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Silicate Antibodies in Women with Silicone Breast Implants: Development of an Assay for Detection of Humoral Immunity

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Silicon, in the form of sodium silicate (Na_2SiO_3), adsorbed onto bovine serum albumin (BSA)-precoated plates served as the solid-phase antigen in an enzyme immunoassay to detect silicate-reactive antibodies in the plasma of 40 symptomatic women with silicone breast implants, 91 asymptomatic women with silicone breast implants, 50 healthy control women, and 52 women with rheumatic diseases and without silicone breast implants. Silicate-reactive antibodies of immunoglobulin G (IgG) or IgM isotypes were detected in the plasma of 30% (12 of 40) of the symptomatic women with silicone breast implants; 9% (8 of 91) of the asymptomatic women with silicone breast implants; 5% (1 of 20) of the women without implants who had systemic lupus erythematosus; and 0% (0 of 32) of the women without implants who had either Sjögren syndrome, scleroderma, or rheumatoid arthritis. Only 2% (1 of 50) of the sera from the healthy control women contained silicate-reactive antibodies. Preincubation of sera with silicate and eight other metal compounds (including SiO_2) demonstrated that the IgG and IgM antibodies bound specifically to silicate, because preincubation with Na_2SiO_3 inhibited more than 90% of the activity, whereas CrO_3, Li_2SO_4, $MgSO_4$, $NiSO_4$, $HgCl_2$, $ZrOCl_2$, $BeSO_4$, and SiO_2 failed to inhibit the IgG or IgM antibody binding to the silicate-BSA plates. Furthermore, the $F(ab')_2$ portion and not the Fc portion of the silicate-reactive IgG was reactive with BSA-bound silicate in the enzyme immunoassay. The assay for silicate-reactive antibodies was quantified by assigning arbitrary units to a standard curve composed of serial twofold dilutions of high-positive (ten times higher than the cutoff) silicate antibody sera. This novel assay is a useful method for detecting and quantifying humoral immune response to silicate.

Organic compounds of silicon (Si) compose the silicone tetramer (polydimethylsiloxane), which is used in breast implant prostheses (2, 3). Although it was once thought that products composed of silicon in various forms were inert and nonimmunogenic, recent studies show that specific immune responses can be generated against silicone (polydimethylsiloxane) solutions (8, 9, 11, 12, 16, 20). Although there are no solid cause-and-effect data as yet, several reports of different autoimmune conditions in women with silicone breast implants (17–19) suggest that silicone may have an adverse effect on the body's immune system. To this end, certain studies have recently focused on developing laboratory methods for detecting cellular (16) and humoral (8, 12, 20) immune responses to Si compounds, including SiO_2, silicate, and silicone, herein collectively referred to as silicon(e). Of the tests for assessment of humoral immunity to silicon(e) previously reported (8, 12, 20), none used silicate (Na_2SiO_3) and none were quantitative. In this study, we developed an enzyme immunoassay (EIA) for detecting and quantitating specific immunoglobulin G (IgG) and IgM antibodies to bovine serum albumin (BSA)-bound silicate in the plasma of women with silicone breast implants and compared the results with those for healthy women and women with rheumatic diseases who did not have silicone breast implants.

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MATERIALS AND METHODS

Reagents. Lithium sulfate (Li_2SO_4), nickel sulfate hexahydrate ($NiSO_4 \cdot 6H_2O$), chromic trioxide (CrO_3), magnesium sulfate ($MgSO_4$), and silicon dioxide (SiO_2) were purchased from Sigma Chemical Company (St. Louis, Mo.). Other reagents and equipment included beryllium sulfate tetrahydrate ($BeSO_4 \cdot 4H_2O$) (Aldrich Chemical Company, Inc., Milwaukee, Wis.), silicon in the form of sodium silicate (Na_2SiO_3) (Spex Industries, Inc., Edison, N.J.), magnesium chloride ($MgCl_2 \cdot 6H_2O$), sodium azide (NaN_3), sodium carbonate (Na_2CO_3), sodium chloride (NaCl), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$) (Spectrum Chemical Company, Gardena, Calif.), a microtiter plate reader and shaking platform (Tecan U.S., Hillsborough, N.C.), alkaline phosphatase-conjugated goat anti-human IgG and anti-human IgM (American Qualex, La Mirada, Calif.), Immulon 1 flat-bottom microtiter plates (Dynatech, Chantilly, Va.), fat-free BSA, Tween 20 (polyoxyethylene sorbitan monolaurate), and *p*-nitrophenyl phosphate disodium substrate (Sigma Chemical Company).

Blood donors: breast implant patients and controls. EDTA plasma was obtained by venipuncture from 50 healthy control women who did not have breast implants, 40 symptomatic women with silicone breast implants, and 91 asymptomatic women with silicone breast implants. Of the 40 symptomatic women's plasma samples, 31 were remnant samples from a previous study (16). These women had various symptoms, including fatigue, fibromyalgia, insomnia, skin disorders, joint pains, muscle cramps, arthritis, allergies, and arrhythmia. The other nine samples were sent to Specialty Laboratories for routine clinical testing. The samples from 91 asymptomatic women were obtained from Y. Shoenfeld (Tel Aviv, Israel) as controls used in a different study for patients with a variety of autoimmune diseases. All assays were done in a blinded fashion.

Plasma samples from 52 women without silicone implants but with different autoimmune disorders were also tested by EIA for the presence of silicate-reactive antibodies of either the IgG or the IgM isotype. Of the women without implants, 20 had systemic lupus erythematosus, 10 had Sjögren syndrome, 10 had scleroderma, and 12 had rheumatoid arthritis.

Assay for silicate-specific antibodies. In preliminary experiments in which silicate was first used to coat plates and then subjected to blocking with BSA, a negative signal was obtained. Thus, it was determined that plates should be precoated with BSA before the addition of silicate. Antibodies to silicate (adsorbed to BSA) were assayed by EIA. Briefly, Immulon 1 microtiter plates were precoated with 200 μl of 0.5% fat-free BSA in distilled water (dH_2O) per well. After incubation at room temperature with shaking for 2 h, the plates were

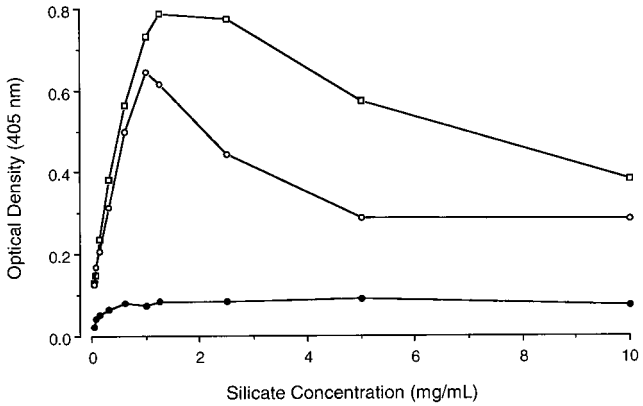

FIG. 1. Silicate concentration-reactivity curves. □, high-positive serum sample for anti-silicate IgG; ○, low-positive serum sample for anti-silicate IgG; ●, normal serum sample from a healthy control woman.

washed three times with dH_2O. One half of each plate was coated with 200 μl of a solution containing 1 mg of Na_2SiO_3 per ml of dH_2O (optimal concentration; data not shown), and the other half (left uncoated and washed with dH_2O) served as control wells without added silicate. After overnight incubation at 37°C in zip-lock bags, the plates were washed three times with dH_2O, air dried, wrapped in Parafilm, and stored at −20°C until used. Portions (200 μl) of plasma, diluted 1:100 in 0.5% BSA in phosphate-buffered saline (PBS)-Tween (a dilution found to be optimal and uniformly used in subsequent assays), were added to the appropriate wells; the plates were incubated overnight with shaking at room temperature. Following incubation, the plates were washed three times with PBS-Tween (0.05% Tween 20). Portions (200 μl) of alkaline phosphatase-conjugated goat anti-human IgG or goat anti-human IgM (diluted 1:2,000 and 1:4,000, respectively) were added to the plates. After room temperature incubation for 1 h with shaking, the plates were washed three times with PBS-Tween, blot dried, and, after addition of 200 μl of substrate buffer containing 1 mg of *p*-nitrophenyl phosphate disodium per ml to all wells, shaken at 300 rpm for 10 to 15 min at room temperature. The plates were read at 405 nm on a Molecular Devices reader linked to the Softmax computer program. The calibration curve was constructed by plotting the optical density (OD) of the standards at 405 nm (*y* axis) versus arbitrary EIA units (*x* axis).

Na_2SiO_3 titration. Various concentrations of Na_2SiO_3, ranging from 0.039 to 10 mg/ml, were added to the BSA-precoated plates. Two silicate antibody-positive plasma samples and one silicate antibody-negative control plasma sample were used to establish an optimal concentration of silicate.

Antibody titration. Three silicate antibody-positive plasma samples (high [80 to 100 units], medium [30 to 40 units], and low [15 to 20 units]) and three negative control plasma samples were serially diluted from 1:50 to 1:400 to find an optimal dilution of plasma. The diluted plasma samples were added to plates coated with a solution containing 1 mg of Na_2SiO_3 per ml and assayed in the EIA.

Conjugate titration. Optimal concentrations of antibody and antigen were used to establish the optimal conjugate concentration. Various dilutions (1:1,000 to 1:8,000) of alkaline phosphatase-conjugated goat anti-human IgG or IgM were tested.

Determination of the interassay and intra-assay variation. The interassay and intra-assay coefficients of variation were determined by running 20 replicates of high-, medium-, and low-positive and negative plasma samples in one experiment on a given day to determine the intra-assay variation and on 10 different days to determine the interassay variation.

Establishment of reference range and standard curve. The standard curves for silicate-specific IgG or IgM antibodies were established from OD values obtained from twofold serial dilutions of a high-positive plasma sample ranging from 1:50 to 1:1,600. An arbitrary value of 100 U was assigned to the highest dilution (1:50), and a value of 3 U was assigned to the lowest dilution (1:1,600). All subsequent OD values were transformed to units from the standard curve.

Determination of antigen specificity. In order to assess whether the antibody reactivity detected in the patient plasma samples was specifically directed to silicate, seven different metal salts (as well as SiO_2 and Na_2SiO_3) were used at different concentrations for absorption of a highly positive silicate-reactive serum sample. $BeSO_4$, CrO_3, Li_2SO_4, $MgSO_4$, $NiSO_4$, $HgCl_2$, $ZrOCl_2$, SiO_2, and Na_2SiO_3 were incubated in plastic tubes for 4 h at 37°C in mixtures with concentrations ranging from 0 to 2.5 mg of a 1:100 dilution of silicate-reactive plasma per ml. After incubation, the mixtures were dispensed into plates coated with BSA or BSA-silicate and the plates were incubated at room temperature overnight with gentle shaking (300 rpm). Following incubation, the plates were washed three times with PBS-Tween and treated thereafter as described for the assay of silicate-reactive antibodies. The percent inhibition was calculated by the following formula:

$$\text{percent inhibition} = \frac{OD_{\text{without absorbent}} - OD_{\text{with absorbent}}}{OD_{\text{without absorbent}}} \times 100$$

Preparation of $F(ab')_2$ and Fc from silicate-specific IgG. An Affi-Gel protein A-agarose column (Bio-Rad) was used to purify IgG anti-silicate antibodies from a positive serum sample (13). The IgG fraction neutralized with 1 M Tris-HCl, pH 8.0, was digested with immobilized pepsin (14). Briefly, the IgG was digested with saturated immobilized pepsin in a tube. The tube was incubated in a shaking water bath at 37°C for 4 h. Separation was accomplished by centrifugation at 1,000 × *g* for 5 min. The supernatant containing the fragments was removed and was separated into $F(ab')_2$ and Fc by using an immobilized protein A column. The Fc fractions eluted with 0.1 M glycine (pH 3.0) were neutralized with 1 M Tris-HCl (pH 8.0). Undigested IgG, Fc, and $F(ab')_2$ fractions were assayed for reactivity with silicate by the EIA described herein.

Effects of serum albumin source on binding of anti-silicate IgG and IgM antibodies to silicate-albumin complex. Six different serum albumins from bovines, sheep, rabbits, chickens, pigs, and humans were used to precoat the plates, which were then coated with silicate. The positive and negative controls were assayed for reactivity with silicate by the EIA described herein.

RESULTS

Antigen concentration curve. Concentration-reactivity curves show that the plates coated with the solution containing 1 mg of Na_2SiO_3 per ml produced optimal reactivity regardless of whether a high or low silicate antibody-positive plasma sample was used or whether the silicate-reactive antibodies were of the IgG or IgM isotype (Fig. 1). The data shown represent the silicate-reactive IgM antibodies. The IgG reaction is similar to the IgM reaction (data not shown).

Silicate antibody titration. Each of the silicate-reactive sera was proportionally positive at dilutions ranging from 1:50 to 1:400 (data not shown). The 1:100 dilution was optimal, and subsequent assays were performed with this dilution.

Conjugate titration. The best results were obtained with a 1:2,000 dilution of the conjugate for IgG and with a 1:4,000 dilution for IgM (data not shown).

Establishment of standard curve. The linearity of the silicate reactivity of the positive control sera allowed arbitrary EIA units to be assigned to increasing dilutions of antiserum ranging from 1:50 to 1:1,600 (Fig. 2).

Determination of specificity of the silicate-reactive antibodies in the sera of some women with silicone breast implants. Of the eight compounds tested, only Na_2SiO_3 inhibited more than 90% of the IgG or IgM antibody binding to Na_2SiO_3 (Fig. 3).

Determination of reference range. On the basis of the mean number of EIA units plus 3 standard deviations for 50 healthy control women, a cutoff of 10 U (OD of 0.18) was established

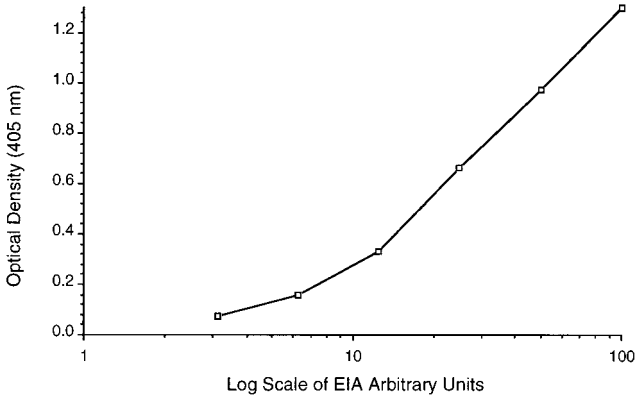

FIG. 2. Establishment of standard curve. The standard curve for detection of silicate IgG antibodies in a high-positive serum is shown.

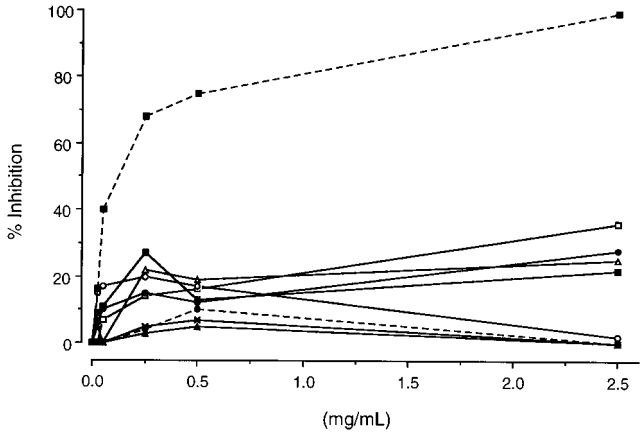

FIG. 3. Determination of silicate specificity by a competitive inhibition assay with different metal salts. ■----■, Na_2SiO_3; ■——■, Li_2SO_4; △, $MgSO_4$; ○, CrO_3; ▲, $NiSO_4$; ×, $HgCl_2$; ●----●, $ZrOCl_2$; □, SiO_2; ●——●, $BeSO_4$.

for both IgG and IgM silicate reactivities. The result for one of the healthy control women was over this range (0.18 standard deviations above the mean). For reasons of confidentiality, no detailed clinical information concerning this patient is available. The frequency distribution curve, based on silicate reactivity observed in the sera of these 50 healthy control women, shows that all of the healthy controls had between 2 and 10 EIA units of silicate-specific IgG or IgM antibodies (Fig. 4).

Determination of interassay and intra-assay variation. The interassay and intra-assay coefficients of variation for detection of silicate-reactive IgG antibodies ranged between 6% and 12%, whereas those for silicate-specific IgM were between 5% and 13% (data not shown). These low coefficients of variation demonstrate the reproducibility of this EIA for the detection of silicate-reactive antibodies.

Presence of silicate-reactive IgG and IgM antibodies in the sera of women with silicone breast implants or women without implants but with different autoimmune diseases. Twelve of the 40 symptomatic women tested (30%) had silicate-reactive antibodies restricted to the IgG isotype and 4 (10%) had antibodies of both the IgG and IgM isotypes. None had antibodies restricted to the IgM isotype (Fig. 5). In comparison, of the 91 asymptomatic women with silicone breast implants, only 8 (9%) had IgG anti-silicate antibodies ($P < 0.001$) and 2 (2%) had antibodies of both the IgG and IgM isotypes. None of the women with Sjögren syndrome, scleroderma, or rheumatoid arthritis had silicate-reactive antibodies of either the IgG or the IgM isotype (Fig. 5). Only one of 20 systemic lupus erythematosus patients tested had low levels of both IgG and IgM silicate-reactive antibodies (Fig. 5).

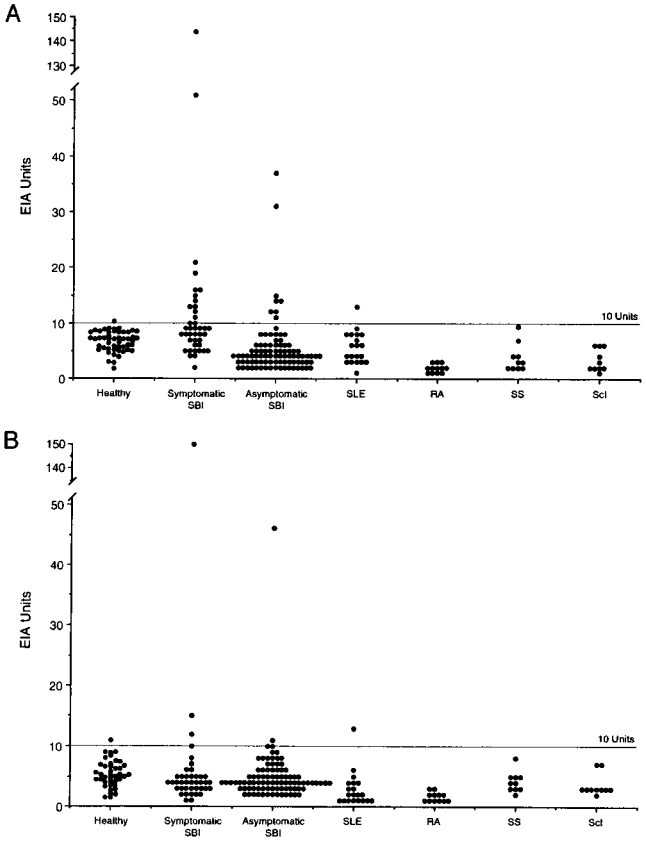

FIG. 5. Demonstration of the relationship between silicate-reactive antibodies in women with silicone breast implants (SBI) and various autoimmune diseases. (A) Silicate-reactive IgG antibodies in healthy women, symptomatic women with breast implants, asymptomatic women with breast implants, and women without implants but with different autoimmune diseases. SLE, systemic lupus erythematosus; SS, Sjögren syndrome; Scl, scleroderma; RA, rheumatoid arthritis. (B) Silicate-specific IgM antibodies in the different groups of women as described for panel A.

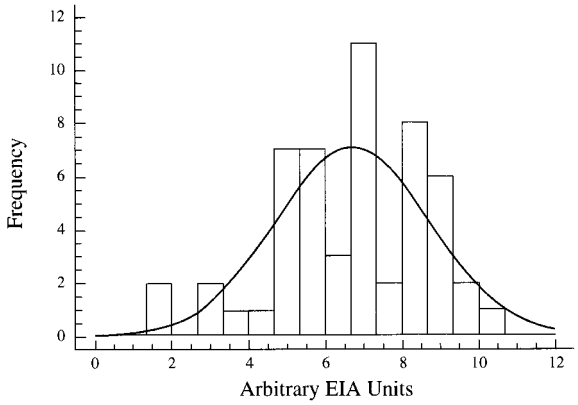

FIG. 4. Normal range frequency distribution curve for silicate IgG antibodies in 50 healthy control women.

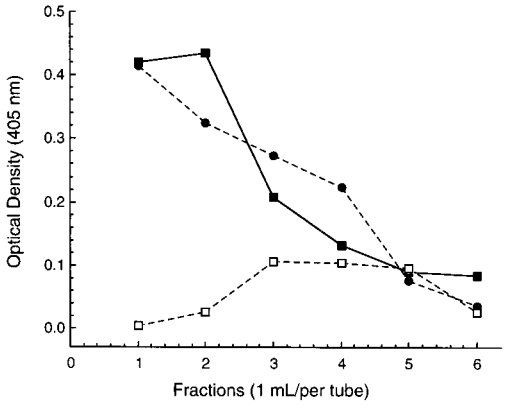

FIG. 6. Effect of pepsin digest on silicate-reactive IgG antibodies in a high-positive plasma sample. ●, $F(ab')_2$ fragment; □, Fc fragment; ■, purified IgG (undigested).

TABLE 1. Effects of order of addition of BSA on binding of anti-silicate antibodies to the silicate-albumin complex

Ig isotype	Protocol	OD of plasma sample			
		Negative control	High positive	Medium positive	Low positive
IgG	BSA/Silicate[a]	0.062	1.105	0.678	0.110
	Silicate/BSA[b]	0.044	0.062	0.047	0.025
IgM	BSA/Silicate[a]	0.104	0.974	0.498	0.246
	Silicate/BSA[b]	0.089	0.103	0.076	0.068

[a] BSA precoated.
[b] Silicate precoated.

Evidence that the silicate activity is mediated by the F(ab′)$_2$ portion of the silicate-reactive IgG molecule. The results depicted in Fig. 6 clearly demonstrate that the silicate-reactive IgG in the plasma sample of one of the women with silicone breast implants who was highly positive for anti-silicate antibodies did interact with silicate through its F(ab′)$_2$ fragment. As expected for an antigen-antibody reaction, no antisilicate activity was mediated by the Fc portion of the silicate-reactive IgG (Fig. 6).

BSA as a carrier for silicate antigen. Table 1 shows that silicate bound the BSA, which acted as a carrier. Silicate could not directly bind to the plates without BSA precoating. The anti-silicate antibody was detected only on the BSA-silicate plates.

Determination that BSA was the best carrier for silicate. Detection of silicate antibodies was performed on the plates which were precoated with serum albumins from different animal species. The best reaction of anti-silicate antibodies was demonstrated by BSA-precoated plates (Table 2). The serum albumins from humans and pigs were not good carriers for silicate antibody detection.

DISCUSSION

Evidence from human and animal models suggests that polydimethylsiloxanes (silicone), the analogs of the silicon-oxygen ions found in silicates, are not immunologically inert but can elicit specific humoral and cell-mediated immune reactions (8, 9, 11, 12, 16, 20). In this report, we describe an EIA method for detection and quantitation of silicate-reactive antibodies in the sera of women with silicone breast implants. Although elevated silicon concentrations are found in sera from some women with silicone breast implants (16), in uremic and dialysis patients (6, 10), in the brains of patients with senile dementia, and in neurofibrillary tangles of patients with Alzheimer's disease (4, 7), no reports of silicate-reactive antibodies (as opposed to silicone antibodies) in humans or animals are available. Herein, we show that 30% of the 40 symptomatic women with silicone breast implants produced silicate-reactive IgG antibodies and 10% had both silicate-reactive IgM antibodies and silicate-reactive IgG antibodies. Nine percent of 91 asymptomatic women with silicone breast implants had IgG anti-silicate antibodies, a percentage which is significantly lower than that observed for symptomatic women. Only 2% (1 of 50) of the 50 healthy control women and 0% (0 of 32) of the women with rheumatic diseases without silicone implants were positive for silicate-specific antibodies. One of 20 women (5%) without implants who had lupus erythematosus had low levels of silicate antibodies. Because false-positive results for IgM isotypes can occur with sera positive for IgM rheumatoid factor (1) in the presence of specific IgG, the four plasma samples with silicate-reactive IgM (plus silicate-reactive IgG) were treated with goat anti-human IgG to rule out potential false positives for silicate-reactive IgM. The treatment failed to remove the silicate-reactive IgM, thus confirming the silicate-reactive IgM positivity of those sera.

The data showed that BSA is the best carrier for silicate binding to microtiter plates to detect anti-silicate antibodies. The reason why human and pig serum albumins could not bind to silicate as carriers (Table 2) is unknown. Further studies are needed to determine the mechanism of silicate-albumin binding.

Previous reports focused on polydimethylsiloxane (silicone)-specific antibodies in women with silicone breast implants (8, 12, 20). However, the nature of the silicon(e) eliciting the specific response is unknown. The fact that the present studies and our previous report (16) show that specific humoral and cell-mediated immune responses can be detected by using inorganic Si compounds (silicon dioxide and sodium silicate) suggests that immune responses elicited by polydimethylsiloxane might in part reflect reaction with one or another compound of silicon. Indeed, the frequency of antibodies detected by our method (30%) is far greater than that (1.7% to 3%) reported by Kossovsky et al., who employed silicone gel adsorbed to a variety of proteins (12).

The mechanism of induction of antibodies or cell-mediated immunity by silicon(e) is unknown. Because silicate is known

TABLE 2. Effect of albumin source on binding of anti-silicate antibodies to the silicate-albumin complex

Albumin source	Silicate	OD of plasma sample with the following antibody isotype:							
		Negative control		High positive		Medium positive		Low positive	
		IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM
Bovine	+	0.050	0.035	1.155	1.052	0.720	0.641	0.295	0.616
	−	0.000	0.001	0.000	0.026	0.000	0.013	0.000	0.036
Sheep	+	0.040	0.035	0.960	1.222	0.617	0.774	0.255	0.582
	−	0.006	0.001	0.015	0.596	0.005	0.219	0.000	0.049
Rabbit	+	0.282	0.059	0.897	0.279	0.509	0.152	0.115	0.104
	−	0.014	0.007	0.203	0.050	0.127	0.023	0.010	0.054
Chicken	+	0.075	0.060	0.460	0.157	0.280	0.070	0.139	0.218
	−	0.024	0.038	0.184	0.052	0.092	0.019	0.051	0.026
Pig	+	0.092	0.083	0.342	0.161	0.193	0.086	0.115	0.117
	−	0.007	0.035	0.056	0.064	0.016	0.025	0.001	0.025
Human	+	0.047	0.031	0.181	0.125	0.125	0.061	0.052	0.067
	−	0.029	0.001	0.057	0.036	0.065	0.034	0.046	0.038

to induce interleukin-1 production in antigen-presenting monocytes and macrophages (5), it is tempting to postulate that silicate can interact directly or indirectly (by binding to exogenous or endogenous peptides) with antigen-presenting monocytes and macrophages and can then be presented to B cells and T cells in conjunction with major histocompatibility complex molecules for induction of a specific immune response. A similar hypothesis has been proposed for Be^{2+} (15).

Because silicone-reactive antibodies are reported to be present in the circulation of about only 1 to 3% of all women with silicone breast implants (12), a subject with an implant might test negative for silicone antibodies but test positive for silicate antibodies (positive rate = 30%) or vice versa (or both could be positive or negative). Thus, addition of a new test for silicate-reactive antibodies could prove valuable for the accurate assessment of effects of silicon(e) on the immune system. Results of preliminary studies in our laboratories (unpublished data) indicate that there is no close correlation between the presence of silicate-reactive antibodies described herein and the T-cell responses to silicate, SiO_2, and silicone gel described elsewhere (16). Of 300 silicone breast implant patients tested in our laboratories for T-cell reactivity with silicate, 140 (47%) were reactive with either SiO_2, silicate, or silicone (or a combination of these antigens) in the T-cell proliferation assay, but only 10% of the 140 patients had silicate-specific IgG antibodies (15a). Therefore, a combined cellular-humoral test for silicon(e) reactivity is likely to increase the sensitivity of laboratory tests for silicon(e) reactivity. The clinical relevance of the presence of silicate antibodies or silicate- and/or silicon(e)-specific T cells in women with silicone breast implants is yet to be determined. Studies designed to answer these questions are currently under way.

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