ELSEVIER

Journal of Immunological Methods 245 (2000) 1–14

JIM
Journal of Immunological Methods

www.elsevier.nl/locate/jim

Induction and detection of antibodies to squalene

Gary R. Matyas*, Nabila M. Wassef, Mangala Rao, Carl R. Alving

Department of Membrane Biochemistry, Walter Reed Army Institute of Research, Silver Spring, MD 20910-7500, USA

Received 18 April 2000; accepted 21 June 2000

Abstract

An enzyme-linked immunosorbent assay (ELISA) utilizing antigen coated on hydrophobic polyvinyldiene fluoride (PVDF) membranes is described for detecting antibodies that bind to squalene (SQE). Because of the prior lack of availability of validated antibodies to SQE, positive controls for the assay were made by immunization with formulations containing SQE to create monoclonal antibodies (mAbs) that reacted with SQE. Among eight immunogens tested, only two induced detectable murine antibodies to SQE: liposomes containing dimyristoyl phosphatidylcholine, dimyristoyl phosphatidylglycerol, 71% SQE, and lipid A [L(71% SQE+LA)], and, to a much lesser extent, an oil-in-water emulsion containing SQE, Tween 80, Span 85, and lipid A. In each case, lipid A served as an adjuvant, but neither SQE alone, SQE mixed with lipid A, liposomes containing 43% SQE and lipid A, nor several other emulsions containing both SQE and lipid A, induced antibodies that reacted with SQE. Monoclonal antibodies produced after immunizing mice with [L(71% SQE+LA)] served as positive controls for developing the ELISA. Monoclonal antibodies were produced that either recognized SQE alone but did not recognize squalane (SQA, the hydrogenated form of SQE), or that recognized both SQE and SQA. As found previously with other liposomal lipid antigens, liposomes containing lipid A also induced antibodies that reacted with the liposomal phospholipids. However, mAbs were also identified that reacted with SQE on PVDF membranes, but did not recognize either SQA or liposomal phospholipid. The polyclonal antiserum produced by immunizing mice with [L(71% SQE+LA)] therefore contained a mixed population of antibody specificities and, as expected, the ELISA of polyclonal antiserum with PVDF membranes detected antibodies both to SQE and SQA. We conclude that SQE is a weak antigen, but that antibodies that specifically bind to SQE can be readily induced by immunization with [L(71% SQE+LA)] and detected by ELISA with PVDF membranes coated with SQE.

Keywords: Squalene; Antibody detection; Enzyme-linked immunosorbent assay; Polyvinyldiene fluoride membranes; Monoclonal antibodies

1. Introduction

Squalene (SQE) is a triterpenoid hydrocarbon oil, $C_{30}H_{50}$, that is widely produced by both plants and animals. In humans, SQE serves a as precursor in the synthesis of cholesterol and all of the steroid hormones (Granner, 1996; Mayes, 1996) (Fig. 1). Both SQE and cholesterol are transported in the blood on very low density lipoproteins (VLDL) and low density lipoproteins (LDL) (Miettinen, 1982; Koivisto and Miettinen, 1988). Squalene and cholesterol are also synthesized in the liver and in the epidermis of the skin where SQE comprises a large amount of the oil secreted by sebaceous glands (Stewart, 1992). Because it is a naturally occurring

*Corresponding author. Tel.: +1-301-319-9477; fax: +1-301-319-9035.

E-mail address: gary.matyas@na.amedd.army.mil (G.R. Matyas).

0022-1759/00/$ – see front matter
PII: S0022-1759(00)00268-4

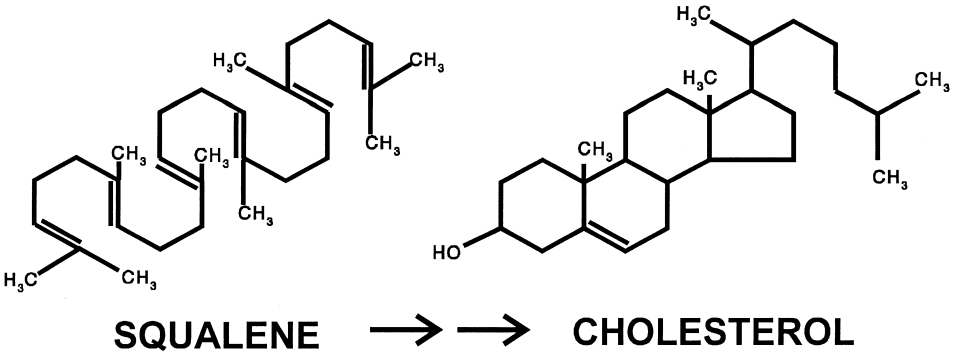

Fig. 1. Squalene, a precursor in the biosynthesis of cholesterol.

biodegradable oil, SQE and its hydrogenated derivative squalane (SQA) have each been proposed for use as the oil component of oil-in-water (o/w) emulsions for new generations of adjuvants for vaccines (Minutello et al., 1999).

Previous studies have demonstrated that cholesterol is highly immunogenic when lipid A is used as an adjuvant, and antibodies to cholesterol can be induced after immunizing mice with cholesterol-loaded liposomes containing lipid A (Swartz et al., 1988; Alving and Swartz, 1991). Using an ELISA assay, with monoclonal antibodies (mAbs) to cholesterol as positive controls, it was discovered that virtually all normal human sera contain naturally occurring antibodies to cholesterol (Alving et al., 1989). It has been proposed that these natural antibodies serve as an important immunomodulating mechanism for regulation of LDL metabolism (Alving and Wassef, 1999).

Using both a mAb and polyclonal antisera containing anti-cholesterol antibodies induced by cholesterol-loaded liposomes containing lipid A, it was found that the critical immunoreactive cholesterol epitope consisted of the 3-β-hydroxy group on the A ring (Dijkstra et al., 1996). It is reasonable that the 3-β-hydroxy should be an immunodominant epitope in cholesterol inasmuch as it is the only polar group on the molecule and would therefore be expressed at the surface of the liposomal membrane. The major purpose of the present study was to determine whether antibodies could be induced to SQE, a precursor molecule that has a rudimentary structural similarity to cholesterol, but which lacks any polar group. If antibodies to SQE could be induced, it would be likely that they might recognize the double bond structure of the molecule, or conformational changes induced by the double bonds, and the antibodies might then be expected to differentiate SQE from SQA.

Immunization against a potential antigen such as SQE presents a particular Catch-22 challenge: *first*, there have never been any previous antibodies developed that could serve as validated positive controls for anti-SQE antibodies, and *second*, there is no validated assay available for detecting antibodies to SQE. This is similar to the problem that we faced in developing antibodies to cholesterol, except in the case of cholesterol an extensive literature suggested that antibodies to cholesterol, and certainly antibodies to many steroids, including steroid hormones, could be induced by immunization (Alving and Swartz, 1991). To overcome this difficult dilemma in the present study, the horns of which are the simultaneous lack of positive antibody controls from immunized animals and lack of a validated assay for antibodies to SQE, our *first* goal was to inject SQE into mice to try to create antibodies that could potentially be validated as having anti-SQE activity. The *second* goal, namely the creation of monoclonal antibodies that could serve as positive antibody controls, was considered to be a requirement in the ultimate *third* goal of development of a valid immunoassay for detection of specific antibodies to SQE.

In view of the success that was previously found using lipid A as an adjuvant for inducing antibodies to cholesterol (Swartz et al., 1988; Alving and Swartz, 1991), immunization strategies using SQE

combined with lipid A were employed in attempting to induce antibodies to SQE. The results demonstrate that murine antibodies to SQE can be induced by injection of SQE-loaded liposomes containing lipid A, and the antibodies can be detected by an ELISA in which the antigen is coated on hydrophobic membranes instead of polystyrene microtiter wells. This has allowed creation of an immunoassay for demonstrating that mAbs to SQE can be produced that differentiate SQE from SQA.

2. Materials and methods

2.1. Lipids

Squalene and squalane oils were purchased from Sigma Chemical, St. Louis, MO, USA. Emulsifiers for creating oil-in-water emulsions consisted of Span 85 and Arlacel A (both from Sigma) and Tween 80 (Aldrich Chemical, Milwaukee, WI, USA). Dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG), both used in the formation of liposomes, were purchased from Avanti Polar Lipids, Alabaster, AL, USA. Lipid A from *Salmonella minnesota* R595 was purchased from List Biological Laboratories, Campbell, CA, USA.

2.2. Immunologic and culture reagents

Aluminum hydroxide gel, Alhydrogel, was purchased from Superfos Biosector, Vedbaek, Denmark. Mouse myeloma X63/Ag8.653 was purchased from American Type Culture Collection, Chantilly, VA, USA. Polyethylene glycol 1500 was from Boehringer Mannheim, GmbH, Germany. Dulbecco's modified Eagle's medium with high glucose (DMEM), MEM sodium pyruvate (100 mM), MEM nonessential amino acids (NEAA) (100×), penicillin (10 000 units/ml) streptomycin (10 000 μg/ml), 200 mM glutamine, 100×HAT (10 mM sodium hypoxanthine, 40 μM aminopterin, 1.6 mM thymidine) 100×HT (10 mM sodium hypoxanthine and 1.6 mM thymidine) supplements, Hank's Balanced Salts Solution, and fetal bovine serum were from GIBCO BRL, Grand Island, NY, USA. Fetal bovine serum was heated at 56°C for 1 h prior to use. Peroxidase-linked goat anti-mouse IgM and peroxidase-linked goat anti-mouse IgG were purchased from The Binding Site, San Diego, CA, USA. ATBS substrate was purchased from Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA.

Gelatin was from BioRad Laboratories, Richmond, CA, USA. Polystyrene Immulon II ELISA plates 'U' and flat bottom were from Dynex, Chantilly, VA, USA. PVDF Multiscreen-IP plates were from Millipore, Bedford, MA, USA and adapted for ELISA. Seal plate adhesive film was from PGC Scientific, Gaithersburg, MD, USA. Sterile Dulbecco's phosphate-buffered saline lacking calcium and magnesium (PBS) was from BioWhittaker, Walkersville, MD, USA. Nonsterile PBS was prepared from standard laboratory salts.

2.3. Manufacture of liposomes

Liposomes containing SQE or SQA were prepared by a modification of the method of Alving et al. (1993). DMPC and DMPG were dissolved in chloroform at 180 mM and 20 mM, respectively. Lipid A was dissolved in chloroform at a concentration of 1 mg/ml. Glassware was depyrogenated overnight at 250°C. Chloroform solutions of lipids, including SQE or SQA, as appropriate, were placed in a pear-shaped flask, and the chloroform was removed by rotary evaporation. The neck of the flask was covered with sterile Whatman 541 filter paper to maintain sterility. The dried lipid film was placed under high vacuum (50 mbar) for at least 1 h. PBS was added to the dried lipid film to give a final phospholipid concentration of 100 mM. After closing with a ground glass stopper, the flask was shaken until all of the dried lipids were in suspension. Liposomes were stored at 4°C.

2.3.1. Liposomes containing 43% squalene for immunization (group 3)

Liposomes containing low amounts of SQE (43 mol %) were made with DMPC:DMPG:SQE in a molar ratio (9:1:7.5). Lipid A was added to give a final dose of 25 μg in 0.2 ml of 100 mM phospholipid. Six ml of DMPC, 6 ml of DMPG, 1.5 ml of lipid A (1 mg/ml), and 0.438 ml of SQE were added to a 100-ml pear-shaped flask. After drying as described in Section 2.3, PBS was added to give a final volume of 12 ml.

2.3.2. Liposomes containing 71% squalene for immunization (group 4)

Liposomes containing high amounts of SQE (71 mol %) were made with DMPC:DMPG:SQE in a molar ratio (9:1:25). Lipid A was added to give a final dose of 25 μg in 0.2 ml of 100 mM liposomal phospholipid. Six ml of DMPC, 6 ml of DMPG, 1.5 ml of lipid A (1 mg/ml), and 1.46 ml of SQE were added to a 100-ml pear-shaped flask. After drying as described in Section 2.3, PBS was added to give a final volume of 12 ml.

2.3.3. Liposomes used for ELISA

Liposomes used for ELISA were made with DMPC:DMPG or DMPC:DMPG:SQE (or SQA, as appropriate), in molar ratios of 9:1 or 9:1:7.5. Twenty ml of DMPC, 20 ml of DMPG, and 1.44 ml of SQE or 1.6 ml of SQA (or no oil antigen) were added to a 100-ml pear-shaped flask. After drying as Section 2.3, PBS was added and the final volume of the liposomes was adjusted to 40 ml. The liposomes are designated L(SQE) for SQE-containing liposomes, L(SQA) for SQA-containing liposomes, or L for liposomes lacking an oil antigen. The final phospholipid concentration was 100 mM.

2.4. Preparation of emulsions for immunization

2.4.1. Emulsion with 40% SQE, 10% Arlacel A, and lipid A (group 5)

Components for this formulation were initially prepared in two separate 2-ml vaccine vials. One vial contained 1 ml of saline. For the second vial, 2.5 mg of lyophilized lipid A was dissolved in 8 ml of SQE; 2 ml of Arlacel A were then added; and 1 ml of the combination was added to the vial. The emulsion was prepared just prior to injection by emulsifying 0.75 ml of saline with 0.75 ml SQE–Arlacel A–lipid A using two 3-ml plastic syringes and a three-way stopcock. The saline was drawn into one syringe and the SQE–Arlacel A–lipid A was drawn into another syringe. The saline was pushed into the SQE–Arlacel A–lipid A. The mixture was passed back and forth at a rate of approximately two passes per second for 5 min to form an emulsion. The emulsion was stable for several hours at room temperature.

2.4.2. Emulsion with 20% SQE, 5% Tween 80, 5% Span 85, and lipid A (Group 6)

Components were vialed in two separate 2-ml vaccine vials prior to emulsification. One vial contained 1.5 ml of saline. The components for the second vial were made by dissolving 12 mg of lyophilized lipid A in 14.4 ml of SQE. Tween 80 (7.2 ml) and Span 85 (7.2 ml) were added to the lipid A in SQE. One ml of the mixture was vialed. The emulsion was prepared just prior to injection by emulsifying 1.05 ml saline with 0.45 ml SQE–Tween 80–Span 85–lipid A using two 3-ml plastic syringes and a three-way stopcock as described in Section 2.4.1. The emulsion was unstable and separated into two layers in approximately 45 min.

2.4.3. Aluminum hydroxide gel mixed with emulsion containing 19% squalene, 1% Tween 80 and lipid A (group 7)

Aluminum hydroxide was diluted in saline to give 1.25 mg Al^{+3}/ml and 1.5 ml was placed in a 2-ml vaccine vial. The components for the second vial were made by dissolving 4 mg of lyophilized lipid A in 6 ml of SQE. Tween 80 (0.32 ml) was added and 1.5 ml of the mixture was added to a 2-ml vaccine vial. The formulation was prepared just prior to injection by emulsifying 1.2 ml of aluminum hydroxide in saline with 0.3 ml of SQE–Tween 80–lipid A, as described in Section 2.4.1. The final aluminum hydroxide concentration was 1 mg Al^{+3} per ml. The mixture was unstable and separated into two layers in less than 30 min.

2.4.4. Aluminum hydroxide gel mixed with emulsion containing 40% squalene, 10% Arlacel A, and lipid A (group 8)

Aluminum hydroxide was diluted in saline to give 2 mg Al^{+3} per ml, and 1.5 ml was added to a 2-ml vaccine vial. The components for the second vial were the same SQE–lipid A–Arlacel A mixture used in group 5. The formulation was prepared just prior to injection by mixing 0.75 ml of aluminum hydroxide in saline with 0.75 ml of SQE–Arlacel A–lipid A, as described in Section 2.4.1. The final aluminum hydroxide was 1 mg Al^{+3} per ml. The mixture was unstable and separated into two layers in less than 30 min.

2.5. Immunizations

BALB/c mice, purchased from Jackson Laboratories (Bar Harbor, ME, USA), were immunized i.p. and bled every 2 weeks under a protocol approved by the institutional Laboratory Animal Care and Use Committee. They were fed standard mouse chow and water ad libitum. Groups of five mice received one of the following immunogens: Group 1 — 0.5 ml SQE; Group 2 — 0.5 ml of SQE containing 25 μg lipid A; Group 3 — 0.2 ml of 43% SQE liposomes; Group 4 — 0.2 ml of 71% SQE liposomes; Group 5 — 0.2 ml of emulsion containing 50% saline (0.9% sodium chloride), 40% SQE, 10% Arlacel A containing 25 μg lipid A per dose; Group 6 — 0.2 ml of an emulsion containing 70% saline, 20% SQE, 5% Tween 80, 5% Span 85 (v/v) containing 25 μg lipid A per dose; Group 7 — 0.2 ml aluminum hydroxide in saline, 19% SQE, 1% Tween 80, containing 25 μg lipid A per dose; Group 8 — 0.2 ml of aluminum hydroxide in saline, 40% SQE, 10% Arlacel A containing 25 μg lipid A per dose (Table 1). Animals were boosted every 2 weeks. Three additional mice were immunized by the intravenous route with 0.2 ml of the high SQE liposomes (group 4). Three days later, the animals were euthanized and the spleens removed for production of monoclonal antibodies.

2.6. Production of monoclonal antibodies

Three days after the primary or boosting immunization, mice were euthanized and spleens obtained. Single cell suspensions of spleen cells were prepared. Spleen cells and mouse myeloma X63/Ag8.653 cells were fused in a 1:1 ratio using polyethylene glycol 1500 (Köhler and Milstein, 1975; Galfré and Milstein, 1981). After fusion, the cells were centrifuged and then suspended in DMEM containing 20% fetal bovine serum, 1 mM sodium pyruvate, 1× NEAA, 4 mM glutamine, 50 units per ml penicillin, 50 μg/ml streptomycin, 1× HT (30 ml per spleen). Cells (0.1 ml per well) were plated in 96-well plates. The next day 0.1 ml of DMEM media containing 1× HAT instead of HT was added to all of the wells. On days 2, 3, 5, 8, and 11, 0.1 ml of media was removed from each well and 0.1 ml of DMEM containing HAT was added. After 8 days culture supernatants were screened for antibodies reacting with SQE and not SQA by ELISA on PVDF plates as described in Section 2.7. Cells from culture supernatants that were positive were expanded and then cloned twice by limiting dilution.

2.7. ELISA for testing serum for antibodies to SQE using polystyrene (PS) plates

Solid-phase ELISAs were performed as described previously with minor modifications (Alving et al., 1996a). For the initial serum screening assays, 10 μg of SQE or SQA in 50 μl of ethanol was placed in PS 'U' bottom plates. The plates were placed overnight in a biological safety cabinet to allow the ethanol to evaporate. The plates were blocked with 0.25 ml of

Table 1
Summary of immunization groups

Group No.	Antigen composition[a]
1.	Squalene alone (0.5 ml)
2.	Squalene (0.5 ml) mixed with 25 μg of lipid A
3.	Liposomes containing both lipid A and 43 mol% squalene
4.	Liposomes containing both lipid A and 71 mol% squalene
5.	Emulsion containing 40% squalene, 10% Arlacel A, and lipid A
6.	Emulsion containing 20% squalene, 5% Tween 80, 5% Span 85, and lipid A
7.	Aluminum hydroxide gel mixed with emulsion containing 19% squalene, 1% Tween 80 and lipid A
8.	Aluminum hydroxide gel mixed with emulsion containing 40% squalene, 10% Arlacel A and lipid A

[a] All injections were administered i.p. in a 0.2-ml dose, except where indicated. Lipid A, when used, was administered at 25 μg of lipid A per dose.

PBS–0.3% gelatin for 2 h. After removal of the blocking buffer, 50 μl per well of serum diluted in PBS–0.3% gelatin was added in triplicate. The plates were incubated at 4°C overnight. The plates were then washed three times with PBS using a plate washer (Skatron, Sterling, VA, USA). Peroxidase-labeled goat IgM (μ chain specific) were diluted 1000-fold in PBS–0.3% gelatin and 50 μl per well was added to the plates.

Following incubation at room temperature for 1 h, the plates were washed three times with PBS. ABTS substrate (50 μl per well) was added and the plates were incubated for 1 h at room temperature in the dark. The absorbance at 405 nm was quantified using a UVmax Kinetic Microplate Reader (Molecular Devices, Palo Alto, CA, USA). Assays were conducted in triplicate. Assay background was determined by incubation with wells lacking antigen. Background was subtracted from experimental values. Endpoint antibody titers were selected as the dilution at which the absorbance was twice background.

2.8. ELISA for testing culture supernatants for antibodies to SQE using PS plates

For assay of culture supernatants of monoclonal antibodies, PS flat-bottom plates were used. The assay was similar to that described in Section 2.7 for the 'U' bottom plates with the following changes. (1) The assay volumes of coating antigen, primary and secondary antibodies and substrate was increased from 50 μl to 100 μl; (2) SQE and SQA were dissolved in isopropanol; (3) incubation of with culture supernatants was for 1 h at room temperature instead of overnight at 4°C. These changes gave less background and somewhat greater reproducibility among triplicate determinations when compared to ELISA on PS 'U' bottom plates. However, better results were obtained using PVDF membranes.

2.9. ELISA for antibodies to SQE Using PVDF plates

The assay for antibodies to SQE was modified from the method described for detecting antibodies to cholesterol by Dijkstra et al. (1996). A volume of 0.1 ml of SQE or SQA, as appropriate, dissolved in isopropanol was placed in each well and the plate was placed overnight in a biological safety cabinet to allow the isopropanol to evaporate. The wells were blocked with PBS–4% FBS, pH 7.4, (0.3 ml per well) and incubated at room temperature for at least 1 h. After removal of the blocking buffer, 0.1 ml of culture supernatant (either undiluted or diluted in PBS–4% FBS) was added to each well. The plate was covered with seal plate adhesive film and placed on a orbital shaker set at 1500 rpm for 1 h. The plates were then washed four times with PBS–4% FBS. Sufficient PBS–4% FBS was added to each well until the air bubble floated off the PVDF membrane. Peroxidase-linked goat anti-mouse IgG or IgM was diluted 1 to 1000 in PBS–4% FBS and 0.1 ml was added to each well. The plates were covered with seal plate adhesive film and placed on the shaker as described above. The plates were washed four times with PBS as described above. ABTS substrate (0.15 ml per well) was added and the plates were covered with seal plate adhesive film. They were placed on the shaker, covered with aluminum foil, and shaken at 1500 rpm. After 1 h, 0.05 ml was transferred from each well and placed in a corresponding well of a 96-well 'U' bottom plate. The absorbance was read at 405 nm using an ELISA plate reader.

2.10. ELISA using L(SQE), L(SQA) and L as capture antigens

ELISAs using liposomes as capture antigens were performed using 'U' bottom PS plates. L(SQE), L(SQA), or L, as appropriate, were diluted to 660 nmol/ml in PBS (equivalent to 10 μg SQE). Fifty μl (33 nmol) were placed in each well. The plate was placed in a biological safety cabinet overnight. The plates containing the dried film of liposomes were processed by ELISA as described in Section 2.7. For serum assays, the plates containing diluted serum were incubated overnight at 4°C. For assays using diluted supernatants from the monoclonal antibodies, the plates were incubated 1 h at room temperature.

3. Results

3.1. Induction and reactivity of polyclonal antisera with SQE by ELISA

Sera from immunized mice were tested by ELISA for the presence of anti-SQE antibodies using SQE as the capture antigen. Among the eight immunization strategies employed (see Materials and methods, and summary in Table 1), only two groups exhibited increased IgM binding activity after injection of the antigen when compared to the preimmunization serum (group 4, Fig. 2A; group 6, Fig. 2B). None of the groups developed IgG binding activity after immunization (data not shown). Mice injected with liposomes containing lipid A and 71% SQE [L(71% SQE+LA)] (group 4, see Table 1) showed progressively increased IgM titers with time when compared to the pre-immunization bleeding (Fig. 2A). The animals were immunized every 2 weeks, and even at 2 weeks after a single injection, an increased IgM titer was evident. To a much lesser extent one of the SQE emulsion groups (group 6, see Table 1) also developed increased titers when compared to the pre-immune sera, but even after multiple injections there was no progressive increase in the antibody titer (Fig. 2B). Because of this, in all further experiments sera from animals immunized with L(71% SQE+LA) was used.

Using an alternative capture antigen in the ELISA, namely liposomes containing SQE, an even higher resolution of positive results was observed when compared to the results obtained with SQE alone (Fig. 3A) as a capture antigen. However, as shown in Fig. 3, after immunization with liposomes containing SQE and lipid A, the antisera reacted not only with

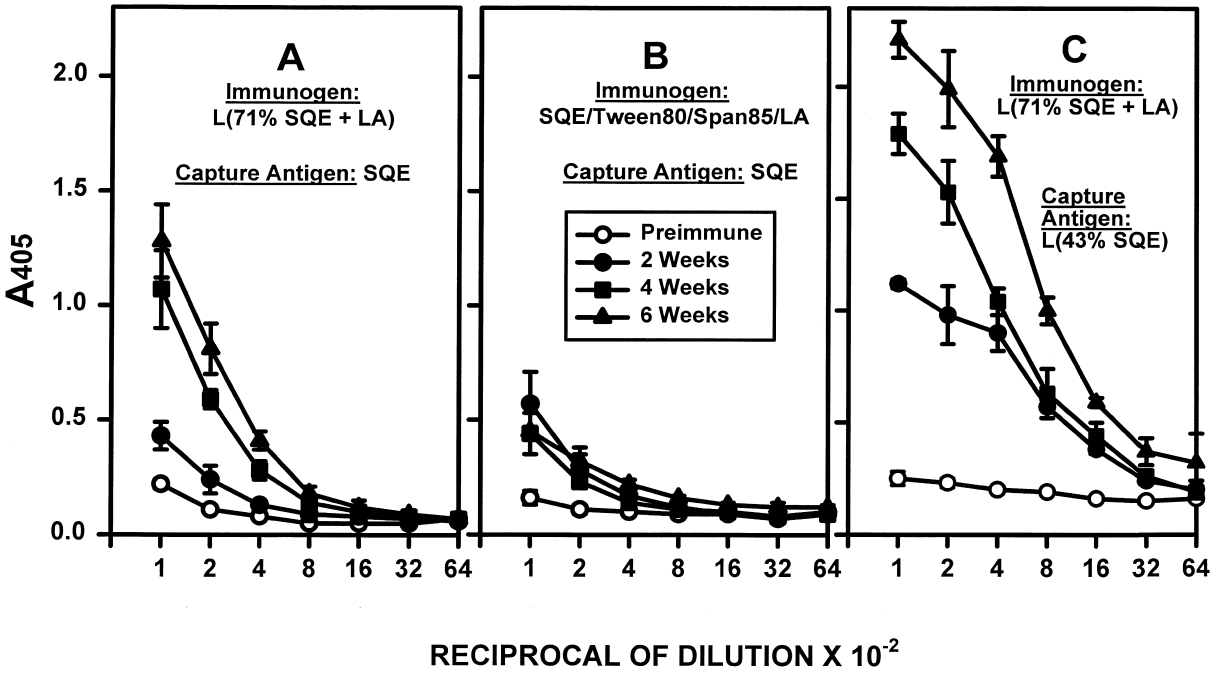

Fig. 2. Binding activity of mouse serum IgM to SQE or L(SQE) by ELISA. Mice were immunized biweekly with: (A) Liposomes containing lipid A as an adjuvant and composed of DMPC/DMPG/SQE in a molar ratio of 9:1:25 (group 4); or (B) an emulsion containing of 20% SQE, 5% Tween 80, 5% Span 85 and lipid A (group 6); or (C) the same liposomes used in (A). Serum obtained from these mice were tested by ELISA as described in the Materials and methods. Polystyrene 'U' bottom plates were coated with 10 μg per well of SQE in ethanol. Binding activity of the indicated dilutions of preimmune and immune serum was assayed at the indicated time points. Results are presented as the mean absorbance from triplicate wells containing squalene subtracted from the absorbance of triplicate wells lacking squalene±S.D.

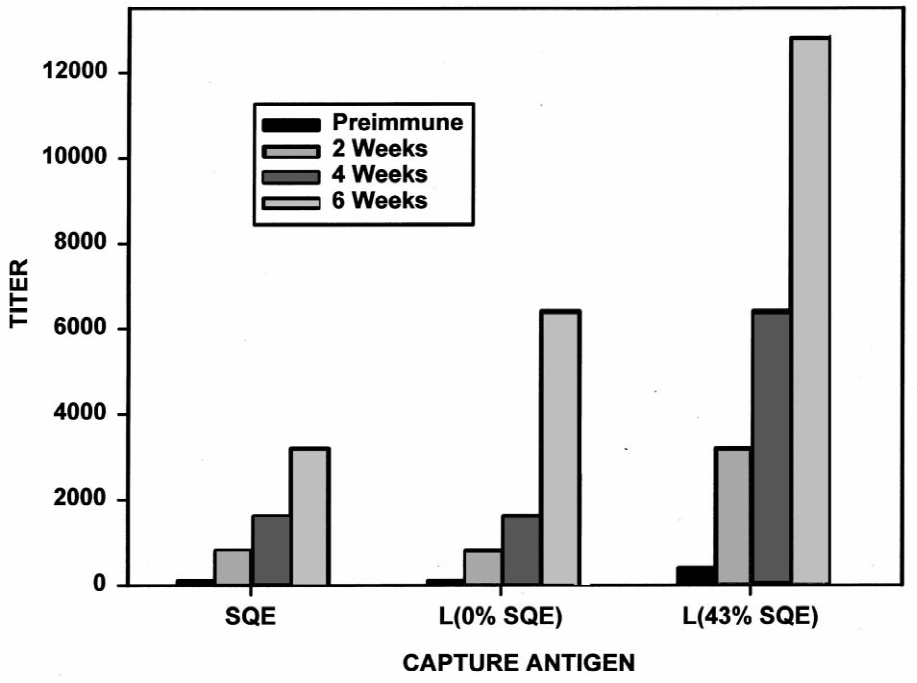

Fig. 3. End point dilution IgM titers of immune mouse serum against SQE, and liposomes containing or lacking SQE. Serum from mice immunized biweekly with liposomes containing lipid A as an additional adjuvant and composed of DMPC/DMPG/SQE in a molar ratio of 9:1:25 (group 4) were tested by ELISA. Capture antigens for the assay consisted of SQE or of liposomes containing or lacking squalene. Polystyrene 'U' bottom plates were coated with 10 μg per well of SQE in ethanol, or with the equivalent amount of L(SQE), or with the equivalent amount of L. The results shown were obtained by subtracting the absorbance of triplicate wells containing the appropriate capture antigen from the absorbance of triplicate wells lacking antigens. Endpoint IgM antibody titers were calculated from the highest dilution of serum giving twice the absorbance of the background.

SQE alone but also with liposomes lacking SQE, albeit to a much lesser extent than with liposomes containing SQE. This latter observation is consistent with previous reports that antibodies to phospholipids are also induced when liposomal lipid A, or even lipid A alone, is used as an adjuvant (Schuster et al., 1979; Banerji and Alving, 1981; Alving, 1986).

The above data suggested that antibodies that could react with SQE were induced in mice by immunization with certain formulations that contained SQE. However, when another oil molecule, SQA, the fully hydrogenated form of SQE, was substituted for SQE as a capture antigen in the ELISA, the polyclonal antiserum to SQE reacted equally well with either SQE or SQA (data not shown). This apparent lack of monospecific binding to SQE could have been due either to extensive cross-reactivity of anti-SQE antibodies with SQA, or to a mixed population of antibodies, some of which cross-reacted with SQA and some of which did not. The possibility of nonspecific binding of IgM antibodies also existed. Because of this, we decided to try to produce monoclonal antibodies that could differentiate between SQE and SQA as antigens. In the course of this work, as shown below, we also refined the ELISA assay to minimize nonspecific effects and increase resolution.

3.2. Development of monoclonal antibodies to SQE

To minimize experimental variation and nonspecific effects observed after coating of hydrophobic antigens on polystyrene microtiter wells, we examined the possible benefits of coating the capture antigens on hydrophobic membranes consisting of

polyvinylidene fluoride (PVDF), as described by Aniagolu et al. (1995). As shown in Fig. 4A and B, when culture supernatants were assayed with PVDF membranes, an IgM anti-SQE mAb was identified that exhibited strong dose-dependent binding to SQE, but displayed little or no cross-reactivity to SQA. When the antigens were coated on flat-bottom PS microtiter wells instead of PVDF membranes, the same anti-SQE mAb showed a complete lack of reactivity with either SQE or SQA (Fig. 4C and D).

Additional clones of anti-SQE mAbs were also produced which, when tested with the PVDF membrane assay, either showed striking specificity for

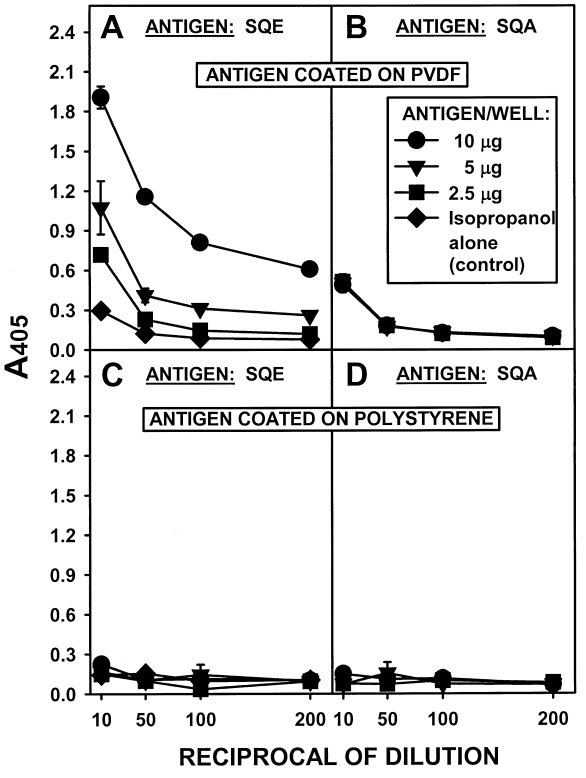

Fig. 4. Comparative binding of a mAb to SQE or SQA coated on PVDF or PS flat bottom plates. Each well contained 10 μg of SQA or SQE dissolved in 0.1 ml of isopropanol, or isopropanol alone (control), as appropriate, at the concentrations indicated. The culture supernatant of a mAb was diluted in PBS–4% fetal bovine serum (PVDF plates) or PBS–0.3% gelatin (PS plates). ELISAs were performed as described in the Materials and methods section for the PVDF and PS plates, respectively. Similar results were observed with eight other clones. Values are the mean±standard deviation of triplicate wells.

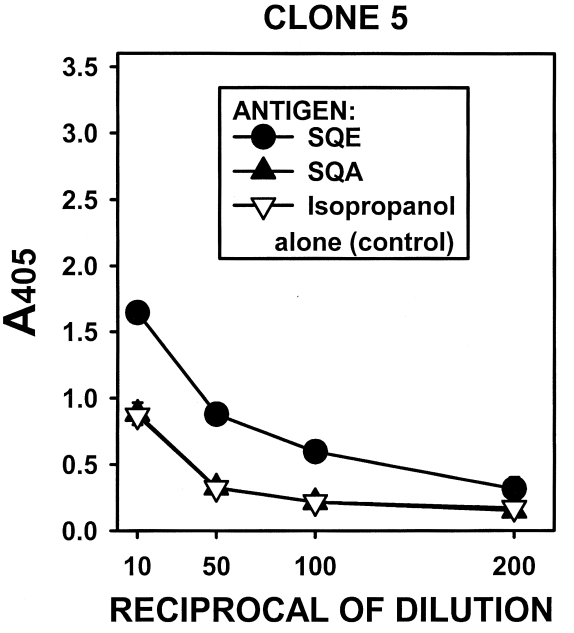

Fig. 5. Specific binding of mAb clone 5 to SQE, but not SQA. The assay was conducted with PVDF plates as described in the legend to Fig. 4.

SQE (clone 5, Fig. 5), or reactivity with both SQE and SQA (clone 15, Fig. 6). These data demonstrate that mAbs can be identified that differentiate free SQE from free SQA by ELISA, particularly when the antigens are coated on PVDF membranes.

3.3. Evaluation of the specificity of mAbs for reactivity with a capture antigen consisting of liposomes containing SQE or SQA

The original immunizing antigen consisted of liposomes containing SQE+LA. Fig. 7 illustrates the results of ELISAs in which PS plates were coated with liposomes containing or lacking SQE or SQA. An irrelevant IgM mAb (anti-asialoG_{M2}) is shown as a negative control (Fig. 7E). When analyzed for reactivity with liposomes containing SQE [L(SQE)], liposomes containing SQA [L(SQA)], or liposomes lacking both SQE and SQA [L], four different patterns of specificity for L(SQE), L(SQA), and L alone were observed, as derived from Fig. 7 and summarized in Table 2. It is noteworthy that we have never obtained a mAb that bound more strongly to

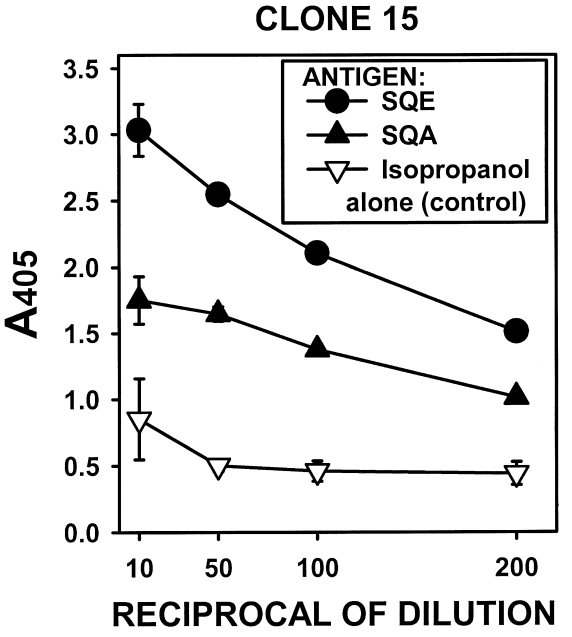

Fig. 6. Binding of mAb clone 15 to SQE and cross-reactivity with SQA. The assay was conducted with PVDF plates as described in the legend to Fig. 4.

SQA than to SQE. This is in keeping with the primary specificity of the antibodies for liposomal SQE.

3.4. Evaluation of the specificity of polyclonal antiserum for SQE and SQA on PVDF membranes

The above studies demonstrate that immunization with SQE induces a mixed population of anti-SQE antibodies that includes some that do not cross-react and some that do cross-react with SQA. In view of this, polyclonal anti-SQE antiserum would be expected to exhibit both SQE reactivity and SQA cross-reactivity on PVDF membranes. As shown in Fig. 8, reactivity with both antigens was observed with polyclonal anti-SQE antiserum.

4. Discussion

We have demonstrated in this study that polyclonal and monoclonal antibodies that bind to SQE can be developed after immunization of mice with liposomes containing 71% SQE and lipid A. Other methods of immunization, including immunizing with liposomes containing 43% SQE or with a variety of SQE-containing emulsions, were either

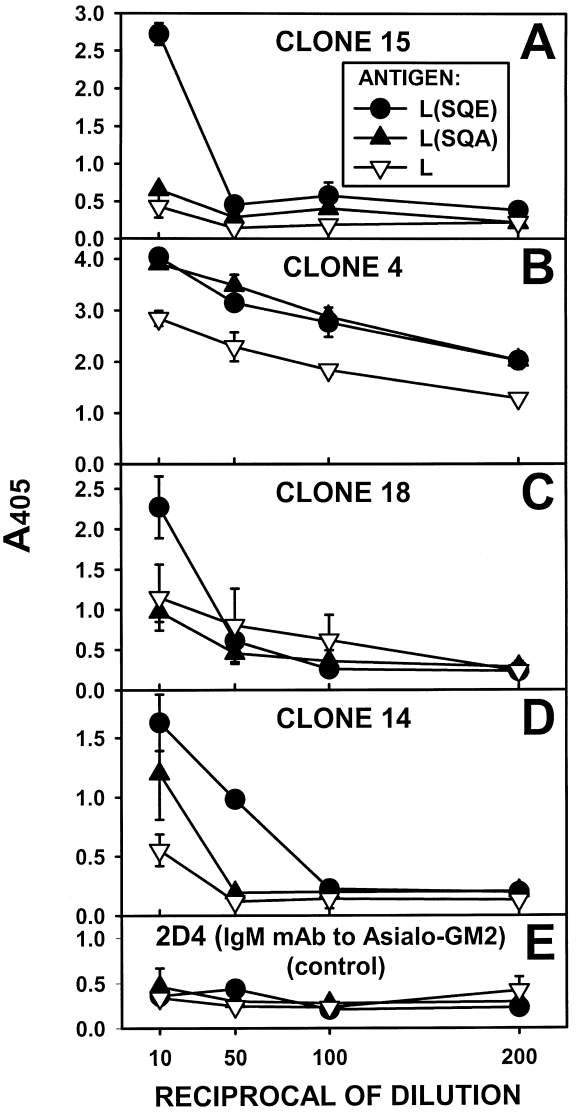

Fig. 7. Reactivity of monoclonal antibodies to liposomes containing or lacking SQE or SQA. L(SQE), L(SQA) and L (33 nmol of phospholipid) in 0.05 ml of PBS was placed in each well of a PS 'U' bottom plate. The plates were processed as described in the Materials and methods. Culture supernatants from the indicated clones were diluted in PBS–0.3% gelatin and 0.05 ml was placed in each well. Values are the mean±S.D. of triplicate wells. A, B, C, D: Binding of the indicated culture supernatants to L(SQE), L(SQA), and L. E: Negative controls consisting of binding of an irrelevant IgM secreting clone 2D4 (IgM anti-G_{M2}).

Table 2
Monoclonal antibody specificities obtained after injection of liposomes containing lipid A and SQE

Clone no.	Binding specificity[a]		
	SQE	SQA	Liposomal phospholipid
15	+	−	−
4	+	+	+
18	+	−	+
14	+	+	−

[a] Based on data from Fig. 7.

completely ineffective, or considerably less effective, as immunogens. The strategy of utilizing liposomes containing 71% SQE and lipid A as an immunogen was modeled after similar success in the induction of antibodies to cholesterol by immunizing with liposomes containing 71% cholesterol and lipid A (Swartz et al., 1988; Alving and Swartz, 1991; Dijkstra et al., 1996). Although we have previously found that simple injection of silicone oil into mice can also cause the induction of antibodies to cholesterol (Alving et al., 1996b), injection of non-emulsified SQE oil mixed with lipid A did not result in the induction of antibodies to SQE (group 2, Table 1). Among four emulsions containing SQE and lipid A as components, only one (group 6, Table 1) induced any immune response to SQE, and this was quite weak even after multiple injections (Fig. 2B). From these data we conclude that SQE is a very poor antigen when used either as an oil or an emulsion, even when lipid A, a potent adjuvant for inducing antibodies to lipids, is included in the immunizing formulation.

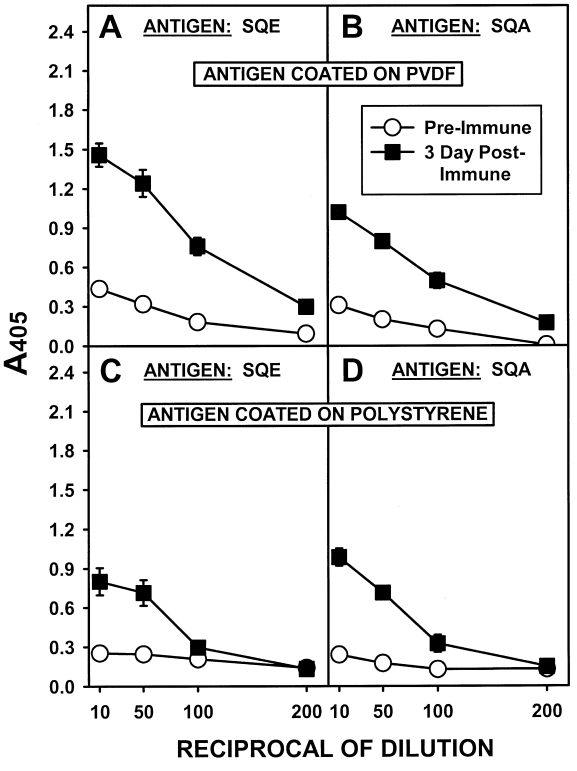

Fig. 8. Binding of antiserum IgM to SQE and SQA on PVDF and PS flat-bottom ELISA plates. Pre-immune or 3-day post-immune serum from mice immunized with liposomes containing 71% SQE was diluted in PBS–4% fetal bovine serum (PVDF plates) or PBS–0.3% gelatin (PS plates). ELISAs were performed as described in the Materials and methods section for the PVDF and PS plates, respectively. Values are the mean±S.D. of triplicate wells.

In our experience, and in the experience of others, liposomal lipid A is required for induction of antibodies to liposomal cholesterol (Swartz et al., 1988; Dijkstra et al., 1996) and in the induction of antibodies to liposomal phospholipids (Schuster et al., 1979; Alving, 1986). In keeping with this, in the present study injection of liposomes containing lipid A and SQE also induced anti-phospholipid antibodies that bound, as determined by ELISA, to liposomes lacking SQE (Fig. 3).

Our previous experience in studying the antigen-binding specificities of antibodies induced by injection of liposomal lipids has led to the conclusion that monospecific antibodies to individual liposomal lipid constituents are unusual. We have proposed that antibodies induced by mixtures of liposomal lipids should be properly considered to be ‘anti-liposome’ antibodies (Banerji and Alving, 1981; Wassef et al., 1984). Antibodies induced by complex lipid mixtures probably represent a spectrum of specificities ranging from immunodominance of a single lipid epitope to subsite reactivities in the antigen binding site that recognize multiple lipid epitopes, or biophysical conformations of lipids, on the liposome surface (Alving, 1986). Although mAbs to cholesterol induced by liposomes containing lipid A were selected for the ability to react with liposomes containing 71% but not 43% cholesterol, some of these antibodies could be partially blocked by binding to nucleotides (Stollar et al., 1989). The results in the present study are consistent with the concept

of induction of anti-liposome mAb antibodies having specificities that include both liposomal phospholipid as well as SQE in the antigen binding site of the antibody (Figs. 7B and C; Table 2). However, as with the anti-cholesterol mAbs, anti-SQE clones were also obtained that did not react with liposomal phospholipid (Figs. 7A and D; Table 2).

It has been previously reported that SQE incorporated into non-phospholipid liposomes has an adjuvant effect on the induction of antibodies to a non-phospholipid liposomal protein, but the adjuvant effect was not enhanced further by simultaneous incorporation of lipid A (Gupta et al., 1996). Although incorporation of lipid A without SQE into non-phospholipid liposomes was not tested in the latter study, the potent adjuvant effect of liposomal SQE for liposomal protein was clearly shown. This adjuvant effect of liposomal SQE therefore may also have played a role in our liposomes in the induction of antibodies to SQE.

As with 71% cholesterol in liposomes, the biophysical conformation of 71% SQE in our liposomes is not completely clear. Previous work has suggested that SQE locates itself in the most disordered region of liposomes, predominately in the center area of the liposomal bilayer (Lohner et al., 1993). Because of this it has been proposed that SQE adopts a coil rather than an extended conformation when it is located in the bilayer interior. Although relatively small amounts of SQE have a disruptive effect on the liposomal bilayer and lead to formation of tubules having the H_{II} conformation in liposomes containing phosphatidylethanolamine (Lohner et al., 1993), the H_{II} conformation does not occur in liposomes, such as ours, that lack phosphatidylethanolamine.

Nonetheless, the reported ability of SQE to lower the transition temperature of phosphatidylcholine and to cause disruption in the stability of the liposomal bilayer (Lohner et al., 1993), together with the high concentrations of SQE combined with lipid A in the liposomes used in this study, may play a role in the potent ability of these liposomes to induce antibodies to SQE.

From a purely structural standpoint, it may not be initially surprising that antibodies to SQE can be induced in a similar manner to those against cholesterol, in view of the striking apparent structural similarity of SQE and cholesterol (Fig. 1). Balanced against this, however, is the observation that the immunogenic epitope of liposome-associated cholesterol is the polar 3-β-hydroxy group in the A ring (Dijkstra et al., 1996), and the fact that SQE not only lacks any closed ring, but is an exceedingly hydrophobic alkene that completely lacks any polar group.

The extreme hydrophobicity of SQE raises an important theoretical problem in demonstrating specificity of antibodies because polyclonal antiserum raised by immunization with SQE shows considerable reactivity with SQA (Fig. 8). Based on serum data alone, it was therefore initially impossible to determine whether the apparent antibody activity in the antiserum is specific to SQE, or if the immunoglobulins are simply nonspecifically binding hydrophobically both to SQE and SQA. Our initial experiments using PS microtiter plates did indeed demonstrate nonspecific hydrophobic binding of IgM molecules to both SQE and SQA, and other alkanes (data not shown).

However, this problem was solved by coating the antigens on hydrophobic PVDF membranes, as described by Aniagolu et al. (1995). Although commercially-available PVDF membranes also present the problem that they are physically located in PS microtiter wells, they apparently do have the salutary effect of blocking most or all of the nonspecific hydrophobic binding sites of the alkane molecules.

To demonstrate that specific antibodies to SQE actually do exist, we created mAbs that were selected for the ability to bind to SQE but had a relative inability to bind to SQA, as determined by ELISA with hydrophobic PVDF membranes. Monoclonal antibodies were successfully created that specifically bound to SQE but to a lesser extent, or not at all, to SQA. However, numerous anti-SQE mAbs were also created that cross-reacted strongly with SQA. It is concluded that specific differentiation of SQE from SQA demonstrates that the unsaturated bonds of SQE can play a major role in the specificity of the antibodies, and such antibodies therefore have a distinctive conformational specificity. However, the extensive cross-reactivity of numerous clones of anti-SQE antibodies with SQA also demonstrates that the unsaturated bonds are not the sole determinant of specificity.

What, if any, are the potential consequences of induction of antibodies to SQE? A recent publication

claims to have detected antibodies to SQE in sick but not in healthy individuals (Asa et al., 2000). However, we believe that such a conclusion may be premature, based on a technical critique of the reported Western blot-type assay that was used (Alving and Grabenstein, 2000).

Turning again to cholesterol for comparison, SQE, as a precursor in the synthesis of cholesterol, is found nearly everywhere that cholesterol is found, with the apparent exception that SQE probably does not have a structural role in promoting the stability of membranes. As with cholesterol, SQE circulates in the blood as a constituent of LDL and VLDL (Miettinen, 1982; Koivisto and Miettinen, 1988). Naturally occurring antibodies to cholesterol have been demonstrated to be present in virtually all human serum samples tested, and they have been proposed to have a vital beneficial role in the normal regulation of LDL and VLDL metabolism (Alving and Wassef, 1999). By analogy, it is possible that antibodies to SQE could have a similar effect on LDL and VLDL metabolism.

With the development of the ELISA using PVDF membranes, as described in this paper, it may now be possible to undertake studies with serum from sick and healthy individuals to determine whether naturally-occurring antibodies to SQE exist, and whether the appearance or amounts of such antibodies have any relationship to normal physiologic functions or whether they are associated with any illness.

References

Alving, C.R., 1986. Antibodies to liposomes, phospholipids, and phosphate esters. Chem. Phys. Lipids 40, 303.

Alving, C.R., Grabenstein, J., 2000. Letter to the editor. Exp. Mol. Path. 86, 196.

Alving, C.R., Swartz, Jr. G.M., 1991. Antibodies to cholesterol, cholesterol conjugates, and liposomes: Implications for atherosclerosis and autoimmunity. Crit. Rev. Immunol. 10, 441.

Alving, C.R., Wassef, N.M., 1999. Naturally-occurring antibodies to cholesterol: a new theory of LDL cholesterol metabolism. Immunology Today 20, 362.

Alving, C.R., Shichijo, S., Mattsby-Baltzer, I., Richards, R.L., Wassef, N.M., 1993. Preparation and use of liposomes in immunological studies. In: Gregoriadis, G. (Ed.), second ed. Liposome Technology, vol. 3. CRC Press, Boca Raton, p. 317.

Alving, C.R., Swartz, Jr. G.M., Wassef, N.M., 1989. Naturally occurring autoantibodies to cholesterol in humans. Biochem. Soc. Trans. 17, 637.

Alving, C.R., Swartz, Jr. G.M., Wassef, N.M., Ribas, J.L., Herderick, E.E., Virmani, R. et al., 1996a. Immunization with cholesterol-rich liposomes induces anti-cholesterol antibodies and reduces diet-induced hypercholesterolemia and plaque formation. J. Lab. Clin. Med. 127, 40.

Alving, C.R., Wassef, N.M., Potter, M., 1996b. Antibodies to cholesterol: biological implications of antibodies to lipids. Curr. Topics Microbiol. Immunol. 210, 181.

Aniagolu, J., Swartz, Jr. G.M., Dijkstra, J., Madsen, J.W., Raney, J.J., Green, S.J., 1995. Analysis of anticholesterol antibodies using hydrophobic membranes. J. Immunol. Meth. 182, 85.

Asa, P., Cao, Y., Garry, R.F., 2000. Antibodies to squalene in gulf war syndrome. Exp. Mol. Path 68, 55.

Banerji, B., Alving, C.R., 1981. Anti-liposome antibodies induced by lipid A. I. Influences of ceramide, glycosphingolipids, and phosphocholine on complement damage. J. Immunol. 126, 1080.

Dijkstra, J., Swartz, Jr. G.M., Raney, J.J., Aniagolu, J., Toro, L., Nacy, C.A. et al., 1996. Interaction of anti-cholesterol antibodies with human lipoproteins. J. Immunol. 157, 2006.

Granner, D.K., 1996. Hormones of the adrenal cortex. In: Murray, R.K., Granner, D.K., Mayes, P.A., Rodwell, V.W. (Eds.), Harper's Biochemistry, 24th ed. Appleton and Lang, Stamsord, p. 547.

Galfré, G., Milstein, C., 1981. Monoclonal antibodies: strategies and procedures. Meth. Enzymol. 73, 3.

Gupta, R.K., Varanelli, C.L., Griffin, P., Wallach, D.F.H., Siber, G.R., 1996. Adjuvant properties of non-phospholipid liposomes (Novasomes®) in experimental animals for human vaccine antigens. Vaccine 14, 219.

Köhler, G., Milstein, C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495.

Lohner, K., Degovics, G., Laggner, P., Gnamusch, E., Paltauf, F., 1993. Squalene promotes the formation of non-bilayer structures in phospholipid model membranes. Biochim. Biophys. Acta 1152, 69.

Koivisto, P.V.I., Miettinen, T.A., 1988. Increased amount of cholesterol precursors in lipoproteins after ileal exclusion. Lipids 23, 993.

Mayes, P.A., 1996. Cholesterol synthesis, transport, and excretion. In: Murray, R.K., Granner, D.K., Mayes, P.A., Rodwell, V.W. (Eds.), Harper's Biochemistry, 24th ed. Appleton and Lang, Stamsord, p. 271.

Miettinen, T.A., 1982. Diurnal variation of cholesterol precursors squalene and methyl sterols in human plasma lipoproteins. J. Lipid Res. 23, 466.

Minutello, M., Senatore, F., Cecchinelli, G., Bianchi, M., Andreani, T., Podda, A. et al., 1999. Safety and immunogenicity of an inactivated subunit influenza virus vaccine combined with MF59 adjuvant emulsion in elderly subjects, immunized for three consecutive influenza seasons. Vaccine 17, 99.

Schuster, B., Neidig, M., Alving, B.M., Alving, C.R., 1979. Production of antibodies against phosphocholine, phosphatidylcholine, sphingomyelin, and lipid A by injection of liposomes containing lipid A. J. Immunol. 122, 900.

Stewart, M.E., 1992. Sebaceous gland lipids. Semin. Dermatol. 11, 100.

Swartz, Jr. G.M., Gentry, M.K., Amende, L.M., Blanchette-Mackie, E.J., Alving, C.R., 1988. Antibodies to cholesterol. Proc. Natl. Acad. Sci. USA 85, 1902.

Stollar, B.D., McInerney, T., Gavron, T., Wassef, N.M., Swartz, Jr. G.M., Alving, C.R., 1989. Cross-reactions of nucleic acids with monoclonal antibodies to phosphatidylinositol phosphate and cholesterol. Mol. Immunol. 26, 73.

Wassef, N.M., Roerdink, F., Swartz, Jr. G.M., Lyon, J.A., Berson, B.J., Alving, C.R., 1984. Phosphate binding specificities of monoclonal antibodies against phosphoinositides in liposomes. Mol. Immunol. 21, 863.