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Review

Quality Control of Diphtheria Tetanus Acellular Pertussis Combined (DTaP) Vaccines in Japan

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SUMMARY: Diphtheria tetanus acellular pertussis combined (DTaP) vaccines have been successfully used in Japan by controlling their potencies and toxicities with animal models. In accordance with the recent practical introduction of DTaP vaccines of various formulations, a question has been raised in other nations as to the efficacy of a quality control system based on animal tests and standard preparations. The World Health Organization issued its guidelines on the production and quality control of acellular pertussis vaccines in 1998 along with the concept of quality control by ensuring that production lots were consistent with clinical trial lots, rather than by comparing them with standard preparations in traditional laboratory tests. However, because it is not feasible to evaluate the combined use of vaccines from different manufacturers in a clinical study, the alternative trend of quality control may give rise to a difficulty in rationalizing the practical immunizations to use vaccines of different brands in a mixed consequence. A standardized national regulation system to ensure the equivalence of approved products would be essential for such an immunization practice. The success of the Japanese DTaP vaccination suggests the possibility of an effective quality control of DTaP vaccines by means of standardized test systems.

1. Introduction

Diphtheria tetanus whole cell pertussis combined (DTwP) vaccine was used effectively to prevent the diseases until it was temporarily discontinued due to two cases of post-vaccination death in the mid-1970s. Subsequently, diphtheria tetanus acellular pertussis combined (DTaP) vaccines were developed and implemented in Japan in 1981. In the course of the acellular pertussis vaccine development, protective antigens were identified by the Kendrick's mouse intracerebral (ic) potency (Kendrick) test, which in the 1950s had been proved to correlate well with clinical protection (1). The efficacy and safety of the DTaP vaccines have been successfully controlled using animal models in Japan. The potency of the pertussis component of the DTaP vaccines has been controlled by the modified Kendrick test with the immunization period extended to 3 weeks. The potency of diphtheria and tetanus toxoids in the DTaP vaccines has been controlled by in vivo or in vitro measurement of the antitoxin titers in the sera of immunized guinea pigs or mice. Animal tests have played crucial roles also in ensuring the safety of the vaccines.

DTaP vaccines of various pertussis antigen compositions and adjuvant gel contents have been developed and put into

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clinical use in other nations during the past 10 years. Accordingly, a question has been raised about the validity of the pertussis ic-potency test in predicting the clinical efficacy of acellular vaccines. An alternative approach relying on the results of clinical trials was suggested for the quality control of acellular pertussis vaccine (2). In this approach, the efficacy of a routine product lot was suggested to be assured by ensuring its consistency with the clinical trial lot. Attempts have also been made to extend this new approach to the potency tests of diphtheria and tetanus toxoids (73) in order to settle the problems concerning the varied production processes and adjuvant gel contents among the products from different manufacturers.

According to the newly proposed concepts of standardization and quality control, the World Health Organization (WHO) issued its guidelines for the production and control of the acellular pertussis component of monovalent or combined vaccines (WHO Guidelines) in 1998 (3). In this paper, the concepts of the guidelines and their influence on the current vaccination system are discussed in comparison with Japanese concepts of quality control of DTaP vaccines.

2. A brief history of Japanese acellular pertussis vaccine development

Pertussis vaccination was temporarily suspended after two cases of post-vaccination death in 1974 and 1975 (4). As a result of the lowered vaccination rate, by 1979 the annual number of cases of whooping cough had increased dramatically, to 13,000 cases (5). Shortly after the cessation of vaccination a research team was set up to improve the vaccine. The research initially focused on isolating protective antigen(s) of *Bordetella pertussis* using the Kendrick test. Two candidate antigens were chosen as potential protective vaccine antigens. One was the 22S protective antigen, which was reported to show purely protective activity (6,7) and the other was an antigen fraction showing histamine sensitizing (HS), lymphocytosis (or leukocytosis)-promoting (LP), hemagglutinating (HA) and protective activities (8-11). In the course of the investigation, an antigen fraction with an appearance similar to that of the 22S antigen was shown to exert no protective activity in the Kendrick test. Accordingly, the research was concentrated on establishing a method for isolating the fraction with HA, LP and HS activities. Although a fraction showing only HA activity was separated from the HA active fraction that also showed LP and HS activities in the early stage of the study (12), attempts to develop a practical vaccine were made along with the efforts to co-purify all the active substances. A soluble fraction rich in the activities was obtained by extracting ammonium sulfate-precipitated culture supernatant or whole culture of *B. pertussis* Phase I Tohama strain with concentrated alkaline salt solution. The extract was found to contain a considerable amount of endotoxin, which was removed by a sucrose-density gradient centrifuge (13). The fraction was later proved to consist of mainly two different antigens, pertussis toxin (PT) and filamentous hemagglutinin (FHA) (14). The toxic activities of the fraction were detoxified by a formaldehyde treatment (13). The DTaP vaccines were formulated using thus developed acellular pertussis vaccines.

Limit values for the residual toxicities of the new vaccine were another subject of the research. An initial goal was set of reducing every measurable toxic activity to one tenth of the level of average whole cell vaccines. Consequently, highly sensitive and quantitative assay methods were required to achieve the goal. Kurokawa et al. (15,16,19) had developed quantitative methods for assaying the toxicity to mouse weight gain (mouse body weight decreasing [BWD] toxicity) and LP toxicity. BWD toxicity was assayed by measuring the toxicity of a vaccine to mouse weight gain over the 16-h post-injection period to calculate the relative toxicity of the vaccine referring to the dose-response of a reference vaccine that have an assigned unit value of the toxicity. LP toxicity was assayed in the same mice by measuring the increase in peripheral leukocyte count on the third day of injection to calculate the relative toxicity of the vaccine referring to the dose-response of the reference vaccine.

At the time of the above researches, the methods available for assaying HS activity were those use the fatal sensitization of vaccinated mice to histamine challenge (histamine-sensitizing death [HSD]) (17,18). To achieve the goal of reducing the activity to one tenth of the average of whole cell vaccines, the assay method was required to have the sensitivity allowing quantitative discrimination of the DTaP vaccines that showed no fatal sensitization of mice to histamine challenge (20). Ishida et al. (21) developed a highly sensitive assay method for HS activity based on the change in rectal temperature of mice after histamine challenge on the fourth day of sensitization. The assay methods for BWD, LP and HS activities played crucial roles not only in the development of the vaccine but also in quality control of the vaccine after its clinical introduction (22). A lyophilized whole cell pertussis vaccine was prepared for use as the reference preparation (Reference pertussis vaccine for toxicity tests, Lot 1) in the toxicity tests. This vaccine was assigned a BWD toxicity value of 1,380 BWDU/vial. Its HS activity was determined to be 60 HSU/vial by referring to the Netherlands Reference 2, whose activity was stated as 4.4 HSU/ml (23,24). The identical value as the HS activity was assigned for the LP activity of the reference vaccine (19). The initial limit values implemented for BWD, LP and HS toxicities of Japanese DTaP vaccines were 10 BWDU, 0.5 LPU and 0.8 HSU per milliliter, respectively (19, 22).

The formulation of adjuvant gel was examined with regard to the safety and potencies of the diphtheria, tetanus and acellular pertussis components of the vaccine. A rather low concentration of the aluminum adjuvant gel was suggested to be adequate for the formulation of safe vaccines that confer sufficinet potencies of diphtheria and tetanus toxoids (68). The upper limit of the adjuvant gel concentration was defined as 0.3 mgAl/ml (22).

The newly developed DTaP vaccine was put into public use in 1981 after a clinical evaluation (25). The vaccine was shown to be clinically safe (5,25). In particular, it was shown to be remarkably less pyrogenic (5). Public confidence in the safety of the vaccine was quickly established, and the number of whooping cough cases quickly returned to the level seen before the suspension of DTwP vaccination (5).

3. A new trend in the quality control of DTaP vaccines and its complications

Following the recent clinical introduction of DTaP vaccines with various antigen and adjuvant gel constituents in the United States, as well as in European and some Asian countries, publications have been made by some researchers to propose alternative concepts of quality control of DTaP vaccines. The newly proposed concepts of quality control were based on ensuring the consistency of a production lot with clinical trial

lots rather than by comparing the production lot with reference or standard preparations (2, 26). In fact, the current ic-potency test might have a validity limitation in detecting the protective potency of certain antigens that have been claimed to confer protection in humans or in mouse respiratory infection models (27,28). The potency tests for diphtheria and tetanus toxoids were also reported to have a complication due to varied production procedures and constituents including adjuvant (29).

In 1998, WHO issued the guidelines for the acellular pertussis vaccine (3) based on the following rationale.

1) Each DTaP product produced by a different manufacturer should be considered unique due to the differences in production processes, antigens and adjuvant-gel constituents.
2) Their quality, therefore, cannot be evaluated in a laboratory test using a universal standard preparation.
3) Accordingly, their quality should be evaluated by ensuring their consistency with a clinical trial lot whose efficacy and safety have been clinically evaluated.

The above concepts have led to remarkable changes in the traditional methods of vaccination and vaccine quality control, as summarized in Table 1. We note that products from different manufacturers should also be considered unique because no chance of clinical evaluation would be probable on the effect of use of vaccines of different brands in a mixed consequence for primary and booster doses. Implementation of such concepts brings an important change to the traditional vaccination practice to allow using a combination of different vaccine brands for primary and booster immunization series. The new concepts require that DTaP vaccines of the same brand be used for all doses in the vaccination series (30). However, full implementation of the new system may cause practical difficulties. Practically speaking, it will be all but impossible to avoid using DTaP vaccines of different brands in a mixed consequence for achieving the full course of vaccination (30).

Although these new concepts seem to be taking hold in the quality control of DTaP vaccines, they will require careful evaluations before full implementation. In particular, certain contradictions inherent in these concepts should be discussed in detail.

3-1. Testing the consistency of products

It has been considered to have a reliable method for directly testing the consistency of the antigen composition of final bulks. Toward this end, intensive studies were made during the development of the acellular vaccine in Japan. However, an applicable method for testing the final bulk could not be established due to the aggregation of antigens by the detoxification process. It was concluded that the test for antigen consistency could be correctly applied only to materials before detoxification. It was assumed necessary, therefore, to consider the possibility of imposing an in-process control test using reference antigens and standardized methods, in spite of the possibility of practical inconsistency between the materials before detoxification and those in final bulk. Another possibility would be to test antibody production by vaccine antigens in appropriate animals. However, this would not be accurate enough for proving the exact consistency of products.

Another concern regarding the test for consistency is the stabilization of a clinical trial lot as the standard preparation. It is stated in the WHO Guidelines that it is necessary to ensure consistency between a routine product lot and clinical trial lot in order to assure efficacy (2,3). A difficulty would arise, however, in preparing stable vials, if by lyophilization. It would not allow ones to use the preparations of exactly the

Table 1. Comparison between the current Japanese quality control system and the WHO Guidelines

	Current Japanese system	New trends in the WHO Guidelines
Basic concept	A quality control system using laboratory models established or evaluated according to clinical relevance.	A quality control system based on ensuring consistency with clinical trial lots whose safety and efficacy has been assured clinically.(2,3)
Role of clinical trials and laboratory tests	Because results of a clinical trial would also have limited accuracy, it is necessary to complement them with results of post-marketing surveillance and of the tests in laboratory model systems.	Should adhere strictly to the production process used for the manufacture of the vaccine lots used to prove efficacy and safety in clinical trials. Laboratory tests should show equivalence between vaccine lots and those lots known to be clinically effective and safe.(3)
Vaccine brand and quality	Under the assumption that appropriate test systems can be effective in predicting the efficacy or safety of a product, all vaccines passing appropriately implemented laboratory tests are considered to have similar levels of safety and efficacy.	Because of the difference in antigen composition (and lack of the chance to evaluate the effect of using vaccines of different brands in a mixed sequence in a clinical trial) vaccines from different manufacturers should be assumed different and unique products.(3)
Vaccine brand and use	All vaccines passing appropriately implemented laboratory tests can be used even in a mixed sequence in the vaccination series.	Because data are insufficient regarding the safety, immunogenicity and efficacy of using vaccines from different manufacturers in a mixed sequence, the same brand of vaccine is recommended to be used for all doses in the vaccination series.(30)
Modifications of production process or formulation	Any modifications of production process must be rationalized clinically. However, it is also acceptable to make the modification according to the results of laboratory tests whose clinical relevance have been proved appropriately.	Any change in manufacture or formulation should be carefully evaluated clinically or, at least, by demonstrating equivalence of appropriately defined criteria of characteristics with that of clinical lots known to be clinically safe and effective.(3)

same characteristics both for a reference and for a clinical trial due to the difficulty in proving the influence of lyophilization as negligible.

3-2. Accuracy of the results of clinical trials

The reliability of the results of a clinical trial is of primary importance for the system prescribed in the Guidelines. In general, however, the results of a clinical trial have limited validity in accurately predicting the outcomes of a mass immunization due to the usually limited sample sizes or ethical restrictions in the inclusion of a placebo. Furthermore, estimates of vaccine efficacy would differ depending on the diagnostic criteria applied. The results would also be affected by the variation in clinical diagnoses, even when made and recorded in accordance with the WHO's case definition (31,32). Furthermore, an estimate of vaccine efficacy, and therefore of the difference in infection rate between the vaccinated and placebo-administered cases, would not be constant even in a single study. Instead, it would vary depending on the selected observation period after vaccination (33). Efficacy estimates on the same product can significantly vary in different trials (34,35). Therefore, we need to be cautious not to rely too much on the absolute figures of the estimated vaccine efficacy or safety. This fact emphasizes the importance of post-marketing surveillance.

In addition, it has become increasingly difficult to carry out clinical trials. Although animal testing is often criticized as a means of evaluating vaccine quality (26), it is necessary to develop useful animal model systems not only for quality control, but also to improve or develop new vaccines (2).

4. Quality control of DTaP vaccines in Japan

One of the fundamental policies for DTaP vaccination in Japan has been to apply a unified vaccination program in which all vaccines are controlled to have the consistent quality irrespective of the manufacturer, as explained in Table 1. This quality control has been realized using laboratory tests with reference or standard preparations. Accordingly, Japanese DTaP vaccines from six different manufacturers have been used in an interchangeable manner in the course of primary and booster immunizations. The system has been accepted as the realistic practice in the routine vaccination.

As no chance would be generally expectable to evaluate the effect of using vaccines of different brands in combination, the Japanese system needs to base upon the assumption that the vaccines from all the six manufacturers are equivalent in their efficacy and safety once they passed the appropriately implemented quality control tests. Effective model systems are of primary importance for quality control and successful routine vaccinations. Japanese quality control of DTaP vaccines has largely depended on animal models and has been successful thus far (5,36-38).

4-1. Potency of the pertussis component

Development of the Japanese acellular pertussis vaccine began with identifying a pertussis bacterial fraction showing protective activity. This was accomplished by means of a Kendrick test in which protective activity was measured as the degree to which a given fraction protected immunized mice against an ic injection of virulent *B. pertussis* at 2 weeks post-immunization. The test had been used throughout the course of developing the vaccine as the only laboratory method for identifying candidate antigens for an effective vaccine. In the process, the interval between immunization and challenge was extended from the original 2 weeks to 3 weeks to improve

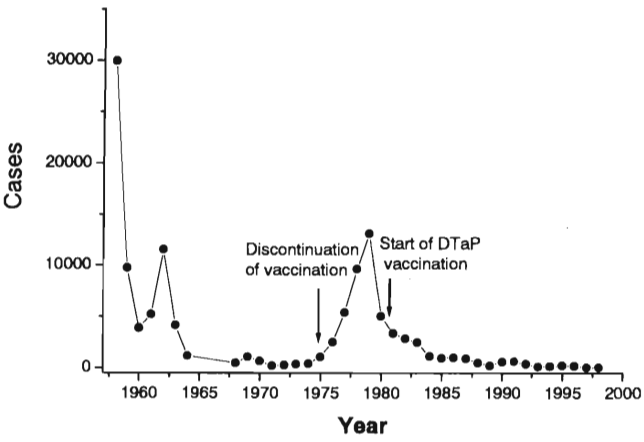

Fig. 1. Annual whooping cough cases in Japan. Pertussis vaccination was suspended in 1975 due to two cases of post-vaccination death. Whooping cough cases quickly increased to over 13,000 per year by 1979, then declined due to the resumed immunization with DTaP vaccine. The annual incidence has declined steadily since 1979, indicating the efficacy of the acellular pertussis vaccine.

reproducibility (modified Kendrick test). This modification was later proved to give an improved immune response to PT constituents of the vaccine in mice (39).

Thus developed Japanese acellular pertussis vaccines were found to comprise mainly FHA and PT. Although the vaccines from different manufacturers had varying contents of FHA, PT and other trace antigens, such as agglutinogens (14), all were found to have equivalent potency in the modified Kendrick test (40). With regard to clinical efficacy, the two Japanese acellular vaccines having the most divergent antigen compositions, namely T-type and B-type vaccines, were both proved to be clinically effective (33,36,38,41-43). Japanese acellular vaccines of T-type, B-type and intermediate antigen compositions approved by the modified Kendrick test showed comparable clinical efficacy (44). As shown in Fig. 1, pertussis immunization resumed with the acellular vaccines effectively reduced the cases of whooping cough in Japan, further suggesting the validity of the modified Kendrick test. However, doubts have been raised in regard to the ability of the ic protection test to predict the clinical protective potency of antigens other than PT. As possible alternatives, intranasal and aerosol infection models have been examined (27,28). No agreement has been reached so far on the immunization doses, schedule or challenge doses for an adequate standardization of the respiratory infection models.

4-1-1. Ic challenge model

The original Kendrick test was approved based on the results of a field trial of the British Medical Research Council (MRC field trial) (1) and has been effectively used to control whole cell vaccines. However, the immune response of mice to acellular vaccines has been suggested to be different from that of mice to whole cell vaccines. Whole cell pertussis vaccines generate both cell-mediated and humoral immune responses, while acellular vaccines induce mainly humoral immune response (27,45,46). T-cell dependent humoral immune response has been suggested to play a crucial role in the protection against ic challenge (27,45). In particular, the DTaP vaccine containing genetically detoxified PT has been shown to be highly protective in the ic potency test and was reported to confer a strong cell-mediated immunity both in mice and humans (52).

Both PT immunization and passive anti-PT antibody are

known to confer sufficient protection against ic challenge (47,48). PT has also been reported to show a synergistic effect when combined with such other antigens as FHA or 69K outer-membrane protein (69k OMP), both of which show marginal protection when given alone (49,50). The role of PT is, therefore, suggested to be crucial for the protection in the Kendrick test. In addition, Pillemer's Stromata Protective Antigen (SPA), which is assumed to comprise mainly bacterial components with HA activity and was, consequently, shown to have HS activity, has been proved to be highly protective both in Kendrick test and clinically based on MRC trial (51).

Regarding the immunization of mice, the optimum immunization period for the maximum anti-PT production by PT toxoid was reported to be 28 days (39). The immunization period for the modified Kendrick test, therefore, is assumed to be more appropriate for optimal anti-PT production comparing than the original Kendrick test. A definite correlation was seen between the logarithmically transformed potency values at 2 weeks and 3 weeks post-immunizations on 13 batches of DTaP and three batches of DTwP vaccines (unpublished). The resulting equation for the relationship between the log-potency values of immunizations of 3 weeks (Y) and 2 weeks (X) was $Y = 0.864X + 0.259$ with a correlation coefficient of 0.671 ($P = 0.0044$). Immunization of 3 weeks was suggested to give slightly higher potency values than that of 2 weeks. However, this difference did not reach the level of statistical significance. The results of the modified Kendrick test obtained by the Japanese National Control Laboratory were highly consistent with those of manufacturers (unpublished).

A method for sequential sampling inspection has been applied for interpreting the results of the test since 1981 so as to avoid approving a vaccine with a potency of below 4.0 units/ml. All Japanese acellular vaccines from six manufacturers, including T-type and B-type (14), were shown to have similar potencies by the modified Kendrick test (40) and have been proved clinically effective (33,38,41-44,53,54).

The ability of the ic potency test to quantitatively predict the clinical protective potency of acellular pertussis vaccines has not been fully established. It is necessary for establishing a quantitative clinical relevance as for whole cell vaccine to carry out a clinical trial similar to the MRC trial using vaccines with widely varied levels of protective potency, including those with very low potency. For ethical reasons, however, it is not possible to conduct such clinical trials. The ic challenge model is assumed worthwhile to be further explored into its mechanisms of protection for its valuable facilitation as the only available potency test method.

4-1-2. Aerosol and intranasal (respiratory) challenge models

In models of respiratory infection, the protective efficacy of a vaccine is evaluated only with respect to the bacterial clearance from lungs, rather than in terms of protection from mortality, since mice generally survive the challenge even after receiving much higher doses of pertussis bacteria than in the ic challenge model. One particular advantage of the respiratory infection model over the ic challenge model is that the susceptibility of mice is not limited to particular challenge strains of *B. pertussis*. As is the case in the ic challenge model, it was reported that anti-PT or PT alone was able to confer protection in the aerosol or intranasal challenge model (55,56). Cell-mediated immunity has been reported to play a crucial role also in bacterial clearance from the lungs of infected mice (46,57). Nonspecific cellular immunity has also been noted to play a role (58). A possible difference of the aerosol or intranasal challenge model from the ic challenge model would be that antigens such as FHA, pertactin or fimbriae could be protective without the aid of PT (59-64). However, caution should be taken when interpreting the results of the respiratory challenge model, since this model was reported to show no correlation with the clinical efficacy of whole cell vaccines and also to have found boiled whole cell vaccines protective (65). In addition, ethical restrictions will prevent quantitative determination of the clinical relevance of this model for predicting the efficacy of vaccines. Further study will be required to evaluate and standardize the respiratory infection model for any of its routine utilizations.

4-2. Potency tests of the diphtheria and tetanus components of combined vaccines

Vaccination with diphtheria and tetanus toxoids has played a key role in the effective prophylaxis against these diseases. The potencies of the toxoids have been controlled by measuring neutralizing antibody production in guinea pigs or mice. The major concerns raised thus far on the reliability of the potency tests have related to 1) immunizing animal species or strains and the clinical relevance of their immune responses; 2) the influence of the adjuvant effect of the aluminum salt or pertussis component; and 3) methods for titration of the serum antitoxin. The results of the assay to measure antitoxin induction might be affected by the species or strains of immunizing animals and by an adjuvant effect of the vaccine constituents. It would also be important to use a method for serum antitoxin titration that can correctly detect the toxin-neutralizing antibody (66).

There is currently a trend to simplify potency tests so as to confirm the potency of a product only to be above the minimally required level. Regarding the safety of the vaccination, however, it is also necessary to establish that the potency is not so high as to cause over-immunizations (67,68). Accurate quantifying of potency is important in this regard.

Guinea pigs have been used for the immunization because their immune response to the toxoid antigens appears to be similar to that of humans (69). Many works have been published regarding mouse models for testing the potency of toxoid vaccines (70,71,73). Care must be taken in selecting the appropriate mouse strains, since the immune response to toxoid antigens may differ from that of guinea pigs (72).

4-2-1. Effect of whole cell pertussis vaccine on the potency test of tetanus or diphtheria toxoid

The adjuvant effect of constituents in combined vaccines would affect the potency of toxoids. The pertussis component in DTwP vaccines shows a significant adjuvant effect on the toxoid immunizations both in animals and humans (68). In the case of tetanus toxoid in DTwP vaccines, Murata et al. (74) proved the efficacy of the potency test in predicting clinical immunogenicity by showing a clear correlation between the amount of immunized international units (IU) of tetanus toxoid and the neutralizing antibody titers in the sera of infants (Fig. 2). They used the potency values of the tetanus toxoid in DTwP vaccines measured against a plain preparation of National Standard Tetanus Toxoid based upon the report on parallelism of the dose-response regressions of the tetanus toxoids in plain and DTwP vaccines (75, 76). Kondo et al. (67) proposed a simple method for estimating the potency of tetanus toxoid in DTwP vaccines using the results measured against the plain standard in accordance with the parallelism of the dose-response regressions. The standardization of tetanus toxoid in DTwP vaccines could proceed using the data depicted in Fig. 2 and the method of Kondo et al. (67), if lyophilized

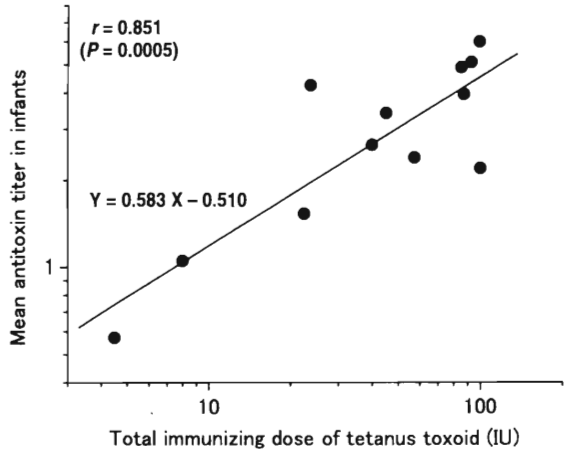

Fig. 2. Relationship between the amount of immunized tetanus toxoid in IU and the serum antitoxin in infants. The clinical relevance of the potency test results was proved for tetanus toxoid in DTwP vaccine. (ref. 74)

reference preparations of DTwP and plain diphtheria tetanus combined toxoids were available. Although no such data have been reported for diphtheria toxoid, it would also seem worthwhile to explore the standardization of diphtheria toxoid in DTwP vaccines in this regard.

4-2-2. Effect of adjuvant gel and animal model on the toxoid potency test

Controlling for the effects of the adjuvant gel would require an additional standardization strategy. The adjuvant effect may differ in different animal species or strains (72,77). Simultaneous dilutions of aluminum adjuvant and toxoid antigens would affect the slope of the dose-response regressions in the potency assays and, consequently, would influence the relative potency values (78). Factors possibly influencing the slope need to be carefully validated so as to minimize the deviation of the potency test results from those reflecting the outcomes of clinical immunization. Although aluminum adjuvant also has a significant effect on the clinical immunizations, differences in its content, so far as within a practical range, no significant difference was detected in the clinical immunogenicity of adsorbed diphtheria and tetanus toxoids probably due to a wide variation in the sensitivity of humans (68). Aluminum salt showed a remarkable adjuvant effect on the immunization of tetanus toxoid in mice and guinea pigs (66,79). Guinea pigs were shown to respond to an almost similar extent to adsorbed tetanus toxoids having markedly different contents of aluminum adjuvant gel (79). In a comparison of the adjuvant effects of aluminum hydroxide and calcium phosphate on tetanus toxoid, guinea pigs showed a much more human-like response to aluminum hydroxide and calcium phosphate than did mice (66). On the other hand, the response of guinea pigs to both these adjuvants on diphtheria toxoid was more similar to that of mice than to that of humans. However, the difference from the response of humans became less evident in guinea pigs after a booster immunization (66). When introducing mouse models, it is of primary importance to select the appropriate test conditions and mouse strains having a responsiveness to adsorbed toxoid vaccines similar to that of guinea pigs.

4-2-3. Effect of the toxoiding process on the immunogenicity and efficacy of adjuvant gel

The process of toxoiding affects the molecular characteristics of the resulting toxoids and has been reported to influence not only the immunogenicity of the toxoid antigen but also the efficacy of the aluminum adjuvant on the toxoid (72,80). This is why it has been difficult to consider the immunochemically assayed antigen contents, such as the value of limes flocculation (LF), as representing immunogenicity. The difficulty of consistently controlling toxoiding processes in turn makes it difficult to estimate the potency of final products using the potency values of bulk materials measured before blending with adjuvant gel because of the influence of inconsistent characteristics of the resulting toxoid molecules on the efficacy of adjuvant (81). Although it may be possible to directly evaluate the structural validity of the toxin neutralizing epitope (82), the results will not be sufficient for quantitatively predicting the adjuvant effect on the toxoid immunization.

4-2-4. Establishing the clinical relevance of toxoid potency tests

The current potency tests evaluate the overall immunogenicity of toxoid vaccines and would be useful to predict clinical efficacy if a correlation between the potency test results and clinical immunogenicity could be demonstrated, as in the case of tetanus toxoid in DTwP vaccines (74). In spite of a possible difference in adjuvant effects between aluminum salt and whole cell pertussis vaccine (79), there seems to be a possibility of correlating the potency estimates and clinical immunogenicity of adsorbed toxoid vaccines. Because a clear correlation between the potency of tetanus toxoid and the clinical immunogenicity of DTwP vaccine was proved even using the tetanus toxoids prepared by various procedures including those suggested to affect differently the characteristics of toxoid molecule (74). In addition, the difference in adjuvant contents was shown to have little effect on the immunogenicity both in guinea pigs (79) and humans (68). Further investigation will be necessary, however, for identifying appropriate animal strains and immunization procedures – including that for making graded doses – so as to maximally reflect the clinical immunization procedure. A potency test involving immunization with serially varied volumes of undiluted adsorbed vaccine would seem to be worth investigating as a possible alternative to the current immunization using serial dilutions of the vaccine because of the possible difference between the effects of dilutions of antigen and adjuvant on the immunization. It would also be important to evaluate boosting in a potency test, since boosting is used clinically and has been shown to result in antitoxin levels in guinea pigs that are highly similar to those in humans and that differ considerably from those obtained with a single dose (66,79).

4-2-5. Other problems

The immunogenicity of toxoid might be affected by other antigens, such as *Haemophilus influenzae* type b (Hib) polysaccharide. Conjugation or simultaneous immunization of Hib polysaccharide and tetanus toxoid was reported to show profound effects on the immunnogenicity of both antigens in mice without affecting the immunogenicities of diphtheria and pertussis components (83,84). However, no such immunopotentiation was noted in infants (85) or guinea pigs (86). Again, selecting appropriate strains would be very important for the potency test in mice. Meaningful potency estimation for Hib-conjugated tetanus toxoid in DTwP-Hib vaccines in mice seems to be possible only on the vaccine before or without conjugation. However, the dose-response curve of tetanus toxoid in DTwP vaccine containing the conjugate of Hib-specific polysaccharide or oligosaccharide did not seem to

deviate significantly from parallelism with that of the control DTwP vaccine in mice of the CBA strain irrespective of the increased extent of antitoxin production (83). If this is true, estimation of the potency of tetanus toxoid in DTwP-Hib conjugate vaccines might be possible by the method of Kondo et al. (67) using a reference preparation of DTwP vaccine and the data of potency measurements on DTwP vaccines with and without the Hib-conjugate.

4-2-6. Antitoxin titration

With regard to efficacy, it is important to evaluate the ability of toxoids to induce toxin-neutralizing antibody. In vivo toxin challenge methods have been losing support for reasons of animal welfare. In vitro assay methods have been developed for measuring the serum antitoxin titer. Passive hemagglutination (87), enzyme linked immunosorbent assay (ELISA) (88), toxin binding inhibition (ToBI) (89) and passive particle agglutination (90,91) assays are the methods based on antigen-antibody binding. The micro Vero cell culture method (92) is an in vitro toxin neutralizing assay method. Because it is essential that the potency test be capable of evaluating the production of toxin-neutralizing antibodies, every in vitro assay method based on binding with the toxin molecule needs to be validated carefully before utilization. Generally, such validation has been made by proving a correlation with an in vivo toxin-neutralizing assay. The immunizing efficacy of toxoid would be affected by various uncontrollable factors in the toxoiding process to give rise to final products of varying potency (80). The micro Vero cell culture method is highly sensitive and appropriate in this regard. The ToBI assay may also become a promising binding assay in this context, through the use of a monoclonal antibody recognizing the epitope of toxic action for sensitizing micro-wells of plates. ELISA and passive particle agglutination assays may also become promising direct binding assays by using specific peptides exhibiting the responsible structure for the toxic action of toxin molecules.

4-3. Toxicity

The toxicity of DTwP vaccines has been controlled as overall toxicity by means of the mouse body weight gain (MWG) test, in which the toxicity of a vaccine is evaluated by the toxicity to weight gain of mice over the first 7 days post-vaccination. Even though the MWG test has a long history of safety control for the pertussis vaccine, it has been discredited by reports that PT toxicity accelerates the weight gain of mice, thereby improving the results for toxic vaccines (106-108). It is therefore considered important to evaluate each of the toxicities separately (16,19) for meaningful toxicity tests.

Toxicity tests for vaccines generally need to be implemented for all measurable toxic activities unless proven to have no clinical relevance. It is difficult to prove the clinical relevance of a biological activity, particularly without its quantitative assay results (94). Kurokawa et al. analyzed bacterial factors of pertussis vaccine influencing the MWG test and reported that endotoxin, heat-labile dermonecrotic toxin (HLT) and PT were the major moieties affecting the test at 1, 2 and from 3 days after injection, respectively (16,19,21). They developed quantitative assay methods for these activities and the methods were later implemented for the quality control of DTaP vaccines in the Minimum Requirements of Biological Products of Japan (Minimum Requirements) in 1981 (19,22).

4-3-1. Endotoxin

One of the characteristics of Japanese acellular pertussis vaccine is that it results in a significantly reduced febrile reaction rate, as low as 1% for a febrile reaction over 37.5°C

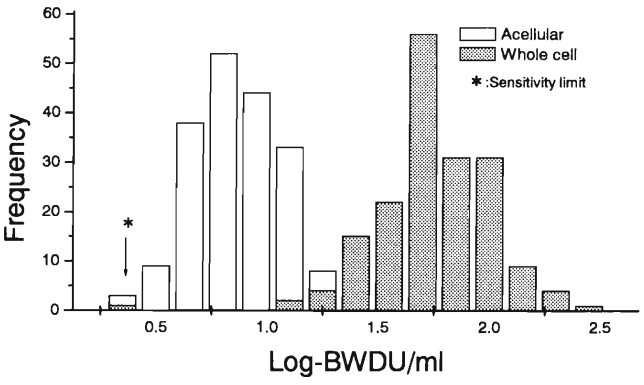

Fig. 3. Mouse body weight decreasing (BWD) toxicity of Japanese DTwP and DTaP vaccines. The average BWD toxicity of the DTaP vaccines was reduced to approximately 1/10 of that of DTwP vaccines.

(95). This had been initially achieved by the BWD toxicity test (21). As represented in Fig. 3, the average BWD toxicity of acellular vaccines was reduced to approximately one-tenth that of whole cell vaccines. The BWD toxicity test is capable of detecting the toxicity of endotoxin to mouse weight gain during the 16 h after injection but is possibly affected by other constituents, such as aluminum adjuvant gel and active PT (106). We should therefore consider replacing the BWD toxicity test with some other test method, particularly for DTaP vaccines (106).

4-3-1-1. The bacterial endotoxin test: The endotoxin content of Japanese DTaP vaccines can also be measured by the endotoxin test method (22) using Limulus amaebocyte Lyzate (LAL) reagents whose reactivity to 1-3-β-D-glucan has been removed or suppressed (96-100) to avoid any possible confusion due to reaction with the non-pyrogenic substance. However, a special precaution has been shown to be necessary when applying the endotoxin test to DTaP vaccines. In an enhancement/suppression experiment to detect endotoxin added to DTaP vaccines, some commercial DTaP vaccine batches significantly suppressed the endotoxin test, as shown in Table 2 (the amount of endotoxin detected is represented as a percent of the added endotoxin). In spite of

Table 2. Interfering effect of DTaP on LAL* activity of endotoxin

DTaP Lot	% detected
A	140.7
B	115.9
C	118.9
D	26.7**
E	120.6
F	126.4
G	161.6
H	135.3
I	135.6
J	8.7**
K	131.2
L	115.0
M	28.4**

1.0 EU/ml of endotoxin spiked to the DTaP vaccine lots was detected by the endotoxin test to express as percent of the spiked endotoxin.
*LAL: Limulus amaebocyte Lyzate
**Significantly suppressed

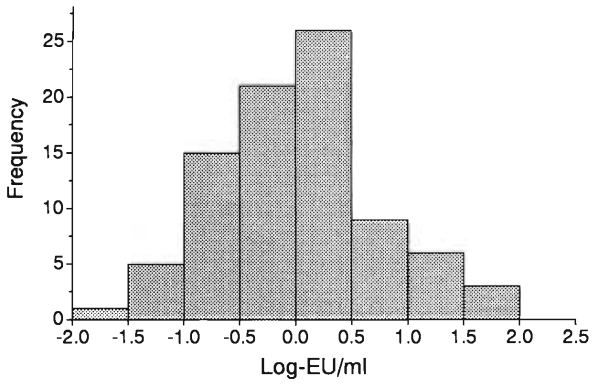

Fig. 4. Endotoxin content of Japanese DTaP vaccines. The endotoxin content showed a diverse distribution. The DTaP vaccines of the early 1980s contained considerable amounts of endotoxin, but these were reduced to a level of 0.1 EU/ml in more recent batches.

the strong suppression of endotoxin test, endotoxin added to vaccine batches D, J and M was found to have retained its biological activity as measured by the pyrogen test, the lethality of D-galactosamine-treated mice and the method of TNF-α induction in a mouse monocytic cell line, RAW264.7 cells (109). Caution is thus suggested when applying the endotoxin test to DTaP vaccines.

Japanese DTaP vaccines show a diverse distribution of endotoxin content, as shown in Fig. 4. A rather high level of endotoxin was detected in the DTaP vaccine batches of the early 1980s, but in more recent batches the level declined remarkably to below 0.1 EU/ml.

4-3-1-2. Clinical relevance of the endotoxin test: The quantitatively estimated endotoxin contents of DTaP vaccines could be utilized for analyzing the relationship with febrile reaction rate of vaccinees, as shown in Fig. 5. Here, the probit transformed rate of vaccinees showing a febrile reaction of 37.5°C or more is shown vertically and the endotoxin content in vaccine batches is shown horizontally. The data summarized in Fig. 5 could be utilized for estimating a vaccine's febrile reaction rate, provided its endotoxin content was available. Clinical studies have become increasingly difficult to conduct. And even in cases where clinical data is available, it is not possible to identify a correlation between a toxic activity of

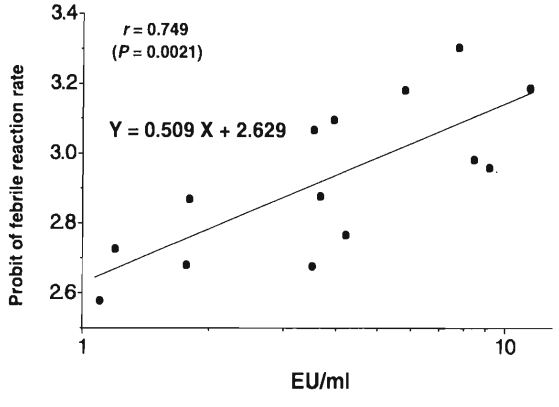

Fig. 5. Relationship of endotoxin content and the rate of febrile response in vaccinees. A significant correlation was demonstrated between endotoxin content in DTaP vaccine batches and the probit-transformed rate of febrile response of 37.5°C or more in the vaccinees. The equation could be utilized for estimating the rate of the febrile response by a vaccine batch with known endotoxin content.

vaccine and a clinical adverse effect without quantitative assay results (94). Qualitative observations in a clinical study may tend to be biased depending on the observing personnel. Quantitative data such as temperature in a febrile reaction can be more reliable and are useful for analyzing the relationship with the quantitative laboratory assay results of biological activities. We believe that the quantitative quality control of final products would be meaningful in this regard.

4-3-1-3. Necessary cautions for substituting a test method: Introduction of a new test method, particularly of a toxicity test, requires careful validation. In the case of the endotoxin test, at very least a limit value for approval or rejection should be implemented so as not to approve a product that should have been rejected in the pyrogen test because safety of such products has not been proved clinically. The results of the different assays will inevitably vary. Thus the new limit should be defined so as not to falsely approve a pyrogenic product even when its endotoxin test result shows a maximal variation. From the point of view of statistics, the extent of variation or accuracy of an assay result would depend on the test design, including the number of doses and their intervals. Use of a smaller number of doses or intervals will produce less accurate results. Consequently, the limit for rejection or acceptance should not be defined according to the ordinal significance-of-difference bases to avoid a better chance of approval due to a less accurate measurement. Instead, the limit should be defined according to the allowable variation of results under a defined accuracy, therefore a defined design, of a test.

In addition, special care should be taken when substituting an in vitro test for an in vivo test. An in vitro test generally cannot detect a possible in vivo synergistic effect of vaccine constituents on the target toxic activity. It may require a preliminary quantitative evaluation of the synergism to define the limit value not only for regulating the amount of toxic substance but also to regulate the extent of the resulting toxic effect. The prescribed limits of endotoxin contamination in interferon injections, human albumin and purified plasma protein fraction injections in the Minimum Requirements were defined by taking the synergistic effect and variation of the test results into consideration so as not to allow the approval of possibly pyrogenic products (22). In the case of endotoxin in DTaP vaccines, no synergystic effect has been detected so far. Therefore, it would seem reasonable to set the limit for endotoxin so as not to allow approval of a batch that has not been empirically shown to be safe – i.e., one that may contain excess endotoxin than previous batches.

4-3-2. LP activity

Leukocytosis or lymphocytosis is a characteristic symptom of whooping cough patients and PT is known as the responsible agent. Leukocytosis can be induced in mice by whole cell pertussis vaccine or PT, and a method for quantitative measurement of the activity was described by Kurokawa et al. (101). The assay was implemented for testing the toxicity of DTaP vaccine in Minimum Requirements in 1981. The activity can be assayed quantitatively in mice by comparing peripheral leukocytosis induced by a vaccine batch with that induced by the serial dilutions of the reference vaccine on the third day of injection. Figure 6 represents accumulated results of the assay on commercial DTaP and DTwP vaccines. The LP activity of the acellular vaccines was also shown to have been reduced to approximately one-tenth that of whole cell vaccines. The LP activities of most of the DTaP vaccines of the early 1980s were over 0.25 LPU/ml, but these values declined to a

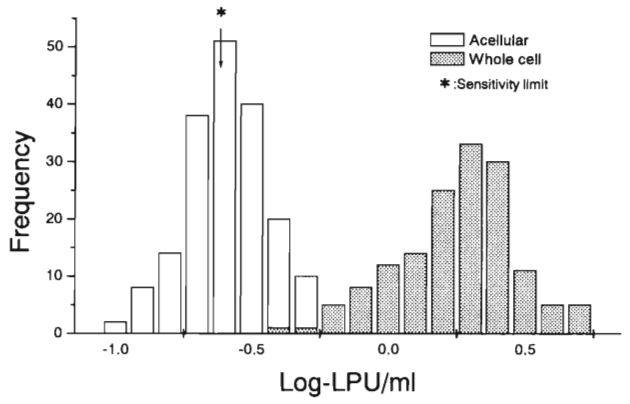

Fig. 6. Lymphocytosis (or leukocytosis)-promoting (LP) activity of Japanese DTwP and DTaP vaccines. The LP activity of the DTaP vaccines was reduced to 1/10 that of DTwP vaccines.

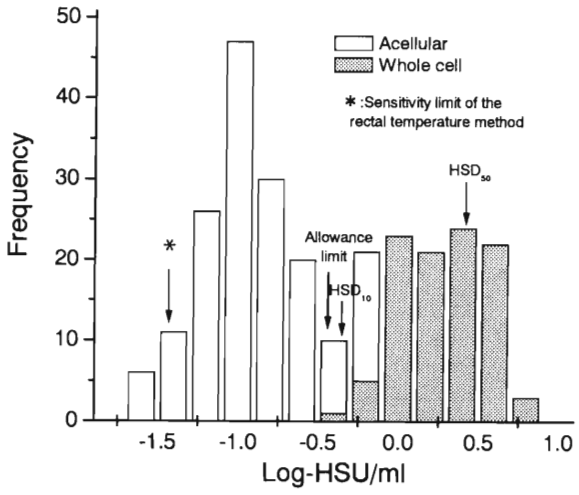

Fig. 7. Histamine sensitizing (HS) activity of Japanese DTaP and DTwP vaccines as measured on the fourth day of sensitization. The sensitivities of assay methods for HS activity are shown for purpose of comparison. The rectal temperature method could adequately evaluate the HS activity of Japanese DTaP vaccines that showed no histamine sensitizing death of mice. The HS activity of DTaP vaccines was shown to be reduced to less than 1/10 the average level for DTwP vaccines.

level below or equal to the sensitivity limit. Accordingly, it would be regarded possible currently to simplify the test adequately, instead of carrying out a quantitative measurement, to prove only that the activity of a batch of DTaP vaccine does not significantly exceed the sensitivity limit under a valid test condition. Furthermore, it would be assumed possible to discontinue the test if LP activity was proved to be more labile to the treatment of detoxification than HS activity under any possible variation of the detoxifying process.

4-3-3. HS activity

As we attempted to develop acellular vaccines with average toxicities of less than one tenth of the average of whole cell vaccines, we faced a serious difficulty. Particularly for HS toxicity, the traditional assay method using HSD was found not adequate due to its insufficient sensitivity and accuracy even in mice of the HSFS/N line of the N:NIH(SW) strain, which are suggested to be the most sensitive to HS activity (102). A highly sensitive and accurate assay method for HS activity in which rectal temperature change is measured after histamine challenge on the fourth day of vaccine injection was developed by Ishida et al. in 1979 (21). The rectal temperature method was sensitive enough for quantifying even the activity of vaccines causing no HSD. Figure 7 shows the frequency distribution of HS activity of DTaP and DTwP vaccines. The sensitivity limit (HSD_{10}) and the median sensitizing dose (HSD_{50}) for the method of HSD and the sensitivity limit for the rectal temperature method are also shown in the figure together with the current acceptance/rejection limit for DTaP vaccines. The regulation limit for HS activity implemented in 1981 was 0.8 HSU/ml, and was revised to the current 0.4 HSU/ml in 1991. The average HS activity of DTaP vaccines was shown to be less than one tenth of the average of whole cell vaccines.

A commercial batch of DTaP vaccine with considerable HS activity was assayed repeatedly five times by the rectal temperature method and by the method of sensitizing death. Although both methods were shown to be highly reproducible, the rectal temperature method was found to have a significantly better accuracy than the sensitizing death method, as seen in Fig. 8. This finding emphasizes the advantage of the measurement on continuous variables, such as rectal temperature, rather than discrete variables, such as survival rate, which gives only one data using several animals. Consequently, the rectal temperature method was expected to give more accurate results using a smaller number of animals.

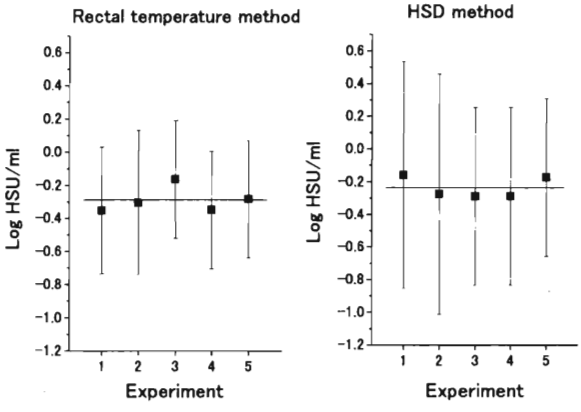

Fig. 8. Comparison of the accuracy of the rectal temperature method to that of the histamine-sensitizing death (HSD) method. A DTaP vaccine batch showing a considerable level of HS activity was assayed five times. The estimated HS activity and its 95% confidence interval obtained in each measurement are indicated by the closed box and attached vertical bar, respectively. Both methods were proven to be highly reproducible. The rectal temperature method was shown to be more accurate than the HSD method and was suggested to require a smaller number of animals.

In the mid-1980s, it was discovered that the chemically detoxified PT in DTaP vaccines may revert to toxicity during long storage, and the Minimum Requirements were revised accordingly in 1991 to implement the relevant toxicity test, namely, the HS test for the incubated preparation in which a vaccine is tested after incubating at 37°C for 4 weeks. Implementation of the new regulation had a considerable effect on the residual HS activity of DTaP vaccines. The level of the residual activity declined remarkably after 1990 without significant change in potency, as measured by the modified Kendrick test. Furthermore, no significant change in the clinical efficacy of DTaP vaccines was noted, as seen in Fig. 1. This might suggest that the modified Kendrick test could be used to evaluate the effects of a modification of the production process, if any, on the potency of acellular pertussis vaccines.

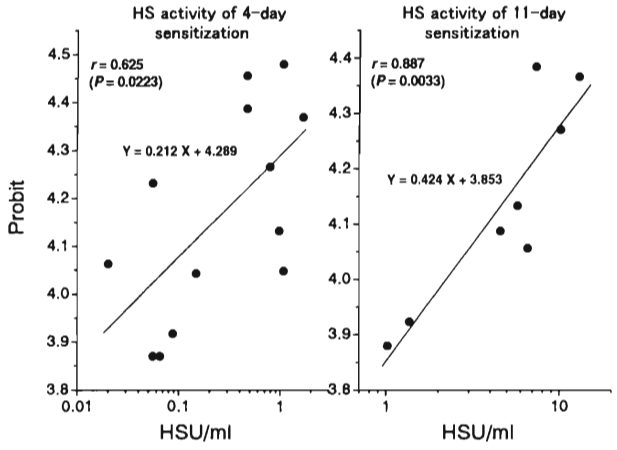

Fig. 9. Correlation between the occurrence rate of erythema and the histamine sensitizing (HS) activities of 4 day- and 11 day-sensitizations. HS activity was shown to be a cause of the local reaction at the primary dose of immunization. The rate of occurrence of erythema of over 10-mm diameter was well correlated to both HS activities measured on the fourth and eleventh days of sensitization.

Iwasa et al. (103) described a novel HS activity of a DTaP vaccine showing a peak sensitization in mice after more than 10 days of injection (late-appearing HS [LHS] activity). Commercial DTaP vaccines were incubated at 37°C for 4 weeks and their HS activity was measured on the fourth and 11th days of sensitization and compared with that of vaccines kept at 4°C. The incubation was shown to markedly increase the LHS activity, while no effect could be detected for the HS activity measured on the fourth day of sensitization.

The clinical relevance of HS activity was also analyzed using the accumulated data of the activity of commercial DTaP vaccines. Both HS activities measured on the fourth and 11th days of sensitization showed a significant correlation with the rate of erythema of over 10 mm in diameter among primary-dose vaccinees, as shown in Fig. 9. A better correlation was seen with LHS activity than with the activity of ordinal four-day sensitization. Although the mechanism of the delayed HS by DTaP vaccines has not yet been clarified, these findings might suggest that four-day sensitization is not adequate for detecting residual HS activity of the DTaP vaccines containing chemically detoxified PT.

4-3-4. Chinese hamster ovary (CHO) cell clustering test

Hewlett et al. (104) described the clustering of CHO cells by PT in 1983. Attempts have been made to apply the method for evaluating the residual PT toxicity in acellular pertussis vaccines (52,105). The assay method was prescribed in the WHO Guidelines in 1998 as a sensitive technique for evaluating the detoxification of PT. However, the assay seemed inadequate for evaluating the safety of acellular pertussis vaccines containing chemically detoxified PT. The CHO-cell clustering activity of PT found decrease to 1/4,000 or less even by mild treatment with 0.1% of formalin at 37°C for 24 h, but to retain a considerable level of its in vivo HS activity (Table 3). Therefore, in spite of its excellent sensitivity, the CHO cell assay was deemed inadequate for evaluating the detoxification of in vivo toxicity of chemically detoxified PT in acellular pertussis vaccines. However, this method might be useful for ensuring the purity of the culture of *B. pertussis* producing genetically detoxified PT. It is important for the validation of a test method to take the possible mechanism and according efficacy limitations into consideration.

Table 3. Comparison of toxicity tests for detecting residual pertussis toxin activity

	CHO cell**clusterization		HS activity
Formalin* treatment	Minimun dose for clusterizing	Relative activity	Relative activity
–	13.7 pg/ml	1.000	1.000
0.1%	58 ng/ml	0.000236	0.164
0.3%	58 ng/ml	0.000236	0.073
0.8%	530 ng/ml	0.000026	0.064

*: Treated at 37°C for 24 h
**: CHO cell: Chinese hamster ovary cell

5. Conclusion

Acellular pertussis vaccines of various formulations are currently available. A clinically relevant method of testing their potency is required in order that vaccines of different brands can be used interchangeably in the course of primary and booster immunizations. A clinical study similar to the MRC trial on vaccines with widely different potencies will be needed to establish the quantitative clinical relevance of potency-testing methods such as the Kendrick test. Currently, however, such clinical trials are not possible. Because the Kendrick test is the only method with proven clinical relevance for testing whole cell vaccines, it will be of particular importance to analyze the mechanism of the Kendrick test to open the way for its application to acellular pertussis vaccines. The role of PT in both the Kendrick test and clinical protection and the fact that the current intra-muscular immunization is not appropriate for the induction of local immunity or secretory immunogulobulin would suggest the efficacy of the Kendrick test.

Eradication of tetanus is desperately hoped for and diphtheria would once again become epidemic if effective immunization was lost (67,93). In addition, because safety is a concern due to the potential for over-immunization, the potency of toxoid vaccines needs to be precisely controlled. And although such control presents many difficulties, there also seem to be viable solutions, as discussed in this paper.

Toxicity tests for vaccines need to be implemented for every detectable toxic activity, with the exception of those shown to have no clinical relevance. The efficacy of every toxicity test also needs to be carefully validated through a consideration of its possible mechanisms. It is of particular importance to ensure a definite correlation with the relevant in vivo test before introducing an in vitro test method. In addition, before replacing a traditional test with a new testing method, it is crucial to define the limit for acceptance/rejection so as not to approve a product that should have been rejected in the traditional test. Quantitative test results are essential for establishing clinical relevance. However, tests could be simplified appropriately to prove only not significantly exceeding the least detectable level if a toxicity of products was reduced to below the detectable limit.

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