Table 2
Results of toxicity tests on aP vaccines.

Vaccines	Toxicity test						
	BWD (BWDU/mL) (95% CI)[a]	LP (LPU/mL) (95% CI)[a]	EHS[b] (HSU/mL) (95% CI)[a]	EHS (37 °C 4 weeks) (HSU/mL) (95% CI)[a]	LHS[c] (HSU/mL) (95% CI)[a]	LHS (37 °C 4 weeks) (HSU/mL) (95% CI)[a]
Imported vaccine						
A (DTaP)	88.40 (27.71–428.95)	0.077 (0.017–0.192)	0.032 (0.012–0.069)	0.117 (0.054–0.23)	0.343 (0.153–0.648)	0.519 (0.25–0.969)
B (DTaP)	84.81 (26.58–405.51)	0.077 (0.017–0.191)	0.053 (0.022–0.108)	0.056 (0.023–0.114)	0.153 (0.054–0.315)	0.184 (0.07–0.364)
C (DTaP-IPV)	480.35 (90.16–194021)	0.219 (0.101–0.396)	0.207 (0.086–0.387)	0.234 (0.096–0.449)	0.121 (0.023–0.312)	0.107 (0.019–0.287)
D (DTaP-IPV)	13.28 (3.14–42.88)	0.060 (0.012–0.155)	0.043 (0.017–0.09)	0.084 (0.037–0.168)	0.262 (0.109–0.502)	0.327 (0.144–0.618)
Japanese vaccines						
Acellular (1999–2005) (N = 158)	9.06	0.112	0.135	0.160	0.078[d]	0.118[d]
Whole cell (~1981) (N = 176)	56.4	1.786	1.918	ND[e]	ND[e]	ND[e]
Control[f]	4.88	0.126	0.135	0.135	0.105	0.105
Japanese Minimum Requirements for DTaP	≤10	≤0.5	≤0.4	≤0.4	-	-

[a] 95% confidence interval.
[b] HS activity of 4-day sensitization.
[c] HS activity of 11-day sensitization.
[d] N = 143.
[e] Not Done.
[f] Mice without sensitization were measured as a central group.

take specimens of 2 injection sites each of rabbits 3 days, 1 week, 2 weeks and 3 weeks after injection for histological examination.

2.6. Mouse intramuscular injection model

We chose a different mouse strain for this model from that for the footpad swelling model in order to minimize the possible influence of the responsiveness of a particular mouse strain on the results. Accordingly, 5 weeks old female mice of ddY strain (Shizuoka Laboratory Animal Center, Shizuoka, Japan) of SPF grade were used. Groups of more than 12 mice each were allocated to test samples and a 50 μL-volume of a sample was injected into their left or right quadriceps muscle. At least 3 mice each of each group were anesthetized and sacrificed at 1–4 weeks after injection and specimens of the muscle tissue at the injection site were submitted for histopathological examination after macroscopic observation.

2.7. Statistic analysis

Analysis of the parallel line assay method was carried out according to Finney's method [20]. Significance and validity tests were made at a level of $P = 0.05$.

3. Results

3.1. Pertussis toxicity tests

Imported DTaP batches A and B, and DTaP-IPV batches C and D were tested for residual toxicities as described in Section 2 and the results were summarized in Table 2. In the table, results on vaccine batches A, B, C and D are shown as toxicity unit values with their 95% confidence intervals and those on Japanese DTaP are shown as geometric mean unit values of batches tested during 1999 and 2005. The level of residual LP toxicity of vaccine batches A, B, C and D was found to be comparable to that of Japanese DTaP batches and did not significantly exceed over the detectable limit [2]. With regard to HS toxicity, measurements were done on the 4th (EHS) and 11th days (LHS) of sensitization. Residual EHS toxicity of vaccine batches A, B, C and D and the extent of reversion to the toxicity after incubating at 37 °C for 4 weeks were also found to be comparable to or lower the detectable limit (a control level) and did not exceed those of Japanese DTaP batches. Regarding LHS toxicity, batches A and D showed excess levels of LHS toxicity over that of Japanese DTaP, while the toxicity levels of batches B and C were comparable to that of Japanese batches. Vaccine batches A, B, C and D showed unexpectedly strong BWD toxicity comparing to that of Japanese DTaP batches. Three of the four imported batches showed the levels of BWD toxicity even comparable or excess to that of Japanese DTwP.

3.2. Mouse footpad swelling reaction

Mouse footpad swelling reactions to imported vaccine batches A, B, C and D and Japanese DTaP batch J were compared and results were represented in Fig. 1A. Mice receiving vaccine batches A, C and D showed an intense swelling that reached a peak at day 5, while batch B induced a less intense but still significant swelling without a clear peak. DTaP batch A and DTaP-IPV batch C consisted of similar DTaP formulation from same manufacturer and induced swellings of equivalent intensity. All the imported batches induced marked swellings varying in intensity, while Japanese DTaP batch J induced no significant swelling until day 5 followed by a slight swelling. In total 18 batches of Japanese DTaP from 5 manufacturers tested induced only slight swellings similar to that by batch J and mean swelling reactions to batches from each manufacturer were calculated for each observation day and depicted in Fig. 1B.

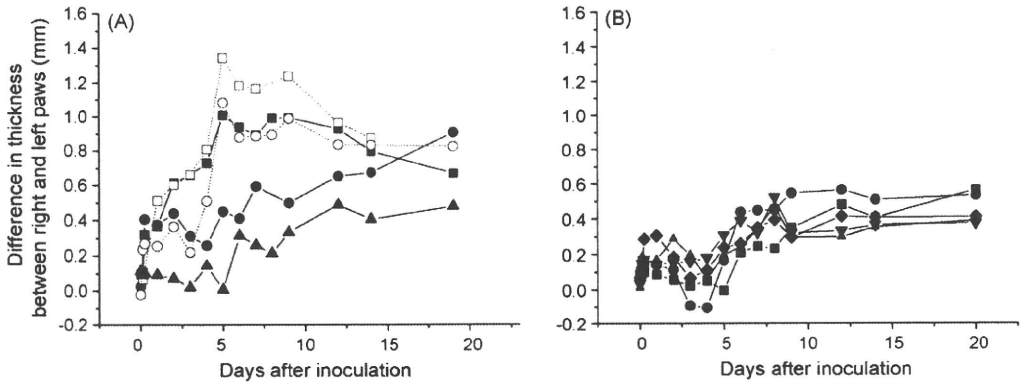

Fig. 1. Mouse footpad swelling response to DTaP and DTaP-IPV of various sources. 50 μL of a vaccine sample and saline were subcutaneously injected to right and left footpads, respectively, and the difference in thickness between the footpads was taken as a swelling response. (A) Mean swelling response for each day of 5–10 mice each to imported DTaP batches A (■) and B (●), DTaP-IPV batches C (□) and D (○) and Japanese DTaP batch J (▲). Batches A, B, C and D induced significant swellings that peaked at around 5 days after injection, while batch J did not cause such significant reaction. (B) Mean swelling responses to several batches each of Japanese DTaPs for 5 manufacturers assessed using 5–10 mice per batch. No significant swelling was seen for any DTaPs from Japanese manufacturers.

After anesthetizing, 2 mice of each group were sacrificed on day 5 to take footpad specimens for histological examination. Fig. 2 represents histological findings of mouse footpads receiving vaccine batches A, B, C, D and batch J. The footpads of mice injected with vaccine batches A, B, C and D showed necrotizing inflammation with mononuclear cells, granulocytes and fibroblasts infiltration. In particular, batch A induced the strongest reaction. In contrast, no such severe necrotizing reaction was seen for batch J.

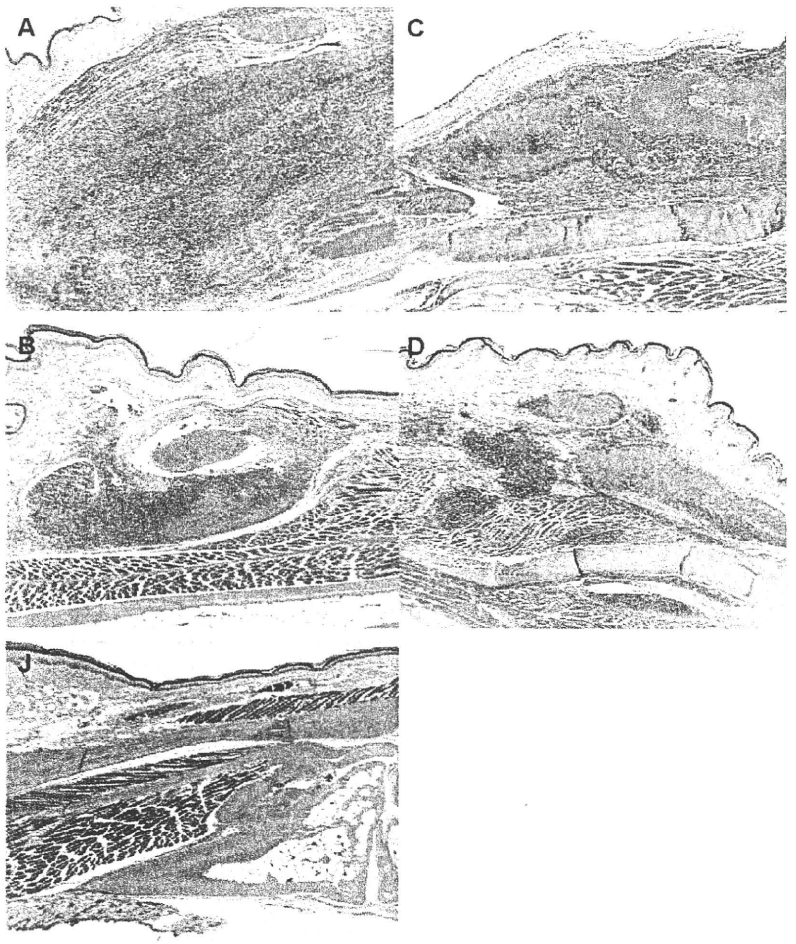

Fig. 2. Histological findings in footpads of mice after injection with DTaPs and DTaP-IPVs. Severe inflammatory infiltration with mononuclear cells and granulocytes was observed on day 5 post-injection under dermis of mouse footpads injected with batches A, B, C and D. No inflammatory infiltration was seen in the footpad injected with batch J (HE; original magnification, 10×).

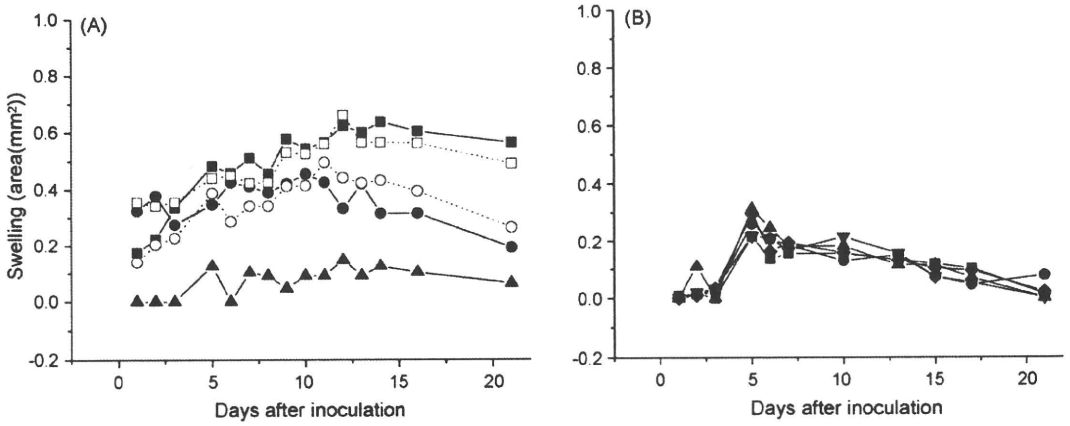

Fig. 3. Rabbit skin swelling reactions to DTaP and DTaP-IPV of various sources. 100 μL of a vaccine sample was intradermally injected to more than 2 different sites each of back skin of 3 rabbits to measure area of swellings. Average area of swellings at the injection sites of all rabbits received a vaccine batch was shown as swelling response to the batch. (A) Mean swelling responses to imported DTaP batches A (■) and B (●), DTaP-IPV batches C (□) and D (○) and Japanese DTaP batch J (▲). (B) Mean swelling responses to several batches each of DTaP of Japanese manufacturers. No significant swelling was seen for DTaP of any Japanese manufacturer.

3.3. *Rabbit skin swelling reaction*

Injection site reaction of rabbits to imported vaccine batches A, B, C, and D and a Japanese batch J was tested as described in Section 2. Fig. 3A represents results of measurements of the swelling responses to vaccine batches A, B, C, D and J. DTaP batch A and DTaP-IPV batch C consisted of similar DTaP formulation from same manufacturer that induced swellings of equivalent intensity, which were strongest among those tested. The swellings or indurations induced by vaccine batches A, B, C and D remained without waning for 3 weeks, while batch J induced only a mild swelling that diminished within a week (Fig. 3A). Injection site reaction of rabbits to 14 batches of Japanese DTaP from 5 manufacturers including batch J was tested in another experiment. All the 14 batches induced only mild swellings and no significant difference was seen among any of the batches. Mean swelling responses to DTaP batches from each

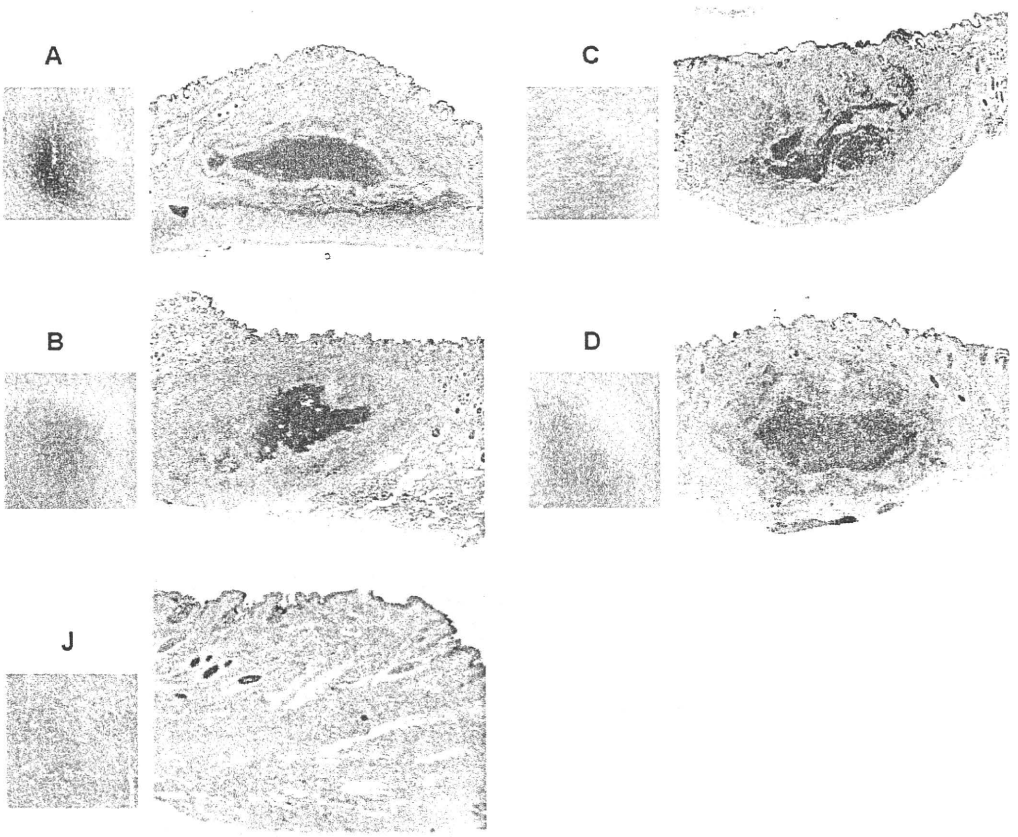

Fig. 4. Macroscopic and microscopic observations of rabbit skin reactions to DTaPs and DTaP-IPVs. Significant swelling responses were seen in rabbit skin even 3 weeks after injecting with batches A, B, C and D. Necrotizing inflammation was seen under dermis of these rabbits 3 weeks after injection. Inflammatory reaction was well localized by fibroblasts infiltration. No swelling reaction and inflammatory infiltrates was seen in the skin after 3 weeks of injection with batch J (HE; original magnification, 5×).

of five Japanese manufacturers were calculated for each observation day and represented in Fig. 3B. The kinetics of swellings seen in Fig. 3B slightly differed from that seen for batch J in Fig. 3A probably due to some variation in responsiveness of rabbits used in the experiments for Fig. 3A and B.

Histopathologically, localized necrotizing inflammation with infiltration of pseudoeosinophils was observed in the dermis of the biopsy specimens of injection sites of vaccine batches A, B, C and D. The necrotizing inflammations were seen persistently from 3 days to 3 weeks after injection as seen in Fig. 4. In contrast, batch J did not cause such severe inflammation. As a result, all the imported vaccines tested induced severe tissue injury at the site of injection due to strong necrotizing inflammation while Japanese batch J did not cause such tissue damage.

3.4. Mouse muscular inflammatory reaction

Inflammatory reactions of mouse quadriceps muscle to the vaccines A, B, C and D and 12 batches of Japanese DTaP from 5 manufacturers were compared by macroscopic and microscopic observations. White nodule could be detected in the muscle tissue by macroscopic inspection. Size of the white nodule gradually increased from 1 to 4 weeks in the muscles injected with A, B, C or D, while only small debris could be seen in the muscle injected with batch J. The results of observations on the reactions to the four imported batches and a representative Japanese batch J at 4 weeks after injection are shown in Fig. 5. Histological examination revealed a circumscribed nodule of inflammatory tissue deposited in the muscles injected with batches A, B, C and D due to severe infiltration of inflammatory cells to cause severe injury of muscle tissues as seen in Fig. 5. No significant difference was seen in severity of the inflammatory reactions between batches A and C. On the other hand, Japanese vaccine did not induce such severe reactions. The difference in size and severity of debris between muscles receiving vaccines A, B, C and D and that receiving vaccine J was obvious.

4. Discussion

Vaccines are required to be consistent to a certain extent irrespective of source manufacturer to be used interchangeably in the primary and booster immunizations. Although acellular pertussis-based combination vaccines have been used for decades in Japan, the U.S.A., Europe and some Asian countries and there is an increasing possibility to use vaccines from multiple sources, little has been reported on direct comparison of the vaccines from different sources. DTaPs and DTaP-IPVs for intramuscular use purchased from foreign markets were tested by the current Japanese laboratory control tests for toxicities to compare with Japanese DTaPs which are for subcutaneous use. This is the first report on such direct comparison of acellular pertussis-based combination vaccines from various sources. All the imported vaccines tested met Japanese criteria for toxicities of aP except for BWD toxicity. Regardless of combination with IPV, 3 of the imported vaccine batches were shown to have the level of BWD toxicity similar to or even significantly higher than that of Japanese DTwP whose use was terminated more than 25 years ago. BWD toxicity test was developed according to the results of analysis on factors affecting the mouse weight gain test [17]. The test was later implemented in Japanese Minimum Requirements to control the toxicity of DTaP to mouse weight gain during 16 h after injection mainly targeting endotoxin control. However, the test has limited sensitivity in detecting endotoxin [2]. The BWD toxicity test is also affected by other constituents such as alum adjuvant gel and characteristics of

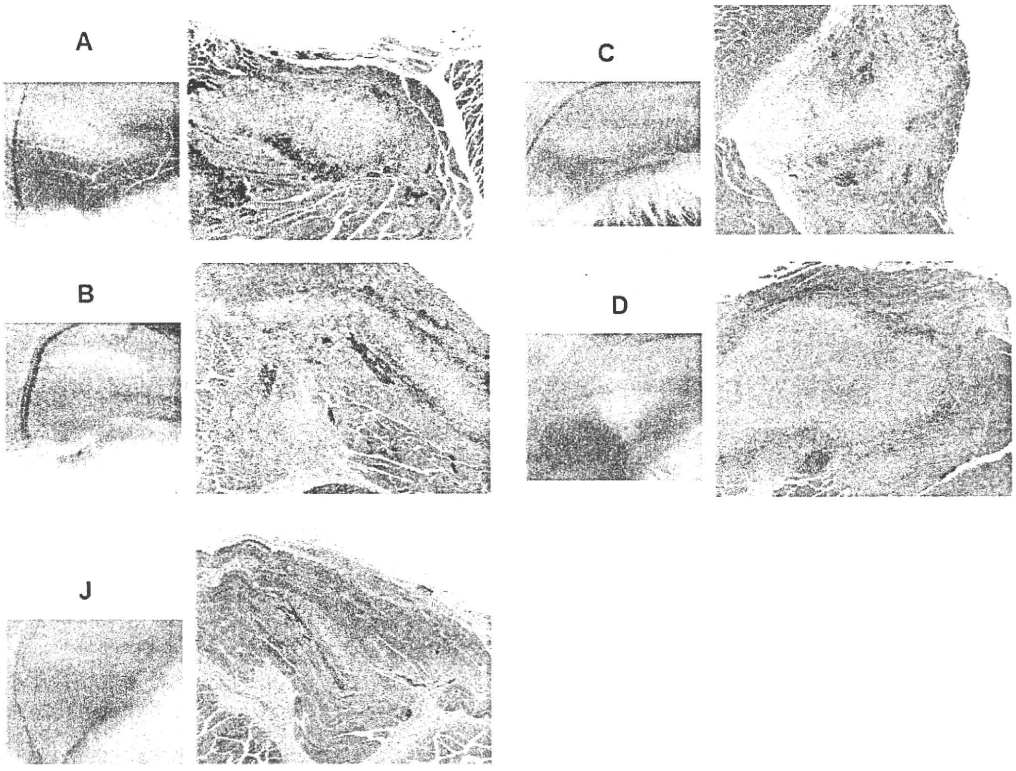

Fig. 5. Macroscopic and microscopic observations of inflammatory reactions of mouse quadriceps muscle to DTaPs and DTaP-IPVs. Severe necrotizing inflammation was seen in the muscles of the mice even 4 weeks after injection with batches A, B, C or D. Slight inflammatory infiltrates was observed in the muscle 4 weeks after injection with batch J (HE; original magnification, 10×).

antigens. Although we measured endotoxin contents in the vaccines by Limulus amaebocyte lysate (LAL) test [23,24], only trace amounts of endotoxin were found (data not shown). The present results of BWD test are not considered to reflect the toxicity of endotoxin but reflecting toxicities of various constituents such as aluminium adjuvant gel and characteristics of antigens [25]. Cause of the excess BWD toxicity of imported vaccines was not identified in the present study.

We examined local reactogenicity of imported DTaPs, DTaP-IPVs and Japanese DTaPs in the mouse footpad swelling, rabbit skin swelling and mouse intramuscular injection models. All the imported vaccines caused stronger swellings of mouse footpad and rabbit skin and larger deposits in mouse quadriceps muscle comparing to Japanese DTaPs. None of Japanese DTaPs from all the 5 manufacturers induced such severe reactions at the injection sites. Histological examinations also showed that the imported vaccines induced stronger inflammations in mouse footpad, rabbit skin and mouse quadriceps muscle comparing to Japanese DTaPs. Although mouse footpad and rabbit skin are not anatomically identical, histopathological lesions caused by the imported vaccines in the both animal models were characterized by tissue injury with inflammatory infiltrates associated with increased neutrophil influx, edema and necrosis.

The mouse footpad swelling model employed BALB/c strain of mice and subcutaneous injection with 50 μL of a vaccine sample. The rabbit skin swelling model employed intradermal injection with 100 μL. The mouse intramuscular injection model employed ddY strain of mice and an injection volume of 50 μL. The injection volumes corresponded to 2.5 mL/kg, 0.01 mL/kg and 2.5 mL/kg, respectively. However it is not clear whether a dose per body weight is relevant to the reaction, vaccine batches A, B, C and D induced stronger swellings and inflammations comparing to Japanese DTaPs irrespective of animal species, injection site and injection volume. The present findings strongly suggest that there is a significant difference in tissue damaging effect between Japanese vaccines and the imported vaccines tested. Therefore, Japanese vaccines and imported vaccines are likely to induce different extent of damages in the tissues at injection site of human subjects. However, all the vaccines used in the present study were approved for clinical use based on clinical evaluations and no significant safety problems have been reported for any of them. Therefore, the present findings of clear difference of vaccines are inconsistent with the clinical evaluations. The reason for the discrepancy has not been clearly known. A possible explanation would be that surface observation of injection sites to evaluate local reactions in clinical trials could not detect or evaluate tissue damages in deeply injected muscles. Although further confirmation would be needed, it would not be likely that only human tissues would be particularly different from those of mice and rabbits in responding to tissue damaging effects of vaccines.

As Japanese vaccines are administered subcutaneously (Japanese Minimum Requirements), there is no difficulty in detecting local reactions at the injection site from the surface. However, other vaccines are given intramuscularly and it is assumed rather difficult to detect a local reaction, if any, only by the surface observation comparing to subcutaneous injections. Therefore, detecting no swelling reactions to intramuscularly administered vaccine in a clinical study may not be the proof of no tissue damage. If tissue damage in infants was as severe as that seen in the animal models, its long-term health impact would need careful monitoring and re-evaluations.

Regarding BWD toxicity, the vaccine batches that showed strong BWD toxicity in mice and those induced severe inflammatory response in the animal models seemed to have correlated but actual relationship of the toxicity and local reactogenicity was not confirmed in the present study.

With regard to causal factors of the strong inflammatory response, Munoz et al. described involvement of pertussigen (currently PT) activity in the mouse footpad swelling of peaking at the 5th day of injection [13]. We confirmed intact PT induced mouse footpad swelling that peaked on around 5 days after injection (data not shown). However, all the vaccine samples used in the present study did not show any significant LP or HS activities. The genetically detoxified PT mutant in the S1 subunit, PT-9K/129G, lacks enzymatic activity and HS activity but still retains binding activity and was reported to have adjuvanticity [26]. Even though having intact S1, PT with mutations in the S3 and S4 subunits loses not only haemagglutinating activity but also LP and HS activities [27]. The LP and HS tests cannot separately evaluate residual activities of A and B subunits but assess overall PT activity. The difference of imported and Japanese DTaPs in induction of inflammation may be reflecting difference in some residual activity that cannot be examined by the LP and HS tests, if PT played a role.

It was also reported that a severe inflammatory response was induced by aluminium adjuvant [28,29]. Japanese DTaPs contain a rather small amount of aluminium adjuvant gel and induced only mild inflammatory responses. However, when the local reactogenicity of aluminium adjuvant gel alone was examined using the swelling models by injecting 1.0 mgAl/mL of aluminium hydroxide gel, no obvious swelling reaction was seen (data not shown). Regarding the role of IPV, no significant difference in severity of the injection site injury was seen between the imported DTaP and DTaP-IPV of the same manufacturer and, furthermore IPV prepared from Sabin strain alone did not induce any swelling reactions in mouse footpad and rabbit skin (data not shown). Although further confirmatory study would be necessary, it was suggested IPV has little impact on the injection site injury.

Difference in severity of inflammatory cell infiltration at the injection sites was significant between the vaccines for intramuscular use and that for subcutaneous use. The difference may lead to different intensities in sensitization of cellular immunity to the vaccine antigens. As an intensified sensitization, if any, may lead to an intensified local reaction to booster doses. Local swelling reactions to booster doses were reported to depend on the vaccines used for primary immunization in children that those primed with DTaP showed stronger local swelling following booster doses with DTaP than those primed with DTwP [9]. Therefore, the correlation of vaccine quality for primary dose and local reaction to booster doses would need careful evaluation.

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An *in vitro* assay system as a potential replacement for the histamine sensitisation test for acellular pertussis based combination vaccines

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ABSTRACT

The histamine sensitisation test (HIST) for pertussis toxin is currently an official batch release test for acellular pertussis containing combination vaccines in Europe and North America. However, HIST, being a lethal endpoint assay, often leads to repeated tests due to large variations in test performance. Although a more precise HIST test based on measurement of temperature reduction after the histamine challenge is used in Asian countries, this test still uses animals. An *in vitro* test system based on a combination of enzyme coupled-HPLC and carbohydrate-binding assays with results analysed by a mathematical formula showed a good agreement with the *in vivo* HIST results based on measurement of temperature reduction after histamine challenge. The new *in vitro* test system was shown to be a potential alternative to the current *in vivo* HIST.

1. Introduction

Pertussis (whooping cough) is still a major cause of morbidity and mortality, especially in infants, and vaccination against *Bordetella pertussis* is a key component of immunisation campaigns worldwide for the prevention of the disease. Two types of pertussis vaccines, namely whole-cell (WCV) and acellular pertussis vaccines (ACV), are recommended by WHO [1]. Both types of vaccine play an essential role in immunisation programmes worldwide. In general, ACVs are less reactogenic and there is an increasing trend towards replacing WCVs with ACVs.

Pertussis toxin in its toxoided form (detoxified) is a major protective antigen and is present in both ACVs and WCVs. Native pertussis toxin (PT) is a protein toxin which has a wide range of biological activities *in vivo* including induction of leukocytosis, histamine-sensitisation, increased insulin production with consequent hypoglycaemia, potentiation of anaphylaxis and lethality in mice [2]. PT also has been reported to increase blood–brain barrier permeability and there has been controversy regarding its possible role in neuropathology [3]. More recently, there is evidence to indicate that PT is able to induce IL-1β production in the mouse brain. IL-1β can affect neuroendocrine functions and modulate release of neurotransmitters, and has been claimed to play a role in neurological reactions observed in children immunised with pertussis vaccines of high PT content [4]. Furthermore, excess residual PT activity may cause intensified sensitisation to components of diphtheria-tetanus-ACV (DTaP) combinations with subsequent severe local reaction to a booster dose in an animal model [5]. Although the biological effects of PT have been under extensive research, its mechanism(s) of toxicity is still unclear. As unmodified PT is considered too toxic for vaccine preparation, it has to be used in a stable non-toxic form. The production of toxoided PT has to be carefully controlled to prevent excessive loss of protective and T_h-epitopes on the one hand, and reversion to toxicity on the other [6,7]. Monitoring chemically toxoided PT for residual toxicity and reversion to toxicity is an essential part of the safety evaluation of ACVs and is required by regulatory authorities [8–11]. The *in vivo* histamine sensitisation test (HIST) is the official pharmacopoeial test used for detecting active PT in such preparations.

Various assay methods have been explored as alternatives to the *in vivo* HIST and most of them are based on either non-lethal animal models or cell culture systems but so far without success [12,13]. The first approach, i.e. the leukocytosis promoting assay (LPA), was shown to have poor reproducibility and large inter-laboratory variation [14]. More recently, attempts have been made to use primary vascular smooth muscle cells from rats and to measure PT induced increase in isoprenaline-induced intracellular cAMP levels or to identify gene expression after administration of PT or WCV [13]. These approaches still need the use of animals and the studies are at a very early stage. Cell culture based assay systems may be useful for

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evaluating antigens in the vaccine bulk stage but their suitability for adjuvant adsorbed final formulation that contains chemically toxoided PT remains questionable because the adjuvant usually affects the cell function/or viability and the cells may not be able to process toxin inside protein aggregates. For example, the Chinese hamster ovary (CHO)–cell assay is used for the determination of residual pertussis toxin activity in the bulk antigen but is not suitable for the assay of pertussis toxin in the final dosage form. Recently, a study has also indicated that this assay does not correlate with HIST [15,16]. Therefore, at the moment HIST is the only test requested by regulatory authorities to determine pertussis toxin activity in final formulation of pertussis containing vaccines [8–11]. However, the precise mechanism of HIST is unclear and it is difficult to standardise, especially for the lethal endpoint method, and large variations in test performance have been observed between laboratories due to mouse strain, age, sex and environmental factors [17]. Therefore, there is an urgent need for a replacement of the HIST.

Two HIST methods are currently used for the determination of residual PT in licensed acellular pertussis vaccines. One is based on lethal end point and the other measures temperature reduction after the histamine challenge [8,18,19]. The lethal endpoint method based on the induction of increased sensitivity to histamine-provoked death in mice has been widely used in North American and European countries [10,11] and its disadvantages have already been reviewed [12]. There is no internationally agreed limit on the permissible level of PT activity in ACVs. The assay remains difficult to standardise and has a history of problems associated with inconsistent results, and inter- and intra-laboratory variability. This often leads to apparently contradictory results between laboratories and to repeat tests, particularly when different mouse strains are used. A more sensitive variant of HIST based on either rectal or dermal measurement of the reduction in body temperature produced by histamine shock (temperature method) has been successfully used in Japan and other countries [8,20]. This test method provides a more precise quantitative estimate of the activity of a test vaccine relative to the activity of a reference standard and this quantitative approach forms the basis of our present study in establishing the relationship between the *in vivo* and *in vitro* assay systems.

PT has the A-B type structure typical of many other bacterial toxins, having an enzyme active A-protomer (monomer), the S-1 subunit, and a binding B-oligomer of subunits S-2 through to S-5 [21–23]. Intact B-oligomer is required for binding of the holotoxin to receptor sites on the cell surface and enables entry of the A-protomer into the cells [24]. The toxin A-protomer catalyses the ADP-ribosylation of eukaryote GTP-binding regulatory proteins, preventing hormonal inhibition of adenylate cyclase and resulting in an increase in intracellular levels of cAMP [25]. Therefore, PT is capable of initiating two types of cellular response. One results from the lectin like action of the B-oligomer moiety, while the other reflects the ADP ribosylation, and hence the abolition of the signal transduction by guanine-nucleotide-binding proteins (G-proteins) [22]. The ADP-ribosylation effects of PT have been attributed to its catalytic transfer of an adenosine diphosphate-ribose (ADP-ribose) moiety from NAD^+ to the α-subunits of signal-transducing G-proteins [25–27]. This activity can be assayed by measuring ADP-ribosylation of a fluorescent synthetic peptide substrate using an enzymatic-HPLC coupled assay (E-HPLC) [28–32].

Although this E-HPLC assay correlates well with the toxicity observed by HIST using native PT preparations [32], it does not take account of the binding activities of the B-subunits. Chemical treatment has been widely used by manufacturers to detoxify PT and different detoxification procedures using various reagents have been shown to result in different amino acid side chain modifications and changes in conformational and epitope binding patterns of the resulting toxoided PT [7,33]. Previous results indicated that different ACV products show differences in ADP-ribosylation activity although a level which would be significant in relation to the reactivity seen in the HIST could not be defined [34]. This was to be expected as toxicity is dependent on the capacity of the enzymic moiety to enter the cell and this will be affected by chemical modification of the B-oligomer. A new functional test for PT based on the carbohydrate-binding and hence cell attachment property of the B-oligomer was developed [35].

In the present study, a refined detection strategy for an *in vitro* test system has been developed for examining both functional domains of PT based on a combination of E-HPLC and carbohydrate-binding assays. This strategy uses the E-HPLC method to measure the toxic activity of the A-subunits of the toxin and uses the carbohydrate-binding assay to measure the B-oligomer carbohydrate-binding function. Investigation was carried out on the relationship between the biochemical activities of PT found in the *in vitro* system with the *in vivo* activity shown in the HIST by the temperature measurement method using a number of ACVs of different types and formulations. A positive correlation of the two assay systems could lead to the establishment of an alternative method to the *in vivo* HIST.

2. Materials and methods

2.1. Materials

The PT used was a freeze-dried reference preparation (National Institute for Biological Standards and Control, UK (NIBSC) code 90/518). Each ampoule contained a nominal 20 μg of PT corresponding to 2100 IU of PT activity in HIST (lethal endpoint method) when calibrated against the WHO First International Standard for Pertussis Toxin (NIBSC Code JNIH-5) and was stored at −20 °C [36]. For use, each ampoule of 90/518 was reconstituted with 0.2% (w/v) ovalbumin (Sigma) in PBS. Aliquots of 100 μl (2 μg/ml; 200 ng) were lyophilised and stored at −20 °C as stock standards.

A two-component ACV, containing toxoided PT and filamentous haemagglutinin (FHA) in combination with diphtheria (D) and tetanus (T) toxoids (NIBSC code 00/486) was kindly donated by the Research Foundation for Microbial Diseases of Osaka University (Biken, Japan) and freeze-dried at NIBSC (nominal potency 11.25 units/vial). This product was used as the positive control in the E-HPLC assay as it was in stable freeze-dried form and known to have ADP-ribosylation activity.

A total of 76 batches (7 different types) of commercially available ACVs (V1–V7) were used in this study (Table 1). They comprised toxoided PT in combination with D and T toxoids (DTaP) ± other antigens such as *Haemophilus influenzae* type b capsular polysaccharide conjugated to T (Hib), inactivated polio vaccine (IPV) and other *B. pertussis* antigens (filamentous haemagglutinin (FHA), pertactin (69 K) and fimbriae 2 and 3 (Fims2/3)). Aluminium phosphate was used as the adjuvant in vaccines V1, V2, V3 while aluminium hydroxide was used in vaccines V4, V5 and V6. Samples of V7 type were adsorbed either to aluminium hydroxide or aluminium phosphate. Positive control vaccines for carbohydrate-binding assay were prepared by pooling several batches of each of the corresponding vaccine type.

The PT enzyme substrate, fluorescein-tagged $G_{\alpha i3}C20$ peptide, F-VFDAVTDVIIKNNLKECGLY-COOH (F-$G_{\alpha i3}C20$) was custom-synthesised by AnaSpec Inc., San Jose, CA, USA and was reported to have >95% purity. Anti-PT sheep serum (Code: 97/572) was prepared at NIBSC. Peroxidase labelled anti-sheep IgG (A-3415) was obtained from Sigma. All other chemicals, unless specified otherwise, are of analytical grade and purchased from either Sigma–Aldrich or VWR-BDH (Poole, UK).

Table 1
Enzymatic and fetuin binding activities in acellular pertussis vaccines.

Vaccines							
Product code	V1	V2	V3	V4	V5	V6	V7
Number of batches	26	10	5	6	14	5	10
PTx ADP-ribosyltransferase activity (E-units/ml)							
G-Mean	3.90	4.43	1.70	2.43	4.33	3.99	2.54
Range	(2.46–6.10)	(3.93–5.64)	(1.29–2.50)	(1.89–3.25)	(2.50–5.80)	(2.93–7.88)	(0.60–10.00)
Fetuin binding activity (B-units/ml)							
G-Mean	4.53	3.85	2.85	3.41	4.60	1.36	4.83
Range	(3.16–8.37)	(3.22–5.18)	(2.17–3.61)	(2.86–3.91)	(1.72–10.16)	(0.37–10.15)	(1.56–23.14)

Detailed description of types of acellular pertussis containing combination vaccines (ACVs) and the determination of their enzymatic and fetuin binding activities are as described in the text. G-Mean: Geometric mean.

2.2. E-HPLC coupled assay

The determination of PT ADP-ribosylation activity in vaccines was performed using a synthetic fluorescein-tagged peptide substrate, F-$G_{\alpha i3}$C20 coupled with an HPLC method for the separation and quantitation of the ADP-ribosylated product, as described in detail previously [32,34]. A standard curve (0–400 ng/ml), constructed from the results obtained with PT reference preparation (90/518) with either $Al(OH)_3$ or $AlPO_4$ adjuvant, according to the type of adjuvant in the test sample, was used to calculate the enzymatic activity in a test sample. Vaccine sample was appropriately pre-diluted in ovalbumin (OVA, 0.2%, w/v) solution so that the ADP-ribosylation activity would be within the range of the PT (90/518) standard curve. The enzymatic activity in the neat test sample (per ml) was calculated after taking account of the sample dilution factor. The enzymatic activity was presented as E-units where 1 unit is equal to fluorescence produced by 1 μg of PT (90/518) under identical assay and analysis conditions. Unless otherwise stated, all assays met the in house assay validity criteria [34] and were performed in duplicate and the results presented as means ± standard deviation (SD) or coefficient of variation (CV%).

2.3. Carbohydrate-binding assay

The PT carbohydrate-binding activity in vaccines using either bovine fetuin (Fet; containing sialylated *N*-glycans) or asialofetuin (AsFet; containing mainly neutral *N*-glycans) as carbohydrate coat was performed as described by Gomez et al. [35]. In brief, 96-well plates (NUNC Maxisorp Microtitre plates) were coated with Fet or AsFet (1 μg) and left overnight at room temperature. At the same time, vaccines and PT were treated with freshly prepared desorption reagent (0.15 M EDTA disodium salt dissolved in 0.52 M di-sodium hydrogen orthophosphate; 1:1 (v/v)) overnight at 37 °C to release the antigen from the aluminium adjuvant. After the desorption process, vaccines were centrifuged at 2000 RPM for 10 min (Centrifuge 5415 D, Eppendorf, Germany) and supernatant, usually as neat solution and up to 3 serial dilutions in diluents (3% BSA-PBS-0.05% Tween 20) were used in the assay. To construct standard binding curves, one ampoule of PT 90/518 (20 μg) was reconstituted and diluted with PBS. The starting concentration of PT for the AsFet binding assay was 1 μg/ml and for the Fet binding assay was 0.2 μg/ml; up to 7 serial dilutions were used. Anti-PT sheep serum (NIBSC Code: 97/572; 1/80,000 dilution) and horse radish peroxidase labelled anti-sheep IgG (Sigma–Aldrich Cat. No. A-3415; 1 in 2000 dilution) were used for detection. All samples were assayed in duplicate. The relative potency of PT binding activity in vaccine to PT (90/518) was calculated using a parallel line assay via the CombiStats software (version 4.0, EDQM) and was expressed as binding (B)-units where 1 B-unit equals to binding activity produced by 1 μg of PT (90/518) under identical assay and analysis conditions.

2.4. Dermal temperature histamine sensitisation test (HIST)

The HIST was carried out according to the method described in previous publications [18,19]. In brief, NIH female mice weighing 9–11 g were randomly distributed in groups of 10 (caged in 5s and individually marked). Mice were inoculated intra-peritoneally (i.p.) with either 0.5 ml phosphate buffered saline containing 0.2% (w/v) gelatin (PBSG) as negative control, PT reference (90/518) in PBSG at doses 5.25, 1.75 and 0.58 IU/dose or with test vaccine at 1 single human dose (SHD, 0.5 ml)/mouse. Five days after the inoculation, mice were challenged i.p. with 0.5 ml of histamine solution (prepared in PBS, equivalent to 2 mg histamine base per dose). The dermal temperature of each mouse was measured 30 min after challenge using an infrared thermometer (IT-550, Horiba Ltd., Japan) according to the method described by Ochiai et al. [18]. The HIST activity in the test vaccine was calculated in comparison with that of the reference toxin groups and expressed as IU/dose.

2.5. Statistical analysis

Data analysis was performed using the statistical program in Microsoft Excel Spreadsheet (2007 version).

Predicted activity (IU) was calculated from the corresponding logarithmic values of the fetuin-binding (Fet) and the enzymatic activities (Enz) using multiple regression analysis. The establishment of consistency of the regression factors $b1$ and $b2$ and constant factor C values for the multiple regression analysis was achieved by estimating population values of b and C for each product type, e.g. batches of a particular product were randomly ordered and $b1$, $b2$ and C were calculated for the first five lots according to the method described in Fig. 5. If the estimated values were found to have sufficiently converged (consistent), that is no significant change in the value observed when additional batches are added, then these estimates of $b1$, $b2$ and C would be considered as the approximating population values.

3. Results

3.1. ADP-ribosylation and carbohydrate-binding activities in different ACVs

ADP-ribosylation and carbohydrate-binding activities were measured in all 76 batches of 7 different vaccine types and the results are presented in Table 1. Both activities were detected in all vaccine types included in this study. There was no consensus value either for enzymatic activity or the binding activity amongst these different vaccine types. Variation of the geometric means of the activities was wide between the products, ranging from 1.70 (V3) to 4.43 (V2) E-units/ml for the enzymatic activity and 1.36 (V6) to 4.83 (V7) B-Units/SHD. The level of residual PT enzymatic activity and the binding activity in these vaccine types appeared to

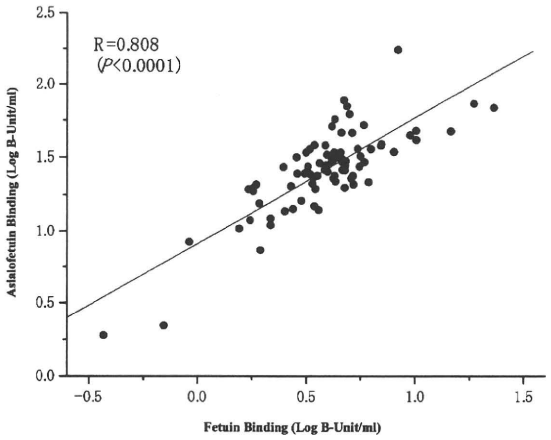

Fig. 1. Evaluation of the relationship between the binding activities to fetuin and asialofetuin in acellular pertussis containing combination vaccines (ACVs).

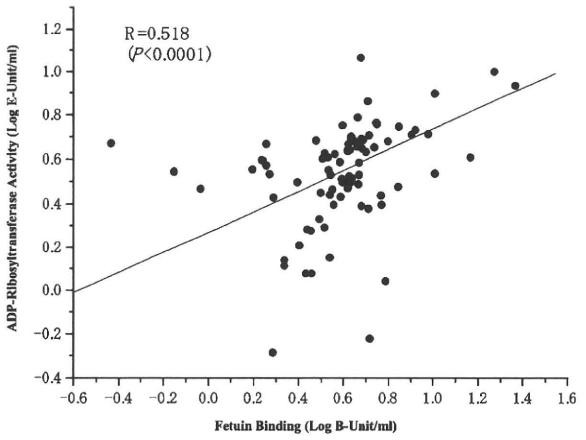

Fig. 2. Evaluation of the relationship between the fetuin binding and ADP-ribosyltransferase activities in acellular pertussis containing combination vaccines (ACVs).

be product specific and batch-to-batch variability (CV%) within a particular product ranged from 18.4% to 50.4% where the numbers of batches tested for a particular type of product were ≥5 which is in line with previous findings [34,35].

3.2. Evaluation of relationship of Fet and AsFet binding activity in ACVs

In the present study, the carbohydrate-binding activity of PT in different types of ACVs to both Fet and AsFet were determined and the relationship between their binding activities was assessed (Fig. 1). Although the values of binding activities differed between Fet and AsFet, there was a highly significant correlation between log values of the two binding activities ($P < 0.0001$) for all products included in this study. However, each different vaccine type had its own specific range of distributions of the unit values for the two binding activities. In addition, variation of the binding activity was wide for each product (results not shown). Since PT binds best toward the fully sialylated multi-antennary structures [35], that is Fet in this study, therefore in subsequent correlation analysis to evaluate the relationship between ADP-ribosyltransferase activity and HIST, only the Fet binding data were used in order to simplify the task.

3.3. Evaluation of the relationship between ADP-ribosylation and carbohydrate-binding activities in ACVs

When the relationship between enzymatic and binding activities was examined, an overall correlation between these activities was significant ($P < 0.0001$) in the range over certain levels of the both activities (approximately 0.1 log unit, Fig. 2) with a few exceptions for individual products. Furthermore, although there was a tendency for overall positive correlation between binding and enzymatic activities ($R = 0.518$), wide batch-to-batch variation was seen for both activities even in the same type of products. This indicates that the detoxification methods used in different product types might have simultaneously affected both the binding and the enzymatic activities, but not to a consistent extent for both activities.

3.4. Establishment of a mathematical model to predict activity observed in the HIST using data from the in vitro biochemical assay system via multiple regression analysis

Direct comparison of *in vivo* HIST activity with the *in vitro* biochemical assays either with the enzymatic or with the binding activity did not show correlation (data not shown). Efforts were then made to develop a mathematical model by multiple regression analysis using the data from the two biochemical assays to predict the activity in HIST. In the mathematical equation, log IU is used to define the activity determined by HIST and log IU (predicted) is the predicted activity calculated from data of the two *in vitro* assays. In the first stage, a three-factor model equation was constructed: log IU (predicted) = $b1 \times \log \text{Fet} + b2 \times \log \text{Fet} \times \log \text{Enz} + b3 \times \text{LogEnz} + C$; where $b1$ = regression coefficient for log Fet (binding); $b2$ = regression coefficient for interaction between log Fet and log Enz (enzymatic activity); $b3$ = regression coefficient for log Enz and C = the constant factor. When data for all products were analysed by this model using common regressions and constant factors (estimated from all the combined data of these products), no correlation was observed (Fig. 3a). However, when product specific regressions and constant factors were applied to the equation, good correlation was obtained between activity observed in HIST and the predicted activity calculated from the data of the *in vitro* assays (Fig. 3b and c). Since different products were usually manufactured using different detoxification processes, which would result in varying degrees of de-naturation of the PT, differences in enzymatic and binding activities between products would be expected. Hence, in a scatter plot for log IU and log IU (predicted), it was not surprising that data for a particular product would distribute in a specific data range of the graph. For example, in the case of products V1 (PT antigen is inactivated by treatment with glutaraldehyde) and V5 (PT antigen is inactivated by treatment with both formaldehyde and glutaraldehyde) (Fig. 3b and c), the log IU (predicted) values of product V1 distribute evenly in the range between −0.6 and −2.0 (Fig. 3b) while the log IU (predicted) values of product V5 distribute in a cluster between −0.2 and −1.0 (Fig. 3c). These results demonstrated that product specific regression coefficients ($b1$, $b2$ and $b3$) and the constant factor (C) have to be applied for the predictive calculation.

In order to assure the adequacy of the model equation, product specific regression coefficient factors were used for assessing the correlation between the predicted activity and the observed *in vivo* activity by HIST for each product separately (data not shown) as well as for the overall correlation for all products using the three-factor equation, log IU (predicted) = $b1 \times \log \text{Fet} + b2 \times \log \text{Fet} \times \log \text{Enz} + b3 \times \log \text{Enz} + C$ (Fig. 4a). A good correlation ($R = 0.916$) between the IU (predicted) and the *in vivo* IU by HIST was demonstrated and the predicted IU estimates were in agreement with measured values by HIST-

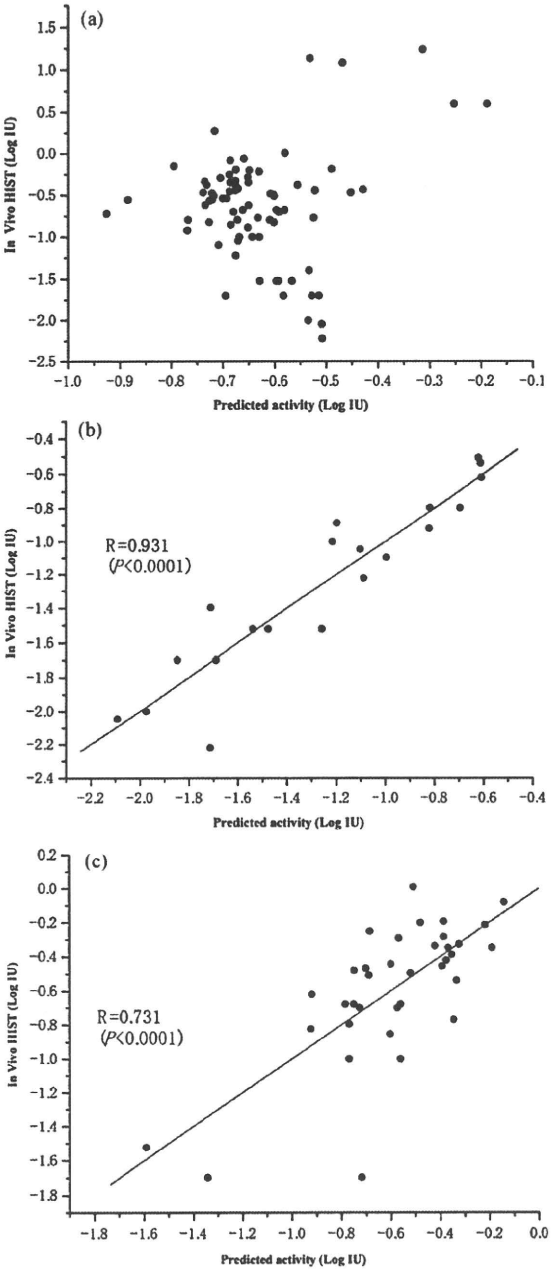

Fig. 3. Evaluation of the relationship between the predicted PT activities (calculated from the *in vitro* assays data) and the activities observed *in vivo* HIST by the temperature method. The predicted PT activities were calculated from the *in vitro* assays data using a three-factors mathematical model equation: log IU (predicted) = $b1 \times \log$ Fet + $b2 \times \log$ Fet $\times \log$ Enz + $b3 \times \log$ Enz + C. log Fet = log B-unit/ml (binding activity of PT to fetuin); log Enz = log E-unit/ml (ADP-ribosyltransferase activity). Panel (a), data for all products were analysed using common regression ($b1$ and $b2$) and constant (C) factors (estimated from all the combined data of all products); Panels (b and c) data for two types of vaccines (V1 and V5) were analysed using the product specific regression and constant factors respectively.

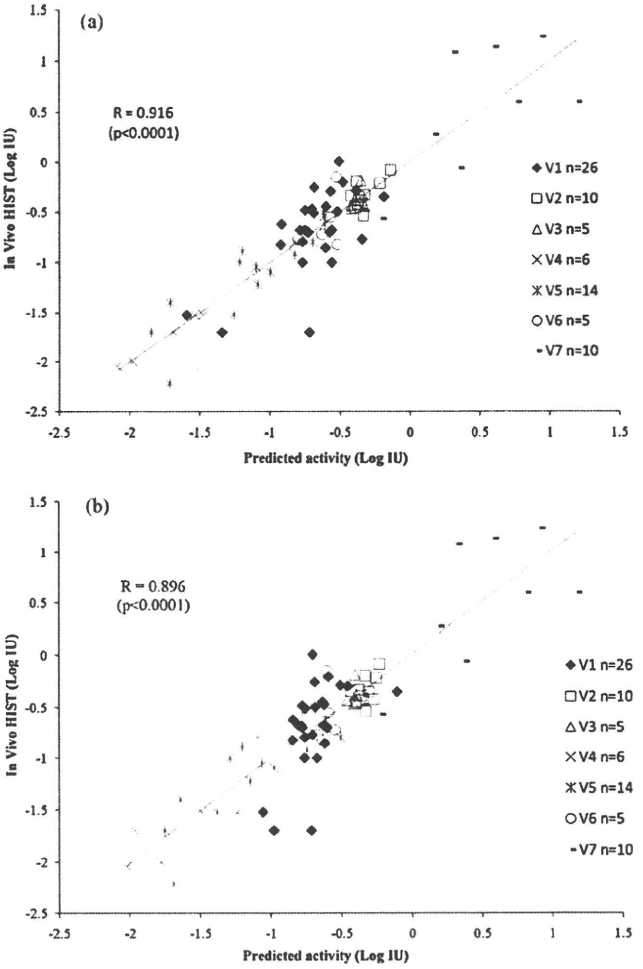

Fig. 4. Comparison of the three-factors mathematical model equation with the two-factors mathematical model equation based on product specific regression and constant factors in the determination of correlations between the *in vitro* assay system and the *in vivo* HIST. (a) data calculated using a three-factors mathematical model equation: log IU (predicted) = $b1 \times \log$ Fet + $b2 \times \log$ Fet $\times \log$ Enz + $b3 \times \log$ Enz + C; (b) data calculated using a two-factors mathematical model equation: log IU (predicted) = $b1 \times \log$ Fet + $b2 \times \log$ Enz + C. Abbreviations used are as described in Fig. 3 and an example how to calculate the predicted PT activity is as described in Fig. 5.

temperature assay. In order to simplify the equation further, calculation was carried out using these data to fit into a two-factor model equation: log IU (predicted) = $b1 \times \log$ Fet + $b2 \times \log$ Enz + C (Fig. 4b). Again, good correlation between the predicted and the measured HIST activities was found ($R = 0.896$). In view of this, the simpler two-factor equation is judged to be adequate in future data analyses. A detailed protocol showing how to calculate those specific regression and constant factors for the two-factor model equation is presented in Fig. 5.

4. Discussion

PT is an A-B type bacterial toxin possessing two functionally distinct domains: the enzymatic A-protomer and the B-oligomer that facilitates host-cell binding and entry of the A-protomer into the cell [16]. PT in its detoxified form is an important antigen present in all current ACVs. It is usually inactivated by chemical treatments either with formaldehyde or glutaraldehyde or both, or with hydrogen peroxide, or tetra-nitromethane [37,38]. These different detoxification procedures could result in different amino acid side-chain modifications and changes in conformational and epitope binding patterns for the resulting pertussis toxoid(s) [7,33]. Glutaraldehyde or formaldehyde treatment has been reported to have differential effects on toxoided PT biological activities [39]. The precise nature and location of the effects of different toxoiding reactions on the PT molecule have not been defined and the toxoided PT present in pertussis vaccines could be modified on either the A-protomer, B-oligomer or both components of the PT

Assay	LogFet	LogEnz	HIST (LogIU)
1	X_{11}	X_{21}	Y_1
2	X_{12}	X_{22}	Y_2
i	X_{1i}	X_{2i}	Yi
n	X_{1n}	X_{2n}	Yn

Measurements on binding, enzymatic and HIST activities of n batches of aP based vaccine were designated as $X_{11}, X_{12} \cdots\cdots X_{1i} \cdots\cdots X_{1n}$, $X_{21}, X_{22} \cdots\cdots X_{2i} \cdots\cdots X_{2n}$ and $Y_1, Y_2 \cdots\cdots Y_i \cdots\cdots Y_n$, respectively.

$$\text{Mean } X_1 = \overline{X_1} = \sum X_{1i}/n, \quad \text{Mean } X_2 = \overline{X_2} = \sum X_{2i}/n, \quad \text{Mean } Y = \overline{Y} = \sum Y_i/n$$

$$Sx_1x_1 = \sum X_{1i}^2 - (\sum X_{1i})^2/n,$$

$$Sx_2x_2 = \sum X_{2i}^2 - (\sum X_{2i})^2/n$$

$$Syy = \sum Yi^2 - (\sum Y_i)^2/n$$

$$Sx_1y = \sum X_1Y - (\sum X_{1i})(\sum Y)/n$$

$$Sx_2y = \sum X_2Y - (\sum X_{2i})(\sum Y)/n$$

$$b_1 = \frac{Sx_2x_2Sx_1y - Sx_1x_2Sx_2y}{Sx_1x_1Sx_2x_2 - (Sx_1x_2)^2}$$

$$b_2 = \frac{Sx_1x_1Sx_2y - Sx_1x_2Sx_1y}{Sx_1x_1Sx_2x_2 - (Sx_1x_2)^2}$$

$$C = \overline{Y} - b_1\overline{X_1} - b_2\overline{X_2}$$

$$Y = b_1X_1 + b_2X_2 + C = b_1LogFet + b_2LogEnz + C$$

Fig. 5. Calculation procedure for the regression factors $b1$ and $b2$ and constant factor C values in the two-factors mathematical model equation. The establishment of consistency of these values was by accumulating population values of b and C for each product type, e.g. batches of a particular product were randomly ordered and $b1$, $b2$ and C were calculated for the first five batches. If no significant change in the value observed when additional batches are added, then these estimates of $b1$, $b2$ and C would be considered as the approximating population values. X_1 = log binding activity; X_2 = log Enz activity; Y_1 = log IU from HIST; i = batch number; n = number of total batches for a particular product; s = Standard deviation; $\sum$ = summation; $\overline{X_1}$ = mean.

molecule. The dual biological function of the PT molecule is likely to be fully reflected in the *in vivo* HIST. To understand better the effect of chemical detoxification on PT, an assay for measuring the B-oligomer binding activity in combination with the E-HPLC coupled assay, which measures the A-protomer activity, should provide a potentially good correlation with the *in vivo* reactivity observed by HIST [34,35].

The assay sensitivity for the HIST lethal endpoint assay varies not only between mouse strain but also between batch of mice used in different experiments. The lethal endpoint assay can only detect the PT activity level above the assay sensitivity baseline as the dose causing fatal sensitisation of mice. In the present study, the HIST based on measuring temperature reduction after histamine challenge was chosen [18,19] because this approach allows more precise estimation of residual PT activity in vaccines with better reproducibility and allows also a better quantitative correlation with the *in vitro* assay system.

All toxoided PTs in the ACVs included in this study were inactivated by treatment with either formaldehyde or glutaraldehyde or both which accounts for most ACV types in the current markets. Aldehyde groups react primarily with the free ε-amino group of lysine residues and form crosslinks with other amino acid-side chain residues, which results in inter- and intra-molecular bridging and structural conformational changes to the B-oligomer [7,40–44]. Since there are no lysine residues present in the S1 subunit, the extent of modification of A-protomer is unclear [43,45]. However, both glutaraldehyde and formaldehyde can still react with other amino acids although to a lesser extent than lysine [46,47]. Several studies showed that both the enzymatic activity and monoclonal antibody binding affinity of S1 subunit were reduced after chemical treatment [7,33,41]. Furthermore, the intensity of the S1 subunit band decreased on SDS–PAGE gels after detoxification with formaldehyde, indicating some modification of the S1 subunit [44,48]. In the present study, a positive relationship between the binding activities and enzymatic activities was found for ACVs (Fig. 2) with the exception of a few batches that showed either very low enzymatic activity (<1.2 E-units) or binding activity (<1.25 B-units). Taking this together with previously reported results [33,41,44,48], it is suggested that the detoxification procedures used for PT in these vaccines could modify both the B- and the A-subunits, although direct evidence of changes in the A-subunit structure remains elusive.

In a previous study [35], a change of carbohydrate-binding characteristic of PT and toxoided PT was observed when using Fet (sialic acid containing glycoprotein) and AsFet (desialylated Fet). It is postulated that by measuring the binding profiles of toxoided PT to both carbohydrate types, it might be possible to differentiate different types of detoxification process in ACVs. In the present study, although we could show that there was an overall positive correlation between the two binding activities, differences in slopes of the relationship between different products were observed. This may be due to differences in proportion or types of the detoxification process amongst different products and product types (data not shown). An extended study into this aspect using well controlled and characterised detoxified samples will be needed to clarify this possibility.

In HIST (both lethal and temperature reduction), batch-to-batch variations from different manufacturers were seldom found. However, significant variation could be observed in the two *in vitro* assays even from the same manufacturer using the same detoxification procedure. This may indicate that the *in vitro* assay system has higher sensitivity and selectivity in measuring the biochemical functions of the PT or that the assay mechanisms are different between the *in vivo* and *in vitro* assay systems. In the *in vitro* assay system, the assays could distinguish HIST negative vaccine samples having very different biochemical assay profiles. For example, HIST negative samples could have a high enzymatic activity but low carbohydrate-binding activity. Conversely, they could have a high carbohydrate-binding activity but low enzymatic activity. These two are complementary functions of PT and both are required for toxicity. Other factors such as toxin translocation into host cells might also be a critical factor for toxicity.

There was no clear direct correlation between *in vivo* HIST and ADP-ribosylation activity nor between HIST and Fet binding in vaccine samples. Similar to previous findings [34], the results indicated that a value of enzymatic activity/or a value of binding activity common to all vaccines could not be defined. Furthermore, the enzymatic and binding activities did not correspond to the total PT protein content as specified in the vaccine formulations. These results are not surprising as different detoxification methods would affect the two PT subunits differently. Since direct comparison of *in vivo* HIST activity with the *in vitro* biochemical assays, either the enzymatic or binding activity, did not show correlation, effort was made to establish a mathematical model to evaluate the relationship between the *in vivo* and *in vitro* activities using multiple regression analysis. Comparison of equations consisting a two-factors (log IU (predicted) = $b1 \times \log$ Fet + $b2 \times \log$ Enz + C) and a three-factors (log IU (predicted) = $b1 \times \log$ Fet + $b2 \times \log$ Fet $\times \log$ Enz + $b3 \times \log$ Enz + C) which includes a factor of interaction of log Fet and log Enz showed no statistical difference in the predicted values between these two equations ($P = 0.782$, by paired t-test). Hence the two-factors equation was chosen for subsequent data analysis. When data for all products were analysed by this mathematical model using common regressions and constant factors (estimated from all the combined data of these products), no correlation was observed

(Fig. 3a). When product specific regression and constant factors were applied to the equation good correlation between the activity from HIST and the predicted activity by the *in vitro* assays was obtained (Fig. 3b and c). The results indicate that HIST activity could be reflected by a combination of functions of enzymatic and binding activities. According to the mathematical model for prediction of HIST activity, the combinations of somewhat varied levels of binding and enzymatic activities may result in similar levels of HIST activity, so that a similar level of HIST activity could result from the combinations of a range of binding and enzymatic activities. Furthermore it is interesting to note that product types which were produced by different detoxification processes resulted in a different population distribution in the correlation curves between the *in vivo* HIST activity and the predicted activity by *in vitro* assays. Since different types of products were usually manufactured by a variety of chemicals and conditions which resulted in varying degrees of detoxification and differences in enzyme and binding activities between products, our results supported the conclusion that the levels of residual PT enzymatic activity, the binding activity and the *in vivo* HIST activities of these vaccine types are product specific.

In the present study, a limited number of batches for each product was examined because of restricted availability. Although results of the model equation for predicting *in vivo* HIST activity by using *in vitro* binding and enzymatic activities of PT proved that this approach worked in principle, for utilising the equation reliably for predicting residual PT toxicity in an ACV, it is essential to establish consistency of the regression factors $b1$ and $b2$ and constant factor C values for the equation: $\log \text{IU (predicted)} = b1 \times \log \text{Fet} + b2 \times \log \text{Enz} + C$. To establish such consistency, it would be necessary to obtain population values of b and C for each product type by accumulating data until they become stable. Since large batch-to-batch variations in both the *in vivo* and *in vitro* activities were observed in this study, further investigation will be necessary for identifying the values of b and C values for monitoring of such consistency. Since ACVs included in this study were inactivated by treatment with either formaldehyde or glutaraldehyde or both, the information on ACVs detoxified by other method, e.g. hydrogen peroxide is not available in the present study. Furthermore, other factors associated with the detoxification procedure such as pH, reactant concentrations, matrix and the location of the reactive amino acid in the protein could also affect the extent of chemical modification. Therefore, these factors need to be established by each manufacturer and may have to be re-evaluated when the production process changes.

The molecular action of PT involves binding of the toxin to its receptors via the B-oligomer, membrane translocation of the S1-subunit and intracellular expression of the ADP-ribosyltransferase activity catalysed by the S1-subunit [6]. The present *in vitro* assay system only addressed the binding activity and the ADP-ribosyltransferase activity but does not take into account the membrane translocation (the internal processing of the toxoided PT *in vivo*). Furthermore, since the detoxification procedure could result in molecule aggregation, the effect of such aggregates on the carbohydrate-binding assay is unclear at the moment. Further investigation is under way to address these questions.

Attempts have been made to develop alternatives to the *in vivo* HIST. Most of these were based on approaches using refinement of animal or cell-based assays. These approaches have had limited success so far and have suffered from problems in assay reproducibility, or are unsuitable for final formulations due to presence of adjuvant or preservative in the final formulations [12,13]. In the present study, the approach using the *in vitro* binding and enzymatic activities of PT in a model equation for predicting *in vivo* HIST activity showed good correlation with the measured activity in HIST which provides good prospects for an alternative to the *in vivo* HIST. However, the successful implementation of this approach as an alternative to current *in vivo* HIST in regulatory requirement for ACVs would depend on further validation of this test system within and between products. An important step would be to organise an international collaborative study between regulatory agencies and manufacturers for method transfer as well as for further validation.

In conclusion, a mathematic model equation is established in principle for predicting *in vivo* HIST activity by using *in vitro* binding and enzymatic activities for ACVs in which PT was inactivated by treatment with either formaldehyde or glutaraldehyde or both. This indicates for the first time that a workable alternative/replacement of *in vivo* HIST is feasible. The reliability of the proposed equation is dependent on establishing a product specific consistency for the regression factors and constant factor C values. In addition, our results showed that chemical detoxification by aldehyde reagents could affect both the enzymatic and binding activities of PT molecules and that both of these activities may play roles in the HIST.

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新時代のワクチン戦略について考える

総論

7．ワクチン行政とサーベイランス

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ワクチン行政とサーベイランス

多屋馨子[1)]

SUMMARY
本邦のワクチン行政を考えるに当たり，現在国内で接種可能なワクチンの種類，現在本邦で実施されている血清疫学調査(感染症流行予測調査)，患者サーベイランス(感染症発生動向調査)，予防接種後副反応報告と接種後健康状況調査を紹介した．予防接種は感染症予防にとって最も基本的かつ重要な手段である．正しく理解して，予防可能な感染症から個人そして社会全体が守られるよう期待したい．
〔臨床検査　54：1263-1271，2010〕

KEYWORDS
予防接種法，感染症発生動向調査，感染症流行予測調査，副反応，VPD

はじめに

ワクチン行政を担当する部署は厚生労働省内でも複数にまたがっている．スケジュールや政策，ワクチンの流通や安全性などにかかわる内容は厚生労働省が担当し，新しい製剤の承認審査などは独立行政法人医薬品医療機器総合機構が担当し，承認後の製剤の国家検定などは国立感染症研究所が担当している．筆者が所属する国立感染症研究所感染症情報センターは，検定業務は行っていないが，予防接種で予防可能疾患(vaccine preventable disease；VPD)や予防接種に関する情報を収集・解析し，予防接種の有効性・安全性に関する研究を実施するとともに，国民に正しい情報を提供する役割を担っている．しかし，近年本邦はワクチン後進国あるいはワクチンギャップと言われており，ワクチン行政の一元化と，米国の予防接種の実施に関する諮問委員会(ACIP)のような予防接種の実施に関する方針を議論する公的な組織の必要性が求められてきた．新型インフルエンザの発生をきっかけとして，2009年12月に厚生科学審議会感染症分科会の中に予防接種部会(座長：独立行政法人国立成育医療研究センター，加藤達夫総長・理事長)が新設された．また，予防接種に関連する専門学会が複数集まって，その代表者から構成される予防接種推進専門協議会(会長：独立行政法人国立病院機構三重病院，神谷齊名誉院長)が設立された．インフルエンザのみならず，既存の予防接種についても戦略が議論されるようになり，ワクチン先進国に向けた取り組みとして期待される．

予防接種の種類
—国内承認・未承認ワクチン

2010年6月現在，本邦で接種可能なワクチンを**表1**に記載する．2008年12月にインフルエンザ菌b型(Hib)ワクチン，2009年6月に乾燥細胞培養日本脳炎ワクチン，同年10月にA型インフルエンザHAワクチン(H1N1株)，同年12月にヒトパピローマウイルスワクチン(子宮頸癌予防ワクチン)，2010年2月に肺炎球菌(7価結合型)ワクチンの接種が可能となり，接種可能なワクチンの数は増加した．

一方，海外の一部の国では，これら以外に**表2**に挙げるワクチンの接種が行われている．麻疹風疹おたふくかぜ混合(MMR)，麻疹風疹おたふく

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表1　日本で接種可能なワクチンの種類(2010年6月現在)

予防接種法に基づく定期接種	**生ワクチン** BCG ポリオ 麻疹風疹混合(MR) 麻疹(はしか) 風疹 **不活化ワクチン** DPT DT 日本脳炎(乾燥細胞培養)：2009年6月接種開始 インフルエンザ
定期外接種(任意接種)	**生ワクチン** 流行性耳下腺炎(おたふくかぜ) 水痘 黄熱 **不活化ワクチン** B型肝炎 破傷風トキソイド 成人用ジフテリアトキソイド A型肝炎 狂犬病 肺炎球菌(23価多糖体) 肺炎球菌(7価結合型)：2010年2月接種開始 ワイル病秋やみ b型インフルエンザ菌(Hib)：2008年12月接種開始 HPV(ヒトパピローマウイルス)：2009年12月接種開始 新型インフルエンザ(H5N1株)：国家備蓄 定期接種対象ワクチンを，対象以外の年齢で受ける場合
国家事業	**不活化ワクチン** A型インフルエンザHAワクチン(H1N1株)：2009年10月接種開始

表2　日本で承認されていないワクチンの種類(2010年6月現在)

国内未承認ワクチン	**生ワクチン** 麻疹風疹おたふくかぜ混合(MMR) 麻疹風疹おたふくかぜ水痘混合(MMRV) 経鼻インフルエンザウイルス 経口ロタウイルス 経口コレラ 経口腸チフス **不活化ワクチン** 不活化ポリオ 不活化腸チフス 髄膜炎菌 ダニ媒介脳炎 そのほかの多価ワクチン(A型・B型肝炎混合他，数種類)

かぜ水痘混合(MMRV)ワクチンは国内にないが，麻疹風疹混合(MR)，麻疹，風疹，おたふくかぜ，水痘のそれぞれの単抗原ワクチンが存在する．そのため医師が特に必要と認めた場合は，これらのワクチンを異なる部位に同時接種することが可能である[1]．以上のことから1つずつ27日以上の間隔を空けて別の日に接種するか，あるいは複数のワクチンを同時に接種するかのいずれかの方法で接種が行われているのが現状である．経口ロタウイルスワクチンに関しては，国内での臨床治験が終了し，承認申請がなされているところである[2]．また，不活化ポリオワクチンに関しては，ジフテリア百日咳破傷風混合(DPT)ワクチンとの混合(DPT+IPV)ワクチンとして，国内

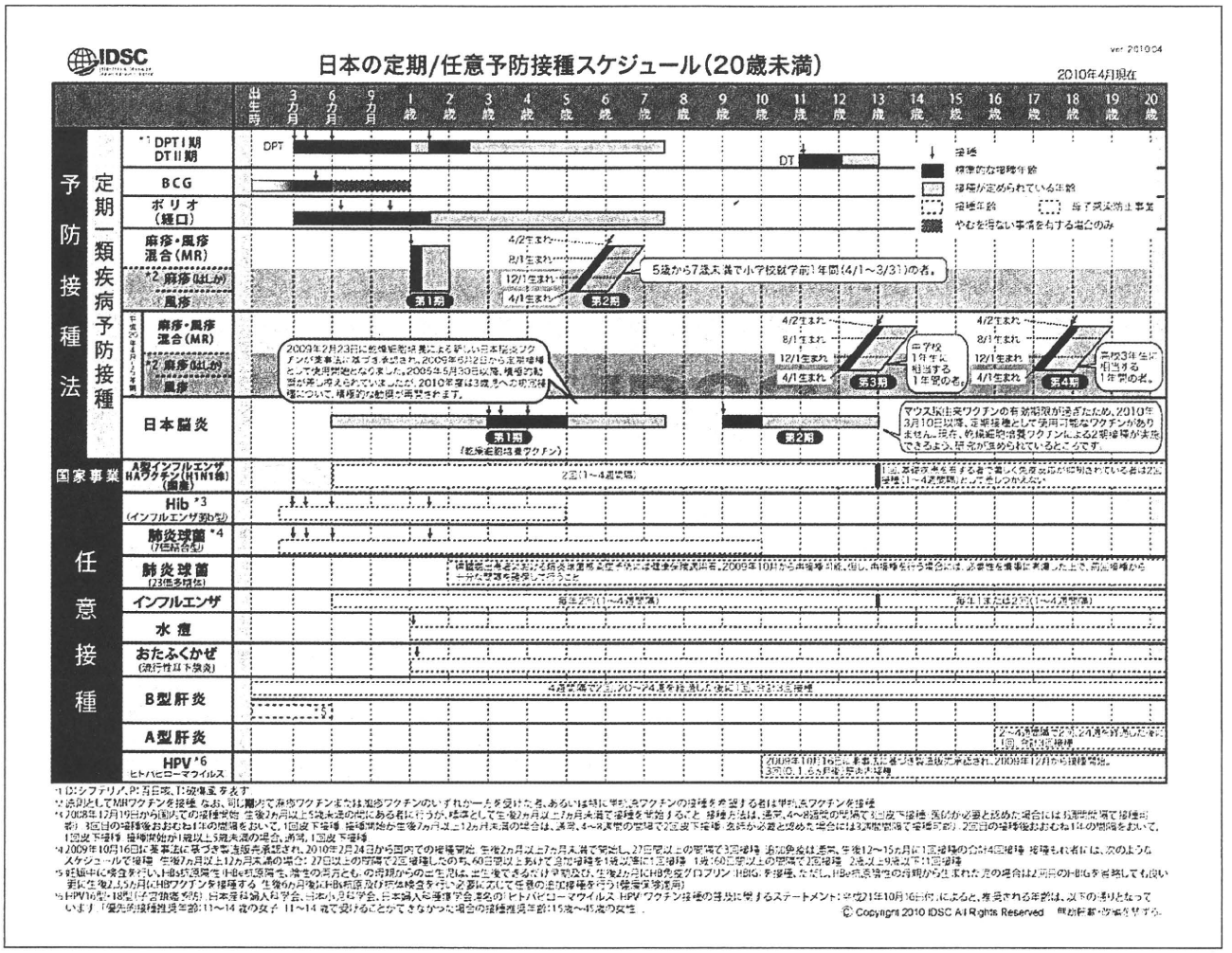
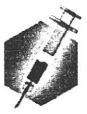


図1　日本の定期/任意予防接種スケジュール(20歳未満)
〔国立感染症研究所感染症情報センターHP(http://idsc.nih.go.jp/vaccine/freport.html)〕

で臨床治験が実施されている．

しかし，それ以外のワクチンについては，医師が個人輸入しなければ国内で受ける方法はなく，希望する場合は，国外で接種を受けることになる．医師による個人輸入は，“医師が患者の治療に際し，国内で販売されている医薬品などでは治療効果が得られず，患者救命のために外国で販売されている医薬品などを個人で輸入する場合”に行われているが，輸入に際しては煩雑な手続きが必要とされている[3]．



予防接種スケジュール

図1に2010年4月現在の20歳未満の予防接種スケジュールを示す．なお，**表1**に記載した国内で接種可能なワクチンのなかで，黄熱ワクチン，狂犬病ワクチン，破傷風トキソイド，成人用ジフテリアトキソイド，ワイル病秋やみワクチン，新型インフルエンザ(H5N1株)ワクチンに関しては，主に渡航時や特別な状況下で接種が行われるワクチンであるため，**図1**には含めていない．また，季節性のインフルエンザワクチンに関しては，65歳以上(一部，60～64歳)に予防接種法に基づく二類疾病として定期の予防接種(定期接種)が行われているが，**図1**は20歳未満を対象としているため，任意接種の枠組みに含めている．

図1の赤矢印は，標準的な接種年齢を一例として挙げたものである．しかし特に乳幼児においては，接種予定日に発熱，感冒などで体調不良となる場合も多く，予定通りに接種が進められないこともよく経験する．その場合は，必ずかかりつけ医と相談して，次の接種日を予定しておくことが接種漏れを防ぐ大切な方法である．

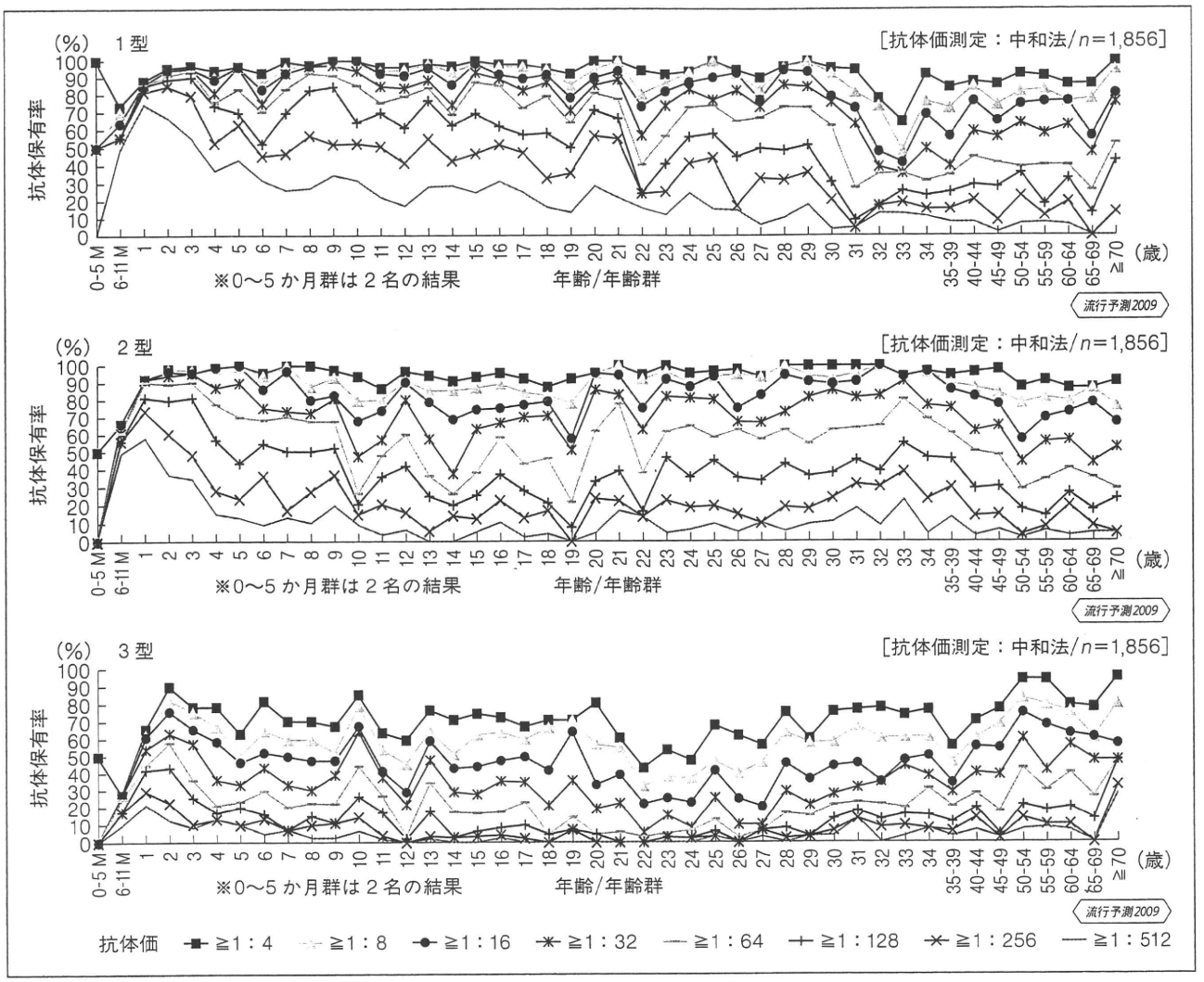


図2　年齢/年齢群別のポリオ中和抗体保有状況，2009年*

*：原則として2009年7〜9月に採取された血清の測定結果(2010年2月現在暫定値)．

〔2009年度感染症流行予測調査より〕

予防接種で予防可能疾患(VPD)のサーベイランス

1．血清疫学調査(感染症流行予測調査)

定期接種対象疾患(麻疹，風疹，インフルエンザ，ポリオ，日本脳炎，百日咳，ジフテリア，破傷風)については，感染症流行予測調査事業として，本邦の国民の抗体保有率が調査されている．

この事業は，厚生労働省健康局結核感染症課が実施主体となり，都道府県，都道府県衛生研究所，国立感染症研究所が協力して行っているもので，麻疹，風疹，インフルエンザについては毎年，ポリオ，日本脳炎については2年に1回，百日咳，ジフテリア，破傷風については5年に1回の頻度で実施されている．結果は年度報告書として報告されるとともに，速報として国立感染症研究所感染症情報センターのホームページに公開している[4]．地域，年齢，性別，予防接種歴別に検討することによって，予防接種の有効性，感受性者数の推計が可能となり，国の予防接種スケジュールを決定するためにも用いられている．

図2に2009年度の事業結果から，ポリオを例として記載する．ポリオは現在，世界中で根絶に向けた取り組みが行われているが，本邦を含めたWHOアジア西太平洋地域では根絶された感染症であり，本邦では1980年の症例を最後に野生株ポリオウイルスによるポリオ患者はいない．そのため，抗体保有はすなわち，ポリオワクチンの接種により獲得されている．乳幼児期の高い接種率により抗体保有状況は高く維持されているが，1型ポリオウイルスについては，1970〜1972年生

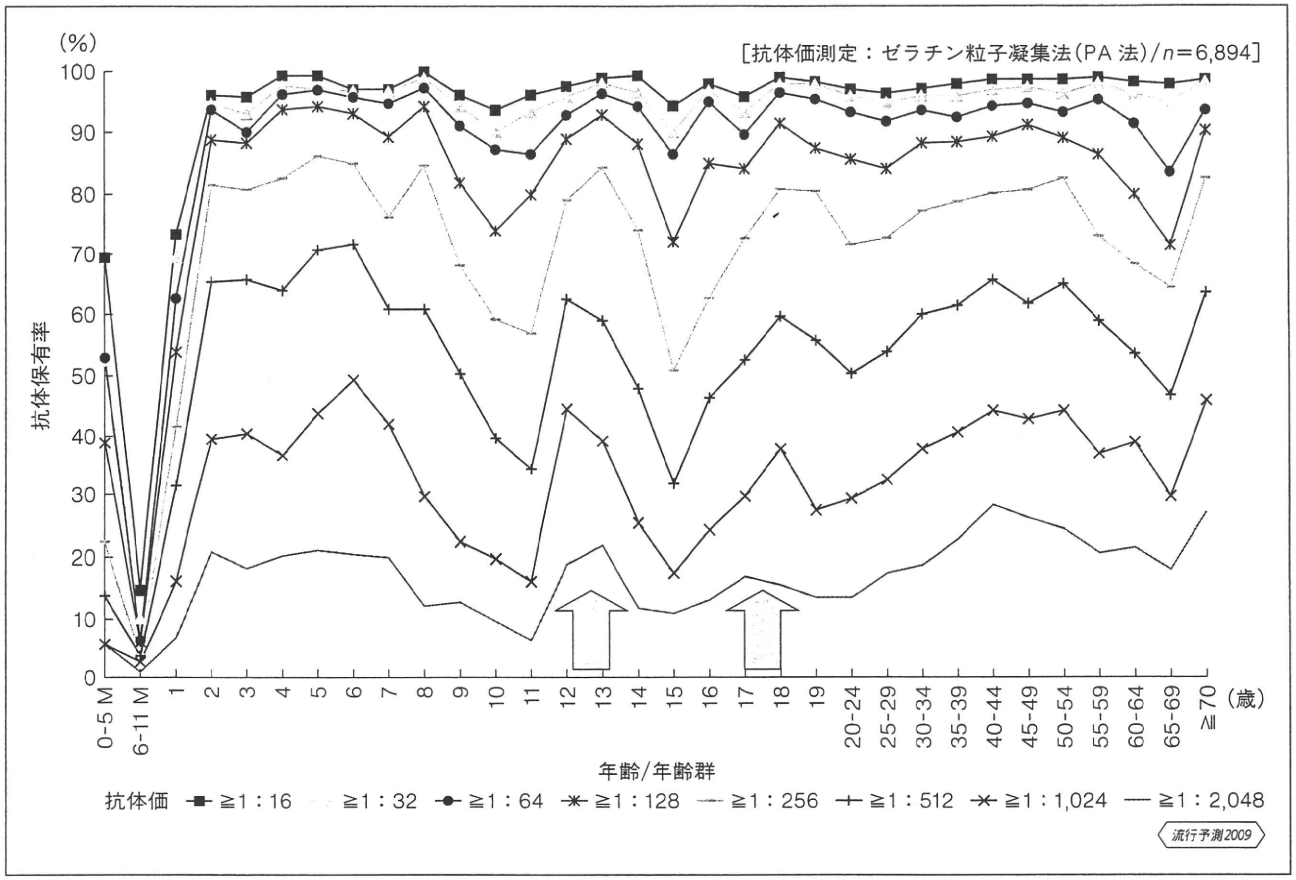


図3　年齢/年齢群別の麻疹 PA 抗体保有状況，2009 年*

*：原則として 2009 年 7～9 月に採取された血清の測定結果(2010 年 2 月現在暫定値)．

〔2009 年度感染症流行予測調査より一部改変〕

まれの抗体保有率が前後の年齢群より低く，この年齢層に対してワクチンの接種が奨められている．

図3には 2009 年度の麻疹のゼラチン粒子凝集(PA)抗体保有状況を示したが，2008 年度から始まった中学 1 年生，高校 3 年生相当年齢に対する 2 回目の接種の効果が結果に反映されている(**図3**矢印)．麻疹発症予防には少なくとも 1：128 以上の抗体を保有していることが必要であり，できれば 1：256 以上の抗体をもっていて欲しい．

2．感染症発生動向調査

表3, 4に国内で発生している VPD の患者報告数を示した．**表3**は感染症法に基づいて全数報告が義務づけられている疾患の一覧であり，**表4**は全数把握は実施されていないが，感染症法に基づく感染症発生動向調査により疾患ごとに定められた定点医療機関を受診した患者数が報告され，サーベイランスが行われている疾患である．

急性脳炎については 2003 年 11 月 5 日以降，定点報告から全数把握に変更となり，痘瘡については，2003 年 11 月 5 日以降新たに全数把握疾患に加わった．また，麻疹(定点報告のときは麻疹と成人麻疹がそれぞれ別の定点から報告されていた)と風疹は 2008 年 1 月 1 日以降，定点報告から全数報告疾患となったため，**表3**と**表4**に分けて記載しているが，**表3**は全数，**表4**は定点把握であるため，数字のみを単純に比較することはできない．

また，麻疹に代表されるように，定期接種として受けるよう努める義務(努力義務)が課せられているにもかかわらず接種率が 95%以上に達しておらず，まだ多くの患者が発生している感染症もあり，日本は輸出国と非難されることも多い．一方，定期接種として実施されていない定期外(任意)接種のワクチンに関しては，水痘や流行性耳下腺炎に代表されるように，水痘は定点報告のみで毎年約 25 万人，流行性耳下腺炎は年によって違いがあるものの，多い年で年間約 25 万人，少ない年でも約 6 万人の患者報告があり，実際には，この数倍の患者が発生しているのが現状である．