

# Inhibition of the Lytic Action of Cell-bound Terminal Complement Components by Human High Density Lipoproteins and Apoproteins

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**ABSTRACT** Human serum lipoproteins are known to participate in or modify several immunologically relevant responses, including the inhibition of target cell lysis initiated by fluid-phase C5b-7 (reactive lysis). We now report that human high density lipoproteins (HDL) can inhibit the complement (C) lytic mechanism after C5b-7, C5b-8, and even C5b-9 have been bound to the target membrane. This inhibitory activity of serum or plasma copurifies in hydrophobic chromatography with antigenically detected apolipoprotein A-I (apoA-I), the major HDL apoprotein, and with HDL in CsCl density gradient ultracentrifugation. Although HDL is more active than its apoproteins in fluid-phase inhibition of C5b-7-initiated reactive lysis, the HDL apoproteins are more effective after C5b-7, C5b-8, or C5b-9 have become bound to human or sheep erythrocytes (E). Highly purified HDL apoproteins, apoA-I and apoA-II, both have greater inhibitory activity than whole HDL on a protein weight basis, and some evidence has been obtained that apoA-I dissociating spontaneously from HDL may be the principal inhibitory moiety in physiological situations. HDL lipids themselves are inactive. The HDL-related inhibitors are ineffective when incubated with EC5b-7 and removed before C8 and C9 are added, and only minimally effective on cell-bound C5b-8 sites before C9 is added. They exert their most prominent inhibitory activity after C9 has been bound to EC5b-8 at low temperature, but before the final temperature-

dependent, Zn<sup>++</sup>-inhibitable membrane damage steps have occurred. Therefore, HDL or its apoproteins do not act to repair already established transmembrane channels, but might interfere either with insertion of C9 into the lipid bilayer or with polymerization of C9 at C5b-8 sites. This heat-stable inhibitory activity can be demonstrated to modify lysis of erythrocytes in whole serum, i.e., it does not depend upon artificial interruption of the complement membrane attack sequence at any of the above-mentioned stages. Contributions of the target membrane itself to the mechanism of inhibition are suggested by the observations that, in contrast to sheep or normal human E, lysis of guinea pig E or human E from patients with paroxysmal nocturnal hemoglobinuria is inhibited poorly.

This is the first description of a naturally occurring plasma inhibitor acting on the terminal, membrane-associated events in complement lysis. Although further study is required to assess the physiologic or immunopathologic significance of this new function of HDL, the HDL apoproteins or their relevant fragments should be useful experimentally as molecular probes of the lytic mechanism.

## INTRODUCTION

Substantial evidence has accumulated to support the concept that complement (C)<sup>1</sup>-mediated cytolysis is

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<sup>1</sup> Abbreviations used in this paper: apoA-I and II, apolipoprotein A I and A II; apoHDL, a mixture of apoproteins A I and A II derived from HDL by chloroform/methanol delipidation; LDL/VLDL, a mixture of low density and very low density lipoproteins; C, complement; HDL, high density lipoproteins; E, erythrocyte; E°, human E bearing C5b-9 sites; gp-guinea pig; GVB, 80 mM barbital buffer, pH 7.4, containing 150 mM NaCl and 0.1% gelatin; GVB<sup>++</sup>, GVB containing 0.5 mM MgCl<sub>2</sub> and 0.15 mM CaCl<sub>2</sub>; GVB-E, GVB containing 20 mM EDTA, pH 7.4; hu, human; PBS-E, 10

produced by the interaction of hydrophobic regions of the terminal components, C5-C9, with membrane phospholipids (reviewed in reference 1). Intermediate complexes of the membrane attack mechanism exhibit binding affinity for phospholipids in increasing order from C5b-7 to C5b-8 through C5b-9 (2). Furthermore, under certain conditions, C activation on the surface of erythrocytes (E) (3), liposomes (4), or bacteria (5, 6) can displace phospholipid from the membranes into the aqueous medium, possibly bound to terminal complement complexes (4). Human serum lipoproteins, presumably by offering alternative hydrophobic sites, are able to compete with membranes for the binding of hydrophobic complement attack complexes generated in the fluid phase. Specifically, it has been shown that the presence of either high density (7) or low density (7, 8) lipoproteins can inhibit the binding of nascent fluid-phase C5b-7 to cell membranes in reactive lysis systems. Another amphiphilic serum protein, the nonlipid-bearing S protein, likewise can bind to C5b-7, and limit its lytic potential for membranes (8, 9). Thus, hydrophobic proteins such as lipoproteins and S protein may serve a regulatory role in limiting injury to bystander cells when C activation, initiated on neighboring cells or in the fluid phase, produces a shower of fluid-phase C5b6 complexes that could, in turn, form the membranophilic C5b-7, C5b-8, and C5b-9 complexes.

Much less is known about the potential for amphiphilic plasma proteins to act on target cell-bound terminal C components to limit lysis. We have obtained evidence that high density lipoprotein (HDL) and, to a much smaller extent, low density lipoproteins (LDL/VLDL) from human serum can modulate the lytic effect of the terminal complement components after they are bound to the target cell membrane. This report describes the purification of the major HDL-associated inhibitory activity and its identification as apoproteins A-I and A-II (apoA-I, apoA-II). We also present evidence that HDL or its apoproteins, when added before full insertion of cell bound C5b-9 complexes into the target membrane, can inhibit not only lysis of human (hu) EC5b-8 or sheep EAC1-8 by hu C9, but also lysis of EC5b-9 (human E bearing C5b-9 sites, E°).

## METHODS

*Cells.* Blood was drawn from normal human donors and from a patient (R) with paroxysmal nocturnal hemoglo-

mM Na phosphate buffer, pH 7.4, containing 150 mM NaCl and 10 mM EDTA; PNH, paroxysmal nocturnal hemoglobinuria; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of 0.1% SDS; Z, the number of hemolytic sites per cell.

binuria (PNH) who possessed a homogeneous population (>95%) of type III PNH E (10). E were either stored in Alsever's solution or frozen in glycerol. The lytic behavior of frozen and fresh E was similar. Sheep E were purchased from Cordis Laboratories Inc., Miami, FL, and guinea pig (gp) E from Rockland Laboratories, Gilbertsville, MD.

*Complement components and intermediates.* C1gp was isolated (11) from fresh-frozen gp serum (Rockland Laboratories). C5 (12), C5b6 (13), and C9 (14) were isolated from fresh hu serum or fresh-frozen plasma. Human C2, C3, C5, C6, C7, C8, and C9 were purchased from Cordis Laboratories. Sheep EAC1gp, 4-7 hu (15) and hu EC5b-7 hu (generated with purified C5b6 and C7 [10]) were prepared according to published methods. For certain experiments, gp C7 (Cordis Laboratories) was used instead of hu C7, with similar results. For some experiments, EC5b-8 were prepared by incubating hu EC5b-7 with C8 for 20 min at 37°C, then washing. For preparation of E°, normal human EC5b-7 were prepared as above.  $4 \times 10^7$  EC5b-7 in 0.2 ml barbital-buffered saline containing 0.1% gelatin and 0.02 M EDTA (GVBE), pH 7.4, were shaken with human C8 at 37°C for 15 min. After washing, pelleted EC5b-8 were shaken with human C9 for 1 h at 10°C. After washing once in ice-cold GVB containing 0.5 mM MgCl<sub>2</sub> and 0.15 mM CaCl<sub>2</sub>, pH 7.4 (GVB<sup>++</sup>), E° were suspended to a volume of 1 ml in cold GVB<sup>++</sup>, and kept on ice until used. Details of individual experiments using E° are given in Results. C8-deficient human serum was kindly supplied by Dr. Bruce H. Petersen, Lilly Research Laboratories, Indianapolis, IN, and Dr. Bruce Gilliland, Providence Hospital, Seattle, WA; and C9-deficient human serum was kindly supplied by Dr. Henry Gewurz, Rush Medical College, Chicago, IL.

*Hemolytic assay for inhibitor.* Dilutions of inhibitor source in GVBE were mixed with hu C8 and/or C9 and added to  $2 \times 10^7$  of the desired cellular intermediate (e.g., sheep EAC1-7 or EAC1-8, or hu or gp EC5b-7 or EC5b-8) in a volume of 1.0 ml. Following incubation for 60 min at 37°C, the degree of hemolysis was determined by measuring the OD<sub>413</sub> of the supernatants. For some experiments, the number of hemolytic sites on the sheep EAC1-7 was limited to 0.4-2.4/cell by restricting input of C6, C7, or C8, with all other components present in excess. The number of effective hemolytic sites per cell (Z) was calculated from the Poisson distribution and a one-hit assumption (16), and the dilution of the inhibitor source producing 50% reduction in Z was determined graphically from a semilog plot of Z vs. inhibitor dilution (inhibitory titer). Fig. 1 demonstrates that similar hemolytic inhibitory titers for a particular preparation (partially purified HDL) are obtained (A) using sheep EAC1-7 bearing C5b-7 sites limited by the C6 input (0.5-1 effective mol/cell), or (B) using sheep EAC1-7 bearing excess C5b-7 sites but with the inhibitor added in the presence of limiting C8 (0.6-2.2 effective mol/cell). This experiment also establishes that the initial number of uninhibited C5b-7 sites per cell is not critical to the inhibitory titer, except possibly at the lowest levels of C6 input (Fig. 1 A). Most of the subsequent hemolytic inhibition assays with sheep EAC1-7 involve limited C6 with approximately one site per cell (uninhibited). When purified inhibitor preparations were obtained, these inhibitory titers were converted to micrograms protein per milliliter required for 50% inhibition (I<sub>50</sub>).

*Hydrophobic chromatography.* Serum was fractionated on a column of phenyl-Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) by methods to be reported in detail elsewhere. Briefly, serum was dialyzed against 10 mM phosphate buffer, pH 6.8, containing 50% (vol/vol) ethylene

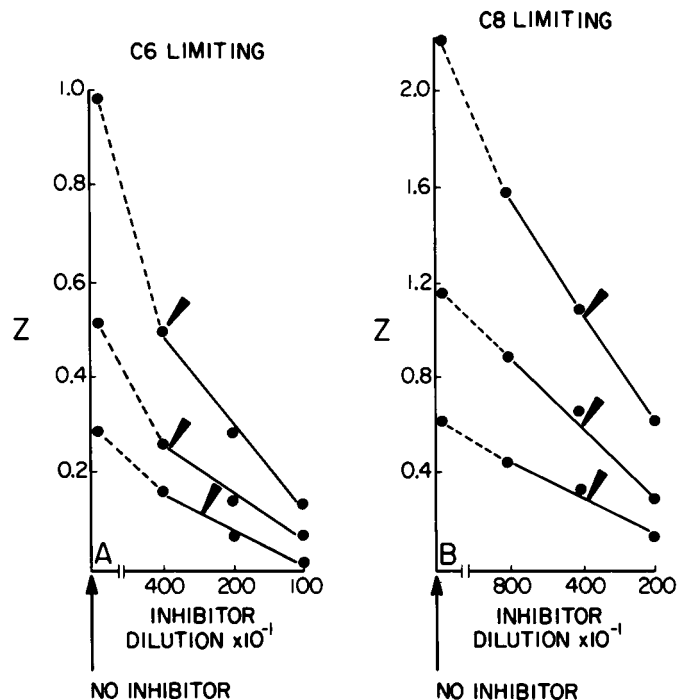


FIGURE 1 (A) Lysis of sheep EAC1-7 (formed with three different limiting dilutions of C6) by human C8 and C9 in the presence of varying inputs of partially purified inhibitor (phenyl-Sepharose pool III). The lysis produced by each dilution of C6 in the absence of inhibitor is given on the ordinate, and the arrows show the points of 50% inhibition. (B) Same basic protocol as in Fig. 2 A, except that three different limiting dilutions of C8 were used.

glycol (certified, Fisher Scientific Co., Pittsburgh, PA) and applied to the column equilibrated in the same buffer. The column was eluted with a linear gradient of starting buffer to deionized water, followed by a second linear gradient of water to 95% ethanol. Fractions were dialyzed against 10 mM phosphate buffer, pH 7.4, containing 150 mM NaCl and 10 mM Na<sub>3</sub>EDTA (PBS-E) and tested for inhibitory activity by the hemolytic assay described above (using sheep EAC1-7), for apoA-I antigens by Ouchterlony analysis, and for C5, C6, C7, C8, and C9 by microtiter adaptation of standard hemolytic assays (15). Inhibitory activity was consistently recovered in two peaks, one eluted with water and a second peak eluted early in the ethanol gradient. These fractions were pooled after dialysis and concentrated (Amicon PM-30, Amicon Corp., Scientific Sys. Div., Lexington, MA), and are designated phenyl-Sepharose pool I and pool III, respectively. Pool I was usually contaminated with small amounts of C7, C8, and C9, and pool III contained traces of C7 and C9. Virtually all of the applied inhibitory activity was recovered in these two peaks, and all fractions containing inhibitory activity also contained apoA-I antigen, whereas apoA-I was detected in no other fractions from the column. By Ouchterlony analysis, pool I contained a trace of antithrombin III but no S protein or  $\beta$ -lipoprotein, whereas pool III contained a trace of  $\beta$ -lipoprotein but no S protein or antithrombin III.

**Density gradient ultracentrifugation.** This technique utilized fresh EDTA plasma drawn after an overnight fast and was performed by a modification of the method of Lux et al. (17), using CsCl (optical grade, Sigma Chemical Co., St. Louis, MO) instead of KBr to adjust density. Following centrifugation at a starting  $d = 1.063$  g/ml, at 40,000 rpm

for 64 h at 15°C in a 50 Ti rotor (Beckman Instruments Inc., Fullerton, CA), the top third of each tube was pooled as the LDL/VLDL pool. The bottom third of these tubes was adjusted to  $d = 1.21$  and recentrifuged at 49,800 rpm for 39 h at 15°C in the same rotor. The upper one-sixth of these tubes contained most of the HDL, which was washed once by flotation through PBS-E at  $d = 1.21$  g/ml. The final HDL pool had a density of 1.185 g/ml before dialysis against PBS-E for functional assays or deionized water for lyophilization.

For ultracentrifugal analysis of various purified lipoprotein-related inhibitor preparations, samples of 1 ml containing 680–3,000  $\mu$ g protein were diluted to 5 ml with PBS-E and adjusted to a density of 1.210 with CsCl. These were centrifuged at 60,000 rpm for 42 h in an SW-65-K rotor at 15°C. The tubes were pierced from the bottom and 40-drop fractions were obtained, dividing each tube into 11 fractions. Densities of each fraction were calculated from refractive index (Bausch & Lomb refractometer, Rochester, NY); then the fractions were dialyzed against PBS-E to remove CsCl before analysis for protein content, inhibitory activity and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) behavior (to assess relative apoA-I and apoA-II content).

**Delipidation of HDL.** 6.5 mg of lyophilized HDL were incubated for 1 h at 0°C with 12 ml chilled chloroform/methanol 2:1 (vol/vol), with thorough mixing. The precipitate was collected by centrifugation, washed once with chloroform/methanol 2:1, dried with nitrogen, redissolved either in 10 mM Tris-HCl, pH 8.6, containing 1 mM Na<sub>3</sub> EDTA or in PBS-E, and stored at 0°C for up to 2 wk, or at -70°C for longer periods. The supernatant and wash solvent containing extracted lipids were pooled, evaporated to 0.5 ml

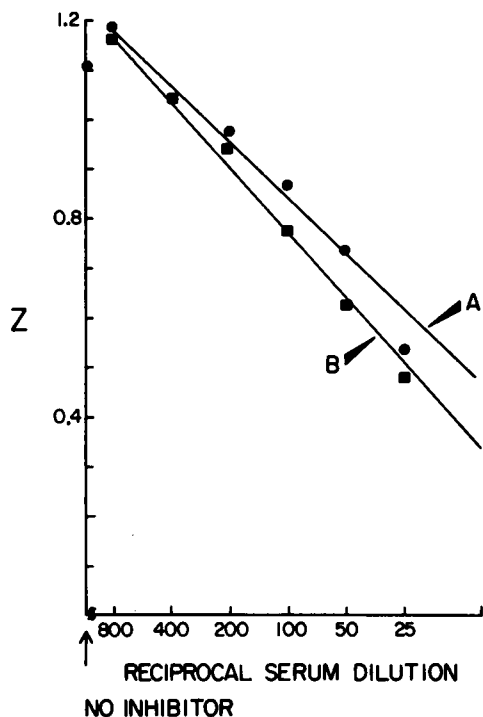


FIGURE 2 Lysis of hu EC5b-7 by hu C8 and C9 in the presence of dilutions in GVB-0.02 M EDTA of (A) fresh normal human serum (circles) and (B) heated (56°C, 180 min) normal human serum (squares). The experiment was performed using excess added C8 and C9, with limited C5b-7 sites. Z, average number of lytic sites per cell.

with nitrogen, and stored at 0°C for the recombination experiments.

**Analytic methods.** Isolation of apoA-I and apoA-II followed a published method (18). Protein determinations were done by the Oyama (19) modification of the Folin method with human serum albumin as standard. Use of the Petersen modification (20) for lipid-containing extracts gave identical values to those obtained by the Oyama method for both lipid-containing and lipid-free protein solutions. PAGE was performed in the presence of 0.1% SDS by the method of Laemmli (21) with a 3% acrylamide stacking gel and a linear gradient of 7.5–20% acrylamide for the running gel. Reduction of disulfide bonds, when desired, was achieved with 5% (vol/vol) 2-mercaptoethanol in the sample buffer.

**Lipid analysis.** Total lipid inorganic phosphorus, reflecting phospholipid content, was analyzed by a minor modification of the method of Harris and Popat (22), and total cholesterol by a modification of Stadtman's method (23).

**Antisera.** Antisera to human apoA, to  $\beta$ -lipoprotein, and to antithrombin III were purchased from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA. Anti-S protein was kindly supplied by Dr. Eckhard Podack, Research Institute of Scripps Clinic, La Jolla, CA. A rabbit was immunized with 0.5 ml of an inhibitor pool from a phenyl-Sepharose column in complete Freund's adjuvant and boosted at 1–2-wk intervals with two subsequent injections of a similar pool in incomplete Freund's adjuvant. The resulting antiserum produced a single precipitin line against normal hu serum in Ouchterlony reactions and crossed immunoelectrophoresis. This line was identical with that produced by

commercial anti-apoA and by a reference anti-apoA-I serum kindly supplied by Dr. H. Bryan Brewer, Jr. (National Heart, Lung, and Blood Institute, Bethesda, MD) and showed non-identity with the lines produced by anti- $\beta$ -lipoprotein and by anti-apoA-II (also furnished by Dr. Brewer).

## RESULTS

During experiments involving reactive lysis, we observed that human EDTA serum was consistently less effective as a C8/C9 source than equivalent or smaller inputs of purified hu C8 and C9 in lysing sheep or hu EC5b-7. This led us to suspect that whole serum possessed an inhibitory activity of a type not previously well characterized. Pilot studies were then conducted, using as a crude inhibitor source hu serum which had been heated at 56°C for 3 h to completely destroy C8 and C9 hemolytic activities.<sup>2</sup> As shown in Fig. 2 (curve B), addition of 3-h heated serum-EDTA with excess hu C8 and C9 does produce a dose-dependent reduction in lysis of hu EC5b-7 (or sheep EC5b-7, data not shown). Similar results were obtained with heated C8- or C9-deficient hu serum-EDTA (data not shown), indicating that altered C8 or C9 was not the inhibitor. Fresh normal serum-EDTA, admixed with C8 and C9 (added in excess), also induces similar dose-dependent inhibition of lysis (Fig. 2, curve A). EDTA is not required for this inhibitory effect; nor is interruption of the membrane attack sequence at the C5b-7 or C5b-8 step. The latter was demonstrated in experiments (not shown) in which a purified inhibitor preparation (apoHDL, see below), added to a limiting dilution (1:200–1:400) of fresh human serum, produced dose-dependent inhibition of lysis of sheep EA.

### *Coisolation of plasma inhibitory activity with HDL or its major apoproteins*

Initial efforts to isolate the protein(s) responsible for this lysis-inhibiting activity of human plasma by ion exchange chromatography or gel filtration were unsuccessful, although starch block electrophoresis localized the activity mainly among the  $\alpha$ -globulins. Hydrophobic chromatography on phenyl-Sepharose was more successful. Virtually all inhibitory activity from serum appeared in two distinct peaks on phenyl-Sepharose hydrophobic chromatography (Methods). Both of these peaks contained apoA-I, the major apoprotein of HDL, which is detectable by immunodiffusion in either intact or delipidated HDL. Furthermore, apoA-I was detectable only in the fractions con-

<sup>2</sup> Heating for 1 h at 56°C destroyed C8 but inactivation of C9 was incomplete, as determined by effective molecule titration. Such residual C9 sometimes enhanced lysis at low serum dilutions, obscuring the inhibition seen at higher dilutions.

TABLE I  
Characteristics of Representative Inhibitor Preparations

Inhibitor preparation*	Lipid Pi (phospholipid)	Total cholesterol	50% inhibitory concentration
	nM/ $\mu$ g protein		$\mu$ g protein/ml $\ddagger$
VLDL/LDL	ND $\S$	ND $\S$	4.8
HDL	0.430	0.520	2.0
Phenyl-Sepharose pool I	0.185	0.013	0.3
Phenyl-Sepharose pool III	0.250	0.200	0.5
ApoHDL	0.019	0.019	0.5
ApoA-I	0 <sup>  </sup>	0 <sup>  </sup>	1.1
ApoA-II	0 <sup>  </sup>	0 <sup>  </sup>	0.9

\* VLDL/LDL pool and HDL were prepared by CsCl ultracentrifugation; apoHDL was derived from HDL by delipidation with chloroform/methanol 2:1; apoA-I and apoA-II were separated by Sephadex G200 filtration in 8 M urea (see Methods). The phenyl-Sepharose pools are described in the text.

$\ddagger$  Final protein concentration producing 50% inhibition of lysis of sheep EAC1-7 by hu C8 and C9 (Methods).

$\S$  ND, not determined.

<sup>||</sup> 0, not detectable.

taining inhibitory activity. The two inhibitory pools (pool I and pool III) from the phenyl-Sepharose column, although both containing apoA-I, differed from each other in their lipid content and in their content of apoA-II, the other major HDL apoprotein. Compared with ultracentrifugally prepared HDL, pool I is more depleted of cholesterol (Table I) and of apoA-II (Fig. 3) than is pool III, but both pools have lower lipid content and higher inhibitory activity on a protein weight basis than does HDL (Table I).

By density gradient ultracentrifugation in CsCl, LDL/VLDL fractions of plasma, which react with anti- $\beta$ -lipoprotein but not with anti-apoA-I or anti-apoA-II, did contain some inhibitory activity, but with less than half the potency (per microgram protein) of HDL (Table I). In a representative experiment, ~10–20% of the total inhibitory activity recovered from 82 ml of plasma was located in the LDL/VLDL pool. At least 80% of the inhibitory activity from the plasma was recovered in the HDL pool.

#### Role of apoproteins vs. lipids of HDL in inhibitory activity

Chloroform/methanol delipidation of ultracentrifugally purified HDL yields apoHDL, a mixture of apoA-I and apoA-II in similar ratio to that of intact HDL (Figs. 3 and 4, tracks a) with small but detectable amounts of residual lipid (Table I). This material has a higher inhibitory activity on a protein weight basis than does intact HDL, similar to the activity seen with the phenyl-Sepharose pools that have also been par-

tially delipidated by hydrophobic chromatography (Table I).

The apoproteins (A-I and A-II) of apoHDL were separated by gel filtration on Sephadex G200 in 10 mM Tris buffer, pH 8.6, containing 8 M charcoal-filtered urea and 1 mM EDTA (18). This procedure yielded pure apoA-I and apoA-II (Fig. 4) with no detectable phospholipid or cholesterol (Table I). The apoA-I contained a small amount of a peptide of smaller apparent molecular weight (Fig. 4, lane b), which probably reflects degradation of apoA-I, since similar bands have appeared in other delipidated apoA-I-containing preparations upon storage at 0°C (but not at -70°C). Nevertheless, despite the somewhat rigorous conditions and prolonged dialysis required for their isolation, the total absence of detectable lipid, and probable self-aggregation (24), both purified apoprotein preparations were quite active in the inhibitory assay (Table I) with activity greater than that of intact HDL, but less than that of unseparated apoHDL.

Purified HDL and the two active phenyl-Sepharose pools were subjected to CsCl density gradient ultracentrifugation (Methods), and fractions were analyzed for density, protein concentration, inhibitory activity and content of apoA-I and A-II (by estimation in SDS-PAGE). For all three inhibitor sources, the lysis-inhibiting activity was highest in high-density fractions ( $d = 1.21$ – $1.33$  mg/ml). These fractions all contained apoA-I, with little or no apoA-II, and are presumably low in lipid. Only a small proportion of the total protein from the HDL preparation appeared in these denser fractions, with the bulk of the apoA-I and apoA-

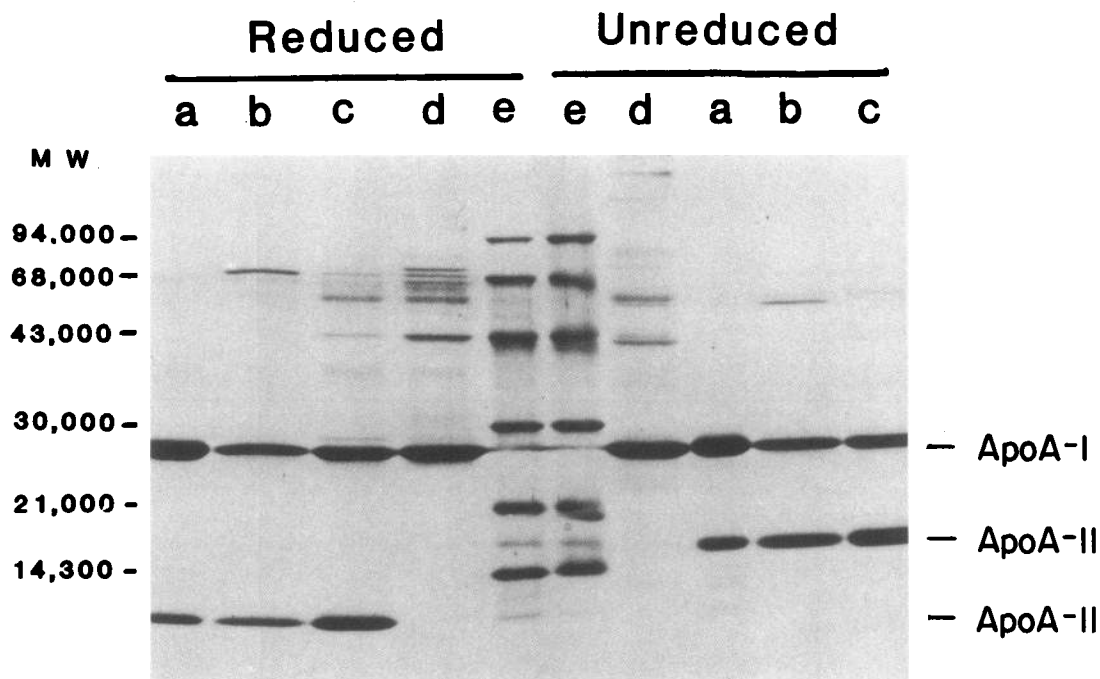


FIGURE 3 SDS-PAGE analysis of some inhibitor preparations. The 7.5–20% acrylamide gradient slab gel was stained with Coomassie blue. Track a: 8  $\mu$ g of HDL; track b: 5  $\mu$ g of a middle fraction from a CsCl gradient ultracentrifugation of phenyl-Sepharose pool III; track c: 8  $\mu$ g of phenyl-Sepharose pool I; track d: 8  $\mu$ g of phenyl-Sepharose pool I; track e: molecular weight standards. The positions of apoA-I, as well as unreduced and reduced apoA-II, are indicated.

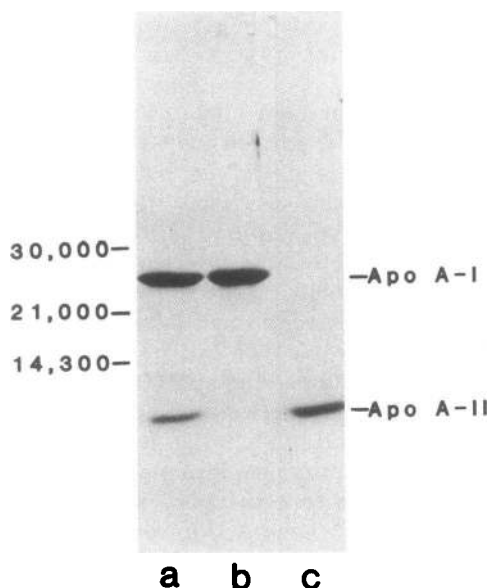


FIGURE 4 Polyacrylamide gradient (7.5–20%) gel electrophoresis, in the presence of SDS (0.1%) and 2-mercaptoethanol (5%), of 5  $\mu$ g apoHDL starting material (track a), 5  $\mu$ g purified apoA-I (track b), and 4  $\mu$ g purified apoA-II (track c). Coomassie blue stain.

II having densities of 1.14–1.16 mg/ml. Although the lighter HDL fractions did have some inhibitory activity, they were less active on an absolute basis (despite their high protein content) than were the fractions with densities of 1.26–1.31. Phenyl-Sepharose pool I, known to be partially lipid depleted (Table I) and deficient in apoA-II (Fig. 3), has the bulk of its apoA-I in this high density range (1.22–1.33), accounting for the high specific inhibitory activity of this pool (Table I). Pool III, eluted from the phenyl-Sepharose column under more hydrophobic conditions, has a more heterogeneous density distribution of inhibitory activity and apolipoproteins, but the most active fractions are in the same high density range as that found in phenyl-Sepharose pool I.

The lipids extracted from HDL were sonicated with buffer and tested for inhibitory activity in the standard assay. The results were negative, although it is quite likely that such lipids were not as well dispersed as are water-soluble lipoproteins. In a few experiments, attempts were made to reconstitute lipoproteins by sonication of delipidated apoHDL with the lipids extracted from HDL. Although full reconstitution was never achieved as indicated by residual immiscible lipid, the “reconstituted” HDL always had inhibitory

activity less than that of apoHDL, and sometimes even lower than that of the original HDL (data not shown).

*Studies on the stage(s) in the membrane attack mechanism inhibited by HDL and its apoproteins*

**Inhibition of formation of EC5b-7.** Previous studies by others had shown that HDL (or LDL) can act in the fluid phase to inhibit formation of EC5b-7 from E, C5b6, and C7 (7, 8), whereas we have found that HDL and particularly its apoproteins can inhibit lysis even after C5b-7 have been bound to the cell membrane. We compared the potency of HDL and delipidated apoHDL for inhibition of the formation of EC5b-7 vs. inhibition of lysis of preformed EC5b-7 by C8 and C9. Human E were incubated with C5b6, C7, and either inhibitor dilutions or buffer (20 min at 37°C) and washed. Tubes that had received inhibitor in the first step then received C8, C9 plus buffer, whereas those receiving buffer in the first step received C8, C9, and inhibitor dilutions for the final incubation at 37°C for 60 min. Lysis was measured and I<sub>50</sub> (based on protein concentration) of both inhibitor preparations determined as in Fig. 1. For 50% inhibition of EC5b-7 formation, only 8.3 µg/ml HDL was required, compared with 23.5 µg/ml apoHDL. For 50% inhibition of lysis of preformed EC5b-7 by C8/C9, however, only 3.4 µg/ml apoHDL sufficed, compared with 41.7 µg/ml of HDL. The ratio of HDL to apoHDL required for inhibition at the C8/C9 step in this experiment is higher than usually observed, but HDL is consistently less active at this stage than is apoHDL, whereas the reverse is true for inhibition of EC5b-7 formation.

**Lack of inhibition of preformed C5b-7 sites in the absence of C8 and C9.** When hu EC5b-7 or sheep EAC1-7 were incubated with HDL, apoHDL, or buffer (45 min, 37°C), then washed, no effect of either inhibitor on subsequent lysis by C8 and C9 was noted (I<sub>50</sub> > 250 µg/ml for both preparations). These same inhibitor preparations exhibited I<sub>50</sub> values (in micrograms protein per milliliter) of 32 and 5 for HDL and 5.9 and 0.9 for apoHDL on these hu EC5b-7 and sheep EAC1-7, respectively, when added to the washed cells together with C8 and C9. The greater sensitivity of sheep E intermediates to inhibition, compared with hu E intermediates, has been consistent, but not always in the 6 to 1 ratio shown in this experiment.

**Inhibition at the C8 step.** No inhibition of lysis was found when apoHDL or HDL were added together with C8 to hu EC5b-7, following by washing and addition of C9. Somewhat variable but minor degrees of inhibition (I<sub>50</sub> generally > 250 µg/ml) were observed when apoHDL or HDL were incubated with pre-

TABLE II  
*Effect of Inhibitors on the Lysis of Cells by Membrane-bound or Fluid-Phase C9*

Inhibitor added to:	Protein concentration (µg/ml) required for 50% inhibition of lysis			
	HDL	apo HDL	apoA-I	apoA-II
Hu EC5b-9 (E* uninserted)*	30.0	1.8	1.5	0.1
Hu EC5b-8 with C9†	ND‡	3.8	9.5	3.4

\* Hu EC5b-9 (E\*) were mixed at 0°C with inhibitor dilutions or buffer, and then warmed to 37°C; lysis was measured after 60 min.

† Hu EC5b-8 were mixed with inhibitor dilutions (or buffer) plus C9, and lysis was measured after incubation for 60 min at 37°C.

‡ ND, not done.

formed hu EC5b-8 or sheep EAC1-8 before washing and development of lysis by C9. In contrast, substantial inhibition was seen when apoHDL or HDL were added together with C9 to the same EC5b-8 or EAC1-8 (I<sub>50</sub> 0.9-1.0 and 10.5-23 µg/ml for apoHDL and HDL, respectively). We had previously reported (25) a significant inhibitory effect on EC5b-8 of partially purified inhibitor preparations (similar to phenyl-Sepharose pool I), but we now attribute this effect principally to the known slight contamination of such preparations by C9. The presence of C9 in the inhibitor during the initial incubation with EC5b-8 allows formation of some C5b-9 sites which are then immediately inhibited, with consequent loss of some C5b-8 sites from the initial EC5b-8. This was supported by the further observation that addition of small amounts of purified C9 to HDL endowed it with an inhibitory effect on human EC5b-8 similar to that seen with phenyl-Sepharose pool I.

**Inhibition of cell-bound C5b-9 sites (not fully inserted).** The foregoing experiments imply that C9 must be present for the HDL-related inhibitors to be effective. This could reflect (a) an interaction between inhibitor and fluid phase C9; (b) interference with the binding of C9 to C5b-8 sites; or (c) inhibition of events in the lytic process subsequent to C9 binding. The only indirect evidence so far obtained against fluid phase interaction of inhibitor and C9 is that preincubation (25°C, 20 min) of apoHDL or HDL with C9 failed to augment inhibition. As an alternative approach, we prepared E\* (EC5b-9, presumably with the C5b-9 sites incompletely inserted), as described in Methods. After adding ice-cold E\* to precooled (0°C) inhibitor dilutions or buffer, the mixtures were warmed to 37°C. The design and results of a typical experiment are presented in Table II. HDL, apoHDL and the purified lipid-free apoproteins of HDL, apoA-I and apoA-II, all effectively inhibited lysis of preformed E\*. Lower concentrations of apoHDL, apoA-I and apoA-II pro-

TABLE III  
Inhibitory Action of ApoHDL on Insertion and Postinsertional Events in Hu EC5b-9

Step I*		Step II*	Steps I and II	
EC5b-9 incubated with:	Lysis	Surviving EC5b-9 incubated with:	Lysis	Total lysis
	%		%	
1. GVB <sup>++</sup>	21.8	GVB <sup>++</sup>	11.5	33.3
2. ApoHDL in GVB <sup>++</sup> (20 μg/ml)†	5.0	GVB <sup>++</sup>	9.0	14.0
3. ApoHDL in GVB <sup>++</sup> (5 μg/ml)	7.4	GVB <sup>++</sup>	9.6	17.0
4. 0.09 M EDTA‡	8.5	GVB <sup>++</sup>	30.5	39.0
5. 0.09 M EDTA‡	9.8	ApoHDL in GVB <sup>++</sup> (40 μg/ml)‡	28.3	38.1
6. 0.09 M EDTA‡	8.9	ApoHDL in GVB <sup>++</sup> (10 μg/ml)	28.8	37.7
7. Zn <sup>++</sup> (2 × 10 <sup>-4</sup> M)‡	9.0	GVB <sup>++</sup>	23.5	32.5
8. Zn <sup>++</sup> (2 × 10 <sup>-4</sup> M)‡	10.5	ApoHDL in GVB <sup>++</sup> (40 μg/ml)‡	20.5	31.0
9. Zn <sup>++</sup> (2 × 10 <sup>-4</sup> M)‡	8.5	ApoHDL in GVB <sup>++</sup> (10 μg/ml)	21.1	29.6

\* After incubation (37°C, 60 min) of ice-cold (uninserted) EC5b-9 in the reagents listed under step I, the unlysed cells were spun down and the supernate removed for determination of lysis. The unlysed cells were suspended and incubated in the reagents listed under step II. Percent lysis for steps I and II is based on the original number of cells in the tube at the beginning of step I. Total lysis is the sum of percent lysis in steps I and II.

† Final concentration of apoHDL, EDTA, or ZnSO<sub>4</sub> in reaction mixture. All reactants were dissolved in GVB<sup>++</sup>.

tein sufficed for inhibition of E° lysis than for inhibition of lysis of EC5b-8 by C9 (Table II). However, the uninhibited lysis of EC5b-8 by C9 was higher than that of E° in these experiments. These results established that HDL-related inhibitors can effectively reduce lysis even after C9 is bound to EC5b-8. While an effect on fluid phase C9 or its binding is not ruled out, such a mechanism cannot be an exclusive mode of action of the inhibitors.

*Inhibition of individual steps subsequent to C9 binding.* We next attempted to determine which events after C9 binding (26, 27) were susceptible to inhibition. By adaptation of the methods of Boyle et al. (27, 28) a two-step incubation system was developed (Table III). In step I, human E° were incubated at 37°C in one of the following: (a) GVB<sup>++</sup> control (line 1); (b) inhibitor dilutions in GVB<sup>++</sup> (lines 2, 3); (c) 0.09 M EDTA (lines 4-6), which prevents lysis by osmotic effects but allows insertion of C5b-9 and subsequent membrane damage to occur (26, 27); (d) 2 × 10<sup>-4</sup> M ZnSO<sub>4</sub> (lines 7-9), which is reported to allow insertion of C5b-9 but block subsequent membrane damage (28). After 60 min, the cells were sedimented in the cold, supernatants were removed as completely as possible for determination of lysis, and the surviving cells resuspended in GVB<sup>++</sup> with or without varying doses of inhibitor (step II). After another 60 min at 37°C, lysis was again determined, and percent lysis calculated on the basis of the number of E° present at the beginning of the experiment.

The experiment shown in Table III is representative

of many similar experiments; it was chosen because all variables were studied simultaneously. Identical experiments using HDL as inhibitor rather than apoHDL gave entirely analogous results. Control lysis in the first and second steps as well as total lysis of the E° generated for this experiment are shown in line 1. Control lysis in the second step of this particular experiment is slightly higher than that usually observed (range 1-12%). ApoHDL, when present during the first incubation (lines 2 and 3), significantly inhibited lysis during the first step, as well as total lysis, whereas lysis during the second incubation (9%) is similar to that in the GVB<sup>++</sup> control (11.5%; line 1, step II). This inhibition by apoHDL appeared to be irreversible, since lysis after removal of apoHDL (step II) never exceeded control lysis in step II. EDTA, 0.09 M, exerts a significant protective effect during the first incubation (lines 4-6); cells then lyse spontaneously when EDTA is removed during the second incubation (line 4, step II), so that total lysis is similar to that in the control (line 1). ApoHDL has no protective effect when added after insertion and damage have proceeded during the first 60-min incubation in EDTA (lines 5, 6).

Lines 7-9 in Table III show that 2 × 10<sup>-4</sup> M Zn<sup>++</sup> exerts a significant protective effect during the first incubation. The protective effect is reversed after removal of Zn<sup>++</sup> during the second incubation (line 7), so that total observed lysis equals that in the control (line 1). In preliminary experiments, this concentration of Zn<sup>++</sup> had given the optimal compromise between



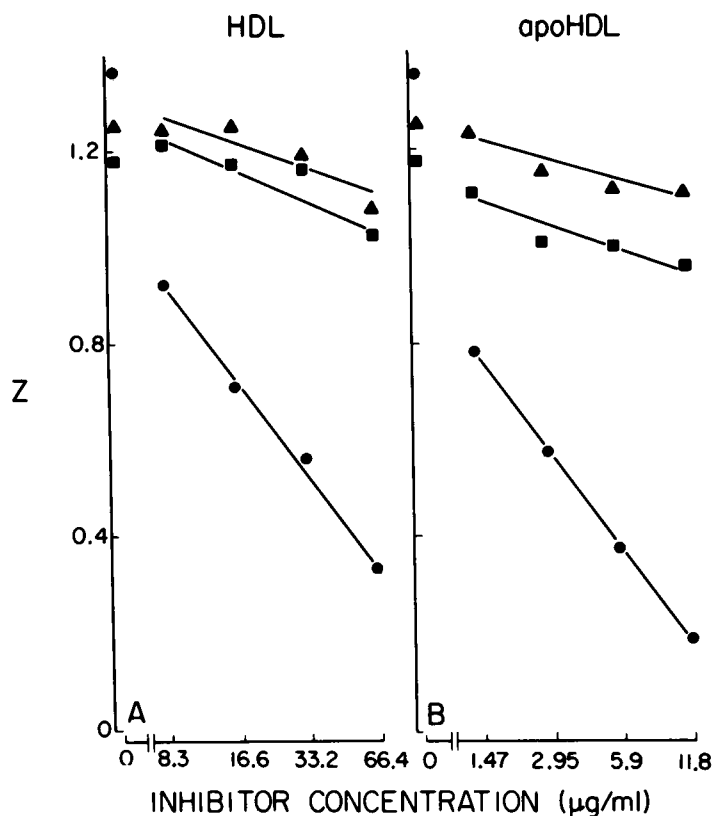


FIGURE 5 EC5b-7 were prepared from normal hu E (●), gpE (▲), and type III E from a patient with PNH (■). EC5b-7 of each type were incubated (60 min, 37°C) with human C8/C9 plus serial dilutions of HDL (panel A) or apoHDL (panel B). The concentration of each inhibitor is expressed in microgram protein per milliliter. Uninhibited lysis, obtained by substituting buffer for inhibitor preparation, was made approximately equal for the three cell types by varying the inputs of C5b6 and C7. Z indicates the average number of lytic sites per cell.

protection and reversibility. After removal of  $Zn^{++}$  the addition of apoHDL in the second incubation (lines 8, 9) did not inhibit further lysis of the unlysed  $E^{\circ}$ , suggesting that the inhibitory action of apoHDL occurs before the damage step(s) influenced by  $Zn^{++}$ . In other experiments not shown, we have demonstrated that apoHDL could inhibit lysis of  $E^{\circ}$  in the concurrent presence of  $2 \times 10^{-4}$   $Zn^{++}$ . Thus, failure of apoHDL to inhibit lysis in step II after  $E^{\circ}$  had been incubated with  $Zn^{++}$  in step I (lines 8, 9) is not attributable to interference with the action of the inhibitor by residual traces of  $Zn^{++}$ . Similarly, apoHDL was effective when incubated with  $E^{\circ}$  in the presence of 0.09 M EDTA during the first step (data not shown).

#### *Role of the target cell in susceptibility to HDL-derived inhibitors*

In the preceding experiments lipoprotein-derived inhibitors were effective in limiting terminal C-me-

diated lysis of both normal human E and sheep E. In similar experiments, HDL, apoHDL, and phenyl-Sepharose pool I were added with human C8 and C9 to EC5b-7 formed with gp E or with the most sensitive (type III) E from a patient with PNH (Fig. 5). The C5b6 and C7 inputs were adjusted to achieve similar uninhibited lysis of all cell types. HDL (panel A) and apoHDL (panel B) had only a minimal effect on terminal lysis of gp EC5b-7 and PNH EC5b-7. In contrast, both preparations were potent inhibitors of terminal lysis of normal human EC5b-7, tested concurrently.

#### *Role of the species source of C8 and C9 in susceptibility to HDL-derived inhibitor*

We studied the ability of apoHDL to limit lysis of normal human EC5b-7 by combinations of gp and hu C8 and C9. The inputs of C8 and C9 in each combination were adjusted to provide similar degrees of uninhibited lysis. Fig. 6 reveals that inhibition was

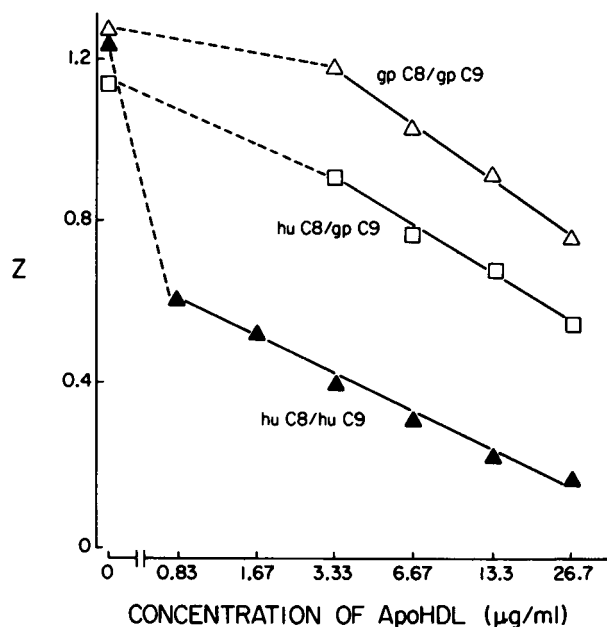


FIGURE 6 Normal human EC5b-7 were lysed by combinations of hu and gp C8 and C9 in the presence of serial dilutions of apoHDL. The ordinate displays lysis expressed as the average number of lytic sites per cell (Z). Uninhibited lysis, obtained by substituting buffer for apoHDL, was made approximately equal for the three cell types by varying the inputs of C8 and C9.

most effective when hu C8 and C9 were used to lyse the cells. The  $I_{50}$  for apoHDL was  $0.78 \mu\text{g/ml}$ . When the combinations hu C8/gp C9 and gp C8/gp C9 were used to lyse the cells, the  $I_{50}$  values were  $23.8 \mu\text{g/ml}$  and  $55.6 \mu\text{g/ml}$ , respectively. The combination gp C8/hu C9, which is known to be ineffective for lysis of normal human EC5b-7 (29), was not used in these studies.

## DISCUSSION

Recent investigations have called attention to several previously unrecognized biological functions of serum lipoproteins in immunological responses. These include (a) the ability of lipoproteins (mainly LDL) to suppress immune responses of human (30) or mouse (31) lymphocytes; (b) a possible precursor role of an apoprotein of some HDL subclasses in the development of secondary (AL) amyloid (32); (c) biologically important actions of HDL: endotoxin complexes in the pathophysiology of endotoxemia and endotoxin shock in rabbits (33, 34); and (d) binding of lipoproteins (HDL and/or LDL) to fluid phase C5b67 complexes to limit reactive lysis of bystander cells (7, 8).

The present report concerns a new function for HDL, and to a greater degree, its apoproteins: the

ability to inhibit C-dependent lysis at a stage after C5b-7, C5b-8, and even C5b-9 complexes, have become bound to the target membrane. This activity is thus distinct from the effects of HDL on fluid-phase C5b-7 complexes studied by the groups cited above (7, 8). In their extensive studies on serum inhibitors of reactive lysis, McCleod, Baker, and Gewurz (35) did note some inhibition of lysis, by relatively crude serum fractions, at a step after C5b-7 binding to target erythrocytes. However, in a subsequent publication from the same laboratory, Lint et al. (7) found that lipoproteins of all classes failed to inhibit the lysis of preformed EC5b67 by limiting amounts of C8 and C9, whereas the same lipoprotein preparations, particularly HDL, were very effective inhibitors of fluid-phase reactive lysis. The latter investigators used gp E to form EC5b67 and we have found that terminal C complexes formed on gp E are far less susceptible to the inhibitory effects of HDL and its apoproteins than are similar complexes formed on sheep or human E (Fig. 5). Thus, an apparent conflict between our data and those of Lint et al. (7) is explained by the type of indicator cell used. Several nonlipid-bearing serum proteins act as inhibitors only on fluid phase C5b-7 or C5b-9. These include antithrombin III (36), S protein (8), and native C8 (37). Two inhibitors, C6 inactivator (38) and the "C8 analogue" of guinea pig serum (39), can inhibit cell bound C1-6 or C5b-7, respectively. Since both of these latter inhibitors are markedly heat labile, and the C8 analogue has not been demonstrated in human serum, it is highly unlikely that such factors account for our findings. Thus, our observations appear to reflect a newly recognized modulatory function influencing the terminal events of the C attack mechanism on a target cell.

In this study, we have used, as a standard assay for screening during purification procedures, inhibition of the lysis of sheep EAC1-7 or hu EC5b-7 by addition of serum inhibitor with excess hu C8 and C9 and, for some studies, we inhibited lysis of EC5b-8 by addition of inhibitor with C9. We have demonstrated that most, if not all, of such inhibitory activity present in human serum is attributable to serum lipoproteins. The bulk of the inhibitory activity adsorbed from serum onto phenyl-Sepharose coelutes with apoA-I of HDL. Although individual fractions were not screened for apoA-II, it is noteworthy that the dominant proteins of the two inhibitory pools from the phenyl-Sepharose columns are apoA-I and apoA-II (Fig. 3).

More complete purification of lipoproteins by sequential CsCl density ultracentrifugation indicated that at least 80% of the inhibitory activity of plasma is associated with HDL and 20% or less with LDL/VLDL. The lysis inhibitory activity per microgram protein of HDL is 2-3 times that of LDL/VLDL

(Table I). Delipidation of HDL with chloroform/methanol significantly increased the inhibitory activity of the apoproteins compared with intact HDL (Table I), and the extracted lipids had no demonstrable inhibitory activity of their own. Furthermore, apoA-I and apoA-II in pure form, free of detectable lipid, were highly (and similarly) active as inhibitors. These observations, together with the observed reduction in inhibitory activity of apoproteins after recombination with extracted lipid, demonstrate that the lysis-inhibiting function of HDL resides in the apoproteins. The theoretical possibility that HDL might act by donating lipids to the target cell membrane is made very unlikely by these findings.

The observation that the ultracentrifugal fractions of HDL with highest inhibitory activity are denser than the bulk of the HDL, and contain apoA-I in excess over apoA-II, suggests that a portion of the HDL-associated apoA-I spontaneously dissociates from the lipoprotein particle, and that this dissociable apoA-I may be the main biologically available inhibitor in plasma. One of the inhibitory peaks from phenyl-Sepharose chromatography of whole serum (pool I) appears to consist mainly of this dissociable apoA-I-rich fraction. Such dissociation of apoA-I from HDL is consistent with the observed exchangeability of apoA-I between lipoprotein classes and subclasses (40).

Additional studies focused on delineation of the stage in the terminal C sequence at which HDL-related inhibitors act to limit lysis. Preincubation of EC5b-7 with purified HDL or apoHDL or with the active peaks eluted from phenyl-Sepharose, followed by washing, did not inhibit subsequent lysis by C8 and C9; nor could apoHDL block the conversion of C5b-7 to C5b-8 sites by C8. Both HDL and apoHDL were only minimally inhibitory when preincubated with human EC5b-8 or sheep EAC1-8 (followed by washing) before addition of C9. Using  $I_{50}$  values, inhibition was 8-100 times less potent when HDL or apoHDL were removed before addition of C9 compared with the inhibition observed when these inhibitors were added together with C9 to EC5b-8 or EAC1-8. Thus, for maximal effectiveness of the HDL inhibitors, fluid phase or cell-bound (see below) C9 must be present simultaneously.

A key observation, illustrated in Table II, is that HDL and purified apoproteins are capable of inhibiting the lysis of preformed EC5b-9 ( $E^\circ$ ), in the absence of any fluid-phase source of C9. These cellular intermediates are formed by allowing C9 to bind to EC5b-8 at reduced temperature and washing away the fluid-phase C9. They are stable in the cold (0-10°C) for at least 1 h, but lyse spontaneously upon warming. It has been shown in studies of sheep EAC1-9 that the hemolytic function of C9 bound at 0°C is susceptible

to inactivation by trypsin before, but not after, the cells have been warmed (27). Hu et al. (41) demonstrated little or no photolabeling of C9 during assembly of C5b-9 at 0°C on liposomes bearing a membrane-restricted photoactivatable probe, whereas C9 was extensively labeled during such assembly at 37°C. Furthermore, Podack et al. (42) state that the binding of C9 to EC5b-8 at 0°C was reversible, with a  $K_a$  of  $\sim 3 \times 10^7 \text{ M}^{-1}$ , whereas the  $K_a$  of C9 for membrane-bound C5b-8 at 37°C was  $\sim 10^{11} \text{ M}^{-1}$ . Collectively, these studies suggest that C9 bound to membranes at low temperatures is not significantly inserted into the lipid bilayer, but that actual insertion of C9 into the membrane, as demonstrated by Hammer et al. (43), occurs only at higher temperatures. The data in Table III demonstrate that the inhibitors can act after C9 binding has occurred, and thus eliminate interference with C9 binding as the major mechanism of inhibition of lysis. For these experiments, inhibitors were added to cold EC5b-9, presumably before complete insertion of C9 into the cell membrane and/or before polymerization of C9 in the C5b-9 complex [42] had taken place. To distinguish among events occurring after C9 binding, the experiments exemplified in Table III were designed utilizing the findings of Frank et al. (26) and Boyle et al. (27, 28) with sheep  $E^\circ$ . These authors found that the presence of 0.09 M EDTA at 37°C allows insertion of C9 into  $E^\circ$  (as indicated by trypsin resistance), and also allows some additional membrane damage step(s) to proceed (as indicated by a kinetic lag between loss of trypsin sensitivity and the onset of  $^{86}\text{Rb}$  release) (27). Although these cells are osmotically protected from lysis by the EDTA, they are permanently damaged and will lyse after removal of EDTA, even at 0°C (26, 27). We found that when inhibitor was added to  $E^\circ$  at 0°C before insertion of C9, whether EDTA was present or absent, lysis was irreversibly inhibited. If the inhibitor was added after the  $E^\circ$  had incubated at 37°C in 0.09 M EDTA to allow insertion of C9 (and presumably the subsequent membrane damage steps) to occur, no inhibition was seen (Table III). This indicates that the apoproteins are not acting to seal already developed transmembrane channels.

Boyle et al. (28) have presented evidence suggesting that  $\text{Zn}^{++}$  ions can reversibly block the postinsertional damage step(s) while still allowing insertion of C9. If these findings using sheep  $E^\circ$  can be extrapolated to the human  $E^\circ$  used in our system, our observation (Table III) that apoHDL was ineffective when added to  $E^\circ$  after preincubation in  $2 \times 10^{-4} \text{ M Zn}^{++}$  would suggest that these inhibitors can act only before the postinsertional damage step(s) have occurred. If the apoproteins actively interfere with insertion of C9 into the membrane, they could do so (a) by displacing reversibly bound C9 before insertion; (b) by binding to

and thus blocking nascent hydrophobic sites on C9 itself (or other nascent hydrophobic portions of the C5b-9 complex); or conceivably (c) by binding to transiently disordered membrane lipids in the vicinity of inserting C5b-9 complexes. Alternatively, if the post-insertional membrane damage step involves polymerization of C9 molecules within the plane of the membrane (42), the apoproteins might interfere with this process. We plan direct binding and membrane extraction studies with radiolabeled C components and apoproteins to resolve some of these issues.

Lipoprotein-derived inhibitors are very inefficient in limiting lysis of gp E and human PNH-III E (Fig. 5), cell types known to be highly sensitive to reactive lysis with human C (7, 10). This observation suggests that the cell membrane may play an active or permissive role in the mechanism of inhibition. The HDL-derived inhibitors are also much less efficient at limiting lysis when gp C8 and C9, rather than human C8 and C9, are used to lyse the cells (Fig. 6). This observed difference in susceptibility to inhibition by human apoHDL lends further support to the hypothesis that gp and hu C9 differ in the mechanism by which they produce cell lysis. The latter hypothesis was suggested by previous studies (29, 44-46) which indicated that differences in the lytic efficiency of gp and hu C9 are related to events occurring after C9 binding. It is of interest that the primary site of action of lipoprotein-derived inhibitors is also at a stage after C9 binding.

The C5b-9 complex is involved in the lysis of bacteria (5, 6) tumor cells (47), parasites (48, 49), and in certain forms of lymphocyte-mediated cytotoxicity (50). Either C9 or the C5b-9 complex has been implicated in several immunopathological processes, including (a) erythrocyte lysis in cold agglutinin disease (51), paroxysmal cold hemoglobinuria (52), and PNH (10, 53); (b) damage to the motor end plate in myasthenia gravis (54); (c) damage to basement membrane of skin in patients with systemic lupus erythematosus and discoid lupus (55); (d) damage to glomerular basement membrane in patients with lupus nephritis (56); and (e) experimental damage to alveolar basement membrane of rabbits in vitro (57). We have shown that the inhibitory activity we are studying is present in whole serum or plasma; that it can protect a human cell (E) from damage by human C5b-9; and that the concentrations of HDL required in our in vitro studies are significantly lower than those present in plasma. Therefore, it is possible that this newly recognized function of HDL and its major apoproteins could play a physiologically significant role in the regulation of both protective and pathologic functions of the membrane attack complex. Furthermore, we believe that the apoproteins or their biologically active fragments will prove to be useful experimentally as molecular probes of the lytic mechanism.

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