

Gelatin-specific cellular immune responses persist for more than 3 years after priming with gelatin containing DTaP vaccine

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Summary

Background Gelatin-specific cell-mediated immunity develops in subjects inoculated with gelatin containing DTaP vaccine. However, it is not yet known whether such established sensitization to gelatin disappears or persists with time.

Objective The aim of this study was to follow the patients with gelatin sensitization elicited by DTaP vaccination for their lymphocyte responsiveness and IgE, IgG antibody specific to gelatin over several years and to compare the activities with those at the time of enrolment into the study.

Methods We studied 28 subjects who developed positive lymphocyte proliferation test (LPT) after receiving gelatin containing DTaP vaccine and eight subjects who had a negative LPT after inoculation of non-gelatin DTaP. Determination of IgE, IgG antibodies and specific lymphoproliferative response directed against gelatin were performed at enrolment and on follow up.

Results None of the subjects had antibody to gelatin at enrolment and none developed gelatin IgE or IgG during follow-up. There was no significant difference in the SIs of the subjects receiving gelatin DTaP ($P = 0.150$, 95% CI, -0.198 – 0.032), whereas lymphocyte activity to gelatin increased between enrolment and follow-up in the subjects with non-gelatin DTaP ($P = 0.011$, 95% CI, 0.063 – 0.338).

Conclusion Gelatin-specific lymphocyte activity persists at comparable levels for more than 3 years in subjects who acquire a positive LPT response to gelatin after receiving primary DTaP vaccine containing gelatin. Furthermore, five out of eight subjects initially with negative LPT to gelatin have been shown to acquire specific LPT with time.

Keywords gelatin hypersensitivity, allergy, vaccine, cellular immunology, lymphocyte transformation, gelatin IgG, gelatin IgE

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Introduction

Gelatin-containing vaccines occasionally cause clinical reactions including anaphylaxis and such sensitized subjects may develop gelatin-specific lymphocyte responses [1–4]. Regarding the mechanism by which children were sensitized to gelatin, Nakayama et al. [5] published an epidemiological observation suggesting that the priming process of this particular hypersensitivity was attributable to serial inoculation of gelatin-containing DTaP vaccine. We also conducted a prospective laboratory-based study that showed that all subjects inoculated with gelatin-containing DTaP vaccine developed positive T cell responses against gelatin [6]. These investigations were followed by the observation by Sakaguchi et al. [7] that the vast majority

of patients with positive gelatin IgE had received gelatin-containing DTaP vaccination. Furthermore, epidemiological studies and our own clinical experience indicated that adverse reactions related to gelatin decreased dramatically once gelatin was eliminated from DTaP vaccine, emphasizing the significant role of gelatin contained in DTaP vaccine in causing gelatin hypersensitivity [8].

However, it remains unknown how long this established sensitization to gelatin caused by gelatin-containing DTaP vaccine persists. As the majority of gelatin-containing DTaP vaccine recipients develop a specific lymphoproliferative response to gelatin [6], there is still a concern that such lymphocyte activity might somehow predispose to harmful events in the future. Thus, it is important to elucidate how long this particular lymphocyte response persists. To address this issue, we determined gelatin-specific IgE and IgG antibody and measured the lymphocyte proliferation response serially for over 3 years in subjects who had a positive gelatin-specific lymphocyte response and a history of receiving gelatin-containing DTaP vaccine.

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Materials and methods

Subjects

The study groups were composed of 28 recipients (21 boys, seven girls) of gelatin-containing DTaP vaccine with mild local reactions at the injection site, with a mean age of 10.4 months (range, 4–24 months) who were found to have a positive lymphocyte response; and eight recipients (four boys, four girls) of non-gelatin DTaP vaccine without any reactions, with a mean age of 9.5 months (range 8–11 months) who did not develop a positive lymphocyte response. The clinical features of the study subjects are summarized in Table 1. The interval between clinical reactions and blood sampling was 2 weeks. Adverse reactions were composed of 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. Of the present study subjects none developed immediate-type reactions. Informed consent was

obtained from the parents of the subjects after the project was thoroughly explained.

Determination of gelatin-specific IgE

The fluorometric ELISA system, used to determine IgE antibody to bovine gelatin (Wako Junyaku, Tokyo, Japan), resembles one described previously [6]. Briefly, microplates were coated with bovine gelatin (10 µg/mL). After incubation of the serum sample with immobilized antigens, anti-human IgE antibody conjugated with β -D-galactosidase (Pharmacia, Upsala, Sweden) was added. The enzyme reaction substrate was 0.1 mmol/L 4-methylumbelliferyl- β -D-galactoside (Sigma Chemical Co., St Louis, MO, USA). The fluorescence unit was measured on a fluorometric microplate reader (*f* max Fluorescence Microplate Reader; Molecular Devices, Sunnyvale, CA, USA). Antibody value was calculated from the standard curve made by diluting a positive serum that was

Table 1. Clinical characteristics of subjects

Patient No.	Age (months)*	Sex	Gelatin (mg/shot)**	Reactions
1	10	M	0.02–0.1	Erythema, swelling at the local injection site
2	5	M	0.02–0.1	Erythema, swelling at the local injection site
3	9	M	0.02–0.1	Erythema, swelling at the local injection site
4	4	M	0.02–0.1	Erythema, swelling at the local injection site
5	6	F	0.02–0.1	Erythema, swelling at the local injection site
6	9	M	0.02–0.1	Erythema, swelling at the local injection site
7	15	M	0.02–0.1	Erythema, swelling at the local injection site
8	9	M	0.02–0.1	Erythema, swelling at the local injection site
9	10	F	0.02–0.1	Erythema, swelling at the local injection site
10	10	M	0.02–0.1	Erythema, swelling at the local injection site
11	6	M	0.02–0.1	Erythema, swelling at the local injection site
12	7	M	0.02–0.1	Erythema, swelling at the local injection site
13	24	M	0.02–0.1	Erythema, swelling at the local injection site
14	7	M	0.02–0.1	Erythema, swelling at the local injection site
15	14	F	0.02–0.1	Erythema, swelling at the local injection site
16	14	M	0.02–0.1	Erythema, swelling at the local injection site
17	8	F	0.02–0.1	Erythema, swelling at the local injection site
18	7	M	0.02–0.1	Erythema, swelling at the local injection site
19	7	M	0.02–0.1	Erythema, swelling at the local injection site
20	8	F	0.02–0.1	Erythema, swelling at the local injection site
21	12	M	0.02–0.1	Erythema, swelling at the local injection site
22	7	F	0.02–0.1	Erythema, swelling at the local injection site
23	10	M	0.02–0.1	Erythema, swelling at the local injection site
24	23	M	0.02–0.1	Erythema, swelling at the local injection site
25	12	M	0.02–0.1	Erythema, swelling at the local injection site
26	8	M	0.02–0.1	Erythema, swelling at the local injection site
27	8	M	0.02–0.1	Erythema, swelling at the local injection site
28	22	F	0.02–0.1	Erythema, swelling at the local injection site
29	9	F	None	None
30	11	F	None	None
31	8	F	None	None
32	10	M	None	None
33	11	M	None	None
34	8	F	None	None
35	11	M	None	None
36	8	M	None	None

*Age at enrolment in the study. **These data were obtained from the manufacturer's literature, which gives the contents and the volume of vaccine administered except for that of DTaP. Amount of gelatin contained in DTaP was estimated by calculation from the basic materials used and the dilution factor of the manufacturing process.

determined properly. The potential detection limit of the test was set at 0.05 U/mL for IgE.

Determination of gelatin-specific IgG antibody

Gelatin-specific IgG antibodies were measured by a photometric ELISA system. Microtitre plates were coated with 100 µL/well of the antigen solution (bovine gelatin 10 µg/mL) at 4 °C overnight. The plates were washed three times with PBST and incubated for 2 h at 37 °C with 300 µL/well of 1% bovine serum albumin in PBST. After washing the plates three times, 100 µL/well of serum samples diluted with 1% bovine serum albumin in PBST were added and incubated for 2 h at room temperature. The plates were washed three times and 100 µL/well of horseradish peroxidase conjugated goat anti-human IgG (Cappel Product, Durham, NC, USA) were added and incubated for 2 h at room temperature. The enzyme reaction substrate was 1 mg/mL o-phenylenediamine solution (Sigma Chemical Co.) and 0.0012% hydrogen peroxide in 0.05 M citrate buffer (pH 5.0). The plates were loaded with 100 µL/well of substrate solution and incubated at 37 °C for 15 min in the dark. The reaction was stopped by the addition of 100 µL/well of 1 M sulphuric acid. The absorbance (at 492 nm) was measured on a photometrical plate reader (Toso, MPRA41, Tokyo, Japan). The levels of specific antibodies were represented as an arbitrary unit (U/mL). The U/mL of each serum was calculated from standard titration curves of a pooled serum known to have highly positive gelatin IgG. The cut-off value of gelatin IgG was determined as mean value $\pm$ 3 SD of OD values in the sera of the negative control that was set at 0.1 U/mL for IgG.

In vitro lymphocyte proliferation test (LPT)

The *in vitro* lymphocyte proliferation test used resembles that described previously [6]. 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). Regarding the positive/negative cut-off point in the LPT, we assayed more than 35 specimens from subjects who had never received gelatin-containing vaccines. The age distribution of these subjects was from 5 to 24 months. Standard deviation of the data was calculated and mean value $\pm$ 3 SD was adopted as our cut-off point. A stimulation index $\geq$ 1.5 was considered to be a positive response. Actually, mean SI and standard deviation were 0.96 and 0.17, respectively, thus 1.5 was 3.18 SD apart from the mean value.

Bovine gelatin was used as conventional gelatin antigen (Wako Junyaku, Tokyo, Japan). The optimal concentration of Wako gelatin 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. The maximal response of lymphocytes was elicited in concentrations of 100 or 250 µg/mL. 100 and 250 µg/mL concentrations have been utilized throughout the present study.

Statistical analysis

All SIs in the LPT were log-transformed prior to analysis and skewness and kurtosis in the distribution of SIs were evaluated. The differences between values of the SIs at the beginning of the study and at follow-up were compared by means of the two-tailed paired *t*-test along with evaluation of 95% confidence intervals (CI) for the mean value of SIs. Statistical significance level was set at $P < 0.05$. All analyses were carried out with SAS software (SAS Institute, Cary, NC, USA) package.

Results

Gelatin-specific IgE and IgG antibody as well as the lymphoproliferative response directed against gelatin was determined at the time of the initial clinical reaction and at 2–3.5 years after enrolment into the study. None of the subjects had gelatin-specific IgE or IgG at enrolment into the study. Also, none developed IgE and/or IgG antibody at follow-up, 2–3.5 years after enrolment.

Regarding the lymphoproliferative response to gelatin, the geometric mean SIs with 95% CI limits to gelatin among subjects with gelatin DTaP vaccine were 3.838 with 95% CI limits of 2.752 to 4.925 (range, 1.52–11.39) and 3.415 with 95% CI limits of 2.331 to 4.499 (range, 0.76–10.65) at enrolment and on follow-up, respectively. For subjects with non-gelatin DTaP they were 1.070 with 95% CI limits of 0.958 to 1.182 (range, 0.82–1.26) and 1.801 with 95% CI limits of 1.203 to 2.400 (range, 1.05–3.09), respectively. There was no significant difference in the SIs of the subjects with gelatin DTaP ($P = 0.150$, 95% CI, -0.198 – 0.032 , (Fig. 1, top), whereas lymphocyte activity to gelatin increased between enrolment and follow-up in the subjects with non-gelatin DTaP ($P = 0.011$, 95% CI, 0.063 – 0.338 , (Fig. 1, bottom). Gelatin-specific lymphocyte activity persists at comparable levels for more than 3 years in subjects who acquire a positive LPT response to gelatin after primary DTaP vaccine containing gelatin. Furthermore, five out of eight subjects initially with a negative LPT to gelatin acquired specific LPT with time.

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

Discussion

Recently published observations from our laboratory demonstrated that gelatin contained in DTaP vaccine sensitizes the lymphocytes of recipients [6]. However, it is not yet well defined how long such sensitization persists or whether it can progress

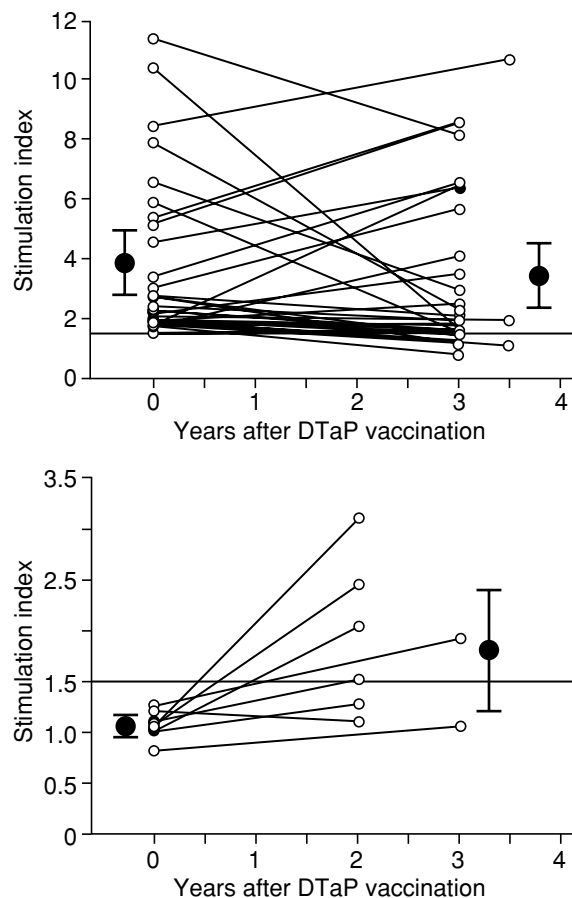


Fig. 1. Lymphoproliferative response to gelatin in recipients of gelatin-containing DTaP vaccine who developed a positive response shortly after vaccination and 3–3.5 years later (top), and in those inoculated with non-gelatin DTaP vaccine shortly after vaccination and 2–3 years later (bottom). Abscissa represents years after vaccination. Ordinate the stimulation index. The horizontal line defines the positive negative cut-off point at SI of 1.5. Vertical bar with closed circle shows geometric mean SI with 95% CI limits.

to IgE sensitization resulting in the potential for anaphylaxis in the future. In the present study, we found that once subjects acquired specific cell-mediated immunity to gelatin, it lasts a relatively long period of time. In addition, a substantial number of subjects who did not receive gelatin-containing DTaP acquire a specific lymphocyte response directed against gelatin with time. To our knowledge this is the first long-term follow-up study of the immune responses specific to gelatin in the same vaccinees.

In spite of the presence of a positive lymphocyte response to gelatin during this observation period, none of the subjects in the present study have yet developed IgE and/or IgG sensitization, indicating that such lymphocyte responses do not directly link to the production of IgE and/or IgG and clinical reactions in the future in most recipients. Numerous factors including HLA class II genetic polymorphism are possibly involved in the process linking specific lymphocyte activation to IgE sensitization [9]. Nevertheless, the induction of persistent cell-mediated immunity to gelatin should be avoided as much as possible, because there remains a valid concern that such activity might be the basis for or underlie generation of IgE in the future. Combining the results from the current study with the previous

observation that elimination of gelatin from DTaP resulted in a marked decline of adverse reactions to vaccines [8], we conclude that elimination of gelatin from all DTaP preparations should be accomplished. The fact that many European countries and the United States have started to employ acellular pertussis vaccine that contains gelatin, makes the resolving of this issue an urgent matter.

It is important not only in the field of vaccinology but also in that of allergology that a substantial number of subjects who did not receive gelatin-containing DTaP acquire a specific lymphocyte response directed against gelatin with time. Those subjects had never received gelatin parenterally, indicating that dietary gelatin might be the possible source of stimulation. According to a report of the Glue & Gelatin Manufacturers Association of Japan and that of WAKODO Co., Ltd. (personal communication), Japanese import of cow bone as raw material for the production of gelatin doubled over the last decade and the consumption of a pre-cooked weaning diet for infants increased rapidly during the same period. Dietary habits surrounding infants and young children in Japan have continuously changed and the rapid westernization of lifestyle of the Japanese people during recent decades might have had an enormous influence on the population-based immune status to food antigens.

In regard to the characteristics of the subjects in the present study, one might consider that the study groups should include four different populations; subjects who have had a local reaction both with gelatin DTaP and non-gelatin DTaP and subjects who did not have any reaction to both gelatin DTaP and non-gelatin DTaP. This consideration might be valid if a local reaction is definitely and only related to gelatin in DTaP preparations. However, symptoms developing after DTaP vaccination are not necessarily related to gelatin allergy and their cause remains unexplained. In our previous paper we have shown that, regardless of the occurrence of adverse reactions, all subjects inoculated with gelatin-containing DTaP vaccine develop positive T cell responses against gelatin, compared to less than 13% of all recipients of DTaP vaccine without gelatin [6]. Thus, we believe it is invalid to divide subjects inoculated with gelatin-containing DTaP into two populations in accordance with whether they experienced a local reaction or not, especially in a follow-up study. The problem of gelatin contained in DTaP vaccine was not that it might have caused a local clinical reaction but that it provided primary sensitization to the recipients. The situation that all subjects in the group of gelatin DTaP in the present study showed a local reaction resulted from the mode of sampling, not from a selected study design. It was relatively easy to persuade parents of the subjects to be enrolled in a follow-up project if their children had had reactions to vaccines.

A potential weakness of our study is its small sample size, especially in that of recipients of non-gelatin DTaP vaccine. Many participants have dropped out from follow-up mainly because that they did not show any clinical symptoms or signs throughout the period monitored. Thus we were unable to convince parents or guardians of vaccinees to participate continuously. This is especially true for those with negative results in their initial laboratory examination. Therefore, the available data were limited, and do not allow a conclusive explanation of the time kinetics of cell-mediated immunity specific to gelatin for the follow-up period. Our data should be considered

preliminary and should be validated and tested. However, consistent positiveness in LPT activity in the group of recipients of gelatin DTaP is a powerful signal even in a small cohort. It is unlikely this consistency would get much more variable in a larger study. It seems to be valid that our estimation is not far from actuality. With respect to the data obtained from the recipients of non-gelatin DTaP, as an inevitable consequence regarding concern about universal sensitization of the general population to specific antigen, additional studies of a larger size are warranted to confirm the findings in subjects with the same background.

In conclusion, in combination with earlier studies [6–8], it is clear that children may develop sensitization to gelatin by inoculation of gelatin-containing DTaP vaccine that lasts a relatively long period of time. It is also possible that universal sensitization to gelatin in the general population might have occurred during recent years. The findings in this report provide useful data that help further understanding of gelatin-related issues in the field of allergology as well as in that of vaccinology.

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