

The lymphoproliferative response to enzymatically digested gelatin in subjects with gelatin hypersensitivity

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Summary

Background This study was designed to evaluate the immunogenic characteristics of enzymatically digested gelatin, 'FreAlagin', employing the lymphoproliferative response in subjects with gelatin hypersensitivity. Our purpose was to assess the response of primed lymphocytes to the newly developed FreAlagin and compare it to the response to conventional gelatin.

Methods A gelatin-specific lymphocyte proliferation test (LPT) was performed in 110 children with adverse reactions to gelatin-containing vaccines, who showed positive gelatin-specific cell-mediated immunity and were thus diagnosed as having gelatin hypersensitivity. Gelatin-specific IgE was measured in all subjects. The antigenic activity of FreAlagin to lymphocytes was compared with that of conventional bovine gelatin. Positive and negative control specimens were obtained from the patients with anaphylaxis and from subjects inoculated with gelatin-free vaccine who showed no adverse reactions in order to establish the fluorometric ELISA system to determine IgE antibody to gelatin and LPT.

Results The lymphocyte activity against FreAlagin was much less than that to Wako gelatin and more than half of the subjects who reacted positively to Wako gelatin had a negative LPT to FreAlagin. Although 47% of the subjects had positive LPTs to FreAlagin, all but two still had lower SIs to FreAlagin compared with Wako gelatin.

Conclusion We conclude that the antigenic activity of FreAlagin as measured by the cell-mediated immune response is significantly less than that of conventional bovine gelatin. However, it is still necessary to perform clinical trials to show a reduced or absent clinical reactivity to FreAlagin in sensitized patients to conventional gelatin.

Keywords: gelatin allergy, hypersensitivity, measles vaccine, mumps vaccine, rubella vaccine, varicella vaccine, cellular immunology, lymphocyte transformation, anaphylaxis, collagenase, FreAlagin

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Introduction

Reports of hypersensitive reactions to gelatin containing vaccines or foods have increased in frequency during the

last few years [1,2]. Although gelatin-specific IgE is a primary cause of immediate reactions including anaphylaxis occurring post-vaccination [3,5], we have also reported that cell-mediated immunity is associated with both immediate and nonimmediate type reactions to gelatin-containing vaccine [6]. It is imperative to develop or to find stable and safe alternative additives for pharmaceutical use to replace

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conventional gelatin. A Japanese company has successfully developed an enzymatically digested gelatin 'FreAlagin', which comes in three types, none of which exhibited any reactivity to gelatin-specific IgG antibody raised in guinea-pigs. They also possessed an extremely low reactivity with gelatin-specific IgE antibody from the serum of patients who had experienced an anaphylactic reaction to gelatin after vaccination or after eating gelatin-containing foods [7]. Because cell-mediated immune responses are also associated with gelatin hypersensitivity [6], this newly developed gelatin must also be tested for gelatin-specific cell-mediated immunity. Employing an *in vitro* gelatin-specific lymphocyte proliferation test (LPT), we investigated the reactivity of FreAlagin to lymphocytes obtained from subjects with adverse reactions to gelatin-containing vaccines and cell-mediated immune responses to gelatin.

Materials and Methods

Subjects

The study was performed from December 1996 to January 1998 at four paediatric outpatient clinics. One hundred and ten infants and children were diagnosed as having a gelatin hypersensitivity after having adverse reactions to inoculation of various live or inactivated vaccines containing gelatin, and subsequently were shown to have a positive lymphocyte proliferation test to gelatin. Of the 110 subjects, 37 received primary DTP vaccine, 30 booster DTP, 21 measles vaccine, seven mumps vaccine, seven rubella vaccine, and eight varicella vaccine. In the primary DTP vaccine group, the DTP vaccine administered was the first gelatin-containing vaccine for all subjects. The diagnosis of gelatin hypersensitivity depended on a lymphocyte proliferation response specific to conventional gelatin. Adverse reactions were varied. Some children had sharply demarcated redness, swelling and induration at the injection site exceeding 1 cm in diameter, appearing more than 12 h after administration of vaccine and persisting for a few days. Another type of adverse reaction consisted of a measles-like miliary rash without itching also appearing more than 12 h after administration of vaccine and disappearing without any pigmentation within a couple of days. Immediate-type reactions included urticarial rashes and symptoms of anaphylaxis. The characteristics of the subjects are summarized in the table. Informed consent was obtained from the parents of the patients.

Determination of gelatin-specific IgE antibody

IgE antibody to bovine gelatin (Wako Junyaku, Tokyo, Japan) was measured by a fluorometric ELISA system. Microplates were coated with bovine gelatin (10 µg/mL).

After incubation of the serum sample with immobilized antigens, antihuman IgE antibody conjugated with β-D-galactosidase (Pharmacia, Upsala, Sweden) was added. The enzyme reaction substrate was 0.1 mmol/L 4-methyl-umbelliferyl-β-D-galactoside (Sigma Chemical Co., St. Louis, USA). The fluorescence unit was measured on a fluorometric microplate reader (f max Fluorescence Microplate Reader; Molecular Devices, Sunnyvale, CA, USA).

To establish the system, positive sera were obtained from the patients with anaphylactic reactions, while control sera were from more than 20 recipients of gelatin-free vaccine who did not show any adverse reactions. The positive/negative cut-off has been set at the point of maximal sensitivity (i.e. 0.05 Ua/mL) in the assay system.

In vitro lymphocyte proliferation test (LPT)

The *in vitro* lymphocyte proliferation test used resembles that described previously [6]. Briefly, peripheral blood mononuclear cells were obtained by Ficoll-Hypaque gradient centrifugation from the subjects after the onset of the reaction. Cells were cultured at a concentration of 5×10^5 cells/well in 200 µL of RPMI 1640 containing 10% autologous serum. Triplicate cultures were set up with medium alone (control) or with varying concentrations of gelatin antigen. Cultures were incubated at 37°C in a 5% CO₂ atmosphere, usually for 5 days. Then 1 µCi of ³H-thymidine was added to each culture and 16 h later the cultures were harvested onto fibreglass filters using a multiple automated sample harvester. Incorporation of radioactive thymidine into harvested cells was determined in an LSC-3500 Aloka liquid scintillation system (Aloka, Tokyo, Japan). The results are expressed as ratios of ³H-thymidine incorporation in antigen stimulated cultures relative to that in control cultures (stimulation index; SI). The positive/negative cut-off value was determined on the basis of comparison with the distribution of the SI values obtained with more than 30 subjects who never had received gelatin containing vaccines and had never experienced adverse reactions. The standard distribution of obtained data was calculated and mean value + 3 S.D. was considered to be the cut-off point. A stimulation index greater than or equal to 1.5 was considered to be a positive response.

Gelatin antigen preparation

Wako gelatin was used as conventional gelatin antigen. Three types of FreAlagin, type R, type AD and type P, have been manufactured and type R (FreAlagin R) was utilized throughout this study [7].

The optimal concentration of Wako gelatin and FreAlagin was predetermined for each batch of antigen by testing various dilutions of antigen (1, 10, 50, 100, 250 µg/mL) in

cultures of lymphocytes obtained from subjects known to react to Wako gelatin and/or FreAlagin. The maximal response of lymphocytes was elicited in concentrations of 100 or 250 $\mu\text{g/mL}$ for both Wako gelatin and FreAlagin.

Thus, 100 and 250 $\mu\text{g/mL}$ concentrations have been utilized throughout the present study.

Statistical analysis

The Kormogorov-Smirnov test was used to check the normality of distribution of the difference in each subject between the SIs against Wako gelatin in LPT and those against FreAlagin. Normal distribution of the data in the subjects inoculated with primary DTP, booster DTP and measles vaccine was confirmed, whereas it was not clearly confirmed in the entire study group and in the subjects inoculated with mumps, rubella and varicella vaccine. Thus, the differences between values of SI in LPT against Wako gelatin and those against FreAlagin were compared by the two-tailed paired *t*-test in the subject groups inoculated with primary DTP, booster DTP and measles vaccine and the Wilcoxon matched-pairs signed-ranks test was used for the entire study group and for the subject groups inoculated with mumps, rubella and varicella vaccine. In addition, according to the results of the Kormogorov-Smirnov test, the two-tailed paired *t*-test was used for the data obtained from the positive IgE group and skin eruption group, whereas the Wilcoxon matched-pairs signed-ranks test was used for the IgE negative group and the group with local swelling. A *P*-value of less than 0.05 was considered statistically significant. All data are expressed as mean $\pm$ 1 S.D.

Results

Fifty-eight subjects (53%) with positive lymphoproliferative responses to conventional gelatins had SIs of less than 1.5 to FreAlagin. The geometric mean SI to Wako gelatin was 6.31 ± 5.88 , whereas that to FreAlagin was 2.12 ± 2.09 . Thus the mean activity of sensitized lymphocytes against FreAlagin was 66.4% less than that against Wako gelatin ($P < 0.0001$). Thus the activity of sensitized lymphocytes against FreAlagin was decreased by 66.4% compared to that against Wako gelatin which was statistically significant ($P < 0.0001$). However, it should be noted that 47% of the subjects' lymphocyte responses still

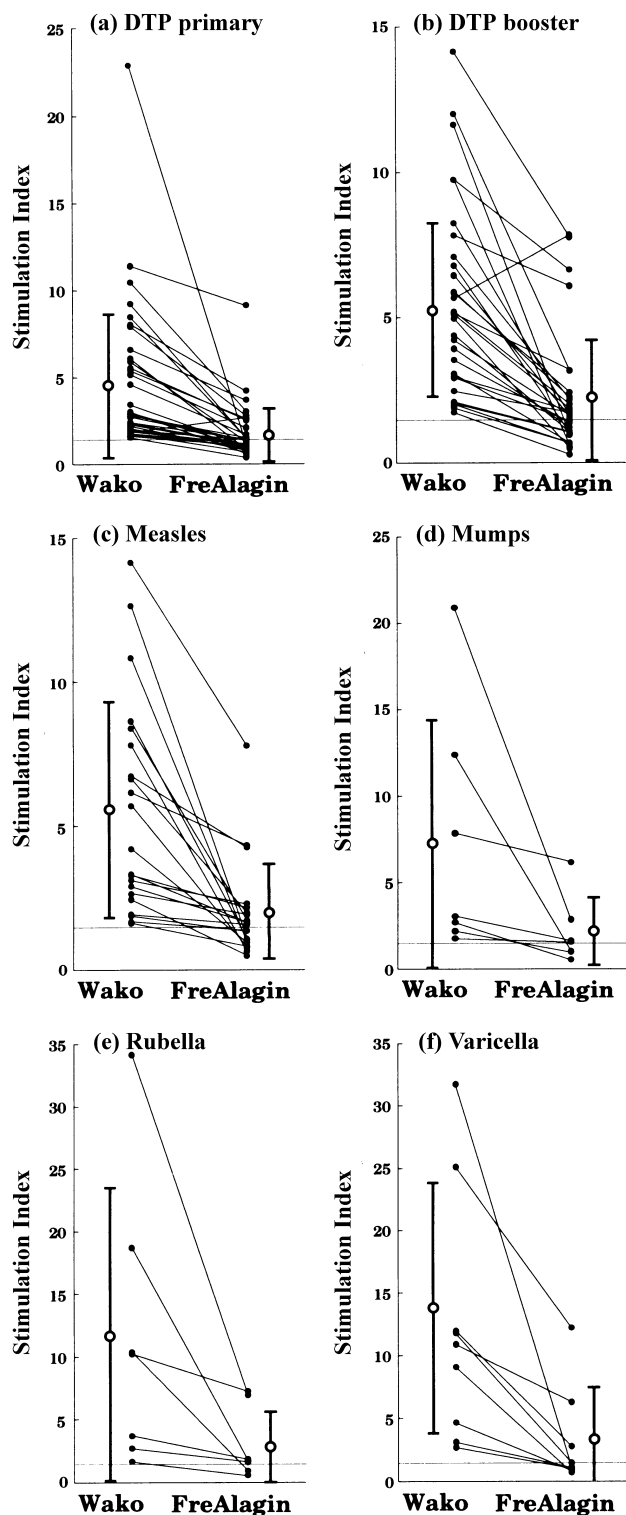


Fig. 1. Lymphoproliferative responses directed against Wako gelatin and FreAlagin. Subjects inoculated with (a) DTP primary vaccination, (b) DTP booster vaccination, (c) measles vaccination, (d) mumps vaccination, (e) rubella vaccination, (f) varicella vaccination. Black dot represents stimulation index to Wako gelatin and FreAlagin in each subject. Vertical bar with open circle shows geometric mean SI $\pm$ 1 standard deviation. The horizontal line defines the positive negative cut off point at SI of 1.5.

remained positive. Among the 52 patients with positive lymphocyte proliferation tests to FreAlagin, only two (one of the primary DTP recipients and one of the booster DTP group) had higher SIs to FreAlagin than to Wako gelatin. Fig. 1 shows the distribution of SIs in the lymphoproliferative response directed against Wako gelatin and FreAlagin among the subjects inoculated with various vaccines. Geometric mean SIs to Wako gelatin were 4.54 ± 4.14 in the recipients of primary DTP vaccine; 5.63 ± 3.27 in the booster DTP group; 5.56 ± 3.73 in measles; 7.27 ± 7.15 in mumps; 11.64 ± 11.57 in rubella; 13.50 ± 9.94 in varicella whereas those to FreAlagin were 1.74 ± 1.52 ; 2.21 ± 2.09 ; 1.95 ± 1.67 ; 2.11 ± 1.93 ; 2.97 ± 2.85 ; and 3.32 ± 4.05 , respectively. Thus, the decrease in the mean SI in the recipients of each vaccine were 61.7%, 60.7%, 64.9%, 71.0%, 74.5%, 75.4%, respectively. The differences between the lymphocyte activities to Wako gelatin and those to FreAlagin among the recipients inoculated with all of these vaccines were statistically significant ($P < 0.0001$ in primary DTP, booster DTP, and measles; $P = 0.0180$ in mumps and rubella; $P = 0.0117$ in varicella). Fig. 2 shows the comparison between SIs to Wako gelatin and those to FreAlagin among the subjects inoculated with various vaccines, and are arranged in accordance with the presence or absence of gelatin-specific IgE and with the type of clinical reaction observed. Of these subjects, 12 were gelatin-specific IgE positive whereas the remaining 98 were negative. Geometric mean SIs to Wako gelatin were 7.31 ± 7.70 in the subjects positive for IgE and 6.19 ± 5.66 in the subjects without IgE, whereas those to FreAlagin were 2.35 ± 3.19 and 2.09 ± 1.93 , respectively. The decrease in SI in the recipients of each vaccine was 67.9% and 66.2%, respectively. The differences between the lymphocyte activities to Wako gelatin and those to FreAlagin among the subjects with and without IgE were statistically significant ($P < 0.0001$) in both groups.

Twenty-two subjects experienced a mild systemic cutaneous reaction, and 77 only developed local swelling and induration at the injection site. Geometric mean SIs to Wako gelatin were 6.24 ± 4.32 in the subjects with a skin eruption and 6.17 ± 6.01 in the subjects with a local reaction, whereas those to FreAlagin were 2.08 ± 3.19 and 2.10 ± 1.95 , respectively. The decreases in SI in the recipients of each vaccine were 56.6% and 66.0%, respectively. The differences between the lymphocyte activities to Wako gelatin and those to FreAlagin among the subjects with skin eruption and with local reaction were statistically significant ($P < 0.0001$) in both groups.

All of the data obtained in this study were re-analysed utilizing actual c.p.m. data instead of SIs, and the results corresponded to those obtained from the SI data. Background activities of lymphocytes in each subject in the present study were quite stable, being around several

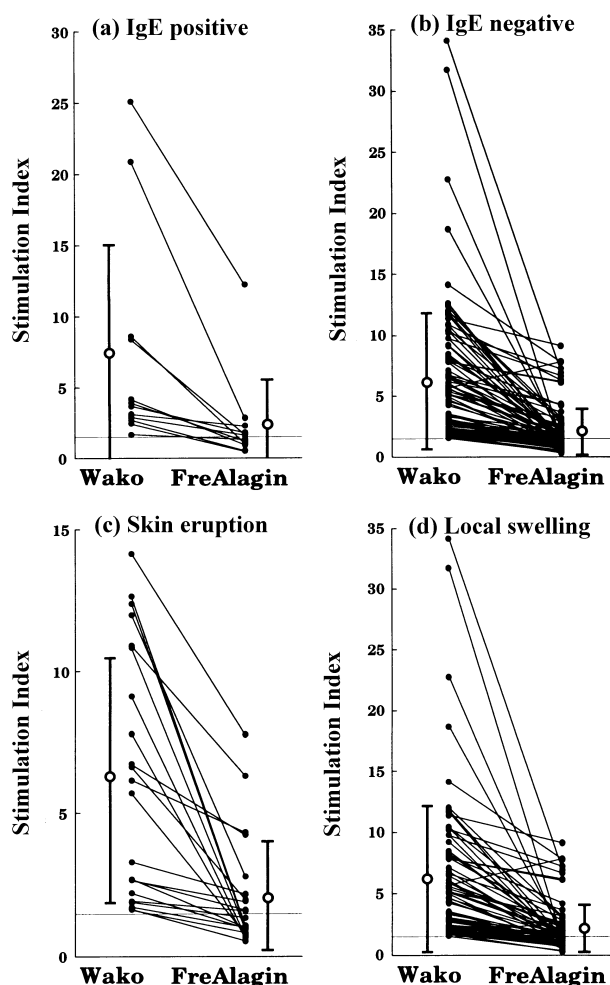


Fig. 2. Lymphoproliferative responses directed against Wako gelatin and FreAlagin. (a) Subjects with positive gelatin-specific IgE, (b) those with negative gelatin-specific IgE, (c) those with skin eruption as adverse reaction, (d) those with local swelling in the injection site as adverse reaction. Black dot represents stimulation index to Wako gelatin and FreAlagin in each subject. Vertical bar with open circle shows geometric mean SI $\pm$ 1 standard deviation. The horizontal line defines the positive/negative cut-off point at SI of 1.5.

hundred to 1500 c.p.m., in spite of culturing lymphocytes in relatively high numbers (5×10^5 /well).

In order to eliminate the possibility that the FreAlagin nonspecifically reduces lymphocyte responsiveness, we have performed another set of experiments. Lymphocytes from 10 control subjects who had never received gelatin containing vaccine have been cultured under stimulation with phytohemagglutinin ($10 \mu\text{g/mL}$) in the presence or absence of FreAlagin (250, 100 $\mu\text{g/mL}$) or Wako gelatin (250, 100 $\mu\text{g/mL}$). Neither a suppressive nor a stimulatory effect of FreAlagin or Wako gelatin to PHA stimulated lymphocytes in culture has been observed.

Table 1. Subject characteristics

Vaccines	Subject no.	Mean age (mo)	Gender		Gelatin* (mg/shot)	Gelatin IgE			Reaction to vaccine			Skin rash	
			Male	Female		Post.	Negat.	Systemic†	Local	Immediate	Non-immediate	Urticarial	Not urticarial
DTP primary	37	11.2	24	13	0.02~0.1	0	37	1	36	0	37	0	37
DTP booster	30	29.6	15	15	0.02~0.1	1	29	0	30	0	30	0	0
Measles	21	15.1	10	11	1.0~2.5	7	14	20	1	7	14	7	13
Mumps	7	50.6	6	1	1.0~2.5	2	5	4	3	2	5	2	2
Rubella	7	76.3	3	4	1.0~2.5	1	6	3	4	1	6	1	2
Varicella	8	28.4	3	5	13	1	7	5	3	1	7	1	4

*These data were obtained from the manufacturer's literature, which gives the contents and the volume of vaccine administered except for that of DTP. Amount of gelatin contained in DTP was estimated by calculation from the basic materials used and the dilution factor of the manufacturing process. †Systemic reactions include a mild systemic cutaneous reaction (22 cases) and anaphylactic reaction (11 cases).

Discussion

Bovine and porcine gelatin have been used as additives in many biological preparations including vaccines [8]. However, an increasing number of adverse reactions in children inoculated with live attenuated virus vaccines containing gelatin have been reported during the last few years [1–6,9–11]. In these reports, investigations have focused on the presence of gelatin-specific IgE, however, we recently showed that cell-mediated immunity is also important in the development of gelatin hypersensitivity [6].

Vaccine manufacturers in Japan have tried to remove gelatin from vaccine preparations, or to use alternative additives including human albumin (Inouye T: personal communication). However, there is still a need to use gelatin as a stabilizer or additive for some biological preparations such as cytokines [12,13], mainly because of its capability to block the adsorption of pharmaceuticals onto vessels or to keep them dispersed. Thus, in an effort to create a gelatin preparation with decreased antigenicity, an improved gelatin called FreAlagin was developed by Sakai *et al* [7] employing a process of enzymatic digestion. They have reported that the binding of FreAlagin to gelatin-specific IgE from patients with gelatin hypersensitivity has been shown to be extremely low. More specifically, the reactivity of FreAlagin R to gelatin-specific IgE obtained from 25 of 46 patients was demonstrated to be less than 10%, whereas Wako gelatin reacted with the same sera from all 46 patients. Because cell-mediated immune responses to FreAlagin have not yet been investigated, we tested whether FreAlagin provokes a decreased response in an *in vitro* LPT.

We found that mean SIs to FreAlagin in the LPT were much less than those to Wako gelatin and that more than half of the subjects who had reacted positively to Wako gelatin had a negative LPT to FreAlagin. Although 47% of the subjects had positive LPTs to FreAlagin, all but two still had lower SIs to FreAlagin compared with Wako gelatin. Nonetheless, epitopes capable of stimulating sensitized T cells in a substantial minority of subjects remain in FreAlagin.

It is important to consider the equivalency of concentration of gelatin that might be present in the vaccines with that under our experimental conditions. The amount of gelatin administered varies from 20 to 100 µg/dose in DTP [14], around 1.0–2.5 mg/dose in measles, rubella, and mumps vaccines and 13 mg/dose in varicella vaccine. In contrast, Wako gelatin and FreAlagin were utilized as an antigen in concentrations of 250 or 100 µg/mL in the present study. Thus, in the microenvironment at the local injection site just after administration of vaccine where lymphocytes and other immunologically competent cells are migrating, concentrations of gelatin administered are approximately

equivalent to those of our experimental conditions *in vitro* allowing for diffusion factors.

Some caution should be used in interpreting our results. We used samples from subjects hypersensitive to conventional gelatin and who showed a decreased responsiveness of the lymphocytes to FreAlagin. However, it is still necessary to perform clinical trials to show reduced or absent clinical reactivity to this product in patients sensitized to conventional gelatin. In addition, it remains uncertain whether FreAlagin itself can prime and cause hypersensitivity in human beings. However, we think this possibility is unlikely for at least one reason. One of the authors (TN) observed that IgG antibodies against gelatin were not generated in animal models after administration of FreAlagin co-stimulated with adjuvant whereas they were detected after immunization with Wako gelatin co-stimulated with adjuvant (unpublished data). Further research will be required to explore the immunogenicity of FreAlagin in human beings utilizing lymphocytes from subjects inoculated with FreAlagin containing vaccines or other preparations. In terms of lymphocyte reactivity, we should continue attempts to devise or develop more satisfactory substances which provoke no response whatever.

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