Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 1902–1906, March 1988
Immunology

Antibodies to cholesterol

(liposomes/lipid A/complement/atherosclerosis)

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Communicated by Keith R. Porter, October 16, 1987

ABSTRACT **Cholesterol-dependent complement activation has been proposed as a factor that might influence the pathogenesis of atherosclerosis. Although antibodies to cholesterol conjugates have been reported, cholesterol is widely regarded as a poorly immunogenic substance. Monoclonal IgM complement-fixing antibodies to cholesterol were obtained in the present study after immunizing mice with liposomes containing high amounts of cholesterol (71 mol % relative to phosphatidylcholine) and lipid A as an adjuvant. Clones were selected for the ability of secreted antibodies to react with liposomes containing 71% cholesterol but not with liposomes containing 43% cholesterol. The antibodies also reacted with crystalline cholesterol in a solid-phase enzyme-linked immunosorbent assay. Binding of monoclonal antibodies to the surface of crystalline cholesterol was demonstrated by electron microscopy by utilizing a second antibody (anti-IgM) labeled with colloidal gold. The immunization period required to induce monoclonal antibodies was very short (3 days) and a high fraction of the hybrid cells (at least 70%) were secreting detectable antibodies to cholesterol. The results demonstrate that cholesterol can be a highly immunogenic molecule and that complement-fixing antibodies to cholesterol can be readily obtained.**

Cholesterol has been widely studied as a fundamental and ubiquitous constituent of cell membranes and lipoproteins (1, 2). Despite occasional reports that conjugates of cholesterol can be immunogenic (3–5), because of its widespread distribution and important biological roles cholesterol has generally been assumed to be a nonimmunogenic or poorly immunogenic molecule. However, since 1977 several laboratories have reported activation of the classical and alternative pathways of complement by cholesterol (6–11). The exact mechanism (or mechanisms) of complement activation by cholesterol has not been determined. In one case human IgG antibodies to cholesterol were implicated in the activation of the classical pathway (9). On the other hand, it has been proposed that nonimmune activation of the alternative pathway of rabbit complement (7) or human complement (8, 9) also occurs. The issue of the immunogenicity of cholesterol is an important one, since antibodies to cholesterol have been reported to protect against induced atherosclerosis in rabbits (4). On the other hand, activation of the classical pathway of complement by cholesterol has been proposed as a possible mechanism in the pathogenesis of atherosclerosis (6, 9, 11).

In previous studies we induced antibodies to the phospholipid constituents of lipid model membranes (liposomes) (reviewed in ref. 12). Production of antibodies to liposomes was achieved by utilizing lipid A as a powerful adjuvant in the liposomal lipid bilayer. In the present work we used lipid A as an adjuvant to induce antibodies to liposomes containing high concentrations of cholesterol. We found that cholesterol is an excellent immunogen and that murine monoclonal antibodies to cholesterol are easily obtained.

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MATERIALS AND METHODS

Liposomes. The liposomal lipids consisted of combinations of dimyristoyl phosphatidylcholine ([Myr_2]PtdCho) (Calbiochem-Behring), cholesterol, dicetyl phosphate (Cet_2-*P*) (K & K Laboratories, Fairview, NJ), and lipid A (isolated from *Escherichia coli* O-111 as described in ref. 13). Different batches of reagent-grade cholesterol received from several suppliers were not completely "pure" and had melting points ranging from 118°C to 145°C. After recrystallization three times from hot ethanol, the published melting point (148°C) was achieved. The effectiveness of the cholesterol-purification procedure was further manifested by the fact that when tested as an antigen by ELISA (see below), after each of the recrystallizations the cholesterol showed progressively improved antigenic activity. When fully purified cholesterol was examined by thin-layer chromatography, only a single spot was present and a contaminant sometimes found in unpurified cholesterol (25-hydroxycholesterol) was not detected. Complete details on the sources of lipids and preparation of liposomes (multilamellar vesicles) have been given previously for liposomes in general (13) and high-cholesterol liposomes in particular (6). The liposomes were multilamellar vesicles prepared by Vortex mixing of dried lipids in isosmotic swelling solution.

For immunization the liposomes contained [Myr_2]PtdCho/cholesterol/lipid A (molar ratio 1:2.5:0.01, where the molarity of lipid A refers to lipid A phosphate). For assays of antibody activity by complement-dependent glucose release, the liposomes contained [Myr_2]PtdCho/cholesterol/Cet_2-*P* (6, 13). The assay liposomes contained [Myr_2]PtdCho and Cet_2-*P* in a ratio of 1:0.11, and cholesterol was present in the concentration indicated for each experiment. The liposomal cholesterol concentration is described as a percentage, and this is calculated as mol % with reference to [Myr_2]PtdCho; e.g., a cholesterol/[Myr_2]PtdCho ratio of 0.75:1 is 43 mol %, 2.5:1 is 71 mol %, etc. The 71% cholesterol liposomes were used immediately after preparation and were quite stable even over 24 hr. As described earlier (6), liposomes containing either 43% or 71% cholesterol had identical light-scattering properties and ability to trap glucose, and they were equally sensitive to complement-dependent damage leading to glucose release in the presence of antibodies to liposomal antigens.

Abbreviations: [Myr_2]PtdCho, dimyristoyl phosphatidylcholine; Cet_2-*P*, dicetyl phosphate.

The liposomes used for immunization were swollen in nonpyrogenic 0.154 M (0.9%) NaCl prepared for i.v. infusion (Cutter). The liposomes used for complement-dependent glucose release were swollen in 0.308 M glucose. In each case the volume of swelling solution used was selected such that the [Myr_2]PtdCho concentration was 10 mM. The liposomes were dialyzed against 0.15 M NaCl to remove untrapped glucose (6, 13).

Complement-Dependent Immune Assay. Complement-dependent immune damage to liposomes resulting in release of trapped liposomal glucose has been described in detail (6, 13). Glucose released from liposomes by the action of complement was measured spectrophotometrically by using a Tris-buffered assay reagent containing hexokinase, glucose-6-phosphate dehydrogenase, ATP, and NADP.

Enzyme-Linked Immunosorbent Assay (ELISA). ELISAs were performed by using crystalline cholesterol as an antigen on the bottoms of the wells of microtiter plates (13). The ELISA procedure was developed by modification of previous techniques for analysis of antibody binding to lipid antigens (14–16). Crystalline cholesterol was coated onto the surface of wells in polystyrene plates (Immulon 96, "U" bottom, Dynatech Laboratories, Alexandria, VA) by addition of an ethanolic solution and evaporation of the solvent by air under a fume hood. Plates were further dried under high vacuum and stored at −30°C when not used the same day. Plates were blocked by addition of phosphate-buffered saline (PBS: 137 mM NaCl/2.7 mM KCl/9.6 mM phosphate, pH 7.2) containing 10% heat-inactivated fetal bovine serum (FBS) (M.A. Bioproducts, Walkersville, MD). This was accomplished by washing the wells three times for 10 min each. Fifty microliters of ascites fluid containing monoclonal antibodies, diluted in PBS containing 10% FBS, was added to the wells and incubated 1 hr at room temperature. Plates were then washed three times for 5 min each with PBS. Fifty microliters of goat anti-mouse IgM (μ chain) alkaline phosphase conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) at 1 μg/ml in PBS containing 10% FBS was added to the wells and incubated 1 hr at room temperature. Plates were again washed three times for 5 min each with PBS. Fifty microliters of the substrate, *p*-nitrophenyl phosphate at 2 mg/ml in diethanolamine buffer (Kirkegaard and Perry Laboratories) was added to the wells and incubated 30 min at room temperature. Plates were scanned for optical activity at 405 nm using a Titertek Multiscan (Flow Laboratories). Values reported were adjusted by subtracting values in blank wells that lacked both antigen and monoclonal antibody.

Immunoelectron Microscopy. A cholesterol crystal suspension was prepared by sonicating cholesterol crystals (20 mg/ml) in PBS containing 0.5% fetal calf serum (FCS) (GIBCO). Aliquots (200 μl) were placed into plastic centrifuge tubes and subjected to the following procedure. For each wash the tubes were centrifuged for 3 min in a Beckman Microfuge B, the supernatant was removed, 150 μl of wash buffer was added, and the tube was agitated on a Vortex to resuspend the pellet. The cholesterol crystals were washed once with PBS, resuspended in PBS with 10% FCS for 30 min at room temperature, and washed once with PBS containing 10% FCS.

The crystals were resuspended for 1 hr at room temperature in a 1:5 dilution of ascites fluid containing murine anti-cholesterol antibody and then washed three times with PBS containing 10% FCS. They were then resuspended with affinity-purified secondary antibody, consisting of 10-nm-gold-labeled goat anti-mouse IgM (Janssen Life Science, Piscataway, NJ) diluted 1:10, for 1 hr at room temperature. The crystals were subsequently washed three times with PBS containing 10% FCS. They were resuspended in 30 μl of PBS containing 10% FCS, and a small aliquot was placed on a glow-discharged Formvar-coated grid, immediately rinsed with 4–5 drops of 2% phosphotungstic acid (pH 6 with KOH), blotted dry with filter paper, and examined with a Philips EM300 electron microscope.

Negative controls for verifying the specificity of the reaction were performed with ascites fluid derived from fusion of cells from a mouse immunized with an antigen unrelated to cholesterol (ascites fluid containing monoclonal antibody to trypanosomal glycoprotein, as described below) as primary antibody. The gold-labeled secondary antibody was also used in further control experiments without the presence of the primary antibody.

Monoclonal Antibodies. The original immunizing liposomes contained a cholesterol/[Myr_2]PtdCho ratio of 2.5:1 (71 mol % cholesterol), and 0.2 ml (containing 2 μmol of [Myr_2]PtdCho) was injected. Three days after immunization (i.v.) of a BALB/c mouse, a single fusion of spleen cells to myeloma cell line P3-X63-Ag8.653 was performed as described (14). After fusion, four lines of cells secreting IgM antibodies were selected for the ability to induce complement-dependent immune damage to liposomes with 71% cholesterol but not to liposomes with 43% cholesterol (see Fig. 1). One of the anti-cholesterol cell clones, designated anti-cholesterol hybridoma 2C5-6 (antibody no. 2 in this paper), has been deposited in the American Type Culture Collection (ATCC) 30-year patent depository. It has ATCC accession number HB 8995 and is available to the scientific public upon request to ATCC.

Ascites fluid that was not expected to contain antibodies to cholesterol was utilized as a control in every immunoassay. The control ascites fluid contained a murine monoclonal IgM antibody (32.2A6.1) produced against the variant surface glycoprotein of the WRAT 1 clone of *Trypanosoma brucei rhodesiense* (17) and was a generous gift of Klaus Esser (Walter Reed Army Institute of Research).

The IgM concentrations in the ascites fluids were determined by a Mancini assay (18) using radial immunodiffusion plates containing anti-IgM (Meloy Laboratories, Springfield, VA). The IgM values were 1.7 mg/ml (antibody no. 1), 3.45 mg/ml (antibody no. 2), 1.35 mg/ml (antibody no. 3), 2.15 mg/ml (antibody no. 4), and 5.15 mg/ml (control antibody, 32.2A6.1).

Purified antibodies, when they were employed, were obtained by the method of "affinity binding to liposomes" (13). In this technique the antibodies were bound to high-cholesterol (71%) liposomes, the liposomes were washed to remove unbound antibodies, the bound antibodies were removed by elution with 1 M NaI, liposomes were removed by centrifugation, and NaI was removed by dialysis.

RESULTS

Complement-Dependent Damage to High-Cholesterol Liposomes. After immunization with liposomes containing 71% cholesterol, 59/84 (70%) of hybridoma cell culture supernatants tested (50 μl each) from two and one-half microtiter plates were secreting anti-cholesterol antibodies as determined by the criterion of at least 10% glucose release from liposomes containing 71% cholesterol but less than 10% glucose release from liposomes containing 43% cholesterol (Fig. 1). Four of the highest-activity cell lines were selected for cloning. Control experiments showed that murine ascites fluid containing an IgM antibody that would not be expected to bind cholesterol (32.2A6.1 against trypanosomal cell surface glycoprotein) did not induce complement-dependent glucose release from liposomes containing 43% or 71% cholesterol.

The degree of immunoreactivity of each monoclonal anti-cholesterol antibody with liposomes was related to the concentration of cholesterol in the liposomes (Fig. 2). Al-

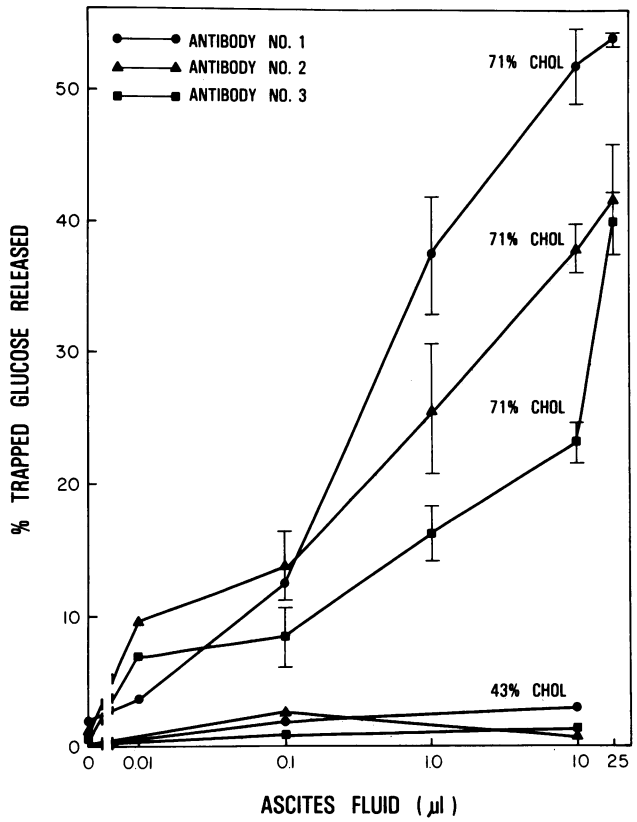

FIG. 1. Complement-dependent immune damage induced by three monoclonal anti-cholesterol antibodies, as measured by glucose release from liposomes containing 71% cholesterol. Each point is a mean (±SD) of single values determined with three separate liposome preparations.

though significant immune damage was not observed at 43% cholesterol, some reactivity at higher levels of antibody was found with liposomes containing 50% cholesterol, and a plateau of maximal damage occurred at a concentration of approximately 62% cholesterol. The degree of immune damage at all levels of cholesterol was proportional to the antibody concentration used. In the absence of antibody, when only complement was present, no significant immune damage occurred (Fig. 2).

Binding of Antibodies to Crystalline Cholesterol. A solid-phase ELISA was developed for detecting IgM immunoreactivity with crystalline cholesterol, and each of four anti-cholesterol antibody clones showed positive results (Fig. 3). Two of the four antibodies (nos. 1 and 2) were still easily detected even when diluted 1:31,250. Antibody no. 3 had the lowest activity both in the glucose-release assay (Fig. 1) and in the ELISA (Fig. 3). As with the two other immunoassays, the control ascites fluid containing IgM antibody to trypanosomal glycoprotein (32.2A6.1) failed to show reactivity in the ELISA.

The binding of monoclonal anti-cholesterol antibodies to cholesterol crystals was clearly demonstrated also by electron microscopy using a two-step labeling technique. After incubation of the cholesterol-crystal suspension with the anti-cholesterol antibody, followed by a secondary antibody conjugated to colloidal gold, gold particles were observed dispersed over the surface of the crystals (Fig. 4*a*). Gold particles were absent from the surface of cholesterol crystals treated with control ascites fluid containing antibody to trypanosomal glycoprotein (Fig. 4*b*). Gold particles were absent also in control experiments (data not shown) in which the cholesterol crystals were treated with the secondary antibody but not with the primary (anti-cholesterol) antibody.

Separate experiments confirmed that affinity-purified anti-cholesterol antibodies (see *Materials and Methods*) gave strongly positive results when tested with crystalline cholesterol either by ELISA or by the immunoelectron microscopy technique.

DISCUSSION

Complement fixation by cholesterol has been previously reported but the complement-activating mechanisms involved (immune or nonimmune) have not been fully clarified (6–11). Activation of complement by cholesterol has been proposed as a mechanism in the pathogenesis of atherosclerosis (6, 9, 11). However, the immunogenicity of cholesterol itself has not been definitively addressed, and cholesterol often has been assumed to be a nonimmunogenic substance. Antibodies to cholesterol have been reported to occur after

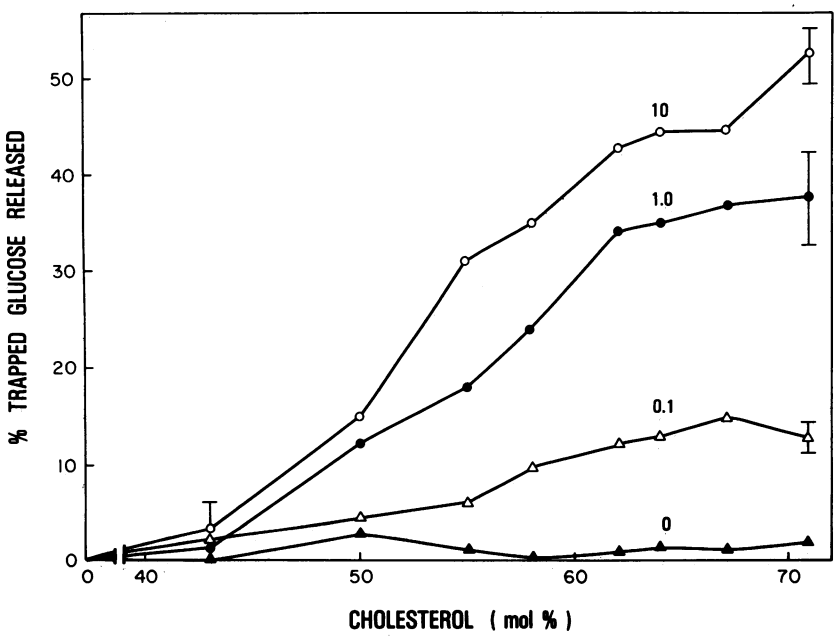

FIG. 2. Complement-dependent immune damage to liposomes containing different amounts of cholesterol. Each curve represents complement-dependent glucose release in the presence of the indicated volume (0, 0.1, 1, or 10 μl) of ascites fluid containing monoclonal anti-cholesterol antibody (this antibody is designated antibody no. 1). Each cholesterol concentration represents a separate liposome preparation tested with the indicated antibody concentrations except the 67% and 71% cholesterol points, each of which represents a mean value obtained with three separate liposome preparations (±SD is indicated for 71% cholesterol). The assays that were run in the absence of ascites fluid (the bottom curve) were performed in the presence of complement only (fresh guinea pig serum).

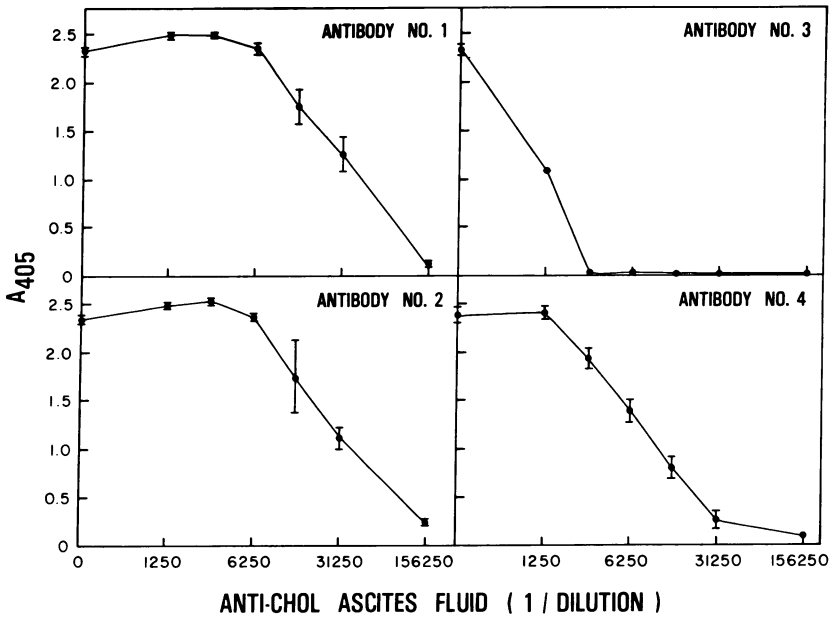

FIG. 3. Binding of four separate anti-cholesterol (anti-chol) antibodies to crystalline cholesterol in a solid-phase ELISA. Each value represents the mean (±SD) of three assays using the indicated dilution of ascites fluid.

immunizing with cholesterol-albumin conjugates (3, 4) or with a synthetic phospholipid, phosphatidylcholesterol (5). Rabbits that were immunized with cholesteryl ester-albumin conjugates were resistant to development of atherosclerotic plaques induced by cholesterol feeding (4). Despite these observations, the ability to produce complement-fixing antibodies to cholesterol has not been widely recognized. The present study demonstrates that when cholesterol is placed in the proper environment it is a highly immunogenic molecule. Cholesterol therefore joins a growing list of lipids that were previously thought to be nonimmunogenic but are now known to be excellent immunogens (12, 19).

Three conclusions from our data are particularly noteworthy. First, the antibodies to cholesterol exerted considerable specificity for the high-cholesterol (71% cholesterol) liposomes used for immunization. Antibodies were originally selected for the absence of detectable crossreactivity with liposomes having "low" cholesterol (43% cholesterol or less) (Figs. 1 and 2). It is evident therefore that above 50 mol %, cholesterol entered into a separate phase in the liposomes that allowed it to become an immunodominant molecule. Second, the induction of antibodies to cholesterol was achieved by utilizing lipid A, the endotoxic moiety of Gram-negative bacterial lipopolysaccharide, as a powerful adjuvant in the liposomes. In the absence of lipid A the liposomes were not immunogenic. The requirement for endotoxin (lipid A) for stimulation of antibodies to cholesterol is consistent with a previous hypothesis that "naturally occurring" antibodies (often present in normal serum) that react with constituents of lipid bilayers are induced by endotoxin (20, 21). Third, high titers of antibodies were readily induced within 3 days after a single primary immunization, and when

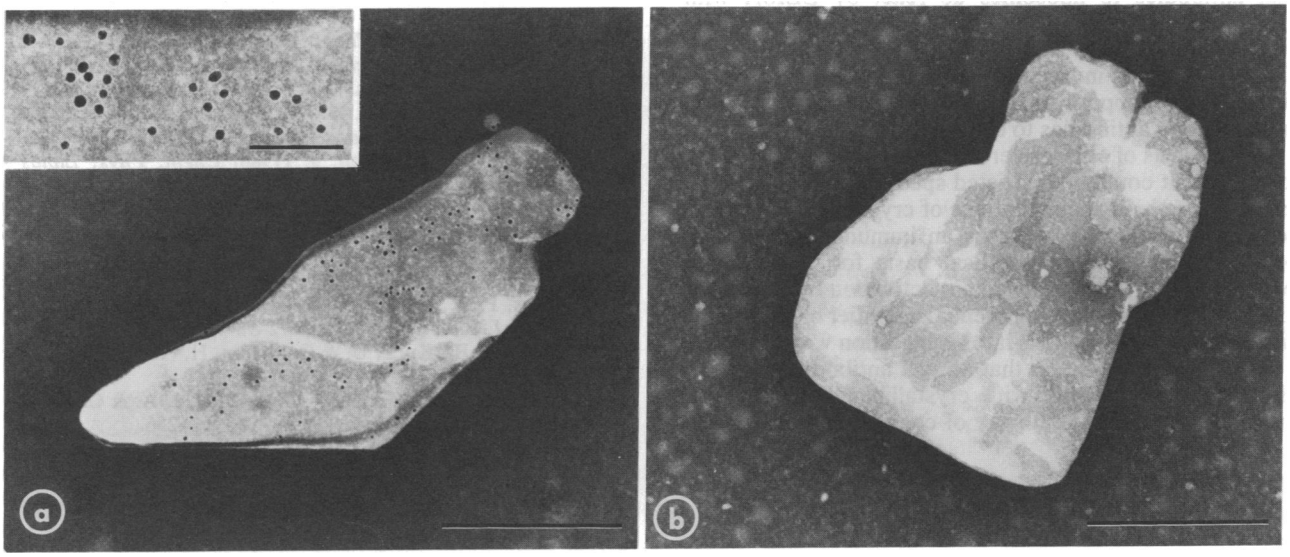

FIG. 4. Binding of anti-cholesterol antibodies to crystalline cholesterol. (*a*) Negatively stained cholesterol crystal treated with mouse anti-cholesterol (antibody no. 2) followed by goat anti-mouse IgM conjugated with 10-nm gold. (Bar = 0.5 μm.) *Inset* shows higher magnification of the gold particles on the crystal surface. (Bar = 0.1 μm.) (*b*) Negatively stained cholesterol crystal treated with control ascites fluid containing IgM antibody to trypanosome glycoprotein (see text for details) followed by goat anti-mouse IgM conjugated with 10-nm gold. (Bar = 0.5 μm.)

the spleens were removed and fused with myeloma cells to produce hybridomas, at least 70% of the fused splenic cells were secreting complement-fixing antibodies to cholesterol.

The exact configuration of the cholesterol that was being recognized by the antibodies in the 71% cholesterol compared to 43% cholesterol liposomes is unknown. There has been considerable discussion in the literature on the biophysical, biochemical, and morphological properties of liposomes containing high concentrations of cholesterol. It is well established that liposomes (22–25) or even cells (26) can have cholesterol/phospholipid ratios of 2:1, or even up to 4:1, and 71% cholesterol liposomes have a cholesterol/phospholipid ratio of 2.5:1. Liposomes having cholesterol/phospholipid ratios >2:1 are in a nonequilibrium metastable state in which cholesterol gradually separates over a course of days or weeks into a different phase from the phospholipid (25). Electron microscopic studies have suggested that crystalline cholesterol may be associated with, and even slowly "grow from," high-cholesterol multilamellar liposomes.

Although it is premature at this juncture to identify a structure of liposomal cholesterol that is recognized by the antibody, there is ample precedent for exquisite discrimination of unique lipid patterns by antibodies to lipids and liposomes (12, 27). For example, specificities of individual antibodies to phosphatidylinositol phosphate were identified that either did or did not crossreact with phosphatidylinositol or phosphatidylinositol bisphosphate (28). Monoclonal antibodies to cardiolipin can react with cardiolipin or even phosphatidylethanolamine in the hexagonal H_{II} phase, but not with the lipids in the bilayer configuration (29). Recently it was shown that the TEPC 15 IgA myeloma antibody, which binds to phosphocholine but also recognizes phosphatidylcholine (30), interacts differently with phosphatidylcholine and phosphatidylethanolamine in a lipid monolayer system (31).

Despite the ability to discriminate between unique lipid patterns, all of our anti-liposome antibodies, including antibodies to cholesterol, have a "subsite" that can recognize soluble phosphorylated haptens in the antibody binding site (12). It has been postulated that the phosphate-binding subsite might be responsible for some of the crossreactivity that is sometimes observed between antibodies to DNA and antibodies to cardiolipin (12). Although some of our monoclonal antibodies to liposomes do react by ELISA with purified lipid A, none of the monoclonal antibodies that recognize lipid A react with purified cholesterol.

The observations in the present paper have several implications. They support the hypothesis that activation of complement by antibodies to cholesterol could play a role in the pathogenesis of atherosclerosis. Furthermore, activation of complement could be restricted specifically only to those sites that have high accumulations of crystalline cholesterol where cholesterol may serve as an immunodominant epitope. This provides a theoretical basis for the previous proposal (6, 9, 11) that complement activation by crystalline cholesterol might serve as a potential amplifier of atherosclerotic ischemic damage. This latter suggestion was supported by the clinical observation that an IgG antibody apparently was reacting specifically with crystalline cholesterol and activating the classical pathway of complement in a patient with ulcerating atherosclerosis (9). Based on our previous successful experience in stimulating antibodies to other types of lipids, it is likely that lower concentrations of membrane cholesterol also could modulate the reactivities of antibodies having specificities to other lipid bilayers having different immunodominant epitopes (12, 19).

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