

Enhanced sensitisation of mice with diphtheria tetanus acellular pertussis vaccine to local swelling reaction to the booster immunisation

Akihiko Yamamoto^{a,*}, Noriyo Nagata^b, Masaki Ochiai^a,
Michiyo Kataoka^a, Hiromi Toyoizumi^a,
Kenji Okada^c, Yoshinobu Horiuchi^a

^a Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases,
4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan

^b Department of Pathology, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan

^c Department of Paediatrics, Minamifukuoka National Sanatorium, 4-39-1 Yakatahara, Minami-ku, Fukuoka 811-1394, Japan

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Abstract

Severe local swelling has been regarded as a serious safety problem for the booster immunisations of diphtheria tetanus acellular pertussis combined (DTaP) vaccine and DT combined toxoids (DT-td). We attempted to search for the factor of DTaP vaccines possibly contributing to the enhanced local reaction by using the mouse hind paw swelling reaction. Mice were immunised intramuscularly with DTaP vaccine twice at 1-month interval and were challenged their hind paw with one of the antigens of DTaP vaccine 2 weeks later. D-td was shown to elicit the strongest swelling among the vaccine antigens. No causal relationship was found between the swelling and the level of immunoglobulin G (IgG) or IgE in mice. Residual pertussis toxin (PT) activity of DTaP vaccines for immunisation was shown to play a role in the enhanced sensitisation of mice to the D-td-related hind paw swelling.

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1. Introduction

Diphtheria tetanus whole cell pertussis combined vaccine (DTwP) played an important role in controlling the diseases until the vaccination was temporarily suspended due to two cases of post-vaccination death in mid 1970s [1]. The number of whooping cough cases dramatically increased, thereafter, to yearly over 13,000 by 1979 due to a lowered vaccination rate [1]. Diphtheria tetanus acellular pertussis combined (DTaP) vaccine was developed successfully and put into practical use in 1981 [2]. The vaccine could gain public confidence in its safety and the once increased whooping cough cases quickly returned to the level before the suspension of DTwP vaccination [2]. The experience strongly suggested the importance of safety control of vaccines for an effective control of diseases.

DTaP vaccines however have been claimed to cause occasionally rather severe local reactions to booster doses

including the additional dose of DT combined toxoids (DT-td) at 12 years of age [3]. The reaction is reported to occur within 24 h after vaccination [3] and has collected particularly practitioner's attention due to its severity. The similar kind of local reaction has been reported also in the USA and Europe [4–6]. Although some workers attempted an analysis of the local reaction, no causal factor of the vaccine has been reported [7–9].

We attempted to quantitatively evaluate the local reactivity of DTaP vaccine antigens using the mice immunised with DTaP vaccine. We analysed factors of DTaP vaccine relating to the local swelling and are presenting the results of the analysis in this paper.

2. Materials and methods

2.1. Antigens

A pertussis antigen fraction containing pertussis toxin (PT), filamentous haemagglutinin (FHA) and other trace antigens at the approximately similar ratio to the antigen

* Corresponding author. Tel.: +81-42-561-0771 ext. 559;
fax: +81-42-567-0740.

E-mail address: yama-aki@nih.go.jp (A. Yamamoto).

Table 1
Composition of immunised antigens

Preparation	D-td ^a (Lf/ml)	T-td ^a (Lf/ml)	PT ^b (µg/ml)	aP-Ag ^c (µg/ml)	Al(OH) ₃ (mg/ml)
DT-PT-4 µg	30	10	4	–	0.15
DT-PT-2 µg	30	10	2	–	0.15
Exp-DTaP	30	10	–	120	0.15
DT-td	30	10	–	–	0.15

^a Purity of D-td was 2760 Lf/mg PN and that of T-td was 1500 Lf/mg PN.

^b Intact purified PT.

^c Acellular pertussis antigens with the similar composition to T-type vaccines, detoxified with 0.8% formalin at 37 °C for 2 weeks.

composition of Japanese T-type acellular pertussis vaccines was extracted and isolated from a liquid culture of *Bordetella pertussis* Phase I Tohama strain by a Cibacron Blue F3GA affinity chromatography [10]. The fraction was detoxified with 0.8% formalin at 37 °C for 2 weeks followed by dialysis to remove formalin and was stored as detoxified acellular pertussis antigen (aP-Ag) at 4 °C until use. Diphtheria and tetanus toxoids were kind presents from Dr. Ohkuma of Chemo-Sero-Therapeutic Research Institute. An experimental DTaP vaccine (Exp-DTaP) was prepared to consist of 120 µg/ml of aP-Ag, 30 Limes flocculation (Lf)/ml of D-td, 10 Lf/ml of tetanus toxoid (T-td) and 0.15 mg Al/ml of aluminium hydroxide gel (Wako Pure Chemicals Co. Ltd., Osaka, Japan) so as to have approximately similar composition to practical DTaP vaccines [11] (Table 1).

PT was a lyophilised preparation (Seikagaku Kogyo Co. Ltd., Tokyo, Japan) purified from Steiner and Scholte's liquid culture of *B. pertussis* Phase I Tohama strain [12].

Fourteen lots of commercial DTaP vaccines were used. Eight of them were manufactured before 1991 and six lots were those manufactured after that year by six Japanese manufacturers.

The reference pertussis vaccine (for toxicity tests) Lot 2 (reference vaccine) was a lyophilised whole cell preparation of pertussis organisms, being used for the National Quality Control Tests on pertussis vaccines in Japan since 1981. Its histamine-sensitising (HS), activity was designated to be 48 HSU per vial [13]. A vial of the vaccine was reconstituted in 12 ml of physiological saline and was serially diluted at four-fold intervals from 1 in 1 to 1 in 64 before use unless stated otherwise [14].

2.2. Mice

Four-week-old female mice of ddY strain (SLC Inc., Sizuoka, Japan) and 8-week-old female mice of BALB/c strain (Charles River Japan Inc., Yokohama, Japan) were used. Randomly grouped five mice each were housed in cages containing wood shavings in an animal room in a barrier system at a temperature of 22–24 °C. They were allowed free access to food pellets and water.

2.3. Mouse hind paw swelling reaction

Each group consisting of five mice was injected intramuscularly with an immunising vaccine sample twice at 1-month interval. They were challenged subcutaneously with a 50 µl dose of D-td, T-td or aP-Ag at their right hind paw 14 days later. They were injected with the same volume of saline at their left hind paw in parallel as the control. Concentrations of each D-td, T-td and aP-Ag solutions used for the challenge were 30 Lf/ml, 10 Lf/ml and 120 µg/ml, respectively, and also contained 0.15 mg Al/ml of Al(OH)₃ gel. The concentrations were approximately similar to those of practical DTaP vaccines. Thickness of right and left hind paws was measured until 100 h after challenge using a laser-equipped gauge for the thickness measurement (Natsume Seisakusho Co. Ltd., Tokyo, Japan) and the differences between the thickness of right and left paws were taken as the swelling reaction.

In the subsequent experiment, mice were intraperitoneally injected with 2.5 µg of indomethacin (Sigma–Aldrich) daily from 3 days before challenge [15] to obtain clearer swelling reactions in the mice received smaller doses of immunisation.

2.4. Serum antibody

Anti-D-td and T-td IgG titres of mouse serum were measured by an enzyme-linked immuno-sorbent assay (ELISA) [16]. Antitoxin titres were calculated referring to the standard antitoxins to express as units per millilitre (U/ml) of serum. Therefore, although employing different methods, the unit values could be assumed to be equivalent with IU values. Mouse anti-D-td and T-td IgE levels were assayed by the passive cutaneous anaphylactic reaction (PCA) in rats [17] and the reciprocal of the highest dilution showing the positive response was taken as the titre.

2.5. Histamine-sensitising (HS) activity

HS activity of vaccines was measured by Isida's rectal temperature method [18–21]. In brief, each group consisting of 10 (4-week-old female) mice of ddY strain was intraperitoneally injected with a 0.5 ml volume of a DTaP vaccine sample or a dilution of the reference vaccine. The mice were intraperitoneally challenged with 4 mg of histamine dihydrochloride (Sigma–Aldrich) in 0.5 ml of saline on the 4th (EHS activity) and 11th days (LHS activity) of the sensitisation. Their rectal temperatures were measured 30 min later [21] using an electric thermometer (Natsume Seisakusho, Tokyo, Japan). HS activity of a vaccine sample was calculated according to the parallel line assay method using logarithmic doses and rectal temperatures referring to the activity of the reference vaccine to express as HS units per millilitre (HSU/ml).

2.6. Statistic analysis

Analysis of the parallel line assays was carried out by Finney's method [22]. Significance and validity tests were made at a level of $P = 0.05$ and confidence intervals were expressed at 95% probability level unless otherwise stated.

3. Results

3.1. Causal antigen of the swelling reaction

Each group consisting of five female mice of BALB/c strain was immunised intramuscularly with 0.3 ml of Exp-DTaP or DT-td twice at 1-month interval. The mice were bled from their tail veins immediately before the second immunisation and challenge to measure their antibodies against D-td and T-td. Anti-D-td titres at the time of challenge and their 95% confidence intervals of mouse groups immunised with Exp-DTaP and DT-td were 20.2 (7.57–54.0) and 17.7 (12.7–24.8) U/ml, respectively. Anti-T-td IgG titres of the mouse groups and their 95% confidence intervals at the time of challenge were 9.90 (7.89–12.4) and 8.64 (4.24–17.6) U/ml, respectively. No significant differences in titres of the antibodies against D-td and T-td were found between the two immunisation groups. Anti-D-td and T-td IgE titres were measured by PCA assay in rats as described in Section 2. Anti-D-td IgE titres and their 95% confidence intervals of the both groups were 17.1 (7.83–37.1) and anti-T-td IgE titres of the DTaP

and DT-td immunisation groups and their 95% confidence intervals were 17.1 (7.83–37.1) and 30.3 (13.9–66.0) U/ml, respectively. No significant difference was seen also for IgE antibody titres between the two groups.

Thus, immunised mice were subcutaneously challenged at their hind paw with D-td, T-td or aP-Ag 2 weeks after the second immunisation as described in Section 2. Changes in swelling of their hind paw were monitored until 100 h after the challenge. The results were as shown in Fig. 1. Some extent of non-specific swelling was seen in both the hind paw received an antigen challenge and that injected with saline of all mice immediately after the challenge irrespective of immunised and challenged antigens. The non-specific swelling was temporal and did not adversely affect the specific swelling reaction that was observed as the difference between the challenged and control paws.

The mice immunised with Exp-DTaP showed stronger swelling than DT-td group irrespective of challenge antigens. The swelling reactions showed a clear peak within 10 h after the challenge except for that by aP-Ag challenge as seen in Fig. 1. D-td was shown to elicit the strongest swelling reaction among the challenge antigens.

3.2. Causal factors of the swelling reaction

According to the result that the mice immunised with Exp-DTaP received an enhanced sensitisation to D-td in the course of primary and booster immunisations, as shown in Fig. 1, we focused on the effect of aP-Ag in immunising vaccines to enhance the swelling reaction. Residual PT

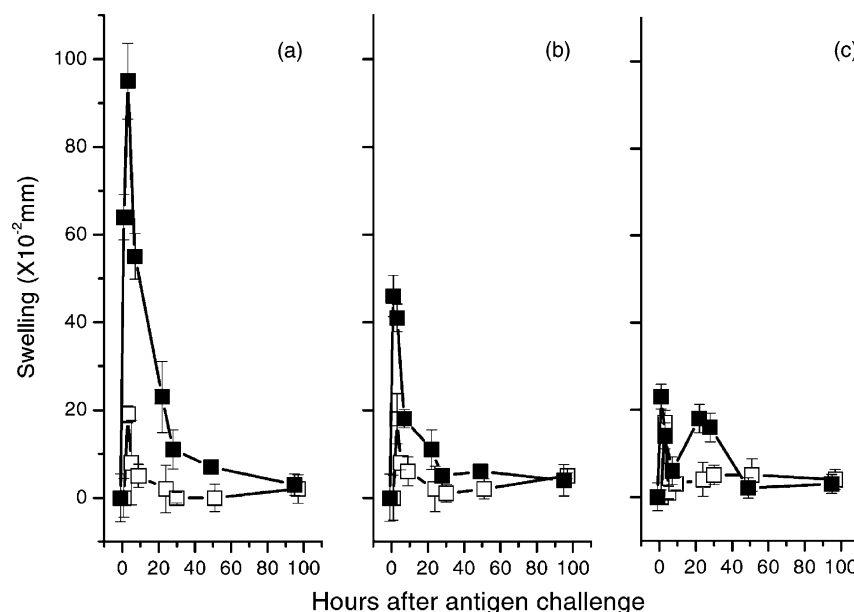


Fig. 1. Causal antigen of hind paw swelling of mice immunised with DTaP vaccine. BALB/c mice were immunised with two intra-muscular doses of Exp-DTaP vaccine (■) or DT-td (□) at 1-month interval. They were challenged with a 50 μ l dose of D-td (a), T-td (b) or aP-Ag (c) at their right hind paws 2 weeks later. Left hind paws were injected with the same volume of saline as the control. The difference in thickness between left and right paws was taken as the swelling response. Each point and attached vertical bar represent mean swelling of five mice and its 95% confidence interval, respectively. D and T toxoids elicited clear swelling reactions that peaked within 10 h after challenge. D-td was found to elicit the strongest swelling among the antigens.

activity in Exp-DTaP, if any, was suggested to be a possible cause for the adjuvant effect [23]. We examined the effect of PT on the D-td-related swelling response by immunising mice with an antigen preparation composed of 30 Lf/ml of D-td, 10 Lf/ml of T-td, 0.15 mg Al/ml of Al(OH)₃ gel and 4 µg/ml (DT-PT-4 µg) or 2 µg/ml (DT-PT-2 µg) of PT. Adsorbed DT-td and that also combined with 120 µg/ml of aP-Ag (Exp-DTaP) were immunised in parallel as listed in Table 1. The immunisation for the experiment shown in Fig. 1 was suggested to have elicited excess antibody titres to D and T toxoids in mice comparing to the practical vaccination in humans [24,25]. Accordingly, we employed a 0.1 ml dose instead of 0.3 ml for the immunisation in the subsequent experiments. A 0.1 ml dose of DT-PT-4 µg, DT-PT-2 µg, Exp-DTaP or DT-td was intramuscularly administered to mice twice at 1-month interval. The mice were challenged with 50 µl of D-td 2 weeks after the second immunisation. Because of the reduced immunisation dose, the mice needed an indomethacin treatment to show a clear swelling reaction. They were intraperitoneally injected with 2.5 µg of indomethacin daily from 3 days before challenge. Swelling reactions were monitored until 48 h after the challenge. The results were shown in Fig. 2. All immunisation groups showed a peak swelling at around 9 h after challenge. The mice of DT-PT-4 µg group showed a clear swelling reaction that was much stronger than the reactions of other groups. The reactions of all the groups became dependent on the immunised antigens from around 12 h and almost diminished by around 48 h after challenge. Order of severity of the response was DT-PT-4 µg, DT-PT-2 µg, Exp-DTaP, DT-td and the unimmunised control groups. The order for

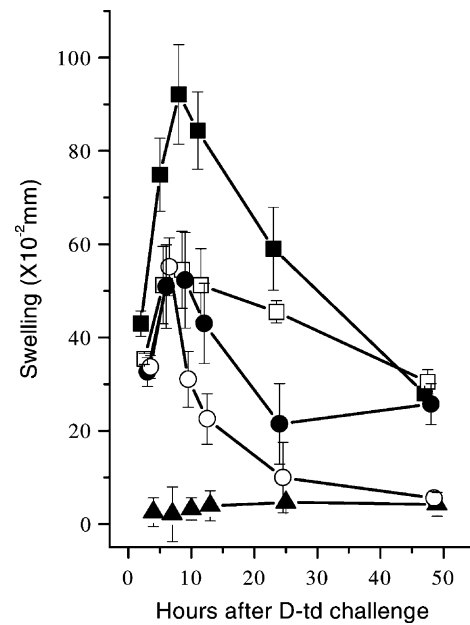


Fig. 2. Effect of active PT on the D-td-related hind paw swelling reaction of mice. BALB/c mice were intramuscularly administered with a 0.1 ml dose of DT-PT-4 µg (■), DT-PT-2 µg (□), Exp-DTaP (●) or DT-td (○) twice at 1-month interval and were challenged with 1.5 Lf in 50 µl of D-td 14 days later after intraperitoneally treated with 2.5 µg of indomethacin for 3 days. Unimmunised mice (▲) were challenged in the same manner as the control. Each point represents mean and 95% confidence interval (vertical bar) of swelling reactions of five mice. DT-PT-4 µg group showed the strongest D-td-related hind paw swelling. The swelling became dependent on the immunised antigens from 12 h of challenge.

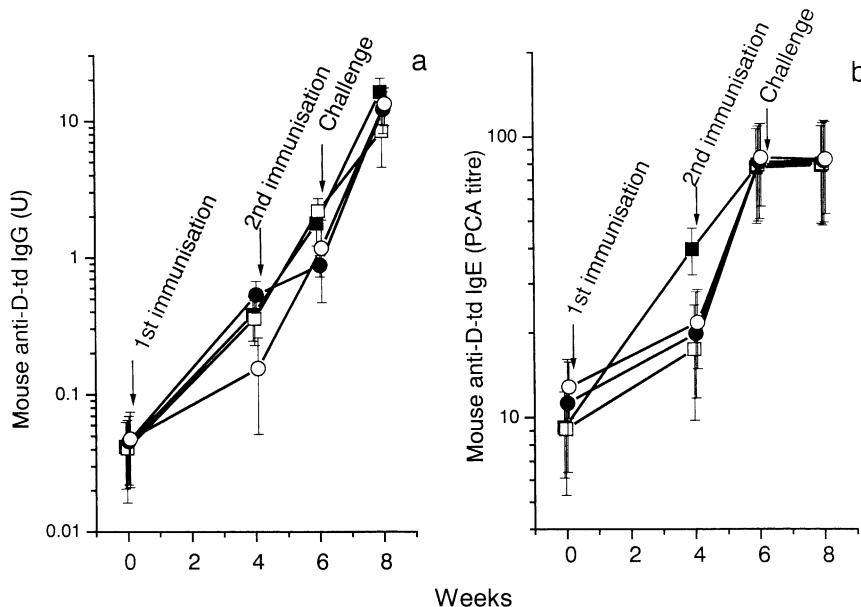


Fig. 3. Serum IgG and IgE antibodies to D-td and hind paw swelling. Serum samples were taken from tail veins of the mice in the experiment shown in Fig. 2 to measure anti-D-td IgG (a) and IgE (b) by ELISA and PCA in rats, respectively. For the marks in the figure, see the legend for Fig. 2. In spite of the significant difference in the extent of swelling reaction among the immunisation groups, no difference was seen for the both antibodies at the time of challenge.

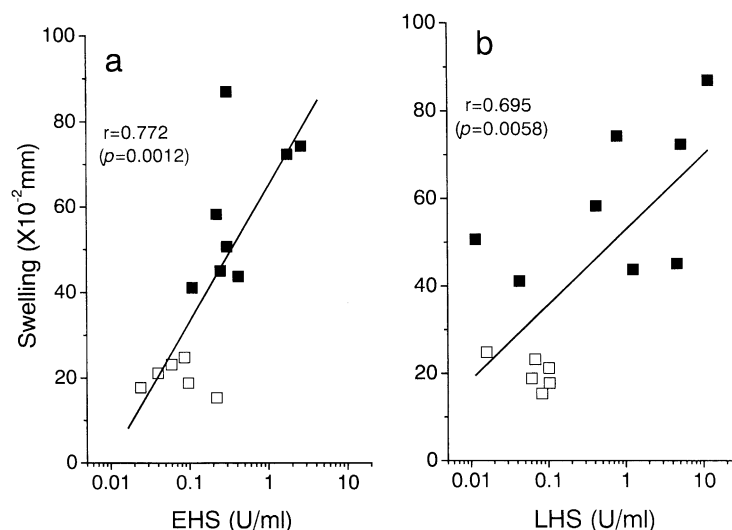


Fig. 4. Correlation between D-td-related hind paw swelling and residual HS activities of DTaP vaccines. Sensitising activity of commercial DTaP vaccines to the D-td-related swelling was measured by the same method described in the legend for Fig. 2. Eight lots of the vaccine were manufactured before 1991 (■) and the other six were those manufactured after 1991 (□). The eight lots showed varied levels of EHS and LHS activities due to reversion to the activities during the long storage and the activities of the six recent lots were equal or below the detectable limits. The sensitising activity to D-td-related swelling showed fine correlations with both EHS (a) and LHS (b) activities with correlation coefficients of 0.772 ($P = 0.0012$) and 0.695 ($P = 0.0058$), respectively.

the former three appeared to have corresponded with their PT activity as measured by the HS test of 4 days sensitisation, which were 3.54, 1.77 and 0.56 HSU/ml, respectively.

The mice were bled immediately before the first, second immunisations and challenge and also 2 weeks after the challenge to monitor the increase in anti-D-td IgG and IgE antibodies. All immunisation groups showed a similar extent of rise in the levels of IgG and IgE antibodies to D-td according to the first, second immunisations and the challenge. No significant difference was seen among all the immunisation groups in both anti-D-td IgG and IgE levels at the time of challenge as shown in Fig. 3 in spite of the significant difference in their swelling reactions. The results, accordingly, suggested that there is no causal relationship between IgG or IgE antibody level and the swelling reaction in mice.

3.3. Swelling reactivity and residual PT activity of DTaP vaccines

As PT activity in the immunised vaccine was suggested to be a causal factor for the enhanced swelling reaction at a booster immunisation with D-td, a further analysis was made using commercial DTaP vaccines that had varied levels of residual PT activity. HS activities of 14 lots of commercial DTaP vaccines were measured on the 4th and 11th days of sensitisation. We chose eight lots from those manufactured before 1991 and the rest from those manufactured after 1991. HS activities on the 4th and 11th days of sensitisation of the eight lots varied from 0.10 to 2.60 and from 0.22 to 11.7 HSU/ml, respectively. While the HS activities of the recent six lots were below or equal to the levels of de-

tectable limits. Each group consisting of five female BALB/c mice were intramuscularly immunised with a 0.1 ml dose of the vaccines twice at 1-month interval. They were intraperitoneally treated with indomethacin for 3 days to receive the challenge with D-td 2 weeks after the second immunisation as described in Section 2. Hind paw swelling of the mice was measured 12 h after the challenge. As similarly as HS activities, the vaccine lots manufactured before 1991 showed varied levels of sensitisation to the D-td-related swelling, while the six recent lots showed the least sensitisation to the swelling reaction. Fine correlations were seen between the D-td-related swelling and both the EHS and LHS activities of the vaccine lots as shown in Fig. 4a and b. The correlation coefficients were 0.772 ($P = 0.0012$) and 0.695 ($P = 0.0058$), respectively.

4. Discussion

Two cases of post-vaccination death after DTwP vaccination in mid 1970s resulted in a lowered rate of the vaccine acceptance. Accordingly, Japanese annual pertussis cases increased to the level over 13,000 by 1979 [1]. This experience suggested importance of safety of the vaccine for a successful control of the disease. After the practical introduction of DTaP in 1981, the vaccine successfully gained public confidence in its safety. However, booster doses of DTaP and DT-td have been claimed to cause occasionally severe local reactions [3–6]. Mechanism or causal factor of the local reaction has not been identified [7–9].

We attempted to quantitatively evaluate local reactivity of DTaP vaccines using mice immunised with DTaP vaccines.

We employed the procedure of intramuscularly immunising mice with a vaccine sample twice at 1-month interval and challenging their hind paws 2 weeks later. Concentrations of all the vaccine antigens, D-td, T-td and aP-Ag, used for immunisation and challenge were approximately equivalent to the concentrations in practical DTaP vaccines except for active PT that was used for the immunisation to examine its effect on the swelling reaction. In a search for the causal antigen of hind paw swelling, mice immunised with a DTaP vaccine were challenged with the vaccine antigens. D-td elicited the strongest swelling reaction among the challenge antigens (Fig. 1). Protein concentration of D-td in the immunising and challenge antigen solutions was higher than that of T-td but almost half the concentration of aP-Ag (Table 1). This might explain the stronger swelling reaction to D-td than to T-td. However, the mildest swelling by aP-Ag comparing to D-td and T-td could not be fully explained in the present study.

In the first experiment, a 0.3 ml volume was employed for the immunisation. The dose was found to induce slightly higher levels of antibodies to the vaccine antigens comparing to the levels seen in the practical immunisation in humans [24]. The immunisation dose was accordingly reduced to 0.1 ml to obtain the antibodies of comparable levels with that of humans in the practical immunisation. Indomethacin treatment of immunised mice was necessary for the reduced immunisation dose to elicit significant swelling [15]. When mice were immunised with DT-PT-4 µg, DT-PT-2 µg, Exp-DTaP or DT-td and were challenged with D-td, DT-PT-4 µg induced the strongest swelling reaction at around 9 h after challenge, which was consistent with the kinetics of the local reaction in humans [26,27]. Then intensity of the swelling reaction became dependent on the immunised vaccines after 12 h of challenge (Fig. 2). Accordingly, in the subsequent experiments, mice were immunised intramuscularly with 0.1 ml of a vaccine sample twice at 1-month interval. Then after treating intraperitoneally with 2.5 µg of indomethacin for 3 days, the mice were challenged at their hind paws with a 50 µl dose of D-td 2 weeks after the second immunisation to measure their hind paw swelling 12 h later. The swelling reaction could not be explained by anti-D-td IgG or IgE because no significant difference was seen among the immunisation groups for the levels of the both antibodies against D-td (Fig. 3). In addition to the antibody levels, the effectiveness of indomethacin treatment for enhancing the D-td-related swelling suggests the involvement of cellular immunity. With regard to the relationship with delayed-type hypersensitivity (DTH), the immunisation of twice at 1-month interval markedly differed from the immunisation procedure for DTH reaction. In addition, swelling reaction in mouse footpad is seen not earlier than 12 h in the ordinal DTH reaction [28,29]. On the other hand, the swelling reaction in the present study peaked within 10 h after challenge which is consistent with the local reaction to booster doses in DTP and DTaP vaccinees [26,27]. In our preliminary

histopathological examination, severe infiltration of neutrophil was seen at the swelled region of the mouse hind paw (unpublished). Further study would be required for fully characterising the D-td-related swelling reaction.

We evaluated 14 lots of commercial DTaP vaccine for their activities to sensitise mice to D-td-related local swelling. The vaccine lots were selected to have varied levels of EHS and LHS activities. Particularly, those manufactured before 1991 had not been regulated their reversion to HS toxicity and showed rather strong HS activities due to the reversion during the long storage. The activity of the vaccines to sensitise mice to D-td-related hind paw swelling showed fine correlations with the both HS activities of the vaccines (Fig. 4). PT is known to have various biological activities including HS and adjuvant activities. B-oligomer of PT was reported to stimulate both Th1 and Th2 typed antigen specific T-cell proliferation to exert adjuvant effect on co-administered antigen [23]. Consequently, the fine correlation between residual HS activity of DTaP and the D-td-related swelling reaction suggests that the residual HS activity may be an indication of the residual adjuvant activity of PT and can be used for predicting the sensitising effect of DTaP to the D-td-related swelling reaction.

Minimum Requirements of Biological Products of Japan (MR) was revised in 1991 to implement a test for reversion to HS activity [30]. DTaP vaccines manufactured after 1991 to comply with the revised MR were found to have lowered levels of HS activity. It is assumed interesting, therefore, to investigate whether the number of the severe local reactions to the booster dose of DT-td at 12 years of age showed any decline.

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