The Main Lytic Factor of *Trypanosoma brucei brucei* in Normal Human Serum Is Not High Density Lipoprotein

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Summary

Natural immunity of humans to the cattle pathogen *Trypanosoma brucei brucei* has been attributed to the presence in normal human serum (NHS) of lytic factors for the parasites. We and others have shown that NHS contains two trypanolytic factors (herein termed TLF1 and TLF2) that can be separated by gel filtration. TLF1 copurifies with a subclass of high density lipoprotein (HDL), whereas TLF2 has a much higher molecular weight and does not appear to be a lipoprotein. We find that the trypanolytic activity of purified TLF1 is totally inhibited by exogenous haptoglobin (Hp) at concentrations (0.1 mg/ml) lower than those present in NHS (0.2–2 mg/ml). In contrast, exogenous Hp (up to 2.5 mg/ml) has no effect on the lytic activity of either NHS or isolated TLF2. Hp-depleted sera from patients with intravascular hemolysis is severalfold more trypanolytic than NHS. These sera contain only TLF1, and their lytic activity is totally abolished upon the addition of Hp (0.1 mg/ml). When NHS containing different Hp allotypes is fractionated by gel filtration, TLF1 activity is either revealed or remains masked, depending on whether it coelutes with Hp. Masked TLF1 activity in the column fractions is revealed if Hp is removed by density gradient ultracentrifugation. We conclude that endogenous Hp inhibits TLF1 activity, and that TLF2 is the main trypanolytic factor in NHS.

Two subspecies of trypanosomes can cause sleeping sickness: *Trypanosoma brucei gambiense* (*T. b. gambiense*) which causes a chronic wasting disease prevalent in West Africa, and *Trypanosoma brucei rhodesiense* (*T. b. rhodesiense*) which is associated with an acute disease in East Africa. Although genetically distinct from each other, *T. b. gambiense* and *T. b. rhodesiense* may be human-adapted forms of the cattle pathogen *Trypanosoma brucei brucei* (1, 2). Normal human serum (NHS)[1] contains cytotoxic factor(s) that lyse the cattle but not the human pathogens.

In 1978, Rifkin (3) identified the trypanolytic factor in NHS as high density lipoprotein (HDL, ~500 kD, e = 1.063–1.216 g/ml). Further studies have shown that lytic activity is associated with a minor subclass of HDL termed TLF (trypanosome lytic factor) (4–6). Trypanosome lysis requires the endocytosis of TLF, followed by the disruption of acidic intracellular vesicles (lysosomes) (7, 8). Recent evidence indicates that the parasites are killed by the acid-induced peroxidase activity of a complex formed from two components of TLF, haptoglobin-related protein (Hpr) and

hemoglobin (Hb) (9). The Hpr is >90% identical to haptoglobin (Hp) (10), a plasma glycoprotein that avidly binds free Hb. Whereas there is a single type of Hpr, there are three main Hp types in humans, namely Hp 1-1, Hp 2-1, and Hp 2-2 (11).

Human serum also contains a second poorly defined trypanolytic factor with properties distinct from HDL (1,000 kD, e >1.25 g/ml). In the present paper we name the HDL-like lytic factor TLF1, and the high molecular weight lytic factor TLF2. Previous reports document the presence of either or both lytic factors (12–15) in different individuals; however, the molecular basis for this heterogeneity is unknown. In this paper, we provide an explanation for this variability, and demonstrate that TLF2 is the main trypanolytic factor in NHS.

Materials and Methods

Parasites and Sera. Bloodstream forms of *T. b. brucei* (Trypanosome resource, Edinburgh University, Edinburgh, Scotland) 667 stock (16) and *T. b. rhodesiense* ETat 1.9R Edinburgh *Trypanozoon* antigen type 1.9 resistant (17) were used in all experiments. The former are sensitive to lysis by human serum; the latter are resistant and were used as the control for serum-specific trypanolysis. The parasites were grown in Swiss Webster mice, harvested mid

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[1]*Abbreviations used in this paper:* Hb, hemoglobin; HDL, high density lipoprotein; Hp, haptoglobin; Hpr, haptoglobin-related protein; NHS, normal human serum; PNH, paroxysmal nocturnal hemoglobinuria; TBS, Tris-buffered saline; TLF, trypanosome lytic factor.

log phase, and purified by the method of Lanham and Godfrey (18). NHS and normal human plasma were obtained from blood collected from healthy volunteers, stored at 4°C, and analyzed within 5 d. No differences in trypanolytic activity were found between plasma and serum. Dr. W. Rosse (Duke University, Durham, NC) kindly provided sera from 19 patients with sickle-cell anemia and from 2 patients with paroxysmal nocturnal hemoglobinuria (PNH). Four sera from PNH patients were kindly provided by Dr. L. Luzzato (Memorial Sloan-Kettering Cancer Institute, New York, NY), and one serum from a PNH patient was collected at New York University Medical Center (New York, NY; patient SB [19]).

Parasite Lysis and Hp Inhibition Assays. Parasite lysis was determined using a fluorescence-based assay as described (13). Dilutions of Hp prepared in 20 mM Tris, pH 7.5, and 130 mM NaCl (Tris-buffered saline [TBS]) were added to 9 vol of serum or serum fractions and incubated for 15 min at 37°C. Typically, 5 μl of Hp dilution was added to 45 μl of test sample. An equal volume (50 μl) of trypanosomes at 2 × 10^7/ml in high glucose DME (GIBCO BRL, Gaithersburg, MD) and 1.0% BSA (Sigma Chemical Co., St. Louis, MO) was then added; parasite survival was determined after a 150-min incubation at 37°C. Purified, lyophilized Hp (Sigma Chemical Co.) was reconstituted to 25 mg/ml in TBS and stored for up to 2 wk at 4°C.

Hp Assay and Typing. Hp concentration in serum and serum fractions was determined by an ELISA. A standard procedure was followed (20) using an IgG fraction of rabbit anti-Hp (Sigma Chemical Co.) as capture antibody (1:100 dilution), and goat anti-Hp (Sigma Chemical Co.) as the second antibody (1:1,000 dilution). Bound Hp–antibody complex was determined using rabbit anti–goat IgG conjugated to alkaline phosphatase, and p-nitrophenyl phosphate disodium (Pierce, Rockford, IL) as chromogenic substrate. Hp phenotyping of serum was determined by the separation of 2 μl of reduced serum proteins on 12% SDS-PAGE followed by their transfer onto polyvinyldifluoride (PVDF) membrane (25 min, 15 V). The membrane was blocked by incubation with 10% BSA in TBS, 0.1% Tween 20 for 1 h at room temperature (milk cannot be used as it contains Hp). The first antibody was an IgG fraction of rabbit anti–human Hp (1:10,000; Sigma Chemical Co.), and the second antibody was goat anti–rabbit conjugated to horseradish peroxidase (1:20,000; Promega Corp., Madison, WI). The immunoreactive proteins were visualized by enhanced chemiluminescence (ECL; Dupont NEN, Boston, MA).

Lipoprotein Isolation. Lipoproteins were isolated from human serum by density gradient centrifugation according to the method of Poumay and Ronveaux-Dupal (21). Fresh serum (12 ml) or pooled gel filtration fractions from NHS (12 ml) were mixed with solid KBr to give a final density of 1.25 g/ml. The lipoproteins were then floated by centrifugation in a NVTi 60.1 rotor (Beckmann Instruments, Inc., Palo Alto, CA) (48 K, 16 h, 10°C). Routinely, the top 2.5 ml, a bright orange layer containing lipoproteins, was collected and dialysed against TBS for 4 h at 4°C in Slide-A-Lyzers (Pierce). Lipoproteins (1 ml) were further fractionated by gel filtration (see below).

Gel Filtration of Lipoproteins and Serum. Fresh serum was fractionated by fast performance liquid chromatography (FPLC) using Superose 6 HR 10/30 column (Pharmacia Biotech Inc., Piscataway, NJ). Lipoproteins were fractionated using Superose 6 HR 16/50 and Superose 12 HR 16/50 columns connected in tandem. Sample volumes of 500 μl and 1 ml were applied to 10/30 and 16/50 columns, respectively. All columns were equilibrated and run in TBS at 0.2 ml/min (10/30 column) or 0.4 ml/min (16/50 columns).

Purification of Hb. Hb was purified based on the method of Williams and Tsay (22), but with modifications to minimize the formation of methemoglobin. Blood was drawn into citrate glucose anticoagulant and erythrocytes were washed six times in 0.9% saline. All subsequent steps were done at 4°C. Erythrocytes were lysed in 2 vol of 0.05 M Tris-HCl, pH 7.6. The lysate was centrifuged at 30,000 g for 15 min, and the center two thirds of the solution was mixed with an equal volume of 0.05 M Tris-HCl, pH 7.6. This solution was then added to an equal volume of 50% DEAE-cellulose preequilibrated with 0.05 M Tris-HCl, pH 7.6, and mixed for 15 min. After centrifugation (3,000 g, 10 min), the supernatant was applied to a DEAE-cellulose column equilibrated with 0.05 M Tris-HCl, pH 8. Hb was eluted with a buffer containing 0.2 M NaCl and 0.05 M Tris-HCl, pH 7.6. Early red fractions were discarded and collected fractions were adjusted to pH 8, aliquoted, and stored at −70°C. Hb concentration was estimated from absorbance at 541 nm [ε(1%) = 8.63] (22).

Fractionation of Hp–Hb Complexes. Hp 1-1 (Sigma Chemical Co., St. Louis, MO) at 10 mg/ml was mixed with 1 vol of Hb at 25 mg/ml (twofold molar excess). The mixture was incubated at 37°C for 30 min, and 100 μl was applied to a Superose 12 HR10/30 FPLC column. The sample was eluted with TBS at 0.2 ml/min. Purified Hp 1-1 and Hb were individually chromatographed under the same conditions. Hp 1-1 was used since the other Hp allotypes form multimers and failed to yield single peaks after fractionation. Hp 1-1 forms dimers of ∼90 kD which combine with Hb dimers to yield tetramers of ∼150 kD. Hp–Hb complexes are thus easily separated from free Hp and Hb.

Lysosomotrophic Agents. Trypanosomes were preincubated with NH$_4$Cl (2.5–15 mM) or chloroquine (10–50 μM) for 30 min at 37°C, followed by incubation with either purified TFL1 (2 U), TLF2 (1 U) or TBS. 1 U is equivalent to the amount of sample that lysed 50% of the trypanosomes in 150 min, as previously defined (5).

Results

Inhibition of Isolated TFL1 by Serum Hp. A recent report suggests that the trypanolytic activity of NHS is mediated by the peroxidase activity of TLF1-associated Hpr–Hb complexes (9). Hp, found in NHS at concentrations of 0.2–2
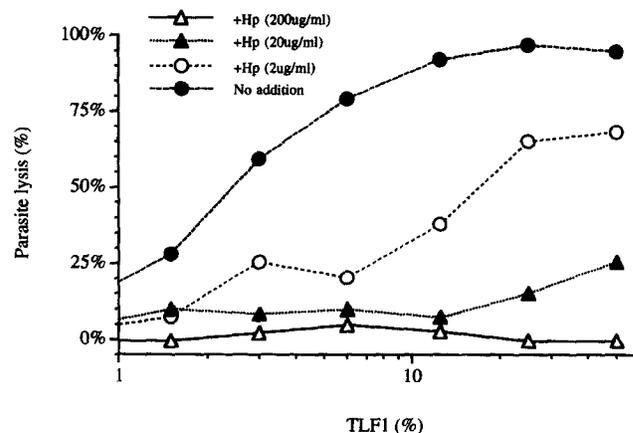

Figure 1. Inhibition of TLF1 by Hp. Increasing concentrations of Hp (mixed type) were added to dilutions of purified TLF1. Trypanosomes *T. b. brucei* were then added (10^7/ml final concentration) and lysis was determined after 150 min at 37°C.

Table 1. *Relationship of Hp Concentration and Type to Lytic Activity in Human Sera*

Serum	Hp allotype	Hp concentration μg ml^{-1}	LU ml^{-1}
NHS 1	1-1	270	47
NHS 2	2-1	320	38
NHS 3	2-1	1,400	10
NHS 4	2-1	512	12
NHS 5	2-2	290	44
NHS 6	2-2	1,800	32
PNH 1	NA	<0.01	550
PNH 2	NA	<0.01	400
PNH 3	NA	10	400
PNH 4	NA	26	360
SCA 1	NA	<0.01	220
SCA 2	NA	<0.01	350
SCA 3	NA	<0.01	380
SCA 4	NA	15	400
SCA 5	NA	20	450

SCA, sickle cell anemia.

mg/ml, is >90% homologous to Hpr (10). This raised the possibility that Hp might inhibit TLF1-mediated trypanolysis, perhaps through the displacement of Hb. Fig. 1 shows that the activity of TLF1, isolated from NHS by density gradient ultracentrifugation followed by gel filtration, is totally suppressed by the addition of exogenous Hp at 0.2 mg/ml.

Comparison of Trypanolytic Activities of NHS and Sera Depleted of Hp. To further substantiate the finding that serum Hp is a potent inhibitor of TLF1, we measured the trypanolytic activity of sera from patients with intravascular
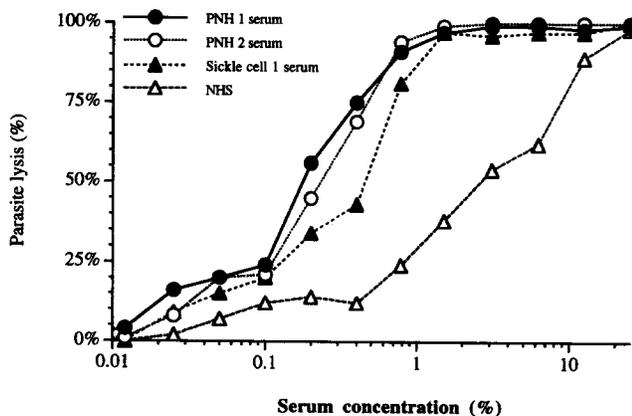

Figure 2. Lytic activity of NHS and Hp-deficient pathological sera. Parasite lysis was determined after addition of trypanosomes *T. b. brucei* (10^7/ml) to dilutions of NHS or of sera from patients with PNH (patients 1 and 2, Table 1) and sickle-cell anemia (patient 1, Table 1).

hemolysis (sickle-cell anemia and PNH). In these patients, the free Hb released in the plasma binds with very high affinity to Hp (dissociation constant >10^{-15} M; 23), and the complex is rapidly removed from the circulation. As shown in Table 1 and Fig. 2, Hp-depleted sera were 10–40-fold more lytic than NHS. Notably, there was no apparent correlation between the lytic activity of NHS and either Hp concentration, or Hp allotype. A total of 12 NHS and 20 pathological sera (4 PNH, 16 sickle-cell anemia) were tested and only representative serum is shown.

Inhibition of Serum Lytic Activity by Hp. We tested whether, in addition to its ability to inhibit purified TLF1, exogenous Hp could also inhibit the lytic activity of whole human sera. The addition of Hp to NHS, even at high concentrations (2.5 mg/ml) had no effect on its lytic activity (Fig. 3 *a*). In sharp contrast however, when purified Hp was added to the highly trypanolytic serum of a patient with PNH, there was a dose-dependent abrogation of activity; complete inhibition was obtained with 0.1 mg/ml
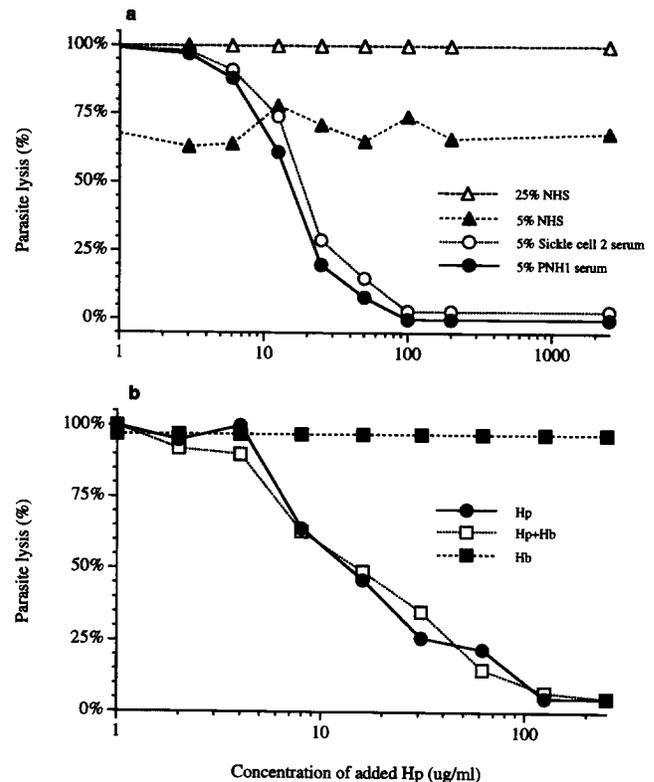

Figure 3. Effect of exogenous Hp and Hb on the trypanolytic activity of NHS and Hp-deficient sera. (*a*) Two dilutions of NHS, causing either 100 or 75% lysis (*triangles*), were incubated with increasing concentrations of Hp (mixed types). Trypanosomes *T. b. brucei* (10^7/ml) were then added and trypanolysis determined. As shown, Hp had no effect on the lytic activity of NHS. Increasing concentrations of Hp were also added to sera from patients with PNH or sickle-cell anemia (*circles*). The trypanolytic activity of the patients' sera was inhibited by added Hp. Sera indicated in this figure are those in Table 1. (*b*) 5% PNH serum 1 (Table 1), which produced 100% tyrpanolysis, was mixed with the indicated concentrations of either purified Hp 1-1 alone, Hp–Hb complexes, or Hb alone, and the trypanolytic activity of the serum subsequently measured.

Hp (Fig. 3 *a*). Both mixed and 1-1 Hp allotypes were effective inhibitors of lysis (not shown).

Inhibitory Activity of Hp–Hb Complexes. Inhibition of TLF1 by Hp could be due to the displacement of Hb from the putative Hpr–Hb complex in TLF1. To test this hypothesis, we compared the inhibitory activity of isolated Hp and purified Hp–Hb complexes of 1:1 stoichiometry. As shown in Fig. 3 *b*, Hp and Hp–Hb complexes were equally inhibitory over a wide range of concentrations. The addition of Hb alone had no effect on the lytic activity of either NHS (not shown) or PNH serum (Fig. 3 *b*), and purified Hp–Hb complexes were nonlytic (not shown).

Trypanolytic Profiles of Sera Fractionated by Gel Filtration. To reveal the origin of the non Hp-inhibitable activity of NHS (Fig. 3 *a*), we fractionated the sera of normal individuals with different Hp phenotypes by gel filtration. NHS containing Hp 1-1 gave two lytic peaks upon size fractionation, one of high molecular weight (∼1,000 kD; TLF2), and one of between 150 and 600 kD (TLF1) (Fig. 4 *a*). Fractionation of NHS containing Hp 2-1 or 2-2 yielded only one lytic peak corresponding to TLF2 (Fig. 4, *b* and *c*). Fractionation of serum from a patient with sickle-cell anemia (Fig. 4 *d*), as well as of sera from other patients with intravascular hemolysis (not shown), also yielded only one major lytic peak, corresponding to TLF1 (Fig. 4 *d*). We then added purified Hp (250 μg/ml) to each serum fraction, and repeated the trypanolytic assay. The added Hp effectively inhibited TLF1 activity, but had no effect on TLF2 (Fig. 4, *e–h*). These findings account for the observation that exogenous Hp inhibits the activity of Hp-depleted pathological sera, which contains little or no TLF2 activity, but has no effect on NHS activity (Fig. 3 *a*).

The distribution of Hp in the NHS fractions corresponded to the known sizes of the different Hp types (Fig. 4, *a–c*; Hp 1-1 is a dimer, whereas Hp 2-1 and 2-2 are polymers (11). Only in NHS containing Hp 1-1 was endogenous Hp effectively separated from TLF1 (Fig. 4 *a*). In NHS of Hp 2-1 and 2-2, endogenous Hp was found in the same fractions as TLF1 (Fig. 4, *b* and *c*). Taken together, these data strongly suggest that TLF1 activity was not detected in Hp 2-2 and 2-1 NHS because endogenous Hp cofractionates with TLF1 and inhibits its activity.

Density Gradient Fractionation of TLF1 and TLF2. To demonstrate that indeed endogenous Hp was inhibiting TLF1 activity in NHS (Hp 2-1), nonlytic fractions corresponding to the expected position of the "masked" TLF1 peak in the
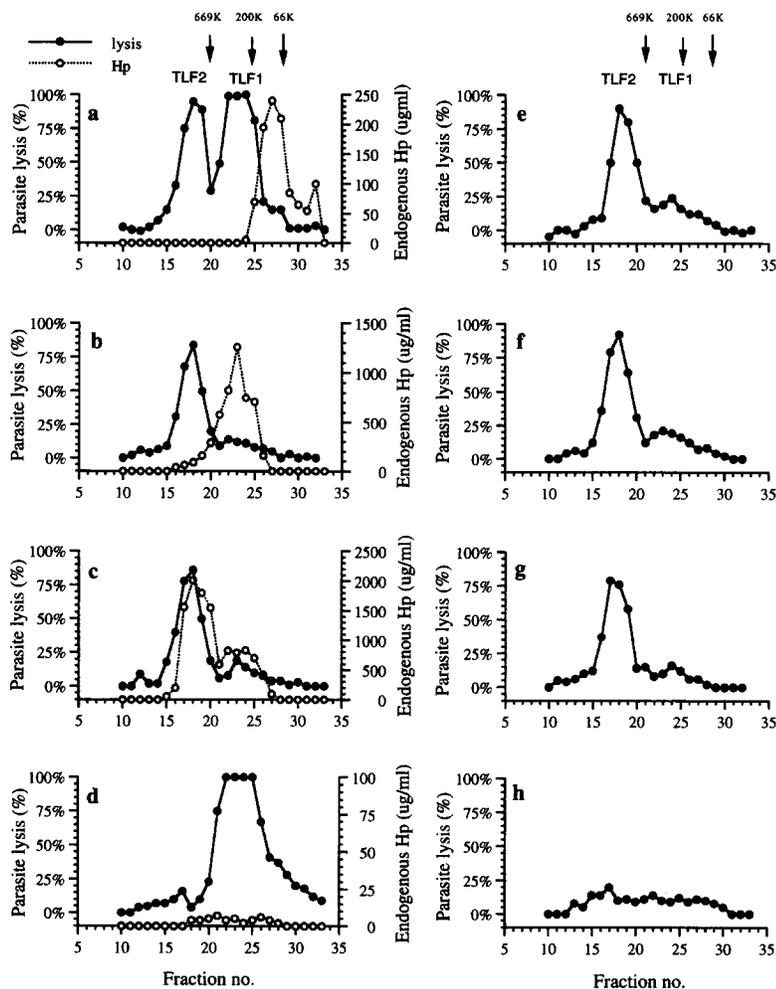
Figure 4. Gel filtration of individual sera containing different Hp allotypes. NHS of Hp type 1-1 (*a* and *e*), 2-1 (*b* and *f*), or 2-2 (*c* and *g*), and Hp-deficient serum from a patient with sickle-cell anemia (*d* and *h*) were fractionated on a Superose 6 FPLC column. Fractions were assayed for lytic activity and Hp concentration (*a–d*). Exogenous Hp (0.25 mg/ml) was then added to each fraction and the lytic activity was again determined (*e–h*).
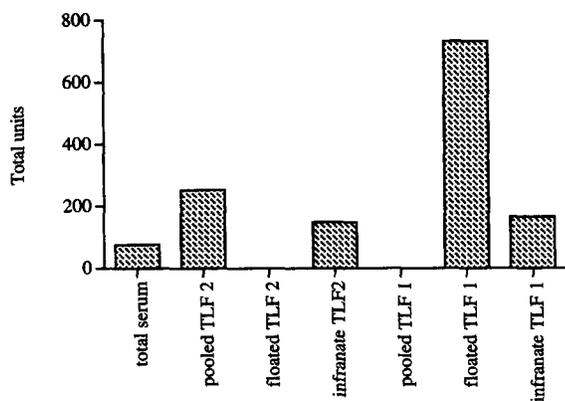
Figure 5. Density gradient ultracentrifugation. NHS Hp type 2-1 was fractionated on Superose 6 and 12 FPLC columns connected in tandem. TLF2 fractions were pooled (13.2 ml), as were fractions where TLF1 would normally elute (13.2 ml). 12 ml of each pool was adjusted to a density of 1.25 g/ml and the lipoproteins floated. The total serum, FPLC pools, and the floated and infranate fractions were titrated to determine the dilution that killed 50% of the trypanosomes *T. b. brucei* (1 U). The total units in each pool or fraction are represented.

gel filtration profile were pooled and adjusted to e = 1.25 g/ml with KBr. After ultracentrifugation, lytic activity was recovered in the floated lipoprotein fraction (e <1.25 g/ml; Fig. 5), whereas Hp was located in the infranate (e >1.25 g/ml). In contrast to TLF1, when TLF2 was subjected to density gradient centrifugation, lytic activity was recovered solely in the infranate (Fig. 5). This activity was not inhibitable by Hp and yielded the high molecular weight peak when fractionated by gel filtration (not shown). As previously noted (5), when TLF1 was purified by density gradient ultracentrifugation, there was a substantial increase in activity over that of whole serum (Fig. 5).

The Effect of Lysosomotrophic Agents on TLF Activity. To determine if TLF2, like TLF1, must be delivered to the lysosomes before trypanolysis (7, 8), we tested the effect of lysosomotrophic agents such as NH₄Cl and chloroquine. Lysis by both purified TLF1 and TLF2 was equally inhib-
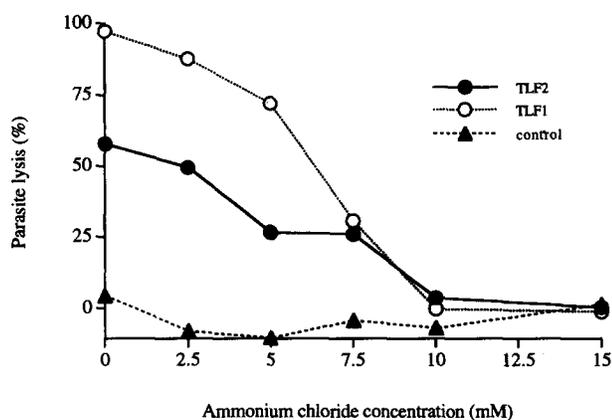

Figure 6. Effect of lysosomotrophic agents on TLF activity. Trypanosomes *T. b. brucei* were preincubated with NH₄Cl (2.5–15 mM) followed by the addition of TLF1, TLF2, or TBS. Trypanosome lysis was then determined.

ited by preincubation of the trypanosomes with either NH₄Cl (Fig. 6) or chloroquine (not shown).

Discussion

The main finding of this paper is that TLF2, and not the better characterized minor HDL subclass, TLF1, is the principal trypanolytic factor in NHS. Even though, as first shown by Rifkin (3), purified HDL (TLF1) particles are strongly trypanolytic, their activity is inhibited by Hp, a major component of normal serum. This conclusion is supported by several findings. (*a*) Purified TLF1 activity is inhibited by concentrations of Hp below those found in NHS (Fig. 1), whereas trypanolytic activity of TLF2 is not affected (Fig. 4). (*b*) Addition of very high concentrations of Hp to NHS does not inhibit trypanolytic activity (Fig. 3). (*c*) Pathological sera depleted of Hp contain only TLF1, and their trypanolytic activity is totally inhibited by low concentrations of Hp (Fig. 3). (*d*) When NHS containing allotypes Hp 1-1, 2-1, and 2-2 are fractionated gel filtration, substantial activity is recovered in the TLF2 fractions. The sole NHS displaying TLF1 activity after fractionation contains the dimeric Hp1-1, which elutes in fractions later than TLF1 (Fig. 4 *a*). In the other sera, TLF1 is inhibited by the coeluting polymeric Hp 2-1 and 2-2 (Fig. 4, *b* and *c*). To confirm this point, TLF1 trypanolytic activity was recovered after removal of Hp (Fig. 5).

These findings provide the explanation for the large gain in trypanolytic activity when lipoprotein fractions are isolated from NHS (5). Flotation of lipoproteins by density gradient ultracentrifugation, a procedure commonly used to purify TLF1, effectively separates HDL from serum Hp. TLF2 can be recovered in the infranate (Fig. 5), but it loses its activity at 4°C within 72 h of isolation; this loss is accelerated by prolonged exposure to the high concentrations of KBr used during lipoprotein separation procedures.

The mechanism by which Hp inhibits TLF1 is not known. It seems unlikely that Hp displaces Hb from Hpr present in TLF1, since the inhibitory activity of Hp–Hb complexes (which form one of the strongest noncovalent interactions known) was as effective as that of Hp alone. Neither free Hb, which at physiological pH stimulates the peroxidation of lipids, nor isolated Hp–Hb complexes, in which the lipid peroxidation is neutralized (24), have trypanolytic activity. However, at acidic pH, the Hp–Hb complex has a high peroxidase activity in the presence of H₂O₂, whereas free Hb is neutralized. Although the predicted amino acid sequence of Hpr (10) should allow Hb binding, there is no biochemical evidence for this complex, or that it contains peroxidase activity (25).

An alternative explanation for the inhibitory effects of Hp, is that Hpr is the TLF1 ligand for trypanosome receptors, and that serum Hp competes with Hpr for these receptors. Consistent with this idea, polyclonal antibodies to Hp (which also recognize Hpr) prevent trypanosome lysis by purified TLF1 (9). Since TLF2 is not inhibited by Hp, the trypanosome receptors for TLF2 and TLF1 may be distinct. Our results indicate that both lytic factors need to en-

ter an intracellular acidic vesicle in order to exert their cytolytic effect, as TLF1 and TLF2 are equally well inhibited by the weak base NH$_4$Cl (Fig. 6) and chloroquine (not shown).

We have noted that TLF2 is either absent or much diminished in some of the highly lytic sera from PNH patients. The reason for this deficit is unknown. Further studies with additional sera are needed to verify whether Hp deficiencies are always associated with TLF2 deficiencies. Of note, Hp deficiencies are frequently observed in Africa and other areas in which trypanosomiasis and malaria are endemic (26), and Hpr gene amplification has been documented in American Blacks (27). Clarification of the issues raised by these observations requires elucidation of the

structure of TLF2 and whether it is a single molecule or a complex sharing components, such as Hpr, with TLF1. Our finding that Hp has such a profound effect on trypanolysis in NHS also raises the possibility that Hp or structurally related molecules modulate the destruction of *T. b. brucei* by sera from some game animals in Africa. Whereas we find no correlation between human Hp types and trypanolytic activity, it is intriguing that Hp from most nonhuman species resembles the dimeric human Hp 1-1, whereas Hp from sera of many African game animals (Artiodactyla) is polymeric, similar to human Hp 2-1 and 2-2 (11). Current efforts in our laboratory are directed at answering some of these questions.

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