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Assessment of Immunologic Mechanisms for Flare Reactions to Synvisc®

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Intraarticular injection of Synvisc® for treatment of knee pain sometimes results in an acute local reaction (flare). We tested the hypothesis that the flare was a Type-1 hypersensitivity reaction as manifested by the presence of Synvisc® antibodies in the synovial fluid and serum and by an increase in the concentration of the mast-cell enzyme tryptase in the synovial fluid. Our second objective was to determine whether the ratio of CD4+ to CD8+ lymphocytes in the synovial fluid was increased, as would be expected in a Type-4 hypersensitivity reaction. The study population was a prospective, consecutive series of 16 patients who had a flare, and 20 control patients. We found no differences in product-specific antibodies in the synovial fluid or serum between patients with flares and patients without flares. The mean tryptase level in the synovial fluid of patients with flares, 3.8 ± 0.8 µg/L, was not different from the corresponding level in the control patients. The CD4+/CD8+ ratio in the synovial fluid was more than eight times greater in patients with flares. Flares that sometimes occur after treatment with Synvisc® are probably not Type-1 (antibody-mediated) hypersensitivity reactions, but may be Type-4 (cell-mediated) hypersensitivity reactions.

Intraarticular injections of high-molecular weight hyaluronan (viscosupplementation) can provide temporary relief from knee pain secondary to osteoarthritis.[1,3,17] The first two hyaluronan products approved by the U.S. Food and Drug Administration for this purpose were Synvisc® (Genzyme Biosurgery, Ridgefield, NJ) and Hyalgan (Sanofi Pharmaceuticals, New York, NY). Subsequent to their approval in 1997,[12,13] a possible complication of hyaluronan treatment was described, manifested by severe pain, warmth, joint swelling, and effusion of joint fluid; the complication was termed severe acute inflammatory reaction (flare) or pseudosepsis.[16] Flares are to be distinguished from granulomatous reactions that occur when the viscosupplement is injected into the fat pad or subsynovial tissue.[8,24,44]

The hyaluronan in Synvisc® is extracted from rooster combs and then cross-linked using formalin to increase molecular weight. Cross-linked hyaluronans, called hylans, are combined in a proprietary formulation to produce Synvisc®. In case reports and retrospective studies, flares were described in 22 patients treated with Synvisc®.[2,5,19,25,30–32,41,42] In two prospective studies, a flare occurred once in 213 injections in patients who received a second treatment of Synvisc®,[39] and once in 171 injections in patients who received an initial Synvisc® treatment[20] (the investigators found four additional flares in a retrospective study of 75 injections in patients who received two or three courses). Flares also were described in case reports involving three patients treated with Hyalgan, which is a noncross-linked hyaluronan extracted from rooster combs.[21,22] Flares usually occurred 24 hours after a second or subsequent hyaluronan injection; fluid aspirated from the joint during the acute reaction contained increased leukocytes, but usually neither crystals nor bacteria.[2,5,19,21,22,25,30–32,41,42]

On the basis of animal studies that documented the systemic production of hylan antibodies and the occurrence of cells of the immune system in the synovial fluid of patients with flares, Goldberg and Coutts hypothesized that the flare associated with Synvisc® was an immunologic reaction,[16] instead of a mechanical, irritant, or inflammatory reaction. Their evidence regarding the initiating immune component was limited to antibodies, indicat-

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ing that they favored a humoral mechanism (Type-1 hypersensitivity), but not ruling out the possibility of a cell-mediated (Type-4) mechanism.

We tested the hypothesis that the flare was a Type-1 hypersensitivity reaction as manifested by the presence of Synvisc® antibodies in the synovial fluid and serum and by an increase in the concentration of the mast-cell enzyme tryptase in the synovial fluid. Tryptase is stored almost exclusively in mast cells,[40] such as those present in the synovial lining,[26,40] and is a key mediator of the Type-1 hypersensitivity reaction.[33] Our second objective was to determine whether the ratio of CD4+ to CD8+ lymphocytes in the synovial fluid was increased, as would be expected in a Type-4 hypersensitivity reaction.[15,43]

MATERIALS AND METHODS

Patients who received Synvisc® for treatment of knee pain because of osteoarthritis and who had a flare reaction were prospectively entered into the study between January 2003 and June 2004. The controls, patients with osteoarthritis who did not have a flare, were entered in the study during the same period. All patients came from one clinical practice (DDW)[38]; between November 1997 and June 2004, the total population treated with Synvisc® consisted of 1660 patients who received 7245 Synvisc® injections in 2415 knees. Approximately 86% of the patients had Grade III or IV osteoarthritis (Kellgren-Lawrence scale); all patients in this study, regardless of whether they had a flare reaction, had Grade IV osteoarthritis.

For the enzyme-linked immunosorbent assays (ELISAs), synovial fluid and serum were obtained prospectively from five consecutive patients who had a flare reaction (Table 1), defined as increased local pain and swelling starting within 24 hours of injection and requiring medical treatment. All patients were seen within 3 days of injection, except for one patient who was seen 7 days later. The synovial fluid aspirated from the joints was highly cellular but devoid of crystals or bacteria. Synovial fluid and serum from patients without flares who were having total knee arthroplasties served as controls (Table 1); one control group previously had been treated with Synvisc® but never had flares, and a second group never received viscosupplementation.

After centrifugation at 10,000 g for 15 minutes, the synovial fluid supernatant and serum were recovered and frozen at −80°C until used. The synovial fluid and serum from one patient with a flare and one control patient who received Synvisc® were studied without freezing to evaluate the possibility that freezing might inactivate putative Synvisc® antibodies. No difference in assay results were seen in these cases and the data were included.

For flow cytometry, synovial fluid was obtained prospectively from 11 consecutive patients who had flare reactions and from 11 patients without flare reactions who were having total knee arthroplasties (Table 2). The fluid samples were maintained at room temperature and examined within 2 hours.

Microtiter plates, goat antihuman immunoglobulin (Ig) (Fab specific)-conjugated antibodies (A 8542, A 0293), and substrate-chromagen solution (liquid Substrate System, N 7653) were obtained from Sigma (St. Louis, MO); the antibody detects all Ig including IgG and IgE. Human IgE was obtained from Chemicon (AG30P, Temecula, CA). Polyclonal rabbit antihuman lung tryptase was obtained from Calbiochem (650367, La Jolla, CA). Tryptase was obtained from ICN (57047, Costa Mesa, CA). Antihuman tryptase mAb Biotin (clone AA5) was obtained from Promega (G3361, Madison, WI). All other reagents were obtained from Sigma, except for Synvisc®, which was obtained commercially from the product distributor. Chicken protein was extracted from the combs of freshly killed roosters using a commercial protein isolation kit (Biochain Institute, Hayward, CA).

For detection of putative product antibodies, wells of microtiter plates were coated by overnight incubation with Synvisc® or rooster-comb protein (50 μL/well or 10 μg/mL, respectively);

TABLE 1. Clinical Data for the Three Groups of Patients Studied Using ELISA

Age (years)/ Gender	Group	Synvisc® (course/injection)	Time* (days)	Volume† (mL)	Leukocytes (cells/mm^3)
73/F	SF	4-3	3	35	44,250
76/F	SF	4-1	0.5	50	17,930
79/F	SF	1-3	7	22	116
78/F	SF	4-1	2	60	4130
76/F	SF	1-2	2	34	4841
71/F	SNF	2 courses	—	—	—
68/F	SNF	2 courses	—	—	—
66/F	SNF	2 courses	—	—	—
48/F	SNF	1 course	—	—	—
70/F	SNF	4 courses	—	—	—
68/M	NS	—	—	—	—
73/M	NS	—	—	—	—
75/F	NS	—	—	—	—
77/F	NS	—	—	—	—

SF = Patients treated with Synvisc® who had a flare; SNF = Patients treated with Synvisc® who did not have a flare; NS = Patients not treated with Synvisc®; *time between injection and joint aspiration; †volume of aspirated fluid

TABLE 2. Clinical Data for the Two Groups of Patients Studied Using Flow Cytometry

Age (years)/ Gender	Group	Synvisc® (course/injection)	Time* (days)	Leukocytes (cells/mm^3)
72/F	SF	5-1	1	2841
77/M (right knee)	SF	4-3	3	2180
77/M (left knee)	SF	4-3	3	2671
74/F	SF	2-1	1	5429
71/F	SF	1-3	1	10,167
74/F	SF	4-3	1	56,000
51/F (right knee)	SF	1-3	1	3500
51/F (left knee)	SF	1-3	1	3000
55/F	SF	2-1	1	13,824
78/F (right knee)	SF	4-1	4	4650
78/F (left knee)	SF	4-1	4	3950
78/M	NS	—	—	128
75/F	NS	—	—	3
70/F	NS	—	—	29
82/F	NS	—	—	162
66/M	NS	—	—	13
81/F	NS	—	—	73
60/F	NS	—	—	36
73/F	NS	—	—	35
72/F	NS	—	—	51
68/M	NS	—	—	41
51/F	NS	—	—	524

SF = Patients treated with Synvisc® who had a flare; NS = Patients not treated with Synvisc®; *time between injection and joint aspiration

coat formation was verified by staining with Coomassie blue. The antigen solutions were replaced with blocking buffer (0.17 mol/L H_3BO_4, 0.12 mol/L NaCl, 0.05% Tween 20, 1 mmol/L EDTA, 0.25% albumin), and the plates were incubated for 30 minutes. The buffer was replaced by various concentrations of the test solutions in phosphate-buffered saline (PBS), and the plates were incubated for 4 hours. The test solutions were replaced by goat antihuman Ig alkaline-phosphatase conjugate, diluted 1:5000 in blocking buffer. After incubation (12 hours), the Ig solution was replaced by a substrate-chromagen solution, and optical density at 405 nm was measured 30 minutes later. All measurements were done in quadruplicate, and all wells were washed three times with buffer each time a well solution was changed. Nonspecific binding of the Ig antibody (assessed using immobilized albumin) yielded an optical density of 0.05, which was subtracted from all optical-density measurements. The positive control consisted of total human Ig immobilized in the wells; binding of the Ig antibody resulted in an optical density of 2.3. The sensitivity of the assay to detect IgE, evaluated using appropriate weight ratios of immobilized IgE and albumin, showed that we would have detected antibodies against antigens immobilized on as few as one per 10,000 binding sites on the ELISA plates (Fig 1).

To measure tryptase concentration, wells of microtiter plates were coated by overnight incubation with polyclonal tryptase antibody in PBS (5 μg/mL). The antibody solution was replaced with blocking buffer, and the plates were incubated for 30 minutes. The buffer was replaced with synovial fluid or tryptase, and the plates were incubated for 3 hours. The test solutions were replaced by antihuman tryptase conjugated with biotin (1 μg/mL). After incubation (3 hours), the antibody solution was replaced by a solution of avidin conjugated to peroxidase, which was replaced after 3 hours by a substrate chromagen solution; the optical density of the solution (450 nm) was measured 60 min-

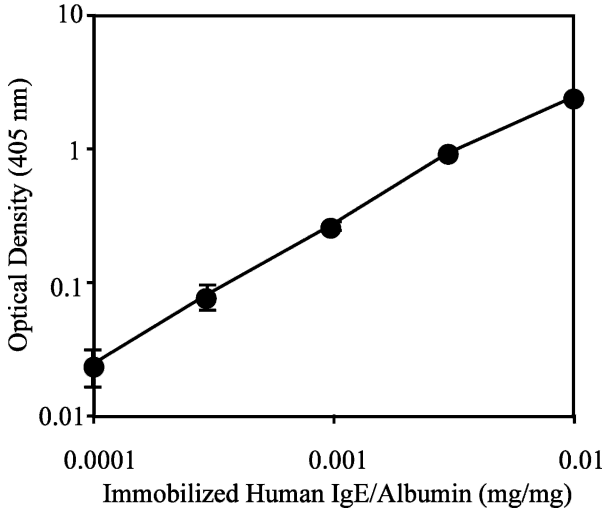

Fig 1. A chart shows the sensitivity of the ELISA for detecting IgE in albumin. Human IgE and albumin were immobilized overnight and detected with IgG enzyme-conjugated antibody (2 hours) and substrate chromagen (2 hours).

utes later. All measurements were done in quadruplicate, and all wells were washed 3 times with blocking solution each time the well solution was changed. Human tryptase was used to generate a standard curve to define the detection limits of the immunoassay. The detection limit of the tryptase assay was approximately 0.2 μg/L (not shown).

For flow cytometric analysis (FACSCaliber, Becton Dickinson, Franklin Lanes, NJ), synovial fluid was diluted 1:20 with PBS, cells were washed twice more with PBS, adjusted to 1×10^6 mononuclear cells/mL with PBS containing 2% fetal calf serum, and incubated with monoclonal antibodies for 30 minutes at 2° to 8°C, followed by one PBS wash. The following monoclonal antibodies (Becton Dickinson) were used: CD45-peridinin chlorophyll protein, CD4-fluorescein (FITC), CD8-phycoerythrin (PE), CD4-allophycocyanin, and CD3-FITC. Lymphocytes were gated by CD45 versus side scatter parameters combined with forward scatter versus side scatter parameters. Isotypic controls were used to set quadrant markers for the fluorochromes. At least 10,000 cells were counted. The percentages of CD4+ and CD8+ cells were expressed relative to CD45+ cells.

Flow cytometry data were evaluated using the Mann-Whitney U test. The antibody titration data were analyzed by calculating the dilution corresponding to 50% of the greatest optical density value and then comparing the means of different groups using the t test.[36]

RESULTS

Antibodies against Synvisc® or against chicken proteins that could explain the occurrence of the flare were not detected in synovial fluid or serum (Figs 2, 3). Nonspecific binding to immobilized Synvisc® and to immobilized avian proteins increased with increasing concentration of synovial fluid and serum, evidenced by the corresponding changes in optical density (Figs 2, 3). However, at a given concentration of synovial fluid or serum, the mean optical density was identical in all three groups of patients. The assay was capable of detecting antigen added to Synvisc® at a concentration at least as low as 0.1% (Fig 4).

The average concentration of tryptase in the synovial fluid of patients who had not been treated with Synvisc® (5.0 ± 0.8 μg/L) was not different from the corresponding level of patients who had received Synvisc®, regardless of whether they had a flare (4.0 ± 0.2 μg/L and 3.8 ± 0.8 μg/L, respectively) (Fig 5).

The CD4+/CD8+ ratio in the synovial fluid of patients who had a flare was greater ($p < 0.05$) than the corresponding value in the control patients (Table 3). The leukocyte concentration in the synovial fluid in the control patients (NS, Table 2) was too low to permit measurement of lymphocyte subpopulations in five patients; in the others, the CD4+/CD8+ ratio was near one (Table 3), as expected.[9,14] During the acute phase of the flare, however, the leukocyte concentration and the relative presence of CD4+ T cells in the synovial fluid were increased (Table 3). There was no correlation between the CD4+/CD8+ ratio and the number of courses of treatment before the flare (not shown).

DISCUSSION

Based on an analysis of studies that showed systemic production of Synvisc®-specific antibodies in animals, the presence of immune-system cells in the synovial fluid of patients treated with Synvisc® who had flares, and on a report of Synvisc® antibodies in the serum of a patient who

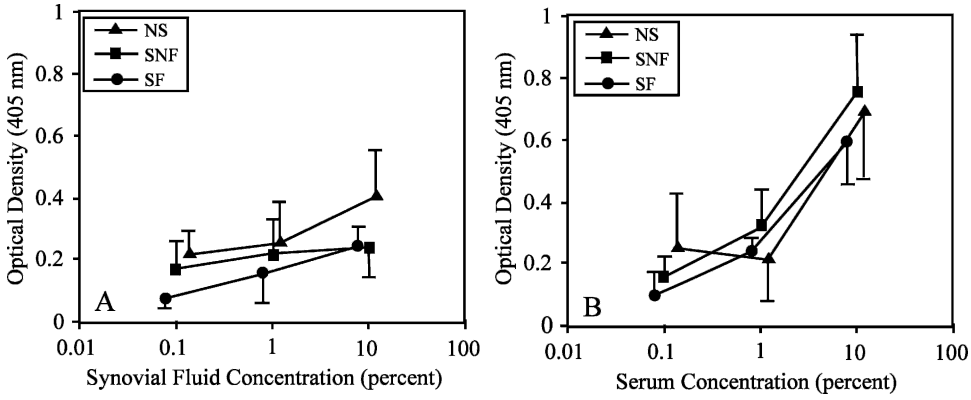

Fig 2A–B. The charts show the results of indirect ELISA (mean ± SD) to detect putative antibodies to Synvisc®. Immobilized Synvisc® was used to screen synovial fluid obtained from patients who had never received Synvisc®, but who did not have a flare (SNF) or had received Synvisc® and had a flare (SF). In the SF group, the (A) synovial fluid and the (B) serum were obtained during the active phase of the flare (Table 1). The samples were diluted (volume/volume) in buffer. At each concentration, the points were displaced slightly along the abscissa to facilitate observation.

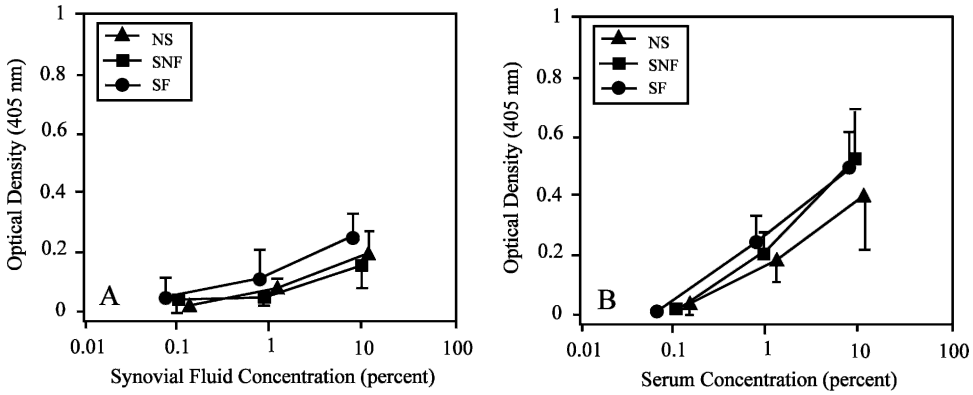

Fig 3A–B. The charts show the results of indirect ELISA (mean ± SD) to detect putative antibodies to chicken proteins. Immobilized rooster comb proteins were used to screen synovial fluid obtained from patients who had never received Synvisc®, had received Synvisc® but did not have a flare (SNF), or had received Synvisc® and had a flare. In the SF group, the (A) synovial fluid and the (B) serum were obtained during the active phases of the flare (Table 1). The samples were diluted (volume/volume) in buffer. At each concentration, the points were displaced slightly along the abscissa to facilitate observation.

had a flare, Goldberg and Coutts suggested that the flare was an allergic reaction of the type mediated by antibodies.[16] We tested for the presence of the antibodies and for the allergic mediator tryptase, both of which would be expected to be elevated during a flare. We also determined whether the ratio of CD4+ to CD8+ lymphocytes in the synovial fluid was increased in patients with flares, as would be expected in a Type-4 hypersensitivity reaction.

An exaggerated, antibody-mediated immune response termed Type-1 hypersensitivity sometimes occurs after a subject's second or subsequent contact with an antigen.[33] In this type of hypersensitivity reaction, IgE antibody produced against an antigen becomes bound to F_C receptors on mast cells. When the antigen is presented again, it interacts with the bound antibody, leading to degranulation of mast cells and release of pharmacologic mediators that produce acute inflammation. In principle, antibodies could have been raised in the patients against the hyaluronan moiety of Synvisc®, epitopes created during product-specific processing of the hyaluronan, or against residual avian proteins. We considered these possibilities by screening patient fluids with Synvisc® and with avian proteins extracted from rooster combs. We found no evidence of the presence of antibodies to Synvisc® or chicken proteins in the synovial fluid or serum of patients who had flares (Figs 2, 3).

One possible interpretation of the results is that there is an antigen in Synvisc® that is responsible for the flare, but that the antigen was not available to react in the ELISAs with product-specific antibodies in synovial fluid or serum because it was prevented from doing so by the hyaluronan molecule. Another possibility is that there were no antibodies present in the synovial fluid or serum that could account for the flare. The question of which interpretation was preferable could be definitively resolved by means of a positive control consisting of human antiSynvisc® antibodies. Antibodies against Synvisc® have been raised in rabbits and mice by injecting large amounts of Synvisc®,[6,34] but it is impossible to inject similar amounts of Synvisc® into patients to obtain the needed antibody titer. Antibodies raised in animals would not be appropriate positive controls because, at best, they could establish only that the ELISA could detect animal antibodies, whereas the question at issue is the ability of the assay to detect human antibodies. To address the problem of the absence of a true positive control antibody, we used a spike ex-

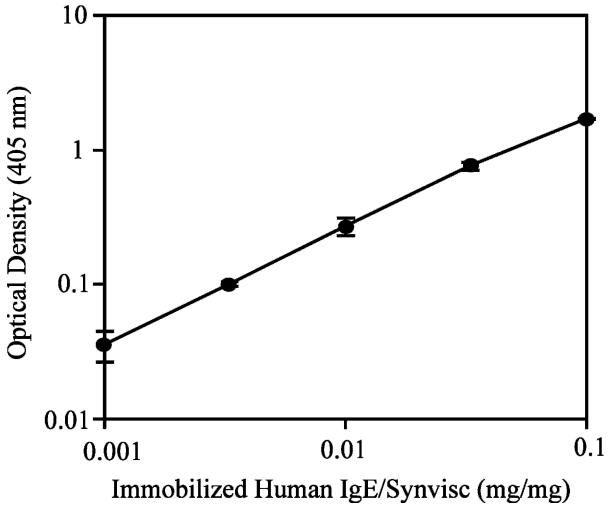

Fig 4. A chart shows the sensitivity of the ELISA for detecting IgE in Synvisc®. Human IgE and Synvisc® were immobilized overnight and IgE was detected with IgG enzyme-conjugated antibody (2 hours) and substrate chromagen (2 hours).

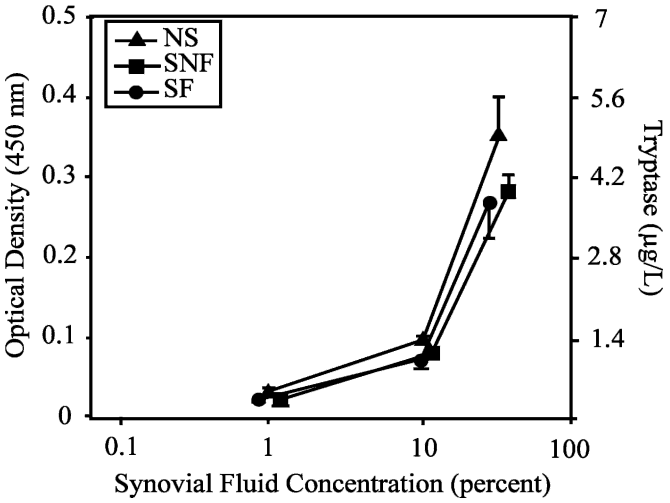

Fig 5. A chart shows the results (mean ± SD) of sandwich ELISA for tryptase. Immobilized tryptase was screened with synovial fluid obtained from patients who had never received Synvisc® (NS), had received Synvisc® but did not have a flare (SNF), or who received Synvisc® and had a flare (SF). In the group that had received Synvisc® and had a flare, the synovial fluid was obtained during the active phase of the flare. The samples were diluted (volume/volume) in buffer. The tryptase scale (expressed in units of micrograms of tryptase per liter of synovial fluid) was computed using a standard curve (not shown) and the optical density measurements obtained at a synovial-fluid concentration of 50%. At each concentration the points were displaced slightly along the abscissa to facilitate observation.

periment to evaluate the possibility that the hyaluronan molecule could effectively sequester an antigen and account for the negative results (Figs 2, 3).

The concentration of the putative antigen in Synvisc® is unknown, but if it is associated with a protein moiety, as suspected,[16] its concentration can reasonably be expected to be in the range 0.5% to 1%.[4] When we added IgE to Synvisc® to mimic the presence of the putative product antigen, we found that the ELISA for determining the concentration of antibodies against IgE was linear, suggesting the absence of an interaction between Synvisc® and the added antigen and indicating that we could detect low levels of antibody and antigen (Figs 4, 5). Therefore, although the results (Figs 2, 3) could mean that antibodies were not present or that they were not detected, the alternatives were not equally probable. First, the development of color occurred in the reaction in a concentration-dependent manner (Figs 2, 3) which precludes some kind of experimental error, such as a dysfunctional substrate solution. Second, the results obtained in the spike experiment, which can be considered a positive control for the ELISAs, indicated that sequestration of an antigen was unlikely. Finally, if product-specific antibodies were present (indicating a Type-1 hypersensitivity response), an increase in tryptase in the synovial fluid would have been expected, but no such increase occurred (Fig 5). Taken together, therefore, the results indicate that product-specific antibodies were unlikely to have mediated the patient's symptoms. Nevertheless, the possibility cannot be completely excluded because a human antiSynvisc® antibody is not available for use as a positive control in the ELISAs.

TABLE 3. Distribution of Lymphocyte Subpopulations in Synovial Fluid from Patients with Osteoarthritis

Lymphocyte Subpopulation	Flare (n = 11)	No Flare †(n = 6)
CD4+	*81.55 ± 5.97	41.17 ± 8.73
CD8+	*13.36 ± 5.28	50.67 ± 11.99
CD4+/CD8+	*7.47 ± 4.01	0.87 ± 0.31

Numbers represent mean percentages with the standard deviation; *p < 0.05, Mann-Whitney U test; †five additional patients had too few cells to evaluate.

It could be argued that the antibody concentrations in the patients who had flares had returned to baseline by the time the patients' joints were aspirated, thereby accounting for the absence of a difference between the flare and control groups. For several reasons, we think that this explanation is unlikely. First, the tissue fluids were obtained from the patients who had flares during the acute reaction (0.5 to 7 days after injection, Table 1), which is when any unbound product-specific antibodies most likely would be present. Second, even if the antibody concentration in the synovial fluid had returned to baseline or had never departed from baseline despite the occurrence of a flare, the presence of elevated tryptase levels in the synovial fluid of patients who had flares would have been expected because mast-cell degranulation and tryptase release is a basic process in the Type-1 reaction. However, the tryptase levels in both groups of patients who received Synvisc® were essentially identical to the levels in patients who had never been treated with Synvisc®, indicating that treatment with Synvisc® did not activate synovial mast cells. The tryptase levels for all three groups were the same as previously reported in patients with osteoarthritis when using clone AA5 as the capture antibody.[7]

Hyaluronan is immunogenic in rabbits.[10,11] The suggestion that Synvisc®-specific antibodies were responsible for the flare[16] was partly based on animal studies that reported detection of such antibodies under experimental conditions in which antibodies to Hyalgan were not detected.[6] The clinically significant issues regarding the antibody-producing propensity of Synvisc® are whether it is antigenic when used according to label instructions and, if so, whether the product-specific antibodies mediate the

flare. Our evidence suggested a negative answer to both questions (Figs 2, 3, 5).

Antibodies to hylan and chicken serum proteins were reported in the serum but not synovial fluid of one patient who had a flare after treatment with Synvisc®.[31] Goldberg and Coutts speculated that the absence of antibodies in synovial fluid might have been an artifact in the ELISA used to detect the antibodies, caused by the presence of high concentrations of endogenous hyaluronans. There are several reasons why their idea can be discounted. First, if Synvisc®-specific antibodies were present in synovial fluid but did not bind to immobilized antigen in the ELISA because they were bound to endogenous hyaluronan, the antibodies also would be bound to hyaluronan in the joint and unavailable to interact with mast cells or lymphocytes. In that case, patients injected with Synvisc® would not have a flare, which is contrary to observation. Second, any specific antibodies also would have been present in the blood, but none were present in our experiments. Third, if specific antibodies triggered the flare, they would have been present throughout the body and therefore should have initiated flares at multiple locations. However, the flares always occurred only in the injected joint, suggesting that our ELISAs were not negative for Synvisc® antibodies for the reason supposed by Goldberg and Coutts.

All of the patients in our study who had flares had Grade IV osteoarthritis. It seems unlikely to us that product-specific antibodies could account for flares in patients with less severe osteoarthritis because that was not the case in the patients we studied, but such a possibility cannot be excluded.

Type-4 hypersensitivity is mediated by antigen-sensitized T cells that release lymphokines after a second or subsequent contact with the same antigen.[33] Although there is some evidence that a subpopulation of B cells can be involved in initiation of a cell-mediated hypersensitivity response,[35] the effector phase (which corresponds to the flare reaction) usually is associated with a predominant infiltration of CD4+ T cells.[15,43] The CD4+/CD8+ ratio reported here for the synovial fluid of control patients (who had osteoarthritis but no flares) is the same as that previously reported for patients with osteoarthritis[9,14] and rheumatoid arthritis.[14,37] The increased CD4+/CD8+ ratio in patients with flares compared with control patients (Table 3) is consistent with the idea that the flare reaction is a Type-4 hypersensitivity response. Such responses are known to be mediated by activated CD4+ T cells in various anatomic locations.[18,27,29] Selective retention of CD4+ T cells has been associated with memory responses to contact sensitizers,[28] a situation analogous to that of patients who have received multiple Synvisc® treatments.

The delay in the appearance of symptoms after injection (usually 24 hours or more) (Tables 1, 2) was also consistent with the possibility the flare is a Type-4 hypersensitivity response. Type-1 responses usually are evident within a few hours, whereas a delay of 24 hours or more is consistent with a Type-4 reaction.[23] Therefore, it is possible that activation of sensitized CD4+ T cells by subsequent injections of Synvisc® in susceptible persons contributed to the flare reaction.

The flare reaction after treatment with Synvisc® probably is not a Type-1 (antibody-mediated) hypersensitivity reaction, but may be a Type-4 (cell-mediated) hypersensitivity reaction.

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