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

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Abstract The African trypanosome, *Trypanosoma brucei brucei* causes a fatal wasting disease in livestock but does not ordinarily infect humans, apparently because this unicellular parasite is lysed by high density lipoproteins (HDL) in human serum. To assess whether there is a specific active constituent in trypanolytic HDL, we have systematically compared the cytotoxic action on *T.b.brucei* in vitro of native and delipidated HDL, and of individual apolipoproteins, from nonpermissive hosts (human and baboon) with their counterparts from susceptible hosts (cattle and sheep). When suspensions of trypanosomes were incubated for 2 h at 37°C with human or baboon plasma most cells were lysed, but not with bovine or sheep plasma. Similarly, HDL isolated from human and baboon plasma were trypanolytic (typically about 95% and 60% lysis, respectively, at 1 mg protein/ml), whereas bovine and sheep HDL were benign (< 8% lysis). Subfractionation of human HDL by serial isopycnic ultracentrifugation and by heparin-Sepharose affinity chromatography established that the denser and smaller particles had greater trypanolytic activity both in vitro and in vivo. When human HDL was delipidated, the trypanocidal activity was associated with the water-soluble protein (apolipoprotein) fraction and not with the lipid constituents. Bovine apolipoproteins were also weakly trypanolytic in free solution (20–40% lysis), but not when complexed with cholesterol-phospholipid liposomes (<10% lysis). The major apolipoprotein of human HDL, apolipoprotein (apo) A-I had full trypanolytic activity (89–95% lysis at 1 mg protein/ml) when purified, whether in solution or incorporated into liposomes, but other apolipoproteins isolated from human HDL, including apoA-II, apoC, and apoE, were nontrypanolytic. Purified baboon apoA-I was also trypanolytic, though less potent than human apoA-I, but apoA-I from permissive hosts (cattle and sheep) was inactive when presented in liposomes. Incubation of bovine or sheep HDL with purified human apoA-I, and subsequent separation of the HDL by ultracentrifugation, produced chimeric HDL containing significant amounts of the human apolipoprotein; these particles showed appreciable trypanolytic activity. By contrast, human HDL particles in which about 70% of the apoA-I had been displaced with apoA-II had markedly reduced lytic properties compared to the native HDL (30% versus 80% lysis at 0.6 mg total protein/ml). We tentatively conclude that the trypanolytic activity of native human or baboon plasma resides in the apoA-I content of the HDL particles and that, conversely, bovine and sheep plasma are inactive because the apoA-I polypeptide present in their HDL lacks trypanocidal activity.—**Gillett, M. P. T., and J. S. Owen**. 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.* 1992. **33:** 513–523.

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The cytolytic activity of normal human serum on *Trypanosoma brucei brucei* was first described by Laveran in 1902 (1) and is thought to underlie the human innate immunity to infection by this species of African trypanosome. Like humans, baboons are resistant to infection by *T. b. brucei;* their plasma is also trypanolytic (2–4) whereas that from susceptible animals such as rabbit (4, 5) and cattle (6) is not. The morphologically indistinguishable trypanosomes of the *brucei* subgroup, *T. b. rhodesiense* and *T. b. gambiense*, the causative organisms of acute and chronic human sleeping sickness, respectively, are relatively resistant to the

Abbreviations: apo, apolipoprotein; HDL, high density lipoprotein; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid; LCAT, lecithin:cholesterol acyltransferase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; VLDL, very low density lipoprotein, LDL, low density lipoprotein.

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trypanolytic activity of human serum. These differences have found practical application in the blood incubation infectivity test (7–9) which detects potentially human-infective African trypanosomes on the basis of their survival and infectivity after incubation with normal human blood. Identification of the trypanocidal factor in human serum and its mode of action may help to elucidate the molecular basis for the host range specificity of African trypanosomes; it might also give insight into new chemotherapeutic strategies to combat human and animal trypanosomiasis.

Early reports suggested that the trypanocidal factor of human serum might be IgM (10) or α_2-macroglobulin (2). But their participation in the lytic process was excluded by Rifkin (5), who also demonstrated that complement activation was not involved. In a subsequent study, she reported that the trypanocidal activity was associated with high density lipoprotein (HDL) particles isolated from normal human plasma by ultracentrifugation (11), a conclusion supported by the finding that baboon HDL, but not rat or rabbit HDL, was trypanolytic (3, 4, 11). Other studies are also consistent with HDL as the trypanolytic factor in human plasma: variability in the trypanocidal activity of human plasma is related to the level of HDL (12), plasma depleted of HDL by nonultracentrifugal methods looses its lytic effect (12, 13), and plasma from two patients with Tangier disease (a very rare familial disorder characterized by a severe deficiency of HDL) is reported to lack trypanocidal activity (11).

Plasma HDL are multimolecular complexes, containing a variety of different polypeptides (the apolipoproteins) as well as phospholipids, free and esterified cholesterol, and triacylglycerols, and are heterogeneous in size, density, and metabolic properties (14–16). Whether the trypanolytic activity of HDL is common to all or only certain subclasses or whether it is a property of a particular apolipoprotein or lipid constituent is unclear. In the present study, we report that the trypanocidal activity in vitro of HDL particles isolated from resistant host species (human and baboon) is a property of their apolipoprotein components and that apolipoprotein (apo) A-I is apparently the specific active factor. The HDL particles from the plasma of susceptible hosts (cattle and sheep) were not trypanocidal and their apoA-I, whilst weakly trypanolytic in free solution, was inactive when complexed with lipids. Such benign HDL does, however, become trypanolytic when human apoA-I is incorporated into the particles.

MATERIALS AND METHODS

Trypanosomes

The trypanosomes used in all experiments were derived from the MIAG 427 (MOVS/UG/60/427) (MITat 1.6) clone of *T. b. brucei* provided by Dr. Roger Klein, Molteno Institute, University of Cambridge, U.K. The parasites were obtained as cryopreserved capillary stabilates that were thawed and injected intraperitoneally into irradiated female BALB/c mice. After 72 h, when rising parasitemia was evident, the mice were killed and their blood was collected on ice into sodium heparin (10 IU/ml). The blood was diluted with phosphate-buffered saline (pH 8.0) containing 56 mM D-glucose (17) and dimethylsulfoxide (10% v/v) to give a final concentration of 2×10^7 trypanosomes/ml as determined by phase-contrast microscopy (18). New capillary stabilates, each containing 10^6 trypanosomes, were prepared from this diluted blood and stored in the vapor phase of a liquid N_2 refrigerator; each capillary was subsequently used to infect one mouse.

Blood was withdrawn from mice under terminal anesthesia 3 days after infection when the parasitemia had reached ~5×10^8 trypanosomes/ml of blood. Trypanosomes were isolated from the blood by the method of Lanham and Godfrey (17), washed 3 times with ice-cold phosphate-glucose buffer, and resuspended at 5×10^7 trypanosomes/ml in Dulbecco's modification of Eagle's medium containing 20 mM HEPES buffer (ICN Flow, High Wycombe, Buckinghamshire, U.K.) and supplemented with 1% (w/v) bovine serum albumin (essentially fatty acid-free; Sigma Chemical Company, Poole, Dorset, U.K.) and 56 mM D-glucose; they were kept on ice until used within the next 1 h.

Plasma samples

Human blood samples were drawn by venipuncture from healthy individuals and collected on ice into an anticoagulant/preservative mixture of disodium EDTA, sodium azide, chloramphenicol, gentamycin sulfate, and kallikrein inhibitor (19). Blood samples from adult cross-bred (Hereford × Dairy Shorthorn) cattle and from 6-month-old South Down sheep were obtained from a local abattoir and were treated exactly as described for human blood; they were centrifuged within 2 h of blood collection at 2,500 *g* for 15 min at 4°C to separate plasma. Samples of plasma containing disodium EDTA (1 mg/ml) from four individual olive baboons (*Papio anubis*) were a kind gift from Syntex Research Centre, Heriot-Watt University, Edinburgh, Scotland and were shipped on ice to arrive the same day.

Separation of plasma lipoproteins was begun on the day of blood collection, whilst another aliquot was stored at 4°C overnight before being tested in trypanocidal assays. Additional aliquots of plasma were stored at –70°C for up to 1 year, but when used in subsequent trypanocidal assays they were thawed once only and then discarded.

Preparation of lipoproteins

Very low density lipoproteins (VLDL, density less than 1.006 g/ml), low density lipoproteins (LDL, d 1.019–1.063 g/ml) and whole HDL (d 1.063–1.21 g/ml) were separated from plasma by sequential isopycnic ultracentrifugation and were washed once by recentrifugation at their limiting density before extensive dialysis against 10 mM sodium phosphate buffer, pH 7.4, containing 145 mM NaCl and 0.3 mM disodium EDTA (20). For some experiments HDL subfractions were similarly prepared, including HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.21 g/ml) and also subfractions with nominal densities of 1.063–1.17 g/ml and 1.17–1.26 g/ml as described by Hajduk et al. (21). Whole HDL was also fractionated by heparin-Sepharose affinity chromatography as described previously (22) and the unbound apoA-rich and retarded apoE-rich subfractions were collected. Lipoprotein concentrations are expressed in terms of their protein content as measured by the method of Lowry et al. (23) using bovine serum albumin as standard.

Isolation of apolipoproteins

Delipidated HDL was used either directly in trypanolytic assays or as the starting material for the purification of apolipoproteins. For both purposes, aliquots of freshly isolated human and animal HDL (<10 mg/ml, d 1.063–1.21 g/ml) were extracted with precooled (–20°C) ethanol–diethyl ether 3:2 (v/v) (24) and the precipitated apolipoproteins were washed twice with ice-cold ether and then partially dried under a stream of N_2. For use in trypanocidal assays, the precipitate was dissolved in Tris-buffer containing 2 M guanidium chloride (25) and allowed to renature by dialysis against phosphate-buffered saline. For apolipoprotein purification by ion-exchange chromatography, the precipitated proteins (20–40 mg protein) were dissolved in 10 mM Tris-HCl buffer, pH 7.4, containing 6 M urea, 1 mM disodium EDTA, and 1 mM dithiothreitol (1 ml per 20 mg protein) and applied to a column (2.6 × 50 cm) of DEAE-Sepharose CL-6B (24, 26) pre-equilibrated with the same buffer. Fractions of 3.5 ml were collected at a flow rate of 0.5 ml/min and the column effluent was continually monitored (E_{280}). After collecting 8–10 tubes, elution was switched to a linear NaCl gradient using a two-chambered gradient apparatus containing 0.125 M NaCl in 250 ml of equilibration buffer connected to 250 ml of NaCl-free equilibration buffer (outlet side). Elution was continued overnight and, after collecting a further 130 tubes, those containing fractions of interest were pooled and immediately dialyzed against 10 mM NH_4HCO_3. Each fraction was examined by 17% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (26); those showing a single band only were considered pure apolipoproteins and were lyophilized in aliquots and stored at –70°C. Prior to use apolipoproteins were solubilized as above or were incorporated into liposomes (see below). The apolipoproteins purified included human apoA-I, apoA-II (monomeric form), apoC-I, apoC-II/C-III, baboon apoA-I and apoA-II, and bovine and sheep apoA-I. Specifically for the purification of human apoE, delipidated apoE-rich HDL was dissolved in 25 mM NH_4CO_3 containing 0.1% 2-mercaptoethanol (1 ml per 5 mg protein) and applied to a column (1.0 × 10 cm) of heparin-Sepharose; after extensive washing with 25 mM NH_4CO_3, pure apoE was eluted with 0.75 M NH_4CO_3 (27). However, no attempt was made to purify apoA-IV even though it has properties similar to apoA-I; both, for example, are able to activate lecithin:cholesterol acyltransferase (LCAT). Much of plasma apoA-IV exists in free solution (28), making it an unlikely trypanocidal agent since lipoprotein-free human plasma is nonlytic (12, 13).

Preparation of liposomes

Liposomes were prepared by co-sonication of 40 mg of cholesterol and 80 mg of dipalmitoyl lecithin (both obtained from Sigma Chemical Co.) with 10 ml of phosphate-buffered saline (29). Bovine serum albumin (50 mg) was added and the solution was clarified by centrifugation at 20,000 *g* for 15 min at 20°C; the upper 4 ml was collected. Delipidated HDL or purified, lyophilized apolipoproteins (1 mg protein) were dissolved in 0.5 ml of liposomes by mixing for 2 h at room temperature. The solution was centrifuged at 13,000 *g* for 5 min and was used on the same day in trypanolytic assays.

Preparation of modified HDL particles

Bovine or sheep HDL (1 mg protein) was added to 1 mg of purified human apoA-I and mixed at room temperature for 2 h. The solution was transferred to an ultracentrifuge tube and, after adjusting the density to 1.21 g/ml, the HDL was reisolated by ultracentrifugation as described above; other aliquots of animal HDL without added human apoA-I were treated similarly for control purposes. In other experiments, human HDL_3 (1 mg protein) was incubated with 10 mg of human apoHDL to prepared apoA-II-enriched HDL_3 exactly as described by Vadiveloo and Fidge (30); substantial displacement of apoA-I from the starting HDL_3 by apoA-II was confirmed by SDS-PAGE. The content of human apoA-I in all these modified HDL particles was determined by radial immunodiffusion using commercially available plates and standards (Immuno Ltd., Sevenoaks, Kent, U.K.; no cross-reactivity was noted with bovine or sheep apoA-I).

Assay of trypanocidal activity

In order to assess their trypanolytic activity plasma, HDL, or apolipoproteins (0.1 ml) were incubated with an equal volume of a freshly prepared suspension of *T. b. brucei* (5×10^6 cells). Control incubations contained phosphate-glucose buffer or liposomes alone in place of the test substance, whilst in each set of incubations one tube contained normal human plasma of known trypanocidal efficacy as a positive control. Incubations were carried out in a 37°C water bath without shaking in disposable plastic test tubes (internal diameter = 6 mm). At the end of the incubation period (usually 2 h), 0.2 ml of ice-cold buffered-formalin solution was added to the incubation tube to fix the trypanosome sample. The percentage cell lysis was determined by counting the number of cell ghosts in the total cell population by phase-contrast microscopy at 400 × magnification. At least 200 cells were counted in each sample. Some variation in the extent of lysis was evident, presumably reflecting differences in the particular trypanosome preparation or in the source of plasma, but all experiments were repeated two or more times and were qualitatively reproducible. However, only single representative experiments are presented in the figures.

In some experiments the trypanocidal activity of HDL subfractions was assessed in vivo by two methods. In the first (11, 31, 32), the lipoprotein (up to 0.5 mg protein in 0.2 ml buffer) or buffer alone were injected intraperitoneally into heavily infected (~5×10^8 trypanosomes per ml of blood) mice; blood trypanosome concentrations were then determined at appropriate time intervals after injection and the survival times of the mice were recorded. In the second method (31), normal mice were injected intraperitoneally with trypanosomes (2×10^6 cells) that had been preincubated for 15 min at 37°C with HDL (up to 0.2 mg protein in 0.2 ml) or buffer alone; the appearance of trypanosomes in the blood of these mice was recorded together with their survival time. At least two experiments of each type were performed and representative results are presented.

RESULTS

Lysis of *T. b. brucei* by plasma and HDL

As expected from published data (1–5), both human and baboon plasma caused extensive lysis at a dilution of 1:2 (**Fig. 1A**) which was preceded by the conversion of slender trypanosomes into swollen forms. The extent of trypanolysis induced by human plasma after 120 min incubation (88.0 ± 3.5% for 10 different plasmas) was greater than that induced by baboon plasma (58.6 ± 2.6% for 4 different plasmas). Neither bovine nor sheep plasma had any significant trypanolytic activity. Similar results were observed when the trypanolytic effects of whole HDL prepared from human and animal plasma samples were compared (Fig. 1B); both bovine and sheep HDL were inactive but human and baboon HDL at 1 mg/ml caused a similar degree of trypanolysis as whole plasma. Human VLDL and LDL had negligible trypanolytic activity (data not shown).

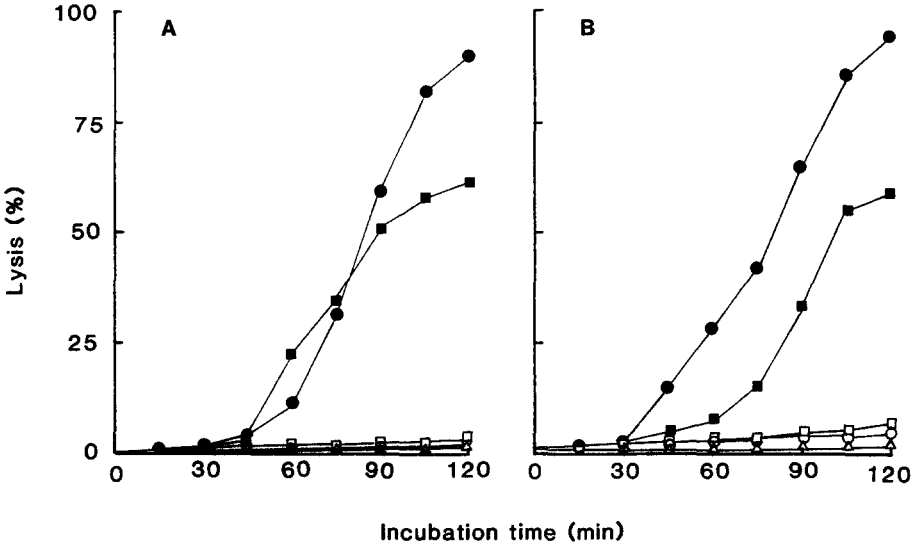

Fig. 1. Lysis of *T. b. brucei* as a function of time in the presence of plasma (A) and isolated HDL (B) from different species. Suspensions of trypanosomes (2.5×10^7/ml final concentration) were incubated at 37°C with plasma samples (final dilution 1:2) or with HDL (1 mg protein/ml final concentration) from human (●), baboon (■), sheep (□), or cattle (○) or with buffer alone (△).

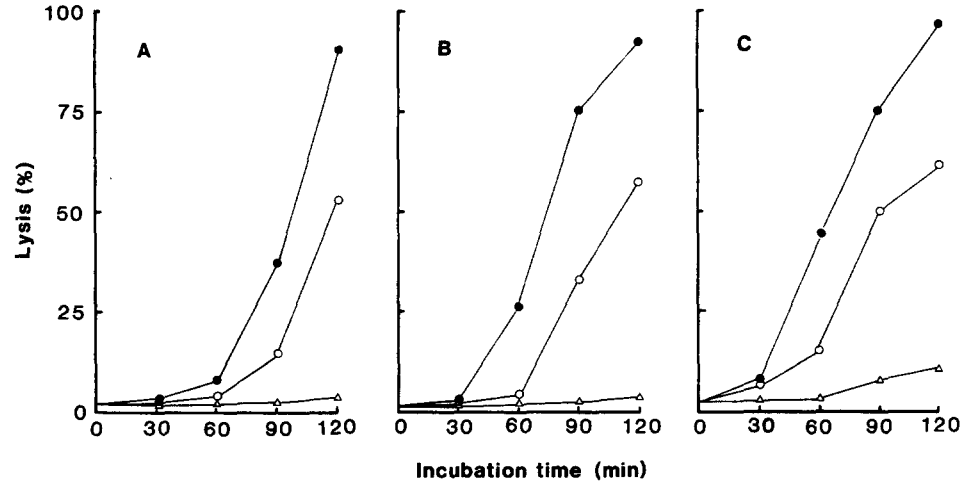

Fig. 2. Lysis of *T. b. brucei* as a function of time in the presence of different subfractions of human HDL. Human plasma HDL was subfractionated: panel A, by conventional isopycnic ultracentrifugation into HDL_2 (○, d 1.063–1.125 g/ml) and HDL_3 (●, d 1.125–1.21 g/ml); panel B, by heparin-Sepharose affinity column chromatography into unbound apoA-enriched (●) and retarded apoE-rich (○) particles; panel C, by the method of Hajduk et al. (21) into fractions of d 1.063–1.17 g/ml (○) and of d 1.17–1.26 g/ml (●). Suspensions of trypanosomes (2.5×10^7/ml final concentration) were incubated at 37°C with each HDL subfraction (1 mg protein/ml final concentration) or with buffer alone (△).

Human HDL was subfractionated by various methods and the trypanolytic activity in vitro of the different subfractions was tested (**Fig. 2**). All HDL subfractions had significant trypanolytic activity, but clear differences were evident in their potency: HDL_3 was more active than HDL_2, the d 1.17–1.26 g/ml subfraction was more trypanolytic than the subfraction of density 1.063–1.17 g/ml, and the large apoE-rich HDL particles bound by the heparin-Sepharose column were less active than the unretained fraction. The HDL subfractions of d 1.063–1.17 g/ml and d 1.17–1.26 g/ml were also tested for trypanocidal activity in vivo. Both fractions showed the ability to clear *T. b. brucei* from the blood of infected mice and both also delayed the onset of patent parasitemia when co-injected with *T. b. brucei* into normal mice; in each case these effects were greater with the d 1.17–1.26 g/ml HDL subfraction (data not shown). This finding was most clearly demonstrated by the increased survival time of mice infected with trypanosomes that had been preincubated with the d 1.17–1.26 g/ml fraction (**Fig. 3A**) and of heavily infected mice that were injected with this denser HDL subfraction (Fig. 3B). The corresponding bovine HDL subfractions were without trypanolytic activity either in vitro or in vivo (data not shown).

Trypanolytic activity of delipidated HDL and of purified apolipoproteins

Delipidated human HDL dissolved in phosphate-glucose buffer appeared to retain the full trypanolytic activity of the native HDL particles, as shown by lysis studies in vitro as a function of time (**Fig. 4A**) and con-

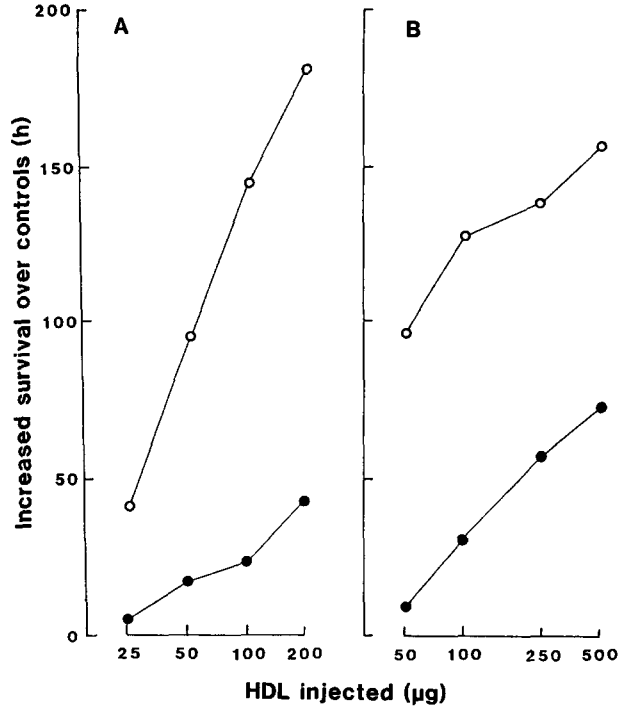

Fig. 3. Ability of human HDL subfractions to increase the survival of mice infected with *T. b. brucei*. Human plasma HDL was subfractionated into light d 1.063–1.17 g/ml (●) and dense d 1.17–1.26 g/ml (○) particles by the method of Hajduk et al. (21). Panel A, trypanosomes (2×10^6 cells) were preincubated for 15 min at 37°C with HDL (25–200 µg protein in 0.2 ml) or with buffer alone (controls). The mixture was then injected intraperitoneally into mice and their survival time was recorded; the mean survival time of control mice was 72 h. Panel B, mice were infected with *T. b. brucei* and when the parasitemia had reached ~5×10^8 trypanosomes per ml of blood, the mice were injected intraperitoneally with HDL (50–500 µg protein in 0.2 ml) or with phosphate-buffered saline alone (controls). Their survival time was recorded; control mice lived for less than 6 h.

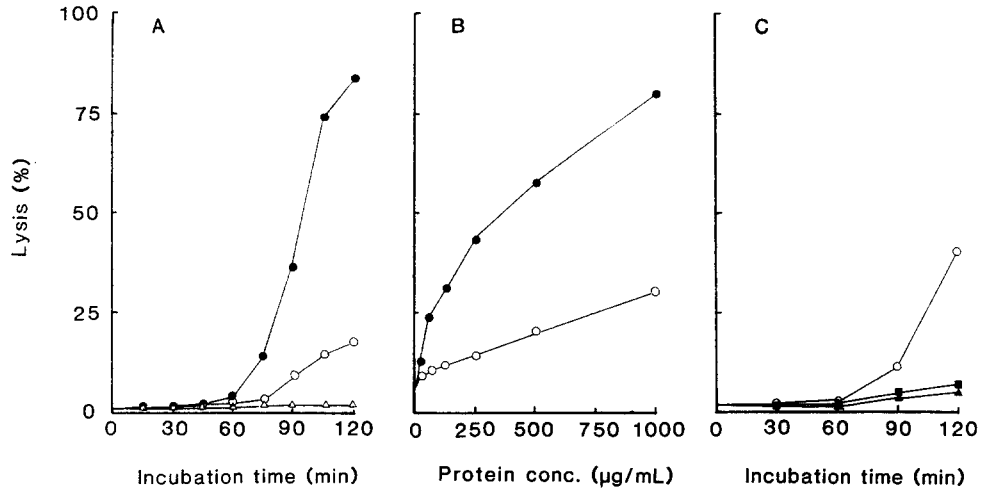

Fig. 4. Lysis of *T. b. brucei* by delipidated human and bovine HDL. Panel A, trypanolysis as a function of time in the presence of delipidated human (●) and bovine (○) HDL (1 mg protein/ml final concentration) or of buffer alone (△). Panel B, trypanolysis after 2 h as a function of the concentration of delipidated human (●) and bovine (○) HDL. Panel C, trypanolysis as a function of time in the presence of delipidated bovine HDL (1 mg protein/ml) in free solution (○) and incorporated into liposomes (●) or of liposomes alone (▲). In all experiments, incubations were at 37°C with 2.5×10^7 trypanosomes/ml (final concentration).

centration (Fig. 4B); similar curves were obtained when this apoHDL was incorporated into cholesterol-dipalmitoyl lecithin liposomes (data not shown). Unexpectedly, a solution of bovine apoHDL showed weak trypanolytic activity (Figs. 4A and 4B), even though the native bovine HDL was inactive, but this was lost when the apolipoproteins were incorporated into liposomes (Fig. 4C).

Purified apolipoproteins from human, baboon, bovine, and sheep HDL were tested for trypanolytic activity both in free solution and when incorporated into liposomes. Trypanolytic activity was associated with apoA-I but not with the other apolipoproteins (**Fig. 5** and **Table 1**). The trypanolytic characteristics of the human and baboon apoA-I appeared comparable to those of the corresponding plasma or HDL (Fig. 1), as indicated by lysis studies both as a function of time (Figs. 5A and 5B) and of concentration (Fig. 5C). However, whilst the apoA-I polypeptides incorporated into cholesterol-phospholipid liposomes (Fig. 5B) had time courses of lysis similar to those of the native HDL (Fig. 1B), their trypanocidal actions were much more rapid when in free solution (Fig. 5A). Bovine and sheep apoA-I showed weak trypanolytic activity in free solution but were inactive as complexes with liposomes. While human apoA-I was consistently trypanolytic, samples from three individuals gave 89, 91, and 95% lysis after 2 h incubation at 1 mg protein/ml,

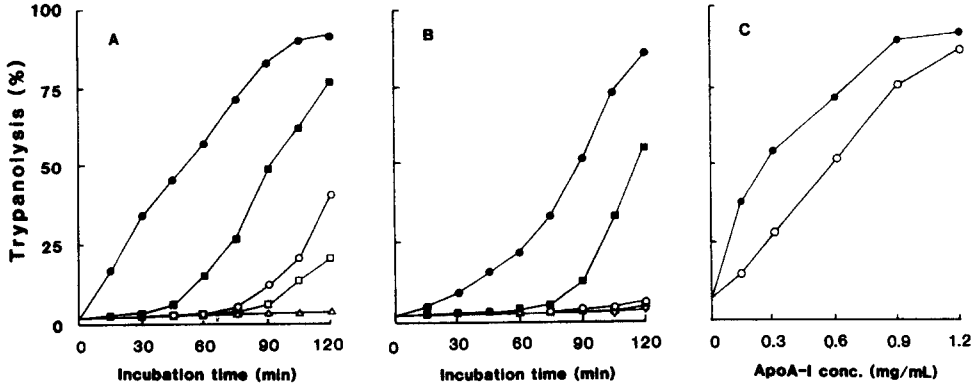

Fig. 5. Lysis of *T. b. brucei* by purified apoA-I from different species. Trypanolysis as a function of time in free solution (A) and incorporated into liposomes (B), or as a function of concentration after 2 h incubation (C). Suspensions of trypanosomes (2.5×10^7/ml final concentration) were incubated at 37°C with apoA-I (1.5 mg/ml final concentration) from human (●), baboon (■), sheep (□), and cattle (○) or with buffer (△) and liposomes (▽) alone (panels A and B) and also with human apoA-I in free solution (●) or incorporated into liposomes (○) (panel C).

TABLE 1. Lytic activity of purified human and animal apolipoproteins incorporated into cholesterol-phospholipid liposomes on *Trypanosome brucei brucei*

Apolipoprotein	Species	Final Concentration	Trypanolysis[a]
		mg protein/ml	%
A-II	Human	1	8.2
A-II	Baboon	1	5.8
A-II	Bovine	1	6.7
C-I	Human	0.5	4.2
C-II/C-III	Human	0.5	3.5
E	Human	0.5	3.0
Liposomes only			2.2

[a]Purified trypanosomes (5×10^6 cells) in 0.1 ml of Dulbecco's modification of Eagle's medium were mixed with 0.1 ml of apolipoprotein-liposome recombinants or with liposomes alone and were incubated for 2 h at 37°C; further details are given under Materials and Methods. The values shown are the mean values of two separate experiments.

the other human apolipoproteins examined, including apoA-II, apoC, and apoE, were all inactive (Table 1) even when they were tested at concentrations several fold higher than their normal plasma levels.

Trypanolytic activity of modified HDL particles

The incorporation of human apoA-I into bovine and sheep HDL particles was estimated to be 32% and 30% of the total HDL apolipoproteins, respectively. As shown by time-course studies of trypanolysis in vitro, such hybrid particles of animal HDL containing human apoA-I had appreciable trypanocidal activity when compared to the control animal HDL samples (**Fig. 6**).

The apoA-I content of human HDL_3 was 83% of its total apolipoproteins and this was reduced to 24%, as judged by radial immunodiffusion, after the enrichment of the particles with apoA-II. This decrease in the apoA-I content of the HDL_3, and its replacement by apoA-II, was confirmed by SDS-PAGE (data not shown). A comparison of the trypanolytic activities in vitro of the two populations of HDL particles is shown in **Fig. 7**. At 300 μg of protein/ml the native HDL_3 showed significant lytic activity whereas the apoA-II-enriched particles were essentially inactive; at 600 μg of protein/ml, however, the apoA-II-enriched HDL_3 did show weak trypanolytic activity.

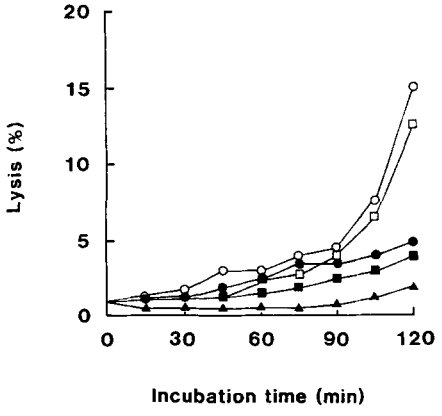

Fig. 6. Lysis of *T. b. brucei* as a function of time in the presence of native and modified animal HDL particles. Suspensions of trypanosomes (2.5×10^7/ml final concentration) were incubated with 1 mg protein/ml (final concentration) of HDL (●, native bovine HDL; ○, bovine HDL containing human apoA-I; ■, native sheep HDL; and □, sheep HDL containing human apoA-I) or with buffer alone (solid triangle).

DISCUSSION

Although the anti-trypanosomal action of human and baboon HDL has been documented by Rifkin (3, 4, 11), there have been few attempts to establish whether a particular subclass of HDL particles or an individual HDL constituent is specifically responsible. In a detailed study, Hajduk et al. (21) separated human HDL between the density limits of 1.17–1.26 g/ml and then used sequential Sepharose CL-4B, hydroxyapatite, and gel filtration chromatography steps to isolate from this a minor subclass of HDL with potent trypanolytic activity in vitro. This particle was reported to be uniquely trypanolytic, since other HDL particles were described as being totally inactive, but neither its presence in baboon HDL nor its absence in nonlytic animal HDL were demonstrated. While the present results confirm that isolated human HDL has trypanolytic activity and that the dense 1.17–1.26 g/ml particles are particularly potent, we also found that appreciable activity was associated with the other HDL subclasses examined. The reason for this difference is unclear, but unlike Hajduk et al. (21) who carried out experiments only in vitro, we confirmed by two different studies in vivo that the lighter fraction with density 1.063–1.17 g/ml had trypanolytic activity. Moreover, our previous report (12) that the trypanolytic activity in vitro of native plasma can be correlated closely with total HDL might argue against an activity confined to <1% of the total HDL particles (21). Conceivably, the relatively complex purification procedure used by Hajduk et al. (21) may have artefactually enhanced the cytotoxic properties of this minor HDL fraction, an effect previously noted by Hawking, Ramsden, and Whytock (2) and by Rifkin (4) during other manipulations of serum or HDL.

When human HDL was delipidated, and initially resolubilized in buffer containing a moderate level of denaturant to dissociate soluble aggregates (25), we found that the trypanolytic activity of the native HDL could be completely recovered with this apoHDL. However, although apoA-I and apoA-II comprise more than 90% of total HDL proteins, a host of other apolipoproteins are also carried by HDL, as are several

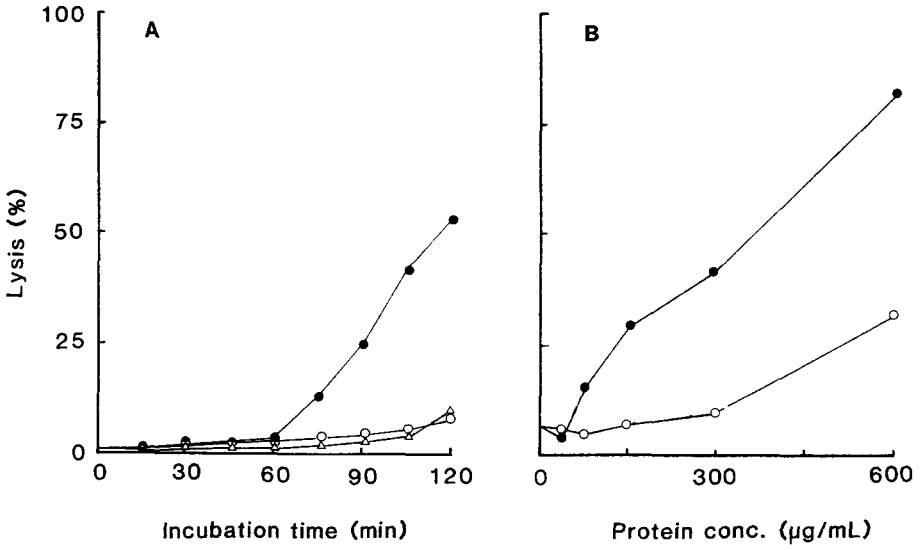

Fig. 7. Lysis of *T. b. brucei* by native and modified human HDL_3. Panel A, trypanolysis as a function of time in the presence of native human HDL_3 (●), of human HDL_3 enriched with human apoA-II (○) and of buffer alone (△). Panel B, trypanolysis after 2 h as a function of the concentration of native (●) or apoA-II enriched (○) HDL_3 particles.

proteins including LCAT, lipid transfer proteins, beta-glycoprotein-I, and the serum AA amyloid protein (14). Nevertheless, our studies with certain purified HDL apolipoproteins were able to implicate apoA-I as the major trypanocidal agent of human HDL; it was the only apolipoprotein examined to have cytotoxic properties and its trypanocidal activity in vitro as a function of time and concentration closely resembled that of apoHDL. ApoA-I is a polypeptide of 243 amino acids (mol wt 28,016) of known sequence, but appears to exist in plasma as a number of nongenetic isoforms whose origins have only recently been investigated (33, 34). It remains to be established whether these individual isoforms of apoA-I have different trypanolytic activities.

This apparent identification of apoA-I as the HDL-associated trypanocidal factor in human plasma clearly requires that the apoA-I of nonlytic plasma should be inactive. But bovine and sheep apoA-I were found to be weakly lytic in free solution and it seems that a certain degree of trypanolysis might occur through a detergent-like action on the trypanosome membrane by these amphipathic polypeptides. However, this non-specific lytic property was lost by complexing the apoA-I (or apoHDL) with cholesterol-phospholipid liposomes; not only were bovine apoA-I/liposomes inert, but the trypanolytic action of human apoA-I more closely resembled that of native HDL when incorporated into liposomes than when in free solution.

Although the use of apolipoprotein/cholesterol-phospholipid complexes appeared to provide clear evidence for apoA-I as the trypanolytic constituent of human HDL, such recombinants have no neutral lipid core and thus differ markedly from native HDL in lipid composition, size, and morphology (30). To circumvent such deficiencies, we manipulated the human apoA-I content of native, spherical HDL particles in two different ways and then measured any change in their trypanolytic properties. Both modifications were consistent with human apoA-I as a trypanolytic agent in native HDL: incorporation of human apoA-I into nonlytic animal HDL conferred trypanolytic activity on the particles, whilst replacement of apoA-I in human HDL_3 with apoA-II was accompanied by loss of lytic activity. These findings are of interest for two reasons. First, because they are consistent with the observed trypanocidal action of the different human HDL subfractions studied; virtually all HDL particles contain apoA-I, and so should have trypanolytic activity. Second, because it seems to us that incubation of lytic HDL_3 with a tenfold excess of lytic apoHDL to produce nonlytic apoA-II-enriched HDL_3, tends to exclude the presence of an active non-apoA-I protein; it would require such a putative trypanolytic factor to be displaced from the initial HDL_3 against its concentration gradient.

We anticipated that additional support, albeit indirect, for the lytic action of human apoA-I might be obtained from a study of purified baboon apolipoproteins; this primate is also reported to be resistant to infection by *T. b. brucei* and to have both plasma (6) and HDL (10, 11, 32) that are particularly effective at lysing this trypanosome in vitro. Unfortunately, although five distinct species of the genus *Papio* are currently recognized (35), these reports did not always record the exact species of baboon used and it is con-

ceivable that each species may have very different trypanocidal activities. Indeed, plasma from *Papio cynocephalus* (yellow baboon) is considered to be relatively ineffective against *T. b. brucei* (and *T. b. rhodesiense*) (36) and, although *Papio papio* (Guinea baboon) is resistant to infection by *T. b rhodesiense*, another species, *Papio hamadryas* (sacred baboon), is fully susceptible (37). Accordingly, we first assayed the trypanocidal activity of plasma and its HDL from the baboon species available to us, *Papio anubis* (olive baboon). Both were shown to have good lytic activity, though less than human plasma and human HDL, but, importantly, purified apoA-I from this species was also found to be trypanolytic. We propose, therefore, that baboons and humans may have a common defence against infection by *T. b. brucei* inasmuch as the apoA-I polypeptides in their HDL appear to possess trypanocidal activity.

It should be noted, however, that the present study was not designed to examine the mechanism by which apoA-I within particles of human or baboon HDL might lyse *T. b. brucei*. Indeed, although exposure of the parasite to human serum is followed by an influx of ions into the cell and lysis by a colloid-osmotic process (38), the initiating event is not understood. Hajduk et al. (21) have proposed that the trypanosomes take up cytotoxic HDL particles by endocytosis and that subsequent intracellular events lead to lysis. Recently, we conducted detailed binding studies of human and bovine HDL by *T. b. brucei* (39), but found negligible uptake or degradation of HDL by the cells. However, we did find that human HDL were bound more avidly than bovine HDL and by a greater number of high-affinity sites (64,000 versus 11,500 per cell). Whether such enhanced binding can be linked to the trypanolytic activity of human HDL merits further investigation, particularly as indirect evidence suggests it may provide an explanation for the different action of lytic and nonlytic apoA-I. Thus, apoA-I is known to be a key mediator of HDL binding by a variety of other cell types (16, 30, 39), an ability presumably dependent on particular amino acid sequences within the polypeptide chain (40–42). This not only suggests that the common cytotoxic effect of human and baboon apoA-I on *T. b. brucei* is due to their close structural similarity, but also that the benign nature of bovine and sheep plasma can be explained by the poor homology of their apoA-I with human apoA-I. A comparison of the primary amino acid sequence of apoA-I from various mammals supports this contention (43, 44). Thus, whilst the apoA-I of human and baboon is highly conserved with 96.4% homology at the cDNA level and 95.1% in the predicted amino acid sequence (45), there is generally poor homology between human and nonprimate apoA-I (42–45), including bovine apoA-I (46); apparently the rate of nucleotide substitution amongst the apolipoprotein genes is considerably higher than the average rate for other mammalian genes (42, 47, 48).

In direct contrast to our findings, Rifkin has recently reported that neither human HDL apolipoproteins (4) nor purified human apoA-I (49) have trypanolytic activity in vitro when tested at 0.6 mg/ml. One explanation for this incongruity is that the strain of trypanosomes used (EATRO 110) was less sensitive to human HDL than ours; unfortunately, no experiments with higher physiological concentrations of apoA-I or apoHDL (about 1.1 and 1.4 mg/ml, respectively) were reported. Alternatively, there may have been differences in renaturation of the apolipoproteins and apoA-I during the solubilization step or, possibly, in oxidation damage or other modifications (33, 34) which might affect biological activity (50). Recognizing such potential problems, Rifkin also carried out trypanolytic assays with sera from two transgenic mice expressing the human apoA-I gene (49). But the lytic activity of this sera did not differ from control sera, even though it contained high levels of undenatured human apoA-I as integral constituents of the mouse HDL (49). However, in a much larger and more detailed study to be reported elsewhere (Owen, J. S., M. P. T. Gillett, and T. E. Hughes, unpublished results), we have found transgenic mouse sera to have significantly, albeit moderately, greater trypanolytic activity in vitro than sera from control mice ($15.1 \pm 1.3\%$ [mean ± SEM] versus $8.5 \pm 1.1\%$, $P < 0.001$) and for it to correlate with the level of human apoA-I in the sera ($r = 0.56$, $n = 40$; $P < 0.001$). This finding not only confirms that human apoA-I has trypanocidal capability but it also effectively excludes another possibility: namely, that the lytic activity of purified apoA-I is due solely to a minor, but highly potent, contaminating protein. Nevertheless, our transgenic mouse sera had considerably less trypanolytic activity than human plasma and we are currently investigating possible reasons for this difference. One explanation is suggested by Rifkin's finding that certain phospholipids have antitrypanocidal properties (4); conceivably the phospholipid constituents of transgenic mouse HDL, unlike those of human HDL, may counteract the trypanocidal action of human apoA-I within the HDL particle. Alternatively, other factors in mouse HDL or in mouse serum may be inhibitory; indeed, it has long been recognized that mouse serum reduces the trypanolytic activity of human serum (2). Clearly, this apparent difference between the action of human apoA-I in human serum and in mouse serum deserves further study, as it can be expected to lead toward a more complete understanding of the trypanolytic process.

In summary, we have attempted to identify a specific active constituent in trypanolytic HDL by carrying out a series of cell lysis assays in vitro in which we have compared the effects of HDL and HDL-apolipoproteins from animals resistant (human and baboon) and susceptible to infection by *T. b. brucei*. Several experimental findings were consistent with our conclusion that HDL particles need to contain molecules of cytolytic apoA-I in order to kill trypanosomes in vitro: trypanolysis by purified human apoA-I as a function of time and concentration was similar to that by apoHDL and by whole HDL; other purified apolipoproteins were inactive; baboon apoA-I was also lytic, whereas the apoA-I of two susceptible hosts was inert; human HDL largely depleted of apoA-I lost much of its lytic activity, whilst incorporation of human apoA-I into inactive animal HDL conferred trypanolytic properties on the particles. Nevertheless, the modest lytic activity of sera from transgenic mice expressing human apoA-I, the report of any unusually toxic HDL subclass (21), and the anti-trypanolytic properties of anionic HDL phospholipids (4) all emphasize that additional factors contribute to the overall specificity and potency of native HDL particles; their relationship to the trypanocidal capability of certain apoA-I polypeptides remains to be defined.

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