

Immunogenicity of Hylan G-F 20 in Guinea Pigs and Mice

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ABSTRACT. Objective. To determine the product-specific immunogenicity of a chemically-modified sodium hyaluronate derivative, hylan G-F 20, that is used in the treatment of osteoarthritis of the knee.

Methods. Guinea pigs were subcutaneously immunized with hylan G-F 20 (Synvisc®) once a week for 3 weeks. After resting, these animals received an intradermal challenge with hylan to elicit allergic skin reactions. Animal sera were tested for the presence of hylan-specific antibodies by homologous passive cutaneous anaphylaxis (PCA) assay and of anti-hylan IgG by ELISA. Further, mice were similarly immunized with hylan, and their sera were analyzed by heterologous PCA assay in rats and by ELISA for anti-hylan Ig(G+M) and anti-hylan IgE.

Results. In the guinea pig studies, acute and delayed erythematous skin reactions were elicited in immunized animals after the intradermal challenge with hylan. The sera of hylan-immunized guinea pigs showed positive reaction in the homologous PCA assay and significantly high amount of anti-hylan IgG, whereas the sera did not show any cross-reactivity against sodium hyaluronate. Hylan also exhibited immunogenicity in mice of 3 inbred strains, and C3H/HeN mice showed higher production of anti-hylan antibodies than Balb/c and C57BL/6 mice.

Conclusion. Hylan G-F 20 exhibited immunogenicity in guinea pigs and mice. Recent reported severe acute inflammatory reactions in human patients after repeated intraarticular injections of hylan G-F 20 might involve product-specific, immune-mediated mechanisms. (J Rheumatol 2004;31:943–50)

Key Indexing Terms:

HYLAN G-F 20 SODIUM HYALURONATE PRODUCT-SPECIFIC IMMUNOGENICITY SEVERE ACUTE INFLAMMATORY REACTIONS

Intraarticular hyaluronan (HA) administration has been widely accepted as a treatment option for the painful symptoms of knee osteoarthritis (OA)¹⁻³. HA products are divided into 2 major preparations, sodium hyaluronate (SH) and hylan G-F 20, and the tolerability of these products, in general, has proved to be very good. The main adverse events after the intraarticular HA injection are transient local reactions in the injected joint. The mean prevalence of the reactions is generally between 2% and 4% of injections, and is similar to other intraarticular injection products². However, a growing number of published reports have suggested the possibility that there is a product-specific adverse event after intraarticular injection of hylan G-F 20.

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The reactions are called severe acute inflammatory reactions and are characterized by non-septic acute articular swelling and effusions without any further detrimental sequelae⁴⁻⁸. Several authors have suggested immunogenicity of the product as one possible cause, because reactions occurred after more than one injection^{4-6,8}. Unlike SH products, hylan G-F 20 is a chemically-modified HA derivative that is produced after protein cross-linking and divinyl sulfone cross-linking⁹. Bucher, *et al*¹⁰ reported that the sera of rabbits immunized with hylan G-F 20 exhibited an anti-chicken protein response, whereas the sera of those immunized with SH did not exhibit such response, suggesting a possible contribution of product-specific immunogenicity to human severe acute inflammatory reactions.

Clinical use of unmodified HA (SH) in orthopedic surgery as well as ophthalmic surgery over the past 30 years and large numbers of pre-clinical animal studies have shown that purified SH preparations are non-immunogenic¹¹⁻¹⁶. However, there is little available information of potential immunogenicity of hylan G-F 20 not only in humans but also in experimental animals.

Our aim was to investigate potential immunogenicity of hylan G-F 20 in guinea pigs and mice. In the guinea pig studies, SH was used as a comparison material in order to distinguish the potential immunogenicity of both products.

MATERIALS AND METHODS

Test materials. Hylan G-F 20 (Synvisc®, 8 mg hylan polymers/ml, denoted hylan hereafter) was obtained from Genzyme Biosurgery, Ridgefield, NJ, USA. Sodium hyaluronate (SH, Supartz™, 10 mg/ml, average molecular weight 0.9×10^6 Da) and egg albumin (EA) were from Seikagaku Corporation, Tokyo, Japan. Each material was diluted at defined concentrations with physiological saline. Complete Freund's adjuvant (CFA, ICN Biomedicals Inc., Aurora, OH, USA) was used to enhance immune reactivity in the test animals.

Animals. Male Hartley guinea pigs at 4 weeks of age were purchased from Japan SLC, Inc. (Shizuoka, Japan). Three strains of inbred female mice at 5 weeks old, BALB/cAnN, C57BL/6J, and C3H/HeN, and male Sprague-Dawley rats (7 wks old) were purchased from Charles River Japan Inc. (Kanagawa, Japan). These animals were acclimatized in the laboratory housing cages at least 7 days before the following experiments. They were maintained on the standard laboratory diet and water *ad libitum*. The research protocols for the animal experiments were approved by the Animal Ethics Committee at the authors' institutions.

Immunization and serum collection in guinea pigs. Guinea pigs are one of the most effective species to detect immunogenicity of non-human macromolecules with molecular weight > 5000 ¹⁷. The immunogenicity studies in guinea pigs were performed as described by Nagami, *et al*¹⁸, with some modifications. Guinea pigs were immunized by subcutaneous injection of test material once a week for 3 weeks. The animals were randomly divided into 7 groups, as follows: (1) hylan 0.5 mg/kg, (2) hylan 2.5 mg/kg, (3) hylan 2.5 mg/kg with equal volume of CFA (+ CFA), (4) SH 2.5 mg/kg, (5) SH 2.5 mg/kg + CFA, (6) EA 1 mg/animal + CFA as a positive control, and (7) non-immunized as a negative control. Each experimental group consisted of 6 to 8 animals, except that the positive controls were 4. Twelve days after the final immunization, blood was drawn from each animal and separated sera were stored at -20°C until performance of the analyses described below.

Skin test and histopathological assessment of the biopsy specimens. Fourteen days after final immunization, each guinea pig was challenged by an intradermal injection of 0.1 ml of corresponding immunized antigen: hylan 0.1 mg (Groups 1–3), or SH 0.1 mg (Groups 4 and 5), or EA 0.01 mg (Group 6). After the elicitation, acute (3 h after challenge) and subacute (24 and 48 h) skin reactions such as swelling, erythema, and other changes at the challenge sites were inspected. The reactions were graded by the mean diameter of erythema as follows: –, less than 1.0 mm; 1+, 1.0 ~ 5.0 mm; 2+, 5.1 ~ 10.0 mm; 3+, 10.1 ~ 15.0 mm; 4+, ≥ 15.1 mm.

For histological study, 4 other guinea pigs immunized with hylan 0.5 mg/kg were used. These animals received an intradermal injection of 0.1 ml of hylan G-F 20 (undiluted material, 0.8 mg/site) 14 days after the final immunization. Twenty-four and 48 h after the injection, a biopsy was performed at the injection site on 2 animals of each group. Specimens were fixed in 10% neutral buffered formalin for 3 days, paraffin embedded, and sectioned. Each section was stained with hematoxylin and eosin, and alcian blue at pH 2.5 with or without hyaluronidase digestion¹⁹. Immunohistochemical staining of anti-guinea pig major T cell subset antibody (Biosource International Inc., Camarillo, CA, USA) was also performed. The antigen of the antibody may be the guinea pig homolog of CD4, according to the manufacturer's product sheet. In brief, the primary antibody was applied to each slide for 1 h at room temperature, after which epitope retrieval was performed using citrate buffer, pH 6.0, for 10 min at 95°C ²⁰. Then the immune complex was visualized by the avidin-biotin peroxidase method using Vectastain Elite ABC kit (for mouse IgG; Vector Laboratories, Inc., Burlingame, CA, USA) and 3,3'-diaminobenzidine (Dojindo Laboratories, Kumamoto, Japan) as a chromogen. These sections were counterstained with Meyer's hematoxylin.

Homologous passive cutaneous anaphylaxis. Guinea pig homologous passive cutaneous anaphylaxis (PCA) is known as a biological assay of sera to detect antigen-specific IgG1 and IgE²¹. PCA has the advantage of

measuring not only the biologically active antibodies but also the consequence of allergen/antibody interaction leading to inflammatory mediator release from mast cells and the expression of cutaneous anaphylaxis. The sera (0.1 ml) from immunized animals were intradermally injected at the dorsal skin of other naïve guinea pigs. Each guinea pig received injections of 6 to 8 sera. About 18 h later, each animal received intravenous injections of a corresponding antigen (hylan 5 mg/kg, or SH 5 mg/kg, or EA 1 mg/animal) and a dye solution. Thirty minutes after the elicitation injection, the animals were sacrificed and the diameters of blue spots that had developed at the serum injection sites were measured on the subcutaneous side. When the blue spot was > 5 mm in diameter, the serum was considered positive and the maximal PCA titer of the serum was determined using the other naïve guinea pigs. Each serum was tested in 2 naïve recipients to confirm the reproducibility of the reactions.

Anti-hylan IgG ELISA. It is difficult to coat plastic microtiter plates with polysaccharides because of their high hydrophilicity²². Therefore, to capture hylan on the ELISA plates, hyaluronic acid binding protein (HABP) from bovine nasal cartilage (Seikagaku Corp.) was used. HABP is capable of binding specifically with HA that has a length at least 10 sugar residues, and has been used to detect serum hyaluronic acid for the diagnosis of various inflammatory diseases such as rheumatoid arthritis²³. Ninety-six-well ELISA plates were coated with 0.5 $\mu\text{g/ml}$ of HABP in carbonate buffer, pH 9.4, and incubated overnight at 4°C . After washing the plates with phosphate buffered saline (PBS), 20 $\mu\text{g/ml}$ of homogenized hylan solution was added to each well and incubated for 1.5 h at 37°C . Hylan G-F 20 consists of water-soluble hylan A fluid and water-insoluble hylan B gel with a ratio of 8:2², and simple dilution of the material with PBS resulted in a suspension containing gel precipitations. Therefore, a mild homogenization was done to make the diluted solution uniform. Plates were subsequently washed with PBS containing 0.05% Tween 20 and blocked with 20% donkey serum (Seikagaku Corp.) and 4% Pluronic F-68 (Sigma, St. Louis, MO, USA) for 1 h at room temperature. Specific coating of the wells with hylan was confirmed by a sandwich binding method using biotinylated HABP and avidin-peroxidase²³. After that, diluted test sera were added to the wells of the plates and incubated for 1 h at 37°C . Peroxidase (horseradish peroxidase, HRP)-conjugated sheep anti-guinea pig IgG(Fc) (Nordic Immunology, Tiburg, The Netherlands) at a 1:2000 dilution and a TMB solution (Moss Inc., Pasadena, MD, USA) were used for color development, and the differential absorbance between 450 and 655 nm was measured. The quantities of anti-hylan IgG were expressed as delta-OD at 10-fold dilution of test sera, where the absorbance of uncoated wells was subtracted from that of those coated with hylan. Similar ELISA plates coated with SH were also prepared to test anti-SH IgG in the SH-immunized groups. These plates were also used to determine the serum cross-reactivity against hylan and SH.

Immunogenicity studies of hylan G-F 20 in mice. Mice of 3 strains mentioned above were divided into the following immunization groups: (1) hylan 10 μg , (2) hylan 100 μg , (3) hylan 100 μg + CFA, (4) EA 10 μg + CFA as a positive control, and (5) non-immunized as a negative control. Each group consisted of 6 animals. Immunization was performed by subcutaneous injections of each antigen once a week for 3 weeks. Fourteen days after the final immunization, blood was withdrawn from the animals, and the sera were tested for the presence of heterocytotropic antibodies using the PCA assay in naïve rats. This assay can detect antigen-specific IgE in mouse sera²¹. The testing procedure was similar to that of the guinea pig homologous PCA assay. The sera were also tested by ELISA for the presence of anti-hylan Ig(G+M) using anti-mouse Ig(G+M)-HRP (Biosource International) as a primary antibody. The sera of C3H/HeN mice were also quantified as anti-hylan IgE using anti-mouse IgE-HRP (Southern Biotechnology, Birmingham, AL, USA).

Statistical analysis. The data of ELISA studies were statistically analyzed with Tukey's multiple-comparison test. P values < 0.05 were considered significant.

RESULTS

Acute and delayed skin erythema after intradermal challenge with hylan. Test animals immunized with hylan 0.5 and 2.5 mg/kg showed acute erythema with grades 2+ to 4+ 3 h after the intradermal challenge (Table 1). These reactions included edematous swelling that peaked at 24 h after the challenge. The size of erythema was maintained at a similar level until 48 h after elicitation. In the animals immunized with hylan + CFA, erythema and edema were somewhat weaker than in the animals solely immunized with hylan, but the changes occurred predominantly 24 h or later after the challenge. These findings assumed that the reaction included both immediate and delayed-type hypersensitivity. In comparison, the animals of SH immunized groups showed no local change after the intradermal challenge (Table 1). Positive control animals that were immunized with EA + CFA exhibited definitive erythema and swelling at each observation point.

Intradermal injection of hylan G-F 20 (undiluted) in the hylan-immunized guinea pigs elicited similar local reactions as those observed in the skin test. However, the macroscopic changes were much more severe because the elicitation concentration of hylan was 8 times higher. After 24 h, the injection site of one immunized animal showed marked swelling and erythema with hemorrhagic patches (Figure 1A). Histological findings suggest an Arthus-like reaction, i.e., involving severe edema, hemorrhage, and a large number of cell infiltrations (neutrophils, macrophages, and lymphocytes) in epidermis and upper dermis (Figure 1B). Another animal in the 48-h biopsy showed a decrease in the change in swelling, whereas the erythema at the center of the lesion intensified (Figure 1C). Palpation confirmed that the lesion became firmer with time. A histological examination revealed a large accumulation of mononuclear cells at the injection site (Figure 1D). Multiple cellular nodules were found in the middle layer of dermis, and a palisading cell accumulation around the amorphous and basophilic cluster material was found in the bottom layer of dermis. The cluster materials were positive for alcian blue (Figure

1E), and were digested by hyaluronidase. An immunohistochemical examination revealed that the cells accumulating around the material were T cells that might express CD4 (Figure 1F). The intradermal injection of hylan G-F 20 in non-immunized guinea pigs never induced such reactions.

PCA reaction. Developments of subcutaneous blue spots were found in the recipient guinea pigs treated with sera of groups immunized with hylan. Maximal PCA titers of the sera were 1 to 25, 5 to 25, and 125 in the groups of hylan 0.5 mg/kg, hylan 2.5 mg/kg, and hylan 2.5 mg/kg + CFA, respectively (Table 2). On the other hand, the sera of comparison groups immunized with SH or SH + CFA did not elicit any reaction. Positive control sera from animals immunized with EA + CFA showed a definitive reaction.

Anti-hylan IgG in sera. Significant increase in anti-hylan IgG was detected in the sera of hylan-immunized animals (Figure 2). The sera of hylan + CFA group showed greater quantities of anti-hylan IgG than those of solely immunized groups. Serum anti-hylan IgG levels in SH immunized groups were the same as those in the non-immunized group, and considered as a background level. Anti-SH IgG was not detected in the sera of SH-immunized groups or in hylan-immunized groups (data not shown). These results indicate that anti-hylan IgG does not have cross-reactivity against SH.

Anti-hylan antibody production in mice. Heterologous PCA assay showed that the sera of hylan-immunized BALB/cAnN and C57BL/6J mice exhibited no reaction (Table 3). In contrast, significant positive reactions of passive anaphylaxis were observed in the recipient rats treated with the sera of hylan-immunized C3H/HeN mice (Figure 3 and Table 3). The sera of C3H/HeN mice immunized with hylan + CFA, however, showed no reaction. In each mouse strain, the positive control sera (immunized with EA + CFA) showed a definitely positive reaction.

In ELISA studies, the sera of hylan-immunized C3H/HeN mice showed a significantly high amount of anti-hylan Ig(G+M) (Figure 4). In contrast, only the sera of groups immunized with hylan 100 µg showed a significantly

Table 1. Skin reactions after intradermal challenge.

Immunization, mg/kg BW	Challenge, mg/site	No. of Animals	No. of Animals with Erythema (Grades ^a)		
			3h	24h	48h
Hylan 0.5	Hylan 0.1	7	6 (2+–4+)	7 (2+–4+)	7 (2+–4+)
Hylan 2.5	Hylan 0.1	6	6 (3+–4+)	6 (3+–4+)	5 (2+–4+)
Hylan 2.5 + CFA	Hylan 0.1	7	7 (2+–3+)	7 (3+)	7 (2+–3+)
SH 2.5	SH 0.1	8	0	0	0
SH 2.5 + CFA	SH 0.1	8	0	0	0
EA ^b + CFA	EA 0.01	4	3 (2+–3+)	4 (3+)	4 (2+–3+)
Non-immunized	Hylan 0.1	4	0	0	0
	SH 0.1	4	0	0	0

^a Mean diameter of erythema: 2+, 5.1–10.0 mm; 3+, 10.1–15.0 mm; 4+, ≥ 15.1 mm. ^b 1 mg/animal. SH: sodium hyaluronate; EA: egg albumin; CFA: complete Freund's adjuvant; BW: body weight.

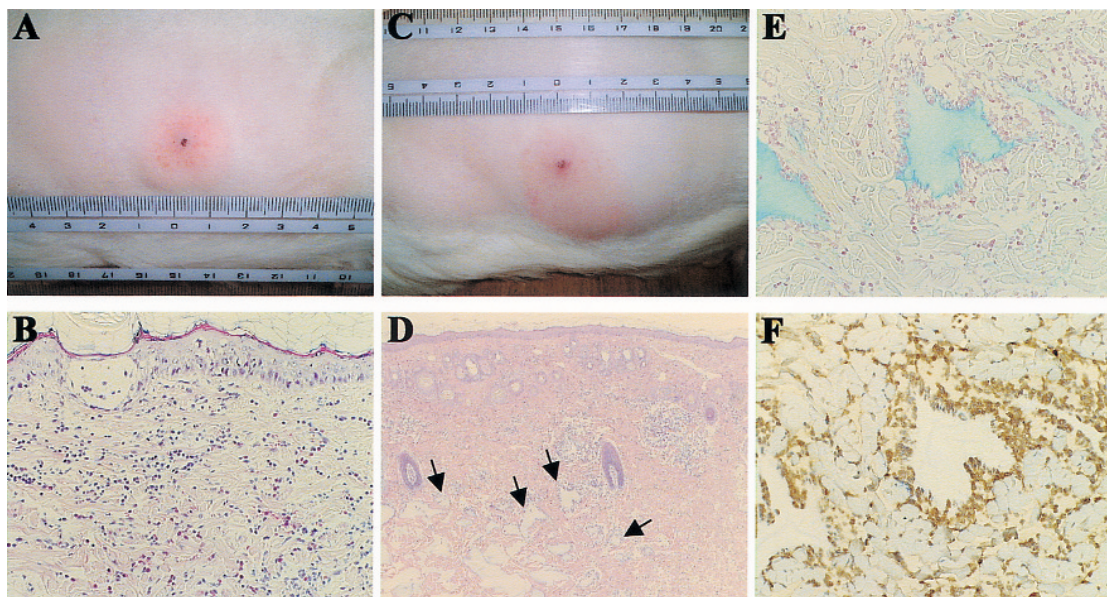


Figure 1. Gross and histological changes after intradermal injection of hylan G-F 20 (undiluted material, 0.1 ml) in hylan-immunized guinea pigs. (A) Gross appearance at 24 h after challenge. Acute allergic erythema and swelling with hemorrhagic patches were found. (B) Photomicrograph of the tissue section of A (H&E stain, magnification 150 \times). Severe inflammatory changes including edema and vacuolar changes in epidermis, numerous cell infiltrations, and microhemorrhage in upper dermis were observed, suggesting Arthus-like reaction. (C) Gross appearance at 48 h after challenge. Swelling change recovered, but erythematous change at the center of the lesion intensified. (D) Photomicrograph of the tissue section of C (H&E stain, magnification 12.5 \times). Note the multiple cellular nodules at the middle layer of dermis, and acellular, basophilic amorphous material surrounded by numerous mononuclear cells at the bottom layer of dermis (arrows). (E) High-power view of the adjacent section of D, stained with alcian blue, at pH 2.5 (magnification 150 \times). The amorphous cluster materials found at the bottom layer of dermis were positive to alcian blue, which were digested by hyaluronidase. The histochemical result proved that these materials were composed of hyaluronan. (F) Immunohistochemical staining showed that the accumulated cells around the alcian blue-positive materials were T cells, suggesting delayed-type hypersensitivity reaction (magnification 150 \times).

Table 2. Homologous passive cutaneous anaphylaxis responses in naïve guinea pigs.

Immunization, mg/kg BW	Challenge, mg/kg BW	No. of Positive Sera	Maximal PCA Titers
Hylan 0.5	Hylan 5	7/8	1–25
Hylan 2.5	Hylan 5	6/7	5–25
Hylan 2.5 + CFA	Hylan 5	4/8	125
SH 2.5	SH 5	0/8	—
SH 2.5 + CFA	SH 5	0/8	—
EA ^a + CFA	EA ^b	4/4	10000
Non-immunized	Hylan 5	0/4	—
	SH 5	0/4	—

^a 1 mg/animal, ^b 5 mg/animal. BW: body weight.

high amount of Ig(G+M) in BALB/cAnN and C57BL/6J. The sera of C3H/HeN mice solely immunized with hylan showed a detectable increase in anti-hylan IgE although the amount of increase was rather small (Figure 5). The sera that exhibited high titer in the PCA assay also showed a high amount of anti-hylan IgE.

DISCUSSION

Severe acute inflammatory reactions or pseudoseptic knee

after injections of hylan G-F 20 were first reported by Puttick, *et al*⁴. They reported 6 of 22 patients (27%) had the reactions, characterized by pain, warmth, and swelling in the injected joint that lasted for up to 3 weeks. Following the report, several authors describing similar adverse events suggested the involvement of immune-reaction mechanisms because the reactions were never found after the first injection^{5,6,8}. Further, the report of Leopold, *et al*⁷ showed that

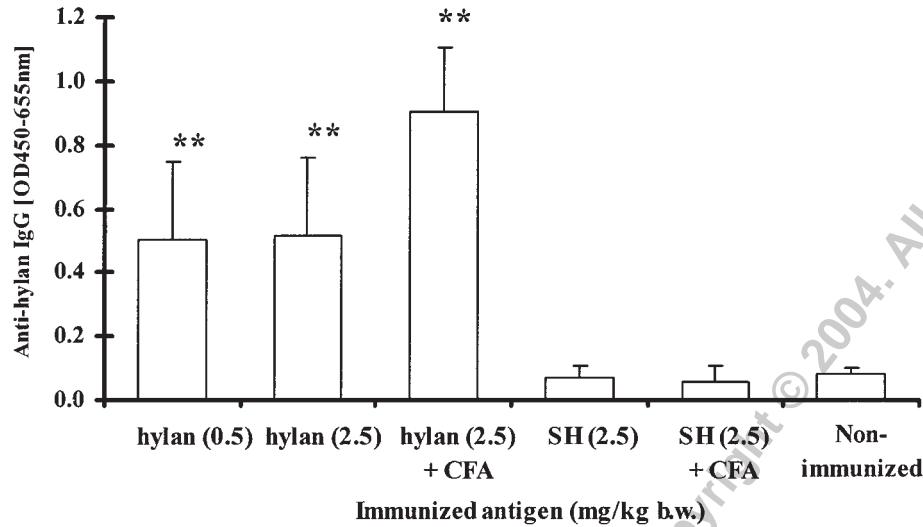


Figure 2. Anti-hylan IgG in the sera of immunized guinea pigs. Values are the mean \pm SD in each group. ** $p < 0.01$ versus the values of non-immunized controls.

Table 3. Heterologous passive cutaneous anaphylaxis responses in naïve rats.

Immunization, μ g/animal	Challenge	No. of Positive Sera (maximal PCA titers)		
		Balb/cAnN	C57BL/6J	C3H/HeN
Hylan 10	Hylan, 5 mg/kg	0/6	0/6	4/6 (5–10)
Hylan 100	Hylan, 5 mg/kg	0/6	0/6	3/6 (10–20)
Hylan 100 + CFA	Hylan, 5 mg/kg	0/6	0/6	0/6
EA 10 + CFA	EA, 1 mg/animal	6/6 ($\geq 5^a$)	6/6 ($\geq 5^a$)	6/6 ($\geq 5^a$)
Non-immunized	Hylan, 5 mg/kg	0/4	0/4	0/4
	EA, 1 mg/animal	0/2	0/2	0/2

^a Maximal titers were not determined. PCA: passive cutaneous anaphylaxis; EA: egg albumin; CFA: complete Freund's adjuvant.

the frequency of acute local reactions were significantly higher in patients who received multiple courses of hylan G-F 20 treatment (21%) than those who received a single

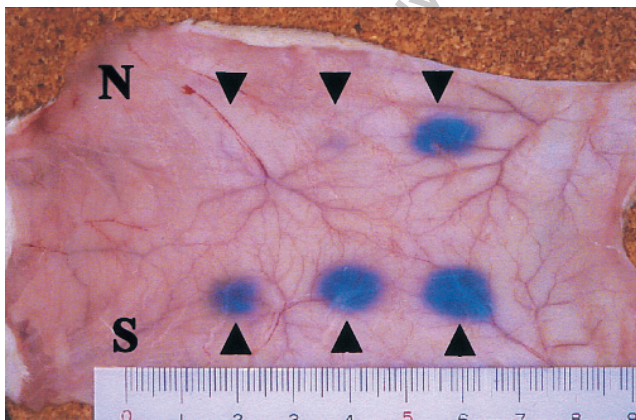


Figure 3. Heterologous passive cutaneous anaphylaxis response in rats treated with sera of hylan-immunized C3H/HeN mice. Sera from 4 of 6 mice showed a positive reaction (arrowheads). The saline control (S) and the serum of non-immunized mice (N) were negative.

course (2%). A recent review of the safety of intraarticular HA products in human patients suggested that there is a considerable difference in safety profiles between SH and hylan G-F 20, especially in the frequency of severe acute inflammatory reactions²⁴.

Unmodified HA has been considered to be non-immunogenic because it distributes widely in mammalian connective tissue and may act as a self-antigen in living tissues. Several polysaccharides including hyaluronic acid are reported to induce immunological tolerance in B cells²⁵. Further, exogenous polysaccharide exposure induces IgM-dominated primary antibody response, but does not induce an antibody isotype switch from IgM to IgG without the presence of co-stimulators such as interferon- γ ²⁶. These events may be the reason that exogenous HA exhibits non-immunogenicity in humans and animals. In contrast, hylan is a synthetic derivative of HA and is not a natural polymer. It seems that exogenous exposure to these products induces different immune responses in humans and animals. Moreover, 2 features are known to be essential for a xenobiotic to be a sensitizer: it must be foreign (non-self) and

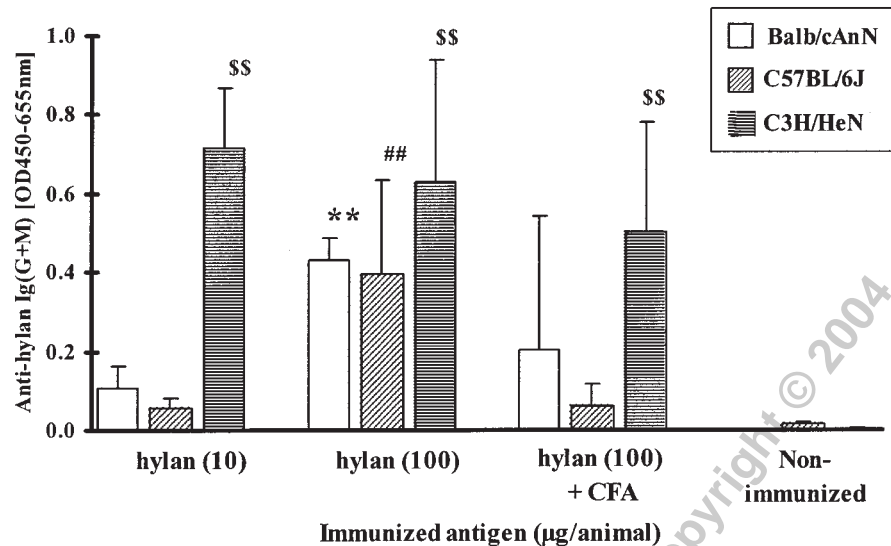


Figure 4. Anti-hylan Ig(G+M) in the sera of immunized mice. Values are the mean \pm SD in each group. Double symbols: $p < 0.01$ versus the values of non-immunized controls of each inbred strain.

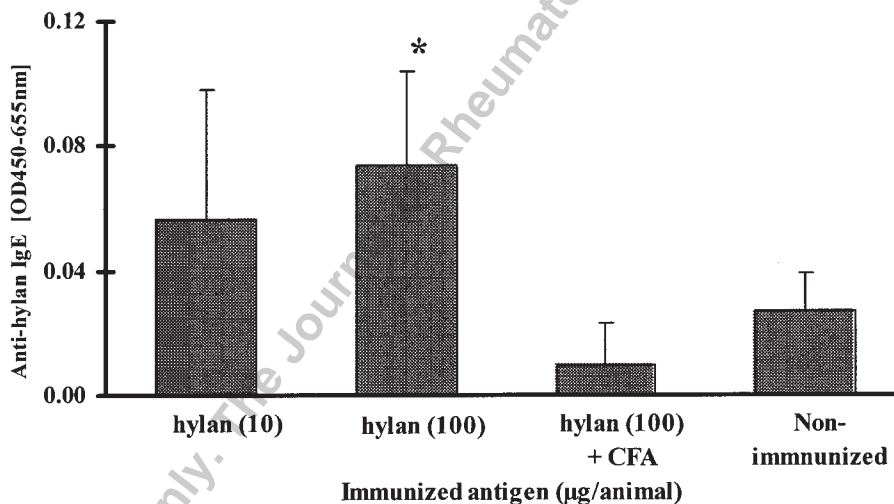


Figure 5. Anti-hylan IgE in the sera of immunized C3H/HeN mice. Values are the mean \pm SD in each group. * $p < 0.05$ versus the values of non-immunized controls.

have large molecular weight¹⁷. These findings also prompted us to investigate the potential immunogenicity of hylan.

In our study, hylan exhibited immunogenicity in guinea pigs, including the induction of immediate and delayed skin erythema, serum transmissible acute local hypersensitivity reactions, and specific IgG antibody productions. Immunization of hylan with CFA predominantly induced delayed-type skin reactions and production of more anti-hylan IgG. Histological analyses of the biopsy specimens also demonstrated immediate and delayed hypersensitivity

reactions. In contrast, immunization of SH did not induce any immune-mediated hypersensitivity reaction and specific antibody production in guinea pigs. This result was consistent with reported antigenicity studies of SH products in guinea pigs^{13,15,16}.

It has been reported that intradermally injected hylan B gel (an insoluble component of hylan G-F 20) was histologically found as variably sized basophilic islands in H&E-stained tissue sections²⁷. In our study, similar cluster materials were observed in the skin lesions of the hylan-immunized guinea pigs. At the injection sites, inflammatory

cell infiltration was found not only in the bottom layer of dermis but also in the upper and middle layer, where the cluster material was not found. It seemed that the specific immune response occurred against both hylan A fluid and hylan B gel. Hylan A fluid could not be observed histochemically by staining with alcian blue, presumably because of its water-soluble character. It was considered that hylan A fluid might have diffused away from the injection site before excision of the tissue, or during processing of the histological sections.

The ELISA studies in guinea pigs indicated several important findings regarding the product-specific immunogenicity of hylan. First, the immunogenic sites of hylan might bind to HA chains because the ELISA plates were coated with hylan polymers via specific binding with HABP. Second, we tested the reactivity of anti-hylan guinea pig sera against the 5 other commercial lots. The sera showed similar reactivity against these products as that against the original immunization lot, suggesting that hylan consistently contains the immunogenic components (data not shown). Further, from the 2 findings mentioned above, the possibility was ruled out that accidental batch contaminants in one lot produced immunogenicity in guinea pigs. Finally, the anti-hylan guinea pig sera did not have cross-reactivity against SH. This indicates that the immune reaction may be hylan-specific and not related to native HA. Hamburger, *et al*²⁴ suggested 6–8 kDa chicken proteins as possible antigens in the rabbit study. Further investigations are needed to confirm the antigenic determinants of hylan.

The immunogenicity of hylan was also definitively found in studies on mice. There was a significant difference in antibody production among the 3 strains and the C3H/HeN mouse was considered to be much more sensitive to hylan than the other strains. In C3H/HeN mice, immunization with hylan alone induced positive PCA reaction and specific IgE production, whereas the immunization with hylan + CFA did not induce such responses. The reason for the inhibitory effect of CFA on the anti-hylan IgE production could not be determined.

Immune reaction depends on many factors, including structure of antigen, dose of antigen, schedule and method of application, and genetic background of individuals²⁸. The method in our study is rather optimized to detect immunogenic potentials of medicinal products¹⁸. Therefore, our results cannot be directly compared with the frequency of allergic adverse reactions in humans. However, the test results may provide useful information to consider the possible participation of immunogenicity to hylan-induced severe reaction in patients with osteoarthritis.

Our experimental results contained several immune-mediated reactions such as IgE-mediated immediate-type hypersensitivity, immune-complex mediated Arthus reaction, and cell-mediated delayed-type hypersensitivity.

There is no report of skin testing in the patients with hylan-induced severe acute inflammatory reactions, and the sensitization condition in these patients is unknown. On the other hand, intradermal skin test has been routinely performed for confirming the immune status of animal models of antigen-induced arthritis²⁹. Our results in guinea pigs suggest that intradermal skin test might be useful for investigating the sensitization status against hylan G-F 20 in human patients and for predicting the occurrence of allergic adverse reactions in future use. We also suggest that ELISA studies for anti-hylan antibodies in the sera and/or synovial fluids of human patients might be valuable for these purposes.

The summary of pre-clinical safety studies of hylan G-F 20 has shown that repeated administration of the product induced anti-hylan and anti-chicken protein antibodies in primates³⁰. Further, Puttick, *et al*⁴ reported that the serum of one patient with acute local reactions had anti-chicken protein antibodies. Despite these findings, to our knowledge there is no clinical investigation to test potential immunogenicity of hylan G-F 20 in human patients. Indeed, the product brochure of hylan G-F 20 states that it is non-antigenic, non-inflammatory, and does not induce foreign body reactions³¹. We question what evidence provided grounds for such assertions. A possible reason for the absence of detailed investigation of immunogenicity may be that both SH and hylan G-F 20 have been approved in the USA as medical devices rather than drugs. In contrast, large numbers of immunogenicity tests of SH products were conducted, especially in Japan¹³⁻¹⁶, because these products have been approved as drugs and the evaluation of immunogenicity was required for a chemical to be registered as a drug in Japan.

Chemical modification of HA is intended to enhance its rheological properties and to achieve prolonged residence time of the injected material at the joint cavity². However, the relationships between physical properties or molecular weight of HA products and symptomatic efficacy in patients with OA have not been clearly shown^{2,32,33}. It should be noted that chemical modifications of native HA may alter not only physicochemical properties but also immunogenic potential. Concerning the product-specific immunogenicity of hylan, we found that the anti-hylan G-F 20 guinea pig sera had reactivity against another hylan product, HylaformTM, which has been used as a skin-filler in esthetic medicine (unpublished data). Clinically, adverse events indicative of allergic reactions after intradermal injection of the product have also been reported^{34,35}. These results suggest that several hylan-related products might exhibit immunogenicity not only in guinea pigs but also in humans.

In conclusion, our study investigated the immunogenicity of hylan G-F 20 in guinea pigs and mice. Despite the well-documented tolerability of the product in human patients with knee OA, clinicians and patients should be made aware

of the occurrence of adverse reactions possibly due to product-specific immunogenicity. It is also desirable that immunogenic potentials of hylan G-F 20 and sodium hyaluronate products should be strictly distinguished.

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