LOW DENSITY LIPOPROTEIN AND VERY LOW DENSITY LIPOPROTEIN ARE SELECTIVELY BOUND BY AGGREGATED C-REACTIVE PROTEIN*

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Low density lipoprotein (LDL)[1] is a major class of plasma lipoprotein, the metabolism and biological interactions of which are of particular interest in view of the association between elevated levels and the incidence of coronary heart disease (1, 2). There is much evidence implicating abnormal deposition of LDL in arterial walls in the pathogenesis of atherosclerosis, although the molecular mechanisms of this deposition are not well understood (3, 4). LDL is derived by metabolism of very low density lipoproteins (VLDL), and both these classes of lipoprotein particles, as well as high density lipoprotein (HDL), consist of apoproteins together with varying proportions of phospholipid, triglyceride, esterified, and nonesterified cholesterol (5).

C-reactive protein (CRP) is a plasma protein the concentration of which increases from normal values of <1 mg/liter to as much as several hundred mg/liter in response to most forms of tissue injury, inflammation, or infection (6, 7). It is known to bind in vitro to phospholipids in the form of liposomes (8) or in modified cell membranes (9), and CRP may be deposited on damaged or necrotic cells in vivo (10–12). These reactions involve two binding sites in the CRP molecule, one, which is calcium-dependent, binds phosphorylcholine residues (13–15), whereas the other binds polycations (16). However, CRP does not bind to the intact membranes of normal healthy cells either in vivo or in vitro, and in experiments with phosphatidyl choline/cholesterol liposomes, in vitro inclusion of a proportion of lysophosphatidyl choline has been found necessary to promote significant binding by CRP (8, 9).

The in vivo function of CRP is not known, though it is presumably both important and generally beneficial to the organism because proteins that closely resemble human CRP both in structure and in their calcium-dependent capacity to bind phosphoryl-

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[1] *Abbreviations used in this paper:* apoB, apolipoprotein B; CNBr, cyanogen bromide CRP, C-reactive protein; HDL, high density lipoprotein; LDL, low density lipoprotein; NHS, normal human serum; PAGE, polyacrylamide gel electrophoresis; SAP, serum amyloid P component; SDS, sodium dodecyl sulphate; Tris-saline-Ca, 0.01 M Tris buffered 0.14 M NaCl pH 8.0 containing 0.002 M $CaCl_2$; Tris-saline-EDTA, 0.01 M Tris buffered 0.14 M NaCl pH 8.0 containing 0.01 M EDTA; VLDL, very low density lipoprotein.

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choline have been stably conserved throughout vertebrate evolution (17, 18). A homologous phosphorylcholine-binding protein has even been recognized recently in an invertebrate, *Limulus polyphemus*, the horseshoe crab (19, 20).

There are reports (21–25) in the earlier literature of association between CRP and serum lipoproteins during isolation of CRP, but estimates of the molecular size of CRP in human or rabbit serum did not suggest that CRP is complexed to a significant extent within the circulation (26). Interactions under other circumstances between CRP and plasma constituents containing phospholipids or other potential ligands for CRP have not previously been investigated. We have reexamined the molecular form of CRP in acute phase serum and confirmed that it exists in the free state and is not complexed with macromolecular ligand(s). However, we report here for the first time that when isolated CRP is aggregated on a solid phase at a sufficient density, it acquires the capacity to selectively bind LDL and traces of VLDL from whole human serum. This interaction, occurring only after aggregation of CRP molecules has taken place, may have important implications both for the function of CRP and the clearance and metabolism of LDL.

Materials and Methods

Proteins. CRP was isolated in pure form as described elsewhere (27, 28). Briefly, CRP was obtained by calcium-dependent affinity chromatography on a column of Sepharose-cyanogen bromide (CNBr)-pneumococcal C-polysaccharide and then gel filtered on Ultrogel AcA44 (LKB Instruments, Ltd., London, England) in the presence of calcium ions. To ensure removal of any trace contaminants, the CRP was passed over a column of Sepharose to which polyvalent anti-normal human serum (NHS) antibodies were coupled and was finally gel filtered on Sephacryl S300 (Pharmacia [G.B.] Ltd., Hounslow, Middlesex, England). The final CRP preparations contained a single component by gradient polyacrylamide gel electrophoresis (PAGE) of the undenatured protein and by sodium dodecyl sulphate (SDS)-PAGE. No contaminants were detectable by immunoelectrophoresis or crossed immunoelectrophoresis against antiserum to NHS, and prolonged immunization with the CRP yielded monospecific antisera.

LDL and VLDL were isolated from plasma by density gradient ultracentrifugation essentially as described by Chung et al. (29). Venous blood from a normal donor who had fasted overnight was anticoagulated with 0.1% wt/vol Na_2 EDTA, and 10 ml of plasma was separated immediately. The plasma was adjusted to a density of 1.30 g/ml, by addition of 4.93 g solid KBr per 10 ml plasma, and layered underneath ~26 ml of a solution of NaCl-Na_2 EDTA pH 7.4, density 1.006 g/ml in a 38.5-ml Beckman centrifuge tube (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). The tube was spun for 2.5 h at 10°C and 50,000 rpm in a Beckman VTi-50 vertical tube rotor, and the contents were then fractionated into 1.0-ml vol by upward displacement with a solution of 30% wt/vol KBr in 20% wt/vol sucrose. The A_{280} of each fraction was measured and they were all assayed for cholesterol and apolipoprotein B (apoB).

Lipid Composition. Triglycerides were determined using the triglycerides kit from Dow Diagnostics (Uniscience Ltd., Cambridge, England). Total and free cholesterol were assayed using a modification of the enzymic procedure described by Allan et al. (30). Lipoprotein phospholipids were assayed as lipid phosphorus by the modification of the Fiske-Subba-Row method described by Marinetti (31).

Antisera. Monospecific rabbit anti-human CRP was raised by immunization with isolated pure CRP. The IgG fraction of the antiserum was prepared by affinity chromatography on protein A-Sepharose (Pharmacia Ltd.) according to the manufacturer's instructions. Affinity-purified anti-CRP antibodies were prepared by passing whole rabbit anti-CRP serum over a column of CNBr-activated Sepharose to which pure CRP had been coupled (see below). After extensive washing with phosphate-buffered isotonic saline, pH 7.4, containing 10 mM EDTA, the column was eluted at 4°C with glycine-HCl buffer at pH 2.2, and the effluent was collected into tubes containing 1.0-ml vol of 1.0 M K_2HPO_4. The eluted material was pure IgG as

evidenced by SDS-PAGE of reduced samples. Polyvalent antiserum to NHS was raised by repeated immunization of sheep. Rabbit antiserum to human β-lipoprotein (Miles-Yeda Ltd., Rehovoth, Israel), goat anti-human apoB (Immuno Diagnostika, Vienna, Austria), and sheep anti-human apoA-I (Seward Labs, London) were obtained as indicated. Monospecific antiserum to human serum amyloid P component (SAP) was prepared by immunization with isolated pure SAP (32, 28).

Immobilized Proteins. Isolated CRP was coupled to CNBr-activated Sepharose beads (Pharmacia Ltd.) according to the manufacturer's instructions. After coupling, the CRP-Sepharose was washed extensively with 0.01 M Tris-buffered 1.14 M NaCl pH 8.0 containing 10 mM EDTA to remove noncovalently associated material. The recommended alternate washes at pH 4.0 and pH 8.0 were avoided to minimize damage to the coupled CRP. Isolated SAP was coupled to Sepharose as reported previously (33). Rabbit IgG anti-CRP preparations were coupled to CNBr-activated Sepharose at pH 7.4 in phosphate-buffered saline (0.65 M). The whole IgG fraction was coupled at 20 mg/ml of Sepharose, and immunopurified IgG anti-CRP antibodies were coupled at 5 mg/ml of Sepharose.

Human Serum. Fresh NHS was obtained by allowing venous blood from laboratory volunteers to clot at room temperature for 2–4 h before centrifugation. All donors were on normal diets and were not fasted. In some experiments serum from a single donor was used, in others different sera were pooled. Acute-phase sera containing raised levels of CRP were obtained during routine investigation of patients with Hodgkin's disease, Crohn's disease, and rheumatoid arthritis.

Protein Assays. Approximate quantitation of total protein was obtained by measurement of A_{280}, and all samples were also examined at 320 nm to check for light scattering. Specific proteins were detected and identified by double immunodiffusion or by immunoelectrophoresis in 1% wt/vol agarose gel (Indubiose A37, IBF SA, Clichy, France) in 0.075 M veronal buffer, pH 8.6, containing 0.01 M EDTA. CRP in fractions from density gradient ultracentrifugation of acute-phase serum was detected and quantitated by electroimmunoassay (sensitivity 1–2 mg/liter; coefficient of variation of replicate assays 10%) (34) or by a solid-phase immunoradiometric assay, which we have reported elsewhere (35) (sensitivity 1 μg/liter; coefficient of variation 5–10%). Isolated pure CRP was used to calibrate both these assays. LDL in the fractions from density gradient ultracentrifugation or ultracentrifugal flotation and after elution from CRP-Sepharose columns was quantitated by electroimmunoassay with anti-apoB serum using samples of the starting material as calibration standards.

PAGE. Gradient PAGE analysis of native proteins was performed in 4–30% gradient gels (Pharmacia Ltd.) run according to the manufacturer's instructions. Standard globular proteins used as markers were: thyroglobulin 669K, ferritin 440K, catalase 232K, lactate dehydrogenase 140K, and albumin 67K, obtained from Pharmacia Ltd. SDS-PAGE analysis was undertaken in slab gels using the method of Laemmli (36). Marker polypeptides were fibronectin 220–225K and α_2-macroglobulin 181K, and the Pharmacia kit of phosphorylase b 94K, albumin 67K, ovalbumin 43K, and carbonic anhydrase 30K as markers. Lipoprotein samples were lyophilized, delipidated with methanol/ether, 1:2 wt/vol, and washed twice with ether before solubilization of the apoproteins by boiling in sample buffer.

Agarose Gel Electrophoresis. Samples to be tested and control samples of NHS and plasma from a patient with type IIB hyperlipoproteinemia were subjected to agarose gel electrophoresis and stained for lipid by standard techniques (37).

Ultracentrifugation Experiments. Linear 10–40% wt/wt sucrose (BDH Chemicals Ltd., Poole, England) gradients were formed by sequential layering of 1.1 ml of sucrose solutions (40%, 30%, 20%, 10%) into 5.5-ml polycarbonate ultracentrifuge tubes (34411-111; MSE Scientific Instruments, Crawley, England). After allowing diffusion for 5 h at 4°, 200-μl samples of acute-phase serum that had been diluted 1:3 with 0.01 M Tris-buffered 0.14 M NaCl containing 0.002 M $CaCl_2$ pH 8.0 (Tris-saline-Ca) or with 0.01 M buffered 0.14 M NaCl containing 0.01 M EDTA pH 8.0 (Tris-saline-EDTA) were layered on top of the gradients. Serum samples containing ~100 mg/liter of CRP from three patients each with Crohn's disease, rheumatoid arthritis, and Hodgkin's disease were tested. Separate sucrose solutions were made up in Tris-saline-Ca and in Tris-saline-EDTA, and each serum sample was run both in calcium and in EDTA. Loaded tubes were centrifuged at 40,000 rpm for 16 h at 4° using a swing out rotor

(43127-126; MSE Scientific Instruments) in an MSE Prepspin 65 ultracentrifuge. After ultracentrifugation, 100-μl fractions were collected from the bottom of the tube. In each experiment the amount of material sedimenting to the bottom of the tube was determined after elution of the pellet with buffer containing 10 mM EDTA. Isolated pure CRP was also run under identical conditions.

A sample (1.0 ml with A_{280} = 1.1) of the material eluted from CRP-Sepharose with EDTA was diluted to 10 ml with NaCl-EDTA, density 1.006 g/ml, and the density adjusted to 1.30 g/ml by addition of solid KBr. It was then subjected to density gradient ultracentrifugation exactly as described above for isolation of LDL from normal plasma (29).

Gel Filtration Experiments. Samples (5-ml) of acute-phase sera containing >100 mg/liter of CRP were run on a 2.6 × 100-cm column of Sephacryl S300 equilibrated with Tris-saline-Ca or Tris-saline-EDTA. The column was eluted with the appropriate buffer at 16 ml/h, and 5.0-ml fractions were collected and assayed for CRP by electroimmunoassay. Isolated pure CRP was also run on the same column both in calcium and in EDTA.

Affinity Chromatography Experiments. The capacity of CRP immobilized on Sepharose to bind serum constituents was tested by passing fresh NHS over columns of CRP-Sepharose at 4°C. The columns were pre-equilibrated with Tris-saline-Ca and, after passing serum over them, were washed with the same buffer until the A_{280} of the effluent was zero. Elution with Tris-saline-EDTA was then started, and all the eluate with significant absorbance at 280 nm was collected, pooled, and concentrated by ultrafiltration on a PM30 membrane in the Amicon Diaflo system (Amicon Corp., Scientific Sys. Div., Lexington, MA). The capacity of CRP-Sepharose to bind isolated LDL was tested in the same protocol, whereas in other experiments, elution of material from the serum-treated CRP-Sepharose with heparin (1 mg/ml in Tris-saline-Ca) or phosphorylcholine (Sigma Chemical Co. Ltd., Poole, Dorset, England) (0.001 M in Tris-saline-Ca) was attempted. Columns of anti-CRP-Sepharose bearing either the whole IgG fraction of anti-CRP serum or the affinity-purified anti-CRP antibodies were saturated with isolated pure CRP. They were then washed extensively with Tris-saline-Ca before passage of NHS over them, followed by further Tris-saline-Ca and finally elution with Tris-saline-EDTA. Controls for the specificity of binding by CRP-Sepharose were provided by columns of SAP-Sepharose, bovine serum albumin-Sepharose, normal sheep IgG-Sepharose, and ethanolamine-Sepharose prepared and tested as described previously (33).

Results

Form of CRP in Acute-phase Serum. The CRP in acute-phase serum was eluted from gel filtration columns of Sephacryl S300 in the same fractions regardless of whether the column was run in Tris-saline-Ca or Tris-saline-EDTA. Isolated pure CRP also eluted in precisely the same position. On density gradient ultracentrifugation, CRP in acute-phase serum and isolated pure CRP both sedimented to the same position on gradients containing calcium or EDTA. In most cases, no significant amount of CRP from acute-phase serum sedimented more rapidly than the main CRP peak, however, in one patient with Crohn's disease and extensive abdominal fistulae, trace quantities of rapidly sedimenting CRP were detected, comprising altogether 0.1% of the total CRP (Fig. 1). In no case were significant amounts of CRP detected that sedimented more slowly than the main peak of CRP, and there were no fractions in which both CRP and LDL were detected (Fig. 2). These results suggest that CRP in acute-phase serum exists almost entirely in a free form, not complexed with any ligand capable of altering its apparent molecular size or sedimentation characteristics.

Calcium-dependent Binding of Serum Proteins by CRP-Sepharose. Although CRP in acute-phase serum is not apparently complexed, the capacity of CRP to bind serum constituents was tested by passing fresh NHS over columns of CRP-Sepharose prepared by covalent conjugation of pure CRP to CNBr-Sepharose. After washing with Tris-saline-Ca until no further material absorbing at 280 nm was eluted, the

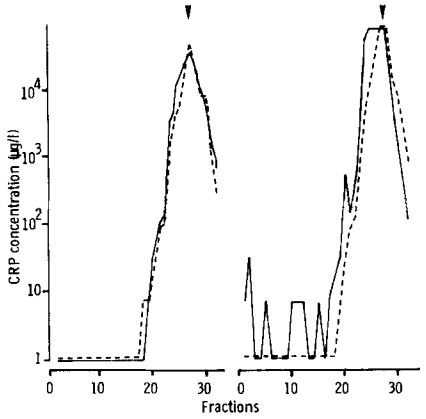

FIG. 1. Sucrose density gradient ultracentrifugation of serum from two patients with severe active Crohn's disease containing >100 mg/liter of CRP. The distribution of CRP in calcium (—) and EDTA-containing (- - -) gradients was identical in all patients tested, as shown on the left, except the individual shown on the right in whose serum ~0.1% of the total CRP sedimented ahead of the main peak. The arrows indicate the peak fraction of isolated pure CRP run on the same gradients.

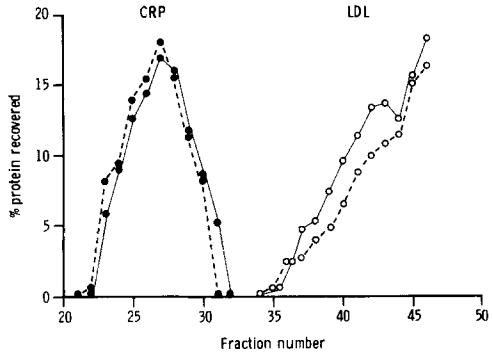

FIG. 2. Sucrose density gradient ultracentrifugation of serum from a patient with septicemia containing 129 mg/liter of CRP. The distribution of CRP (●) and of LDL (○) in calcium- (—) and EDTA-containing (- - -) gradients is shown.

TABLE I

Calcium-dependent Binding of Serum Proteins by CRP-Sepharose

Quantity of CRP coupled (mg/ml of Sepharose)	0.1	1.0	2.0	5.0
A_{280} of EDTA eluate	0.017	0.037	0.109	0.489

2-ml vol of NHS were offered to separate 1.0-ml vol of Sepharose bearing the quantities of CRP shown. After extensive washing with Tris-saline-Ca each column was eluted with 2 ml of Tris-saline-EDTA.

columns were eluted with Tris-saline-EDTA. Significant amounts of protein were always obtained, provided there was 1 mg or more of CRP coupled per ml of Sepharose (Table I) and increased sharply as the density of CRP on the solid phase was increased. The eluates did not show any absorbance or light scattering at 320 nm. Passage of >2.0 ml of NHS over the column of 1 ml of Sepharose, bearing 5 mg of CRP, did not yield increased amounts of protein in the eluate. The yields obtained from CRP-Sepharose always exceeded those obtained from control columns of bovine

serum albumin-Sepharose, normal sheep IgG-Sepharose, or ethanolamine-Sepharose, the eluates of which contained only traces of protein as we have reported previously (33).

Characterization by Gradient PAGE of the Serum Proteins Bound by CRP-Sepharose. Analysis in gradient PAGE, without denaturation, of the material eluted with EDTA after passing NHS over CRP-Sepharose, always revealed two components, the major one comprising ~80% of the total protein (Fig. 3). The minor band, with an apparent molecular weight of ~250,000, which was not seen in lightly loaded gels (Fig. 4, track 3) migrated in the same position as isolated pure SAP. This protein is a normal serum constituent with a known calcium-dependent binding affinity for agarose, and it is therefore always obtained when Sepharose exposed to NHS in the presence of calcium is eluted with EDTA (32). The other component eluted from CRP-Sepharose, which gave a trail from the origin leading to a discrete band corresponding to an apparent molecular weight of 2×10^6 or more (Fig. 3), was apparently specifically bound by the immobilized CRP. It was never seen in eluates from any of the control columns mentioned above (33), neither was it present in the eluate from SAP-Sepharose columns (33) (Fig. 4).

SAP is closely related to, though distinct from, CRP, and they are known to have different calcium-dependent ligand-binding specificity (27). When aggregated, immobilized SAP is exposed to NHS, it selectively binds fibronectin and C4-binding protein (33), which yield a quite distinct pattern in gradient PAGE from that given by the material bound by CRP (Fig. 4).

Identification of the Material Selectively Bound by CRP-Sepharose as Lipoprotein. When the proteins eluted from CRP-Sepharose were subjected to electrophoresis in agarose gel and then stained with Sudan Black, a band corresponding in mobility to normal plasma β-lipoprotein was revealed (Fig. 5). Traces of material with pre-β mobility were also present (Fig. 5). Immunoelectrophoretic analysis of the eluate using antiserum raised against it demonstrated three components (Fig. 6): (*a*) there was a protein

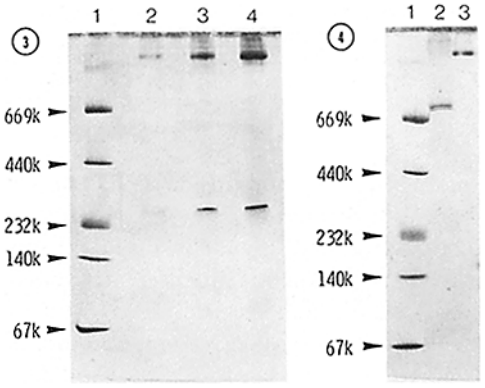

FIG. 3. 4–30% gradient PAGE of undenatured proteins eluted with EDTA after passage of NHS over immobilized CRP. Track 1, marker proteins of known molecular weight; track 2, eluate (A_{280} = 0.28) from CRP immobilized on immunopurified anti-CRP-Sepharose; tracks 3, 4, two separate preparations of eluates (A_{280} = 1.1 and 1.4, respectively) from CRP-Sepharose bearing 4 mg CRP/ml of Sepharose.

FIG. 4. 4–30% gradient PAGE of undenatured proteins eluted with EDTA after passage of NHS over SAP-Sepharose (track 2) or CRP-Sepharose (track 3). Track 1, marker proteins of known molecular weight.

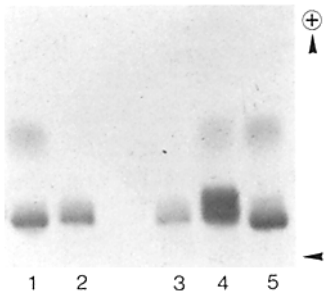

FIG. 5. Electrophoresis in agarose gel of proteins eluted with EDTA after passage of NHS over CRP-Sepharose. Gel stained for lipid with Sudan Black. Tracks 1, 5, whole NHS; tracks 2, 3, different eluates from CRP-Sepharose; track 4, serum from a patient with type IIB hyperlipoproteinemia.

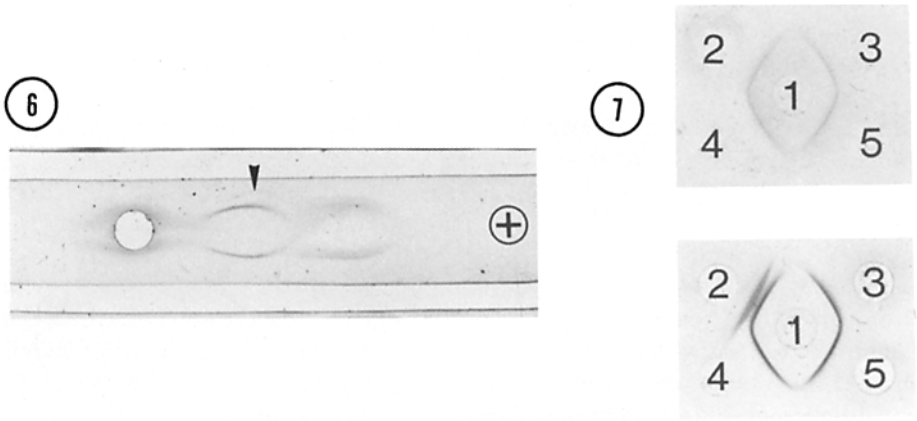

FIG. 6. Immunoelectrophoresis of proteins eluted with EDTA after passage of NHS over CRP-Sepharose. Upper trough, polyvalent anti-NHS serum; lower trough, antiserum raised by immunization with the EDTA eluate. The arrow shows the position of a faint arc with pre-β mobility.

FIG. 7. Immunodiffusion analysis of proteins eluted with EDTA after passage of NHS over CRP-Sepharose. Above, gel stained for lipid with Sudan Black; below, same gel subsequently stained for protein with Coomassie Blue. 1, proteins from NHS eluted from CRP-Sepharose with EDTA; 2, antiserum raised against 1; 3, antiserum to 1 absorbed with isolated SAP; 4, anti-β-lipoprotein serum; 5, anti-apo B serum.

with γ-mobility, identifiable by immunodiffusion as IgG, traces of which can always be found in eluates from any Sepharose column; (*b*) there was a protein with α-mobility, which was identified as SAP (see below); and (*c*) there was an arc with β-mobility which, alone of the three components present, stained with Sudan Black as well as with Coomassie Blue. Immunoelectrophoresis against a strong polyvalent sheep anti-human serum gave these same three arcs and also a weaker one with pre-β mobility (Fig. 6).

In double immunodiffusion tests, the antiserum raised against the serum proteins eluted from CRP-Sepharose gave two distinct precipitation lines with the eluate (Fig. 7). One of these, which did not stain with Sudan Black, was absent after absorption of the antiserum with SAP-Sepharose and therefore was due to SAP in the eluate and anti-SAP in the antiserum (Fig. 7). The second precipitation line stained strongly with Sudan Black, and therefore contained lipid, and also gave a reaction of complete

identity with the lines formed between the eluate and antisera to β-lipoprotein or apoB (Fig. 7). These results indicate that the material from serum selectively bound in a calcium-dependent fashion by CRP-Sepharose was lipoprotein in nature with β and possibly some pre-β electrophoretic mobility and contained apoB.

Characterization of the Material Selectively Bound by CRP-Sepharose as LDL and VLDL. The EDTA eluate from CRP-Sepharose exposed to NHS in the presence of calcium was analyzed by density gradient ultracentrifugation. A major peak of material absorbing at 280 nm, containing all the detectable cholesterol, was obtained in the same fractions in which LDL was found when normal human plasma was run under identical conditions (Fig. 8). Some absorbance at 280 nm was also present in fractions corresponding to the position of VLDL. Traces of absorbing material in fractions corresponding to high density lipoprotein (HDL) probably represented trailing from the origin.

Analysis by SDS-PAGE of the material bound by CRP-Sepharose revealed three protein bands. The major one migrated in the same position as apoB from isolated LDL, a faint band corresponding to apoE from VLDL was seen, and the subunit of SAP was also present. There were no bands corresponding to any other apolipoproteins. Also, in double immunodiffusion analysis of the eluted proteins against antiserum to apoA-I, no precipitation lines were seen. The relative amounts of phospholipid, triglyceride, esterified, and nonesterified cholesterol found in the eluate from CRP-Sepharose differed slightly from the typical composition of LDL particles, but were compatible with the presence of a mixture of LDL with some VLDL (Table II).

Binding of Isolated LDL by CRP-Sepharose. A quantity of isolated LDL corresponding to 0.7 ml of normal plasma was passed over CRP-Sepharose (5 mg CRP on 1 ml Sepharose) in the presence of calcium. It all bound to the column and was quantitatively recovered by subsequent elution of the column with EDTA.

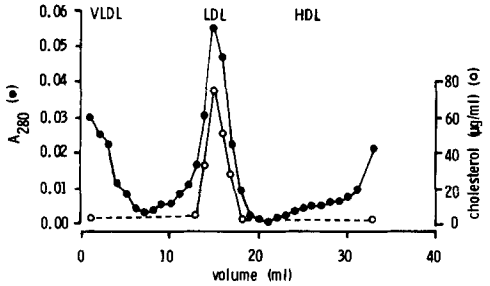

FIG. 8. Analysis by density gradient ultracentrifugation of proteins eluted with EDTA after passage of NHS over CRP-Sepharose. The horizontal bars mark the fractions in which VLDL, LDL, and HDL were located when normal human plasma was run under identical conditions.

TABLE II

Lipid Composition of Serum Lipoproteins Bound by CRP-Sepharose

Relative quantity of lipid	Lipoprotein bound by CRP	Typical LDL
Phospholipid	1	1
Triglyceride	0.9	0.3
Total cholesterol	0.9	1.3
Esterified cholesterol (%)	43	75

TABLE III

Calcium-dependent Binding of Serum Proteins by CRP Immobilized on Anti-CRP-Sepharose

Anti-CRP coupled	CRP bound	Vol NHS offered	A_{280} in EDTA eluate
	mg	*ml*	
Whole IgG fraction (120 mg on 6 ml Sepharose)	8.4	50	0.18*
Immunopurified anti-CRP antibody (10 mg on 2 ml Sepharose)	7	50	1.45‡
Immunopurified anti-CRP antibody (10 mg on 2 ml Sepharose)	7	5	1.40‡

The anti-CRP Sepharose columns shown were saturated with the quantities of pure CRP indicated and then exposed to NHS. After extensive washing with Tris-saline-Ca, bound protein was eluted with Tris-saline-EDTA.

* No LDL was detectable in this eluate.

‡ These eluates contained the same lipoproteins as the eluate from CRP-Sepharose (Fig. 3).

Elution by Phosphorylcholine of Lipoproteins Bound by CRP-Sepharose. After passing NHS over CRP-Sepharose in the presence of calcium, all the bound lipoprotein was eluted with 1 mM phosphorylcholine in Tris-saline-Ca. No further lipoprotein was obtained by subsequent passage of Tris-saline-EDTA over the column. In contrast, after exposing CRP-Sepharose to NHS in calcium, heparin at 1 mg/ml in Tris-saline-Ca eluted no lipoprotein at all. Subsequent passage of Tris-saline-EDTA led to quantitative recovery of the LDL.

Calcium-dependent Binding of Serum Proteins by CRP Immobilized on Anti-CRP-Sepharose. The IgG fraction of rabbit anti-CRP serum was coupled to Sepharose at 20 mg/ml, and a 6.0-ml column of this material was saturated with 8.4 mg of pure isolated CRP. However, when NHS was passed over this column and the column then washed with Tris-saline-Ca, subsequent elution with Tris-saline-EDTA did not yield significant amounts of protein (Table III). In contrast, when 10 mg of immunopurified anti-CRP antibodies was coupled to 2.0 ml of Sepharose and then saturated with a total of 7.0 mg of CRP, this column was able to bind and yield appreciable amounts of protein from NHS. Analysis of the eluate showed that it contained precisely the same proteins as the eluate from CRP immobilized at a sufficient density directly on Sepharose (Fig. 3, Table III). The total quantity of protein in the eluate from the CRP-anti-CRP-Sepharose column was the same regardless of whether 50 or 5 ml of NHS was offered (Table III). The total A_{280} of the eluate was 1.4, and because the quantity of SAP present was only 60 μg, contributing A_{280} of 0.10, the remainder represents the capacity of the 7.0 mg of immobilized CRP to bind lipoproteins.

Discussion

Our findings with regard to the size and sedimentation behavior of CRP in acute-phase human serum confirm those previously reported by Kushner and Somerville (26) for CRP in both human and rabbit serum. Although they used calcium-free phosphate and citrate buffers, the present experiments comparing calcium-containing with citrate or EDTA buffers did not reveal significant amounts of complexed CRP.

Pontet et al. (38) have suggested that CRP in acute-phase rabbit serum exists entirely in a high molecular weight form due to complexing with LDL, but like Kushner and Somerville (26), we have been unable to reproduce this observation using either gel filtration or density gradient ultracentrifugation (M. L. Baltz and M. B. Pepys, unpublished observations).

In contrast to the behavior of free CRP in the serum, CRP that had been aggregated by immobilization on Sepharose beads acquired a selective binding capacity for LDL and traces of VLDL from whole normal human serum. Expression of the binding depended upon a sufficient degree of aggregation of CRP molecules and only occurred appreciably when >1 mg of CRP was coupled per ml of Sepharose. Similarly, CRP immobilized by uptake onto anti-CRP-Sepharose failed to bind LDL unless immunopurified anti-CRP antibodies were used to produce the anti-CRP-Sepharose.

This situation is quite distinct from that observed with immobilized SAP. SAP is closely related to CRP in structure, having 60–70% homology of amino acid sequence (39, 40), but its calcium-dependent ligand-binding specificity is different (27). SAP immobilized directly on Sepharose or by uptake onto anti-SAP-Sepharose selectively binds fibronectin and C4-binding protein from NHS but does not bind detectable amounts of lipoprotein (33). SAP-anti-SAP-Sepharose binds fibronectin more efficiently than directly coupled SAP-Sepharose, and each pair of SAP molecules taken up by a single anti-SAP molecule is capable of reacting with one fibronectin molecule (33). In contrast, aggregation or close association of many CRP molecules on the solid phase was required to permit expression of the binding reactivity for LDL. In view of this fact, the large size of LDL, which partly excludes it from the Sepharose beads, and the difficulty in quantitating LDL particles in molar terms, it is not possible to calculate the reaction ratio of aggregated CRP and fluid phase LDL. However, from the experiments with CRP immobilized on immunopurified anti-CRP coupled to Sepharose, and assuming an LDL particle mass of $\sim 2 \times 10^6$, a maximal estimate of 100 CRP molecules involved in binding 1 LDL particle can be derived.

LDL that had been bound by CRP-Sepharose was completely eluted either by chelation of calcium ions or by exposure to free phosphorylcholine, but heparin had no effct on the binding. This suggests that the reaction involved primarily the calcium-dependent phosphorylcholine-binding site of CRP, although a contribution from the cation-binding site cannot be excluded. The basis for the specificity of CRP-Sepharose for LDL rather than VLDL, which was bound only in trace amounts, and HDL, which was not bound at all, is not clear. However, it is worth noting that LDL is the most cationic lipoprotein and HDL the least. All the lipoproteins contain phospholipids and therefore potential ligands for CRP. The selective binding of LDL may depend both on the precise nature and/or accessibility of phospholipids on the particle surface and on the presence, nature, or distribution of accessory ligand sites, particularly cationic groups. Perhaps apoB, which is the predominant apoprotein of LDL and is also present in VLDL, but not in HDL, may play a part (5).

Regardless of their precise molecular mechanism, the present observations have important physiological and pathophysiological implications. By virtue of its ligand-binding properties, CRP can form complexes in vitro and presumably also in vivo with a wide variety of materials both of extrinsic and autogenous origin. If CRP aggregated in such complexes can then selectively bind LDL, there could be significant effects both on the fate of the complexes and of the LDL. CRP-ligand complexes

formed in the circulation that subsequently bind LDL are unlikely to circulate freely and would probably be rapidly cleared by the reticuloendothelial system. This may explain our failure to find complexed CRP in acute-phase sera from patients with active inflammatory, neoplastic, and infective conditions in which potential ligands are released into the circulation.

Aggregated or complexed human CRP is known to efficiently activate the classical complement pathway (41, 42) and such complement activation may also contribute to clearance from the circulation. However, neither rabbit nor rat CRP is capable of activating its respective autologous complement (43, 18), so it seems unlikely that complement activation is a necessary, integral part of the function of CRP. Also, it is noteworthy that CRP-Sepharose selectively bound LDL but not C1 from fresh NHS, in contrast to IgG-Sepharose, which bound C1 very efficiently under identical conditions (44).

CRP binds to damaged cells both in vitro (9) and in vivo (10–12), and it has been suggested that by then activating complement, at least in humans, it may contribute to the inflammation required for resolution and repair (45). It is not clear what the beneficial effects would be of trapping LDL at sites of tissue injury, but a possible harmful effect may be the deposition of LDL in vessel walls after endothelial injury. This is a purely speculative concept at present, but is nonetheless susceptible to further experimental investigation, which is currently in progress. Whatever the results of these particular studies, the fact that CRP molecules that have been aggregated acquire a new and selective reactivity for LDL, which they do not express when they are free in the serum, seems likely to have biological relevance.

Summary

C-reactive protein (CRP), the classical acute-phase protein, can bind phospholipids by virtue of its specific, calcium-dependent reactivity with phosphorylcholine residues. However, analysis of acute-phase serum by gel filtration and by density gradient ultracentrifugation showed that the CRP was in a free, uncomplexed form, despite the coexistent presence of the various classes of serum lipoproteins, all of which contain phospholipids. In contrast, when isolated CRP was aggregated by immobilization at a sufficient density on a solid phase and then exposed to normal human serum, it selectively bound low density lipoprotein (LDL) and traces of very low density lipoprotein. The reaction was calcium dependent and reversible by free phosphorylcholine but not by heparin. LDL isolated from normal plasma was also bound by aggregated CRP.

CRP reacts in vitro with a wide variety of different ligands both of extrinsic and of autogenous origin, e.g., microbial products and damaged cell membranes, respectively. If CRP aggregated in vivo by complexing with these ligands then acquires the capacity to selectively bind LDL, the phenomenon may have significant implications for the function of CRP and for the metabolism, clearance, and deposition of LDL.

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