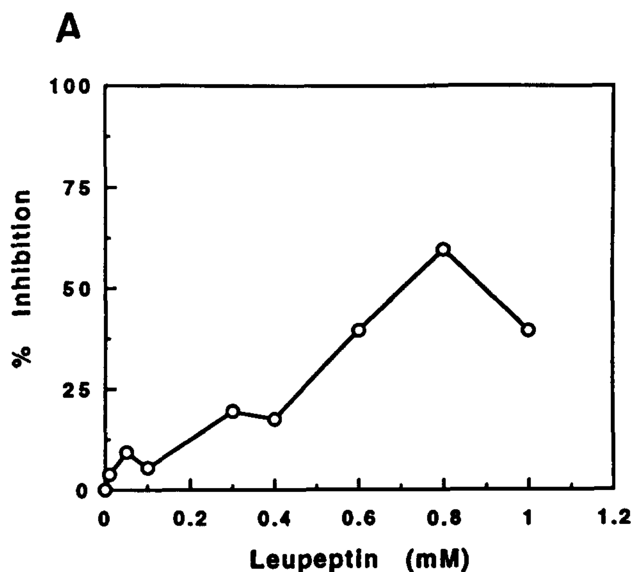


Figure 9. Inhibition of vesicle breakdown by pH-raising agents. Shown are fluorescence and light photomicrographs of cells treated with the acidification inhibitors (as indicated) Am Chl, Chor, and Mon in the absence (*A, left, −TLF*) or in the presence (*B, right, +TLF*) of 2 U of TLF. In each panel, the left column shows the phase contrast microscopy of the trypanosomes while the right column shows the fluorescence microscopy of the same cells. In each experiment, the cells were first incubated for 30 min at 4°C with the appropriate inhibitor, and then with 3 mg/ml dextran for 30 min at 37°C, and then ± TLF for 45 min at 37°C. Inhibitor concentrations were NH_4Cl (*Am Cl*) = 8 mM; chloroquine (*Chor*) = 50 μM; Monensin (*Mon*) = 0.1 μM. (Note: All micrographs are same magnification with the exception of Monensin-treated cells). Bars, 5 μm.



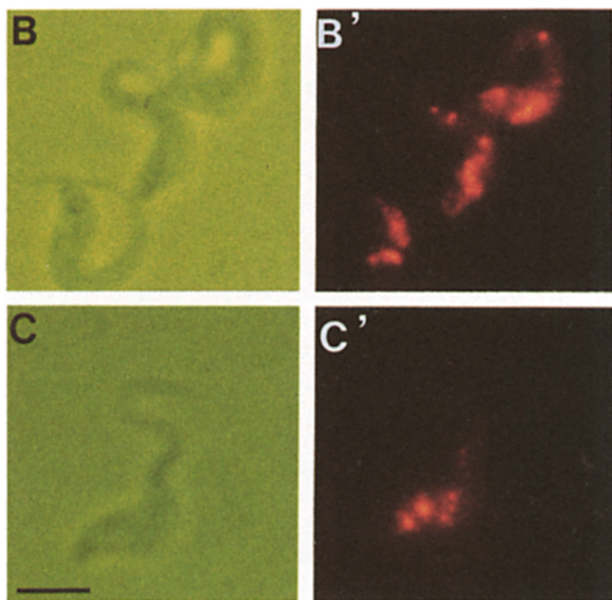


Figure 10. Requirement for a thioprotease is implicated. (*A*) Leupeptin effects on the lysis of *T. b. brucei*. *T. b. brucei* was pre-treated with 0.01 to 1.0 mM leupeptin for 1 h at 37°C. Cells were then incubated with ~2 U of TLF for an additional 2 h at 37°C. (*B–C*) Cells were incubated with 0.6 mM leupeptin (*B*). Phase contrast of cells mock treated with buffer instead of TLF. (*B'*) Fluorescence of the cells in *B*. (*C*) Phase contrast of cells treated with TLF. (*C'*) Fluorescence of the cells in *C*. Bar, 5 μm.

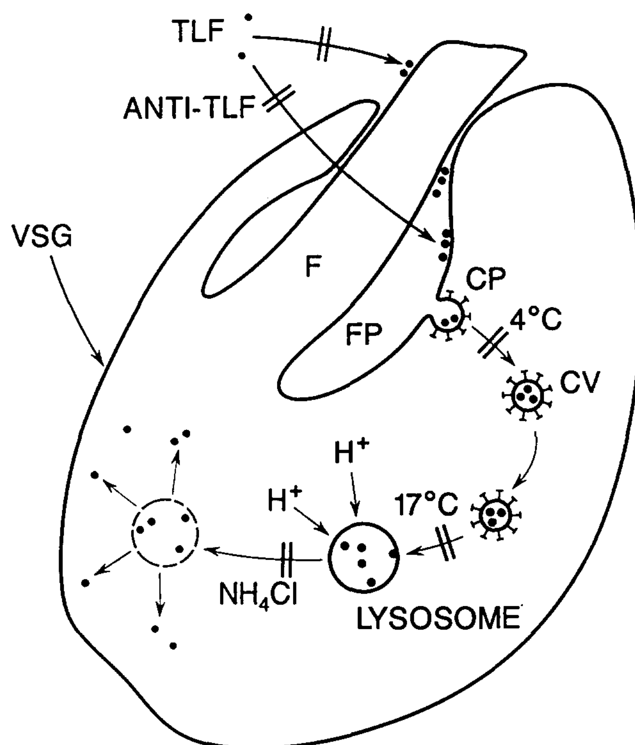


Figure 11. Proposed model for TLF-mediated lysis in *T. b. brucei*. The lytic pathway can be blocked at several steps: (*a*) anti-TLF blocks binding to the flagellar pocket (*FP*) binding site; (*b*) 4°C binding occurs within the flagellar pocket but endocytosis does not occur; (*c*) at 17°C endocytosis occurs but TLF does not move from small coated vesicles (*CV*); or (*d*) treatment with ammonium chloride blocks the acidification of endosomes or lysosomes and blocks cell lysis at 37°C. At 37°C, after a lag of ~30 min, lysis occurs, and is correlated with the breakdown of vesicle membranes. *VSG*, variable surface glycoprotein; *F*, flagellum; *CP*, coated pit.

copy (Figs. 5 and 7) their size, morphology, and intracellular location are highly suggestive of lysosomes. One hallmark of the lysosome is that it is an acidic compartment. To determine whether TLF passes through an acidic compartment following endocytosis by trypanosomes, and whether acidification is necessary for TLF-mediated lysis, we examined the effect of pH-elevating agents on TLF activity (Fig. 8; reference 34). Incubation of trypanosomes with ≥8 mM ammonium chloride inhibited TLF lysis by >83% (Fig. 8 *A*). Chloroquine, another lysosomotropic amine, at concentrations of ≥50 μM inhibited lysis by 98% (Fig. 8 *B*). The ionophore monensin, at a concentration of 0.1 μM, inhibited TLF-mediated lysis by >80% (Fig. 8 *C*). The inhibitor concentrations used above were not lethal to the trypanosomes however, high concentrations of these inhibitors are toxic to them (see Materials and Methods). These findings indicate that TLF requires an acidic environment to become active.

These studies suggested to us that prevention of acidification might also prevent TLF-mediated disruption of the dextran-loaded vesicles as seen in Fig. 7 *C*. We tested this idea using the same lysosomotropic amines as above in conjunction with fluorescence microscopy (Fig. 9). In the presence of the lysosomal inhibitors, dextran accumulated in the large vesicles (Fig. 9, *−TLF*). Thus, these agents do not block endocytic uptake. Upon exposure to TLF for 45 min, the cells pre-treated with chloroquine, ammonium chloride, or monensin maintained punctate fluorescence in the perinuclear region of the cell (Fig. 9, *+TLF*). This is in striking contrast to the breakdown of fluorescent vesicles seen in TLF-treated cells not pre-treated with lysosomal inhibitors (Fig. 7 *D'*).

Thioproteases Implicated in TLF Lytic Mechanism

What is the mechanism of cell lysis? Since TLF appears to

localize to the lysosome and membrane disruption precedes cell death, cell lysis might be a consequence of the release of lysosomal hydrolases into the cytoplasm. These enzymes would then lead to cell lysis by actively degrading the cytoplasm of the trypanosome. Inhibition of some of the hydrolases might make TLF less toxic to the cell. Alternatively, lysosomal proteases might be directly involved in vesicle disruption and subsequent cell lysis. To test these possibilities, we incubated trypanosomes with leupeptin, a thioprotease inhibitor. Cells were pre-incubated with leupeptin then treated with TLF for 2 h at 37°C. Leupeptin inhibited TLF lysis by ~60% at 0.8 mM (Fig. 10 *A*). Fluorescence studies were carried out to determine whether TLF-mediated disruption of the dextran-labeled vesicles was also inhibited by leupeptin (Fig. 10, *B* and *C*). Control experiments with cells treated with leupeptin alone indicated that it does not block endocytosis of rhodamine dextran (Fig. 10, *B* and *B'*). To examine the effects of the inhibitor on TLF action, cells were pre-treated with leupeptin and dextran before incubation with TLF at 37°C for 45 min. Leupeptin blocked TLF-induced breakdown of the rhodamine-labeled endocytic vesicles (Fig. 10 *C*). Higher concentrations (≥1.0 mM) of leupeptin were toxic to the cells raising background lysis (inhibitor alone) to >20%. These results suggest thioproteases are involved in the action of TLF.

Discussion

The purification of TLF from human serum has enabled us to study the interaction of this minor subclass of human HDL with trypanosomes. In agreement with the studies of Gillett and Owen (12), we find that human HDL binds to *T. b. brucei* in a rapid and saturable fashion. Binding studies with purified TLF indicate there are sites on the trypanosome surface that can bind TLF (Fig. 2). These sites are proteins since trypsin treatment (3) abolishes ^{125}I-TLF binding by >65% (Hager, K. M., unpublished data). One site binds non-lytic HDL (ρ = 1.063–1.17 g/ml) and is present at ~22,000 molecules per cell. A second, higher affinity site, present at about 350 molecules per cell, appears to be specific for TLF since this binding was not inhibited by high concentrations of non-lytic HDL (Fig. 2). Consistent with the inability of non-lytic HDL to completely block TLF binding, we find non-lytic HDL does not compete for lysis while heat inactivated TLF does (Fig. 3). Since TLF represents a minor subclass of human HDL, comprising <1% of the total serum HDL, it is likely that the binding of TLF in intact serum is mediated by this TLF-specific binding site. Further, binding occurs exclusively in the flagellar pocket and to the flagellum (Fig. 4). These results are consistent with LDL and transferrin studies on receptor-mediated endocytosis in trypanosomes which suggest that trypanosome receptors are located on the flagellar membrane and flagellar pocket membrane (2, 6, 7, 21, 26, 33).

After binding, TLF is internalized by *T. b. brucei*. This is the first evidence that TLF is taken-up by trypanosomes before cell lysis (Figs. 5 and 6). Electron micrographs show that at 37°C, cytoplasmic vesicles are disrupted prior to cell lysis. The vesicles are large and lysosome-like. Fluorescent microscopy shows native TLF disrupted endocytic vesicles after a lag period of 20 min. It is intriguing that cells show vesicle disruption after being exposed to TLF for 45 min even though they have not yet exhibited a loss in osmoregulation. It is likely that these cells represent the step between vesicle disruption and the loss of osmoregulation that ultimately leads to cell death.

Studies in mammalian systems have previously shown that endosomal fusion with the lysosome is blocked at 17°C (9). When trypanosomes are incubated at 17°C, gold-labeled TLF is observed within small endocytic vesicles or tubules contiguous with the flagellar pocket. A temperature of 17°C is a nonpermissive temperature for TLF lysis, despite TLF's internalization to endosomes. At 37°C, a permissive temperature for TLF-mediated lysis, TLF is seen in large lysosome-like structures. The preservation of the integrity of this large lysosome-like compartment is intimately linked with the maintenance of osmoregulation. Taken together, these temperature studies indicate that TLF not only has to be internalized but also implicates a lysosomal compartment.

Our inhibitor studies with the pH-raising agents (34) further implicated a lysosomal compartment. They revealed that TLF must pass through an acidic compartment in order to disrupt endocytic vesicles and be cytotoxic to trypanosomes (Figs. 8 and 9). Since TLF appears to be capable of disrupting internal membrane compartments, why is TLF not active on the plasma membrane of the trypanosome? TLF might be inactive outside of the trypanosome and activated only when internalized. Activation might be due either to modification of a TLF component (protein or lipid) or alteration of its environment (pH or salt concentration). It is likely to be affected if it is within the acid protease rich environment of the lysosome. Further, it is highly probable that the disruption of lysosomal membranes by TLF and the subsequent release of their hydrolases would lead to cell death by autodigestion (Fig. 11).

The actual mechanism of lysosomal disruption by TLF remains a mystery. However, the leupeptin inhibitor studies shed some light on the process. When cells are pre-incubated with leupeptin, cells are spared TLF-mediated lysis and TLF-mediated vesicle disruption is inhibited. It is possible TLF requires a thioprotease-mediated activation step. It is important to recall TLF has two unique apolipoproteins, L-I and L-III, that exist as disulfide-linked oligomers (15). Perhaps these apolipoproteins require processing to a "toxic" form in order to act on membranes. Future in vitro experiments will address the mechanism of vesicle breakdown and better define the "activation" process. A number of specific enzymes have been found associated with serum HDLs, including both proteases and lipases (20). It is tempting to speculate that one of the unique components of TLF (either apo L-I and/or apo L-III) might be capable of disrupting intracellular membranes upon activation following endocytosis.

We propose the following pathway for TLF mediated lysis (Fig. 11). TLF binds specifically to sites in the flagellar pocket of the trypanosome. TLF binding can be blocked by the action of monoclonal antibodies against the TLF-specific apolipoproteins apo L-I and apo L-III. Binding is followed by endocytosis and delivery of the endocytosed molecules to a lysosomal compartment. Finally, TLF disrupts lysosomal membranes causing release of the contents into the cytoplasm. Thus, the primary site of TLF action is inside the trypanosome and the previously observed osmotic instability of the trypanosomes (29, 30) is a late event in the lytic path-

way. In this respect, TLF represents a unique host defense mechanism. As a natural killing factor, TLF is unique in that it not only requires internalization but also a putative activation step.

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References

1. Bangs, J. D., D. Hereld, J. L. Krakow, G. W. Hart, and P. T. Englund. 1985. Rapid processing of the carboxyl terminus of a trypanosome variant surface glycoprotein. *Proc. Natl. Acad. Sci. USA.* 82:3207–3211.
2. Borst, P. 1991. Tranferrin receptor, antigen variation and the prospect of a trypanosome vaccine. *Trends Genet.* 7:307–309.
3. Brickman, J. J., and A. E. Balber. 1990. *Trypanosoma brucei rhodesiense* bloodstream forms: surface ricin-binding glycoproteins are localized exclusively in the flagellar pocket and the flagellar adhesion zone. *J. Protozool.* 37:219–224.
4. Brickman, M. J., and A. E. Balber. 1993. *Trypanosoma brucei rhodesiense*: membrane glycoproteins localized primarily in endosomes and lysosomes of bloodstream forms. *Exp. Parasitol.* 76:329–44.
5. Burleigh, B. A., C. W. Wells, M. W. Clarke, and P. R. Gardiner. 1993. An integral membrane glycoprotein associated with endocytic compartment of *Trypanosoma vivax*: identification and partial characterization. *J. Cell Biol.* 120:339–352.
6. Coppens, I., F. R. Opperdoes, P. J. Courtoy, and P. Baudhuin. 1987. Receptor-mediated endocytosis in the bloodstream form of *Trypanosoma brucei. J. Protozool.* 34:465–473.
7. Coppens, I., P. Baudhuin, F. R. Opperdoes, and P. J. Courtoy. 1988. Receptors for the host low density lipoproteins on the hemoflagellate *Trypanosoma brucei*: purification and involvement in the growth of the parasite. *Proc. Natl. Acad. Sci. USA.* 85:6753–6757.
8. Cross, G. A. M. 1990. Cellular and genetic aspects of antigenic variation in trypanosomes. *Annu. Rev. Immunol.* 8:83–110.
9. Dunn, W. A., A. L. Hubbard, and N. N. Aaronson Jr. 1980. Low temperature selectivity inhibits fusion between pinocytic vesicles and lysosomes during heterophagy of ^{125}I-asialofetuin by the perfused rat liver. *J. Biol. Chem.* 255:5971–5978.
10. Eisenberg, S. 1984. High density lipoprotein metabolism. *J. Lipid Res.* 25:1017–1058.
11. Gillett, M. P. T., and J. S. Owen. 1991. *Trypanosoma brucei brucei*: differences in the trypanocidal activity of human plasma and its relationship to the level of high density lipoproteins. *Trans. Roy. Soc. Trop. Med. Hyg.* 85:612–616.
12. Gillett, M. P. T., and J. S. Owen. 1992. Characteristic of the binding of human and bovine high-density lipoproteins by bloodstream forms of the African trypanosome, *Trypanosoma brucei brucei. Biochim. Biophys. Acta* 1123:239–248.
13. Gillett, M. P. T., and J. S. Owen. 1992. Comparison of the cytolytic effects *in vitro* on *Trypanosoma brucei brucei* of plasma high density lipoproteins and apolipoprotein A-I from hosts both susceptible (cattle and sheep) and resistant (human and baboon) to infection. *J. Lipid Res.* 33:513–523.
14. Goldstein, J. L., S. K. Basu, and M. S. Brown. 1983. Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *In* Methods and Enzymology. B. Fleischer, and S. Fleischer, editors. Academic Press, Inc., New York. 98:241–251.
15. Hajduk, S. L., D. R. Moore, J. Vasudevacharya, H. Siqueira, A. F. Torri, E. M. Tytler, and J. D. Esko. 1989. Lysis of *Trypanosoma brucei* by a toxic subspecies of human high density lipoprotein. *J. Biol. Chem.* 264: 5210–5217.
16. Hajduk, S. L., P. T. Englund, and D. H. Smith. 1990. African Trypanosomiasis. *In* Tropical and Geographical Medicine. 2nd ed. K. S. Warren and A. A. F. Mahmoud, editors. McGraw-Hill, Inc. N.Y. pp 268–281.
17. Hajduk, S. L., K. M. Hager, and J. D. Esko. 1992. High-density lipoprotein-mediated lysis of trypanosomes. *Parasitol. Today.* 8:95–98.
18. Handley, D. A., C. M. Arbeeny, L. D. Witte, and S. Chien. 1981. Colloidal gold-low density lipoprotein conjugates as membrane receptor probes. *Proc. Natl. Acad. Sci. USA.* 78:368–371.
19. Hoare, C. A. 1972. The Trypanosomes of Mammals: A Zoological Monograph. Cecil A. Hoare, editor. Blackwell Scientific Publications, Oxford. 4, 484 pp.
20. Hoener, M. C., and U. Brodbeck. 1992. Phosphatidylinositol-glycan-specific phospholipase D is an amphiphilic glycoprotein that in serum is associated with high-density lipoproteins. *Eur. J. Biochem.* 206:747–757.
21. Langreth, S. G., and A. E. Balber. 1975. Protein uptake and digestion in bloodstream and culture forms of *Trypanosoma brucei. J. Protozool.* 22:40–53.
22. Lanham, S. N. 1968. Separation of trypanosomes from the blood of infected rats and mice by anion-exchangers. *Nature (Lond.).* 218:1273–1274.
23. Laveran, A. 1902. De l'action du serum humain sur le trypanosome de Nagana (*Tr. brucei*). *Comp. Rendus de l'Acad. Sci* 134:734–739.
24. Owen, J. S., M. P. T. Gillett, and T. E. Hughes. 1992. Transgenic mice expressing human apolipoprotein A-I have sera with modest trypanolytic activity *in vitro* but remain susceptible to infection by *Trypanosoma brucei brucei. J. Lipid Res.* 33:1639–1646.
25. Pownall, H. J., and A. M. Gotto Jr. 1992. Human plasma apolipoproteins in biology and medicine. *In* Structure and Function of Apolipoproteins. Mrayvonne Rosseneu, editor. CRC Press, Inc., Boca Raton, FL. 1–32.
26. Prioli, R. P., J. M. Ordovas, I. Rosenberg, E. J. Schaefer, and M. E. A. Pereira. 1987. Similarly of cruzin, an inhibitor of *Trypanosoma cruzi* neuraminidase, to high density lipoprotein. *Science (Wash. DC).* 238: 1417–1419.
27. Rickman, L. R., and J. Robson. 1970. The testing of proven *Trypanosoma brucei* and *T. rhodesiense* strains by the blood incubation infectivity test. *Bull. Wld. Hlth. Org.* 42:911–916.
28. Rifkin, M. R. 1978. Identification of the trypanocidal factor in normal human serum: high density lipoprotein. *Proc. Natl. Acad. Sci. USA.* 75: 3450–3454.
29. Rifkin, M. R. 1978. *Trypanosoma brucei*: some properties of the cytotoxic reaction induced by normal human serum. *Exp. Parasitol.* 46:189–206.
30. Rifkin, M. R. 1983. Interaction of high-density lipoprotein with *Trypanosoma brucei*: effect of membrane stabilizers. *J. Cell. Biochem.* 23:57–70.
31. Rifkin, M. R. 1984. *Trypanosoma brucei*: biochemical and morphological studies of cytotoxicity caused by normal human serum. *Expt. Parasitol.* 58:81–93.
32. Rifkin, M. R. 1991. *Trypanosoma brucei*: cytotoxicity of host high-density lipoprotein is not mediated by apolipoprotein A-I. *Exp. Parasitol.* 72: 216–218.
33. Schell, D., R. Evers, D. Preis, K. Ziegelbauer, H. Keifer, F. Lottspeich, A. W. C. A. Cornelisseu, and P. Overath. 1990. A transferrin-binding protein of *Trypanosoma brucei* is encoded by one of the genes in the variant surface glycoprotein gene expression site. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1061–1066.
34. Seglen, P. O. 1983. Inhibitors of lysosomal function. *In* Methods in Enzymology. B. Fleischer, and S. Fleischer, editors. Academic Press, Inc., New York. 737–764.