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Identification of the trypanocidal factor in normal human serum: High density lipoprotein

(*Trypanosoma brucei*/*Trypanosoma rhodesiense*/Tangier disease)

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ABSTRACT **The differentiation of *Trypanosoma brucei* from *T. rhodesiense*, the causative agent of human sleeping sickness, depends on their relative sensitivities to the cytotoxic effects of normal human serum. The molecule responsible for the specific lysis of *T. brucei* has now been isolated. Serum lipoproteins were fractionated and purified by ultracentrifugal flotation and chromatography on Bio-Gel A-5m. Trypanocidal activity was recovered in the high density lipoprotein fraction (density, 1.063–1.216 g/ml). Contamination by other serum proteins was checked by crossed immunoelectrophoresis and sodium dodecyl sulfate/acrylamide gel electrophoresis. Only a trace of β-lipoprotein was found. The trypanocidal activity of pure human high density lipoprotein was identical to that of unfractionated serum when the following were tested: (*i*) time course of *in vitro* lysis of *T. brucei*; (*ii*) *in vivo* destruction of *T. brucei*; (*iii*) relative resistance of *T. rhodesiense* to lysis. Rat or rabbit high density lipoprotein had no trypanocidal activity. Identification of the trypanocidal factor as high density lipoprotein was confirmed by the finding that serum from patients with Tangier disease, an autosomal recessive disorder characterized by a severe deficiency of high density lipoprotein, had no trypanocidal activity.**

The toxic effect of normal human serum on *Trypanosoma brucei* was first described in 1902 (1). Since that time, many reports (2–4) have appeared on the selective lytic properties of human serum on the morphologically indistinguishable trypanosomes of the *brucei* subgroup. From these studies it became apparent that, whereas *T. rhodesiense* and *T. gambiense*, the causative agents of African sleeping sickness in humans, were relatively resistant to the cytotoxic action of normal human serum, *T. brucei*, which is noninfective to man, was killed by human serum. Identification of the trypanocidal serum factor might therefore help to elucidate the cellular basis for the host range specificity of these trypanosomes.

Previous work (unpublished) on the characterization of the trypanocidal serum factor indicated that activity was associated with a large protein having a molecular weight of ~500,000 as estimated by gel filtration. The elution profile of the active factor from a Sepharose 6B column overlapped but did not coincide with the elution of α-lipoprotein (α-lp). Because serum α-lp comprises a heterogeneous class of molecules, it seemed possible that the active factor might be associated with a subclass of α-lp. Also, the report (5) that Cohn fraction IV-1, which contains α-lp, possessed trypanocidal properties is consistent with the idea that serum lipoproteins might be involved in the trypanocidal reaction. For these reasons a more detailed investigation into the role of lipoproteins in the serum trypanocidal reaction was undertaken.

The findings reported here indicate that trypanocidal activity can be recovered in a purified high density lipoprotein (HDL) fraction. The trypanocidal properties of human HDL are identical to those of unfractionated serum. Identification of the trypanocidal factor in normal human serum as HDL was confirmed by the demonstration that sera from patients with Tangier disease, an autosomal recessive disorder characterized by a severe deficiency of HDL (6), had no *in vivo* or *in vitro* trypanocidal activity against *T. brucei*.

MATERIALS AND METHODS

Mice. Male NCS mice, weighing 20–25 g, were obtained from the colony maintained at The Rockefeller University. Mice were infected by intraperitoneal injection of trypanosomes from a freshly thawed capillary stabilate (one capillary per mouse). Each capillary contained 5–10 × 10^5 trypanosomes.

Trypanosomes. *T. brucei.* A clone prepared from strain EATRO 110 (7) was used. Large numbers of capillary stabilates were prepared from the fourth and fifth mouse passages since cloning and stored at −70°. Details of the cloning procedure and the preparation of capillary stabilates will be described elsewhere.

T. rhodesiense. Capillary stabilates of strain EATRO 1895 (8) were prepared from the sixth mouse passage since isolation from a naturally infected male patient.

Trypanosomes were harvested from mice 72 hr after infection, purified by passage through DEAE-cellulose columns, washed, resuspended in Eagle's minimal essential medium (Grand Island Biological Co.) at 1 × 10^8 trypanosomes per ml.

Sera. Normal human blood was obtained by venipuncture from a healthy human volunteer (M.R.R.) after overnight fasting. Serum was prepared and passed through a sterile 0.45-μm Millipore filter. Rabbit serum was prepared from normal rabbit blood obtained by cardiac puncture of a white New Zealand rabbit. Rat serum was prepared from rat blood obtained by exsanguinating Sprague–Dawley rats (Charles River).

For routine trypanocidal assays, small aliquots of sera were frozen and stored at −70°. For lipoprotein fractionation, sera were processed as quickly as possible (always within 24 hr from the time of bleeding) without freezing. When unfractionated serum was to be compared to its derived lipoprotein fractions in trypanocidal assays, it was stored sterilely at 4° for the same length of time as was necessary to prepare the lipoprotein

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Abbreviations: α-lp, α-lipoprotein; β-lp, β-lipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein. The terms α-lp and β-lp will be used to designate lipoproteins showing immunological crossreactivity with rabbit anti-human α-lp or anti-human β-lp antiserum; VLDL, LDL, and HDL will be used when lipoproteins have been fractionated according to density or elution position from Bio-Gel columns. T_{50} unit, unit of trypanocidal activity (see text).

fractions. In all dialysis steps, unfractionated serum was dialyzed in the same flask as the various lipoprotein fractions.

Sera from two patients homozygous for Tangier disease were kindly provided by H. B. Brewer, Jr. (National Institutes of Health). These sera arrived frozen and were stored at −70° until needed.

Trypanocidal Assays. Standard *in vitro* incubation conditions were 37° for 2 hr at a cell concentration of 1–2 × 10^7 trypanosomes per ml in Eagle's minimal essential medium containing 25% (vol/vol) serum or serum fractions. Control tubes contained 25% (vol/vol) normal rat or rabbit serum or serum fractions. The percentage cell lysis was determined by counting the number of cell ghosts in the total cell population by phase-contrast microscopy. At least 200 cells were counted in each sample. A unit of trypanocidal activity (T_{50} unit) was defined as the amount of serum or serum fractions necessary to produce 50% lysis of the trypanosomes in 1 ml under standard incubation conditions.

In vivo trypanocidal assays were performed as described by Hawking (9). This consisted of injecting mice with known numbers of trypanosomes simultaneously with different amounts of human serum and then determining the length of the prepatent period. A second method for assessing *in vivo* trypanocidal activity was to inject different amounts of serum and serum fractions intravenously into infected mice and then estimating the number of parasites circulating in the blood by wet mount examination of drops of tail blood (10).

Serum Lipoprotein Fractionation. Sequential preparation of very low density lipoproteins (VLDL), low density lipoproteins (LDL), and HDL by ultracentrifugal flotation (11) was carried out using a Spinco SW 40 rotor run at 35,000 or 40,000 rpm at 18°. Lipoprotein fractions were collected by a tube-slicing technique, and densities of the resultant fractions were determined by pycnometry. Lipoproteins were further purified by chromatography on Bio-Gel A-5m (Bio-Rad Laboratories) (12). All fractions were dialyzed extensively against 3 mM phosphate-buffered saline, pH 7.4, containing 0.01 M glucose and 1 mM EDTA and concentrated, if necessary, by ultrafiltration through a PM-10 or PM-30 membrane (Amicon Corp.) before testing for trypanocidal activity.

Immunoelectrophoresis. Crossed immunoelectrophoresis (13) was performed in 1% agarose (Marine Colloids, Inc.) dissolved in 0.087 M barbital buffer, pH 8.7, containing 1.7 mM calcium lactate. Antisera were incorporated into the agarose for the second dimension at a final concentration of 2% (vol/vol). Electrophoresis in the first dimension was carried out at 4° at 5 V/cm for *ca* 2 hr and in the second dimension at 4° at 1–2 V/cm for 18–20 hr. Lipoproteins were visualized by staining the washed and dried plates with Sudan black [0.1% (wt/vol) in 60% (vol/vol) ethanol] at room temperature for 2 hr. To check for the presence of other serum proteins, the plates were subsequently stained with Coomassie blue.

Rocket immunoelectrophoresis was used to quantitate the amount of α-lp or β-lp in human serum and serum fractions. Standard curves were prepared with a β-lp standard (Behring Diagnostics) (for β-lp) and setting the amount of α-lp in unfractionated serum at an arbitrary value of 100 units/ml (for α-lp).

Rabbit antisera against whole human serum and human α-lp and β-lp were purchased from Behring Diagnostics.

Sodium Dodecyl Sulfate/Acrylamide Gel Electrophoresis. Lipoprotein fractions were analyzed without prior delipidation in 10% slab gels by the method of Neville (14). Gels were stained with Coomassie blue.

Protein Assay. Protein was determined by the method of Lowry *et al.* (15) with bovine plasma albumin as a standard.

RESULTS

Fractionation of Serum Lipoproteins. The three major lipoprotein classes of human serum were separated by sequential ultracentrifugal flotation runs at increasing densities. The top lipoprotein fractions, as well as the infranatant lipoprotein-free sera, were tested for trypanocidal activity against *T. brucei* (Table 1). It is evident that trypanocidal activity was recovered in all fractions containing HDL—i.e., VLDL-free serum, LDL-free serum, and, in particular, the 1.067–1.216 g/ml fraction. That the trypanocidal activity was not an artifact due to an effect of concentrated lipoprotein on the parasite is shown by the lack of trypanocidal activity in an equivalent HDL fraction prepared from rat serum. Unfractionated rat serum is not cytotoxic for *T. brucei*.

The α-lp content of the human HDL fraction was 86.1 units of α-lp per mg of protein and that of unfractionated serum was 1.06 units/mg of protein, indicating an 8.7-fold enrichment on a mg of protein basis over whole serum; however, significant contamination by β-lp, albumin, and other serum proteins was found. Therefore, lipoproteins were further purified by Bio-Gel A-5m chromatography (Table 2). Again, only the HDL fraction had trypanocidal activity. Although more than 70% of the serum α-lp was recovered in this fraction, recovery of trypanocidal activity was roughly 10%. It was not possible to increase the trypanocidal activity of this HDL fraction by adding VLDL, LDL, or lipoprotein-free serum to it. This suggests that the low recovery of trypanocidal activity was due to destruction, during the purification procedure, of the biological activity associated with HDL rather than to loss of another serum component that is necessary for optimal trypanocidal activity. It has been reported (16) that the high-speed and high-salt centrifugation conditions required in the initial purification step can lead to partially delipidated forms of HDL. The delipidated HDL still crossreacts with anti-α-lp serum. Thus, recovery of α-lp as estimated by immunological procedures need not necessarily correspond to recovery of biological activity, if biological activity requires HDL in its native state.

Purity of the Active HDL Fraction. Rabbit anti-human α-lp and β-lp antisera were used to quantitate the concentration of these proteins in the HDL fraction by rocket immunoelectrophoresis. Slight contamination of the HDL fraction by β-lp was found (Table 2). Because anti-β-lp serum adsorbed with LDL

Table 1. Trypanocidal activity of serum and serum fractions prepared by ultracentrifugal flotation

Fraction	% lysis	
	Human	Rat
Whole serum	65	4
1.0063 g/ml top (VLDL)	4	—
1.0063 g/ml bottom (VLDL-free serum)	70	—
1.067 g/ml top (LDL)	2	1
1.067 g/ml bottom (LDL-free serum)	50	3
1.216 g/ml top (HDL)	69	5
1.216 g/ml bottom (lipoprotein-free serum)	4	2

Human VLDL were isolated after centrifugation at 40,000 rpm for 18 hr at a density of 1.0063 g/ml. Part of the bottom fraction was saved for testing for trypanocidal activity; the rest was adjusted to a density of 1.067 g/ml and centrifuged at 40,000 rpm for 18 hr. Rat serum was immediately adjusted to 1.067 g/ml; therefore, the rat 1.067 g/ml top fraction contained both VLDL and LDL. Part of the 1.067 g/ml bottom fraction was adjusted to 1.216 g/ml for HDL isolation by centrifugation at 35,000 rpm for 44 hr. All top fractions were concentrated 4-fold with respect to the original serum volume and all bottom fractions were adjusted to the original serum volume from which they were derived. All trypanocidal assays were carried out on the same *T. brucei* preparation under standard *in vitro* assay conditions.

Table 2. Composition and trypanocidal activity of human serum and purified lipoprotein fractions

	Protein, mg/ml	β-lp, mg/ml	α-lp, units/ml	Trypanocidal titer, T_{50} units/ml
Serum	71.7	1.95	100	20
VLDL	1.3	1.87	12.5	1
LDL	8.0	31.2	15.0	1
HDL	20.2	0.11	1325	40

Human serum (18.7 ml) was subjected to lipoprotein fractionation by ultracentrifugal flotation followed by Bio-Gel A-5m chromatography, as described by Rudel *et al.* (12). Three column fractions, corresponding to the reported elution positions of VLDL, LDL, and HDL, were obtained. These fractions were dialyzed and concentrated to a volume of approximately 1 ml. The HDL fraction was used for crossed immunoelectrophoresis (Fig. 1), acrylamide gel electrophoresis (Fig. 2), and trypanocidal assays (Figs. 3 and 4).

gave no precipitin reaction with the HDL fraction, we can conclude that the crossreactivity between anti-β-lp serum and the HDL preparation is due to traces of β-lp in the HDL fraction rather than contamination of the anti-β-lp serum by anti-α-lp immunoglobulins.

Fig. 1 shows the crossed immunoelectrophoretic patterns obtained when unfractionated serum (*a* and *b*) and the active HDL fraction (*c* and *d*) were analyzed against anti-whole human serum (*a* and *c*) or anti-α-lp serum (*b* and *d*). The heterogeneity of the serum α-lp is evident. The precipitates in Fig. 1 *b–d* all stained with Sudan black, indicating their lipoprotein content. The antigen sample in Fig. 1*c* was deliberately overloaded in an attempt to demonstrate other serum contaminants in the HDL fraction. None was found.

Sodium dodecyl sulfate/acrylamide gel analysis of the fractions shown in Table 2 is presented in Fig. 2. Again, deliberate overloading of the gel (lane 4) failed to disclose any serum protein contaminants in the HDL fraction; only one band, having a molecular weight of about 22,000, was found. This corresponds closely to the reported molecular weight, 28,000, for Apo-AI, the major apoprotein of human HDL (17).

Trypanocidal Properties of Purified Human HDL. In *in vitro* trypanocidal assays, the purified HDL fraction was indistinguishable from unfractionated serum in its effect on *T. brucei* (Fig. 3). In both cases, actual lysis was preceded by a lag period of about 45 min during which the cells rounded up.

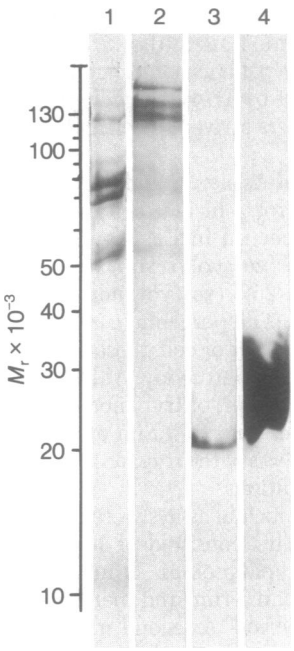

FIG. 2. Sodium dodecyl sulfate/acrylamide gel electrophoresis of the lipoprotein fractions described in Table 2. Samples were as follows: lane 1, VLDL (53 μg of protein); lane 2, LDL (80 μg of protein); lane 3, HDL (20 μg of protein); lane 4, HDL (186 μg of protein).

Neither rabbit serum nor rabbit HDL exhibited any trypanocidal activity.

Purified human HDL was also trypanocidal when tested *in vivo* in *T. brucei*-infected mice (Fig. 4). Intravenous injection of sterile saline into heavily infected mice (5×10^8 trypanosomes per ml of blood at 0 time) had no effect on the rising parasitemia: by 15 hr after injection, trypanosomes were too numerous to count accurately and by 19 hr this control mouse was dead. Injection of whole human serum or purified human HDL, however, resulted in a slow but complete disappearance of trypanosomes from the bloodstream. Little change was noticed in the appearance of the trypanosomes at 6 hr after injection. By 15 hr the trypanosomes were sluggish in their movements, and stained blood smears demonstrated a preponderance of stumpy forms. Twenty-four hours after injection, only occasional trypanosomes were seen and these were always

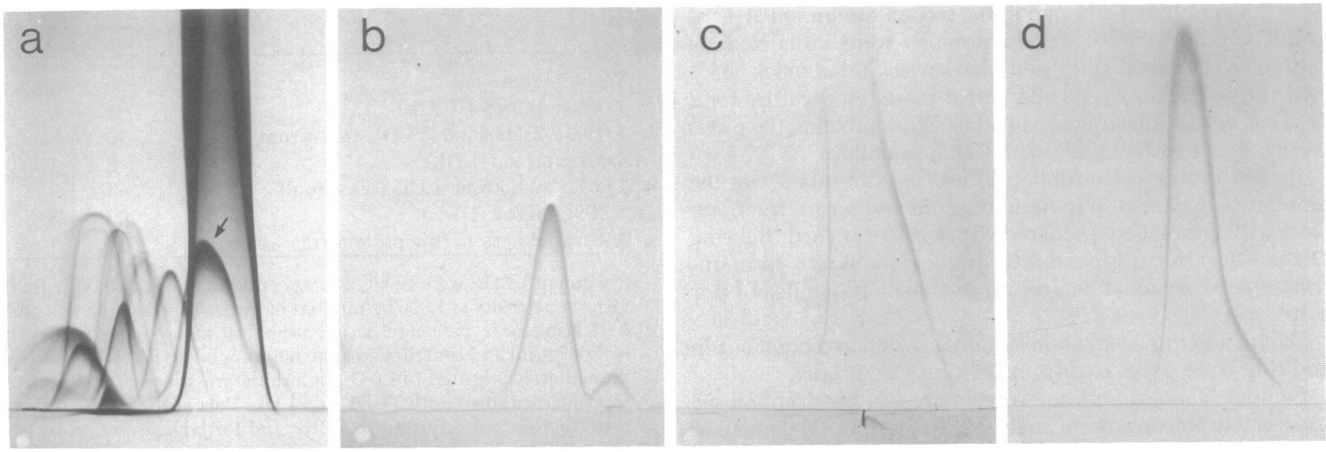

FIG. 1. Crossed immunoelectrophoresis of unfractionated human serum and purified human HDL (Table 2 preparation). (*a*) Antigen, unfractionated human serum (36 μg of protein); antiserum, anti-whole human serum. Arrow indicates α-1p as judged by Sudan black staining of the same plate. (*b*) Antigen, human serum (36 μg of protein); antiserum, anti-human α-lp. (*c*) Antigen, HDL (2.3 μg of protein); antiserum, anti-whole human serum. (*d*) Antigen, HDL (2.3 μg of protein); antiserum, anti-human α-lp. All plates were stained with Coomassie blue.

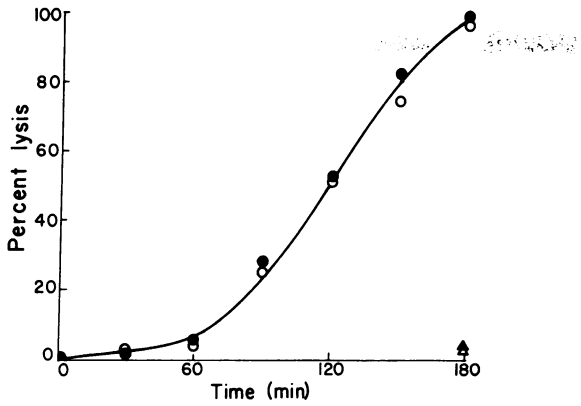

FIG. 3. Time course of lysis of *T. brucei* by unfractionated serum and HDL fractions. Standard *in vitro* trypanocidal assay conditions were used. Incubation tubes contained 25% (vol/vol) human serum (●), 12.5% human HDL (Table 2 fraction) (○), 25% rabbit serum (▲), or 25% rabbit HDL (△).

stumpy forms. By 41 hr the numbers of trypanosomes were below the level of detection (less than 2.5×10^5 trypanosomes per ml of blood). However, 4–5 days after the apparent disappearance of circulating trypanosomes, parasites reappeared and eventually killed the mice. No attempt was made to completely eradicate the infection by repeated injections of serum or HDL.

The relative resistance of *T. rhodesiense* to lysis by human

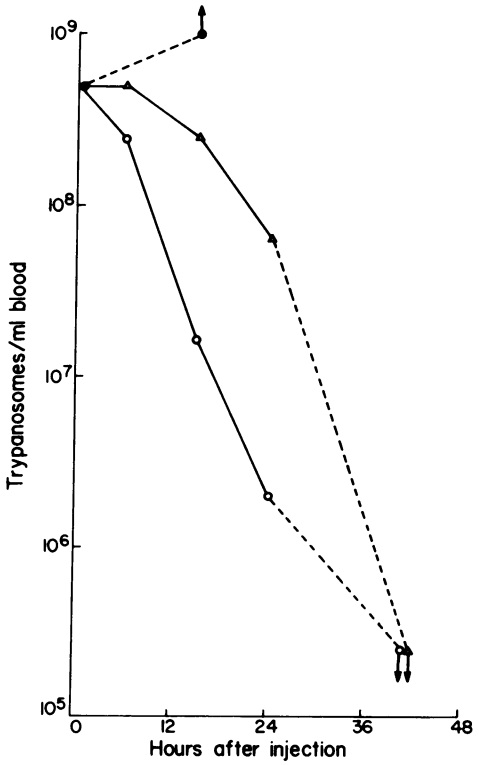

FIG. 4. Effect of intravenous injection of human serum, human HDL, or saline on the bloodstream trypanosomes in infected mice. Six mice were infected 3 days prior to the experiment with 4×10^6 *T. brucei*. Mice were randomly grouped in three groups of two mice each. Each mouse was then injected with 0.2 ml of sterile 0.9% NaCl (●), 0.2 ml of unfractionated human serum (△), or 0.1 ml of human HDL (Table 2 fraction) (○). The parasitemia was followed by wet amount examination of drops of tail blood. Data from only one mouse in each group are plotted; the results were similar between mice within each group.

Table 3. Comparison of susceptibility of *T. brucei* and *T. rhodesiense* to lysis by human serum and human HDL

Organism	% lysis	
	Serum	HDL
T. brucei	86	88
T. rhodesiense	17	23

Standard *in vitro* incubation conditions were used. HDL was prepared by two cycles of ultracentrifugal flotation and was concentrated roughly 9-fold relative to the original serum from which it was prepared. Both unfractionated serum and the HDL fraction were tested at 25% (vol/vol) final concentration.

serum is shown in Table 3. Purified human HDL was similar to whole serum in its differential effects on *T. brucei* and *T. rhodesiense in vitro*. *In vivo* trypanocidal assays, performed according to the method of Hawking (10), indicated that, at equivalent serum and HDL doses, the *T. brucei* preparation contained at most 1 resistant trypanosome in 10^6, while the *T. rhodesiense* showed 10^4–10^5 resistant trypanosomes in 10^6.

Thus, by all parameters available for testing, the trypanocidal activity of purified HDL was identical to that of unfractionated serum.

Trypanocidal Activity of Tangier Disease Serum. Tangier disease is a rare autosomal recessive disorder characterized by a severe lack of HDL. Serum from patients homozygous for Tangier disease has no more than 5% of the normal concentration of HDL (6). Fig. 5 shows that Tangier disease serum is almost completely lacking in trypanocidal activity. In this experiment a tube containing $\frac{1}{10}$th the concentration of human serum (i.e., 2.5% final concentration) showed 18% lysis. Thus, Tangier disease serum contained less than 10% of the trypanocidal activity in normal human serum. When Tangier disease serum was injected intravenously into *T. brucei*-infected mice, no decrease in parasitemia was found and the course of infection resembled that of mice that had been injected with saline. These data confirm the conclusion that trypanocidal activity in normal human serum is associated with HDL.

DISCUSSION

The data presented in this paper indicate that the trypanocidal factor in normal human serum is associated with HDL. All the cytotoxic effects of normal human serum can be duplicated with a preparation of purified human HDL. Moreover, serum deficient in HDL has no trypanocidal activity.

Initial studies (unpublished data) indicated that the absolute

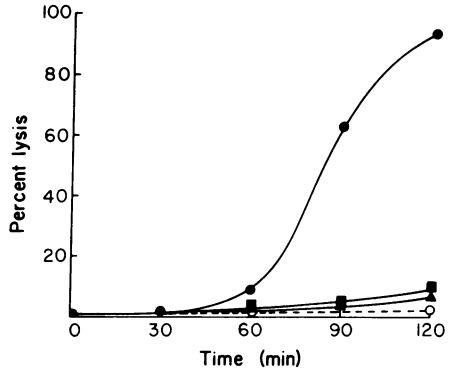

FIG. 5. Comparison of lysis of *T. brucei* by human serum, Tangier disease serum, and rabbit serum. Standard *in vitro* incubation conditions were used; all sera were tested at 25% (vol/vol) final concentration. ●, Normal human serum; ■ and ▲, Tangier disease serum; ○, rabbit serum.

amount of α-lp in serum from a given individual could not be correlated with the trypanocidal titer of that serum. This would appear to contradict the conclusion that α-lp (or HDL) is the trypanocidal substance. However, in other studies (unpublished data) it was found that sera with negligible *in vitro* trypanocidal activity contained an inhibitor of the trypanocidal reaction. Removal of β-lp from relatively inactive human sera increased the trypanocidal titer of those sera, except that with Tangier disease serum removal of β-lp had no effect. Conversely, addition of excess β-lp to active sera decreased the trypanocidal titer. These data suggest that β-lp modulates the cytotoxic effect of human serum. The measured β-lp:α-lp ratio in many human sera could be correlated with the level of trypanocidal activity. However, in those sera in which no correlation was found, it is possible that the lipid moieties of the lipoproteins may also influence the trypanocidal titer. Thus, the trypanocidal titer of a given serum may be determined both by the β-lp:α-lp ratio (or LDL:HDL ratio) and by the lipid content of the lipoproteins, rather than by the absolute concentration of α-lp (or HDL). An antagonistic effect of different lipoprotein classes on each other has also been reported in cultured cells; HDL inhibited the binding and uptake of LDL. (18, 19)

In tissue culture experiments, Stein and Stein (20) found that HDL was capable of removing cholesterol from arterial smooth muscle cells. Although cholesterol removal in these and other cells is probably minimal, excessive cholesterol removal in erythrocytes can lead to increased membrane permeability, osmotic fragility, and eventually lysis (21). HDL apoproteins have also been shown to remove phospholipids from Landschutz ascites cells (22) and, in model systems, to solubilize synthetic multilamellar phospholipid liposomes (23). The finding that lysis of trypanosomes by normal human serum is the result of acute damage to the membrane permeability properties of the parasite (unpublished data) is consistent with the hypothesis that the interaction of human HDL with the surface of *T. brucei* may result in a lethal alteration in the lipid composition of the plasma membrane.

Because the cholesterol to phospholipid ratio in HDL is lower than that in most membranes, it is believed that passive diffusion may account for uptake of cholesterol by HDL. However, the mechanism of adsorption of HDL to cell membranes or of net removal of cholesterol remains unknown. Adsorption of HDL to the trypanosome cell membrane might be affected by the surface coat glycoprotein which is reported to be present in sufficient quantity to form a unimolecular layer over the entire cell (24).

The extent of cell damage might depend on the magnitude of the difference between the cholesterol to phospholipid ratio of HDL and the cholesterol to phospholipid ratio of the plasma membrane of trypanosomes. The cholesterol to phospholipid ratio of HDL from different hosts would then determine the extent of cellular lipid removal among trypanosomes of a given strain. Thus, HDL from a permissive host (rat) would be expected to remove relatively less lipid from *T. brucei* than HDL from a restrictive host (human). Likewise, it is possible that differences in the cholesterol to phospholipid ratio of the plasma membranes of *T. brucei* and *T. rhodesiense* would influence the effect of human HDL on these two species.

Alternatively, the relative resistance of *T. rhodesiense* to lysis by HDL might be due to the enhanced ability of these trypanosomes to repair the membrane lesion caused by HDL. A direct correlation between the resistance of certain tumor cells to lysis by antibody and complement and the ability of the resistant cells to synthesize complex lipids has been demonstrated (25). Complex lipid synthesis may thus serve as a mechanism to repair membrane lesions. A systematic investigation into the relative lipid synthetic capacities of *T. brucei* and *T. rhodesiense* may reveal a basic metabolic difference between these species.

Identification of the particular lipid moieties that may be essential to the ability of human HDL to kill *T. brucei* as well as elucidation of the basis for the resistance of *T. rhodesiense* to lysis by human HDL may further our understanding of the biochemical basis of parasitism by trypanosomes. This could lead to new insights for a rational approach to chemotherapy of trypanosomiasis.

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