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Induction and detection of antibodies to squalene II. Optimization of the assay for murine antibodies

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Abstract

An improved high throughput assay for measuring murine antibodies to squalene (SQE) is described. The assay is highly reproducible and sensitive and can detect 80 ng/ml of antibody to SQE. The assay, an ELISA, is similar to our previously described assay in which plates containing PVDF membranes were used [J. Immunol. Methods 245 (2000) 1]. The PVDF plates worked well for detection of murine monoclonal antibodies (mAbs) to SQE, but substantial PVDF plate variation was observed, resulting in significant loss of signal and reproducibility between different lots of plates. In the new assay, the PVDF plates were replaced with Costar round bottom 96-well sterile tissue culture plates. These latter plates, which are not normally used for ELISA assay, gave high absorbances for monoclonal antibodies and anti-SQE serum binding to SQE and low absorbances for solvent-treated wells. Other commercially available polystyrene ELISA plates were unsuitable, in that either the background was high or the absorbance for antibodies binding to SQE was low, or both. This change in plate from PVDF to polystyrene allowed the use of an ELISA plate washer, which dramatically increased the throughput rate over the hand-washed PVDF plates. The improved assay also replaced fetal bovine serum (FBS), which contained SQE in lipoproteins, with fatty acid-free bovine serum albumin (BSA) as the blocker/diluent. Fifteen nanomoles of SQE were selected as the optimal amount of SQE to add to the wells. The binding of monoclonal antibodies and anti-SQE serum was dependent upon both the amount of antibody added to the wells and the amount of SQE added to the wells. Antibody concentration curves were hyperbolic in shape, as seen with most other antibodies. Antibody binding first increased with SQE amount and then reached a plateau around 10 nmol of SQE/well. At high SQE amounts (>75 nmol/well), antibody binding decreased with the amount of SQE added. Using ^{3}H-SQE, the amount of SQE bound to the wells increased linearly, up to 50 nmol of SQE added. Approximately 90% of the added SQE bound to the well. When amounts greater than 100 nmol of SQE were added, the amount of SQE bound to the wells was greatly reduced to approximately 5–10% of the added SQE. The assay was highly reproducible both from lot to lot of plates and from experiment to experiment.

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

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1. Introduction

Squalene (SQE), a triterpenoid hydrocarbon oil, is a precursor of cholesterol and the steroid hormones (Granner, 1996; Mayes, 1996; for review see Fulco et al., 2000). It is widely produced in animals and plants and, consequently, is present in many foods. SQE is also widely used in skin cosmetics. It is synthesized in the liver and is a major component of oil secreted from skin sebaceous glands (Stewart, 1992). SQE is a constituent of human blood as part of low density lipoproteins (LDL) and very low density lipoproteins (VLDL) (Koivisto and Miettinen, 1988; Miettinen, 1982). Due to its natural occurrence, SQE has been used as an oil in vaccine emulsion formulations (Ott et al., 2000). Although not included in any vaccines licensed in the United States, a SQE emulsion is present as part of an influenza vaccine licensed in Italy and has been given to thousands of people without adverse effects (Podda, 2001).

Antibodies to SQE have stimulated great interest in the popular press. This has been initiated by a claim that antibodies to SQE were present in the serum of sick Gulf War Veterans (Asa et al., 2000). The assay utilized in the later study has been criticized for technical reasons, including the lack of positive and negative controls (Alving and Grabenstein, 2000) and it was concluded that the reported results did not provide evidence that antibodies to SQE were successfully measured (Fulco et al., 2000). In order to create a reliable assay for antibodies to SQE, we have developed murine monoclonal antibodies (mAbs) to SQE that serve as positive controls (Matyas et al., 2000). For the assay of monoclonal antibodies to SQE, we have used 96-well plates with PVDF membranes. This assay was highly reproducible, but very labor-intensive. Because of this, the number of samples that can be assayed at one time is limited. In addition, we have found that different lots of PVDF plates gave variations in assay results. A theoretical problem has also arisen in that the assay uses PBS–4% fetal bovine serum (FBS) as a blocker/diluent buffer. Since all types of serum are known to contain SQE, this SQE might theoretically compete for antibody binding in the assay. In order to overcome these shortcomings, we now describe a modified, highly reproducible, improved assay for measuring murine antibodies to SQE.

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 Chemical, St. Louis, MO. Isopropanol (ISP) (BAKER ANALYZED® A.C.S. Reagent; cat. #9084-03) was purchased from J.T. Baker, Phillipsburg, NJ. PVDF plates (Multiscreen-IP, 0.45 μm, hydrophobic, high protein binding Immobilon-P membrane) were from Millipore, Bedford, MA. Immulon 2 round and flat bottom and Immulon 4HBX 96-well ELISA plates were from Dynex, Chantilly, VA. F96 Maxisorp 96-well ELISA plates were from Nalge Nunc International, Naperville, IL. Costar flat and round (cat. #3799) bottom 96-well sterile tissue culture plates were from Corning, Corning, NY. Mouse IgM secreting myelomas 1B2–1B7, which reacts with *N*-acetyllactosamine, and 2D4, which has a specificity for the carbohydrate on asialo-GM2, were purchased from American Type Culture Collection, Chantilly, VA. FBS was from GIBCO BRL, Grand Island, NY and was heated at 56 °C for 1 h prior to use. Gelatin was from BioRad Laboratories, Richmond, CA. Seal plate adhesive film was from PGC Scientific, Gaithersburg, MD. Affinity-purified and adsorbed peroxidase-linked sheep anti-mouse IgM was from The Binding Site, San Diego, CA. ABTS substrate was purchased from Kirkegaard and Perry Laboratories, Gaithersburg, MD. Squalene [4, 8, 12, 13, 17, 21-^{3}H] (20 Ci/mmol) was from American Radiolabeled Chemicals, St. Louis, MO. OptPhase SuperMix liquid scintillation fluid was from Wallac Oy, Turka, Finland. Female Balb/c mice were purchased from Jackson Laboratories, Bar Harbor, ME and were used under an institution approved protocol. Mouse IgM quantitation kits were from Bethyl Laboratories, Montgomery, TX and were used on the culture supernatants containing mAbs to SQE as per the enclosed instructions.

2.2. Positive control antibodies

Culture supernatants containing mAbs to SQE (clones SQE #14, 16 and 18) and negative control IgM secreting myelomas were grown in Dulbecco's modified Eagle's medium as described (Matyas et al.,

2000). Mice were injected i.p. with liposomes containing 71 mol% SQE and lipid A, as described (Matyas et al., 2000). Anti-SQE positive serum was obtained by terminal bleeding of the mice 3 days after immunization. Normal mouse serum (NMS) was obtained by terminal bleeding of unimmunized mice. The serum was aliquoted and frozen at −20 °C. As we have shown previously (Matyas et al., 2000), only IgM, not IgG, antibodies to SQE were detected in mice. The experiments described in this paper used several different lots of mAb supernatants and anti-SQE serum which accounts for the variations in absorbance among figures.

2.3. ELISA assay on PVDF plates

The ELISA assay was conducted in plates containing PVDF membranes as described (Matyas et al., 2000). Briefly, SQE was diluted in ISP and placed in the wells of the plate. After drying overnight, the wells were blocked with PBS–4% FBS, pH 7.4, for 2 h. Serum and supernatants containing mAbs to SQE were diluted in PBS–4% FBS and 0.1 ml was placed in a well. Following incubation for 1 h, the plate was washed four times with PBS–4% FBS by hand using a 25-ml pipet. A total of 0.1 ml of peroxidase-linked sheep anti-mouse IgM diluted 1:1000 in PBS–4% FBS was added to each well. The plate was incubated for 1 h and then washed four times with PBS by hand. ABTS substrate (0.15 ml) was added to each well and the plates were incubated for 1 h. A total of 0.1 ml/well was transferred to an Immulon 2 round bottom plate and the absorbance was read at 405 nm with a Uvmax Kinetic Microplate Reader (Molecular Devices, Palo Alto, CA). In some experiments, PVDF plates were washed four times with 0.5 ml of PBS/well with an ELISA plate washer (Skatron, Sterling, VA). The vacuum probes of the washer were positioned just above the PVDF membrane.

2.4. ELISA assay on polystyrene plates

The ELISA assay for polystyrene plates was performed as described (Matyas et al., 2000). The assay

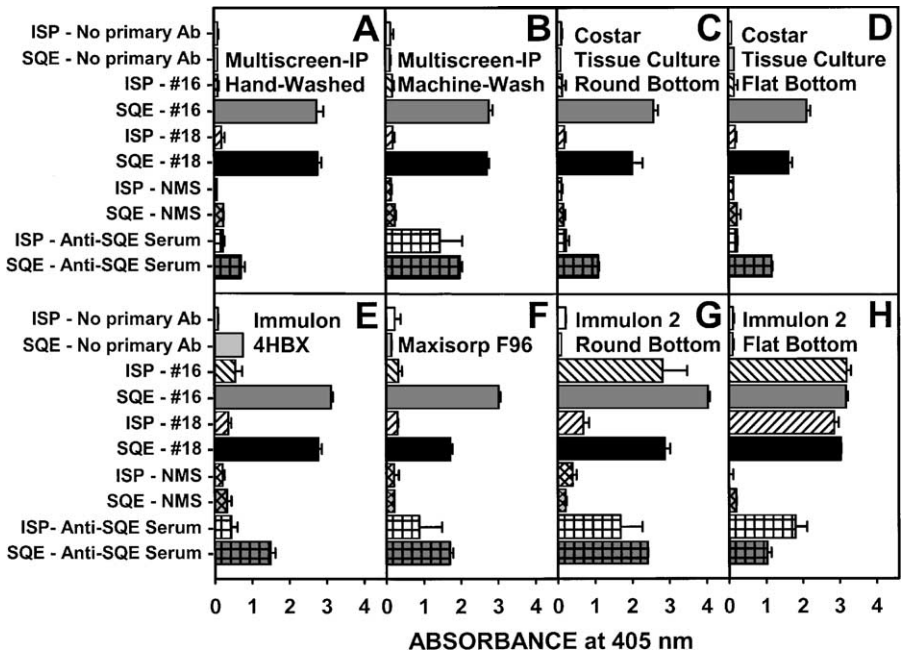

Fig. 1. Comparison of ELISA values obtained with machine-washed plates from different manufacturers with hand-washed Multiscreen-IP plates. PBS–4% FBS was used as the blocker/diluent. Plates were coated with 100 nmol of SQE. mAbs SQE #16 and SQE #18 were diluted 1:10. The normal mouse serum (NMS) and anti-SQE serum were diluted 1:50. The ELISA was performed as described in Materials and methods using PBS–4% FBS. Each panel represents a different plate. Values are the means of triplicate determinations ± S.D.

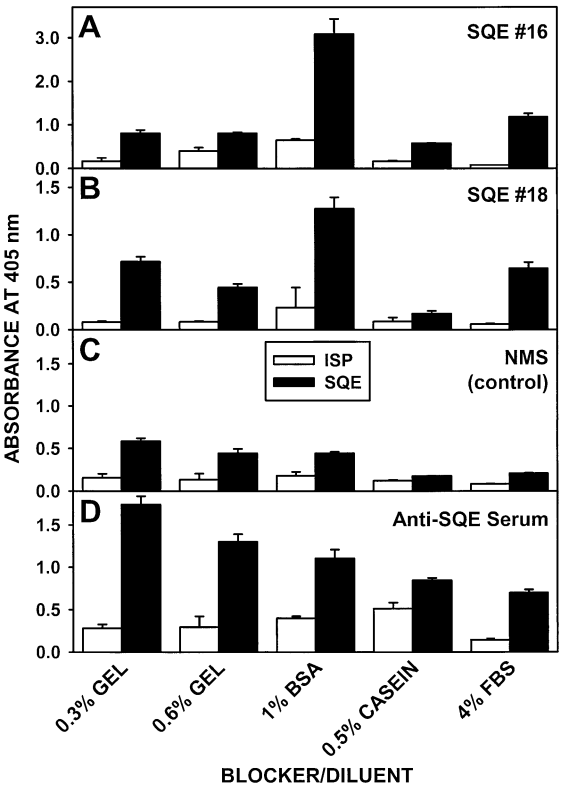

Fig. 2. Comparison of different blocker/diluents on the binding of antibodies to SQE. Costar tissue culture plates were used. Plates were coated with 100 nmol of SQE. mAb SQE #16 (A) and SQE #18 (B) were diluted 1:10 in PBS, pH 7.4 containing the blocker/diluents indicated. Normal mouse serum (NMS) (C) and anti-SQE serum (D) were diluted 1:50. The ELISA was performed as described in Materials and methods. Values are the means of triplicate determinations ± S.D.

for a Costar round bottom 96-well sterile polystyrene tissue culture plate (Costar tissue culture plate) is described in detail. SQE was diluted in ISP (0.15 μmol/ml; 7.2-μl SQE/100 ml) and 0.1 ml (15 nmol) was placed in each well. Control wells contained ISP alone. The plates were placed in a biological safety cabinet and incubated overnight to allow the ISP to evaporate. PBS–2% BSA, pH 7.4, was added to each well (0.3 ml/well) to block unbound binding sites. After incubation at room temperature for 2 h, the buffer was dumped and the plates tapped on paper towel to remove the blocker. Culture supernatants containing mAbs to SQE or mouse serum were diluted in PBS–2% BSA and added to the plates in triplicate (0.1 ml/well). Following incubation for 1 h at room temperature, the plates were washed four times with 0.5 ml of PBS/well using a Skatron plate washer. Peroxidase-linked sheep anti-mouse IgM was diluted 1:1000 in PBS–2% BSA and 0.1 ml was added to each well of the plate. The plates were incubated for 1 h at room temperature and washed four times with PBS. 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. It should be noted that polystyrene pipets, tubes, etc. should not be used in this assay. The use of polystyrene, especially with isopropanol, caused high background (ISP) absorbances (almost equal to the absorbance for antibody binding to SQE) and greatly increased the variability of the results. Highly purified

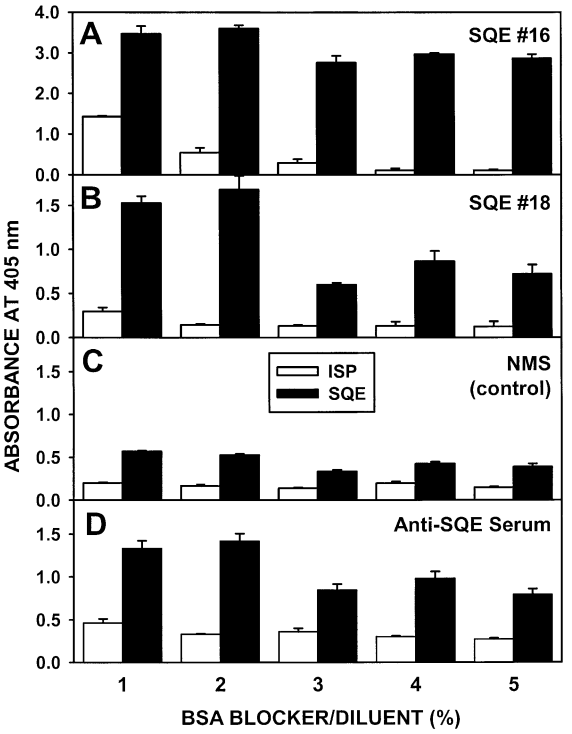

Fig. 3. Comparison of different amounts of BSA used as the blocker/diluent. Costar tissue culture plates were used. Plates were coated with 100 nmol of SQE. Antibodies were diluted in PBS containing the percent BSA indicated. mAbs SQE #16 (A) and SQE #18 (B) were diluted 1:10. Normal mouse serum (NMS) (C) and anti-SQE serum (D) were diluted 1:50. The ELISA was performed as described in Materials and methods. Values are the means of triplicate determinations ± S.D.

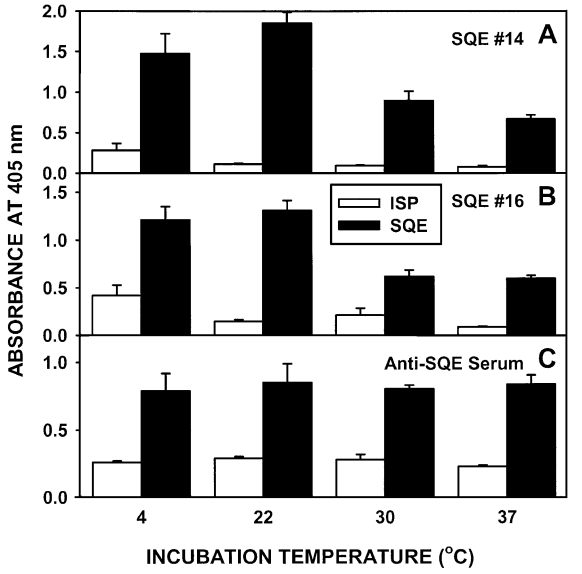

Fig. 4. Binding of anti-SQE antibodies to SQE-coated plates as a function of incubation temperature. Plates were coated with 15 nmol of SQE. PBS–3% BSA was used as a blocker/diluent, which was equilibrated to the temperatures indicated. mAbs SQE #14 (A), SQE #16 (B) and the anti-SQE serum (C) were diluted 1:50, 1:100 and 1:100, respectively. The ELISA was performed as described for the standard assay except that the incubations were at the temperature indicated. Values are the means of triplicate determinations ± S.D.

ISP was also required to ensure low background absorbances.

2.5. Measurement of SQE bound to wells

SQE was diluted in ISP to give the desired amount in 0.1 ml. ^{3}H-SQE (50,000 dpm) was added to each 0.1 ml of ISP to give 50,000 dpm/well. A total of 0.1 ml was added to each well of a Costar tissue culture plate and allowed to dry in a biological safety cabinet overnight. The plates were processed as described above for polystyrene plates using PBS–3% BSA instead of PBS–2% BSA (different lot of BSA). Plates were washed and aspirated by hand to prevent radioactive contamination of plate washing equipment. mAb SQE #16 diluted 1:200 was used as the primary antibody. Liquid scintillation fluid (0.3 ml/well) was added instead of the ABTS substrate. The radioactivity was determined using a microbeta liquid scintillation and luminescence counter (Wallac Oy, Turka, Finland). Total ^{3}H-SQE added was measured by adding ^{3}H-SQE to a control plate. After the ISP evaporated, liquid scintillation fluid was added and the radioactivity determined.

2.6. Statistical analysis

Data were expressed as the mean of triplicate determinations ± S.D. Statistical differences between the means were compared using the paired *t*-test and reported as *p* values. Values ≤ 0.05 were considered significant.

3. Results

3.1. Effect of polystyrene plate type on the measurement of antibodies to SQE

Seven different plates, that were coated with SQE as an antigen and washed with a plate washer, were compared with hand-washed Multiscreen-IP plates for

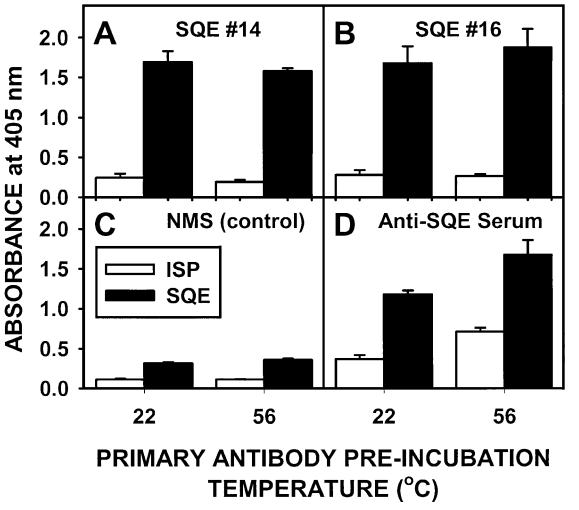

Fig. 5. Effect of heat inactivation of primary antibody on antibody binding to SQE. Primary antibody was incubated at room temperature (22 °C) or 56 °C for 45 min prior to dilution for the ELISA assay. Plates were coated with 15 nmol of SQE. PBS–3% BSA was used as a blocker/diluent. mAb SQE #14 (A), mAb SQE #16 (B), normal mouse serum (NMS) (C) and anti-SQE serum (D) were used as primary antibodies and diluted 40-, 50-, 50- and 200-fold, respectively. The ELISA was performed as described in Materials and methods. Values are the means of triplicate determinations ± S.D.

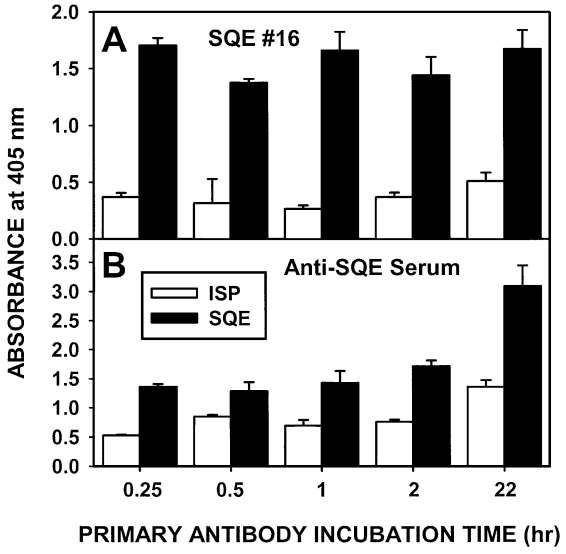

Fig. 6. Binding of anti-SQE antibodies to SQE-coated plates as a function of primary antibody incubation time. Plates were coated with 15 nmol of SQE. PBS–3% BSA was used as a blocker/diluent. mAb SQE #16 and the anti-SQE serum were diluted 1:100. The ELISA was performed as described in Materials and methods except for the primary antibody incubation time. Values are the means of triplicate determinations ± S.D.

their ability to detect mAbs (SQE #16 and SQE #18) and serum antibodies to SQE. Control wells (ISP) had low backgrounds on hand-washed Multiscreen-IP plates (Fig. 1A and Matyas et al., 2000). When the wells were coated with SQE, high anti-SQE absorbances were exhibited for the mAbs (Fig. 1A; SQE-#16; SQE-#18), but low absorbances to SQE-coated wells were obtained for anti-SQE serum (Fig. 1A; SQE-anti-SQE serum). Furthermore, when the Multiscreen-IP plates were washed with an ELISA plate washer, the background absorbances for serum significantly increased (Fig. 1B; ISP-anti-SQE serum). In contrast, Costar round and flat-bottom tissue culture plates had low background absorbances (Fig. 1C and D; ISP-no primary Ab; SQE-no primary Ab; ISP-#16; ISP-#18; ISP-NMS; SQE-NMS; ISP-anti-SQE serum) and high absorbances for SQE-coated wells with both the mAbs and anti-SQE serum (Fig. 1C and D; SQE-#16; SQE-#18; SQE-anti-SQE serum).

Four other types of plates were deemed unsatisfactory because of high backgrounds in the negative controls. These included elevation of the absorbance in wells not incubated with primary antibody (Fig. 1E; SQE-no primary Ab), or in wells lacking SQE (Fig. 1F; ISP-anti-SQE serum; Fig. 1G and H; ISP-#16; ISP-#18; ISP-anti-SQE serum). Because there were no real differences in ELISA values observed between the round bottom and flat bottom Costar tissue culture plates and the other plates that gave unsatisfactory results, all further experiments for optimizing the assay of antibodies to SQE were performed with Costar tissue culture plates.

3.2. Effect of blocking reagents

Various blocker/diluents were used to minimize background and maximize antibody binding to SQE (Fig. 2). The most effective of these consisted of PBS containing BSA (Figs. 2 and 3). Further experiments demonstrated that for both mAbs and anti-SQE serum, the best results were obtained with PBS containing 2% BSA (Fig. 3A, B and D). When compared to 2% BSA, different concentrations of BSA (Fig. 3) or other blocker/diluents (Fig. 2) either raised nonspecific background absorbance levels, or lowered (or even abolished) binding to SQE-coated wells, or both.

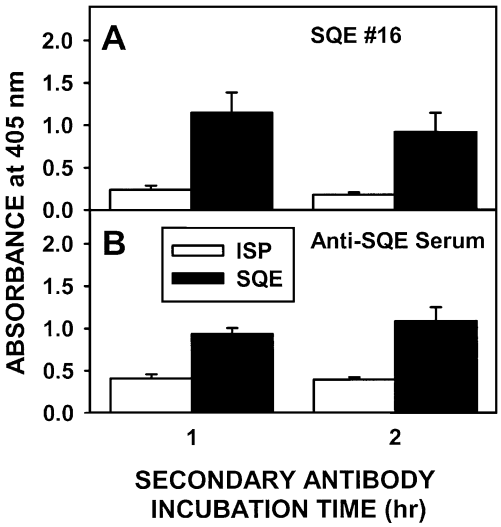

Fig. 7. Effect of secondary antibody incubation time on ELISA absorbance of antibodies binding to SQE. Plates were coated with 100 nmol of SQE. PBS–2% BSA was used as a blocker/diluent. mAb SQE #16 and the anti-SQE serum were diluted 1:40 and 1:50, respectively. The ELISA was performed as described in Materials and methods except for the secondary antibody incubation time. Values are the means of triplicate determinations ± S.D.

3.3. Effect of incubation temperature

The binding of mAb SQE #14 and SQE #16 to SQE-coated wells were dependent on the incubation temperature (Fig. 4A and B). Compared to incubation at room temperature (22 °C), binding of SQE #14 (Fig. 4A) was significantly decreased at 30 °C ($p = 0.02$) and 37 °C ($p = 0.007$). Similarly, binding of SQE #16 (Fig. 4B) was significantly reduced at 37 °C ($p = 0.032$), but the apparent reduction of binding at 30 °C was not significant ($p = 0.073$). There were no significant differences observed between incubation at 22 and 4 °C for both SQE #14 ($p = 0.098$) and SQE #16 ($p = 0.300$). When anti-SQE serum was substituted for mAb, maximal specific binding to SQE-coated wells was independent of temperature (Fig. 4C). For convenience, room temperature was chosen as the incubation temperature for the standard assay.

Pre-incubation of the primary antibody at 56 °C for 45 min prior to the ELISA assay did not significantly change the binding of mAbs SQE #14 and SQE #16 to

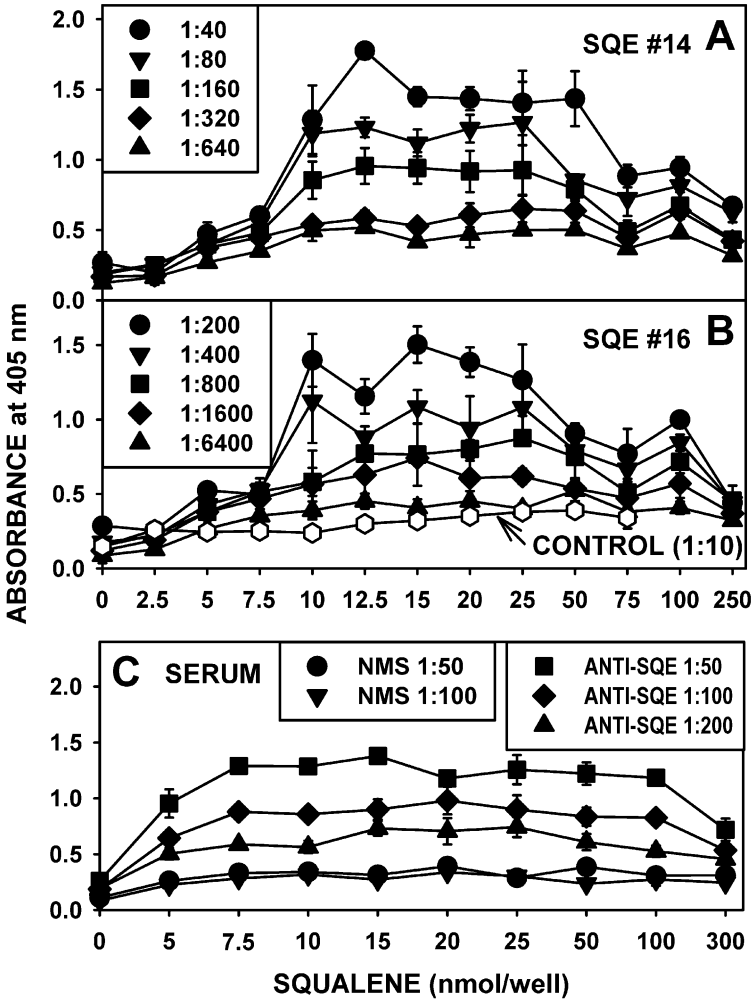

Fig. 8. Binding of anti-SQE antibodies as a function of SQE coated on the well. Plates were coated with the amount of SQE indicated in 0.1 ml ISP/well. PBS–2% BSA was used as a blocker/diluent. mAbs SQE #14 (A), SQE #16 (B) and serum (C) were diluted as indicated. The control was a 1:10 dilution of the culture supernatant from 1B2–1B7, which contained a mouse IgM monoclonal antibody to *N*-acetyllactosamine. The ELISA was performed as described in Materials and methods. Values are the means of triplicate determinations ± S.D.

ISP-treated or SQE-coated wells (Fig. 5A and B). There were no significant differences observed between NMS pre-incubated at room temperature and 56 °C for 45 min (Fig. 5C). Similarly, pre-incubation of anti-SQE serum at 56 °C for 45 min did not significantly change the binding to SQE-coated wells ($p = 0.075$). Furthermore, the apparent increase in binding seen after heating of the anti-SQE serum at 56 °C is due to an increase in background binding (Fig. 5D, ISP). Background binding (Fig. 5D, ISP) was significantly different between the anti-SQE serum pre-incubated at room temperature and 56 °C ($p = 0.021$), but not for NMS (Fig. 5C, ISP) ($p = 0.37$).

3.4. Effect of incubation time

A comparison of the time required for primary antibody binding to SQE-coated wells indicated that overnight incubation did not increase the binding of mAb SQE #16 to SQE-coated wells (Fig. 6A). Although increased binding of anti-SQE serum to SQE-coated wells occurred with overnight incubation ($p = 0.048$ compared to 1 h incubation), this was mostly offset by an increase in nonspecific background binding to ISP-treated wells (Fig. 6B). Consequently, binding of both SQE #16 and anti-SQE serum was independent of incubation time. There were no significant differences seen among the incubation times tested for SQE #16. With the exception of the 22 h incubation time, there were no significant differences among the incubation times for the anti-SQE serum. Similarly, there was no difference between incubating with secondary antibody for 1 or 2 h for both mAb SQE #16 and anti-SQE serum (Fig. 7). Consequently, 1 h was selected for the incubation time for the primary and secondary antibodies. This gives sufficient time for conducting the assay on a large number of samples. However, for small assays, a primary incubation of 15 min would be sufficient.

3.5. Effect of antigen concentration

The binding of mAbs SQE #14 and SQE #16 was dependent upon the amount of SQE added to the wells (Fig. 8A and B). Maximal bindings of mAbs were observed from 10 to 25 nmol of SQE (Fig. 8). Control mouse IgM monoclonal antibody (having a specificity for *N*-acetyllactosamine) did not bind to any amount of SQE coated on the plate (Fig. 8B). The binding of anti-SQE serum was also dependent upon the amount of SQE added to the wells (Fig. 8C). Maximal binding occurred from 7.5 to 100 nmol. NMS did not bind to SQE-coated wells. Based on the above, 15 nmol of SQE was judged to be the optimal amount for the assay when using either mAb or anti-SQE serum.

The binding of different amounts of SQE to the wells was measured using ^{3}H-squalene. A total of 88 ± 4% of SQE added to the wells bound to the well at SQE amounts up to 50 nmol (Fig. 9). Binding of SQE to the well was linear with a slope of 0.82. Linear regression analysis gave $R^2 = 0.997$. At SQE amounts greater than 50 nmol, binding was no longer linear. Only 60 ± 6% of the SQE added to the well bound when 100 nmol was added. SQE amounts greater than 100 nmol had greatly reduced binding of SQE to the wells. Percent binding was 6 and 3 for 300 and 500 nmol of SQE added, respectively. The standard amount of SQE added to the plate in our assays, 15 nmol, resulted in binding of 13.3 ± 0.6 nmol of SQE to the well.

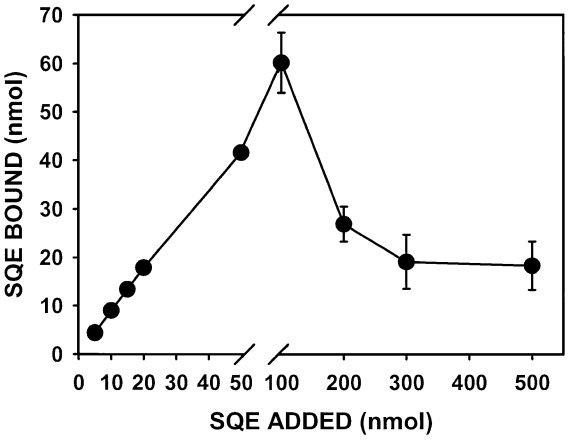

Fig. 9. Quantification of SQE bound to the well as a function of SQE added. SQE containing 50,000 dpm of ^{3}H-SQE in 0.1 ml ISP was added in the amount indicated. The plate was processed as described for the ELISA assay using PBS–3% BSA instead of PBS–2% BSA. mAb SQE #16 was used as the primary antibody. After the last wash, scintillation fluid was added and the radioactivity determined by liquid scintillation counting. Values are the means of triplicate determinations ± S.D.

3.6. Reproducibility of the assay

Several different lots of Costar tissue culture plates were tested under the assay conditions deemed to be optimal (see above). There were no differences between the absorbances obtained with the mAbs on different lots of plates (Fig. 10A and B). Only slight differences in specific absorbances were observed with different lots of plates with anti-SQE serum on SQE-coated wells (Fig. 10C). Background absorbances were the same on the different lots of plates for both the mAb and the anti-SQE serum. The assay was highly reproducible on a daily basis both with the mAb SQE #16 (Fig. 11A) and with anti-SQE serum (Fig. 11B).

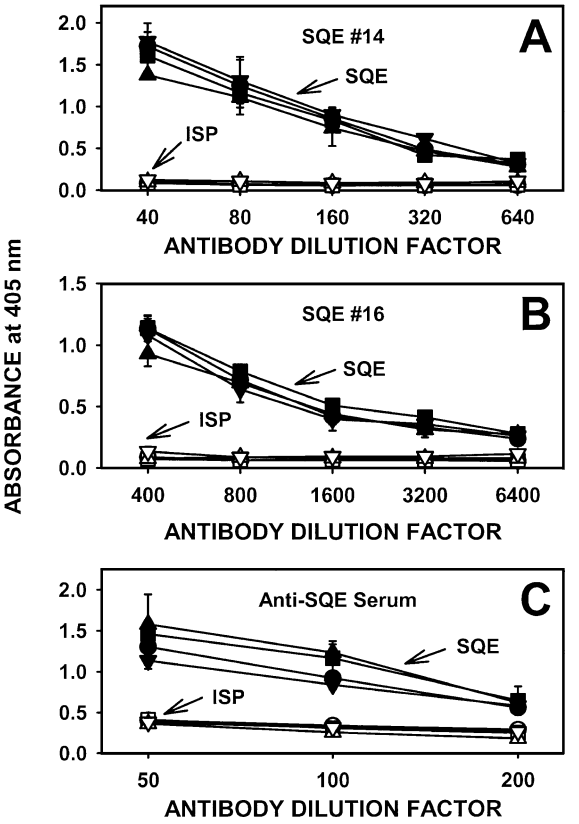

Fig. 10. Comparison of different lots of plates. Plates were coated with 10 nmol of SQE. PBS–2% BSA was used as a blocker/diluent. mAbs SQE #14 (A), SQE #16 (B) and anti-SQE serum (C) were used as primary antibodies. The ELISA was performed as described in Materials and methods. Each symbol type represents a plate with a different lot number. Values are the means of triplicate determinations ± S.D.

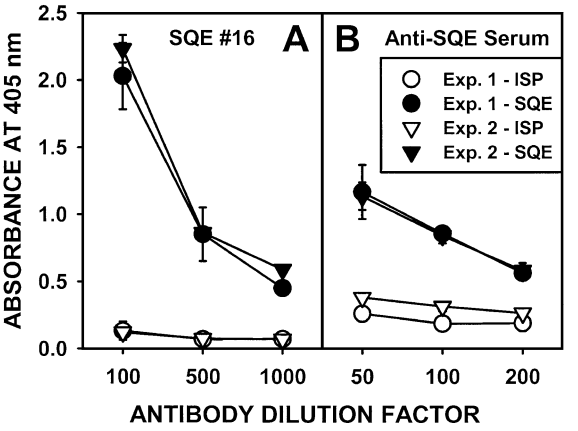

Fig. 11. Day-to-day reproducibility of the ELISA assay for antibodies to SQE. Plates were coated with 10 nmol of SQE. PBS–2% BSA was used as a blocker/diluent. Experiments 1 and 2 were done on separate days as described in Materials and methods. Values are the mean of triplicate determinations ± S.D.

3.7. Effect of antibody concentration

The binding of mAb SQE #16 to SQE-coated wells was dependent upon the concentration of the antibody

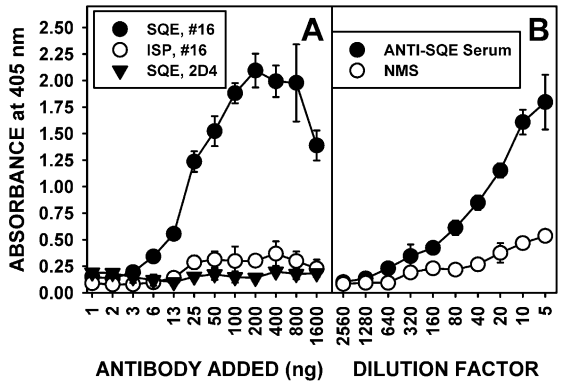

Fig. 12. Effect of antibody concentration on the ELISA assay. Culture supernatant containing mAb SQE #16 and 2D4 (specificity for the carbohydrate on asialo-GM2) were quantified by an IgM quantitation kit and diluted in PBS–3% BSA to give the amount indicated in 0.1 ml. Anti-SQE serum was diluted as indicated. The ELISA assay was performed as described with 10 nmol SQE/well using PBS–3% BSA as the blocker/diluent. Values for ISP-treated wells were subtracted from the data for SQE #16.

(Fig. 12A). The assay was nearly linear from 3.125 to 50 ng of antibody ($R^2 = 0.893$). At concentrations higher than 200 ng, the binding decreased and was variable, as indicated by the higher standard deviations. Control mAb (2D4, specificity for the carbohydrate on asialo-GM2) did not bind to SQE at any concentration tested (Fig. 12A). The binding of anti-SQE serum to SQE-coated wells was also dependent upon concentration (Fig. 12B). After subtracting the NMS background and using the curve for mAb SQE #16 as a standard curve, the concentration of antibodies to SQE in the anti-SQE serum was calculated to be 5.4 μg/ml.

4. Discussion

A highly reproducible, high throughput assay for measuring murine antibodies to SQE is described. The assay has a number of advantages over our previously described assay (Matyas et al., 2000). The PVDF plates were replaced with Costar tissue culture plates, which are not routinely used for ELISA assay. None of the standard ELISA plates tested was useful for this SQE antibody assay. Most gave high background absorbances on control (ISP-treated) wells using PBS–4% FBS (Fig. 1) or PBS–0.3% gelatin as the blocker/diluent. Even when the SQE amount was decreased to 10 nmol, the Costar tissue culture plates were still superior to the Immulon 2HB plates. This change from PVDF plates to polystyrene plates allowed the use of an ELISA plate washer. This eliminated the need for the tedious hand-washing of the plates and greatly increased the throughput of the assay. Furthermore, there were no assay variations when different lots of Costar tissue culture plates were used as had been observed with the different lots of PVDF plates.

Our previous assay utilized FBS as a blocker/diluent. Since serum contained SQE and we were concerned about possible competition of the SQE in the serum with SQE on the plate, FBS was replaced with essentially lipid-free BSA. Various concentrations of BSA were tested to find the concentration that gave the lowest absorbance on ISP-treated wells, but the highest on SQE-coated wells. Two-percent BSA was found to be the optimal concentration. However, in experiments with two other lots of BSA, the optimal concentration of BSA was found to be 3% (Figs. 4, 5, 6, 9 and 12). Therefore, each lot of BSA should be tested prior to use.

The binding of mAbs and anti-SQE serum to SQE was dependent upon the amount of SQE coated on the well (Fig. 8). Binding was reduced at both low and high amounts of SQE. Binding of mAbs and anti-SQE serum to SQE was expected to be similar to that of mAb binding to cholesterol, which bound with a linear or hyperbolic increase in absorbance with increased cholesterol coated on the well (Aniagolu et al., 1995). The reason for the decreased binding of antibody at high SQE amounts was due in part to the loss of high percentages of SQE when high amounts of SQE are added to the wells (Fig. 9). One possible explanation for the inability of large amounts of SQE to bind to the wells may have been that at high amounts of SQE, the SQE was bound to other SQE molecules through hydrophobic interactions, which were then bound to the polystyrene well. Thus, droplets of SQE may have been formed, which then interacted with only a small surface area of the plate. When the plate was washed, the droplets were dislodged from the well surface and removed. When 200 nmol or more of SQE was added to the well, approximately 20 nmol still remained bound to the well after washing (Fig. 9). Since 20 nmol of SQE gave maximal antibody binding (Fig. 8), the 20 nmol remaining from the 200 nmol or more SQE added should also have given maximal antibody binding. However, lower antibody binding was observed (Fig. 8B, 250 nmol). One explanation of this discrepancy may be that the mAbs that reacted with the SQE droplets were washed away, leaving only small amounts of mAb to react with the SQE bound to the well. Another possibility may be that the SQE remaining bound to the well was packed tightly and, consequently, was only accessible to a small amount of antibody. Regardless of the mechanism responsible for the lower antibody binding at high SQE amounts, antibody binding cannot be increased by increasing the amount of SQE as the capture antigen.

The binding of the mAbs SQE #14 and SQE #16 was dependent upon the incubation temperature of the assay (Fig. 4A and B). At temperatures above room temperature, decreased binding was observed. One possible explanation for the decreased binding maybe that at elevated temperatures, the conformation of the

SQE is changed so that the mAbs have reduced affinity in binding or no longer properly recognize the SQE. These mAbs were selected for their ability to bind to SQE at room temperature (Matyas et al., 2000). Consequently, as an inadvertent part of the selection process, clones may have been selected that had reduced binding at elevated temperatures. Since the binding of the polyclonal anti-SQE serum to SQE was independent of temperature (Fig. 4C), there are antibodies which can efficiently bind to SQE at temperatures above room temperature. Unlike antibody binding to other lipids such as cardiolipin, heat-inactivation of the anti-SQE or the mAbs did not increase the antibody binding to SQE (Fig. 5). Furthermore, the heat-inactivation did not decrease the binding of mAbs or anti-SQE serum to SQE. This implies that the decreased binding observed at assay temperatures above 22 °C (Fig. 4) is most likely due to changes in SQE conformation, not permeant heat-induced changes in antibody.

This improved assay was very sensitive and readily detected 125 ng/ml of antibody to SQE (Fig. 12). It was essentially linear over a 10-fold range of antibody concentrations. The new assay was as sensitive as our previously described assay that used PVDF plates (Matyas et al., 2000), but lacked the lot-to-lot differences seen with the PVDF plates. Although we did attempt to use the mAbs to SQE to try to compare our new assay with that described by Asa et al. (2000), the methods of Asa were incomplete (Alving and Grabenstein, 2000; Fulco et al., 2000), and Asa's assay could not be reproduced with murine mAbs to SQE. We conclude that careful adjustment of assay conditions is required for detecting murine antibodies to SQE and that after optimizing the assay, we have now developed a reliable, sensitive, convenient and reproducible assay for detecting antibodies to SQE in murine antiserum.

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