Biochimica et Biophysica Acta 812 (1985) 107–115
Elsevier

BBA 72355

High-density lipoprotein and its apolipoproteins inhibit cytolytic activity of complement. Studies on the nature of inhibitory moiety

Charles H. Packman *, Stephen I. Rosenfeld and John P. Leddy

Department of Medicine, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY 14642 (U.S.A.)

(Received June 29th, 1984)

Key words: High-density lipoprotein; Complement inhibitor; Apolipoprotein; (Erythrocyte)

Human high-density lipoprotein (HDL) and its apolipoproteins A-I and A-II inhibit complement-mediated lysis of human and sheep erythrocytes. This inhibitory activity under study is exerted after C9 is bound to membrane-associated C5b-8 complexes but prior to completed assembly and insertion of the C5b-9 complex. In this paper, we define some structure-activity relationships of the inhibitory moiety. With the exception of weak lytic inhibitory activity found in LDL/VLDL pools and in some unconcentrated minor fractions of plasma obtained by hydrophobic chromatography, all inhibitor activity was found in fractions which contained either apolipoprotein A-I, apolipoprotein A-II, or both. Intact HDL has a high level of inhibitor activity but delipidation by chloroform-methanol extraction was associated with an increase in activity on a protein-weight basis. Purified apolipoprotein A-I and apolipoprotein A-II exhibited equal inhibitory activity, greater than that exhibited by intact HDL. Nevertheless, ultracentrifugal fractions in which no free apolipoproteins could be demonstrated still possessed inhibitory activity. These experiments suggest that delipidation of HDL is not necessary for expression of inhibitor activity, although we could not rule out the possibility that apolipoproteins in dynamic equilibrium with HDL are responsible for the inhibitor activity observed in whole serum and plasma and in HDL preparations. Limited proteinase digestion completely abolished the inhibitory activity of partially delipidated HDL. Phospholipase C had little or no effect on the inhibitory activity of delipidated HDL, apolipoprotein A-I or apolipoprotein A-II, but reduced the inhibitory activity of intact HDL. These data suggest that the phospholipid polar headgroups are not necessary for inhibitory activity. However, the loss of these headgroups is associated with decreased activity, possibly due to increased hydrophobicity of HDL, or increased association among HDL micelles, and subsequent decrease in effective molar concentration of the inhibitory moiety.

Introduction

We previously described the capacity of fresh or heat-inactivated (56°C, 3 h) human serum to limit terminal events in complement (C)-mediated lysis of normal human or sheep erythrocytes, and presented evidence that this inhibitory activity resides chiefly in high-density lipoproteins (HDL) and their apolipoproteins [1]. This inhibitory activity of HDL and apolipoproteins was most prominently exerted after C9 had bound to C5b-8 sites on erythrocytes at 0°C, before cell-bound C5b-9 complexes were allowed to produce irreversible membrane damage at 37°C. HDL (or apolipoproteins) were found to limit complement-mediated lysis of either antibody-coated erythrocytes in diluted fresh

* To whom correspondence should be addressed.

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein.

whole serum or unmodified erythrocytes in 'reactive lysis' initiated by purified C5b6 acting in concert with C7–C9. The target membrane itself may influence the C-inhibitory effect of HDL, since lysis of both erythrocytes from normal guinea-pigs and abnormal erythrocytes from humans with paroxysmal nocturnal hemoglobinuria is poorly inhibited, in contrast to cells from normal humans and sheep [1].

This paper describes in detail the isolation and identification of the HDL-related complement inhibitory activity, and explores the relationships between inhibitory capacity and the physicochemical state of the HDL/apolipoprotein molecules including their degree of lipid association.

Materials and Methods

Cells, complement components, and cellular intermediates. Blood from normal human donors was either stored in Alsever's solution at 0°C or frozen in glycerol at −70°C. Sheep and guinea-pig erythrocytes were purchased from Cordis Laboratories (Miami, FL) and Rockland Laboratories (Gilbertsville, PA), respectively. $C1^{gp}$ was isolated [2] from fresh-frozen guinea-pig serum. C5 [3], C5b6 [4], and C9 [5] were isolated from fresh human serum or fresh-frozen plasma. Human C2, C3, C5, C6, C7, C8, and C9 were purchased from Cordis Laboratories. Sheep $EAC1^{gp}$, $4\text{-}8^{hu}$ (sheep EAC1-7) [6] and human $EC5b\text{-}7^{hu}$ [7] were prepared according to published methods. C assays were performed in barbital-buffered saline (pH 7.4) containing 0.1% gelatin and 0.02 M EDTA (barbital buffer-EDTA) or in phosphate-buffered saline (pH 7.4) containing 0.005 M EDTA (phosphate buffer-EDTA).

Hemolytic assay for inhibitor. Inhibitor activity was assayed as previously described [1]. Briefly, inhibitor, human C8 and C9 were added to sheep EAC1-7 (or, for some experiments, to human EC5b-7). Following incubation for 60 min at 37°C, the degree of hemolysis was determined by measuring the absorption of the supernatants at 413 nm. The number of effective hemolytic sites per cell (Z) was calculated [1]. The concentration of inhibitor source producing 50% reduction in Z was determined graphically from a semilog plot of Z versus inhibitor concentrations (inhibitory titer).

Hydrophobic chromatography. A 1.6×70 cm column of phenyl-Sepharose CL-4B (Pharmacia, Piscataway, NJ) was prepared. Preliminary experiments established that all inhibitory activity was retained on the column when serum made 50% (by volume) in ethylene glycol was applied and eluted with 50% ethylene glycol in 10 mM phosphate buffer (pH 6.8). Subsequent purifications therefore utilized 25 ml fresh fasting human serum which had been dialyzed overnight at 4°C against 10 mM phosphate buffer (pH 6.8) containing 50% (v/v) ethylene glycol (Fisher Certified, Fisher Scientific Company, Pittsburg, PA) (starting buffer). This dialyzed serum was applied to the phenyl-Sepharose column equilibrated in the same buffer and run at 25 ml/h. After washing with 400 ml starting buffer, a linear gradient of 200 ml starting buffer and 200 ml deionized H_2O was applied. After washing with an additional 200 ml H_2O, a second linear gradient of 200 ml H_2O and 200 ml 95% ethanol was applied. Fractions were dialyzed against phosphate buffer-EDTA and assayed for apolipoprotein A-I antigen by Ouchterlony analysis, for inhibitory activity by hemolytic assay, and for C5, C6, C7, C8, and C9 by microtiter hemolytic assay. In some experiments, HDL obtained by CsCl density gradient ultracentrifugation (see below) was applied to the column instead of serum, and the column was eluted as described above.

Purification of HDL and apolipoprotein A-I and A-II. Human HDL was purified by CsCl ultracentrifugation of fresh EDTA plasma using a modification [1] of the method of Lux et al. [8]. The LDL/VLDL-containing fractions were tested for inhibitory activity without further purification. In representative runs, 80–90% of the inhibitory activity was in the HDL pools and only 10–20% was found in LDL/VLDL pools. Some HDL samples were dialyzed against phosphate buffer-EDTA and stored for up to 2 weeks at 0°C for functional inhibitory assays. Other aliquots were lyophilized and delipidated with chloroform/methanol (2 : 1, v/v) to make delipidated HDL [1] from which apolipoproteins A-I and A-II were isolated [9].

Ultracentrifugal analysis of inhibitor samples. Using CsCl ultracentrifugation, purified HDL was compared with inhibitor-containing pools from phenyl-Sepharose chromatography. 1-ml samples of HDL or inhibitor pools (680–3000 μg protein)

were mixed with 4 ml phosphate buffer-EDTA and adjusted to a density of 1.210 with cesium chloride. After centrifugation at 60 000 rpm in a Beckman SW-65-K rotor for 42 h at 15°C, 11 fractions were prepared from each tube. Densities of each fraction were calculated from the refractive index (Bausch and Lomb refractometer, Rochester, NY). The fractions were then dialyzed against phosphate buffer-EDTA to remove CsCl prior to assays for protein content, inhibitory activity and electrophoretic behavior.

For some experiments, we ultracentrifuged fresh plasma at *d* 1.063 in CsCl. The bottom fraction containing HDL and plasma proteins was recentrifuged at *d* 1.21. Of five fractions from this run, the bottom four were saved for crossed immunoelectrophoresis (see below). The top fraction was washed once by recentrifugation at *d* 1.21 and was saved for crossed immunoelectrophoresis. A concentrated pool from phenyl-Sepharose was similarly ultracentrifuged at *d* 1.21 and divided into five fractions. The above fractions, plus whole serum, were analyzed by crossed immunoelectrophoresis against anti-apolipoprotein A-I. Apolipoprotein A-I in serum and in EDTA plasma exhibited similar electrophoretic behavior.

Electrophoretic methods. Polyacrylamide gel electrophoresis in 0.1% SDS was performed by the method of Laemmli [10] using a 3% acrylamide stacking gel and a linear gradient of 7.5–20% acrylamide for the running gel. In some experiments, 5% 2-mercaptoethanol was included in the sample buffer to reduce disulfide bonds. Crossed immunoelectrophoresis was performed on microscope slides, using 1% agarose in 0.02 M barbital buffer (pH 8.4) containing 0.01 M EDTA. The second-dimension gel contained 3–10% rabbit antiserum to apolipoprotein A-I [1].

Enzyme treatment. Highly purified phospholipase C from *B. cereus* was obtained from the laboratory of Dr. L.L.M. Van Deenen, University of Utrecht, The Netherlands, through the kind cooperation of Dr. Guido Marinetti, Department of Biochemistry, University of Rochester. This preparation had activity of 137 I.U. per mg protein, and contained a single protein band by SDS-polyacrylamide gel electrophoresis. Phospholipase C (final concentration 1–20 I.U./ml) was incubated with various inhibitor preparations (final concentrations 28–340 μg protein/ml) at 37°C for 20–60 min in phosphate buffer-EDTA; the mixture was then assayed for inhibitory activity (see below) and for changes in electrophoretic mobility. Phospholipase C alone had no effect on the hemolytic assay for inhibition. Phospholipase C contained no detectable proteolytic activity by the denatured hemoglobin method [11]. The effects of TPCK trypsin (Millipore Corp.) and *S. aureus* proteinase V-8 (Miles Pharmaceuticals) were assayed in a similar manner.

Antisera. A potent monospecific rabbit antiserum to apolipoprotein A-I was produced as described [1]. Goat anti-human-α_1-lipoprotein, and anti-human-β-lipoprotein were purchased from Calbiochem-Behring Corporation (La Jolla, CA).

Results

Initial physicochemical characterization and isolation

Attempted batch preparation (euglobulin or $(NH_4)_2SO_4$ precipitation), gel filtration (Sephadex G-200), or DE-52 cellulose chromatography of either 3-h heated or fresh human serum were ineffective in isolating proteins possessing the inhibitory activity. Starch-block electrophoresis revealed that the bulk of the inhibitory activity was found in the α-globulin region, but smaller peaks were found in other electrophoretic zones. A number of these inhibitory fractions from chromatographic or electrophoretic procedures displayed, in addition to their ability to inhibit lysis at the C8-C9 step, the capacity to inhibit uptake of nascent, fluid phase C5b-7 by human or sheep erythrocytes, as had been reported by others for lipoproteins and for S-protein [12,13]. These initial observations, plus the knowledge that the terminal lytic events in the complement sequence depend largely upon hydrophobic interactions, led us to adopt methods better suited to proteins with hydrophobic or lipoprotein properties.

Hydrophobic chromatography

Fig. 1 shows the elution profile of human serum proteins from a typical phenyl-Sepharose column developed as outlined in Materials and Methods, together with the peaks of lysis-inhibiting activity. We also performed hemolytic assays for native

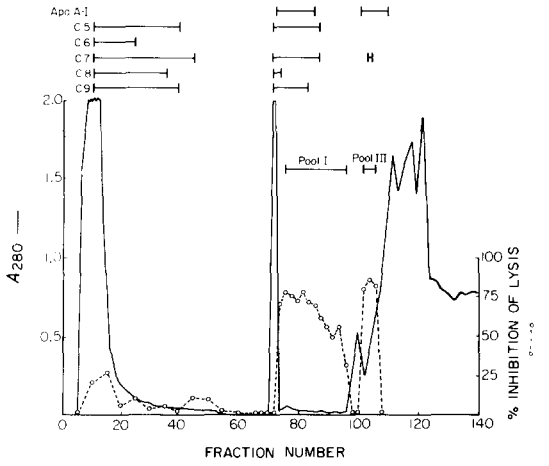

Fig. 1. Hydrophobic chromatography of fresh human serum on phenyl-Sepharose. The conditions employed are given in Materials and Methods. The starting buffer-distilled water gradient was started at fraction 42. The distilled water-95% ethanol gradient was started at fraction 88. Ethanol wash began at fraction 111. Absorbance (————) and percent inhibition of lysis (— — —) (see text) are indicated, as are the elution position of apolipoprotein A-I (apo A-I) and complement components C5 through C9. The latest fractions eluted (starting around fraction 110) were very turbid but contained minimal protein by folin analysis.

complement components, C5 through C9, shown as bars in Fig. 1. The main inhibitory activity is eluted in a broad peak (fractions 76–96, pool I) during the distilled-water gradient and subsequent wash. This is followed by a few fractions containing no inhibitory activity (pool II). A second sharp peak of inhibitory activity is found during the early portion of the water-to-ethanol gradient (fractions 102–106, pool III). Smaller peaks of inhibitory activity found in the main fall-through fraction and during the early part of the ethylene glycol-to-water gradient were inconsistently found in several similar fractionations and have not been further characterized. Later fractions (after 110) were turbid and thus exhibited a high absorbance at 280 nm, but demonstrated little protein by Folin analysis and presumably contained lipids. By Ouchterlony immunodiffusion, apolipoprotein A-I antigen eluted from the column only in the fractions containing inhibitory activity, although some unconcentrated fractions possessing weak inhibitory activity did not contain detectable apolipoprotein A-I antigen by this method. Pools I and III were concentrated (Amicon PM10). Pool II (fractions 97–101), similarly concentrated, contained no apolipoprotein A-I or lysis inhibitory activity. After concentration, some C5, C7, C8, and C9 were detected in pool I and traces of C7 and C9 were found in pool III. Pool III, but not pool I, gave a faint reaction in Ouchterlony immunodiffusion against anti-β-lipoprotein.

Electrophoretic analysis

Analysis of unreduced samples of representative inhibitor preparations in a 7.5–20% polyacrylamide gradient gel with 0.1% SDS is shown in Fig. 2. A major band at approx. 27 kDa, present in all preparations, corresponds to apolipoprotein A-I [9]. The other major band at approx. 16 kDa in the unreduced state (and approx. 8 kDa in the reduced state, not shown) corresponds to apolipoprotein A-II [9]. Apolipoprotein A-I appears in somewhat higher concentration than apolipoprotein A-II in the ultracentrifugally purified delipidated HDL shown in track a. HDL prior to delipidation ex-

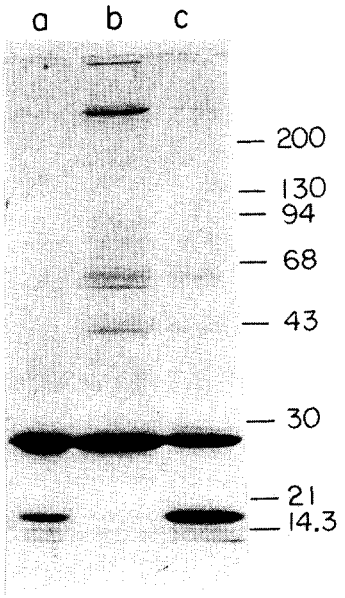

Fig. 2. SDS-polyacrylamide gel electrophoresis (unreduced) 7.5–20% gradient, Coomassie blue stain of inhibitor preparations. Track a, ultracentrifugally purified, delipidated HDL. Track b, inhibitor pool I eluted from phenyl-Sepharose. Track c, inhibitor pool III eluted from phenyl-Sepharose. The positions of the molecular weight ($\times 10^3$) markers are indicated.

hibited identical electrophoretic bands (not shown). Inhibitor pool I from the phenyl-Sepharose column (Fig. 1) has a major band of apolipoprotein A-I, but only traces of apolipoprotein A-II (Fig. 2, track b). The higher molecular weight bands in this preparation presumably represent other hydrophobic proteins eluted by distilled water from the phenyl-Sepharose column. This pool was eluted under less hydrophobic conditions than pool III, and was relatively deficient in lipid (particularly cholesterol), as well as in apolipoprotein A-II, compared to HDL [1]. Pool I possesses high activity in the inhibition assay, slightly higher than that of delipidated HDL on a protein-weight basis. Inhibitor pool III (track c), which was eluted from the phenyl-Sepharose column at the beginning of the ethanol gradient, displays approximately equal amounts of apolipoprotein A-I and apolipoprotein A-II and is enriched in apolipoprotein A-II compared to the HDL preparation. Pool III contains approximately half the phospholipid and cholesterol of HDL and its inhibitory activity is equal to that of delipidated HDL. Similar pools from other phenyl-Sepharose columns sometimes contained more apolipoprotein A-I than apolipoprotein A-II, but were still enriched in apolipoprotein A-II relative to HDL. Purified apolipoprotein A-I and apolipoprotein A-II are approximately equal in inhibitory activity per protein content, intermediate between HDL and delipidated HDL [1].

Ultracentrifugal analysis of HDL and phenyl-Sepharose-derived inhibitor pools

Table I shows the density, inhibitory activity, and relative quantities of apolipoprotein A-I and apolipoprotein A-II in fractions derived from CsCl density gradient ultracentrifugation of HDL and phenyl-Sepharose pools I and III. Apolipoprotein A-I and apolipoprotein A-II concentrations were estimated from the density of Coomassie blue-stained bands in SDS-polyacrylamide gel electrophoresis. The inhibitor assay for this experiment employed washed human EC5b-8, whose lysis was tested by addition of C9 with or without the simultaneous addition of inhibitor. The inhibitor activity in each preparation is highest in fractions having d of 1.21–1.31 g/ml; with HDL, considerable inhibitory activity occurs in lighter fractions as well. The relatively dense fractions, presumably

TABLE I

DENSITY DISTRIBUTION OF INHIBITORY ACTIVITY AND APOLIPOPROTEINS A-I AND A-II IN HDL AND PHENYL-SEPHAROSE-DERIVED INHIBITOR POOLS

Fract. No. [a]	HDL					Phenyl-Sepharose pool I					Phenyl-Sepharose pool III				
	d (g/ml)	Inh. act. [b]	apo A-I [c]	apo A-II [c]	protein (μg/ml) [d]	d (g/ml)	Inh. act.	apo A-I	apo A-II	protein (μg/ml)	d (g/ml)	Inh. act.	apo A-I	apo A-II	protein (μg/ml)
11	<1.10	33%	++	+	410	<1.10	0%	0	0	0	<1.10	18%	+	tr	170
10	1.11	52	+++	++	975	1.11	0	0	0	0	1.10	0	tr	0	20
9	1.14	61	++++	+++	1970	1.13	0	0	0	0	1.12	3	tr	0	60
8	1.16	68	++++	+++	1760	1.17	0	0	0	0	1.15	18	+	+	100
7	1.18	71	+++	++	620	1.19	3	tr	0	20	1.17	40	++	+	200
6	1.21	74	++	tr	260	1.22	55	tr	0	50	1.21	55	+	+	120
5	1.26	82	++	0	230	1.28	85	+++	0	580	1.26	78	+	tr	130
4	1.31	80	++	0	230	1.33	53	+	0	490	1.31	81	++	0	370
3	1.36	18	tr	0	22	1.38	0	0	0	80	1.36	14	tr	0	100
2	1.42	4	0	0	10	1.44	2	0	0	10	1.43	0	0	0	20
1	1.47	0	0	0	10	1.51	0	0	0	0	1.50	3	0	0	40

[a] Drop-wise fractionation from bottom of tubes. HDL and the phenyl-Sepharose pools were run concurrently (see Materials and Methods).

[b] Inhibition activity as percent inhibition of lytic sites (Z) produced by coaddition (with C9) of a 1/5 dilution of each dialyzed fraction to hu EC5b-8.

[c] Visual estimation from Coomassie blue-stained SDS polyacrylamide gel electrophoresis analysis. apo, apolipoprotein; tr, trace.

[d] Folin reaction (human serum albumin standard).

low in lipid, contain little or no apolipoprotein A-II. Although fractions of density greater than 1.21 from the HDL preparation contain a small fraction of the total protein, they contain a large proportion of the inhibitory activity from this preparation. Phenyl-Sepharose pool I, known from other experiments to be partially lipid-depleted and deficient in apolipoprotein A-II, has the bulk of its protein in this density range, consistent with its high-specific inhibitory activity. Pool III was more hydrophobic than Pool I by phenyl-Sepharose chromatography. As opposed to pool I, pool III contains some inhibitory activity and apolipoproteins in the lightest fractions, similar to HDL. Furthermore, apolipoprotein A-II and apolipoprotein A-I are both found in fractions of density 1.15–1.17 in pool III along with inhibitor activity. Neither apolipoprotein nor inhibitor activity is found in this density range in pool I. Nevertheless, the most active inhibitory fractions from pool III are in the same density range (1.21–1.31) as that found for phenyl-Sepharose pool I.

In similar experiments, fresh serum and fractions from ultracentrifugation of partially purified HDL and pool III were analyzed by crossed immunoelectrophoresis using anti-apolipoprotein A-I (see Materials and Methods for details). In Fig. 3, fraction 5 is the lowest density fraction, approximately analogous to fractions 9–11 in Table I. Fraction 1 in Fig. 3 is the most dense fraction, analogous to fractions 1 and 2 in Table I. Apolipoprotein A-I in serum and in plasma (the HDL starting material) exhibits a single peak of anodal mobility, whereas apolipoprotein A-I in pool III exhibits two peaks with more cathodal mobility. Crossed immunoelectrophoresis of phenyl-Sepharose pool I (not shown) gave a pattern similar to that of pool III. Apolipoprotein A-I antigen of pool III was found by this method only in relatively dense fractions of the gradient (fractions 2 and 3); in contrast, the HDL preparation contained apolipoprotein A-I antigen of anodal mobility in the lightest fractions (5 and 4) and antigen of more cathodal mobility in heavier fractions (4, 3 and 2). Apolipoprotein A-I in both freshly prepared and stored HDL (0°C, up to 6 weeks) exhibited similar anodal mobility, and was consistently slightly more anodal than apolipoprotein A-I in serum. Delipidation of HDL by chloroform-methanol caused total conversion of apolipoprotein A-I from anodal to cathodal mobility (not shown).

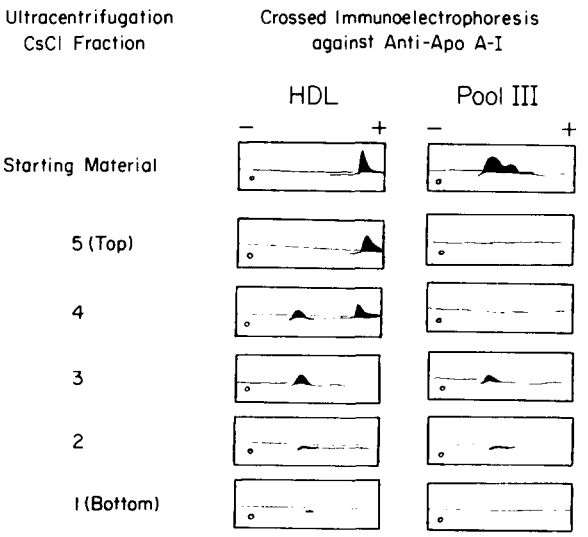

Fig. 3. CsCl ultracentrifugation of HDL and phenyl-Sepharose pool III: analysis of fractions by crossed immunoelectrophoresis against anti-apolipoprotein A-I. Fresh EDTA plasma, the starting material for HDL, yielded an electrophoretic pattern identical to that shown above for serum. See Materials and Methods for sources of fractions. Direct tracings of the dried, Coomassie blue-stained gels are shown.

The role of protein-bound lipid

In an effort to evaluate a possible role for lipid in the complement inhibitor activity, we studied the effect of phospholipase C on various inhibitor preparations. Several initial experiments indicated that 60 min incubation (at 37°C) of HDL with highly purified phospholipase C (0.007–0.12 I.U./μg HDL protein) produced dose-dependent reduction in inhibitory activity. Higher concentrations of phospholipase C (0.04–0.6 I.U./μg protein) were required to inactivate most of the C inhibitory function of delipidated HDL. SDS-polyacrylamide gel electrophoresis revealed that phospholipase C treatment of delipidated HDL caused a partial shift of the 28 kDa apolipoprotein A-I band to one or two bands of slightly faster mobility. With minimal phospholipase C treatment, only the slower of these two bands was seen; however, progressive conversion of native apolipoprotein A-I to the faster of the two bands

occurred with longer incubation or higher phospholipase C doses. Similar bands appear progressively in delipidated HDL or purified apolipoprotein A-I upon storage at 0°C, but not at −70°C. Mild phospholipase C treatment (or storage) of HDL does not produce these changes, but more vigorous phospholipase C treatment (0.05 I.U. phospholipase C/μg HDL for 60 min at 37°C) of HDL does yield small amounts of both faster bands in SDS polyacrylamide gel electrophoresis (not shown).

The experiment shown in Fig. 4 employed more limited phospholipase C treatment: HDL, delipidated HDL, purified apolipoprotein A-I and apolipoprotein A-II were incubated for 20 min at 37°C with 0.006 and 0.018 I.U. phospholipase C/μg inhibitor protein. Appropriate dilutions of each inhibitor/phospholipase C mixture were added to EAC1-7 and C8 and C9, and the percent inhibition of effective lytic sites (Z) was tested as a function of phospholipase C added. Phospholipase C alone had no effect on the hemolytic assay. Although significant loss of inhibitory activity of HDL (65% inhibition to 42%) occurred, no change in electrophoretic pattern is seen. On the other hand, although little or no loss of inhibitory activity was seen with phospholipase C treatment of delipidated or highly purified apolipoproteins, apolipoprotein A-I in these preparations showed conversion to one or two slightly faster moving bands. No low molecular weight peptides were visualized on this 7.5–20% acrylamide gel, however. No effect of phospholipase C on apolipoprotein A-II was seen by SDS-polyacrylamide gel electrophoresis or inhibitory assay.

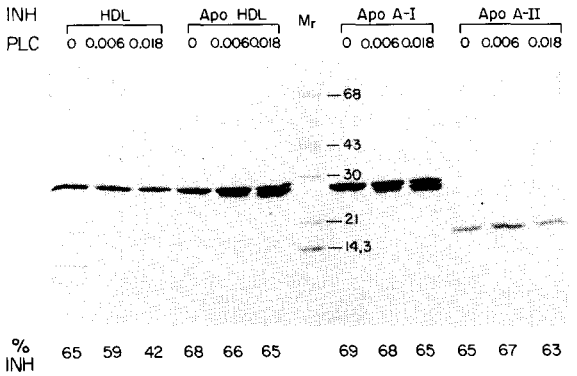

Fig. 4. SDS-polyacrylamide gel electrophoresis of inhibitor (INH) preparations treated with phospholipase C (PLC). The 7.5–20% acrylamide gradient slab gel was stained with Coomassie blue. Inhibitor preparations and doses of phospholipase C (in I.U./μg inhibitor protein) are indicated above the tracks (apo HDL, chloroform-methanol delipidated HDL; Apo A-I and Apo A-II, apolipoproteins A-I and A-II). The percent inhibition of lysis following treatment of the inhibitor preparations by phospholipase C is indicated below each track. The positions of molecular weight ($\times 10^3$) markers are indicated in the center track.

The hemoglobinase assay [11] of phospholipase C for proteinase activity was negative. In this regard, very limited treatment of delipidated HDL with TPCK trypsin or *S. aureus* proteinase V-8 (1 : 100, w/w, for 1 min at 37°C) produced total loss of inhibitor activity with complete loss of native apolipoprotein A-I and the appearance of lower molecular weight products similar to that seen with phospholipase C, but without apparent effect on apolipoprotein A-II (not shown). This suggests that trace contamination of the phospholipase C with a proteinase could have produced the observed changes. However, longer incubation with these two known proteinases (more than 10 min) produced extensive breakdown of both apolipoprotein A-I and apolipoprotein A-II to smaller fragments, whereas even 60 min incubation with 4 : 1, w/w of phospholipase C led to complete conversion of apolipoprotein A-I to the slightly faster product, but without further breakdown of apolipoprotein A-I or visible change in apolipoprotein A-II.

Discussion

We have previously demonstrated that HDL can inhibit complement-mediated lysis of erythrocytes at a late stage of the membrane attack sequence, even after C9 is bound [1]. Isolated apolipoproteins A-I and A-II are more potent inhibitors on a protein-weight basis than intact HDL but the most potent inhibitory activity is expressed by HDL partially delipidated by chloroform/methanol and by phenyl-Sepharose pools I and III. This paper explores the lipid-protein interactions within HDL fractions separated by various means and attempts to determine structure-activity relationships.

Three different procedures were used to purify inhibitor activity from human serum or plasma:

density gradient (CsCl) ultracentrifugation of fresh plasma, hydrophobic chromatography of fresh serum on phenyl-Sepharose and isolation of apolipoprotein A-I and apolipoprotein A-II from HDL using gel filtration chromatography in 8 M urea. With the exceptions of modest inhibitor activity in VLDL/LDL fractions from density gradient ultracentrifugation and minor fractions obtained by phenyl-Sepharose chromatography, all inhibitor-containing fractions exhibited either apolipoprotein A-I, apolipoprotein A-II or both.

That delipidation of HDL caused increased inhibitory activity is best illustrated by ultracentrifugal analysis of HDL and phenyl-Sepharose pools I and III (Table I). Maximal inhibitor activity was found in fractions with density of 1.26–1.33 mg/ml. In the phenyl-Sepharose pools (pools I and III), these fractions contained the bulk of the protein applied to the CsCl and the bulk of apolipoprotein A-I, suggesting that much of the native HDL in serum was partially delipidated during isolation of inhibitor activity. Most or all of the apolipoprotein A-II in HDL and phenyl-Sepharose pool III remained in fractions of density 1.10–1.21, more typical of intact HDL, suggesting that apolipoprotein A-II remained more tightly bound to lipid than apolipoprotein A-I during hydrophobic chromatography. Crossed immunoelectrophoresis against anti-apolipoprotein A-I revealed that all apolipoprotein A-I in phenyl-Sepharose pool III migrated cathodally with reference to apolipoprotein A-I in whole serum (Fig. 3). When the densest fraction of serum obtained from an ultracentrifugation at density 1.063 was recentrifuged at density 1.21, anodally migrating apolipoprotein A-I appeared in the light fractions and cathodally migrating apolipoprotein A-I in denser fractions. In whole serum and in purified HDL, all detectable apolipoprotein A-I antigen is of anodal mobility. This is consistent with other evidence that apolipoprotein A-I and HDL lipid are in dynamic equilibrium [14,15], and during ultracentrifugation, these two molecules become physically separated to a significant degree. This dissociated apolipoprotein A-I has a distinctive beta electrophoretic mobility.

Intact HDL as found in the lightest fractions obtained by CsCl ultracentrifugation retains considerable inhibitory activity. This preparation contains no apolipoprotein A-I with the cathodal mobility characteristic of dissociated apolipoprotein A-I, suggesting that apolipoprotein A-I does not have to be lipid-free to exert inhibitory activity. Whole serum and plasma, both of which possess inhibitory activity, do not in fact, contain detectable cathodal (free) apolipoprotein A-I by crossed immunoelectrophoresis. Nevertheless, this does not eliminate the possibility that apolipoprotein A-I in dynamic equilibrium with intact HDL is the actual inhibitory moiety affecting C lysis under the assay conditions we employ. Evidence for heterogeneity in the dissociability of serum apolipoprotein A-I was obtained by phenyl-Sepharose chromatography (Fig. 1). Pool I appears to contain the portion of apolipoprotein A-I that is most easily dissociated from its lipid (Table I). Pool III contains apolipoprotein A-I that appears to be more tightly bound to lipid, and is eluted together with apolipoprotein A-II (Table I) and lipid, particularly cholesterol [1]. The high inhibitory activity of the easily dissociable fraction of apolipoprotein A-I might account for the bulk of inhibitory activity in whole serum and HDL, even though free apolipoprotein A-I is not detectable by the methods employed. The missing ingredient could be a hydrophobic surface to enhance and stabilize dissociation of apolipoprotein A-I from HDL. Both phenyl-Sepharose and cell membranes containing C5b-9 could provide such a surface. Analogous observations have been made with exchange of apolipoproteins with liposomes [16,17].

Experiments testing inhibitor sensitivity to lipolytic and proteolytic enzymes were intended to screen for the relative contributions of lipid and protein components, respectively, to inhibitory activity. Although phospholipase C treatment of HDL reduced its inhibitory activity, there was no change in SDS-polyacrylamide gel electrophoretic behavior of apolipoprotein A-I and apolipoprotein A-II in phospholipase C-treated HDL. In contrast, phospholipase C treatment of purified apolipoprotein A-I or delipidated HDL resulted in progressive alteration of the electrophoretic migration of apolipoprotein A-I (but not apolipoprotein A-II), suggesting an apparent reduction in molecular weight of 3000–5000. This was not accompanied by a change in inhibitor activity (Fig. 4). These observations are not likely to be caused by con-

taminating proteolytic enzymes in this highly purified phospholipase C, since (a) there is a lack of concordance between changes in molecular weight of apolipoprotein A-I and inhibitor activity; (b) there was no detectable cleavage of acid-denatured hemoglobin by phospholipase C, and (c) even very limited digestion of delipidated HDL with TPCK trypsin and *S. aureus* proteinase V-8 produced complete conversion of apolipoprotein A-I to lower molecular weight fragments and complete loss of inhibitor activity. We suspect that the apparent change in molecular weight is due to a change in tertiary structure of the apolipoprotein A-I molecule brought about by cleavage of tightly bound phospholipid. As another intriguing possibility, these data could suggest that there is a small amount of lipid covalently linking a small peptide subunit to apolipoprotein A-I by a bond susceptible to phospholipase C, but this lipid and peptide are not necessary for inhibitory activity.

In these experiments, the greater amount of lipid in intact HDL seemed to protect apolipoprotein A-I from cleavage by phospholipase C. To account for the loss of inhibitor activity, we postulate that the removal of polar headgroups from HDL phosholipid by phospholipase C resulted in increased hydrophobicity and thus, greater self-association among HDL micelles. If micellar size were increased by coalescence of several particles, this could reduce the effective molar concentration of the inhibitor moiety. The sparse distribution of terminal C lytic sites on the erythrocyte surface makes it unlikely that one particle could interact with more than one lytic site. Alternatively, if dissociation of apolipoprotein A-I from the HDL micelle is required for inhibition, the increased hydrophobicity of the lipid moiety of HDL produced by phospholipase C treatment might alter the equilibrium to enhance apolipoprotein A-I binding to HDL. These alterations would not be detectable by electrophoresis.

Acknowledgements

The authors thank Amy McNally, Patricia Theim and Deborah Beyer for excellent technical assistance and Beth Bush for typing the manuscript. This work was supported by the U.S. Public Health Service Grants, PO1-HL-18208, R23-HL-22742, Biomedical Research Support Grant RR-0-5403 to the University of Rochester, P50-AI-15372, RO1-AI-16089, and by the David Welk Memorial Fund.

References

1 Rosenfeld, S.I., Packman, C.H. and Leddy, J.P. (1983) J. Clin. Invest. 71, 795–808
2 Nelson, R.A., Jr., Jensen, J., Gigli, I. and Tamura, N. (1966) Immunochemistry 3, 111–135
3 Nilsson, U.R., Tomar, R.H. and Taylor, F.B., Jr. (1972) Immunochemistry 9, 709–723
4 Yamamoto, K. and Gewurz, H. (1978) J. Immunol. 120, 2008–2015
5 Biesecker, G. and Muller-Eberhard, H.J. (1980) J. Immunol. 124, 1291–1296
6 Rosenfeld, S.I., Kelly, M.E. and Leddy, J.P. (1976) J. Clin. Invest. 57, 1626–1634
7 Packman, C.H., Rosenfeld, S.I., Jenkins, D.E., Jr., Thiem, P.A. and Leddy, J.P. (1979) J. Clin. Invest. 64, 428–433
8 Lux, S.E., Levy, R.I., Gotto, A.M. and Fredrickson, D.S. (1972) J. Clin. Invest. 51, 2505–2519
9 Scanu, A., Toth, J., Edelstein, C., Kogh, S. and Stiller, E. (1969) Biochemistry 8, 3309–3316
10 Laemmli, U.K. (1970) Nature (Lond.) 227, 680–685
11 Cohn, Z.A. and Weiner, E. (1963) J. Exp. Med. 118, 991–1008
12 Lint, T.F., Behrends, C.L. and Gewurz, H. (1977) J. Immunol. 119, 883–887
13 Podack, E.H., Kolb, W.P. and Muller-Eberhard, H.J. (1978) J. Immunol. 120, 1841–1848
14 Shepherd, J., Patsch, J.R., Packard, C.J., Gotto, A.M., Jr. and Taunton, O.D. (1978) J. Lipid Res. 19, 383–389
15 Grow, T.E. and Fried, M. (1977) Biochem. Biophys. Res. Commun. 75, 117–124
16 Tall, A.R., Small, D.M., Deckelbaum, R.J. and Shipley, G.G. (1977) J. Biol. Chem. 252, 4701–4711
17 Massey, J.B., Gotto, A.M., Jr. and Pownall, H.J. (1981) Biochemistry 20, 1575–1584