A novel monoclonal antibody (ASH1a/256C) that recognizes atherosclerotic lesions in human and Watanabe heritable hyperlipidemic (WHHL) rabbit aortae is described. When \(^{125}\)I-labeled ASH1a/256C antibody is injected intravenously into WHHL rabbits, it associates specifically with fatty streaks on the aorta. The antigen recognized by the antibody is lipid, based on extraction with chloroform and methanol from WHHL rabbit tissues. The antigen, purified by high performance liquid chromatography, was shown to be phosphatidylcholine (PC), which contains unsaturated fatty acyl groups.

Intracellular and extracellular accumulation of neutral lipids in the arterial intima is a typical feature of atherosclerotic lesions. In the early stages of atherosclerosis, foam cells that accumulate cholesteryl ester (CE) droplets in their cytosol are formed from macrophages and smooth muscle cells (1–3). Several types of scavenger receptors, which are capable of binding and taking up modified low density lipoproteins (LDL), have been shown to play crucial roles in foam cell formation (4, 5). In advanced lesions, neutral lipids are also accumulated in the extracellular space, and cholesterol crystals can form (1, 3, 6–8). Neutral lipids may be deposited in the extracellular spaces when foam cells eventually die either by necrosis or apoptosis (9). However, little is known of the mechanisms of extracellular deposition of neutral lipids or the fate of foam cells. Furthermore, it is not known whether lipid accumulation affects cellular responses in the lesions.

Multiple factors are closely involved in the formation of these lesions, including lipoprotein metabolism, smooth muscle cell proliferation, endothelial cell malfunction, formation of modified LDLs, and accumulation of foam cells (4, 5, 10–12). To establish useful tools for the investigation of the mechanisms of atherogenesis, a series of monoclonal antibodies using homogenates of human atheroma as immunogen has been raised. Through characterization of these anti-atheroma antibodies, the presence of vintroenctin (13, 14), oxidized phosphatidylcholine (PC) (15, 16), and cross-linked proteins (17) in human and rabbit atherosclerotic lesions have been demonstrated.

In this study, a monoclonal antibody was selected that bound to fatty streaks using an in vitro artery wall binding assay. Strips of aorta from Watanabe heritable hyperlipidemic (WHHL) rabbits were incubated with hybridoma culture media followed by \(^{125}\)I-labeled second antibody. The antibodies that bound to the surface of fatty streak but not to the normal endothelium were selected. The monoclonal antibody ASH1a/256C (a murine monoclonal antibody against surface of human atheroma), which bound atherosclerotic lesions \textit{in vivo} and immunohistochemically, recognized PC containing polyunsaturated fatty acyl groups (PUFA). The content of PC in atherosclerotic lesions was at most twice that of normals, although the antigenicity of the lesion homogenates was more than eight times higher than that of the normal aortae. The reactivity of this antibody to PC was greatly increased in the presence of...
neutral lipids, suggesting that certain complex structures of PC and neutral lipids are present in atherosclerotic lesions.

**MATERIALS AND METHODS**

**Preparation of Monoclonal Antibody—Atherosclerotic areas of human abdominal aorta were cut into pieces and homogenized with a Polytron® homogenizer in SVE solution (0.25 M sucrose, 1 mA EDTA, 1% ethanol, pH 7.4). After centrifugation at 220 × g for 10 min at 4 °C, the supernatant was recovered and used as immunogen. BALB/c mice (8 weeks old, female) were immunized three times with the homogenate of human atheroma over a period of 3 months (18). Spleens were removed from the immunized mice 3 days after the final injection. The spleen cells were fused with the murine myeloma cell line P3/U1 using polyethylene glycol-4000 and cultured in HY medium (DMEM: spleen cells were fused with the murine myeloma cell line P3/U1 using polyethylene glycol-4000 and cultured in HY medium (DMEM: NCTC109 medium = 8.1 containing 1 m sodium pyruvate, 5 μg/ml insulin, 0.16 mg/ml oxaloacetate, 7% fetal calf serum) containing hypoxanthine, aminopterin, and thymidine (19). Antibody titers in the culture medium of hybridomas were tested by enzyme-linked immunosorbent assay (ELISA) and an *in vitro* binding assay to WHHL aorta. Hybridomas showing anti-atheroma reactivity were cloned by limiting dilution procedure twice.

To select anti-atheroma antibodies, homogenates of human atheroma from WHHL rabbits, and normal aorta obtained from control rabbits as well as human and rabbit sera were used as antigens for ELISA. For those clones that were positive to homogenates of human and rabbit atheroma and negative to the other antigens, immunohistochemical staining of frozen sections (4–6 μm) of WHHL rabbit aorta and human atheroma were performed. Then the *in vitro* binding assay to WHHL aorta was performed for the selected clones that stained atherosclerotic lesions immunohistochimically. Strips of WHHL rabbit aorta (4 × 15 mm) were incubated with the culture medium of the selected hybridomas clones followed by 125I-labeled goat anti-mouse IgG(M) (New England Nuclear Co.). After rinsing the strips with phosphate-buffered saline (PBS) five times, autoradiography was performed. The ascites obtained from mice bearing P3 U1 myeloma, which was not hybridized with any cells, was used as control. One of the clones that produced the highest number of spots corresponding to the areas of fatty streaks was isolated and was named ASH1α/256C. The antibody produced by this clone was partially purified from ascites of mice bearing the hybridoma using ammonium sulfate precipitation. Its immunoglobulin class was IgM. During investigating this antibody, the hybridoma clone has been recloned five times so far, and no change in the reactivity of the antibody has been observed.

**Identification of Antigen Recognized by ASH1α/256C—**Aorta or kidney from WHHL rabbits were cut into pieces and homogenized using Polytron® homogenizer as described previously (14). After removal of cellular debris by centrifugation at 220 × g for 10 min, the supernatant was collected. Lipids were extracted from the homogenate using the method of Bligh and Dyer (21). The lipid extracts were dried under an argon atmosphere when applied on silica gel columns and then eluted with chloroform-methanol-water (6:4:1) to remove phospholipids from neutral lipids. After washing the column with chloroform followed by chloroform:methanol:water (9:1) to remove neutral lipids, polar lipids including those with antigenic activity were eluted with chloroform:methanol:water (6:4:1). The eluate was then fractionated using straight phase high performance liquid chromatography (HPLC) (column: LiChrosorb Si60, 4 × 250 mm, Merck, Germany) by gradient elution with hexane:2-propanol-water (44:55:1 to 33:55:12). The flow rate was 0.5 ml/min. The antigenic activity, which was eluted at 45 min, was separated completely from neutral lipids and glycolipids by this purification step. The antigen recovered from the HPLC was rechromatographed on the same column with another solvent system chloroform:methanol:water (4:5:1) at a flow rate of 0.2 ml/min. The antigenic activity was eluted as a single peak at 23 min.

Molecular species of PC were separated using a reverse phase HPLC (column: LiChrosorb RP-18, 4 × 250 mm, Merck) with isocratic elution of methanol:water:acetonitrile (60:7.2:3.66) with a final concentration of choline chloride of 20 mM. The flow rate was 1.0 ml/min.

**TLC Immunostaining—**Partially purified antigens eluted from the first HPLC separation were spotted onto a TLC plate (Polygram Sil-G, 80001600, Macherey-Nagel Co.). The plate was developed with hexane:diethylether (1:1) followed by chloroform:methanol:water (6:4:1) in the same direction. TLC immunostaining was carried out using the method described by Karasawa et al. (22). Briefly, after the plate was soaked for 4 min in 0.4% polyisobutylmethacrylate (Aldrich), it was incubated with 1% ovalbumin in 15% buffered saline (25 mM Tris-HCl, 100 mM NaCl, pH 7.4) for 2 h at room temperature to avoid nonspecific binding. The plate was incubated with ASH1α/256C antibody diluted 1:1,000 with PBS containing 1% ovalbumin and 1% polyclinviniliprolidone (average molecular weight, 40,000; Sigma). The plates were then incubated with biotin-conjugated goat anti-murine IgG(M) antibody (AM3759; Bio Source International Inc., Camarillo, CA) followed by peroxidase-conjugated streptavidin (Dako Japan). After extensive washing with PBS containing 0.05% Tween 20, the immunostained bands were visualized by incubating the plate with diaminobenzidine hydrochloride (Wako Pure Chemicals, Osaka, Japan) and H2O2.

**Two-dimensional Thin Layer Chromatography—**The purified antigen and PC standard (4 μg each) were spotted onto a silic gel TLC plate. The plates were developed with hexane:diethylether (1:1) following a linear gradient of methanol:water (4:6:1) in the same direction. The plate was then developed in the direction perpendicular to the first run with chloroform:methanol:acetic acid:acetone:water (6:2:4:2:1). The samples were visualized by spraying molybdophosphoric acid onto the plate (23).

**Structural Analyses—**Proton NMR spectra of the purified antigen (2.7 mg) and sn-1-palmitoyl-2-oleinoyl PC (2 mg) dissolved in (CD3)2SO were obtained using a GSX-400 spectrometer (Jeol) with 512-pulse scanning at 400 MHz (24). Two-dimensional cross-relaxation spectra (NMR-COSY) were obtained using 512-pulse scanning at 400 MHz. Proton chemical shifts were indicated in ppm downfield from tetramethylsilane. 13C NMR spectrum was obtained using 61,440-pulse scanning at 100 MHz using the same spectrometer. Carbon chemical shifts were indicated in ppm downfield from the internal solvent (CD3)2SO. Fourier transform infrared spectra of the antigen were obtained using a Fourier transfer infrared spectrometer 8000 spectrometer (Jasco, Japan). Fast atom bombardment mass spectrometry of the antigen were examined using JMS-LX2000 spectrometer (Jeol) with a Hiber LC/ChroCAST RP-18 column (4 × 250 mm; 7 μm; Merck) under the same conditions as described above.

**Measurement of Antigenic Activity—**Reactivity of ASH1α/256C to various materials was determined by ELISA. Aqueous samples, such as homogenates of atheroma, were coated onto 96-well microtiter plates (Falcon number 3912) that had been pretreated with 2% glutaraldehyde for 2 h. After incubating the plates at 37 °C for 1 h, the surfaces of the microtiter wells were blocked by incubating with Tris-buffered saline containing 2% skimmed milk. The plates were incubated with ASH1α/256C antibody diluted with Tris-buffered saline containing 2% skimmed milk followed by alkaline phosphatase-conjugated goat anti-murine IgG(M) antibody (Tago Inc., AM3750). After washing extensively with Tris-buffered saline containing 0.05% Tween 20, the plates were incubated with p-nitrophenyl phosphate (0.4 mM) at 37 °C for 30 min in 1 M diethanolamine-HCl buffer, pH 9.8 at 37 °C for the appropriate time periods. The absorbance at 405 nm was measured photometrically using an ELISA plate reader (Bio-Rad).

When the antigenic activities of the lipids were tested, their methanol solutions were placed into microtiter wells without the pretreatment with glutaraldehyde. The plates were incubated at 37 °C for 5–10 min to remove the methanol in which the surfaces of the microtiter wells were blocked with 0.3 M sucrose. Oxidized PC was prepared by incubating sn-1-stearoyl-2-oleinoyl PC (400 nmol in 1 ml of PBS) with ferrous sulfate (40 μM) and ascobic acid (0.4 mM) at 37 °C for various periods of time. Total oxidized lipids were extracted by the method of Brigh and Dyer (21). Two aldehydehyde-containing oxidized PC, sn-1-palmitoyl-2-(9-oxonanoyl) PC (9-CHO PC) and sn-1-stearoyl-2-(5-oxopentanoyl) PC (5-CHO PC) were prepared by reductive osmium tetroxide treatment of sn-1-palmitoyl-2-oleoyl PC and sn-1-stearoyl-2-arachidonyl PC, respectively. The oxidized lipids suspended in PBS were incubated with BSA (ratio, 1 nmol of PC/1 μg of BSA) at room temperature for 30 min, and then they were placed in microtiter wells (6.5 nmol of oxidized PC/well).

**Density Gradient Ultracentrifugation of Atheromatous Lipids—**Homogenates of atherosclerotic lesions from WHHL and normal rabbit aorta (6 mg of protein) were fractionated using a sucrose density gradient ultracentrifugation according to the method described previously (20). Briefly, a linear gradient of SVE solutions containing 53 to 0% sucrose was layered on top of the homogenates containing 64% sucrose. Ultracentrifugation at 89,000 × g for 75 min at 4 °C using RPS-27 rotor (Hitachi), samples were collected from each ml from the bottom to the top of the gradient.

**Histochemical Study of WHHL Rabbit Aorta—**Frozen sections (4–6 μm) of WHHL rabbit fatty streaks were obtained and fixed with 10% neutral formalin immediately after autopsy. The sections were incubated with ASH1α/256C (ascites) followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG(M) (Organin Teknika Corp.,
Durham, NC). The adjacent WHHL section was stained with 0.1% oil-red O in 60% 2-propanol for 10 min, and the section was counterstained with Mayer’s hematoxylin for 5 min after washing off any excess oil-red O with 2-propanol.

**Other Analytical Methods**—The amounts of total cholesterol were measured by a cholesterol oxidase method using the Cholestase-V kit (Nissui, Co.) (25, 26). Levels of phospholipids were determined by measuring phosphorus in organic extracts using malachite green according to the method of Zhou and Arther (27). Protein concentrations were measured by the Bradford method using the Bio-Rad protein assay kit with BSA as the standard (28).

**RESULTS**

**A New Antibody That Binds Specifically to Atheromatous Lesions**—In an attempt to obtain monoclonal antibodies against atherosclerotic lesions, hybridoma clones from mice immunized with homogenates of fatty streak lesions of human atheroma were prepared. Anti-atheroma clones were selected by ELISA using homogenates of atheroma from humans and WHHL rabbits for initial screening, followed by immunohistochemical staining using frozen sections of WHHL rabbit aorta. Then candidate clones were further tested using a binding assay to WHHL rabbit aorta strips. Clones reactive to materials in human and rabbit sera proteins were omitted. A clone was finally established after these selections and was named ASH1a/256C (atheroma, surface, human).

123I-Labeled ASH1a/256C antibody was injected intravenously into normal and WHHL rabbits. These rabbits were sacrificed 48 h after injection, and the distribution of the labeled antibody in isolated aortas was visualized by autoradiography (Fig. 1). Fatty streaks were observed in the WHHL rabbit aorta but not in the normal rabbit aorta. Lesion formation was prominent in the aortic arch and at the points of vessel branching. The radioactivity was co-localized with the atherosclerotic plaques in the WHHL rabbit aorta. In contrast, the area that was free of visible lesions in the WHHL aorta and the aorta from normal rabbit were negative. The antibody reacted strongly to atheromatous homogenates from human and WHHL rabbits but did not react to homogenates from normal rabbits (Fig. 2). Furthermore, this antibody also bound to atheromatous lesions in WHHL rabbit aortae as shown by an in vitro binding assay (see “Materials and Methods”). These results show that this monoclonal antibody recognizes atherosclerotic lesions both in vivo and in vitro.

**Antigen Purification**—The antigen of ASH1a/256C was effectively extracted with chloroform and methanol from homogenates of rabbit aorta with the residual fractions having no antigenicity, suggesting that the antigen is likely to be lipid (Fig. 2). The reactivity of the antibody to the lipid extracts from the WHHL rabbit aorta was 8-fold greater than that from the same amount (10 μg of protein) of normal rabbit aorta homogenate. When the same amount of phospholipid extracted from either the WHHL aorta or normal rabbit aorta was used as antigen, the antigenic activity in WHHL extract was 3.9-fold higher than the extract from normal rabbit by phospholipid basis (data not shown).

When the antigenicity of homogenates of several tissues to ASH1a/256C was examined by ELISA, kidney and xanthoma as well as aorta from WHHL rabbits showed strong activities (data not shown). The lipid extracts obtained from atheroma and kidney of WHHL rabbits were fractionated by silica gel column chromatography followed by HPLC. The identity of the antigens obtained from atheroma and kidney was investigated by the following experiments. First, when the partially purified antigens were analyzed by TLC immunostaining using ASH1a/256C, both samples showed single bands with the same retention times.
Finally, the same molecular mass numbers were obtained for these antigens by liquid chromatography-mass spectrometry analysis (data not shown). Therefore, the antigens in atheroma and kidney could be identical.

The antigen was purified from both the aorta and kidney of WHHL rabbits. The antigen purified from the kidney was used to perform structural analyses (see below), because the quantity of the antigen purified from rabbit aorta was very limited.

Fraction III was then applied to a straight phase HPLC with a gradient elution using hexane:2-propanol:water (44:55:1 to 33:55:12). The antigenic activity was eluted at 44 min as a single peak (Fig. 4B). This fraction was further purified on the same HPLC column using a different solvent system (Fig. 4C). The purified antigen, which was eluted at 23 min as a single peak by the second HPLC, showed a single spot on two-dimensional TLC (data not shown).

Structural Analyses of the Antigen—The antigen purified from WHHL rabbit kidney underwent a number of structural analyses. No signal corresponding to either ketone, aldehyde, acid anhydride, or free carboxylic acid was observed by Fourier transfer infrared spectrum of the antigen; however, the spectrum did suggest the presence of ester bonds (C=O; 1735 cm$^{-1}$) (data not shown). The presence of two ester bonds (C=O; 172 ppm) was confirmed by a $^{13}$C NMR spectrum (Fig. 5A). Signals corresponding to two C=O double bonds (127 and 129 ppm) were also observed in the $^{13}$C NMR spectrum.

Furthermore, one-dimensional and two-dimensional NMR analysis (NMR-COSY) of the antigen was performed to identify its molecular structure. The signals marked in alphabets in the $^{1}$H NMR spectrum of the antigen were identified as described in the legend of Fig. 5. The spectrum of the antigen was found to be very similar to that of sn-1-palmitoyl-2-linoleoyl PC (Fig. 5, C and D). One particular signal (d = 3.1 ppm; 9H, marked with asterisks in Fig. 5 (C and D), corresponds to signal e) did not interact with any other signal, suggesting that there is no proton in close proximity to the nine hydrogen atoms in the
Fig. 5. **NMR analyses of the purified antigen.** A, $^{13}$C NMR spectrum of the purified antigen in (CD$_3$)$_2$SO. B, $^1$H NMR spectrum of the purified antigen in (CD$_3$)$_2$SO. C and D, two-dimensional cross-relaxation spectra (NMR-COSY) of the purified antigen (2.7 mg) (C) and authentic sn-1-palmitoyl-2-linoleoyl PC (D) in (CD$_3$)$_2$SO are shown. The signals at 3.1 ppm (asterisk) that do not interact any other peak were identified as...
antigen, as is the case with the N-trimethylamino group of the authentic sn-1-palmitoyl-2-linoleoyl PC. These results strongly suggest that the antigen is PC.

Analyses of the antigen by fast atom bombardment mass spectrometry showed several peaks ranging from m/z = 756–808. One of the peaks (m/z = 756) corresponds to palmitoyl-linoleoyl PC. The molecular species of the antigenic PC were separated by reverse phase HPLC (Fig. 6). Several antigenic peaks appeared, and a major antigenic peak at 21 min and a large peak at 29 min were identified by liquid chromatography-mass spectrometry as palmitoyl-linoleoyl PC and stearoyl-linoleoyl PC, respectively. It appears that the antigenic PC consists of several molecular species with different combinations of fatty acids. The possibility that certain compounds other than PC are present in the purified antigen is very unlikely for two reasons: first, the antigen was purified to homogeneity by two-dimensional TLC by which most of the phospholipid classes were separated, and, second, all of the signals (apart from one corresponding to the N-trimethylamino group in the NMR-COSY analysis) interacted with other signals. Therefore all of the signals were related to one structure. These results confirm that the monoclonal antibody ASH1a/256C recognizes PC molecules containing PUFA.

Specificity of the Antigen Recognition—To investigate specificity of ASH1a/256C to recognize PC, reactivity of the antibody to various phospholipids, neutral lipids, and PC-related compounds was examined by ELISA (Table I, experiment 1). The antibody did not react to phosphatidylethanolamine, monomethyl phosphatidylethanolamine, or dimethyl phosphatidylethanolamine, indicating that the binding was specific for the choline-containing head group. Because these three phospholipids were prepared from egg PC by a head exchange reaction, their fatty acid compositions are essentially the same (palmitic acid, 50%; oleic acid, 25%, palmitoleic acid; linoleic acid, 16%; stearic acid, 8%). Other phospholipids such as phosphatidylserine and phosphatidylinositol had no reactivity with the antibody. All the neutral lipids tested were also negative. Platelet-activating factor and sphingomyelin were not antigenic, although they share the choline head group. It seems that not only the choline head group but certain combinations of acyl groups are necessary for antigen recognition.

Reactivity of the antibody to various molecular species of PC was examined using chemically synthesized PCs (Table I, experiment 2). The positional isomers sn-1-stea royl-2-linoleoyl PC and sn-1-linoleoyl-2-stearoyl PC had almost equal reactivity, suggesting that the position of PUFA is not important. Inability of the antibody to bind to lysoPC and glycerophosphocholine further supports this previous observation. Concerning PC species without PUFA, dipalmitoyl PC and distearoyl PC did not react with the antibody, and dioleoyl PC reacted only slightly. Dilinoleoyl PC was as active as sn-1-stearoyl-2-linoleoyl PC, suggesting that PUFA itself is necessary for the recognition.
reduction in reactivity of the antibody to bind with 1-stearoyl-2-linoleoyl PC (data not shown).

When human LDL, copper-oxidized LDL, or high density lipoprotein, was not found to be a good antigen. Capable of increasing the binding of the antibody to PC.

The Effect of Neutral Lipids on the Antigenicity of PC—As mentioned above, the antigenic activity was effectively extracted with chloroform and methanol from homogenates from rabbit tissues. Recovery of the antigenic activity was, however, reduced significantly during the purification of the antigen. The final yield of antigen activity was approximately 6.6%. It is noteworthy that the specific activity of the antigen was normalized by the amount of phosphorus decreased during the purification. A possibility to be considered is that there may be activators of antigen-antibody interaction in the homogenates. One of the major characteristics of atherosclerotic lesions is accumulation of neutral lipids; to see whether neutral lipids enhance the antigenicity of PC, the reactivity of ASH1a/256C to PC in the presence of neutral lipids was measured using ELISA. Addition of cholesterol, CE, or triacylglycerol markedly increased the binding of the antibody to PC. LDL, a huge particle containing phospholipids, neutral lipids, and apolipoprotein B, was not found to be a good antigen. When human LDL, copper-oxidized LDL, or high density lipoprotein were coated onto microtiter wells, no reactivity was observed with the antibody ASH1a/256C (data not shown).


d-1-Stearoyl-2-linoleoyl PC (0.4 mM) was incubated with ferrous sulfate (40 μM) and ascorbate (0.4 mM) in PBS at 37 °C for indicated periods. Oxidized lipids extracted from the reaction mixture were mixed with BSA and then placed onto microtiter wells as antigen (6.5 nmol PC/well). ELISA was performed using ASH1a/256C and FOH1a/DLH3 as described under “Materials and Methods.” The results are expressed as relative reactivity to the highest values obtained by these antibodies. The absorbance obtained with ASH1a/256C (0 min) and FOH1a/DLH3 (3 h) were 0.685 and 0.579, respectively. Note that the reaction with ASH1a/256C was less effective under this experimental condition than the data in Table I, because the antigen suspended in PBS as lipid-BSA mixture was coated onto microtiter wells without glutaraldehyde pretreatment. The decrease in the ELISA reaction of ASH1a/256C during the oxidation of PC was equally observed under the other experimental conditions.

FIG. 7. Effect of neutral lipids on the antigenicity of PC. Various amounts of cholesterol olate (closed circles), triolein (open circles), and cholesterol (open squares) were coated onto microtiter wells together with 1.3 nmol of d-1-stearoyl-2-linoleoyl PC. The antigenic activities of these PC-neutral lipid mixtures as demonstrated by ELISA are shown as relative percentages of those without neutral lipids.

The lipid droplets in aorta homogenates were separated from cellular membranes and proteins by sucrose density gradient ultracentrifugation. As shown in Fig. 8B, the antigenic activity in WHHL rabbit atheroma separated into two peaks, the top fractions and the middle fractions. These fractions correspond to lipid droplets and cellular membranes, respectively. The distribution of the antigenic activity corresponds to the

TABLE I
Reactivity of various lipids against ASH1a/256C

| Experiment 1 | Egg PC | 1.00 |
|--------------|--------|------|
|              | Egg dimethyl PE | 0.00 |
|              | Egg monomethyl PE | 0.00 |
|              | Egg PE | 0.05 |
|              | Egg FA | 0.00 |
|              | Egg lysoPC | 0.00 |
|              | Yeast phosphatidylserine | 0.00 |
|              | Yeast phosphatidylinositol | 0.00 |
|              | Soy bean cardiolipin | 0.00 |
|              | Brain sphingomyelin | 0.06 |
|              | Platelet activating factor | 0.00 |
|              | Phosphatidylglycerol | 0.00 |
|              | Ceramide | 0.02 |
|              | TC (Triolein) | 0.00 |
|              | CE (Cholesterol olate) | 0.00 |
|              | Linoleic acid | 0.05 |
|              | Stearic acid | 0.00 |
| Experiment 2 | sn-1-Linoleoyl-2-stearoyl PC | 1.00 |
|              | sn-1-Stearoyl-2-linoleoyl PC | 0.80 |
|              | sn-1-Palmitoyl-2-linoleoyl PC | 0.83 |
|              | Dilinoleoyl PC | 0.82 |
|              | Distearoyl PC | 0.02 |
|              | Dioleoyl PC | 0.19 |
|              | sn-1-Oleoyl lysoPC | 0.01 |
|              | Glycerophosphocholine | 0.00 |
| Experiment 3 | sn-1-Stearoyl-2-linoleoyl PC | 1.00 |
|              | sn-1-Palmitoyl-2-(9-oxooxanoyl) PC | 0.00 |
|              | sn-1-Stearoyl-2-(5-oxooxentanoyl) PC | 0.00 |
|              | sn-1-Stearoyl lysoPC | 0.00 |
|              | sn-1-Oleoyl lysoPC | 0.00 |

TABLE II
Effect of peroxidation of PC on its antigenicity to ASH1a/256C

| Incubation time | ASH1a/256C | FOH1a/DLH3 |
|----------------|------------|------------|
| 0 min          | 1.00       | 0.03       |
| 10 min         | 0.83       | 0.15       |
| 60 min         | 0.81       | 0.37       |
| 3 h            | 0.68       | 1.00       |
| 24 h           | 0.68       | 0.89       |
amounts of both phospholipid and cholesterol. In the case of normal rabbit aorta, there was no antigenic activity, although phospholipids were localized in fraction 7. The cholesterol content in fraction 7 was about \(\frac{1}{100}\) of that of the corresponding fraction of WHHL rabbit (Fig. 8A). These results suggest that antigenicity in rabbit aorta is greatly affected by cholesterol accumulation in the tissue.

To confirm the effect of cholesterol on the reactivity of PC in atherosclerotic lesions, an aliquot of cholesterol was added to each fraction obtained from normal rabbit aorta by sucrose density gradient centrifugation (Fig. 9). The ASH1a/256C antibody strongly reacted to the top and middle fractions following the addition of cholesterol, especially to fraction 6, which contained cellular membrane phospholipids. Similar enhanced antigenicity was also observed by addition of either cholesteryl olate or triolein (data not shown). These results show that addition of neutral lipids to normal vessel wall increases the antigenicity of PC as observed in WHHL rabbit atheroma.

**Immunohistochemical Analysis**—Serial sections of WHHL rabbit atherosclerotic aorta were stained with ASH1a/256C and oil-red O to study the localization of antigenic PCs and lipid deposits. Large intracellular lipid droplets related to foam cells and small lipid droplets in extracellular matrix were observed when stained with oil-red O (Fig. 10A). ASH1a/256C stained the area where small lipid droplets were profusely deposited (Fig. 10B), whereas the antibody did not stain the endothelium and the media. These immunohistochemical observations, together with the other results, strongly suggest that the ASH1a/256C antibody does not recognize normal cellular membranes but rather that certain structures of PCs complexed with neutral lipids formed in atherosclerotic lesions.

**DISCUSSION**

This paper describes the preparation of a novel monoclonal antibody that recognizes fatty streaks in human atherosclerotic
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aorta. This antibody was selected by reactivity to homogenates of atheroma using ELISA and to atheromatous plaques in aortic strips using an in vitro binding assay. The antibody also recognized atherosclerotic lesions of WHHL aorta in vivo.

The antigen is a lipophilic compound, based on the effective extraction from WHHL rabbit aortae by use of organic solvents. The antigen was purified by repetitive HPLC to a single spot on two-dimensional TLC. From extensive spectrometric analyses the purified antigen was identified as PC. Other phospholipids and neutral lipids were inactive. By reverse phase HPLC, the purified antigen was shown to contain several antigenic molecular species of PC. One major antigenic species was confirmed to be sn-1-palmitoyl-2-linoleoyl PC, by comparison with authentic PC and by use of liquid chromatography-mass spectrometry. Judging from the reactivity of the antibody to various molecular species of PC and PC analogs, it was concluded that the choline head group is necessary for antigen recognition and that at least one PUFA is also required.

It is intriguing that the monoclonal antibody that recognizes PC binds to atherosclerotic lesions in vivo and in vitro binding assays, despite PC, a major component of cellular membranes, having a ubiquitous distribution in whole animal tissues. It is possible that the microenvironments of PC molecules in normal aorta and atherosclerotic lesions are different. The current data indicate that PC mixed with neutral lipids such as cholesterol was highly reactive with the antibody, although the neutral lipids themselves were not antigenic. Fractionation of aortic homogenates by density gradient centrifugation showed that fractions rich in both phospholipids and neutral lipids were antigenic, and, furthermore, addition of neutral lipids to the PC-rich fraction from normal aorta markedly increased its antigenicity. From these observations, it is proposed that the monoclonal antibody ASH1a/256C is likely to recognize particular conformations or packing structures of PC molecules that are formed in the presence of high concentrations of neutral lipids.

In atherosclerotic lesions there are a number of foam cells that accumulate neutral lipids as cytoplasmic and lysosomal droplets (1–3). Immunohistochemical studies showed that the ASH1a/256C antigen present in atherosclerotic lesions of WHHL aorta preferentially found in areas rich in small lipid droplets. The scenario suggested above is supported by our recent experiments showing that ASH1a/256C reacts to the small lipid droplets left after the foam cells die in culture.

The possibility that antigenic-PC neutral lipids form complexes without being accumulated in macrophages and smooth muscle cells cannot be ruled out. From a series of extensive electromicroscopic studies Guyton and co-workers (2, 3, 7, 8, 29) proposed that free-cholesterol-rich particles in the extracellular space could be formed without prior accumulation of lipids in foam cells. This group has shown that extracellular lipid vesicles accumulate in early lesions prior to the appearance of lipid-laden foam cells.

A number of monoclonal antibodies that recognize atherosclerotic materials have been prepared by many investigators; however, few of them have succeeded in identifying their antigenic materials. An anti-oxidized LDL monoclonal antibody, FOH1a/DLH3, that recognizes foam cells has previously obtained (15). Its antigen was identified as oxidized products of PC including 9-CHO PC (16). The specificity of ASH1a/256C is clearly different from that of FOH1a/DLH3. The former does not bind to OxPC or oxidized LDL, and the latter does not recognize native PC species. Another monoclonal antibody recognizing atherosclerotic lesions prepared in a previous study, EMR1a/212D, specifically stained extracellular regions of atheroerotic intima from WHHL rabbits in immunohistochemical studies (18). The antibody was shown to recognize rabbit vitronectin (13), and, using this antibody, accumulation of subtypes of vitronectin with small molecular masses was demonstrated (14).

The antibody reported in the present study is unique in that it represents unusual structures of common lipid complexes. Further study is needed to understand the physical properties of the putative antigenic PC-neutral lipid complex in the lesions. Finally, this antibody can bind to atherosclerotic lesions in vivo, thus applications for immuno-diagnosis and drug delivery systems may be possible in the future.

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