Isolation and Characterization of a Complement-activating Lipid Extracted from Human Atherosclerotic Lesions

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Summary

The major characteristics of human atherosclerotic lesions are similar to those of a chronic inflammatory reaction, namely fibrosis, mesenchymal cell proliferation, the presence of resident macrophages, and cell necrosis. Atherosclerosis exhibits in addition the feature of lipid (mainly cholesterol) accumulation. The results of the present report demonstrate that a specific cholesterol-containing lipid particle present in human atherosclerotic lesions activates the complement system to completion. Thus, lipid could represent a stimulatory factor for the inflammatory reaction, whose underlying mechanistic basis may be, at least in part, complement activation. The complement-activating lipid was purified from saline extracts of aortic atherosclerotic lesions by sucrose density gradient centrifugation followed by molecular sieve chromatography on Sepharose 2B. It contained little protein other than albumin, was 100–500 nm in size, exhibited an unesterified to total cholesterol ratio of 0.58 and an unesterified cholesterol to phospholipid ratio of 1.2. The lipid, termed lesion lipid complement (LCA), activated the alternative pathway of complement in a dose-dependent manner. Lesion-extracted low density lipoprotein (LDL) obtained during the purification procedure failed to activate complement. Specific generation of C3a desArg and C5b-9 by LCA indicated C3/C5 convertase formation with activation proceeding to completion. Biochemical and electron microscopic evaluations revealed that much of the C5b-9 present in atherosclerotic lesions is membraneous, rather than fluid phase SC5b-9. The observations reported herein establish a link between lipid insudation and inflammation in atherosclerotic lesions via the mechanism of complement activation.

Atherosclerosis shares in common with chronic inflammation the features of leukocyte infiltration, fibrosis, mesenchymal cell proliferation, and tissue necrosis. Complement plays a central role in many inflammatory and immune diseases, and most of the complement components have now been identified in human atherosclerotic lesions (1). Of particular importance is the presence of C5b-9 terminal complement complexes since their assembly indicates that complement activation with C3/C5 convertase formation has taken place. The respective convertases cleave C3 and C5 generating the anaphylatoxins C3a and C5a, which in turn mediate proinflammatory leukocyte functions (2). The C5b-9 complex, in addition to its potential cytolytic effect, can influence cell physiology in sublytic doses via activation of various calcium-dependent pathways (3).

In a previous report, we demonstrated a temporal and spatial colocalization of C5b-9 complexes and lipid in the aortic tunica intima of cholesterol-fed rabbits (4). Lipid vesicles rich in unesterified cholesterol are an early and consistent feature of human and animal atherosclerotic lesions (5–11). In vitro, liposomes containing a >50 mole percent of unesterified cholesterol activate complement to completion (12, 13). We therefore suspected that lipid vesicles enriched in unesterified cholesterol are capable of activating the complement system in situ in the arterial wall. In this paper we describe the isolation and characterization of a lipid particle extracted from human atherosclerotic lesions and demonstrate its potent complement-activating properties. The data establish a link between the insudation of lipid and the inflammatory features of atherosclerotic plaques.

Materials and Methods

Reagents and Antibodies. PBS contained 150 mM NaCl and 10 mM phosphate buffer, pH 7.4. For transporting and washing tissues, PBS was supplemented with 5 mM EDTA. Veronal-buffered saline

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(VBS^{2+})[1] consisted of 2.5 mM barbital, 73 mM NaCl, 0.1 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.4.

Polyclonal rabbit antibodies against human: serum proteins; albumin, C3; and apolipoprotein B were obtained from Dakopatts (Copenhagen, Denmark). Polyclonal and monoclonal antibodies against neoantigens of the C5b-9 complex were produced in our laboratory (14, 15).

Human low-density lipoprotein (LDL) was purchased from Sigma Chemical Co. (Münich, FRG). Trypsin, chymotrypsin, staphaureus protease, and neuraminidase were purchased from Worthington Biochemicals Corp. (Freehold, NJ).

Tissue Specimens and Extraction. Carotid endarterectomy specimens were obtained at surgery and transported to the laboratory on ice in PBS/EDTA. After rinsing away residual blood, the tissue was finely minced with a scissors using PBS as the extraction medium (~1 ml PBS per gram of tissue). The mincate was centrifuged at 740 *g* for 8 min. The resulting supernatant was recentrifuged at 11,000 *g* for 10 min and/or passed through a 0.45 μm filter to clear particulates. The final supernatant was then either tested for complement-activating ability by immediately mixing it with normal human serum (NHS), or it was applied to a Sepharose 4B column (see chromatography methods below).

Human aortic tissue was obtained at autopsy 10–48 h after death. A longitudinal incision was made to expose the lumenal surface of the aorta, which was then cleaned of residual blood. The intima and inner tunica media were stripped-off to obtain predominantly lesional tissue. Aortas with large areas of grossly normal tissue were used to obtain control material. The intimal/medial tissue pieces were finely minced with scissors, using PBS as the extraction medium. After centrifugation at 740 *g*, the supernatant was harvested and the tissue pellet was re-extracted one or two more times overnight at 4°C.

For both carotid and aortic specimens, tissue areas containing calcification, thrombosis, or ulceration were omitted. 6 carotid lesions and 15 aortic specimens were examined. Many extracts of the aortic lesions were eventually pooled. Patient ages at the time of death varied from 27 to 91 years with the majority of specimens deriving from patients in their seventh or eighth decade. Cause of death was variable and the only causes of death omitted from sample gathering were those due to highly infectious agents.

Sucrose Density Gradient Fractionation. Sucrose density gradients ranging from 10 to 50% (wt/vol) were prepared in 20 mM Tris-HCl, 100 mM NaCl, pH 8.0 (35 ml total volume) and 5 ml of lesion extract was applied to the top of each gradient. Centrifugation was performed at 240,000 *g* for 3 h (4°C) in a vertical rotor (type VTi-50; Beckman Instruments, Fullerton, CA). 10 equal fractions were then collected for further analyses.

Molecular Sieve Chromatography. Carotid lesion extracts were fractionated on a 0.9 × 60 cm Sepharose 4B column (Pharmacia Fine Chemicals, Uppsala, Sweden) and eluted with 1.5 M NaCl containing 5 mM EDTA and 15 mM NaN_3, pH 7.4, at 4°C. 1-ml fractions were collected and the flow rate was 8 ml/h. Aortic lesion extracts were either directly applied to a 2 × 100 cm Sepharose 2B column or first fractionated by sucrose density gradient centrifugation and then applied to the column. In the latter case, pooled fractions 9 and 10 from the sucrose gradient were used. The flow rate was 25 ml/h and 8–9-ml fractions were collected.

No qualitative or striking quantitative differences were observed in the fractionation of lipid or complement components relative to the source of tissue used for extraction or the columns used. Fractions from the Sepharose 2B column containing complement-activating activity were pooled and concentrated 15–25-fold by ultrafiltration using Filtron omega cells (Filtron, Karlstein, FRG). This concentrate represents the purified lesion lipid complement activator (LCA).

Electroimmunoassays. Rocket and fused immunoelectrophoresis of fractionated lesion extract was performed in the conventional manner using 1% (wt/vol) Litex agarose (type HSA; Copenhagen, Denmark) dissolved in 0.1 M glycine, 0.038 M Tris, pH 8.7.

Immunoelectrophoresis was performed in the same buffer system at 2–3 V/cm, overnight. For analysis of fractions collected from the Sepharose column, the application was supplemented with 0.2% Triton X-100 in order to liberate proteins from the lipid particles. Detergent was not added to the antibody-containing gels. SDS-PAGE and immunoblotting were performed as described (15).

Complement Activation Tests. Generally, one volume of sample was incubated with one volume of fresh human serum at 37°C for 45–60 min. Fractions recovered from the sucrose gradients were dialysed against VBS^{2+} overnight at 4°C prior to testing. Due to their lower LCA content, fractions from the Sepharose columns were admixed with only one-tenth the volume of NHS.

Complement Assays. C3a desArg was measured using a radioimmunoassay kit supplied by Amersham (Braunschweig, FRG). The C5b-9 complex was quantified by an ELISA as described (15). Concentrations were calculated on the basis of the original volumes of NHS present in the reaction mixtures.

Lipid Analysis. Cholesterol and triacylglycerides were measured using commercial kits from Boehringer Mannheim (Mannheim, FRG). Standard curves were generated using the lipid standards provided by the same company.

Fatty Acid Analyses. Long chain fatty acids were analyzed by gas-liquid chromatography (GLC) and combined gas-liquid chromatography/mass spectrometry (GLC-MS). The dialyzed and lyophilized sample (2.5 mg) was dissolved in 2.5 ml of 4 M HCl and 25 μg of heptadecanoic acid (17:0) was added to serve as an internal standard. The mixture was hydrolyzed (100°C, 4 h) and the fatty acids were extracted three times with 3 ml of chloroform. The organic layers were combined, dried over Na_2SO_4, and finally concentrated under a stream of nitrogen. After esterification with diazomethane in ether (Diazald, Aldrich Chemical Co., Milwaukee, WI), the sample was concentrated again and an aliquot (1/20) was subjected to GLC analysis. GLC was carried out on a gas-chromatograph (model 3700; Varian Associates, Palo Alto, CA) equipped with a fused silica capillary column (25 m, 0.2 mm internal diameter) with chemical bonded SE-54. The temperature program was set to 150°C for 3 min, then raised to 250°C at a rate of 3°C/min. The amounts of the fatty acids were calculated on the basis of peak areas relative to the internal standard (17:0). GLC-MS was performed on a 5890A gas-chromatograph equipped with a 5970 mass selective detector (Hewlett-Packard Co., Palto Alto, CA) using the same temperature program as for GLC.

Amino Acids, Amino Sugars, and Ethanolamine. Amino acids, amino sugars, and ethanolamine were liberated from 2 mg of the LCA by acid hydrolysis in 2.5 ml of 6 M HCl (100°C, 16 h) in sealed ampules. They were then identified and quantitated by an amino acid analyzer (Alpha Plus 4151, LKB-Pharmacia Fine Chemicals, Piscataway, NJ) using post-column derivatization with ninhydrin.

Electron Microscopy: Negative Staining. Samples of LCA in PBS were applied to thin carbon-coated Formvar films carried on copper grids, exposed to glow-discharge in atmospheric air shortly before use, and negatively stained with 2% sodium silicotungstate, pH

[1] *Abbreviations used in this paper:* LCA, lesion lipid complement activator; LDL, low density lipoprotein; NHS, normal human serum; VBS, veronal-buffered saline.

7.0 (16). Attempts to demonstrate the presence of cholesterol by reaction with filipin were performed as follows. Filipin (batch U 5956, obtained as a gift from the Upjohn Company, Kalamazoo, MI) was dissolved in DMSO (50 μl/mg) and diluted with PBS to a final concentration of 4 mg/ml. Aliquots of LCA in PBS were mixed with this filipin solution at final filipin concentrations of 0.8, 1.2, 1.6, and 2.0 mg/ml, and left to stand in the dark at 23°C for 1 h before negative staining. Other aliquots were incubated with trypsin or α-chymotrypsin (type II; Sigma Chemical Co., St. Louis, MO) in PBS, pH 8.0, at individual enzyme concentrations of 0.1 mg/ml for 1 h at 23°C.

Electron Microscopy: Thin Sectioning. LCA in PBS was mixed with an equal volume of 2% OsO_4 in 0.2 M sodium cacodylate buffer, pH 7.0, and left to stand for 15 min at 23°C. It was then centrifuged at 10,000 *g* for 30 min in volumes of 150 μl with a sedimentation distance of 5 mm. The resulting pellets were dehydrated in ethanol, followed by propylene oxide, and embedded in Epon 812. Ultrathin sections were cut on a diamond knife and stained with uranyl acetate and lead citrate.

Electron Microscopy: Freeze-Fracture Replication. LCA in PBS was mixed with an equal volume of 60% glycerol, mounted as drops on gold stubs, and frozen in Freon 22, cooled in liquid nitrogen. In addition, some LCA specimens were frozen without the addition of glycerol. Negative staining of glycerinated samples showed no detectable structural changes of the LCA relative to nonglycerinated samples. Freeze-fracture was performed by standard techniques in a Balzers 301 unit (17).

All electron microscope specimens were examined and photographically recorded in a JEOL 100CX electron microscope equipped with an ultra high resolution objective lens pole piece (Cs 0.7 mm), operated at 80 kV.

Results

Atherosclerotic Lesion Extracts Activate Complement in Normal Human Serum. Terminal C5b-9 complement complexes have been detected in human atherosclerotic lesions by immunochemical techniques (18–20), thus implicating in situ activation of complement. However, direct demonstration of complement activation by lesion material has not been shown, nor has the element(s) responsible for triggering activation been identified. We therefore prepared saline extracts of freshly removed carotid artery atherosclerotic lesions and incubated them with normal human serum (vol/vol) at 37°C for 60 min. After subtracting out baseline (time 0) levels and the nonspecific generation of C3a in serum incubated with buffer alone, 13.3 ± 4.5 μg/ml of C3a desArg was found to have been generated. Each sample preparation was similar in terms of the tissue weight (0.6–0.8 g), extraction volume (1 ml), and cholesterol concentration (243 ± 44 μg/ml). The extracts were viewed by light microscopy and found to contain few, if any, crystals.

The generation of C3a in serum mixed with lesion extract indicated that a component(s) is present in atherosclerotic lesions that can generate C3 convertases. Extracts of normal arterial tissue failed to generate significant amounts of C3a under the same conditions. Without exception, however, extracts obtained from 15 atherosclerotic aortic lesions exhibited similar complement activating properties as the material derived from freshly isolated carotid lesions.

Isolation of a Lipid from Extracts that Activates Complement. To determine the lesional component(s) responsible for triggering complement activation, saline extracts of aortic atherosclerotic lesions were fractionated by sucrose density gradient centrifugation. Fig. 1 depicts the characteristic fractionation profile for lipid, protein, apoprotein B, complement component C3, and the C5b-9 complex. The pattern was invariant between all the samples analyzed and exhibited only minor quantitative differences from sample to sample. Lipid, as measured by cholesterol (Fig. 1, *A*), immunoreactive apoprotein B (Fig. 1 *B*), and triacylglycerides (data not shown), floated at the top of the gradient and was therefore detected in fractions 9 and 10. The bulk of the protein was present in fractions 6–9 (*A* and *C*), and C3 was identified in fractions 7 and 8 (*D*). The C5b-9 complex sedimented towards the bottom of the gradient, exhibiting a peak in fraction 3 (*E*). The sedimentation behavior of this entity differed from that of fluid-phase SC5b-9, which peaked in fraction 5, corresponding to 23 S in these gradients (position indicated by an arrow in Fig. 1).

To determine whether a specific lesion component was present that could activate complement, each fraction from the sucrose density gradient was dialyzed against VBS^{2+} and reacted with NHS. It was found that fraction 10, and to a lesser extent, fractions 6 and 7, exhibited the capacity to activate complement as assessed by the specific generation of C3a desArg (Fig. 2). The top fractions (9 and 10) containing the bulk of activity were applied to a Sepharose 2B column. The resulting elution profile, which was typical of all aortic specimens examined, is shown in Fig. 3. A lipid peak devoid of apoprotein B eluted in the void volume, whereas the majority of protein was detected towards the included (end) volume (Fig. 3, *A–C*). A small apoprotein B immunoreactive peak was observed towards the end volume (*C*); however, cholesterol could not be detected in these fractions without prior concentration.

When each fraction from the Sepharose column was reacted with serum, a peak of complement-activating activity was observed that coincided with the void volume peak (Fig. 3 *D*). Fractions containing the void volume peak were pooled and concentrated 15–25-fold. This pool constitutes the purified lesion LCA. In addition, the fractions containing the apoprotein B peak were pooled and concentrated. Both of the pooled concentrates were subsequently analyzed for cholesterol and tested for their complement-activating capacity. As shown in Fig. 4, the LCA activated serum complement in a dose-dependent manner. Half-maximal C5b-9 levels occurred at a final LCA/cholesterol concentration of ~0.25 mg/ml, and specific C5b-9 generation was still observed at LCA/cholesterol levels as low as 0.06 mg/ml. In contrast, the rear, apoprotein B-containing peak from the column failed to activate complement even when the amount of cholesterol added was 0.7 mg/ml.

Further experiments were undertaken to determine whether LDL or modified LDL has the potential to activate serum complement. Kinetic and dose-response experiments were performed with purified LDL wherein the LDL was incubated in Hams F-10 media containing 5 μM $CuSO_4$ or with cul-

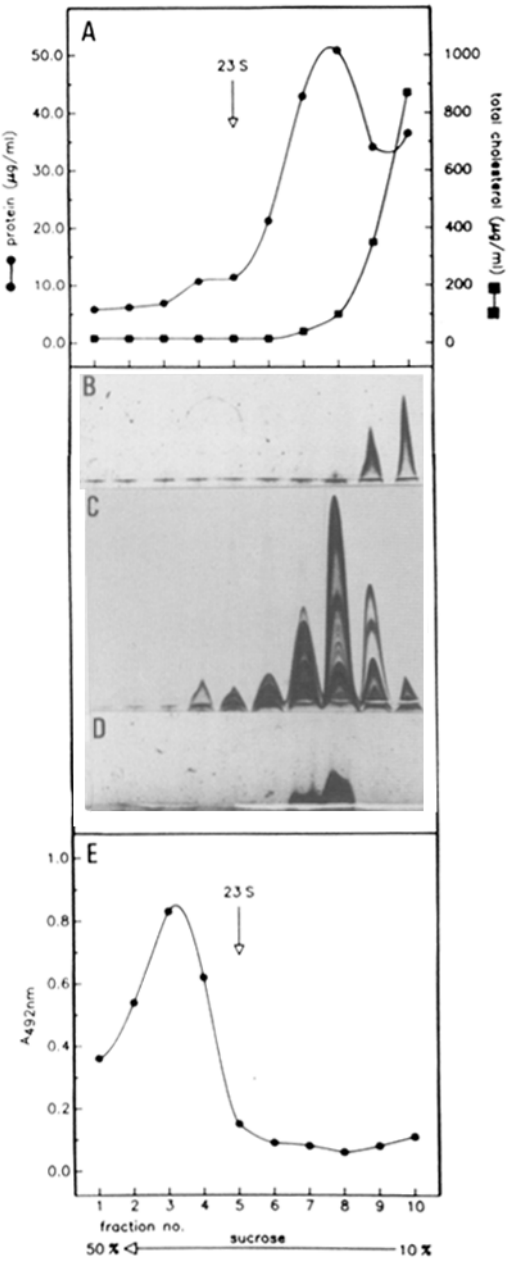

Figure 1. Sucrose density gradient profile of aortic atherosclerotic lesion extract. Fresh lesion extract (5 ml) was layered onto a 10–50% sucrose gradient (35 ml). After centrifugation, 10 equal fractions of 4 ml were collected. Cholesterol was detected biochemically in fractions 9 and 10 (*A*). Rocket immunoelectrophoresis demonstrated: the presence of apoprotein B in fractions 9 and 10 (*B*); serum proteins predominantly in fractions 7–9 (*C*) (which corresponded well with total protein determined biochemically [*A*]); and complement C3 in fractions 7 and 8 (*D*). The C5b-9 complex (measured by ELISA) sedimented towards the bottom of the gradient peaking in fraction 3 (*E*). The sedimentation position of purified SC5b-9 (23 S) is depicted by an arrow.

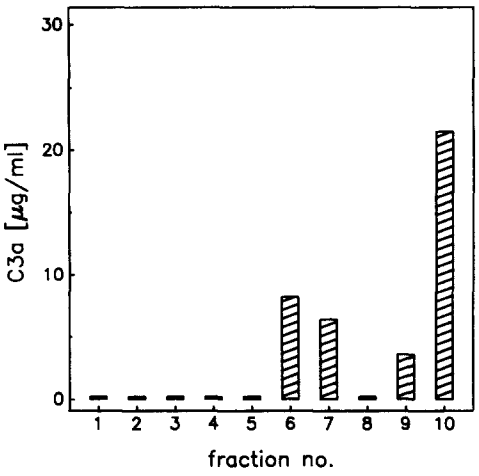

Figure 2. The complement-activating capacity of fractionated atherosclerotic lesion extract. All 10 fractions collected from the sucrose density gradient were dialysed against VBS^{2+} and mixed with normal human serum (vol/vol) for 60 min at 37°C. The specific C3a desArg generated in the serum was then measured by RIA.

tured human endothelial, monocyte, or vascular smooth muscle cells to induce oxidative/degradative changes (21–24). Additionally, purified LDL (500 μg/ml) was subjected to treatment with: trypsin (50 μg/ml), chymotrypsin (50 μg/ml), neuraminidase (27 U/ml), staphaureus protease (100 μg/ml), or cholesterol esterase (2.5 U/ml). Under none of these circumstances was complement-activating activity ever observed upon addition of LDL or modified LDL to NHS (final concentration of maximal LDL/cholesterol, 0.5 mg/ml).

Characteristics of Complement Activation by LCA. The dose-response relationship of LCA-mediated complement activation was alluded to above and shown in Fig. 4. In the experiment depicted in Fig. 5, LCA was added to NHS to yield a final LCA/cholesterol concentration of 0.75 mg/ml, and the kinetics of complement activation were studied. C3a desArg levels peaked rapidly, reaching a plateau within 30 min (corresponding to 30–40 μg/ml C3a desArg production). C5b-9 generation was slower and more sustained, reaching a plateau at ~60 min (Fig. 5). The relative efficiency of the terminal sequence activation was high, with molar ratios of C3a to C5b-9 approaching 20:1, typical of complement activation on particulate surfaces (25). C5b-9 levels were remarkably high in the extract-reacted serum samples, reaching levels exceeding 300 μg/ml SC5b-9, when calibration curves were generated with purified SC5b-9. Preliminary results indicate that a substantial portion of the C5b-9 generated by LCA is in the form of C5b-9(m) complexes. Membranous C5b-9 exhibits a higher reactivity in the ELISA as compared with SC5b-9 complexes (15) (used as the calibrating standard throughout). The present values may therefore represent overestimates of the true C5b-9 levels.

The ability of LCA to activate complement did not change upon standing for up to several weeks at 4°C. There was no difference in the complement-activating capacity of LCA isolated from aortas obtained 10 h after death as compared with those obtained 48 h after death. These results indicate a high stability of the LCA particle and its complement-activating property. They also argue that oxidative and/or proteolytic modifications are not involved in generating or rendering this lipid particle a complement activator.

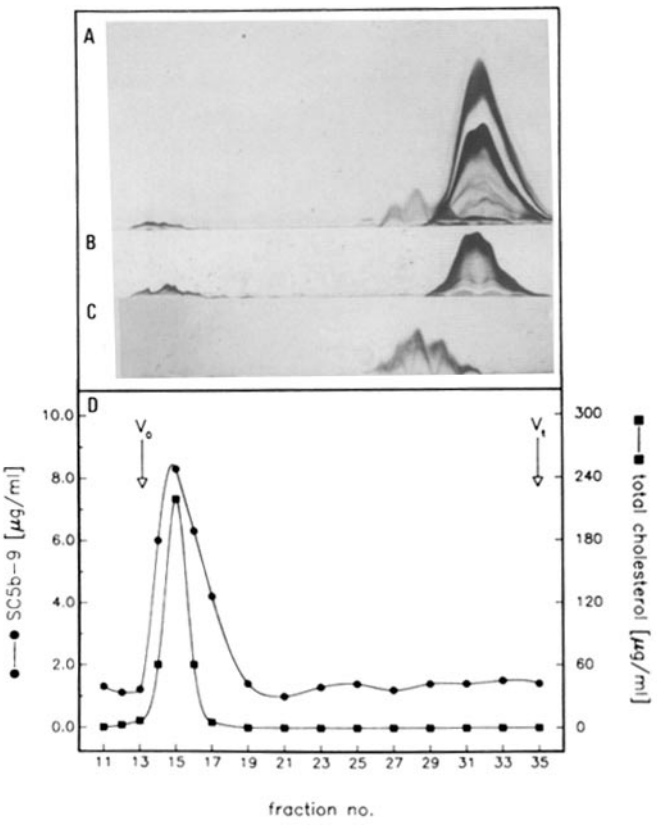

Figure 3. Molecular sieve chromatography of the complement-activating fractions from the sucrose density gradient. Fractions 9 and 10 were applied to a 20 × 100 cm Sepharose 2B column and eluted with VBS^{2+}. Fractions of 8 ml were collected and analyzed by rocket immunoelectrophoresis for serum proteins (*A*); albumin (*B*) and; apoprotein B (*C*). Cholesterol was detected biochemically and found to elute in the void volume (*D*). Each column fraction was mixed with normal human serum for 60 min at 37°C and the degree of complement activation was assessed by measuring the C5b-9 generated. A C5b-9 peak was evident that coincided with the void volume lipid peak (*D*).

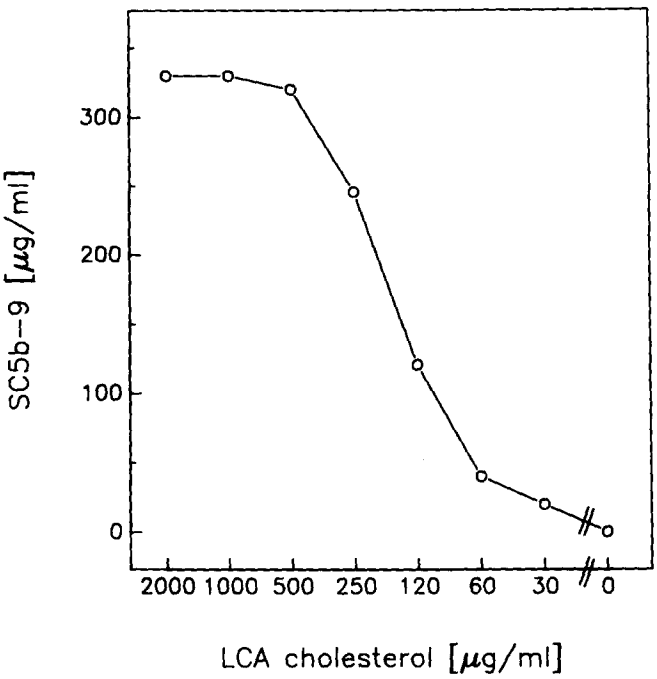

Figure 4. The dose-response relationship of LCA-mediated complement activation. Varying concentrations of LCA were mixed with normal human serum for 60 min at 37°C and the amount of C5b-9 generated was quantitated by an ELISA. C5b-9 values were calculated from a standard curve generated with purified SC5b-9 complexes. In the same experiment, the pooled concentrated apoprotein B peak from the Sepharose 2B column was analyzed and found to generate no C5b-9.

To test whether complement activation occurred via the classical or alternative pathway, activation tests were conducted in the presence of 2 mM Mg^{2+} and 5 mM EGTA. Complement activation was found to occur to an undiminished degree despite blockade of the classical pathway, indicating a predominant involvement of the alternative pathway.

Characterization of the LCA. The LCA exhibited an unesterified to total cholesterol ratio of 0.58, a small amount of triacylglyceride, and an unesterified cholesterol to phospholipid ratio of 1.2 (Table 1). When extracted with chloroform/methanol, 95% by weight of the LCA partitioned to the organic phase. Phospholipids and cholesterol accounted for 43% by weight of the organic phase material. Palmitate, oleate, linoleate and most likely, nervonic acid (or nervonate) constituted the major fatty acids esterified to phospholipid (the technique did not permit analysis of cholesterol esterified fatty acids) (Table 1). The residual material represented a mixture of amino acids that were not further differentiated.

A mixture of amino acids plus two amino sugars, glucosamine and galactosamine, were detected in the water phase of the chloroform/methanol extracts.

As depicted in Fig. 3 *B*, the major immunoprecipitable serum protein present in the LCA Sepharose 2B column fractions was albumin. In SDS-PAGE, a single major protein band of 68,000 daltons was observed in the LCA material (Fig. 6) and was identified as albumin by immunoblotting using antialbumin-specific antibodies (data not shown). A number of faintly staining protein bands were also observed that have not been identified. No immunoprecipitation was observed when concentrated LCA was solubilized with 0.2% Triton X-100 and double diffusion was performed using antibodies against apoprotein B.

Ultrastructural Analyses of LCA. By far the dominating component of the LCA preparations observed in negative staining were spherical lipid particles, mostly 100–500 nm in diameter. These particles exhibited a characteristic blurred contour that distinguishes them from liposomes bounded by phospholipid bilayers, which exhibit sharp surface boundaries. Typical liposomes were present only in minute amounts (Fig. 7 *A*). Examination of a series of fractions from the Sepharose columns used for the purification of the LCA revealed that the particles with blurred contours were confined to the strongly complement-activating fractions, and these particles will henceforth be referred to as the specific LCA particles. Most of these particles carried on their surface tufts of non-

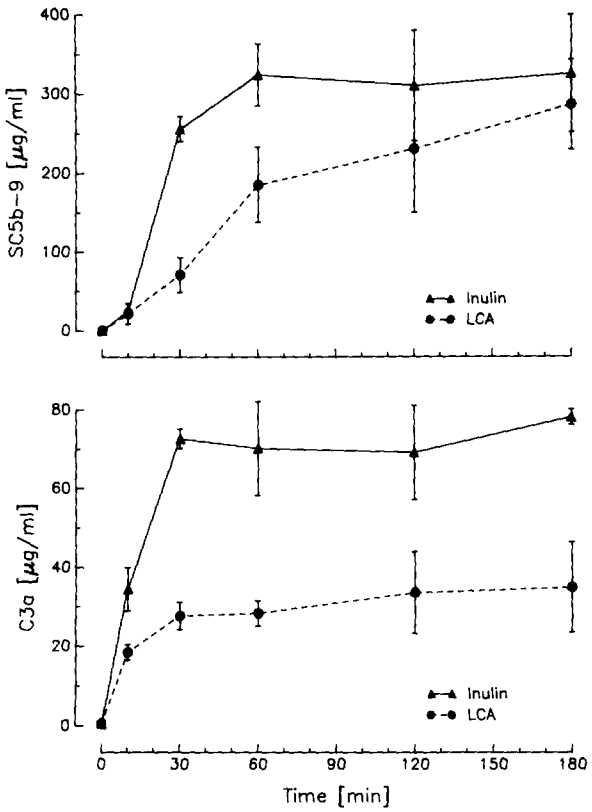

Figure 5. The kinetics of LCA and inulin-mediated complement activation. LCA was mixed with normal human serum at a final cholesterol concentration of 0.75 mg/ml. The amounts of C3a desArg and C5b-9 generated were determined at varying time intervals.

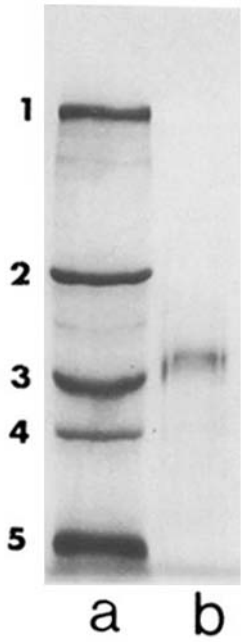

Figure 6. SDS-PAGE of purified LCA. A single band at 68 kD was stained with Coomassie Blue under reducing conditions (lane *b*). Several weakly staining bands were observed of varying molecular mass. Molecular mass markers are shown in lane *a* (from top: 205,000; 92,000; 60,000; 43,000; 29,000).

lipid material (Fig. 7 *A*). This material appeared loosely associated with the particles, evidenced by the finding of such material in partially or fully detached form. Incubation with trypsin and α-chymotrypsin did not remove this material to a detectable extent.

Incubation of the specific LCA particles with filipin failed to result in the development of characteristic filipin-cholesterol complexes. A few filipin lesions were observed associated with the sporadic phospholipid bilayer structures in the preparations. At the higher filipin concentrations applied, the specific LCA particles tended to dissolve.

The nonbilayer organization of the specific LCA particles was confirmed in the thin sections where the dominating structures were hollow "bodies" bounded by a 20–50 nm broad rim with no apparent bilayer configuration and with poor staining properties relative to the multilamellar phospholipid structures that were found sporadically in the sections (Fig. 7 *B*). Many of the hollow bodies carried tufts of weakly stained material on the surface. The hollow bodies were most probably identical with the specific LCA particles observed by negative staining. The density of their interior was identical to that of the background, suggesting that they were indeed hollow. It could not be excluded, however, that they contained lipid that was not preserved by the fixation, and accordingly was extracted in the embedding procedure.

Freeze-fracture replication of LCA preparations demonstrated almost exclusively smooth fracture faces (Fig. 7 *C*). A few of the particles were seen in cross fracture, and appeared to have solid nonaqueous cores. These particles could represent a less common subpopulation of lipid particles.

Analysis of Fresh Plaque Extract. To further exclude that the LCA represents a post-mortem artifact, endartectomy specimens (n = 4) were extracted into PBS and directly applied to a Sepharose 4B column. Two lipid peaks were again resolved (Fig. 8). The void volume peak exhibited the same lipid characteristics as that found for the LCA, i.e., the unesterified to total cholesterol ratio was ~0.5, and no apoprotein B could

Table 1. *Hydrophobic Constituents of the Lipid Complement Activator*

Cholesterol and phospholipid		Fatty acids		
	μg/mg		μg/mg	
Cholesterol total (TC)	287.0	Myristic	14.0	9.5
		Palmitic	16.0	40.2
Cholesterol unesterified (UC)	167.8	Stearic	18.0	5.0
Phospholipid	137.7	Oleic	18.1	24.9
UC/TC	0.58	Linoleic	18.2	12.4
UC/PL	1.20	Arachidonate	20:1	5.8
		Lignoceric	24:0	5.0
		Nervonic	24.1	13.7

Approximately 10 mg (cholesterol) of purified LCA was chloroform/methanol extracted and analyzed by GLC and GLC-MS. All lipids shown were present in the organic phase of the extracts. Only fatty acids with concentrations of ≥5 μg/mg are shown.

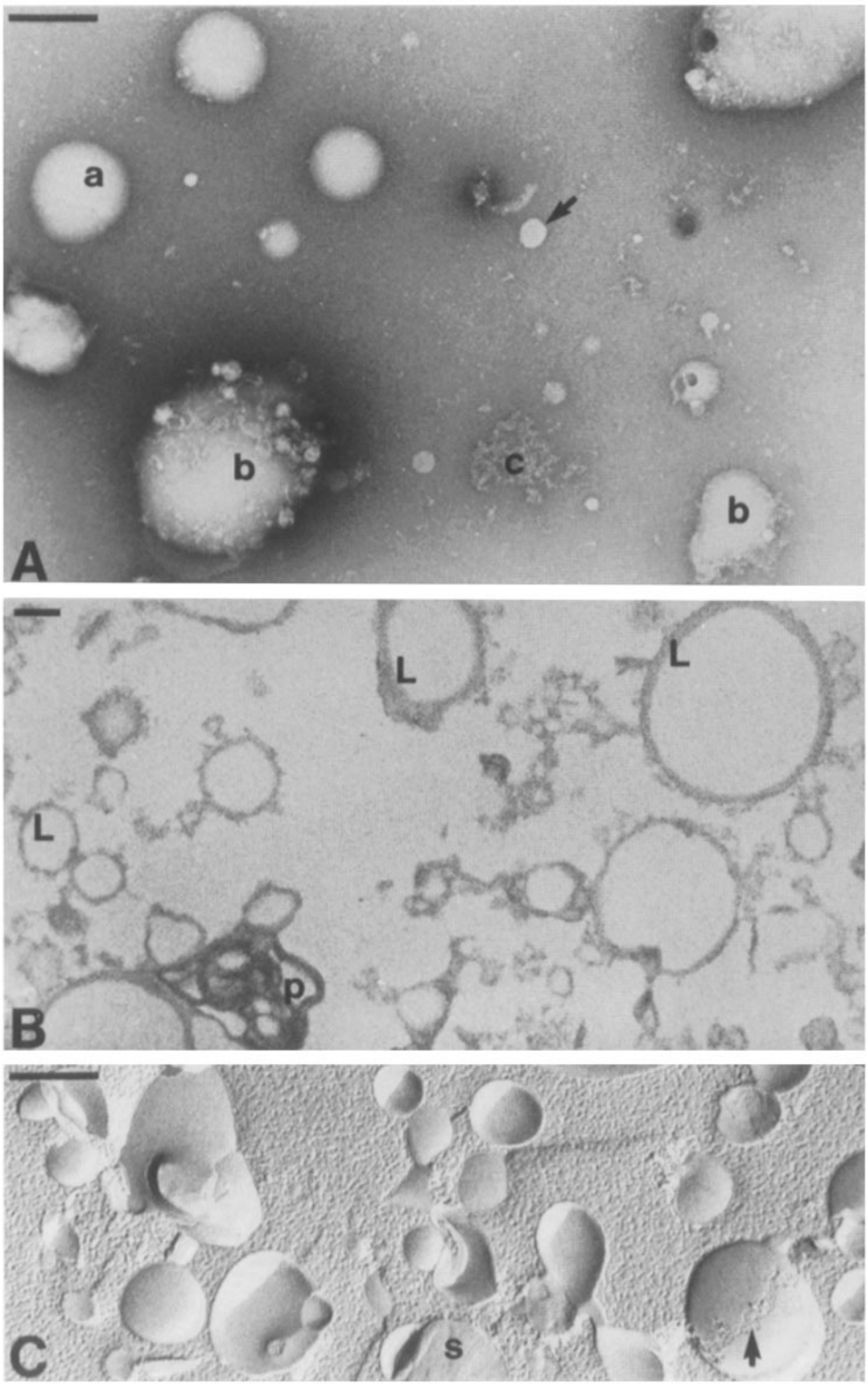

Figure 7. (*A*) Negatively stained preparations of purified LCA from Sepharose 2B fractions 14–16. The dominant constituent is lipid particles with a characteristic blurred contour (*a*). Some of these particles (*b*) are associated with non-lipid material, which may be found detached from the particles as well (*c*). Sharply contoured particles (*arrow*), probably bordered by a phospholipid bilayer, are found only as a minor constituent in these fractions. (*B*) Thin sections of the same preparation. The major constituent is represented by hollow bodies (*L*) bordered by a broad rim, whose dimensions and staining properties differ from that of phospholipid bilayers, that may be found sporadically as multilamellar particles (*P*). (*C*) Freeze-fracture replication of the same LCA preparation showing mostly smooth fracture faces. Irregularities in the fracture face (*arrow*) are occasionally seen, that may represent irregularities in lipid structure rather than incorporated non-lipid material. Cross-fractures of particles revealing a solid nonaqueous core (*S*) are rare and may represent a subpopulation of particles. Scale bars indicate 200 nm.

be detected. The second lipid peak eluted in the same fractions as did purified LDL (data not shown), reacted with polyclonal antibodies to apoprotein B (Fig. 8), and exhibited a lipid composition similar to that of LDL (unesterified to total cholesterol ratio of 0.32). Both the crude extract and the void volume peak eluting from the Sepharose 4B column exhibited complement-activating activity similar to that found for the LCA derived from aortic lesions.

Presence of Membranous C5b-9 Complexes in Atherosclerotic Lesions. C5b-9 neoantigens were detected in the high molecular weight fractions of the sucrose density gradients (Fig. 1). When this material was analyzed by SDS-PAGE/immu-

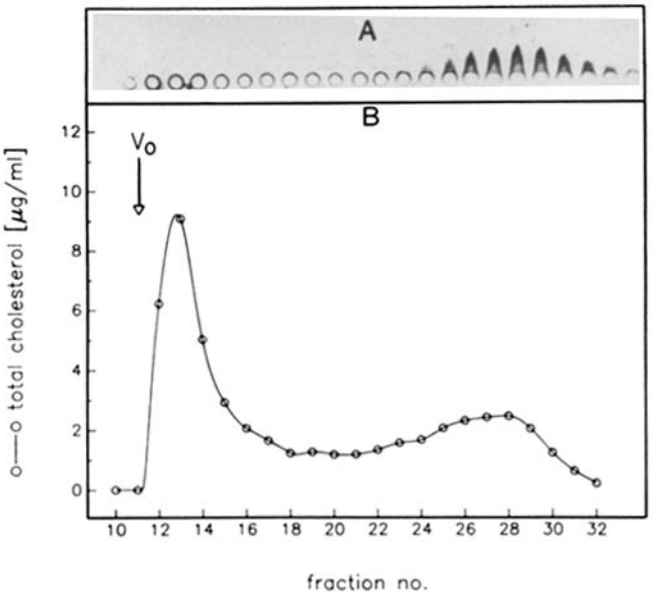

Figure 8. Elution profile of carotid artery atherosclerotic lesion extract fractionated on a 0.9 × 60 cm Sepharose 4B column. Apoprotein B was detected by rocket immunoelectrophoresis (*A*). Cholesterol was detected biochemically (*B*).

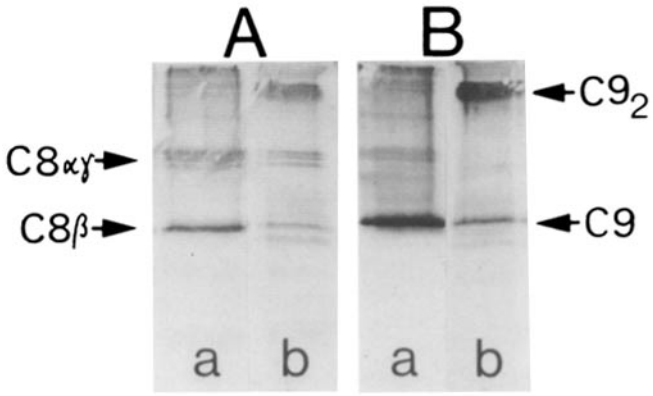

Figure 9. C8 and C9 analysis of lesion extracted C5b-9 complexes by SDS-PAGE followed by immunoblotting. Fraction 3 from sucrose density gradient fractionation (contains C5b-9 complexes, see Fig. 1 and 10) was analyzed for C8 (*A*, lane *b*) and C9 (*B*, lane *b*) using the respective antibodies. Purified SC5b-9 complexes were analyzed in parallel (lanes *a*) for comparison. The subunits within the terminal complex have been previously identified (3, 34).

noblotting, both C8 and C9 components were detected (Fig. 9). Some nonspecific staining of protein bands was observed (high molecular weight band in lane *b*, Fig. 9 *A*, and bands below C8β and C9 in the lanes of both blots), the origins of which were not investigated. No bands developed when the primary antibodies were omitted (not shown). There was a conspicuously strong staining of the C9 dimer ($C9_2$), which is virtually absent in fluid-phase SC5b-9 (Fig. 9 *B*, lane *a*, and reference 26) and whose presence is characteristic of membrane C5b-9 complexes (26). Fluid-phase SC5b-9 was analyzed for comparison (Fig. 9, lane *a*), and it is apparent that the major C8 and C9 protein bands correspond to those detected in the plaque extract. When the high molecular weight fractions of the sucrose density gradients were examined by electron microscopy, they were found to contain large quantities of morphologically characteristic cylindrical C5b-9(m) complement complexes. Many of these carried a girdle of material at their terminus. The bottom fractions (1 and 2, Fig. 1) contained fragments of membranes carrying massive numbers of C5b-9(m) complexes (Fig. 10, *B* and *C*). In fractions 3 and 4, C5b-9(m) was present mostly as single cylinders (Fig. 10 *A*). C5b-9(m) complexes were also sporadically found in very low numbers in the preparations of specific LCA particles (not shown).

Discussion

This paper describes the isolation and characterization of a complement-activating lipid particle from extracts of human atherosclerotic lesions. The lipid, termed LCA, contained no immunoreactive apoprotein B, was 100–500 nm in size, exhibited a unesterified to total cholesterol ratio of 0.58, and an unesterified cholesterol to phospholipid ratio of 1.2. The LCA activated the alternative complement pathway in a dose-dependent manner. Kinetic studies revealed that C5b-9 complex formation was slower than C3a generation, implying that the C5 convertase was stabilized and/or there was a delay in its formation. In addition to the LCA, one other major lipid particle was isolated from lesion extracts. This lipid had the characteristics of LDL and was not an activator of complement, nor could we make purified LDL into an activator by a variety of oxidative or enzymatic means. Since the vast majority of lesion-deposited lipid is derived from LDL, it would appear that within the arterial wall a profound metabolic and physiochemical transformation of LDL occurs, resulting in the evolution of a unique lipid particle capable of activating complement.

Several features of the composition of the LCA suggest that its complement-activating capacity derives from its high content of unesterified cholesterol. Unesterified cholesterol in the crystalline state activates the alternative pathway of complement via an interaction between complement component C3 and the free hydroxyl group of cholesterol (27, 28). Liposomes containing a greater than, but not less than, 50 mole percent unesterified cholesterol spontaneously activate the alternative complement pathway in vitro (12, 13). Data have been reported indicating that unesterified cholesterol in equimolar concentration with phospholipid sequesters to form patches of liquid crystalline structure (29, 30). Hence, it is possible that on the surface of the LCA, the free hydroxyl groups of cholesterol are clustered, thus providing a site for C3 convertase formation.

Regarding possibilities other than unesterified cholesterol as responsible for LCA-mediated complement activation, protein is highly unlikely since albumin was the only protein detected in significant quantities. By electron microscopy a material was observed adherent to the LCA that is probably glycosaminoglycan and/or proteoglycan. This was also sug-

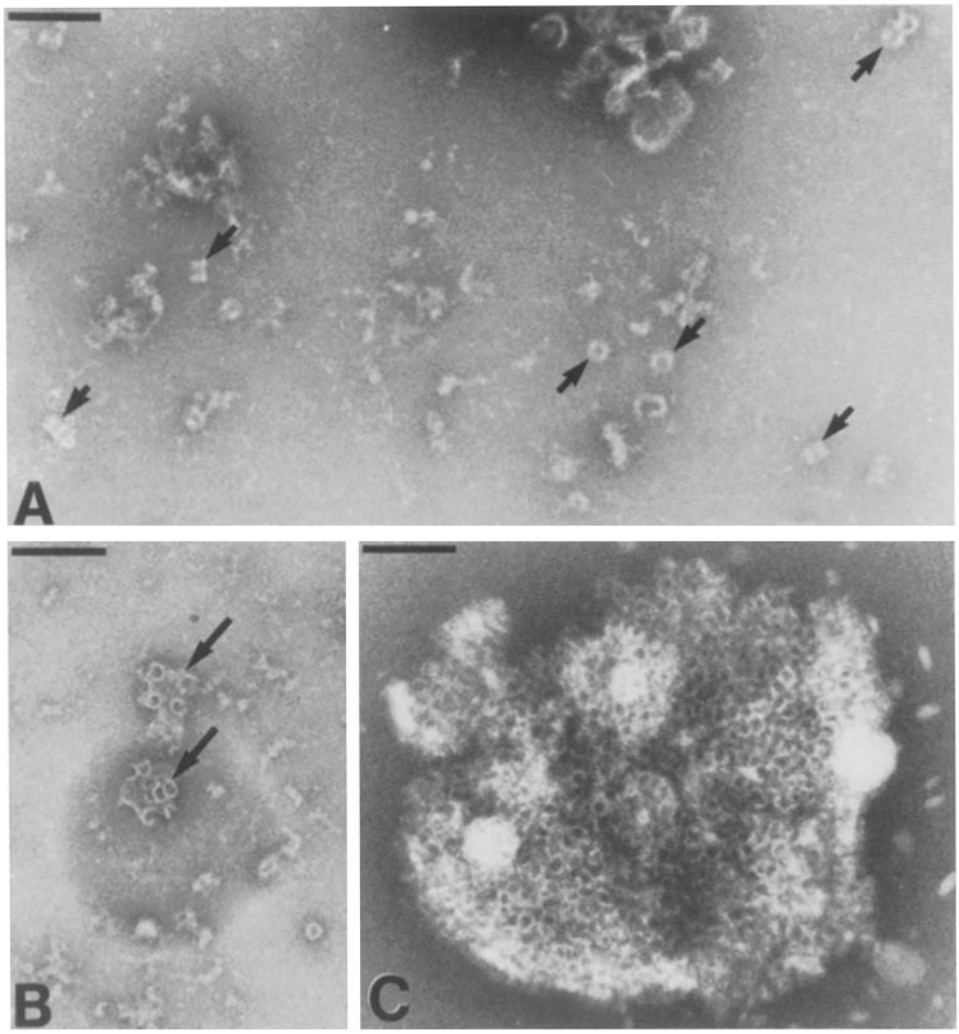

Figure 10. Electron microscopic examination of C5b-9 complexes present in fractionated lesion extract. (*A*) Negative staining of sucrose density gradient fraction 4 (see Fig. 1) demonstrating many single copies of cylindrical C5b-9(m) complexes (*arrows*). (*B* and *C*) Bottom fractions 2 and 3 of the sucrose density gradients contain many membrane fragments carrying C5b-9(m) complexes (*arrows*), sometimes in massive numbers (*C*). Scale bars indicate 100 nm.

gested by the detection of amino sugars in the water phase of LCA chloroform/methanol extracts. Lipid particles are well known to exhibit an affinity for glycosaminoglycans and proteoglycans (31) and have been demonstrated in void volume lipid fractions of lesion extracts (10). Treatment of the LCA with chondroitinase ABC did not, however, interfere with its complement-activating properties (data not shown). This suggests that these substances, if present, were not responsible for the activation of complement. Furthermore, glycosaminoglycans derived from human atherosclerotic lesions (kind gift of Dr. German Camejo, Gothenburg, Sweden) did not activate complement alone or in combination with LDL (data not shown).

Large lipid particles (70–300 nm in diameter) rich in unesterified cholesterol have been isolated from human and rabbit atherosclerotic lesions by Chao et al. (10); however, certain characteristics of these particles differ from what we observed with the LCA. For example, the LCA unesterified cholesterol to phospholipid ratio was 1.2, whereas that described by Chao and colleagues was roughly twice that amount. Additionally, their particles were reported to exhibit uni- and multilamellar phospholipid membrane structure and form complexes with filipin to form the characteristic lesions. The latter difference may in fact be related to the absence of bilayer structure in the LCA, which may in turn be related to its phospholipid content.

One possible explanation for the dissimilarities between the particles described herein and those found by others is that different subpopulations within a larger, heterogeneous population have been isolated due to differences in the techniques used. The results of Chao et al. support this possibility in that within their large (70–300 nm) lipid fraction, they found subpopulations with significantly different lipid compositions after saline density gradient centrifugation (10).

Fractionation of lesion extracts by sucrose density gradient centrifugation revealed that C3, C5b-9, and lipid did not cosediment (Fig. 1). This does not necessarily contradict the finding that lipid represents the major complement-activating entity in lesions. Several studies have demonstrated, for example, that the majority of cleaved C3 formed during activation is released into the fluid phase, and that for certain activators, little if any C3 becomes bound to the activator (32, 33). Furthermore, activated C3 is rapidly degraded by specific complement regulatory proteins, several of which are known

to be present in human atherosclerotic lesions (20). Also, since lesion macrophages possess receptors for C3b and iC3b (20), opsonized lipid might be expected to be phagocytosed. Hence, the fraction of lipid in a plaque carrying bound C3 might be below the detection limits of our assay procedures.

Regarding the C5b-9 complex, there is as yet no evidence that it would remain associated with the lipid particles following assembly at their surfaces. Of significance is the fact that LCA may not be bounded by a lipid bilayer into which C5b-9 insertion could effectively occur. In this connection, it is noteworthy that C5b-9(m) complexes purified from target membranes can post-bind S protein, resulting in the appearance of a girdle of material (presumably the S-protein) at the apolar terminus of the cylinder (34). Such girdles were regularly observed on free C5b-9(m) complexes present in fractionated lesion extracts (Fig. 7). Thus, C5b-9(m) may first assemble on the LCA but then dissociate to bind S-protein. That C5b-9(m) forms on the LCA and subsequently dissociates was also suggested by the kinetics of LCA-mediated complement activation, the strong reaction of C5b-9 in the ELISA, and the finding of occasional C5b-9 complexes in the LCA fractions.

The origin(s) of C5b-9 aggregates attached to membrane-like structures in atherosclerotic lesions requires further study. They may represent secondarily formed conglomerates of terminal complexes attached to LCA, or they may be derived from additional complement attack on genuine plasma cell membranes or membranes of intracellular organelles such as mitochondria. Irrespective of their origin, the presence of C5b-9 complexes in atherosclerotic lesions betrays that complement activation, inevitably accompanied by the generation of large quantities of C3a and C5a anaphylatoxins, occurs at these sites.

Complement C5b-9 complexes are co-deposited with lipid very early in atherosclerotic lesion formation in rabbits (4). In the present paper we have demonstrated the presence of a lipid in human atherosclerotic lesions capable of activating complement. Taken together, the results indicate that lipid-mediated complement activation is intimately associated with lesion progression. It seems reasonable to propose that the cholesterol that accumulates in the arterial tunica intima serves as the nidus for promoting a chronic inflammatory response, and that the activation of complement plays an important role due to generation of proinflammatory complement activation products.

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