

# Human High Density Lipoproteins Are Platforms for the Assembly of Multi-component Innate Immune Complexes\*

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Human innate immunity to non-pathogenic species of African trypanosomes is provided by human high density lipoprotein (HDL) particles. Here we show that native human HDLs containing haptoglobin-related protein (Hpr), apolipoprotein L-I (apoL-I) and apolipoprotein A-I (apoA-I) are the principle antimicrobial molecules providing protection from trypanosome infection. Other HDL subclasses containing either apoA-I and apoL-I or apoA-I and Hpr have reduced trypanolytic activity, whereas HDL subclasses lacking apoL-I and Hpr are non-toxic to trypanosomes. Highly purified, lipid-free Hpr and apoL-I were both toxic to *Trypanosoma brucei brucei* but with specific activities at least 500-fold less than those of native HDLs, suggesting that association of these apolipoproteins within the HDL particle was necessary for optimal cytotoxicity. These studies show that HDLs can serve as platforms for the assembly of multiple synergistic proteins and that these assemblies may play a critical role in the evolution of primate-specific innate immunity to trypanosome infection.

High density lipoprotein (HDL)<sup>2</sup> has several well established physiological activities. The best understood of these is the ability of HDL to protect against atherosclerotic cardiovascular disease by promoting the efflux of excess cholesterol from peripheral tissues and its subsequent transport to the liver for excretion. In addition, apolipoproteins and enzymes carried by HDL have antioxidant activities that inhibit the oxidative modification of low density lipoprotein (LDL), thereby reducing the atherogenicity of these lipoproteins. A less well known activity of HDL is an antimicrobial property that protects some primates from infection by certain eukaryotic pathogens. In these primate species, HDL apolipoproteins have been proposed to have novel innate immune protective functions that provide selective protection against infection.

One such innate immune activity in the serum of humans, apes, and old world monkeys limits the host range of *Trypanosoma brucei brucei* to non-primate mammals (1). This activity fractionates with a minor subclass of human HDL termed trypanosome lytic factor 1 (TLF-1) that,

like all HDLs, is composed of apolipoproteins, phospholipids, and neutral lipids (2, 3). Trypanosome killing by these cytotoxic HDLs requires high affinity binding to receptors on the trypanosome surface, endocytosis, and lysosomal localization (4–8). Following lysosomal acidification, destabilization of the lysosome membrane is facilitated by either free radical-mediated lipid peroxidation or pore formation (9–11). Two HDL-associated proteins have been implicated in the toxicity of TLF-1, haptoglobin-related protein (Hpr) and apolipoprotein L-I (apoL-I) (12, 13).

Haptoglobin-related protein differs by only 27 amino acids from human haptoglobin, an acute phase serum protein that binds hemoglobin released from red blood cells during trauma or infection and transfers the hemoglobin to liver cells for detoxification (14, 15). However, Hpr does not bind hemoglobin, and its physiological function is unknown (9, 16). The role of Hpr in *T. b. brucei* killing was initially proposed based on its fractionation with TLF-1 and its presence in lipid-deficient human serum fractions that also contain trypanosome killing activity (TLF-2) (12, 17, 18). Additionally, the *Hpr* gene evolved during primate evolution and is present in all primate species with trypanolytic sera. An apparent exception is the chimpanzee, which have the *Hpr* gene but lack trypanolytic sera and are susceptible to infection by *T. b. brucei*. When chimp sera were tested for the presence of Hpr none was detectable, indicating that although chimps retain the *Hpr* gene they lack Hpr in their serum (19).

Another primate-specific protein, apoL-I, has recently been shown to possess cytotoxic activity against *T. b. brucei*. ApoL-I is a member of a multi-gene family on human chromosome 22 consisting of *APOLI-APOLVI* (20–22). The *APOL-I*, *APOL-II*, *APOL-III*, and *APOL-IV* genes form a distinct cluster and may be the result of recent tandem gene duplications. Comparative sequence analysis indicates that the *APOL-V-APOL-VI* cluster is distinct and has a different origin (23). Consistent with this view, the *APOL-I-APOL-IV* cluster is only detected in primates (21–23). ApoL-I was initially isolated from normal human serum as a full-length 42-kDa protein and a 38-kDa truncation product, both of which are found associated with large HDL particles (20, 22, 23). Secondary structure predictions based on cDNA sequences identify four lipid-binding amphipathic  $\alpha$ -helices (20). Based on its proposed structure, apoL-I has been suggested to function as a catalyst for lipid transfer, although this activity has not been demonstrated (20, 22). ApoL-I is expressed in a wide range of tissues, and apoL-I mRNA is most abundant in lung, pancreas, liver, placenta, and spleen (22). Studies with purified apoL-I from human serum and recombinant apoL-I expressed in mammalian cells have shown that apoL-I kills *T. b. brucei* (13). A role for apoL-I in *T. b. brucei* killing was supported both by the lack of apoL-I in the serum of chimpanzees, which are susceptible to *T. b. brucei*, and its presence in the serum of gorillas, which are resistant to *T. b. brucei*. However, other *T. b. brucei*-resistant primates such as the baboon and the sooty mangabey do not contain apoL-I in their serum, although Hpr

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<sup>2</sup> The abbreviations used are: HDL, high density lipoprotein; apoL-I, apolipoprotein L-I; apoA-I, apolipoprotein A-I; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Hpr, haptoglobin-related protein; LDL, low density lipoprotein; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PBSE, phosphate-buffered saline with EDTA; TLF, trypanosome lytic factor.

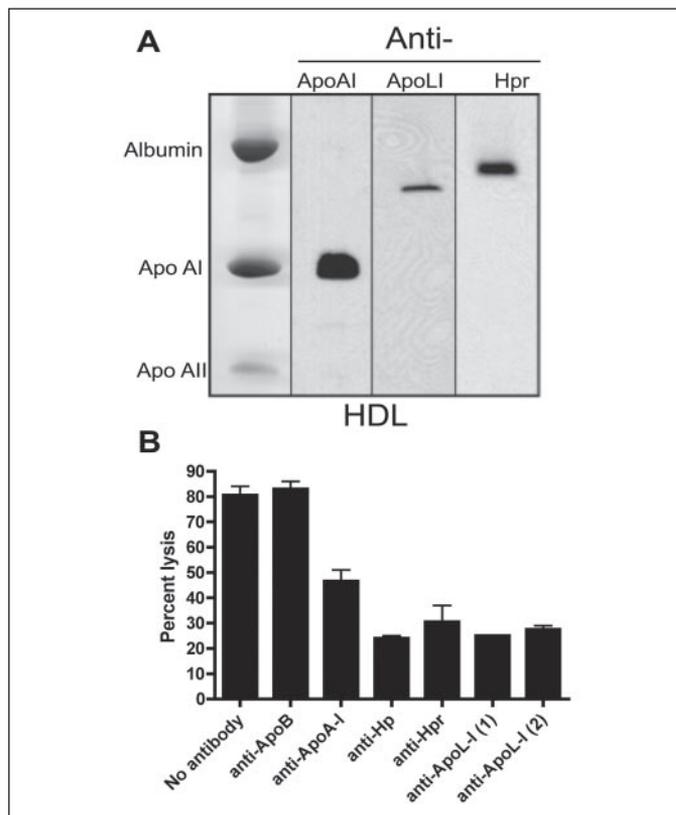
is present (19, 24). These inconsistencies have led us to re-investigate the role of Hpr and apoL-1 in *T. b. brucei* killing.

In the studies reported here we show that Hpr and apoL-1 assemble into the same native human HDL particle, that purified apoL-1 and Hpr are both *T. b. brucei*-specific toxins, and that assembly of these proteins into the same HDL particle resulted in a synergistic increase in trypanolytic activity. Our results suggest that HDL assembly and remodeling play important roles in the formation of multi-component assemblages in the circulation that may accentuate innate immune protection against infectious agents.

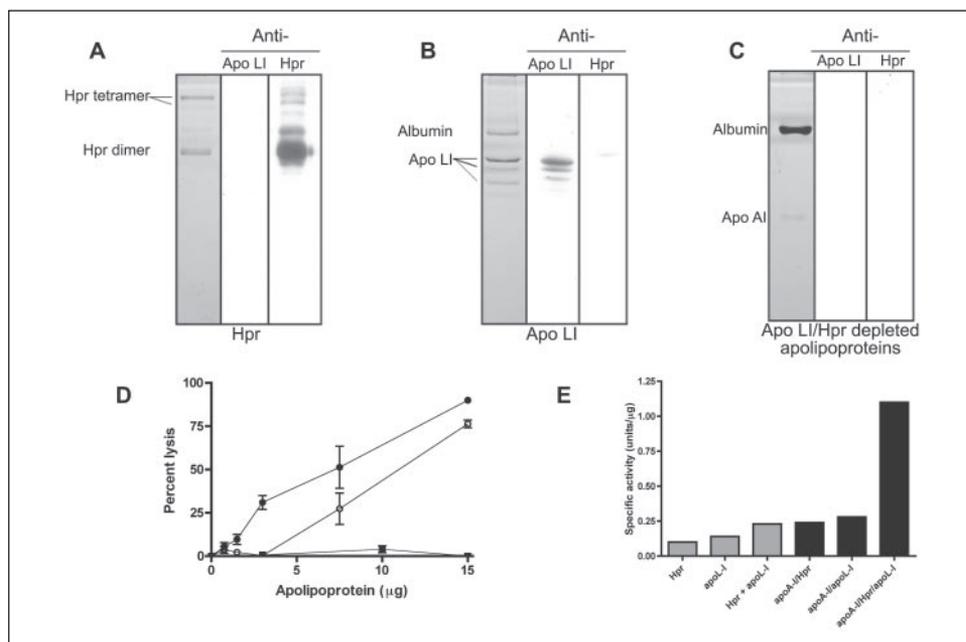
**MATERIALS AND METHODS**

**Purification of Human HDLs and Apolipoproteins**—Human HDLs were purified by differential flotation on sodium bromide gradients as described previously (3). Briefly, normal human blood was obtained from healthy fasted donors, and serum was immediately separated from blood cells by centrifugation at 4 °C for 10 min at 9,500 rpm in a Sorvall SS-34 rotor. The serum was adjusted to 0.5 mM EDTA, and density was adjusted to 1.063 g/ml by the addition of sodium bromide. Very low density lipoprotein (VLDL) and LDL were removed by centrifugation at 18 °C for 23 h at 50,000 rpm in a Beckman Ti70 rotor. HDL was sequentially isolated by adjusting the LDL-deficient serum to 1.26 g/ml followed by centrifugation at 18 °C for 42 h at 50,000 rpm in a Beckman Ti70 rotor. The HDL-containing float was diluted with phosphate-buffered saline containing 3 mM EDTA (PBSE) and dialyzed against PBSE to remove the sodium bromide. These total human HDLs were then fractionated by immunoaffinity adsorption with anti-Hpr or anti-apoL-1. Bound HDL fractions were washed with PBSE, eluted using 100 mM glycine at pH 2.0, and immediately neutralized by the addition of 100 mM Tris (pH 7.5). A fraction of HDL was identified that was deficient in both apoL-1 and Hpr after sequential immunoaffinity selection with anti-Hpr and anti-apoL-1 columns. This unbound fraction of HDL represented >99% of the starting HDL and lacked trypanolytic activity. All HDL fractions were characterized by *in vitro T. b. brucei* lysis assays and Western blots. For purification of apolipoproteins, total human HDLs were first solubilized in PBSE by the addition of either deoxycholate or CHAPS to a final concentration of 10 mM. Individual apolipoproteins were immunoaffinity-purified by anti-Hpr or anti-apoL-1 column chromatography. Purity of apolipoprotein fractions was determined by SDS-PAGE and Western blotting as described (3).

**Antibodies and Affinity Columns**—Antibodies used in these studies were either purchased commercially (anti-apo-B, anti-apoA-I, and anti-haptoglobin from Sigma; anti-apoL-1 (anti-apoL-1 (1) in Fig. 1B) from Santa Cruz Biotechnology, Inc.) or were prepared by



**FIGURE 1. Lysis of *T. b. brucei* by human HDLs is inhibited by anti-apolipoprotein antibodies.** A, apolipoprotein L-1 and Hpr fractionate with human HDL particles. Unlabeled lane, Coomassie-stained sample. Labeled lanes, Western blots probed with monoclonal antibodies to apo-AI (ApoAI), apoL-1 (ApoLI), and Hpr. B, antibody inhibition of lysis of *T. b. brucei* by human HDL. The addition of monoclonal antibodies against apoA-I, apoL-1 (anti-apoL-1 (2)), Hpr, or polyclonal sera against haptoglobin or apoL-1 (anti-apoL-1 (1)) inhibited HDL lysis of *T. b. brucei*, whereas the addition of antibodies against the LDL apolipoprotein B had no effect on lysis.



**FIGURE 2. Lytic activity of purified and reconstituted Hpr and apoL-1.** A, purified Hpr was analyzed by SDS-PAGE, Coomassie staining (far left lane), and Western blot. B, purified apoL-1 was analyzed by SDS-PAGE, Coomassie staining (far left lane), and Western blot. C, unbound apolipoproteins analyzed by SDS-PAGE, Coomassie staining (far left lane), and Western blot were found to be efficiently depleted of Hpr and apoL-1. D, the trypanosome lytic activity of purified apoL-1 (●) was 0.14 units/µg and the lytic activity of Hpr (○) was 0.10 units/µg, whereas HDL apolipoproteins depleted of apoL-1 and Hpr were not lytic to *T. b. brucei* (■). E, specific activities of purified Hpr and apoL-1 and mixtures of the purified apolipoproteins (gray bars). Specific activities of reconstituted Hpr, apoL-1, and both proteins into HDL particles (black bars). Reconstituted HDL subclasses all contained 10 µg of nonlytic human HDL and the following amounts of purified apolipoproteins: 12.5 µg of purified Hpr and 2.5 µg of purified apoL-1 (5:1 ratio) for apoA-I/Hpr/apoL-1; 12.5 µg of Hpr for apoA-I/Hpr; and 2.5 µg of apoL-1 for apoA-I/apoL-1.

TABLE ONE

## Trypanolytic activity of purified Hpr and apoL-I, reconstituted HDL subclasses, and native HDL subclasses

	Protein $\mu\text{g}$	Specific activity $\text{units}/\mu\text{g}$	Recovered activity $\text{units}$	Recovered activity %
Purified apolipoprotein <sup>a</sup>				
Hpr	562	0.10	56	75.7
ApoL-I	130	0.14	18	24.3
Reconstituted HDL subclass <sup>b</sup>				
ApoA-I/Hpr/ApoL-I		1.10		
ApoA-I/Hpr		0.24		
ApoA-I/ApoL-I		0.28		
Native HDL subclass <sup>c</sup>				
ApoA-I/Hpr/ApoL-I	330	83.3 <sup>d</sup>	27,498	99.3 <sup>e</sup>
ApoA-I/Hpr	240	0.5 <sup>d</sup>	115	0.4 <sup>e</sup>
ApoA-I/ApoL-I	116	0.8 <sup>d</sup>	89	0.3 <sup>e</sup>
ApoA-I	2.51e5	0.0 <sup>d</sup>	0	0.0 <sup>e</sup>

<sup>a</sup> Apolipoproteins purified from HDLs as described in the Fig. 2 legend.

<sup>b</sup> Reconstituted HDL subclasses all contained 10  $\mu\text{g}$  of non-lytic human HDL and the following amounts of purified apolipoproteins: 12.5  $\mu\text{g}$  of purified Hpr and 2.5  $\mu\text{g}$  of purified apoL-I (5:1 ratio) for apoA-I/Hpr/apoL-I; 12.5  $\mu\text{g}$  of Hpr for apoA-I/Hpr; and 2.5  $\mu\text{g}$  of apoL-I for apoA-I/ApoL-I.

<sup>c</sup> Apolipoproteins as described in the Fig. 5 legend.

<sup>d</sup> A lytic unit is defined as the amount of protein needed to lyse 50% of the trypanosomes in a standard lysis assay following a 2-h incubation at 37 °C (3).

<sup>e</sup> Recovered activity was calculated from total activity purified from a unit of blood.

hyperimmunization of mice with purified human TLF and the production of hybridoma cell lines expressing monoclonal antibodies against apoL-I (*anti-apoL-I* (2) in Fig. 1B) and Hpr (3). Affi-gel 10 (Bio-Rad) was used as described by the manufacturer to couple purified fractions of monoclonal anti-apoL-I and anti-Hpr to make affinity chromatography columns.

**Trypanosome Lysis Assays**—Trypanosome lysis was evaluated by phase-contrast microscopy following the incubation of *T. b. brucei* clonal cell line II<sup>T</sup> at 1.3 for 2 h at 37 °C in the presence of either purified HDLs or individual apolipoproteins. For antibody inhibition studies, 15  $\mu\text{g}$  of the indicated antibody or sera was included. For assays with individual apolipoproteins, 15  $\mu\text{g}$  of anti-apoL-I was added to Hpr assays, and 15  $\mu\text{g}$  of anti-Hpr was added to apoL-I assays. Each lysis assay contained  $3 \times 10^6$  trypanosomes in 300  $\mu\text{l}$  of assay buffer. Results presented in this paper were from samples assayed in triplicate, and S.E. is indicated. Methods for the purification of trypanosomes, standard lysis assay conditions, and the specificity of this assay for *T. b. brucei* have been described previously (3).

**Mixing and Reconstitution Experiments**—For the mixing of Hpr and apoL-I without reconstitution, 2.5  $\mu\text{g}$  of purified Hpr and 0.75  $\mu\text{g}$  of purified apoL-I were incubated together in the presence of 5  $\mu\text{g}/\text{ml}$  inactive human HDLs for 3 min on ice prior to the addition of trypanosomes and incubation at 37 °C for 2.5 h. For reconstitution, inactive human HDLs at 10  $\mu\text{g}/\text{ml}$  were incubated with 12.5  $\mu\text{g}$  of purified Hpr, 2.5  $\mu\text{g}$  of purified apoL-I or 12.5  $\mu\text{g}$  of Hpr plus 2.5  $\mu\text{g}$  apoL-I (5:1) in the presence of 10 mM sodium deoxycholate. Detergent concentration was reduced to 0.08  $\mu\text{M}$  by dialysis to allow particles to re-form (25, 26). Reconstituted HDL samples (1  $\mu\text{g}$ ) were tested in standard lysis assays as described. Reconstituted particles were characterized by affinity chromatography using anti-Hpr and anti-apoL-I, and the efficiency of apoL-I and Hpr reconstitution into an HDL particle was evaluated by SDS-PAGE and Western blot.

**Radioiodination and Binding of HDL Subclasses**—The different HDL subclasses were purified as described above. Each subclass was radiolabeled with 1 mCi of <sup>125</sup>I using 1,3,4,6-tetrachloro-3 $\alpha$ ,6 $\alpha$ -diphenylglycylcoulil (Iodogen) as the catalyst (Sigma). Labeled HDLs were separated from unincorporated <sup>125</sup>I by filtration on PD-10 columns (Amersham Biosciences). SDS-PAGE gels and autoradiography confirmed incorporation of the label with the apolipoproteins. Labeled HDL was added at

the indicated concentrations to *T. b. brucei*,  $3 \times 10^7/\text{ml}$ , in F12 media containing 5% fetal bovine serum and incubated at 4 °C for 16 h (6). Incubations included a 100-fold excess of unlabeled, nonlytic human HDLs (Hpr- and apoL-I-deficient fraction of the total HDL) to compete for general HDL binding. Cells were washed three times with PBSE, and the amount of bound HDL was measured by scintillation counting.

**MALDI-TOF Mass Spectrometry and Sample Preparation**—Highly purified TLF was prepared by sequential flotation on sodium bromide gradients followed by immunoaffinity purification with anti-Hpr. Samples were separated under non-reducing conditions on 12% SDS-polyacrylamide gels. Gels were stained with Coomassie Blue and destained with 20% isopropanol and 5% methanol, and protein bands were manually excised for MALDI-TOF analysis. Gel pieces were macerated and washed with 50% aqueous acetonitrile to remove SDS, salts, and stain, dried to remove solvent, rehydrated in a solution containing trypsin (12 ng/ $\mu\text{l}$ ; Roche Applied Science), and incubated overnight at 37 °C. Tryptic peptides were recovered, concentrated with a Centricon device (Millipore), and desalted using a Zip-Tip (Millipore), and peptides were eluted with acetonitrile. Samples were spotted onto a gold target plate for MALDI-TOF analysis using a PE-Biosystems Voyager Elite instrument (Framingham, MA). Peptide peaks were submitted for analysis with publicly available “Mascot” software (www.matrixscience.com).

## RESULTS

Previous studies have shown that the majority of trypanosome killing activity in human serum fractionates with HDLs (2, 3). We have presented evidence that a minor subclass of HDL containing Hpr was trypanolytic, whereas others have shown that apoL-I is toxic to *T. b. brucei* (12, 13). These apparently disparate findings were initially investigated by testing the ability of antibodies raised against apoA-I, Hpr, and apoL-I to neutralize trypanosome killing. Antibodies against these apolipoproteins were incubated with total human HDL, and the specificity of each antibody was shown by Western blotting (Fig. 1A). Each of the antibodies showed a characteristic inhibition of trypanosome lysis by human HDLs (Fig. 1B). Even at high antibody concentrations (data not shown) we failed to observe complete inhibition of trypanosome lytic activity using only a single antibody against Hpr or apoL-I. This suggested that multiple toxins might be present in the human HDL preparation. Inhibition of lysis by anti-apoA-I was expected, because

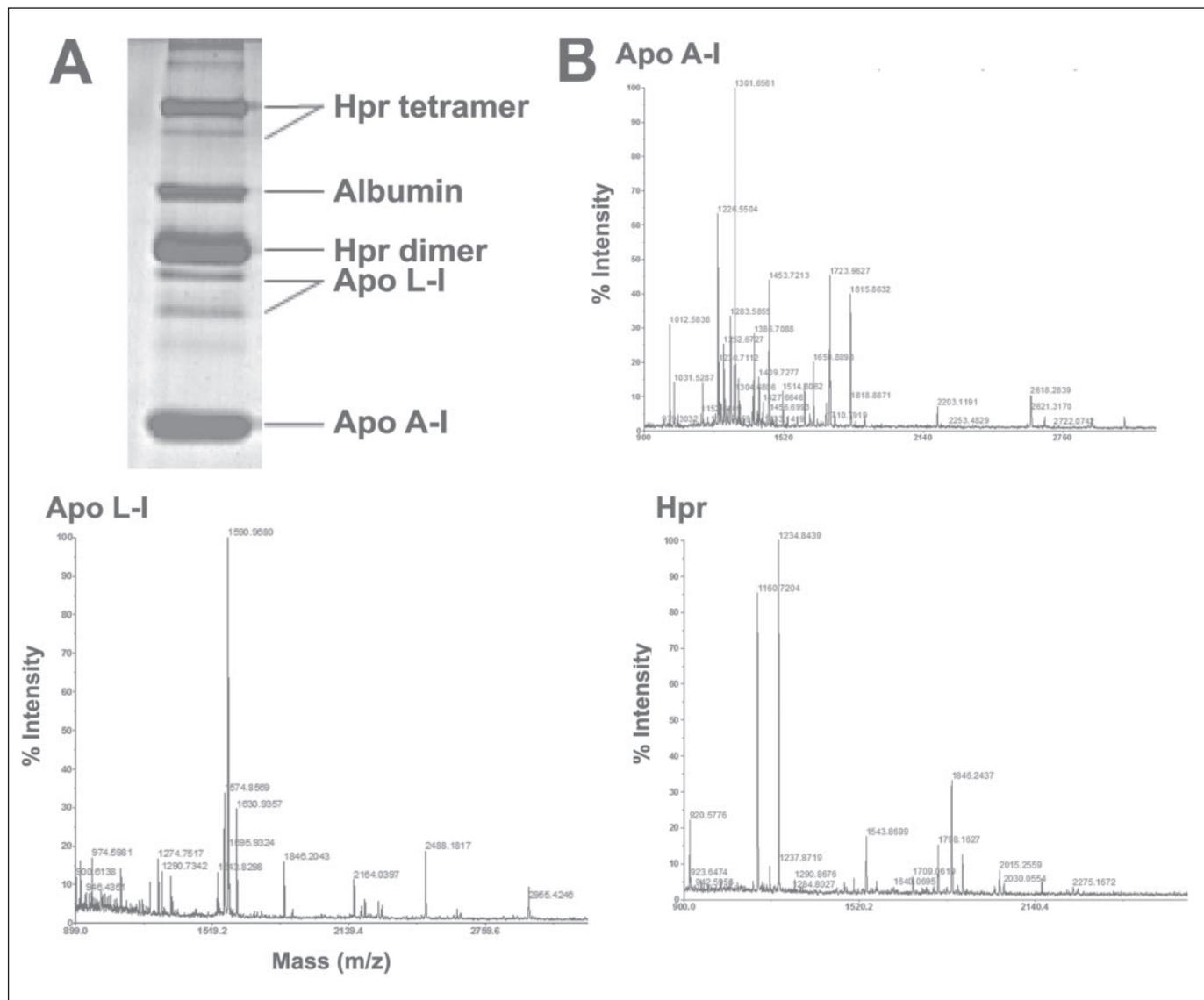


FIGURE 3. MALDI-TOF analysis of apolipoproteins from Hpr-containing human HDL. *A*, Coomassie-stained SDS-polyacrylamide gel of apolipoproteins fractionated from human HDL and purified by immunoaffinity chromatography with anti-Hpr. *B*, MALDI-TOF spectra of apoA-I, Hpr dimer, and apoL-I full-length bands. The tryptic peptides identified for each of these proteins is shown in TABLE TWO.

apoA-I is a ubiquitous component of all HDL particles. However, it is unlikely that apo A-I is the active toxin for trypanosomes in HDLs because previous studies using both purified human apoA-I and recombinant apoA-I have shown that alone it lacks the ability to lyse trypanosomes (26, 27). In addition, >99% of the total human serum apoA-I is associated with HDLs that lack trypanolytic activity (28). Antibodies to proteins absent from lytic HDLs, such as apolipoprotein B, the major protein component of LDL particles, did not inhibit trypanosome lysis (Fig. 1*B*). Based on these results we focused on the potential role of both Hpr and apoL-I as active toxins to *T. b. brucei*.

Because both Hpr and apoL-I have been implicated as toxins to *T. b. brucei*, the relative killing activity of these apolipoproteins was evaluated. We immunoaffinity purified Hpr and apoL-I from detergent-solubilized human HDLs and tested their activity in trypanosome lysis assays (Fig. 2). The purity of the fractions was determined by Coomassie staining and Western blotting (Fig. 2, *A–C*). The Hpr fraction contained no detectable apoL-I, whereas the apoL-I fraction contained a trace amount of Hpr (Fig. 2, *A* and *B*). The apoL-I- and Hpr-depleted fraction contained predominantly apoA-I, apolipoprotein A-II, and albumin by Coomassie and Western blotting (Fig. 2*C*). Purified Hpr and apoL-I

were tested for trypanosome lytic activity (Fig. 2*D*). Trypanosomes incubated with increasing concentrations of purified Hpr or apoL-I were killed in a dose-dependent manner with specific activities of 0.10 units/ $\mu\text{g}$  and 0.14 units/ $\mu\text{g}$ , respectively (TABLE ONE). The apoL-I- and Hpr-depleted fraction, containing all other proteins present in human HDLs, had no lytic activity. These results demonstrate, for the first time, that Hpr and apoL-I are both toxic to *T. b. brucei* and account for all lytic activity observed in human serum HDLs.

Although our analysis of purified Hpr and apoL-I indicated that both are toxins, we observed a significant reduction in lytic activity as compared with native HDL particles. The purified proteins have specific activities  $\sim 600$ – $800$ -fold less than those of native HDLs, indicating that maximal activity may require assembly into an HDL particle or a possible synergistic interaction between the two proteins. We first assayed mixtures of purified Hpr and apoL-I (3:1 molar ratio) in trypanosome lysis assays and found only an additive increase in trypanosome killing (Fig. 2*E* and TABLE ONE). These results suggested that assembly of Hpr and apoL-I into an HDL particle may be required for maximal trypanolytic activity. To test this possibility we used conditions established previously for the reconstitution of HDLs (25, 26).

TABLE TWO

Peptide fragments identified by MALDI-TOF mass spectrometry of trypsin-digested apolipoproteins present in Hpr-containing human HDL

	Predicted mass	Observed Mass	Amino acid sequence <sup>a</sup>	Amino acid numbers
	<i>Da</i>	<i>Da</i>		
Hpr	2014.314	2013.04	<u>FPKPPEIANGYVEHLFR</u>	34–50
	1845.175	1844.04	VVLHPNYHQVDIGLIK	178–193
	1630.966	1499.88	<u>SDLGAVISLLLWGR</u>	2–15
	1543.656	1542.75	<u>VGYVSGWGQSDNEK</u>	220–233
	920.035	920.51	GSFPWQAK	113–120
ApoL-I	2956.348	2955.42	<u>VSTQNLLLLLTDNEAWNGFVAAAELPR</u>	64–90
	2773.051	2487.18	EFLGENISNFLSLAGNTYQLTR	241–262
	1630.729	1630.94	<u>VTEPISAESGEQVER</u>	291–305
	1595.821	1595.93	<u>WWTQAQAHDLVIK</u>	219–231
	1590.762	1590.97	<u>ANLQSVPHASASRPR</u>	276–290
	1349.428	1331.74	NWHDKGGQYQR	113–122
	1274.455	1274.75	<u>VNEPSILEMSR</u>	306–316
	1254.454	1236.75	NWFLKEFPR	123–131
	1105.259	1105.68	LNILNNNYK	367–375
	975.023	974.60	<u>SKLEDNIR</u>	134–141
	918.061	900.61	KALDNLAR	98–105
ApoA-I	2636.855	2618.28	EQLGPVTQEFWDNLEKETEGLR	86–107
	1833.929	1815.86	DSGRDYVSQFEQSALGK	48–64
	1668.864	1650.89	DLATVYVDVLKDSGR	37–51
	1612.756	1612.80	LLDNWDSVTSTFSK	70–83
	1532.754	1514.81	VSFLSALEEYTKK	251–263
	1485.66	1453.72	VDPLRAELQEGAR	143–155
	1429.61	1411.70	KWQEEMELYR	131–140
	1386.565	1386.71	VSFLSALEEYTK	251–262
	1252.389	1380.72	VQPYLDDFQKK	121–131
	1301.422	1301.66	THLAPYSDELRL	185–195
	1283.421	1283.59	WQEEMELYR	132–140
	1252.389	1252.67	VQPYLDDFQK	121–130
	1230.470	1230.71	QGLLPVLESFK	240–250
	1175.307	1157.63	LEALKENGGAR	202–212
	1031.192	1031.53	LSPLGEEMR	165–173
	1012.174	1012.58	AKPALEDLR	231–239

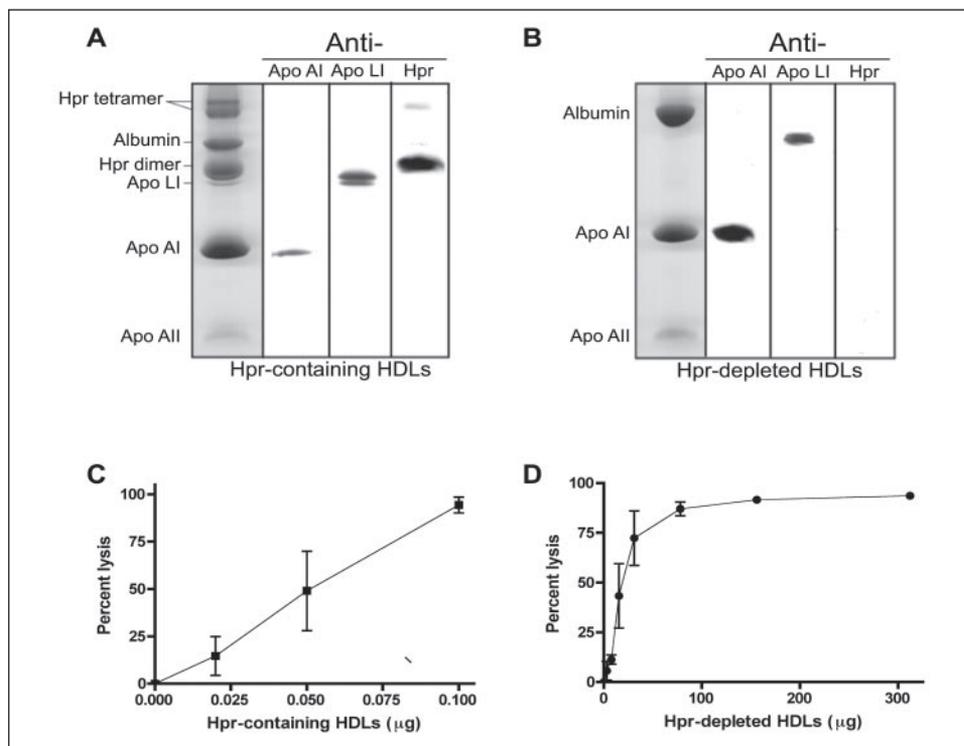
<sup>a</sup> Unique Hpr peptides as compared to haptoglobin and unique apoL-I peptides as compared to apoL-II through apoL-VI are underlined.

Hpr and apoL-I were reconstituted with nonlytic human HDL at a molar ratio of 5:1, mimicking the ratio of Hpr to apoL-I seen in HDL. Reconstituted particles containing only Hpr or apoL-I had specific activities of 0.24 units/ $\mu\text{g}$  or 0.28 units/ $\mu\text{g}$ , respectively, whereas particles containing both Hpr and apoL-I showed an  $\sim$ 5-fold increase in specific trypanosome killing activity to 1.10 units/ $\mu\text{g}$  (Fig. 2E and TABLE ONE). These findings demonstrate that the assembly of Hpr and apoL-I into the same HDL particle results in enhanced killing of *T. b. brucei*. This observation suggested that native HDL subclasses containing both Hpr and apoL-I likely have a high specific activity for *T. b. brucei* killing and play a major role in human innate protection from trypanosome infection. We therefore decided to fractionate native HDLs into subclasses containing either Hpr or apoL-I or both proteins and to examine the lytic activity of these native HDL particles.

Human HDL was initially fractionated into Hpr-containing and Hpr-depleted particles by immunoabsorption with anti-Hpr. Analysis of the Hpr-containing HDLs by MALDI-TOF mass spectrometry and Western blot identified apoA-I, apoA-II, apoL-I, and Hpr as components of this subfraction of human HDL (Fig. 3, A and B, TABLE TWO, and Fig. 4A). The unambiguous identification of Hpr and apoL-I was complicated, because both proteins are encoded by multi-gene families in humans. Hpr was distinguished from human haptoglobin based on

detection of three tryptic fragments unique to Hpr (amino acids 2–15, 34–50, and 220–233). Similarly, apoL-I was distinguished from other apoL family members (II–VI) based on six tryptic fragments unique to apoL-I (amino acids 64–90, 134–141, 219–231, 276–290, 291–305, and 306–316) (TABLE TWO). Hpr was present both as a heterodimer ( $\alpha/\beta$ ) (45 kDa) and heterotetramer ( $(\alpha/\beta)^2$ ) (92 kDa), and both the full-length apoL-I (42 kDa) and a previously described N-terminal truncation (39 kDa) were detected (20). Based on Coomassie staining, this subclass of human HDL contains roughly stoichiometric amounts of apoA-I and Hpr and sub-stoichiometric amounts of apoA-II and apoL-I (Fig. 4A). Hpr-containing HDLs account for nearly 98% of the recoverable lytic activity present in human HDLs with an estimated specific activity of 20 units/ $\mu\text{g}$  (Fig. 4C). The major apolipoproteins in the Hpr-depleted HDLs were apoA-I and apoA-II; a small amount of apoL-I was also detected by Western blot (Fig. 4B). The Hpr-depleted HDLs contained  $\sim$ 2% of the recovered *T. b. brucei* lytic activity with an estimated specific activity of 0.04 units/ $\mu\text{g}$  (Fig. 4D).

Two critical conclusions can be made based on these studies. First, a significant fraction of apoL-I is associated with Hpr-containing HDL. Second, most of the trypanocidal activity of human HDL is associated with Hpr-containing subclasses. However, these experiments also demonstrated that a small but reproducible portion of the trypanosome lytic



**FIGURE 4. The trypanosome lytic activity fractionates with Hpr-containing HDLs.** Total human HDLs were separated by immunoaffinity absorption with anti-Hpr. *A*, Hpr-containing HDLs were analyzed by SDS-PAGE, Coomassie staining (far left lane), and Western blot with anti-apoA-I, anti-apoL-I, and anti-Hpr. *B*, Hpr-depleted HDLs were separated by SDS-PAGE, stained with Coomassie (far left lane), and probed with monoclonal antibodies to apoA-I, apoL-I, and Hpr. *C*, Hpr-containing HDLs were assayed for concentration-dependent killing of *T. b. brucei*. The specific activity of the Hpr-containing HDLs was estimated to be 20 units/ $\mu\text{g}$  Hpr-containing HDL. *D*, Hpr-depleted HDLs were assayed for concentration-dependent killing of *T. b. brucei*. The specific activity of the Hpr-depleted HDLs was estimated to be 0.04 units/ $\mu\text{g}$  Hpr-depleted HDL.

activity was associated with apoL-I-containing HDLs that are deficient in Hpr (Fig. 4D). Additionally, the stoichiometry of the apolipoproteins in the Hpr-containing subclass suggested that it contained a mixture of particles containing apoA-I, apoL-I, and Hpr as well as particles containing apoA-I and Hpr but lacking apoL-I (Fig. 4A).

Based on these initial fractionation studies, we wanted to better establish the distribution of Hpr and apoL-I in HDL subclasses as well as the relative distribution of trypanosome lytic activity in these subclasses. HDL was purified by sequential immunoabsorption first with anti-Hpr immediately followed by anti-apoL-I separation (Fig. 5A). Four distinct classes of HDL were fractionated based on apolipoprotein composition, namely HDLs containing the following: 1) apoA-I, Hpr and apoL-I; 2) apoA-I and Hpr; 3) apoA-I and apoL-I; and 4) apoA-I but not Hpr or apoL-I. The composition of each of these HDL subclasses was determined by SDS-PAGE and Western blot analysis with anti-apoA-I, anti-Hpr, or anti-apoL-I (Fig. 5, B–E). Because apoA-I is a ubiquitous component of HDL subclasses we anticipated that all subclasses would contain apoA-I. Based on the recovery of protein in the different subclasses, it appeared that most of the HDL-associated Hpr and apoL-I co-purified as a complex (TABLE ONE). *In vitro* lysis assays indicated that this HDL subclass contained 99% of the recovered trypanosome lytic activity and had a specific lytic activity of 83.3 units per  $\mu\text{g}$  of protein (Fig. 5F and TABLE ONE). The HDL subclasses containing either apoA-I and Hpr or apoA-I and apoL-I also had lytic activity but contributed only 0.4% and 0.3% of the recovered lytic activity, respectively, and the specific activity of both the apoA-I and Hpr and the apoA-I and ApoL1 subclasses was reduced >100-fold to 0.5 and 0.8 units/ $\mu\text{g}$ , respectively (Fig. 5G and TABLE ONE). HDLs not containing either Hpr or apoL-I but containing apoA-I were inactive, confirming that apoA-I itself is not trypanolytic (Fig. 5G and TABLE ONE). Together, these studies indicate that the majority of trypanolytic activity of human HDLs is associated with particles containing both Hpr and apoL-I and that the specific activity of this HDL subclass is higher than that of HDLs containing either Hpr or apoL-I alone.

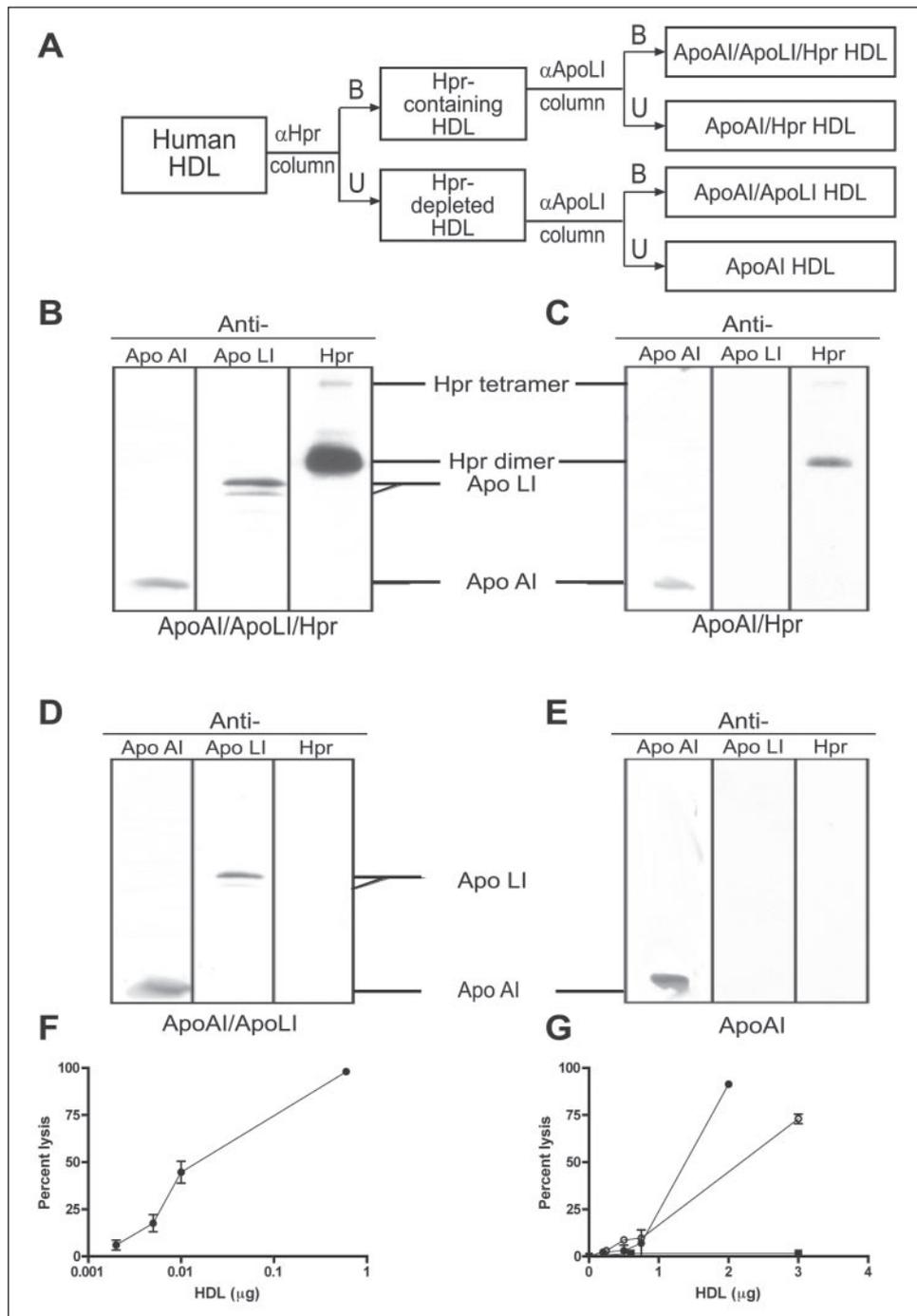
Studies were initiated to investigate the mechanism of synergy between Hpr and apoL-I. The cellular pathways for Hpr and apoL-I

killing of *T. b. brucei* may share common characteristics, because both require cell surface receptors and intracellular trafficking to the lysosome (6, 13). To determine whether the enhanced killing activity by HDLs containing both Hpr and apoL-I was a consequence of preferential recognition by trypanosome receptors, the binding of HDLs containing either apoL-I alone, Hpr alone, or both apolipoproteins was compared. When *T. b. brucei* was incubated with the three subclasses of HDL, no significant differences in binding were observed after 3 and 16 h at 1 and 5  $\mu\text{g}/\text{ml}$  (Fig. 6; data not shown for the 3-h time point). These results indicate that the increased lytic activity of HDL containing both Hpr and apoL-I is a consequence of intracellular events following cell surface binding.

## DISCUSSION

Innate immunity confers first-line host defense against microbial pathogens and generally operates in conjunction with adaptive immunity to combat infection (29). African trypanosomes pose a unique challenge to the mammalian host because a single parasite can establish infection, and the adaptive immune response is largely ineffective because of antigenic variation (30). Therefore, human resistance to *T. b. brucei* infection is entirely dependent on innate immunity and must provide sterile protection against challenging parasites. We show that the human serum HDL apolipoproteins apoL-I and Hpr are both toxic to trypanosomes and that assembly of these proteins into the same HDL particle is necessary for maximal protection against infection by *T. b. brucei*. Therefore, the results presented here establish a new paradigm for our understanding of the role of HDL as antimicrobial complexes.

Haptoglobin-related protein and apoL-I have both been previously proposed as the human serum proteins responsible for *T. b. brucei* killing (12, 13). Although the role of apoL-I was directly evaluated with recombinant and native affinity-purified protein, the potential toxicity of Hpr was based largely on inference (13, 19). When Hpr and apoL-I were purified from total human serum HDL preparations by immunoaffinity chromatography, we were able to confirm that both were toxic to *T. b. brucei* and that the specific activities of the two proteins were comparable (Fig. 2). However, the specific activity of the individual puri-



**FIGURE 5. Distribution of lytic activity in human HDL subclasses.** *A*, schematic illustrating the sequential chromatographic purification of the HDL subclasses from total human HDLs. Bound (*B*) and unbound (*U*) fractions are indicated. *B*, Western blot of HDL subclass containing apoA-I (*Apo AI*), apoL-I (*Apo LI*), and Hpr. *C*, Western blot of HDL subclass containing apoA-I and Hpr. *D*, Western blot of HDL subclass containing apoA-I and apoL-I. *E*, Western blot of HDL subclass deficient in both Hpr and apoL-I but containing apoA-I. Lysis assays were performed as described. *F*, lytic activity of HDLs containing apoA-I, apoL-I, and Hpr was 83.3 lytic units/ $\mu$ g (●). *G*, lytic activity of HDLs containing apoA-I and Hpr was 0.5 lytic units/ $\mu$ g (○), and the lytic activity of HDLs containing apoA-I and apoL-I was 0.8 lytic units/ $\mu$ g (●). HDLs lacking both Hpr and apoL-I were completely deficient in lytic activity (■).

fied apolipoproteins was low relative to that of native HDLs containing Hpr and apoL-I (Fig. 2 and TABLE ONE), suggesting that assembly into HDL particles may enhance trypanolytic activity. We found that reconstitution of each protein individually into an apoA-I-containing HDL had little effect on specific activity but that assembly of both apoL-I and Hpr into an apoA-I-containing HDL increased the specific activity for *T. b. brucei* killing by ~5-fold (Fig. 2 and TABLE ONE). These findings led us to examine native HDL subclasses to determine whether a naturally occurring HDL subclass existed that contained both apoL-I and Hpr (Fig. 4). Using monoclonal antibodies specific for Hpr and apoL-I, we were able to physically fractionate four subclasses of human HDLs. Subclasses of HDL containing either Hpr or apoL-I alone were trypanolytic but had low specific activities (0.5 and 0.8 units/ $\mu$ g) and were low in abundance (0.1% and 0.05% of the total HDL, respectively). An HDL

subclass containing both Hpr and apoL-I, though also low in abundance (0.13% of the total HDL), had enhanced lytic activity (83.3 units/ $\mu$ g). This subclass of HDL containing apoA-I, Hpr, and apoL-I accounts for 99% of the total recovered trypanosome lytic activity in human HDL.

Previous studies have shown that the killing of *T. b. brucei* by human HDLs requires high affinity binding to receptors at the trypanosome cell surface, endocytosis, lysosomal localization, and acidification (4–7, 16). The *in vitro* reconstitution studies and the purification of native HDL subclasses reported in this paper show that assembly of Hpr and apoL-I into the same HDL has a synergistic effect on trypanosome lytic activity. The increased lytic activity of HDL particles containing both Hpr and apoL-I could be due to a number of cellular or molecular properties. We have tested the possibility that this HDL subclass binds more efficiently to trypanosome cell surface receptors than HDLs containing only Hpr

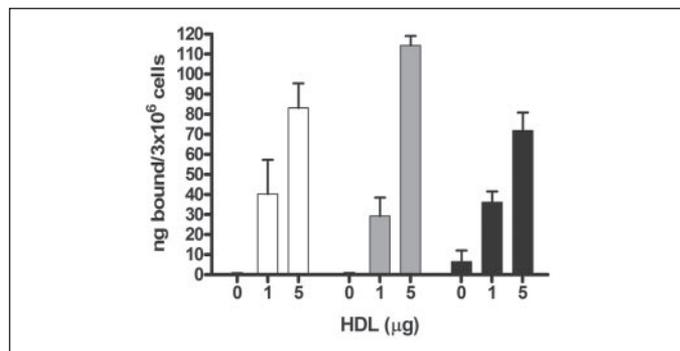


FIGURE 6. **Binding of human HDL subclasses to *T. b. brucei*.** Human HDL subclasses were radioiodinated and incubated with live *T. b. brucei* in the presence of a 100-fold excess of unlabeled nonlytic human HDL at 4 °C for 16 h. Following incubation, unbound HDL was removed by washing with PBSE, and bound HDL was measured by scintillation counting. The amount of bound HDL per  $3 \times 10^6$  trypanosomes was determined at a concentration of 1 and 5  $\mu\text{g}/\text{ml}$  for each HDL subclass. HDL subclasses containing Hpr and apoL-I (black bars), Hpr (white bars), and apoL-I (gray bars) are shown.

or apoL-I (Fig. 6). Because there is no difference in the relative binding of the HDL subclasses, it is likely that increased killing activity is a consequence of activities subsequent to recognition on the trypanosome cell surface. One possible explanation for the enhanced lytic activity of the Hpr- and apoL-I-containing HDLs is the stability of these particles in the trypanosome lysosome. When *T. b. brucei* was incubated with an HDL subclass containing both Hpr and apoL-I, the HDL rapidly accumulated in the lysosome but remained largely undegraded (7). Thus, assembly of Hpr and apoL-I may contribute to resistance to lysosomal proteases. Alternatively, the increased lytic activity of the HDL particles containing both Hpr and apoL-I could result from stimulation of the lytic activities of the individual proteins. Conflicting results on the mechanism of HDL killing of trypanosomes suggest that there might be multiple mechanisms involved (9, 10). We have previously proposed that cytotoxic human HDL kills *T. b. brucei* by an iron-dependent, lipid peroxidation mechanism that leads to lysosomal membrane breakdown (9). It has also been proposed that HDL killing may be mediated by an iron-independent killing that involves lysosomal membrane pore formation, and recent studies have shown that recombinant apoL-I is able to form anionic pores (10, 31). The results presented here clearly demonstrate that Hpr and apoL-I can act independently to kill *T. b. brucei*, and it is interesting to speculate that these proteins may act by different mechanisms. How these activities might act synergistically is completely unknown.

The genes for both Hpr and apoL-I are absent in non-primate mammals and evolved during primate evolution through a series of gene duplication and recombination events (14, 22, 23). Hpr is present in the serum of all primates that are resistant to *T. b. brucei* infection (19). ApoL-I has been reported to be present in the serum of humans and gorillas but is not present in sera from the baboon or the sooty mangabey, both of which have trypanolytic activity (24). Recent results from studies on chimpanzee sera are consistent with a role for Hpr and apoL-I in trypanosome killing (19, 24). Chimpanzees contain both the *Hpr* gene and the *apoL-I* gene, but, based on Western blot results, do not express either protein, and their serum is not lytic to *T. b. brucei*. The lack of expression of both lytic genes is surprising, and the evolutionary events leading to this loss of expression have not been examined.

Our findings raise the important question of why two serum proteins have evolved as toxins for African trypanosomes. The evolution of the haptoglobin and apoL gene families during primate evolution bears some superficial similarities, suggesting common selective pressures.

We favor a pathway of independent evolution of these proteins in response to different selective pressures. Although the notion that both Hpr and apoL-I may have evolved as antimicrobial molecules is appealing, the conservation of their genes and expression in all humans argues against African trypanosomes being the major selective agent. Although the selective pressures leading to the evolution of these proteins are not known, the results presented here demonstrate that enhanced innate host defense is mediated by the assembly of multiple defense molecules onto a serum HDL platform.

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