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Detection of Antibodies to Squalene III. Naturally Occurring Antibodies to Squalene in Humans and Mice

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Research Paper

Detection of antibodies to squalene III. Naturally occurring antibodies to squalene in humans and mice[☆]

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Abstract

An ELISA-based assay is described for the measurement of antibodies to squalene (SQE) in human serum and plasma. The assay was adapted from the previously described assay for murine antibodies to SQE (J. Immunol. Methods 267 (2002) 119). Like the murine SQE antibody assay, the human antibody assay used sterile cell culture 96-well plates coated with SQE (20 nmol/well). Phosphate-buffered saline (PBS)–0.5% casein was used as both a blocking agent and dilution buffer. The assay has a high through-put capacity and is reproducible and quantitative. This assay was used to evaluate samples from three different human cohorts. The first cohort was retired employees of the United States Army Medical Research Institute of Infectious Diseases (USAMRIID alumni). The mean age was 68 ($N=40$; range 58–82). Most were vaccinated with the U.S. licensed anthrax vaccine (AVA) and most had received several other vaccines through a USAMRIID special immunization program. The second cohort was of similar age ($N=372$; mean age 67; range 54–97) from the normal population of Frederick, MD and were not vaccinated with AVA. The third cohort ($N=299$) was from Camp Memorial Blood Center, United States Army Medical Department Activities, Fort Knox, KY. (No additional volunteer information is available.) Using this new ELISA method, antibodies to SQE were detected in all three of the cohorts. IgG antibodies to SQE were detected in 7.5% and 15.1% of the samples from the USAMRIID alumni and Frederick cohorts, respectively. These differences were not significantly different ($\chi^2_{(1)}=1.69$, $p=0.19$). In contrast, no IgG antibodies to SQE were detected in the Fort Knox cohort which is significantly different than the Frederick cohort ($\chi^2_{(1)}=49.25$, $p<0.0001$). IgM antibodies to SQE were detected in 37.5% and 32.3% of the samples from the USAMRIID and Frederick cohorts, respectively, but there was no significant difference between the cohorts.

Abbreviations: ABTS, 2,2′-azino-di-(3-erthylbenzthiazoline-6-sulfonate); AVA, anthrax vaccine absorbed (licensed U.S. anthrax vaccine); BSA, bovine serum albumin; FBS, fetal bovine serum; GEL, PBS–0.3% gelatin, pH 7.4; ISP, isopropanol; LDL, low density lipoprotein; PBS, Dulbecco's phosphate buffered saline without calcium and magnesium, pH 7.4; PVDF, polyvinyldiene fluoride; SQE, squalene; USAMRIID, United States Army Medical Research Institute of Infectious Diseases; VLDL, very low density lipoprotein.

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In the Fort Knox cohort, 19.4% of the samples were positive for IgM antibodies to SQE, which was significantly different from the Frederick cohort ($\chi^2_{(1)}$= 14.23, p = 0.0002). Although the age of the volunteers from the Fort Knox cohort is unknown, the demographic of the donors at the blood bank volunteers is 85% 17–21 years of age. This suggested that the prevalence of antibodies to SQE may increase with age. This was confirmed with mouse studies in which the presence of antibodies was monitored as a function of time. No antibodies to SQE were detected in female BALB/c, B10.Br and C57BL/6 mice at 2 months of age, but they reached a maximum prevalence with 100% and 89% of animals testing positive for IgG and IgM antibodies to SQE, respectively, in the C57Bl/6 mice at 18 months of age. BALB/c and B10.Br mice also developed antibodies to SQE over time, but were at a lower prevalence than those observed in the C57BL/6 mice. Thirty-five of the 40 volunteers in the USAMRIID were vaccinated with AVA (mean no. doses = 26; range 3–47). Comparison of the prevalence of antibodies to SQE from the AVA immunized group with the Frederick cohort revealed that there was no statistical differences for IgG ($\chi^2_{(1)}$= 2.3, p = 0.13) or IgM ($\chi^2_{(1)}$= 0.33, p = 0.56). When the data from the USAMRIID and Frederick cohorts were combined and analyzed for the presence of antibodies to SQE with respect to the sex of the volunteer, females (40.8%) were found to have a higher prevalence of IgM antibodies to SQE than men (28.4%) ($\chi^2_{(1)}$= 6.59, p = 0.01). No significant difference was observed in the prevalence for IgG antibodies to SQE in females (17.7%) and males (12.5%). We conclude that antibodies to SQE occur naturally in humans; have an increased prevalence in females; are not correlated with vaccination with AVA; and appear to increase in prevalence with age.
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Keywords: Squalene; Antibody detection; Enzyme-linked immunosorbent assay; Anthrax vaccine adsorbed

1. Introduction

Squalene (SQE) is a triterpenoid hydrocarbon oil, $C_{30}H_{50}$ (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexene) (Fig. 1), that is widely produced in plants and animals and is present in the food supply. In mammals, SQE serves as a precursor for the synthesis of cholesterol and steroid hormones (Granner, 1996; Mayes, 1996). SQE is synthesized in the liver and in the epidermis of the skin by sebaceous glands (Stewart, 1992). Both SQE and cholesterol are transported in the very low density lipoprotein (VLDL) and LDL (Miettien, 1982; Koivisto and Miettinen, 1988).

Because it is a naturally occurring biodegradable oil, SQE, and its hydrogenated derivative squalane, each has been proposed as the oil component of oil-in-water emulsions to be used as new adjuvants for vaccines. Although not included in any vaccines licensed in the United States, a SQE-containing emulsion is part of an influenza vaccine licensed in Italy and has been given without adverse effects to hundreds of thousands of people (Podda, 2001; Ott et al., 2000). Numerous other human clinical trials for influenza (Ott et al., 2000; Nicholson et al., 2001; Gasparini et al., 2001), cytomegalovirus (Drulak et al., 2000), hepatitis B (Heineman et al., 1999), papillomavirus (Harro et al., 2001), HIV (Kahn et al., 1994; Mitchell et al., 2002; McFarland et al., 2001; Cunningham et al., 2001), and malaria (Stoute et al., 1997, 1998; Kester et al.,2001; Rickman et al., 1991) have used SQE emulsions. Vaccine reactions were typically mild. However, some moderate to severe reactions, which can be attributed to other adjuvants in the formulations, were reported (Kahn et al., 1994; Rickman et al., 1991; Stoute et al., 1997; Kester et al., 2001). Furthermore, no adverse reactions were reported following intravenous injection of humans with chylomicron-like lipid emulsions containing SQE (Relas et al., 2001).

Antibodies to SQE have generated great interest in the popular press and have been the subject of U.S. congressional hearings. This was initiated by a report that antibodies to SQE were present in the serum of sick Gulf War veterans, but not in healthy veterans (Asa et al., 2000). The assay used in the later report was severely criticized for technical reasons; including lack of positive and negative controls (Alving and Grabenstein, 2000). The Institute of Medicine concluded that the reported results did not provide evidence that antibodies to SQE were successfully measured (Fulco et al., 2000). Using the same assay, a report was published that claimed that military service members who were sick following vaccination with certain lots of the U.S. licensed anthrax vaccine had antibodies to SQE (Asa et al., 2002). However, in

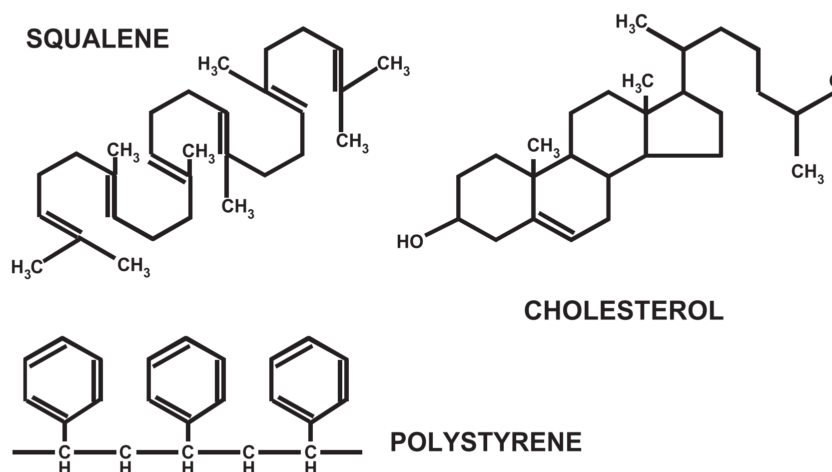

Fig. 1. Structure of squalene, cholesterol and polystyrene.

blinded testing, no correlation was found between individuals who received AVA and those in whom purported antibodies to SQE were observed (Asa et al., 2002). In view of the above observations and controversies, we undertook a study to develop a standardized assay for detecting antibodies to SQE and to establish background levels, if any, for naturally occurring antibodies to SQE.

In order to develop a reliable assay for antibodies to SQE, we developed murine monoclonal antibodies to SQE that could serve as a positive antibody control (Matyas et al., 2000). Our original assay used 96 well plates containing hydrophobic PVDF membranes. The assay was highly reproducible, but was very labor intensive. A new assay was developed that utilized 96 well polystyrene tissue culture plates (Matyas et al., 2002). The assay had a high through-put capacity, was reproducible and quantitative, and had increased sensitivity. We now describe the adaptation of this latter assay for the measurement of human antibodies to SQE in cohorts of nonselected military and civilian populations, and in populations selected for their exposure to numerous vaccines, including AVA.

2. Materials and methods

2.1. Materials

Squalene oil and bovine serum albumin (BSA) (essentially fatty acid free; cat. # A-7030) were purchased from Sigma-Aldrich, St. Louis, MO. Isopropanol (BAKER ANALYZED® A.C.S. Reagent; cat. # 9084-03) and casein were purchased from J.T. Baker, Phillipsburg, NJ. Costar round bottom 96-well sterile tissue culture plates (cat.# 3799) were purchased from Corning, Corning, NY. FBS was from GIBCO BRL Grand Island NY and was heated at 56 °C for 1 h prior to use. 10 × Dulbecco's PBS without Ca^{2+} and Mg^{2+} was also purchased from Gibco BRL. Human myelomas were purchased from the American Type Culture Collection, Chantilly, VA and were grown as described in the supplier's instructions with culture medium and additives from Gibco BRL. The human antibody secreting myelomas were IM-9 (IgG of unknown specificity obtained from a myeloma) (Fahey et al., 1971), SA13 (IgG to tetanus toxoid) (Larrick et al., 1986), 16M3F10 (IgG to diphtheria toxoid) (Gigliotti et al., 1984), C5 (IgM to lipid A of Gram negative bacteria (Teng et al., 1985), L612 (IgM to gangliosides GM3 and GM4) (Irie, 1995) and RPMI 1788 (IgM to tumor necrosis factor beta) (Aggarwal et al., 1984). Mouse myeloma SQE #16, which secretes a monoclonal antibody to SQE, was grown as described (Matyas et al., 2000, 2002). Human IgG and IgM and mouse IgM quantitation kits were purchased from Bethyl Laboratories, Montgomery, TX and were used on the culture supernatants containing mAbs as per the enclosed instructions. Affinity purified and adsorbed peroxidase-linked sheep anti-human IgG (γ-chain specific), anti-human IgM, anti-mouse IgG (γ-chain specific)

and anti-mouse IgM were purchased from The Binding Site, San Diego, CA. ABTS substrate was purchased from Kirkegaard and Perry Laboratories, Gaithersburg, MD. Female BALB/c, B10.Br and C57BL/6 mice were purchased from Jackson Laboratories, Bar Harbor, ME. Retired breeder mice were purchased at 10 months of age. Animal research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition.

2.2. Volunteer population and serum preparation

Volunteers were recruited according to U.S. Army regulations under human use protocols approved by the Walter Reed Army Institute of Research and the United States Army Medical Research Institute of Infectious Diseases Human Use Review Committees and the U.S. Army Human Subjects Research Review Board. Written informed consent was obtained from all volunteers prior to enrollment into the study. U.S. Army human use guidelines were strictly adhered to throughout the course of this study.

Three cohorts were studied. The first cohort consisted of retired employees of the United States Army Medical Research Institute of Infectious Diseases (referred to as USAMRIID alumni). Most had received numerous doses of AVA and other vaccines through a USAMRIID special immunization program (Pittman et al., 2002). All individuals ($N = 40$) were greater than 58 years old (mean age 68; range 58–82; 87.5% male) and most had received multiple doses of AVA (mean of 24 doses, range 0–47 doses). Three individuals were not vaccinated with the anthrax vaccine and the vaccine status of 3 others was unknown. The second cohort was of similar age (mean age 67; range 54–97; 61.8% male) from the normal population of Frederick, MD. 372 individuals were enrolled, and none had been vaccinated with AVA. Blood was collected by standard clinical laboratory methods and the serum was removed and frozen at − 80 °C.

For the third cohort, outdated fresh frozen plasma was purchased from Camp Memorial Blood Center, United States Army Medical Department Activities, Fort Knox, KY. No donor information was provided to the investigators of this study. However, the overall donor population of the Fort Knox Blood Center is comprised of 85% basic trainees, ranging in age from 17 to 21. Their immunization status with respect to AVA administration is unknown. Pooled normal human serum, which on analysis was found to be slightly positive for IgG and IgM antibodies to SQE, was purchased from United States Biological, Swampscott, MA. All of the above serum and plasma were thawed, aliquoted and refrozen at − 80 °C prior to assay.

2.3. ELISA assay for antibodies to SQE in human serum and plasma

SQE was diluted in ISP (0.2 μmol/ml; 9.6 μl SQE/100 ml) and 0.1 ml (20 nmol) was placed in each well of a Costar 96-well sterile round bottom tissue culture plate. Control wells contained ISP alone. The plates were placed in a biological safety cabinet and incubated overnight to allow the ISP to evaporate. PBS–0.5% boiled casein, pH 7.4 (0.3 ml) was added to each well. After incubation at room temperature for 2 h, the buffer was removed and the plates were tapped on paper towels to remove the residual blocker. Human serum was diluted serially in PBS–0.5% casein starting at a 1:25 dilution. Diluted serum (0.1 ml/well) was added to the plate in triplicate. The plates were covered with plastic wrap and incubated overnight at room temperature. The plates were then washed 4 times with 0.5 ml/well of PBS, pH 7.4. Peroxidase-linked sheep anti-human IgG (γ-chain specific) and IgM (γ-chain specific) was diluted 1:1000 in PBS–0.5% casein and 0.1 ml/well was added to the plate. Following a 1-h incubation at room temperature, the plates were washed as described above. ABTS substrate (0.1 ml/well) was added and the plates were incubated at room temperature for 1 h. Absorbance was read at 405 nm. Each plate contained culture supernatant from the C5 cell line, pooled normal human serum and blocking buffer as controls. It should be noted that polystyrene pipets, tubes, or other objects containing polystyrene, should not be used in this assay. The use of polystyrene caused high background (ISP) absorbances and greatly increased the variability of the results. Highly purified ISP also was required in order to ensure low background absorbances.

2.4. ELISA assay for antibodies to SQE in mouse serum

The assay was performed as described by Matyas et al. (2002) with 10 nmol SQE per well. Briefly, the mouse serum assay was similar to the human assay except for the following: (1) PBS–3% BSA was used as a blocker and diluent. (2) Culture supernatant from hybridoma SQE#16 containing a monoclonal antibody to SQE was included as a positive control on each plate. (3) Diluted mouse serum was incubated for 1 h on the plates. (4) Peroxidase-linked sheep anti-mouse IgG (γ-specific) and IgM (μ-chain specific) (The Binding Site) were used a secondary antibodies.

2.5. Interpretation of data and statistical analysis

Sera were judged to be positive for antibodies to SQE if two dilutions (i.e., 1:25 and 1:50 dilutions for human serum and 1:50 and 1:100 for mouse serum) had absorbances that were greater than 3 times baseline. Baseline was defined as the absorbance at which the dilution curve became horizontal. Statistical analyses were performed using Minitab Statistical Software, Release 13 and StatXact, Version 5, Cytel Software, Cambridge, MA. Continuous variables were compared using *t*-tests, and categorical data were compared using Pearson's chi-squared tests. To estimate the probability of antibodies to SQE, logistic regression was used to assess the cohort effect adjusted for the possible confounding effects of gender. The between cohort odds ratios for IgG and IgM antibodies to SQE were estimated.

3. Results

3.1. Effects of blocking agents on the ELISA

Several different blocking and diluent agents were investigated for use in developing the ELISA for human samples. In the absence of primary antibody, either 0.5% casein (CASEIN) or 0.6% gelatin (GEL/CASEIN) as blockers followed by 0.5% casein as the diluent, worked best for blocking

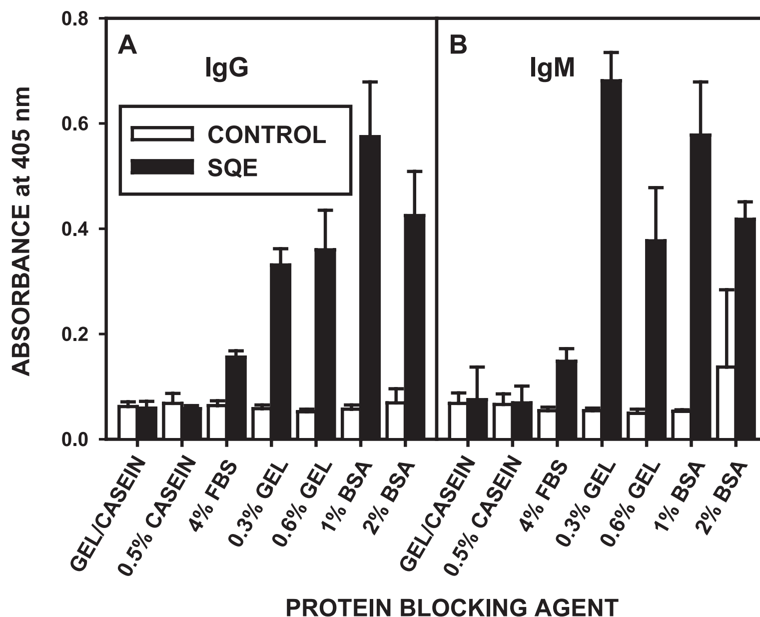

Fig. 2. Comparison of blocking and diluent agents on secondary antibody binding to control (ISP-treated) and SQE-coated wells (20 nmol/well). Assays were conducted in PBS, pH 7.4 containing the blocker and diluent protein indicated. GEL/CASEIN wells were blocked in 0.6% gelatin and the secondary antibody was diluted in 0.5% casein. All other samples were blocked and diluted in the protein buffer indicated. Values were the mean of triplicate determinations ± standard deviation.

of binding of the peroxidase-linked anti-human IgG (Fig. 2A) and anti-human IgM (Fig. 2B) to the plate. The binding of peroxidase-linked anti-human IgG and anti-human IgM to SQE-coated wells was only slightly elevated when 4% FBS was used as blocker and diluent. Because we were concerned that the SQE, which is known to be present in lipoprotein, might inhibit the binding of anti-SQE antibodies to SQE-coated wells, FBS was not considered for further use. Based on these data, 0.5% casein was chosen as the best diluent for human serum and the secondary antibodies.

In order to determine the best blocking agent, wells either were coated with SQE or not coated with SQE (ISP treatment alone) and then blocked with 0.6% gelatin or 0.5% casein. Human serum was diluted in 0.5% casein and plated on the wells A control human monoclonal IgG (IM-9) did not bind to control (ISP-treated) or SQE-coated wells when 0.6% gelatin or 0.5% casein were used as the blocking agent (Fig. 3A,B). 0.6% gelatin did not effectively block the binding of human serum IgG antibodies to control (ISP-treated) wells (Fig 3A). Absorbance values for serum numbers 39, 43, 99, and 109 were >1.0, when incubated on control wells. Furthermore, when 0.6% gelatin was used as the blocker, the absorbance of serum IgG antibodies binding to SQE-coated wells was detectable, but was only slightly above that of

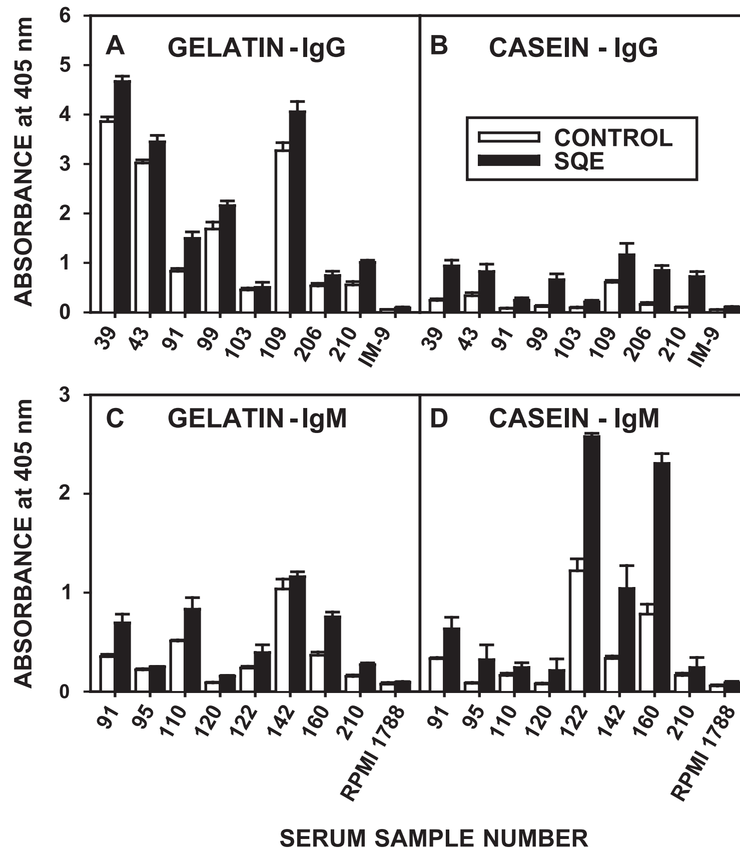

Fig. 3. Comparison of blocking agents on antibodies binding to control (ISP-treated) and SQE-coated wells (20 nmol/well). Wells were blocked with PBS–0.6% gelatin (A, C) or PBS–0.5% casein (B, D). The serum and secondary antibodies were diluted in PBS–0.5% casein. Values were absorbances from sera diluted 1:25 and assayed for IgG (A, B) and IgM (C, D) and are the mean of triplicate determinations ± standard deviation. Culture supernatants of control human monoclonal antibodies IM-9 (IgG) and RPMI 1988 (IgM) were used at a concentration of 4 μg/ml.

control wells (Fig. 3A). In contrast, when 0.5% casein was used as the blocker, the binding of serum IgG antibodies to control wells was greatly reduced for all sera studied (Fig. 3B). The absorbance for serum IgG antibodies incubated on SQE-coated wells was readily detectable when 0.5% casein was used as the blocker (Fig. 3B). Consequently, 0.5% casein was used in all further experiments for the measurement of IgG antibodies to squalene in human serum.

Gelatin and casein were also tested as blocking agents for the measurement of serum IgM antibodies to SQE (Fig. 3C,D). Control human IgM monoclonal antibody (RPMI 1788) did not bind to control (ISP-treated) or SQE-coated wells with either the casein or gelatin blocker (Fig. 3C,D). The absorbances for serum containing IgM antibodies incubated on control wells were essentially the same for gelatin and casein blocked wells (Fig. 3C,D; open bars). However, the absorbance of serum containing IgM antibodies incubated on SQE-coated wells was dramatically higher when the casein blocker was used than when gelatin was used as a blocker (Fig. 3C,D; solid bars). Conse-

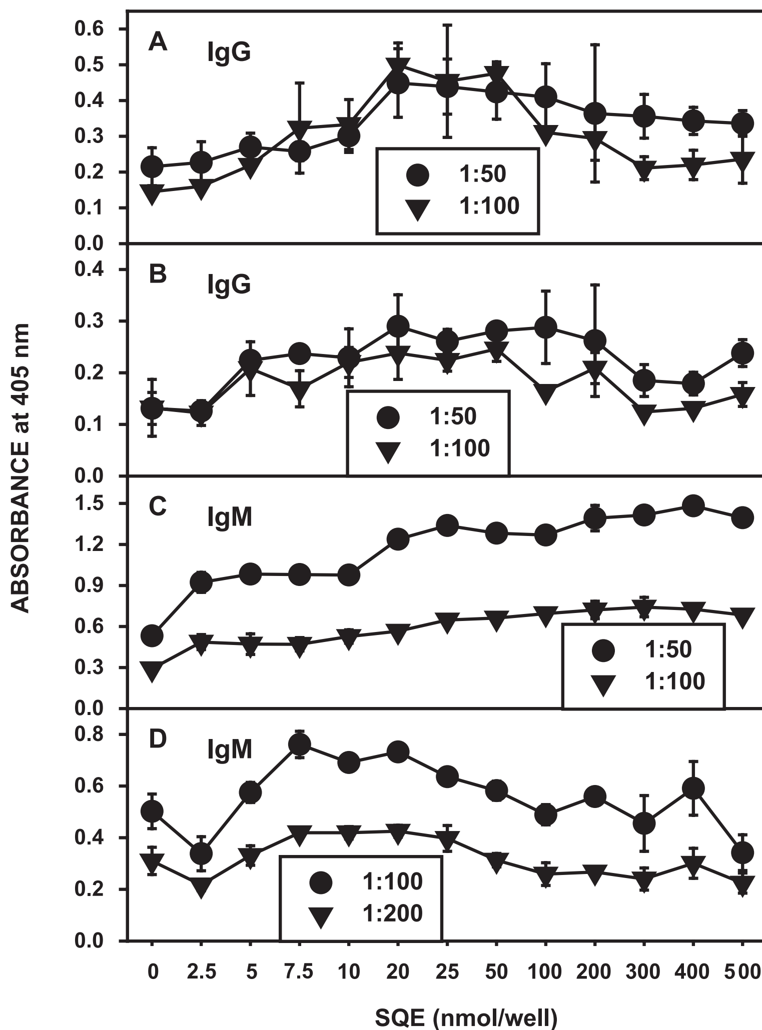

Fig. 4. Binding of serum anti-SQE antibodies as function of the amount of SQE added to the well. Wells were incubated with the amount of SQE indicated in 100 µl of ISP. The ELISA was performed using the protocol described in Section 2 with PBS–0.5% casein as the blocker and diluent. Values were the mean of triplicate determinations ± standard deviation.

quently, casein was chosen as the preferred blocker for the ELISA assay on human IgM antibodies to SQE.

3.2. Effect of the amount of SQE coated on the well

The binding of human serum antibodies to SQE-coated wells was dependent upon the amount of SQE (Fig. 4). Three types of binding curves were observed. The first type had an optimal absorbance range in which the absorbance increased with SQE, reached a plateau, and then decreased (Fig. 4A,D). The plateau region typically ranged from 5 to 50 nmol, but was dependent upon the serum assayed. The second type was relatively independent of SQE (Fig. 4B). The third type of curve continued to increase in absorbance over the entire range of SQE assayed (Fig. 4C). These 3 types of absorbance curves were observed for both IgG and IgM. Since

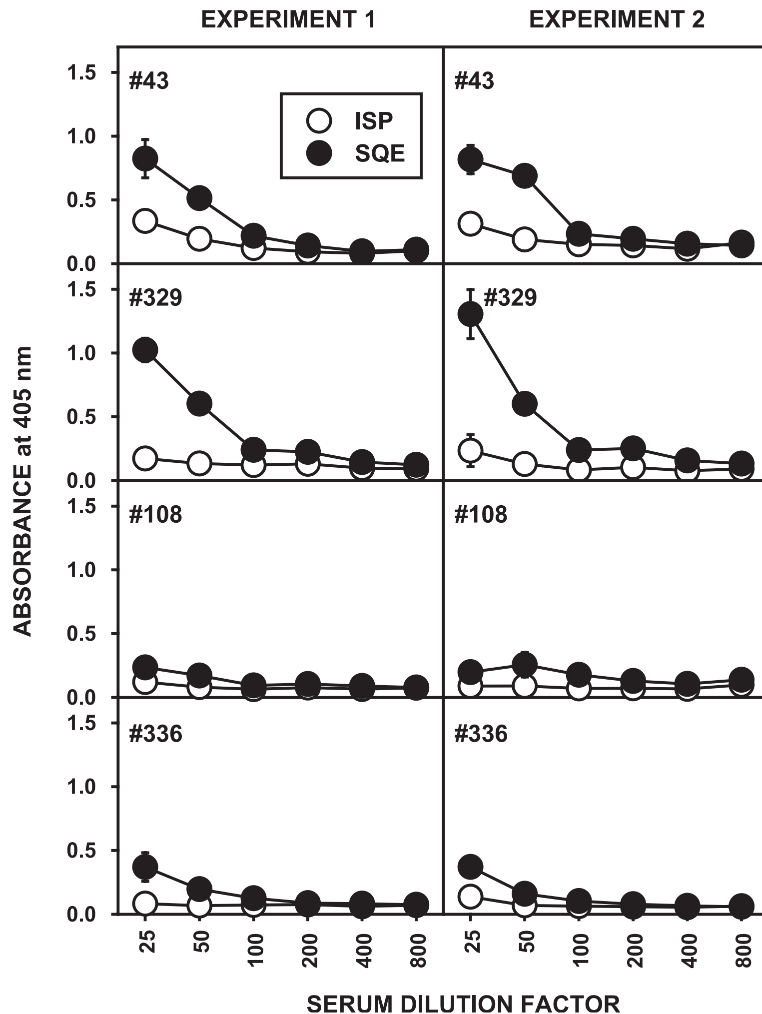

Fig. 5. ELISA dilution curves from representative human sera assayed in two different experiments for IgG antibodies to SQE. Samples positive of antibodies to SQE are serum #43 and 329. Samples negative for antibodies to SQE are serum #108 and 336. The ELISA assay was performed using the protocol described in Section 2 with PBS–0.5% casein as the blocker and diluent. Values were the mean of triplicate determinations ± standard deviation. Sera were judged to be positive for antibodies to SQE if two dilutions (i.e., 1:25 and 1:50) had absorbances that were greater than 3 times baseline. Baseline was defined as the absorbance at which the dilution curve became horizontal.

the absorbances at 20 nmol were high in all of the serum assayed, 20 nmol was chosen as the optimal amount of SQE to use for the detection of antibodies to SQE in human serum.

3.3. Antibody binding to SQE in human cohorts

When serum samples were assayed for antibodies to SQE, seven types of IgG (Fig. 5) or IgM (Fig. 6) binding patterns were observed. (1) Sera were positive for IgG antibodies to SQE (Fig. 5; sera 43, 329). Absorbances for serum IgG binding to SQE-coated wells were elevated above control wells at more than one dilution of serum. (2) Sera were negative for IgG antibodies to SQE (Fig. 5; sera 108). Absorbances for serum IgG binding to SQE-coated wells were not different from control wells, which had very low absorbance (Fig. 5, serum 108).

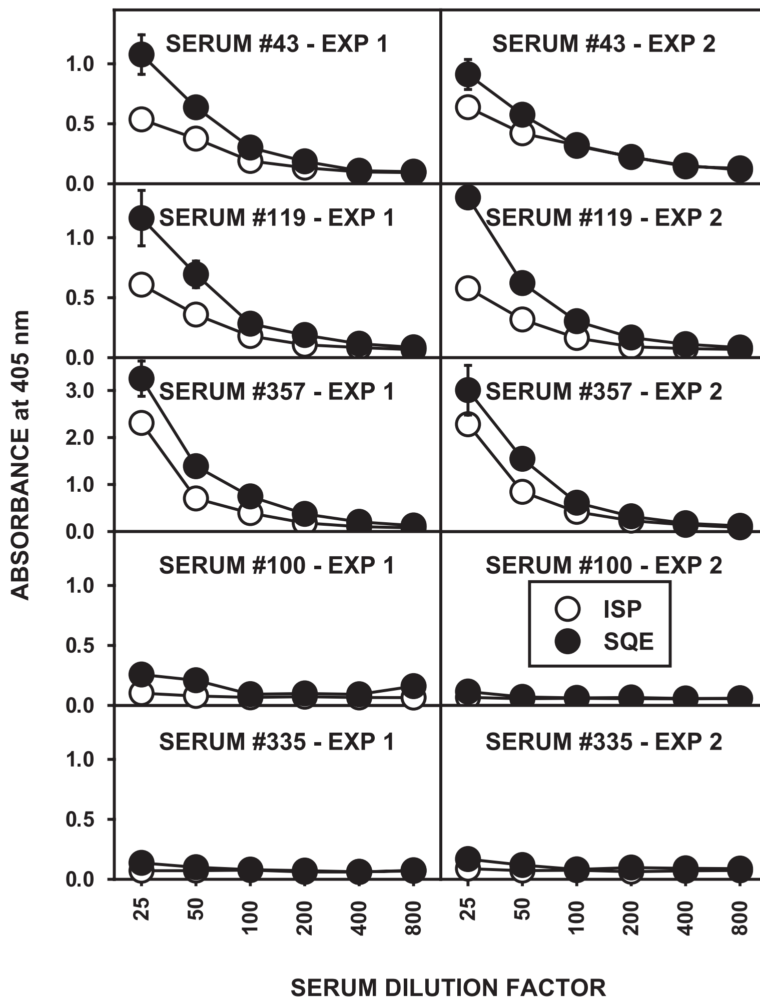

Fig. 6. ELISA dilution curves from representative human sera assayed in two different experiments for IgM antibodies to SQE. Samples positive for antibody to SQE are serum #43 and 119. Sample #357 has high background. Samples negative for antibodies to SQE are serum #100 and 335. The ELISA assay was performed using the protocol described in Section 2 with PBS–0.5% casein as the blocker and diluent. Values were the mean of triplicate determinations ± standard deviation. Sera were judged to be positive for antibodies to SQE if two dilutions (i.e., 1:25 and 1:50 dilutions) had absorbances that were greater than 3 times baseline. Baseline was defined as the absorbance at which the dilution curve became horizontal.

(3) Absorbances for serum IgG binding to SQE-coated wells were elevated at only the 1:25 dilution (Fig. 5; serum 336) and were considered negative. (4) Sera were positive for IgM antibodies to SQE (Fig. 6; sera 43, 119). Absorbances for serum IgM binding to SQE-coated wells were elevated above serum IgM binding to control wells at more than one dilution of serum. Absorbances on control wells were approximately 50% of the absorbance of that on the SQE-coated wells (Fig. 6; sera 43, 119). (5) Sera contained high absorbance values for IgM binding to both control and SQE-coated wells (Fig. 6; serum 357). (6) Sera were negative for IgM antibodies to SQE (Fig. 6; sera 100, 335). Absorbances for serum IgM binding to SQE-coated wells were not different from control wells, which had very low absorbances. (7) Absorbances for serum IgM binding to SQE-coated wells were elevated at only slightly at the 1:25 dilution (not shown) and therefore were considered negative.

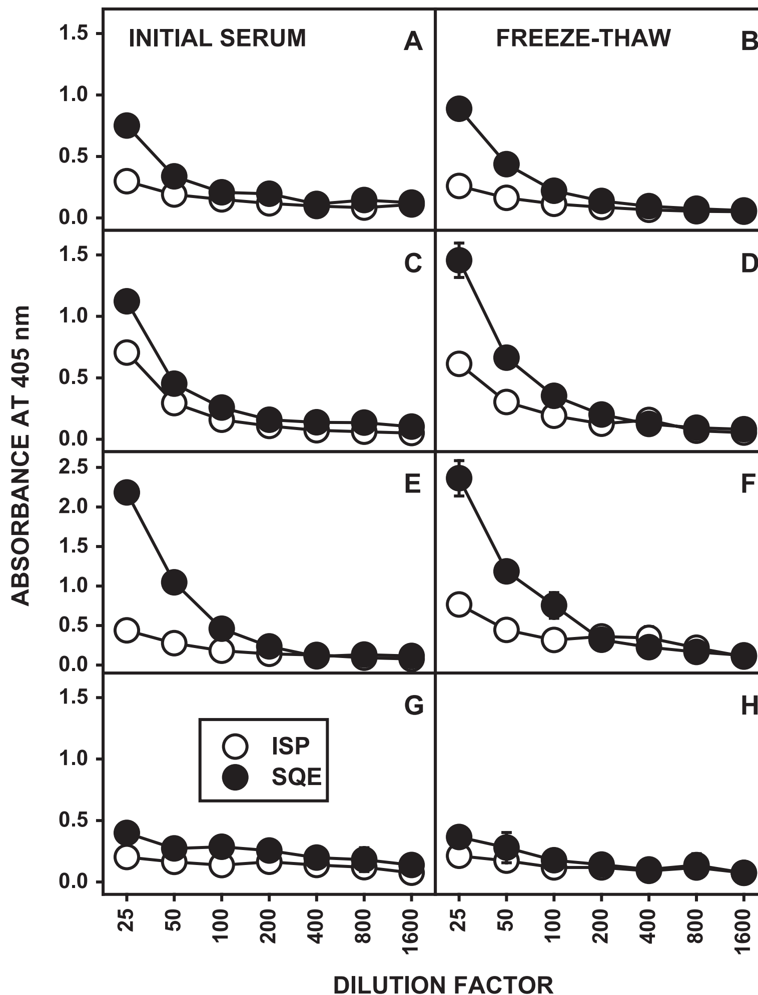

Fig. 7. Effect of freeze–thaw on the ELISA dilution curves for antibodies to SQE from representative human serum samples. Serum was obtained frozen, thawed and assayed. An aliquot was frozen at − 70 °C overnight and assayed the next day. The ELISA assay was performed using the protocol described in Section 2 with PBS–0.5% casein as the blocker and diluent and peroxidase-linked anti-mouse IgM as the second antibody. Values were the mean of triplicate determinations ± standard deviation. Sera were from the USAMRIID alumni cohort #4 (A, B), #5 (C, D), #21 (E, F) and the Frederick cohort #329 (G, H).

3.4. Reproducibility of the assay

The assay was highly reproducible for both positive and negative responses for IgG (Fig. 5) and IgM (Fig. 6) antibodies to squalene. Some sera were assayed 3 times with similar results on each assay. Slight differences were observed in the absorbances for positive responses from assay to assay, but positive response were always positive (Fig. 5; serum 43, 329). High absorbances on control wells also were highly reproducible (Fig. 6; serum 43, 357). Freeze–thaw had no effect on the measurement of antibodies to squalene (Fig. 7). Positive samples remained positive after freeze–thaw with approximately the same dilution curve (Fig. 7A–F) and negative samples remained negative (Fig. 7G–H). The absorbances on control wells also were not affected by freeze–thaw.

3.5. Prevalence of antibodies to SQE in human sera

To establish a basis for comparison of the prevalence of antibodies to SQE in different cohorts, we defined what constitutes a positive result: A serum sample was scored as positive if it had absorbances greater than 3 times baseline at two dilutions. Dilutions consisted of 1:25, 1:50, 1:100, 1:200, 1:400 and 1:800. As shown in Table 1, 42.6% of the sera assayed from the cohort from Frederick, MD were positive for IgG or IgM antibodies to SQE. The predominant antibody to SQE was IgM, which was two-fold greater than IgG (Table 1). Only 4.9% of the sera assayed from the Frederick cohort were positive for both IgG and IgM antibodies to SQE.

The Frederick cohort served as a control cohort of similar aged individuals for the USAMRIID alumni cohort. None of the individuals in the Frederick cohort had been employees at USAMRIID. There were no significant differences between these two cohorts in the prevalence of antibodies to SQE (Table 1, 95% confidence interval). There is a two-fold difference in the prevalence of IgG antibodies to SQE observed in the USAMRIID alumni (7.5%) and Frederick cohorts (15.1%), but this difference is not statistically significant ($\chi^2_{(1)} = 1.69$, $p = 0.19$). IgM antibodies to SQE were 37.5% and 32.3% for the USAMRIID and Frederick cohorts, respectively ($\chi^2_{(1)} = 0.43$, $p = 0.51$). Forty percent of the sera from the USAMRIID alumni were positive for IgG or IgM antibodies to SQE compared with 42.6% from the Frederick cohort (Table 1) ($\chi^2_{(1)} = 0.10$, $p = 0.75$). Only 5% of the sera from the USAMRIID alumni and 4.9% of the Frederick cohort had both IgG and IgM antibodies to SQE (Table 1) ($\chi^2_{(1)} = 0.00$, $p = 0.97$).

Of the 299 samples from the Fort Knox blood bank cohort, only 19.4% were scored as positive for antibodies to SQE (Table 1). Compared to the samples from the Frederick cohort, the samples from the blood bank at Fort Knox had a significantly reduced prevalence of antibodies to SQE (Table 1; 95% confidence interval). The most pronounced difference between the two cohorts was in IgG antibodies to SQE. There were no detectable IgG antibodies to SQE in the samples from the Fort Knox blood bank compared with 15.1% in the Frederick cohort (Table 1) ($\chi^2_{(1)} = 49.25$, $p < 0.0001$). The incidence of IgM antibodies to SQE was also significantly reduced in the Fort Knox Blood Bank cohort (19.4%) compared to

Table 1
Frequency of antibodies to SQE in human serum

Antibody type	SQE-coated wells (percent positive)			Wells lacking SQE (percent positive)		
	USAMRIID ($N = 40$)	Frederick ($N = 372$)	Fort Knox ($N = 299$)	USAMRIID ($N = 40$)	Frederick ($N = 372$)	Fort Knox ($N = 299$)
IgG	7.5 [1.6–20.4][a]	15.1 [11.6–19.1]	0.0 [0.0–1.0]	0.0	0.5	0.0
IgM	37.5 [22.7–54.2]	32.3 [27.4–37.4]	19.4 [15.1–24.3]	2.5	7.3	13.0
IgG or IgM	40.0	42.6	19.4	2.5	7.8	13.0
IgG and IgM	5.0	4.9	0.0	0.0	0.0	0.0

Sera having absorbances of >3 times baseline on SQE-coated wells or wells lacking SQE (ISP-treated) at the 1:25 and 1:50 dilutions were scored as positive.

[a] 95% confidence intervals for binomial proportions.

the Frederick cohort (32.3%) (Table 1) ($\chi^2_{(1)}=14.23$, $p=0.0002$). It should be noted that the estimated average age of the Fort Knox cohort (age range 17–21) was considerably lower than the Frederick control or USAMRIID cohorts (mean 68, age range 58–82).

The titers of the positive samples in each of the three cohorts were generally low. Only one serum sample was positive both for IgG and IgM at the 1:100 dilution in the USAMRIID alumni. In this one serum sample, the endpoint titers were 100 for the IgG and 400 for the IgM. There was only one positive IgG serum sample out of 372 at the 1:100 dilution in the Frederick cohort, and that serum had an endpoint titer of 400. There were 27 positive IgM samples at the 1:100 dilution in the Frederick cohort. Titers ranged from 100 to 1600, with 12 samples having endpoint titers of 100, 4 samples having endpoint titers of 200, 3 samples having endpoint titers of 400, and 1 each with endpoint titers of 800 and 1600. Only three of the samples from the Fort Knox cohort had IgM endpoint titers ≥ 100, with titers of 100, 200 and 400, respectively.

3.6. Antibodies to SQE in USAMRIID alumni who received anthrax vaccine

As a condition for employment most of the USAMRIID employees were vaccinated with anthrax vaccine AVA before access was granted to biocontainment laboratories where *Bacillus anthracis* was studied. Most of the USAMRIID alumni received several other vaccines as well as AVA through the USAMRIID special immunization program (Pittman, 2002). Six of the 40 individuals in the cohort were not vaccinated or the AVA vaccination status was unknown, and all 6 have been removed from analysis in this section. One of the latter six samples removed from analysis was positive for IgG to SQE and two out of six were positive for IgM (Table 2). The mean number of doses of AVA that had been administered to the remaining individuals was 26 (range 3–47 doses) (Table 2). After removal of the six samples, the prevalence of antibodies to SQE was basically unchanged from that shown in Table 1 for the USAMRIID alumni. 5.7% were positive for IgG or both IgG and IgM antibodies to SQE. 37.1% were positive for IgM alone or IgG or IgM antibodies to squalene. There were no statistical differences between the USAMRIID alumni who received AVA and the controls of similar ages from Frederick (IgG—$\chi^2_{(1)}=2.3$, $p=0.13$; IgM—$\chi^2_{(1)}=0.33$, $p=0.56$; IgG or IgM—$\chi^2_{(1)}=0.39$, $p=0.53$). Analyses of the health information from the USAMRIID alumni revealed no obvious relationship between the health of the individuals and antibodies to SQE. Many of the individuals in both the USAMRIID and the Frederick control cohorts did

Table 2
Age, sex, AVA doses, and antibodies to SQE among the USAMRIID alumni

ID no.	Age	Sex	AVA # doses	SQE IgG	SQE IgM
1	59	M	3	0	0
2	66	M	33	0	0
3	63	F	0	0	0
4	82	F	31	Pos	Pos
5	68	M	Unknown	0	Pos
6	71	M	4	0	0
7	69	F	0	0	Pos
8	59	M	37	0	Pos
9	72	M	25	0	0
10	70	M	Unknown	Pos	0
11	66	M	47	Pos	Pos
12	65	M	16	0	0
13	79	M	27	0	Pos
14	70	M	0	0	0
15	Unknown	M	41	0	0
16	60	M	24	0	0
17	74	M	39	0	Pos
18	59	M	14	0	0
19	65	M	40	0	Pos
20	75	M	28	0	0
21	68	M	20	0	Pos
22	69	M	15	0	0
23	64	F	8	0	Pos
24	72	M	32	0	Pos
25	70	M	31	0	Pos
26	61	F	8	0	0
27	71	M	30	0	0
28	58	M	26	0	0
29	Unknown	M	35	0	0
30	70	M	36	0	0
31	76	M	36	0	0
32	78	M	33	0	0
33	67	M	27	0	0
34	71	M	29	0	0
35	59	M	Unknown	0	0
36	64	M	14	0	0
37	71	M	45	0	Pos
38	Unknown	M	9	0	Pos
39	70	M	24	0	0
40	72	M	20	0	Pos

have various illnesses or chronic diseases, but this was expected to be the case for any cohort from the general population with an average age of approximately 68 and an age range of 58–82.

3.7. Increased prevalence of antibodies to SQE in women

The Frederick cohort contained 230 males and 142 females. There were 35 males and 5 females in the USAMRIID cohort. The data from these two cohorts were combined for analysis of the effect of sex on the prevalence of antibodies to SQE. Thus, there were 265 males and 147 females enrolled in the study. Thirty-three (12.5%) of the male samples and 26 (17.7%) of the female samples were positive for IgG antibodies to SQE. There was no statistical difference in the prevalence of IgG antibodies to SQE between males and females ($\chi^2_{(1)}=2.07$, $p=0.15$). However, there was a statistically significant increase in prevalence of IgM antibodies to SQE in females as compared to males. Seventy-five (28.4%) of the male samples and 60 (40.8%) of the female samples were positive ($\chi^2_{(1)}=6.72$, $p=0.01$). Female subjects were 1.75 times more likely to be positive for IgM antibodies to SQE (95% confidence interval on the odds ratio: lower limit = 1.14, upper limit = 2.66). Similarly, females were 1.67 times more likely to be positive for IgG or IgM antibodies to SQE (95% CI on the odds ratio: 1.11,2.51). There were 100 (37.7%) samples from males and 74 (50.3%) samples from females having either IgG or IgM antibodies to SQE ($\chi^2_{(1)}=6.16$, $p=0.013$). In order to ensure that this observed increase in prevalence of antibodies to SQE in females did not alter the conclusions drawn from the comparison of the USAMRIID and Frederick cohorts as homogeneity of Odds Ratio test was conducted. There was a common odds ratio across gender and it was concluded that no effect modification occurred due to the differences in gender distribution between the two cohorts.

3.8. Antibody binding to wells lacking SQE

In assaying sera for antibodies to SQE, absorbances to wells lacking SQE served as controls.

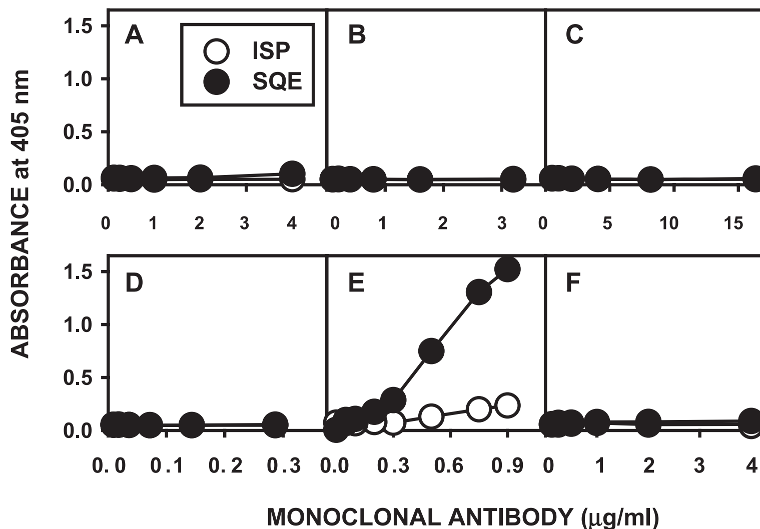

Fig. 8. Culture supernatants containing human monoclonal antibodies were assayed by ELISA for reactivity to SQE or polystyrene. The ELISA assay was performed using the protocol described in Section 2 with PBS–0.5% casein as the blocker and diluent. Values were the mean of triplicate determinations ± standard deviation. Panel A was IM-9, which secreted an IgG antibody of unknown specificity cloned from a human myeloma (Fahey, 1971). Panel B was 16M3F10, which secreted an IgG antibody to diphtheria toxoid) (Gigliotti, 1984). Panel C was SA13, which secreted an IgG antibody to tetanus toxoid) (Larrick, 1986). Panel D was L612, which secreted an IgM antibody to the gangliosides GM3 and GM4 (Irie, 1995). Panel E was C5, which secreted an IgM antibody to lipid A of Gram negative bacteria (Teng, 1985). Panel F was RPMI 1788, which secreted an IgM antibody to tumor necrosis factor beta (Aggarwal, 1984).

However, upon examination of all of the human samples assayed ($N = 711$), absorbances for IgM antibodies binding to control wells were elevated at the 1:25 and 1:50 dilutions in 32.5% and 9.4%, of the samples, respectively. In contrast, only 2.9% (1:25 dilution) and 0.3% (1:50 dilution) of the samples assayed were elevated for IgG antibodies binding to control wells. Overall, these elevations typically were approximately 50% of the absorbance levels observed for binding to SQE-coated wells (Fig. 6; sera 43 and 119). In a small number of samples, the absorbances for control wells were almost equal to the absorbance for SQE-coated wells (Fig. 6; serum 357). These elevations in absorbance in control wells lacking SQE were invariably observed only in samples which also were scored as positive for antibodies to SQE. When culture supernatants containing human monoclonal IgG or IgM antibodies to other antigens were assayed in the SQE ELISA, no binding was detected either to control or SQE-coated wells (Fig. 8A–D,F), except for the antibody from the clone that secretes an IgM antibody to lipid A (Fig. 8E). This latter monoclonal antibody bound to SQE-coated wells, but not to control wells.

The binding of the latter monoclonal anti-lipid A antibody to SQE was approximately 10% of the binding of the antibody to lipid A (data not shown). The above data suggest that antibody binding to control wells was not due to a nonspecific interaction between the polystyrene well and the antibody. Although the precise region of cross-reactivity of the anti-lipid A monoclonal antibody between lipid A and SQE is unknown, several possible regions of similarity could be identified. In any case, the existence of the binding specificity to SQE by this human monoclonal antibody permitted its use as a positive control for standardizing and validating the assays with unknown human serum samples.

In order to investigate the nature of the elevated absorbance on control wells, diluted serum was plated on ISP-treated wells lacking SQE, or on SQE-coated wells, to adsorb the antibodies. After two rounds of adsorption, the supernatant was placed on ISP-treated wells lacking SQE or on SQE-coated wells, followed by ELISA. Two rounds of absorption

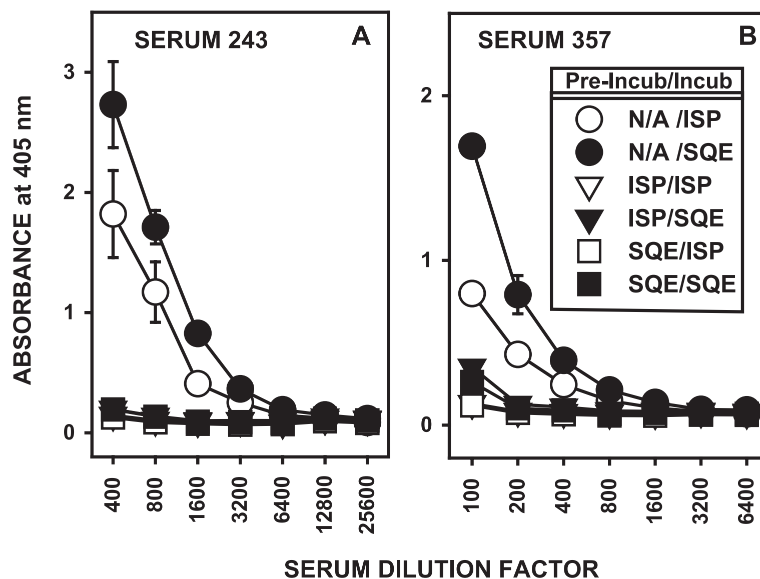

Fig. 9. Effect of pre-incubation of serum on control (ISP-treated) or SQE-coated wells on the binding to control or SQE-coated wells. Sera was diluted in PBS–0.5% casein and plated on 96-well plates treated with ISP or coated with SQE. Following incubation for 2 h at room temperature, the contents of the wells were transferred to a new plate containing wells treated with ISP or SQE. Following incubation overnight at room temperature, the supernatant were removed and assayed by the standard ELISA for IgM antibodies to SQE. Unabsorbed sera (circles) were serum samples assayed by ELISA without prior adsorption. Values were the mean of triplicate determinations ± standard deviation.

removed both the polystyrene and SQE binding activities from serum 243 (Fig. 9A). Absorption on ISP-treated control wells lacking SQE greatly diminished the binding of serum 357 to SQE-coated wells (Fig. 9B).

3.9. Antibodies to SQE in mouse serum

IgG and IgM antibodies to SQE were not observed in the individual sera of 2-month-old mice in any of the strains studied (Table 3). However, many individual mice developed either IgG or IgM antibodies to SQE as a function of time (Table 3). The degree of tendency to develop antibodies was strain specific: C57BL/6>B10.BR>/BALB/c.

The incidence of IgG antibodies to SQE was 100% at 18–19 months of age for the C57BL/6 mice. B10.Br had a maximal incidence of 85% at 18 months of age. This incidence decreased for both strains, with none of the B10.Br mice testing positive, and 46% for the C57BL/6 mice testing positive, at 24 months of age. Ninety-five and 100% of B10.Br and C57BL/6 mice, respectively, tested positive for IgG antibodies to SQE some time during the course of the experiment. Only a low incidence of IgG antibodies to SQE was observed in the serum of BALB/c mice, and low absorbances with positive sera were only slightly above 3 times baseline. Although 35% of BALB/c mice tested positive for IgG antibodies to SQE at some time in the course of the experiment, this only occurred at one time point for each positive mouse.

As with IgG antibodies, IgM antibodies to SQE were not observed in the serum from mice at 2 months of age (Table 3). The prevalence of mouse serum testing positive for IgM antibodies to SQE increased with the age of the mice in all three strains assayed. There was a higher incidence of IgM antibodies to SQE in the BALB/c and C57BL/6 stains as compared to the B10.Br mice, and the IgM antibodies to SQE were observed earlier in the BALB/c and C57BL/6 mouse sera than the B10.Br mouse sera. Only 65% of the B10.Br mice were positive for IgM antibodies to SQE sometime during the experiment. In contrast,

Table 3
Appearance of antibodies to SQE in mouse serum as function of age

Age (months)	BALB/c % positive (# positive/total) [95% confidence interval]		B10.Br % positive (# positive/total) [95% confidence interval]		C57BL/6 % positive (# positive/total) [95% confidence interval]	
	IgG	IgM	IgG	IgM	IgG	IgM
2[a]	0 (0/49) [0.0–5.9]	0 (0/60) [0.0–4.9]	0 (0/25) [0.0–11.3]	0 (0/25) [0.0–11.3]	0 (0/25) [0.0–11.3]	0 (0/25) [0.0–11.3]
10	11 (2/18) [1.4–34.7]	32 (6/19) [12.6–56.6]	0 (0/18) [0–15.3]	10.5 (2/19) [1.3–33.1]	12 (2/17) [1.5–36.4]	30 (6/20) [11.9–54.3]
16	10 (2/19) [1.3–33.1]	37 (7/19) [16.3–61.6]	10 (2/20) [1.2–31.7]	0 (0/20) [0.0–13.9]	74 (14/19) [48.8–90.9]	90 (18/20) [68.3–98.8]
17	0 (0/19) [0.0–14.6]	63 (12/19) [38.4–83.7]	5 (1/20) [0.1–24.9]	5 (1/20) [0.1–24.9]	65 (13/20) [40.8–84.6]	80 (16/20) [56.3–94.3]
18	N.D.	39 (7/18) [17.3–64.3]	85 (17/20) [62.1–96.8]	5 (1/20) [0.1–24.9]	100 (17/17) [83.8–100]	89 (16/18) [65.3–98.6]
19	6 (1/17) [0.1–28.7]	65 (11/17) [38.3–85.8]	60 (12/20) [36.1–80.9]	25 (5/20) [8.7–49.1]	100 (18/18) [84.7–100]	72 (13/18) [46.5–90.3]
21	10 (1/10) [0.3–44.5]	62 (8/13) [31.6–86.1]	45 (9/20) [23.1–68.5]	55 (11/20) [31.5–76.9]	94 (16/17) [71.3–99.9]	62 (10/16) [35.4–84.8]
24	17 (1/6) [0.4–64.1]	86 (6/7) [42.1–99.6]	0 (0/17) [0.0–16.2]	50 (9/18) [26.0–74.0]	46 (6/13) [19.2–74.9]	58 (7/12) [27.7–84.8]
At any time point	35 (7/20)	85 (17/20)	95 (19/25)	65 (13/20)	100 (20/20)	100 (20/20)

Mice were bled at the time intervals indicated and the sera were assayed for antibodies to SQE. Serum was scored as positive for IgG or IgM antibodies to SQE if the absorbance was >3 times the baseline at both the 1:50 and 1:100 dilutions. Baseline absorbances ranged from 0.1 to 0.18 in different assays.

[a] Sera from 2 month old mice were from different animals than the retired breeders used for the remaining time points.

85% and 100% of BALB/c and C57BL/6 mice, respectively, developed IgM antibodies to SQE.

Although the data in Table 3 is not designed to illustrate the titers of the IgG or IgM antibodies to SQE, endpoint titers (absorbance >3 times assay baseline) of positive samples, when examined in detail, were typically 100–400. However, a small number of samples were observed to have positive absorbances at a 1:800 dilution (highest dilution assayed). There were three types of individual animal responses for antibodies to SQE. (1) Animals were negative for IgG or IgM antibodies to SQE (Fig. 10A,C,D). (2) Animals were initially negative for antibodies to SQE and later developed antibodies to SQE which remained elevated (Fig. 10B,E–H). (3) Animals were initially negative or slightly positive for antibodies to SQE developed higher titers to antibodies to SQE and then became negative for antibodies to SQE at later time points (Fig. 10I–J). There was no observed relationship between IgG and IgM

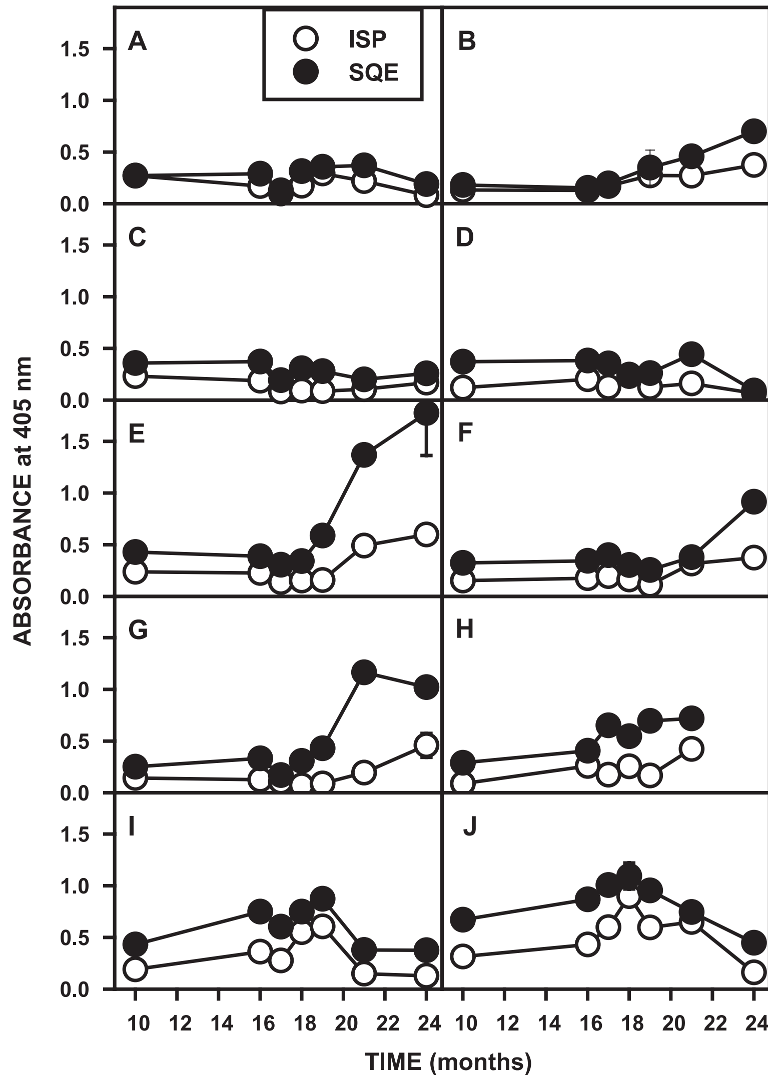

Fig. 10. Occurrence of naturally occurring antibodies to squalene in mice as function of age. Each panel represents a different mouse. Panels A and B are IgG and panels C–J are IgM. Values were the mean of triplicate determinations ± standard deviation of the serum diluted 1:100.

antibodies to SQE. Twenty-two animals died during the course of the experiment with the deaths occurring after 17 months of age. Most of the deaths occurred in the BALB/c mice. There appeared to be no relationship between antibodies to squalene and death. As with the human serum, some of the mouse sera had elevated absorbances on control wells. This was observed for both IgG and IgM antibodies (Fig. 10).

4. Discussion

A highly reproducible, high throughput, and quantitative assay for measuring antibodies to SQE in human serum is described. The assay was modified from our previously described assay for murine antibodies to SQE (Matyas et al., 2002). Like the mouse assay, the human assay used Costar tissue culture 96 well plates, which were plates that are not routinely used for ELISA assay. As with murine antibodies to SQE, none of the standard ELISA plates were useful for this human SQE antibody assay (data not shown). The use of the standard ELISA plates resulted in high background absorbances in control (ISP-treated) wells.

The murine assay used BSA as a blocker/diluent. However, BSA did not effectively block the binding of peroxidase-linked anti-human IgG and IgM to SQE-coated plates. Casein effectively blocked this non-specific binding in the human assay. The optimal amount of SQE added to the plates was similar for both the human and mouse assays. The human assay used 20 nmol of SQE and the mouse assay used 15 nmol. The quantification of the amount of SQE bound to the wells under the conditions used in the human antibody assay was virtually identical to that described for the mouse (Matyas et al., 2002). In the mouse assay, the primary serum was incubated on the assay plate for 1 h. Early experiments during assay development suggested that an increased incubation time (overnight) increased the sensitivity of the assay for human antibodies. The optimal dilution of the secondary antibody was determined to be 1:1000 (data not shown). There were no differences observed with different lots of plates (data not shown).

Elevated absorbances were observed on control wells of some of the samples testing positive for antibodies to SQE in both the human and mouse serum (Figs. 3D; Fig. 5-sample 43; 6-samples 43, 119 and 357; 7C,D; and 10B,E,G,H,I,J). This was most prevalent for IgM antibodies at 1:25 and 1:50 dilutions for the human and mouse samples, respectively. This was not a result of the ISP treatment itself, since similar results were obtained when control wells were untreated. Furthermore, only 32.5% of the human samples that were positive for antibodies to SQE had elevated absorbances on control wells. With any given serum, absorbance in the control wells never exceed those found in the SQE-coated wells.

We hypothesize that the above binding of SQE-positive serum samples to the control wells was due to cross-reactivity of SQE antibodies to polystyrene. This is supported by the following pieces of evidence. (1) None of the samples that tested negative for antibodies to SQE from humans or mice had elevated absorbances on control wells. (2) As shown in Fig. 8, human monoclonal IgG or IgM antibodies did not bind nonspecifically to control wells (ISP-treated). Two additional antibodies, one IgG and one IgM (not shown), were also assayed and no reactivity was observed to control wells or SQE-coated wells. Similarly, control murine monoclonal antibodies (six IgM antibodies) did not have elevated absorbances on control wells (Matyas et al., 2000, 2002; data not shown). (3) Pre-incubation of serum for two rounds on ISP-treated wells removed not only the absorbance seen on control wells, but also effectively all of the SQE binding activity (Fig. 9). Thus, it appears that the binding to control wells was a specific binding of IgG and IgM antibodies to the wells. (4) Polystyrene is a polymer of styrene ($CH_2=CH-C_6H_6$) (Fig. 1), and sterilization of the cell culture plates by gamma irradiation can lead to cleavage of one or more of the double bonds in the benzyl rings and the hydrocarbon backbone. Gamma irradiation may also induce fusion of the benzyl rings. Consequently, irradiated polystyrene may have elements that are structurally similar to SQE in that irradiated polystyrene has open ring structures that still contain double bonds. One interesting possibility may be that the observed antibodies to SQE may be antibodies to polystyrene that cross-react with SQE. Styrene is a widespread environmental contaminant that is particularly derived from cigarette smoke or is present in food after having been leached from polystyrene food containers, especially containers containing yoghurt or fat products

(Newbook and Caldwell, 1993). Although we have no direct evidence for the induction of antibodies to styrene or polystyrene, the structural similarities of styrene and polystyrene with SQE (Fig. 1) raise this as a hypothetical possibility.

Sera from three human cohorts were assayed for antibodies to SQE. Antibodies to SQE were detected in some of the samples in all three cohorts. The volunteers from Frederick were an age-restricted cohort from Frederick, MD. The presence of antibodies to SQE in this cohort implies that antibodies to SQE are a naturally occurring phenomenon in humans. This is not surprising, since previous studies have demonstrated naturally occurring human antibodies to cholesterol (Alving et al., 1989) and to phospholipids (Alving, 1984). Naturally occurring antibodies to cholesterol have been found in essentially 100% of volunteers studied (Alving and Wassef, 1999). Antibodies to cholesterol do not appear to have an adverse impact on health and may play a role in the regulation of LDL (Alving and Wassef, 1999). Since SQE is present on LDL particles in plasma, it is possible that naturally occurring antibodies to SQE may also contribute to the regulation of LDL.

Although no IgG antibodies to SQE were detected in the sera from the Fort Knox cohort, 7.5 and 15.1% of the samples from the USAMRIID alumni and Frederick cohorts, respectively, were positive for IgG antibodies. With respect to IgM, the samples from the USAMRIID alumni and the Frederick cohorts were 37.5% and 32.3% positive, respectively, while the Fort Knox samples were 19.4%. It should be noted that there is a 9.3-fold difference between the number of volunteers in the USAMRIID alumni ($N=40$) and the Frederick cohort ($N=372$). The low number of volunteers in the USAMRIID alumni greatly reduced the power to determine significant differences between the two cohorts. Unfortunately, we were unable to obtain additional volunteers in the USAMRIID alumni cohort. The samples from the USAMRIID alumni and the Frederick cohorts were from volunteers >58 years old. Compared to the Frederick cohort, the prevalence of both IgG and IgM antibodies to SQE was significantly reduced in the Fort Knox cohort. Although no individual age information was available from the volunteers of the Fort Knox cohort, the demographics of the volunteer population at the Camp Memorial Blood Center, Fort Knox (85% of the individuals are 17–20 years of age) suggested to us that antibodies to SQE may increase as a function of age.

There are numerous circumstances in which natural antibodies are related to age. One example of this is that reported by Pinto and Rimon (1970) that demonstrated the presence of antibodies to hydrocortisone hemisuccinate in 26% of the serum samples from normal human adults aged 25–65; but in only 3% of infants 9 months–3 years of age. In support of this hypothesis, we found that young mice (2 months old) had no antibodies to SQE. Ten-month-old mice had a 10–30% incidence of antibodies to SQE. The incidence increased as a function of age, with 100% of the C57BL/6 mice testing positive for antibodies to SQE at 18 and 19 months of age. This mouse study also demonstrated that antibodies to SQE developed naturally without vaccination with AVA or other vaccines. The development of antibodies to SQE varied with the strain of the mouse, thus suggesting that genetic factors may also be involved.

Thirty-four volunteers from the USAMRIID alumni cohort had been immunized with the anthrax vaccine AVA. Most had received numerous vaccinations (mean = 26 doses, range 3–47 doses). Comparison of the AVA-immunized USAMRIID alumni with the similar aged cohort from the normal population of Frederick, none of whom received AVA, revealed that immunization with AVA did not significantly increase the prevalence of antibodies to SQE (IgG—$\chi^2_{(1)}=2.3$, $p=0.13$; IgM—$\chi^2_{(1)}=0.33$, $p=0.56$; IgG or IgM—$\chi^2_{(1)}=0.39$, $p=0.53$). Although not significant, the prevalence of IgG antibodies to SQE in the AVA immunized group (5.1%) was approximately threefold less than the unimmunized Frederick control group. Asa et al. (2002) also found that there was no statistical difference in the incidence of antibodies to SQE between AVA immunized and unimmunized individuals. They also reported a 15.8% prevalence of IgG antibodies to SQE in their controls, which was similar to the 15.1% prevalence of IgG antibodies to SQE in the Frederick cohort. However, no IgG antibodies to SQE were observed in the Fort Knox cohort. Asa et al. (2002) did not report the age of their control population, but in our study the control group had a similar age distribution to that of the AVA immunized group.

The existence of naturally occurring antibodies to SQE raises the issue of whether such antibodies are

produced as a result of immunization with SQE (for example, SQE present as a contaminant of AVA), or by immunization with a substance similar to SQE in the environment that might induce cross-reacting antibodies. Induction of antibodies to SQE by SQE contaminants of AVA or other vaccines seems highly unlikely. Using an HPLC assay with a detection limit of 70 ng/ml, Spanggord et al. (2002) reported that squalene was not present in 17 different lots of AVA. However, the FDA provided written testimony to Congress that several lots of AVA and several other vaccines had squalene contamination in the "low parts per billion" (ng/ml) as detected by gas chromatography (Metcalf, 2000). The data from which these latter determinations were not provided to Congress, nor were the experiments published in the scientific literature, making the scientific validity of the testimony difficult to evaluate. However, even if the FDA data were correct and an individual was given 10 ng of SQE in an AVA dose, it is difficult to believe that this minute amount of SQE contaminant could induce antibodies to SQE. We previously reported an inability to induce antibodies to SQE in mice with multiple immunizations with large amounts of SQE alone or mixed with alum and other potent adjuvants and immuno-stimulatory formulations (Matyas et al., 2000). Only with two of eight formulations were low to moderate titers of IgM antibodies to SQE observed. No IgG antibodies to SQE were observed. Furthermore, the amount of SQE present as an infinitesimal contaminant in vaccines or other injectables pales by comparison with the huge amounts of SQE that are normally continually synthesized and that are present in skin and liver, and normally circulating in blood. It has been hypothesized that naturally occurring antibodies to cholesterol might be derived from inflammatory processes involving the skin (Alving and Wassef, 1999), and antibodies to SQE might be derived in a similar manner.

Although increased prevalence of natural antibodies is often seen in the serum of women, increased prevalence of antibodies to SQE was an unanticipated finding in the present study. However, this increased prevalence of antibodies to SQE in females did not alter the conclusions drawn between the Frederick and USAMRIID (and AVA subcohort), since the crude and gender adjusted odds ratios estimated using logistic regression for IgG, IgM and IgG or IgM antibodies to SQE were not significant. It is unclear as to the reasons for this increased prevalence in women. One possibility may be that some women may have developed antibodies to steroid sex hormones such as progesterone, testosterone or estrogens and that these antibodies may cross-react with SQE. Antibodies to estradiol (Counihan et al., 1991), 16-α-hydroxyestrone (Bucala et al., 1987) and ethinylestradiol (Beaumont et al., 1989) were reported in sera from women who used oral contraceptives or had systemic lupus erythematosus. Patients with systemic lupus erythematosus also were reported to have antibodies to β-estradiol (Mionuddin, 1998). Antibodies to testosterone have been reported in a woman with hypergonadotropic hypogonadism (Kuwahara et al., 1998). Furthermore, using human lymphocyte-derived DNA libraries, antibodies to digoxigenin, estradiol, testosterone and progesterone were found (Dorsam et al., 1997). Since the steroid sex hormones are synthesized from cholesterol (which, in turn, is synthesized from SQE) and since they have the fused cholesterol ring structure, antibodies to these hormones may cross-react with SQE. Murine monoclonal antibodies to steroid hormones have been extensively studied (Franek, 1987; Grover and Odell, 1977). They generally cross-reacted to varying degrees with a small number of other steroid hormones (Fantl and Wang, 1984; Mais et al., 1995; Brochu et al., 1984). Limited analysis of the specificity of our murine monoclonal antibodies to SQE (Matyas et al., 2000, 2002) indicated that some of the clones cross-reacted with cholesterol and/or testosterone (unpublished data).

In summary, we have described a highly reproducible, high throughput, and quantitative assay for measuring antibodies to SQE in human serum. Using this newly developed assay, both naturally occurring IgG and IgM antibodies to SQE were observed in human serum. The antibody titers were generally low. There was an increased prevalence of antibodies to SQE in serum from females as compared to males. There was no correlation between the presence of antibodies to SQE and previous immunization with AVA. The data further suggested that the prevalence of antibodies to SQE in human serum increased with the age of the volunteer. Longitudinal studies in three different strains of mice confirmed the presence of

naturally occurring IgG and IgM antibodies to SQE and the prevalence of antibodies to SQE increased with the age of the mice.

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