Immunization with cholesterol-rich liposomes induces anti-cholesterol antibodies and reduces diet-induced hypercholesterolemia and plaque formation

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Immunization of rabbits with a protein-free formulation consisting of liposomes containing 71% cholesterol and lipid A as an adjuvant induced anticholesterol antibodies that caused complement-dependent lysis of liposomes lacking lipid A. The antibodies, immunoglobulin G (IgG) and immunoglobulin M (IgM), also recognized nonoxidized crystalline cholesterol as an antigen by enzyme-linked immunosorbent assay (ELISA). The effects of immunization against cholesterol on elevations in serum cholesterol and development of atherosclerosis were examined in rabbits fed a diet containing 0.5% to 1.0% cholesterol. Although the mean serum cholesterol level, mainly in the form of very-low-density lipoprotein cholesterol, rose as much as 60-fold in the nonimmunized rabbits, the elevation was significantly less—as much as 35% lower—in the immunized rabbits. Elevation of serum cholesterol was accompanied by an apparent drop in the level of antibodies on initiating the diet, followed by a rebound on stopping the diet, thus suggesting that the antibodies were adsorbed to cholesterol that was present in circulating lipoproteins. When lipoprotein fractions—composed of either very-low-density and intermediate-density lipoproteins derived from cholesterol-fed nonimmunized rabbits or human low-density lipoproteins—were tested as capture antigens by solid-phase ELISA, reactivity was observed with IgG and IgM antibodies present in the serum of immunized rabbits. Immunization also resulted in a marked decrease in the risk of developing atherosclerosis. Analysis of aortic atherosclerosis by quantitative histologic examination and fatty streaks by automated morphometric probability-of-occurrence mapping showed diminished atherosclerosis in most areas of the aorta in vaccine recipients. It is proposed that immunization with liposomes containing 71% cholesterol and lipid A can reduce diet-induced hypercholesterolemia and atherosclerosis. (J LAB CLIN MED 1996;127:40-9)

Abbreviations: ELISA = enzyme-linked immunosorbent assay; IDL = intermediate-density lipoprotein; IgG = immunoglobulin G; IgM = immunoglobulin M; LDL = low-density lipoprotein; VLDL = very-low-density lipoprotein

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A long and interesting literature that originated in Germany in 1925 has addressed the immunologic properties of cholesterol as an antigen or hapten.[1-3] In 1988, liposomes containing high concentrations of cholesterol (71 mol %) and lipid A (the lipid adjuvant moiety of gram-negative bacterial lipopolysaccharide) were used as immunizing formulations in mice to induce polyclonal or monoclonal antibodies to liposomal cho-

lesterol.[4] Anticholesterol antibodies produced by this method had the ability to cause complement-dependent lysis of 71% cholesterol liposomes that lacked lipid A.[4] The antibodies also reacted directly with highly purified nonoxidized crystalline cholesterol as an antigen, as determined by ELISA and by immunogold electron microscopy.[4] Subsequent studies showed that varying titers of naturally occurring IgG and IgM antibodies to nonoxidized cholesterol were present not only in almost all normal human serum samples[5] but also in serum samples from certain other animals, including pigs.[6] The presence of these antibodies to cholesterol in normal serum raises an important question: Do antibodies to cholesterol have a role in regulating cholesterol levels—and by extension, atherosclerosis—in vivo?

Numerous studies have demonstrated that atherosclerotic lesions can develop in animals fed a high concentration of dietary cholesterol and that these can serve as models for human atherosclerosis.[7] Two previous groups of investigators have reported that immunization of rabbits with a cholesterol-containing antigen, either β-LDL[8] or a cholesterol ester antigen in which cholesterol sebacate was esterified to a heterologous protein,[9,10] reduced serum cholesterol and strongly inhibited the development of diet-induced aortic atherosclerosis. Although the previous studies did not directly consider the issue of antibodies to cholesterol in the absence of a protein carrier, it was concluded that immunologic techniques might be used to influence diet-induced hypercholesterolemia and atherosclerosis.

The present study demonstrates that immunization of rabbits with a protein-free liposomal vaccine containing high levels of unconjugated cholesterol induces antibodies that recognize either crystalline cholesterol or a mixture of VLDL and IDL. As in previous work in which cholesterol lowering was accompanied by decreased atherosclerosis, immunization with liposomal cholesterol results in a marked beneficial effect that dramatically reduces atherosclerosis induced by dietary cholesterol. Our data suggest that diet-induced hypercholesterolemia and atherosclerosis can be immunologically modulated by the use of a protein-free liposomal anticholesterol vaccine.

METHODS

Liposome preparation. The complete methods for manufacturing multilamellar liposomes containing 71% cholesterol are described elsewhere.[11] The liposomes contained dimyristoyl phosphatidylcholine, dimyristoyl phosphatidylglycerol, and cholesterol (all from Avanti Polar Lipids, Alabaster, Ala.) in molar ratios of 0.9 : 0.1 : 2.5. The cholesterol, which was highly purified, was recrystallized three times from hot ethanol, had a melting point of 148.5° C, and was free from oxidation products and other contaminants on thin-layer chromatography.[4] The liposomes also contained lipid A from *Salmonella minnesota* R595 (List Biological Laboratories, Campbell, Calif.), which was present at a concentration of 100 μg per μmol of liposomal phospholipid. The lyophilized lipids were hydrated to form liposomes with pyrogen-free 0.154 mol/L NaCl at a concentration of 10 mmol/L phospholipid with respect to the saline solution. The liposomes were injected into rabbits within 2 hours after hydration.

Isolation and characterization of lipoproteins. Lipoprotein fractionation from nonimmunized hypercholesterolemic rabbit serum was performed under contract by Organon Teknika Biotechnology Research Institute, Rockville, Md., with preparative sequential ultracentrifugation as described by Havel et al.[12] The major lipoprotein fraction separated at a density of 1.006 gm/cm^3 and was considered to contain mostly VLDL. Agarose gel electrophoresis of isolated lipoprotein fractions performed according to the method of Noble[13] revealed the presence of IDL in addition to VLDL in the lipoprotein fraction. LDLs purified from human plasma[12] were purchased from Akzo Nobel (PerImmune, Inc., Rockville, Md.) and had a density range of 1.019 to 1.063 gm/ml.

Serum cholesterol. Serum cholesterol was measured with a colorimetric assay kit (catalog no. 352; Sigma Chemical Co., St. Louis, Mo.). A cholesterol calibrator certified by the Centers for Disease Control and the National Cholesterol Education Program, Laboratory Standardization Panel (catalog no. C7921; Sigma), was used as a standard. Serum cholesterol levels of rabbits fed a 1% cholesterol diet became so high that they exceeded the linear range of the assay. However, dilution of the serum samples with saline solution or buffer produced results that were often erratic and unreliable. For this reason, after initiation of the diet, each serum sample was diluted, when necessary, with its corresponding pretreatment serum to bring the cholesterol value into the linear range of the assay. The concentration of cholesterol in the pretreatment serum sample used for dilution was subtracted from the data reported. This procedure reduced the variability and greatly increased the reliability of the cholesterol determination when high concentrations of cholesterol were present.

Immunization and diet protocols. Three experimental protocol schedules were carried out as outlined in Fig. 1. In the first protocol schedule, rabbits (New Zealand white male, approximately 1.8 to 2.3 kg) were immunized intramuscularly with 0.5 ml of a formulation consisting of 0.25 ml of liposomes, containing lipid A and 71% cholesterol, that were adsorbed with 0.25 ml of aluminum hydroxide (Rehsorptar, containing 1.8 mg of aluminum per ml) at 0, 2, 4, and 6 weeks. Five weeks after immunization was initiated, the immunized and nonimmunized control rabbits

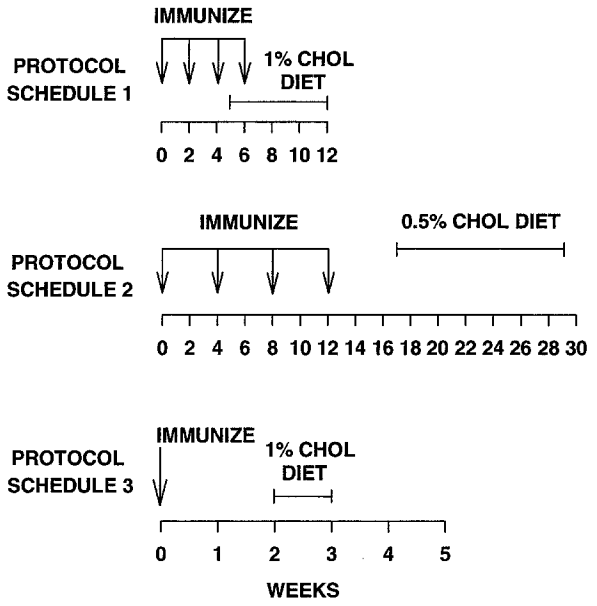

Fig. 1. Experimental protocols for rabbit immunization *(arrows)* and high-cholesterol diet.

were given free access to a 1% cholesterol diet for 7 weeks (Rabbit Grain-Base Diet, Fiber (HI), in which powdered 1% cholesterol was homogeneously mixed into the feed; Bio-Serv, Frenchtown, N.J.). Although the animals remained healthy during this interval on the 1% cholesterol diet, during longer experimental periods hypercholesterolemia and accelerated atherosclerosis caused physical deterioration of the animals and reduced dietary intake.

To avoid this, in the second protocol schedule reduced dietary cholesterol was used. Rabbits were immunized with the same type of antigen at 0, 4, 8, and 12 weeks. Starting 5 weeks after completion of immunization, the immunized and nonimmunized control rabbits were given free access to a 0.5% cholesterol diet for 12 weeks. This diet was prepared by suspending cholesterol (Sigma) in peanut oil (Planter's) and thoroughly mixing the suspension by hand with Purina High Fiber Lab Rabbit Chow (Purina Mills, St. Louis, Mo.). The final mix contained 0.5% cholesterol and 2.0% of the added oil. The diet was stored at 4° C and made fresh on a weekly basis. To ascertain the relative sensitivities of individual rabbits in protocol schedule 2 to dietary cholesterol, 11 days before immunization was started, the animals were first fed a test diet of 0.5% cholesterol for a period of 4 days. In these experiments the animals remained healthy and maintained normal weight gain throughout the 12 weeks of high cholesterol diet.

A third protocol schedule was designed to provide immunized animals with a very short exposure to 1% dietary cholesterol (prepared as in the second protocol schedule) to study the acute effects on antibody titers of initiating and stopping the diet.

Assay of antibody activity. The antibody-mediated complement-dependent lysis of liposomes was assayed by the release of trapped glucose from 71% cholesterol liposomes (5 μl) in the presence of rabbit serum (25 μl) as a source of antibody and fresh guinea pig serum (100 μl) as a source of complement.[4,11] Solid-phase ELISAs were performed with either 10 μg of crystalline cholesterol or 0.1 μg of rabbit VLDL/IDL or human LDL as an antigen to coat each well.[4] Phosphate-buffered saline solution containing 0.3% gelatin was used as blocking buffer and as diluent for the rabbit serum and the secondary antibody. Secondary antibodies were either peroxidase- or alkaline phosphatase–linked goat anti-rabbit IgG (γ-heavy chain specific) or IgM (μ-heavy chain specific) purchased from Fisher Biotech, Pittsburgh, Pa. (for data in Figs. 5 and 6), or from Southern Biotechnology Associates, Birmingham, Ala. (for data in Fig. 7). After development with the respective substrates, absorbance was read at 405 nm with a UVmax Kinetic Microplate Reader (Molecular Devices, Palo Alto, Calif.).

Analysis of aortic atherosclerosis. Analysis of the effects of immunization on the development of diet-induced atherosclerosis in experiment 2 was performed on the excised aorta of each of the immunized and control animals 12 weeks after initiating the 0.5% cholesterol diet. Two methods of analysis were used: (1) automated morphometric probability-of-occurrence mapping of sudanophilia of the entire length of the dissected aortas after staining with sudan IV[14-17] and (2) direct measurement and quantitative analysis of plaque morphology on microscopic sections from the ascending aortas from each of the rabbits.[18]

For probability-of-occurrence mapping, the entire aorta was removed, fixed, stained with Sudan IV, and photographed with Ektachrome film. Images were digitized from 35-mm color slides at a resolution of 400 × 100 pixels with an Eikonix 78/99 digital scanner and were then transferred to an image-processing system consisting of a Microvax II (Digital Equipment Corp.) and a Gould IP8400 display processor. The stored digital images were subdivided into a mosaic of triangular subsections based on anatomic landmarks, which were then used to stretch individual images to remove anatomic variation among animals and produce a composite representation.[14,15] Binary images were stored to calculate percent surface area involved and to create composite topographic probability-of-occurrence maps of sudanophilia at each point on the entire aortic surface.

For histologic analysis, sections of the upper aorta were scanned at a resolution of 1024 × 512 pixels and transferred to the Gould IP8400 display processor. The lumen, internal elastic lamina, and external elastic lamina were identified. By using specialized software the external elastic lamina circumference, lesion area, and lesion maximum thickness were computed. A representative anatomic specimen illustrating computerized quantitative analysis of histologic lesions in a transverse section of atherosclerotic ascending aorta from a rabbit is shown in Fig. 2.

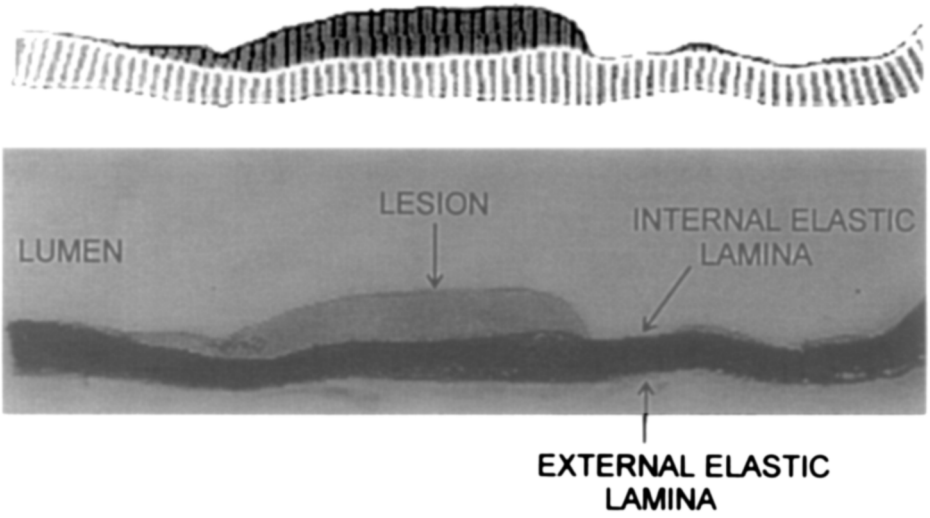

Fig. 2. Quantitative histologic measurement of atherosclerotic rabbit aorta. The *bottom frame* illustrates a transverse section of ascending aorta obtained from a representative cholesterol-fed rabbit. The image was scanned and stored, and the internal elastic lamina, external elastic lamina, and lesion were identified for quantification as shown in the *upper frame*. See text for further details.

RESULTS

Binding of antibodies to cholesterol and VLDL/IDL or LDL.

As previously found with mice,[4] biweekly (protocol schedule 1) or monthly (protocol schedule 2) immunization of rabbits with 71% cholesterol liposomes induced anticholesterol antibodies that caused release of liposome-encapsulated glucose caused by complement-dependent lysis of liposomes containing 71% cholesterol (Fig. 3). There were wide variations among the 40 rabbits in protocol schedule 2 in the levels of antibodies produced, resulting in a range of 3% to 39% glucose release. Serum samples from the control (nonimmunized) rabbits and preimmunization serum samples from the 40 experimental rabbits had little or no activity (range 0% to 3.5% glucose release; mean ± SD, 1.6% ± 0.8%).

Antibodies induced by immunization with 71% cholesterol liposomes also reacted with highly purified nonoxidized crystalline cholesterol as a solid-phase antigen, as observed by ELISA (Fig. 4). In the intensive biweekly schedule of immunization performed in protocol schedule 1, IgM antibodies developed rapidly and reached a maximum level after a single immunization (Fig. 4). During the course of four immunizations (initial injection and three boosts) a possible trend of gradually decreasing IgM levels may have occurred over a period of 10 weeks (Fig. 4, *inset*). However, after the 1% cholesterol diet was initiated at the fifth week the antibodies decreased sharply, despite a further boost at the sixth week, and reached baseline values at the eighth

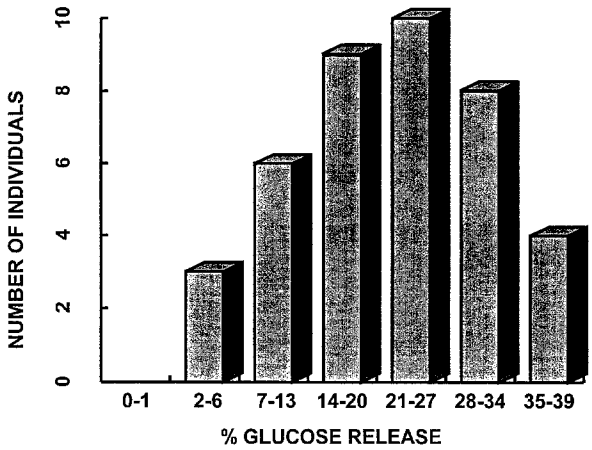

Fig. 3. Distribution of anticholesterol activities in rabbits immunized with liposomes containing 71% cholesterol (experimental protocol 2). The immune activities of 40 individual rabbit serum samples were tested at week 10 after immunization by assaying complement-dependent immune lysis of liposomes containing 71% cholesterol.

week. A similar striking removal of IgG antibodies, again reaching baseline values, was also separately found after the cholesterol diet was initiated. Over the same 10-week time interval no reduction, and no trend of reduction, of induced IgG antibodies was observed with serum samples from control rabbits that were immunized but not fed the 1% cholesterol diet. The decreased IgM and IgG antibody levels that occurred rapidly after initiating the diet sug-

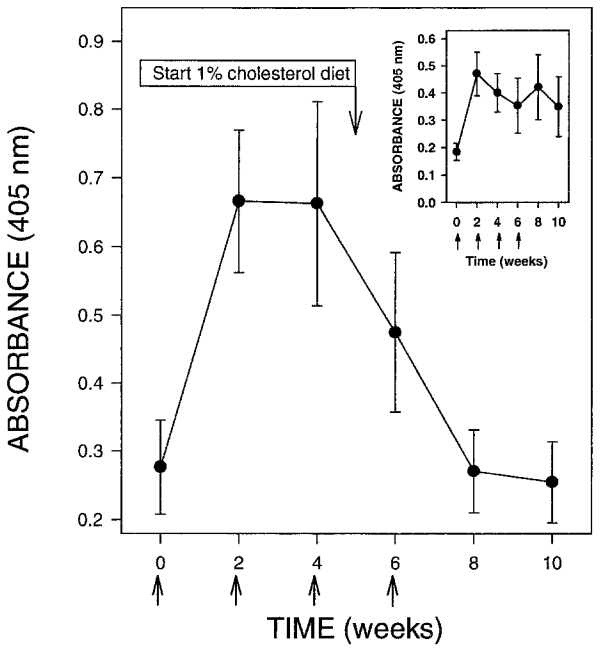

Fig. 4. Time course of anticholesterol IgM antibodies as detected by ELISA with crystalline cholesterol as antigen. Each data point represents the average absorbance value (± SD) obtained for serum samples obtained from rabbits (n = 4, protocol schedule 1) immunized intramuscularly with liposomes containing 71% cholesterol. *Arrows* indicate the times of primary and boosting immunizations. Rabbit serum samples were tested at a dilution of 1:100. Four immunized rabbits were maintained on a normal diet not supplemented with cholesterol *(inset)*.

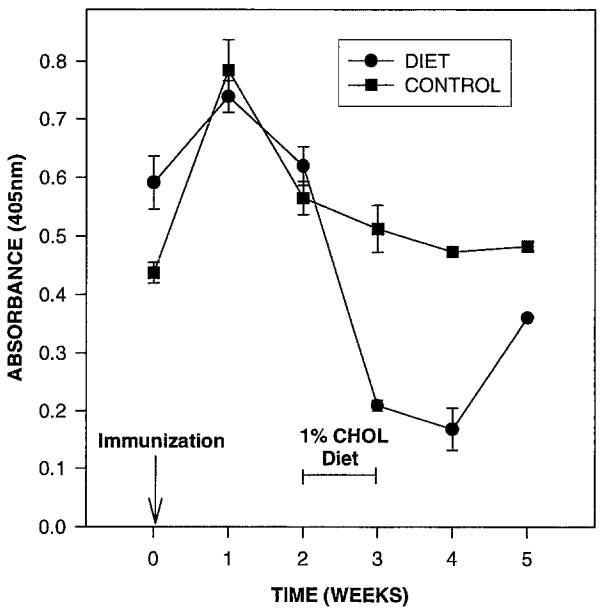

Fig. 5. Time course of binding of IgM antibodies as detected by ELISA with human LDL as antigen. The capture antigen consisted of 0.1 μg of human LDL per well. Each data point represents the average absorbance value ± SEM (n = 3 for rabbits fed 1% cholesterol diet for 1 week in protocol schedule 3; n = 2 for control rabbits kept on normal chow). All five rabbits were immunized once intramuscularly with liposomes containing 71% cholesterol. Rabbit serum samples were tested at a 1:100 dilution.

gested that the dramatic increases of serum cholesterol, VLDL, IDL, and LDL caused by the diet were adsorbing the antibodies induced by immunization.

To test the above hypothesis that the antibodies were being adsorbed by very high levels of lipoprotein, a shortened experiment was performed (protocol schedule 3) that was designed to reduce the duration of the diet and thereby minimize the deleterious effects of prolonged feeding of cholesterol (Fig. 5). In this protocol, a single immunization was administered, followed 2 weeks later by a 1% cholesterol diet for 1 week only, followed by normal chow for 2 weeks. When the antisera were tested with purified LDL (human) as an antigen, the activity rose within 1 week of immunization, followed by a sharp drop of activity on initiation of the diet, followed by a significant rebound of activity on cessation of the diet. The rebound of activity was consistent with the hypothesis that constant intake of dietary cholesterol resulted in the apparent removal of free antibodies that could bind to LDL and that the process was reversible.

The above data provide the further important information that the phenomenon was observed with antibodies that reacted with purified heterologous LDL (human) as the capture antigen. Rabbit antibodies induced by immunization also had the capacity to bind to pooled serum lipoproteins (mainly VLDL/IDL) from hypercholesterolemic rabbits. Solid-phase ELISA experiments demonstrated significant ($p = 0.03$ at the 1/50 dilution) IgG antibodies in the randomly selected immunized rabbit serum samples to the lipoprotein fraction that was used as a capture antigen (Fig. 6) (see *Author's note* at the end of the article).

Serum cholesterol levels. The cholesterol diets caused rapid increases in serum cholesterol values. However, as shown in Fig. 7, cholesterol levels that developed in protocol schedule 1 were significantly lower (as much as 979 mg/dl lower) in the immunized animals when compared with nonimmunized animals ($p = 0.004$ at 6 weeks, $p < 0.05$ at 8 weeks, $p = 0.001$ at 10 weeks, by t test). Under the intensive 1% cholesterol dietary pressure of protocol schedule 1, the differences between the groups disappeared at week 12. This corresponded to the time of maximum hypercholesterolemia and also corresponded to the time after which the animals in both groups deteriorated physically because of adverse effects caused by the highly enriched cholesterol

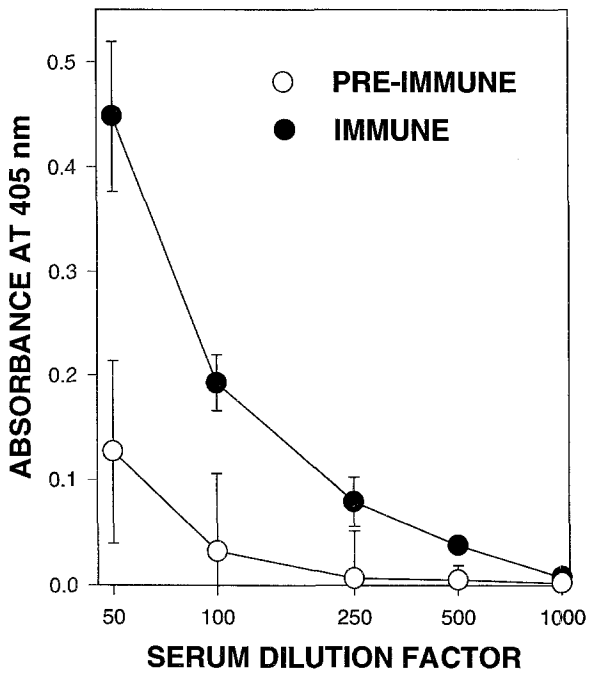

Fig. 6. Detection of IgG antibodies to VLDL/IDL by ELISA. Rabbit antiserum at 14 weeks after immunization (n = 5, protocol schedule 2) was tested for the presence of IgG antibodies to isolated VLDL/IDL fraction (0.1 μg per well) as antigen. The data represent the geometric mean ± SEM (p = 0.03 at 1:50 dilution).

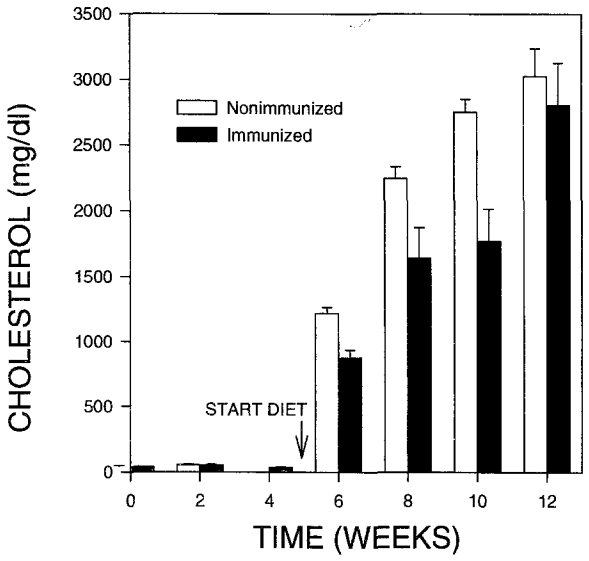

Fig. 7. Time course of serum cholesterol levels of immunized and nonimmunized rabbits (n = 4 for each group, protocol schedule 1). Each *bar* represents the mean ± SEM.

diet. Although not shown in Fig. 7, after 10 weeks both groups of animals had markedly decreased dietary intakes and decreased serum cholesterol levels at weeks 14 and 16. A pattern, similar to that in Fig. 7, of significantly delayed hypercholesterolemia in immunized animals was also seen in a separate experiment in which the animals were fed a lower concentration of cholesterol (0.5%). In this latter experiment the animals never exhibited physical deterioration, and the dietary intake and cholesterol levels remained high even at the end of the experiment. The data therefore demonstrate that a significant delay in the onset of hypercholesterolemia occurred in the immunized animals. Apparently, under the conditions of a 1% (or 0.5%) cholesterol diet, elevation of the serum cholesterol levels—although significantly delayed in the immunized animals—eventually became too high to be controlled fully by the immunization procedure.

Inhibition of atherosclerotic lesions. Immunized animals showed marked diminutions of atherosclerotic plaque formation after extended exposure to a high-fat diet. Fig. 8 illustrates composite probability-of-occurrence maps of aortic sudanophilia (fatty streaks) from the rabbits fed 0.5% cholesterol in protocol schedule 2. Aortas from immunized rabbits had substantially less overall probability of exhibiting fatty streak lesions when compared with the nonimmunized controls. Four regions of interest were examined in detail, based on predicted differences in the patterns of atherosclerosis as determined by morphometric and histologic analyses[14,18]: region 1, ascending aorta, including the aortic arch; region 2, descending thoracic aorta; region 3, middle descending aorta, including the origins of the celiac and superior mesenteric arteries; and region 4, abdominal aorta above the iliac bifurcation, including the left and right renal arteries. Quantitative analysis of the extent of sudanophilia in the immunized animals as compared with nonimmunized controls revealed strong trends toward reduced values in the immunized animals in the entire vessel and in each of the four regions of interest (Table I). Significant reductions (62% and 57% reduction, respectively) were achieved in regions 2 and 4 (p = 0.03 and p = 0.05, respectively).

The results in region 1 of the aortas are of particular interest because this region contains the origins of the coronary arteries. Although reduction of sudanophilia was observed, statistical significance was not achieved in region 1. However, this region also had the greatest overall involvement of sudanophilia (Table I), and it is probable that the amount of pathology was so large, both in the immunized and nonimmunized

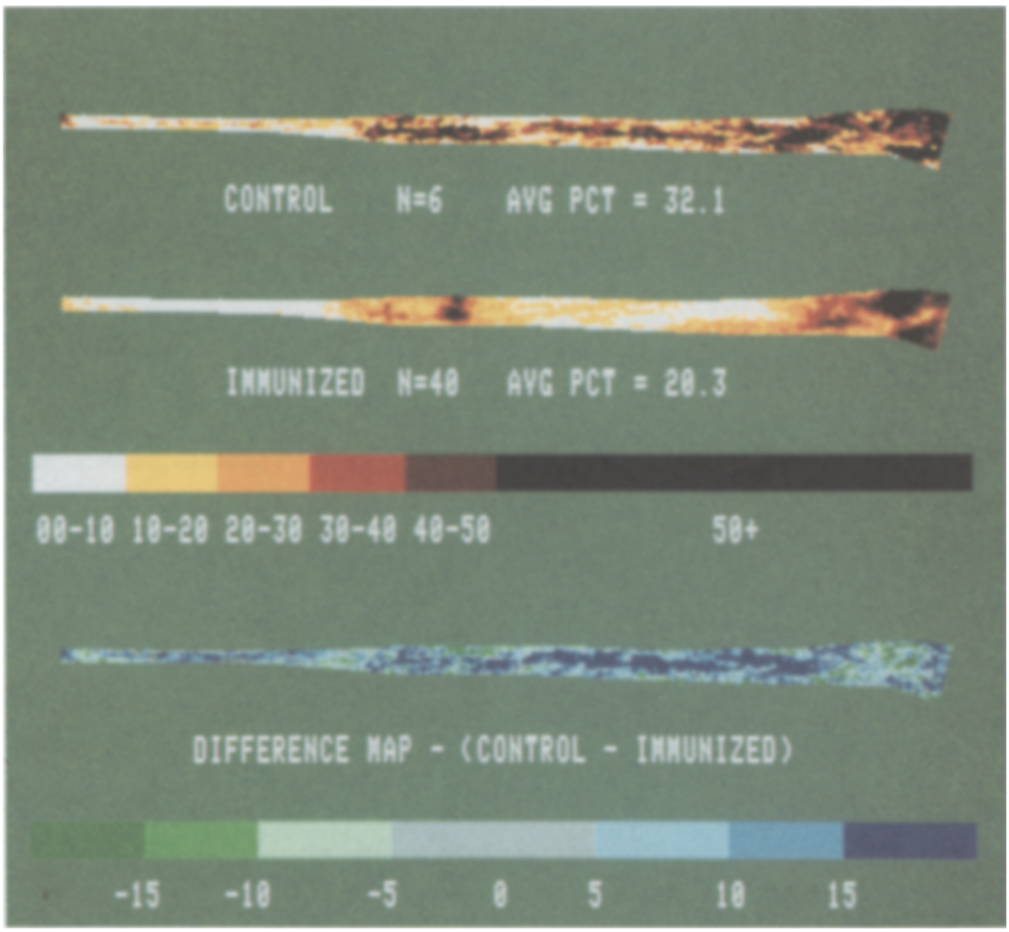

Fig. 8. Composite probability-of-occurrence maps of sudanophilic areas in the aortas of nonimmunized (*CONTROL*, n = 6) or immunized (n = 40) cholesterol-fed rabbits (protocol schedule 2). The ascending aorta (region 1) is at the *right*, and the distal abdominal aorta above the iliac bifurcation (region 4) is at the *left* (see text for details). The upper two maps are displayed in banded incidence isopleths according to the scale shown in the *middle*. The difference map *(bottom)* represents the point-by-point differences between the probability-of-occurrence map of control animals minus the probability-of-occurrence map of immunized animals. The differences in these probabilities are represented by different colors according to the scale shown at the *bottom*. Regions in which the control animals had a greater probability for sudanophilic lesions than immunized animals are presented in increasing shades of blue.

animals, that it had a saturating effect that partially obliterated differences of surface staining by sudan IV. Because of this, direct histologic analysis of cross-sections of region 1 was undertaken with each rabbit. Analysis of deeper lesions, based on overall plaque morphology as determined by direct microscopic analyses of cross-sections of region 1, is shown in Table II. When compared with results in nonimmunized controls, significant reductions were observed in the external elastic lamina circumference ($p = 0.0005$), total lesion area ($p = 0.01$), and lesion maximum thickness ($p = 0.057$) in region 1 of the immunized animals. The morphologies of the cross-sectioned lesions in region 1 were extremely consistent and demonstrated significant reduction of atherosclerotic potential caused by the immunization procedure.

DISCUSSION

Nonoxidized purified crystalline cholesterol, which is nonimmunogenic or poorly immunogenic in high concentrations by itself in liposomes, is known to become highly immunogenic when the liposomes contain lipid A, the adjuvant derived from the lipid moiety of gram-negative bacterial lipopolysaccharide.[4] In the present study it has been demonstrated that immunization of rabbits with protein-free liposomes containing lipid A to induce antibodies

Table I. Analysis of sudanophilic extent in aortas from nonimmunized or immunized rabbits fed a 0.5% cholesterol diet for 3 months

Region of interest	Percent sudanophilic area				p value of difference
	Nonimmunized (n = 6)		Immunized (n = 40)		
	Mean	SEM	Mean	SEM	
Entire vessel	32.1	0.069	20.3	0.027	0.13
Region 1	44.2	0.112	38.5	0.043	0.63
Region 2	32.0	0.088	12.3	0.031	0.03*
Region 3	32.3	0.047	22.1	0.029	0.2
Region 4	12.0	0.057	5.2	0.010	0.05*

*Significant value.

Table II. Histologic analysis of microscopic cross-sections of ascending aortas (region 1) of rabbits fed a 0.5% cholesterol diet for 3 months

Lesion parameter	Mean value		p value of difference
	Nonimmunized (n = 6) Mean ± SEM	Immunized (n = 38) Mean ± SEM	
External elastic lamina circumference (mm)	16.7 ± 1.49	12.8 ± 0.35	0.0005*
Lesion area (mm^2)	2.81 ± 0.77	1.41 ± 0.17	0.01*
Lesion maximum thickness (mm)	0.47 ± 0.09	0.29 ± 0.03	0.057*

*Significant value.

against nonoxidized cholesterol causes delayed onset of diet-induced hypercholesterolemia and reduced atherosclerosis.

Although the underlying basis for the immunologic modulation of serum cholesterol levels and atherosclerosis has not yet been completely elucidated, we propose a logical three-part mechanism based on recognition of serum cholesterol by antibodies. First, liposomes containing 71% cholesterol induce antibodies that recognize LDL cholesterol, IDL cholesterol, or VLDL cholesterol. In support of this, we observed by ELISA that antibodies in rabbit antiserum could bind to purified lipoproteins (mainly IDL and VLDL) derived from rabbits fed a cholesterol-rich diet (Fig. 6). Second, the antibodies bind to VLDL cholesterol, IDL cholesterol, or LDL cholesterol in the plasma, causing opsonization and uptake of the lipoproteins by Kupffer cells or splenic macrophages via the crystallizable fragment or complement receptor, thereby causing reduction of plasma cholesterol present in IDL, VLDL, and LDL. In support of this, we observed the reduction of anticholesterol antibody levels that occurred after initiation of the diet, presumably caused by adsorption of antibodies (Figs. 4 and 5), and subsequently diminished levels of hypercholesterolemia (Fig. 7). Third, reduced plasma LDL, IDL, or VLDL levels cause up-regulation of LDL receptors on hepatocytes that recognize the apoprotein B/apoprotein E of these lipoproteins, resulting in additional removal of the lipoproteins by hepatocytes. Although we do not yet have direct evidence for these latter events, they seem logical based on established mechanisms.[19-21]

It should be noted that the above proposed mechanism would be expected to be sensitive to the availability of antibodies capable of binding to circulating lipoproteins. With the dynamically increasing serum VLDL/IDL cholesterol levels that occur with time in the cholesterol-fed rabbit model, the availability of free (unbound) antibodies would be expected to become diminished because of increased binding to lipoproteins. Fig. 7 suggests that although immunization promotes a significant delay in hypercholesterolemia, continued feeding of cholesterol apparently results in such high levels of serum cholesterol that the serum antibodies become saturated, resulting in eventual disappearance of the beneficial effect of cholesterol reduction. Therefore, although the capacity of the vaccine to block hypercholesterolemia may have limits, the data illustrated in Fig. 7 suggest that the capacity may be quite large, resulting in a mean reduction of serum cholesterol by as much as 979 mg/dl at 10 weeks in protocol 1.

It is important that the cholesterol used both for immunization and for immunoassays in this study

lacked detectable oxidation products, or any other contaminants. Although contaminants were present in the original unpurified cholesterol, as determined by a melting point that was lower than the published value for cholesterol,[4] they were completely removed by recrystallization of the cholesterol three times from hot ethanol. The identification of nonoxidized cholesterol instead of oxidized cholesterol as the antigenic material was further indicated by the fact that murine monoclonal anticholesterol antibodies showed improved binding ability to cholesterol by ELISA after the purification of the cholesterol by recrystallization.[4] This is the opposite of the result that would have been expected if the antigenic activity were due to a contaminant in the cholesterol. The specificities of the antibodies that bound to cholesterol in the present study therefore differed from the previously described antibodies that bound to oxidized or modified LDL.[22-24] Antibodies to oxidized LDL recognized malondialdehyde-lysine-modified, 4-hydroxynonenal-lysine-modified, and delipidated oxidized lipoprotein peptide moieties.[22-23] This is potentially an important distinction because uptake of opsonized oxidized or modified LDL by monocyte-derived macrophages in the subendothelial tissue of arteries has been proposed as a pathogenic mechanism that may be responsible for increased atherosclerosis.[25,26] It is likely that after prophylactic active immunization against nonoxidized cholesterol in the present study, the Kupffer cells in the liver served effectively as a scavenger sink for removal of circulating opsonized lipoproteins that would otherwise have had deleterious effects in subendothelial locations in arteries.

The findings in this study are consistent with previous observations that decreased atherosclerosis can be achieved by immunologic intervention with an antigen consisting of either heterologous low density β-lipoprotein[8] or a heterologous protein–cholesterol ester conjugate.[9,10] In contrast to the previous work, the immunizing antigens in our experiments were completely protein-free. The immunologic basis for generation of T cells that recognize cholesterol in the absence of presentation of a heterologous protein or peptide remains obscure.[27] However, it has been proposed that involvement of a normal serum protein (β_2-glycoprotein I) occurs in the generation of IgG antibodies to phospholipids,[28-30] and it is conceivable that an unidentified normal serum protein could also play a role in the generation of antibodies to liposomal cholesterol. Regardless of the detailed immunologic and physiologic mechanisms involved, the results suggest that immunization of rabbits with protein-free liposomes containing 71% cholesterol induces protection against hypercholesterolemia and atherosclerosis.

It should be pointed out that in contrast to cholesterol that is present in lipoproteins, it is highly unlikely that the cholesterol that is present in any intact cell membranes exposed to circulating antibodies would be easily accessible to the binding of the antibodies. This is because the presumed major binding site, the C3-OH group, which represents the only polar group on the cholesterol molecule, is exceedingly small when compared with the size of the other adjacent polar groups on the surface of the lipid bilayer of the cell membrane. It has been well established that the small sizes of the antigenic epitopes of lipids such as cholesterol and small phospholipids, and steric hindrance caused by the relatively large sizes of surrounding lipids (such as glycolipids) and macromolecules, prevent the binding of antibodies to cholesterol on intact cell membranes.[3,31]

Author's note: During review of this manuscript, the question was raised whether lipoproteins could compete with liposomal cholesterol for binding of antibodies to cholesterol. There are theoretical problems associated with performing a simple competition experiment with two complex insoluble and dissimilar particulate antigens such as liposomes and lipoproteins because the number of epitopes available on each of these particles cannot be rigorously standardized to allow a meaningful affinity competition. However, by sequential adsorption of one serum (rabbit no. 406) first with liposomes lacking cholesterol and then with liposomes containing cholesterol,[11] it was determined with at least one serum that antibodies that bound to purified human LDL by ELISA were not removed even by six absorptions with cholesterol-free liposomes but were removed by three absorptions with cholesterol-containing liposomes. Therefore, although the relative "affinity" for liposomal cholesterol versus lipoprotein cholesterol cannot be determined in a meaningful way, it is concluded that antibodies that recognize liposomal cholesterol can also recognize lipoprotein cholesterol.

Conflict-of-interest note: Carl R. Alving has a significant financial interest in EntreMed, Inc., including stock ownership, and serves as a director. Glenn M. Swartz, Jr., is an employee of EntreMed, Inc. EntreMed owns the intellectual property rights to commercialization of the immunizing formulation described in the above article for use as a vaccine.

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