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Characteristics and potency of an acellular pertussis vaccine composed of pertussis toxin, filamentous hemagglutinin, and pertactin

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In an attempt to develop a safer pertussis vaccine, we successfully purified 3 pertussis protective antigens-pertussis toxin, filamentous hemagglutinin, and a 69-kDa outer membrane protein (also named pertactin), from *Bordetella pertussis* strain ATCC 9340. The toxicity of pertussis toxin could be effectively reduced by the treatment with formaldehyde 0.07% while preserving of a high degree of immunogenicity. By mixing purified pertussis antigens with diphtheria and tetanus toxoids (DT), we have formulated a DT acellular pertussis (DTaP) vaccine. Toxicity studies on body-weight gain in mouse, histamine sensitization, lymphocyte promoting, and Chinese hamster ovary cell clustering tests suggested that this DTaP vaccine is safer than a whole cell vaccine produced in France (DTP[F]). The formulated vaccine elicited high levels of anti-pertussis toxin antibodies in both mice and monkeys. In mice, a 2-fold neutralization of anti-pertussis toxin antibodies was produced by DTaP compared with DTP(F) vaccine and an acellular vaccine manufactured in Japan (DTaP[J]). More importantly, in intracerebral challenge assay in mouse, this vaccine also provided a better protection than DTaP(J).

Key words: Acellular pertussis vaccine, immunogenicity, potency, toxicity, whole cell vaccine

Whole cell pertussis vaccine has been demonstrated to be quite effective in reducing the morbidity and mortality of whooping cough caused by *Bordetella pertussis* [1-4]. The adverse reactions following the administration of these vaccines, however, had led to public concern with regard to its safety [5,6]. In attempts to minimize the side-effects and possibly also to improve the protective efficacy against both disease and infection, substantial efforts have been made to develop a new and safer acellular vaccine.

Whooping cough is a toxin-mediated disease [7]. Previous studies have shown that injection of inactivated pertussis toxin (PT) could protect mice against subsequent challenge with *B. pertussis* [8,9]. Since the first acellular pertussis vaccine was developed and used in Japan in 1981 [10], all of the proposed acellular pertussis vaccines combined with diphtheria and tetanus toxoids (DTaP) have contained a detoxified PT [11] as the major antigen. This protective antigen is a hexameric protein composed of 5 different subunits (S1-S5) [12]. The subunits S2 through S5 are for target cell receptor binding, allowing the entry of the enzymatically active subunit S1 to the target cells. The S1 subunit displays an adenosine diphosphate (ADP)-ribosyltransferase activity [13] that is believed to be responsible for the numerous toxic activities of PT, including lymphocytosis promotion, histamine sensitization, islet activation, and many others [14]. To eliminate the various harmful side-effects resulting from the toxicity of S1, most acellular vaccines detoxify PT by chemical treatment before formulation [8,16], except for one or 2 commercial vaccines [15] that use genetic detoxified PT by chemical treatment before formulation. Although detoxification reduces the immunogenicity [17], it may not completely eliminate the risk of reversion to toxicity [18,19]. Despite this fact, a multicenter acellular pertussis trial [11] found that all acellular vaccines produced significant increases in antibody for the induced antigens, and were associated with significantly fewer adverse reactions than the control whole cell vaccine.

Pertussis toxin has been considered the major antigen that will induce protection against whooping cough and prevent the systemic effects of the disease

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[20,21]. Satisfactory efficacy has been obtained in some clinical trials using DTaP that included PT as the only pertussis antigen [22,23]. Based on those findings, some researchers proposed that PT alone should provide sufficient protection, and recommended the use of monocomponent DTaP (containing only PT) to reduce the manufacturing cost [23,24]. This recommendation, however, has recently been challenged by the findings that the addition of some non-toxic molecules, such as filamentous hemagglutinin (FHA) [25], the 69 kDa outer-membrane protein (also named pertactin) [26], and fimbriae antigens [27], enhanced the immunity of DTaP and conferred a higher efficacy. The immune responses to these cell adhesion proteins have been suggested to be able to prevent bacterial colonization and have an important role in preventing pathogen infection [28]. In spite of these findings, the superiority of multicomponent vaccines has been difficult to evaluate, because the reported vaccine efficacies were obtained from different field trials. Systematic study is thus needed to address this issue. In this report, we described a complete procedure for the formulation of a 3-component acellular pertussis vaccine (containing purified PT, FHA, and pertactin) combined with diphtheria and tetanus toxoids. The purpose of this study was to perform a series of toxicity and immunology tests to evaluate the safety and protection potency of this prototype vaccine, compared with a whole cell vaccine from France (DTP[F]) and an acellular vaccine from Japan (DTaP[J]; containing copurified PT and FHA).

Materials and Methods

Pertussis cultivation and protective antigen preparation

Phase I *B. pertussis* strain ATCC 9340 (obtained from the Food Industry Research and Development Institute, Taiwan) was cultured in a modified SS-medium as described in the literature [29]. Highly purified PT and FHA were prepared by a dual-column method (ie, hydroxyapatite and fetuin-sepharose chromatography) developed in the laboratory of the Institute of Biotechnology, National Chiayi University [29]. Another antigen, the 69-kDa pertussis outer membrane protein (pertactin), was purified by a procedure based on the binding affinity of pertactin to an immobilized metal affinity column (IMAC) [30]. Briefly, following the cultivation, pertussis cells were washed with phosphate buffered saline (PBS; pH 8). After heat extraction at 60°C for 1 h and centrifugation at 7000 rpm for 30 min, the supernatant was loaded onto a Ni^{2+} chelated affinity column (Pharmacia, Uppsala, Sweden).The nonspecific binding proteins were removed by extensively washing the column with PBS, pH 8, then with PBS of pH 7. The bound pertactin was finally eluted with PBS of pH 6. The obtained pertactin was further purified by passing through a hydroxyapatite column pre-equilibrated with PBS of pH 7. All purified antigens were characterized by sodium dodecylsulfate polyacrylamide gel (SDS-PAGE) and Western blotting.

Detoxification of pertussis toxin and formulation of DTaP

Before the preparation of DTaP, the purified PT and FHA (which may have been contaminated with trace amounts of active PT) were detoxified. After the addition of L-lysine 25 mM (final concentration) (Sigma Chemical Co., St. Louis, MO, US) to prevent the aggregation of antigens, the detoxification process was performed at either 37°C or 4°C in various concentrations of formaldehyde. At the indicated time interval (Table 2), a detoxified sample was withdrawn, dialyzed, and subjected to toxicity analysis using a Chinese hamster ovary (CHO) cell-clustering test [31, 32]. According to the obtained results and the results of a preliminary test (data not shown), we chose to use a mild condition, that is, treatment with formaldehyde 0.07% for 3 weeks at 37°C, to inactivate PT and FHA.

After detoxification, a 3-component DTaP was formulated. Each dose of DTaP was composed of PT 10 μg, FHA 10 μg, pertactin 8 μg, diphtheria toxoid 15 Lf, tetanus toxoid 5 Lf, and the gel form of aluminum phosphate 0.5 mg as adjuvant. Results of serial chemical tests, including protein content, pH value, aluminum content, and residual formaldehyde (data not shown), indicated that the formulated DTaP has met the criteria for a prototype vaccine [33].

Toxicity tests

To evaluate the safety of the formulated DTaP, a series of tests were conducted using procedures described in the literature[34] to investigate the vaccine toxicity effects on body-weight gain in mouse, lymphocyte-promoting, and CHO cell clustering. As in our previous study [34], CHO cell-clustering activity was represented by cytopathogenic unit (CPU). For comparison of the safety, 2 commercialized vaccines, DTP(F) and DTaP (J), were also analyzed.

Histamine sensitization [35] was also performed to further investigate the cytotoxic effect associated with the residual toxic PT in the formulated DTaP. Briefly, ICR mice (6 mice per group) received intraperitoneal

injection of detoxified PT 10 μg, FHA 10 μg, pertactin 8 μg, or one human dose of the test vaccines. Four days after injection, all mice were challenged with 1 mg of histamine base through the same route, and the number of deaths in the next 24 h was counted.

To ensure the stability of antigens and the safety of vaccine, the toxicity reversion test was also conducted. The detoxified PT, FHA, pertactin, the formulated DTaP, and the commercial vaccines were stored at 4°C for 1 year and then subjected to toxicity tests for CHO cell clustering and histamine sensitization.

Immunogenicity

Groups of 10 female ICR mice were intraperitoneally immunized on days 0 and 21 with 1/25 human dose of the respective vaccines. After immunization of 2 weeks, mice were bled and blood samples were tested for anti-PT antibody titers by enzyme-linked immunosorbent assay (ELISA). Except the use of Tris-buffered-saline (TBS) solution 0.1 mL (Tris-hydrochloric acid [HCl] 20 mM, pH 9, NaCl 0.15 M, bovine serum albumin [BSA] 1%, Gelatin 0.1%, blocking reagent (Boehringer Mannheim(0.5%) as a blocking solution, ELISA was performed following a similar procedure as described in the literature [29]. Two Formosan rock monkeys (*Macaca cyclopis*) raised in the laboratory were also tested for their immune response against the formulated DTaP. Each monkey was intramuscularly injected 4 times (at 1.5, 3, and 9 months after the primary immunization), with each DTaP injection corresponding to one human dose. Including the pre-immune sera, the antisera that were collected 1 day before each injection and 2 weeks after the last booster were used for the measurement of anti-PT antibody titers.

The obtained antisera (from both mice and monkeys) were also analyzed for toxin-neutralizing activity using the CHO cell assay. Two-fold serial dilutions of antisera in PBS (50 μL each) were mixed with active PT 50 μL (2 ng/mL) and subjected to clustering activity studies as previously described [34]. The neutralizing antibody titers were expressed as the reciprocal of the highest serum dilution that completely inhibited the cell clustering.

Vaccine potency

Groups of 16 female ICR mice were separately vaccinated intraperitoneally with 1/25 human dose of the formulated DTaP, DTaP(J), or DTP(F). Two weeks after vaccination, mice were challenged intracerebrally with 5×10^4 (approximately 200 lethal dosage (LD_{50}) of *B. pertussis* 18323. Deaths within 14 days after the challenge were recorded. The relative median effective dose (ED_{50}) and international unit were also detected. The ED_{50} was calculated in a similar experiment using 1/5, 1/25, and 1/125 dose of each vaccine, using statistical method described by Tiru *et al* [36]. The relative international unit per dose for DTP(F) and DTaP were defined by arbitrarily setting the international unit per dose for DTaP(J) to 8 (minimum potency for a DTaP to pass the required criteria in Japan). To test whether the DTaP composed of 3 protective antigens provided better protection, the relative potencies of other formulated DTaPs containing only one (PT alone) or 2 pertussis antigens were analyzed using the same procedure.

Results

Purification of pertussis antigens

A prototype DTaP vaccine containing 3 pertussis antigens, PT, FHA, and pertactin, was designed in this study. Pertussis toxin and FHA were purified from a

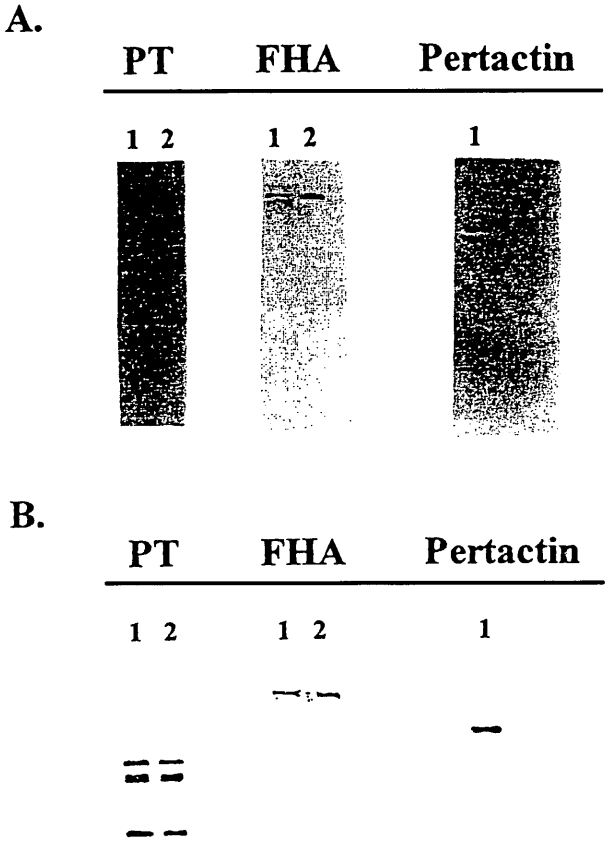

Fig. 1. Sodium dodecylsulfate polyacrylamide gel and immunoblotting analysis of purified pertussis antigens. The purified pertussis antigens were analyzed by sodium dodecylsulfate polyacrylamide gel (panel A) and by immunoblotting (panel B). Lane 1, purified antigens; Lane 2, commercial antigens (pertussis toxin or filamentous hemagglutinin).

phase I *B. pertussis* culture using a procedure described in a previous study [29]. Pertactin was prepared by a newly developed procedure using the methodology of IMAC followed by hydroxyapatite chromatography. The purity and the immunospecificity of PT, FHA, and pertactin were verified by SDS-PAGE with Coomassie Blue staining (Fig. 1, panel A) and immunoblotting with polyclonal antibodies against each antigen (Fig. 1, panel B), respectively. At least 90% purity for each antigen was obtained. The good quality of these antigens has facilitated the development of a prototype vaccine formulation for use in the subsequent studies.

Detoxification of pertussis toxins

To optimize the conditions for detoxification of purified antigens before formulation, the purified PT was treated with various concentrations of formaldehyde at either 37°C or 4°C (Table 1). The residual toxicity was detected by CHO cell clustering assay. Higher concentration of formaldehyde (≥0.105% at 37°C or ≥0.21% at 4°C) eliminated the cytotoxicity of PT in a short period of time. However, lower concentrations of formaldehyde could not accomplish an effective detoxification unless the treatment was prolonged. Since stronger detoxification might greatly decrease the immunogenicity, the inactivated antigens were prepared by treating with formaldehyde 0.07% for 3 weeks at 37°C.

Toxicity tests of DTaP vaccines

As a fundamental procedure for safety evaluation, the formulated DTaP was first tested for toxic effects on the body-weight gain in mice (Fig. 2). Similar to previous results using native PT [34], mice that were injected with DTP(F) showed a significant decrease in body-weight gain on the next day after injection. In contrast, mice that were given either the formulated DTaP or DTaP(J) showed a similar pattern of weight increase to that of mice that were given PBS.

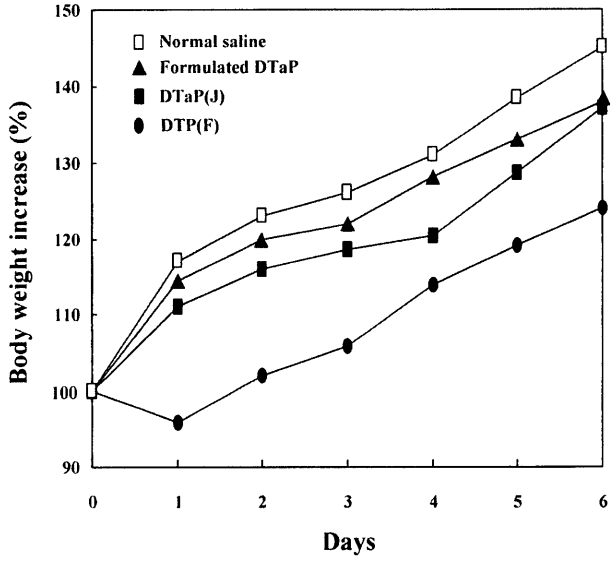

Fig. 2. Mouse body-weight gain test. The toxic effects of DTaP (J), DTP(F), and the formulated DTaP on body weight increase in mice were analyzed. The mean weight of each testing group (10 mice) before injection with one human dose of vaccine was normalized to 100%.

The residual toxicity resulting from an incomplete detoxification of formulated PT was also analyzed by lymphocyte-promoting activity assay. As shown in Fig. 3, mice injected with DTaP or DTaP(J) had a slightly increased white blood cells count and lymphocytes compared with mice injected with normal saline. In contrast, mice injected with the same dose of DTP(F) had a 2.8-fold increase of lymphocytes and a 5.3-fold increase of white blood cells.

Table 1. Detoxification of purified pertussis toxin by formaldehyde

Temperature	Time	% of formaldehyde used for detoxification								
		0	0.035	0.042	0.05	0.07	0.105	0.14	0.21	0.42
37°C	Day 1	+	+	+	+	+	-	-	-	-
	Day 4	+	+	±	±	±	±	-	-	-
	Day 7	+	+	±	±	±	-	-	-	-
	Day 10	+	±	-	-	-	-	-	-	-
	Day 14	+	-	-	-	-	-	-	-	-
4°C	Week 3	+	+	+	+	+	+	+	-	-
	Week 4	+	+	+	+	+	+	+	±	-
	Week 6	+	+	+	±	+	-	-	-	-
	Week 8	+	-	-	-	-	-	-	-	-

Note: Pertussis toxin was treated with formaldehyde, and the resulting solution was tested for Chinese hamster ovary cell clustering activity. The cell clustering was observed by microscope.
"+" indicates a complete cell clustering.
"±" indicates a partial clustering.
"-" shows an apparently no clustering.

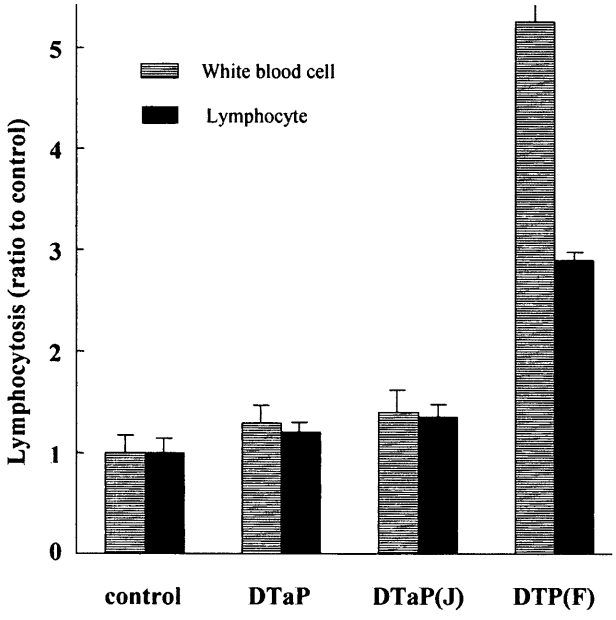

Fig. 3. Promotion of lymphocytosis associated with vaccine injection. The white blood cells and lymphocytes determined from the blood sample of the control group (mice were injected with solvent vehicle, normal saline) were normalized to one.

Chinese hamster ovary cell assay was performed to detect the residual toxicity of the formulated DTaP as previously described [31,32]. Defining 1 CPU as the lowest concentration of PT that caused 50% CHO cell clustering, we determined the relative CHO cell-clustering activities of the purified antigens (20 μg each) and the testing vaccines (one human dose each). The mean of 3 trials of clustering activities caused by detoxified PT, detoxified FHA, pertactin, DTaP, DTaP (J), and DTP(F) were 12, 3, 3, 12, 60, and 300 CPUs, respectively. Since detoxified FHA and pertactin were not reported to cluster CHO cells [37], the obtained activities (3 CPUs) were assumed to represent experimental background levels. The formulated DTaP had a much lower toxicity in clustering CHO cells (12 CPUs) than the whole cell vaccine DTP(F) (300 CPUs).

Histamine sensitization assay was performed to detect the residual toxicities mainly attributed to PT and other contaminated endotoxins. None of the individual antigens nor the formulated DTaP or DTaP (J) has caused death in mice after histamine challenge. On the contrary, DTP(F) and whole cell vaccine exhibited a profoundly lethal effect, resulting in 2 to 3 deaths in groups of 6 mice in 3 independent trials.

In contrast to the study of Storsaeter *et al* [19], there was no evidence of a significantly increased toxicity caused by toxicity reversion after long-term storage. The tested antigens and vaccines, which had been stored for more than 1 year, exhibited a negligible difference in activity in both CHO cell clustering and histamine sensitization (data not shown).

Immunogenicity of DTaP vaccines

Immunogenicity is of considerable importance for a newly developed vaccine. In a preliminary study, we found that the elicited antibody levels against diphtheria and tetanus toxoids after immunization with DTaP were comparable to those with the corresponding doses of diphtheria and tetanus toxoids individually (data not shown). This finding indicated that the pertussis antigens did not interfere with the immunogenicity of the combined diphtheria and tetanus antigens. As shown in Fig. 4, the same dosage of DTaP generated a 4-fold higher anti-PT antibody titers than that of DTaP(J) or DTP(F). Neutralizing anti-PT antibody titer, which is known to be correlated to the protective immune response, was elicited by vaccination with all tested vaccines. The formulated DTaP was superior to the commercial vaccines in this regard (2-fold neutralizing antibodies were obtained). In monkeys, DTaP also generated high levels of anti-PT antibodies and toxin-

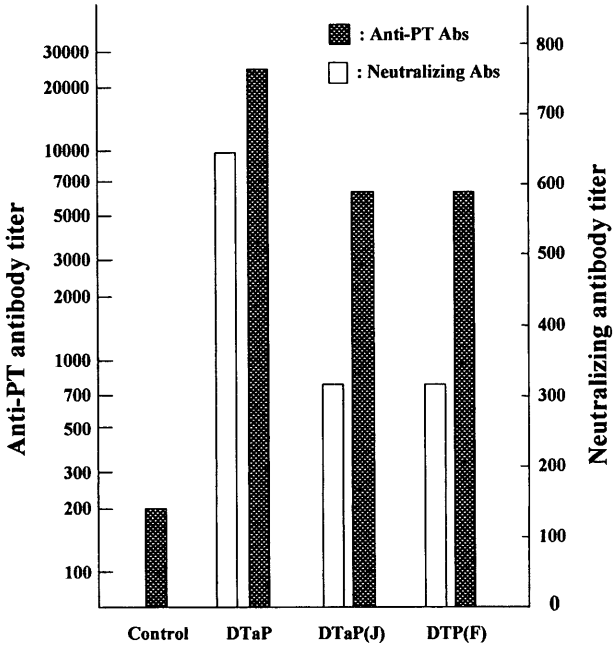

Fig. 4. Anti-pertussis toxin antibody levels and toxin-neutralizing titers in mice. Groups of 10 mice were intraperitoneally injected with 1/25 human dose of DTaP(J), DTP(F), formulated DTaP, or normal saline. Sera were collected and the antibody titers were determined. Neutralizing titers are expressed as reciprocals of the highest serum dilutions resulting in 100% inhibition of the clustering effect on Chinese hamster ovary cells induced by wild-type pertussis toxin.

neutralizing titers (Fig. 5). The immune response was dramatically enhanced after booster injections.

Vaccine potency

To estimate the vaccine potency, the ability of the vaccines to protect mice from a lethal intracerebral challenge assay with virulent *B. pertussis* 18323 was studied. The test was correlated with the protective activity of the cellular vaccine [38]. At 1/25 human dose, the formulated DTaP, DTaP(J), and DTP(F) resulted in mean survival rates of 60.4%, 39.6%, and 56.3% in 3 trials, respectively. When the relative ED_{50} and international unit per dose were calculated [36], DTaP showed a vaccine potency comparable to that of DTP (F), but superior to that of DTaP(J) (Table 2).

The potencies of DTaP preparations containing monovalent, divalent, and trivalent pertussis antigens (Table 3) were also evaluated by the challenge assay mentioned above. Consistent with the findings of a previous study [39], DTaP containing all 3 pertussis antigens provided the best protection against the challenge with virulent pertussis (ED_{50}, 20.1; 14.2 IU per dose), whereas the monovalent DTaP (ie, DTaP/△pertactin△FHA) and the divalent DTaP vaccines were less protective. The vaccine composed of no PT (ie, DTaP/△PT; ED_{50}, 8; 5.7 IU per dose) was least protective.

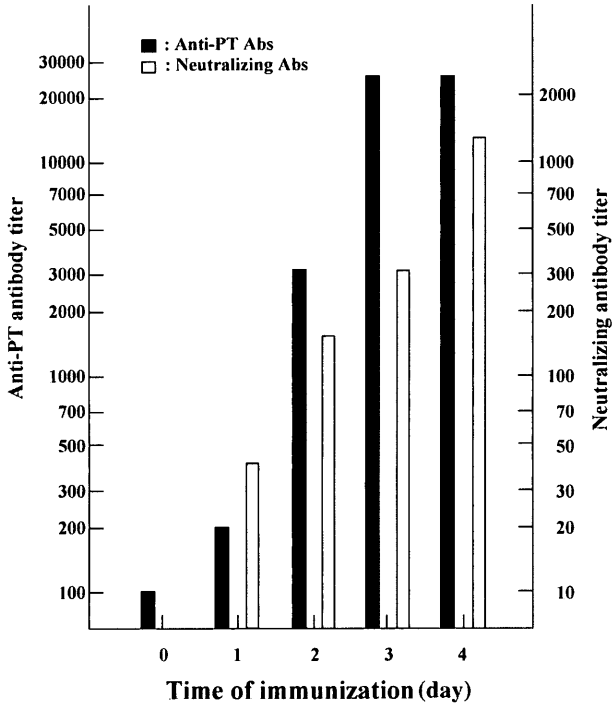

Fig. 5. Enzyme-linked immunosorbent assay levels and toxin-neutralizing titers in monkeys. Anti-pertussis toxin antibody titers were raised in monkeys by the injection of formulated DTaP as shown by enzyme-linked immunosorbent assay. Including the pre-immune serum, the antisera obtained after 4 sequential injections, with one human dose each time, were analyzed. Neutralizing titers of monkey serum were determined by the same method used in the experiment with mice

Table 2. Vaccine potency

Vaccines	ED_{50}[a]	IU/dose[b]
DTaP(J)	10.1	8
DTP(F)	17	13.5
Formulated DTaP	18.3	14.6

Note: Vaccine potency was determined by intracerebral challenge studies as described in "Materials and Methods". The data are expressed as mean with less than 5% experimental error from 3 independent trials.

[a] ED_{50} was calculated by a method proposed by Tiru *et al* [36].

[b] The potency of DTaP(J) was normalized to 8 IU/dose, according to manufacture instruction. The international unit per dose for DTP(F) and formulated DTaP were calculated from the relative ED_{50} to that of DTaP(J).

Discussion

To develop a prototype vaccine for whooping cough prevention, we purified 3 pertussis antigens and formulated a DTaP vaccine. A series of laboratory toxicity tests were conducted to evaluate the safety of the formulated DTaP. Compared with 2 commercial vaccines, the formulated DTaP was safer and exhibited a relatively lower residue toxicity. Although DTaP and DTaP(J) showed similar toxicity in most tests, the superiority in safety of DTaP was demonstrated by its much lower toxic effect in cell clustering test compared with commercial DTaP(J) (12 vs 60 CPUs). Since CHO cell assay is an extremely sensitive method in detecting the toxicity of native PT (the limit of detection can be up to 0.1 ng), the PT detoxification used in this report was efficient even in making DTaP formulation (12 vs 12 CPUs). This study has also demonstrated that acellular vaccines such as DTaP and DTaP(J) are much safer than whole cell vaccines, which has been reported to be associated with significantly more adverse reactions in clinical trials [40]. Since CHO cell-clustering activity is mainly attributed to the presence of toxic PT, either incomplete detoxification or toxicity reversion of PT may account for the toxicity of DTaP (J). The whole cell vaccines (eg, DTP[F]) that contain various cellular toxins (eg, lipopolysaccarides) have been reported to be associated with significantly more adverse reactions in clinical trials compared with acellular vaccines such as DTaP and DTaP (J) [40]. This fact is illustrated by the observations that DTP(F) caused a retardation in body-weight gain in mice, a drastic

promotion in lymphocytosis, and a much higher toxic effects in both histamine sensitization and CHO cell clustering. Similar to previous finding in the reversion of toxicity [19], significant increase in toxicity was not observed in any of the vaccines stored for 1 year. It is unknown whether a longer storage period would cause such effects.

When compared with the 2 commercial vaccines, the formulated DTaP produced a 4-fold increase in anti-PT antibodies in mice. In addition, the relative antitoxin titer correlated well with the neutralizing activity in CHO cell clustering (2-fold neutralizing antibodies produced by DTaP as compared with DTaP[J] and DTP[F]). Since the antibody level is closely related to the quantity of antigen used for immunization, the antigen contents are difficult to accurately quantify from a whole cell vaccine (ie, DTP[F]) or form an acellular vaccine containing copurified PT and FHA. Although the relative ELISA levels obtained in this study probably reflect only dose-dependent immune response, the results are justified on the basis of same dosage (1/25 of human dose) in the tests, and especially by the finding that the antigen contents of DTaP (10 μg for PT and 10 μg for FHA) were not higher than that of most acellular vaccines (10 to 25 μg for PT and 2.5 to 35 μg for FHA). Because the same dosage was used in the tests, the relative immune responses are meaningful.

In spite of a relatively higher titer of anti-PT antibodies (humoral immunity) being elicited by DTaP, previous clinical trials [21,41] have suggested that the anti-PT levels do not correlate with protection, and hence the result does not represent that DTaP will confer a better protection than the tested commercial vaccines. To further evaluate the protective potency of DTaP, the intracerebral challenge test that was used to show the efficacy of vaccine in whole cell DTP assay was performed. According to the mouse survival rate and the calculated ED_{50} (Table 2), DTaP showed a comparable protection activity to that of DTP(F), whereas DTaP(J) provided a somewhat poorer protection against the challenge with virulent *B. pertussis* strain 18323. These results suggested that acellular vaccines, which have been demonstrated to be much safer, may not be superior to whole cell vaccines in terms of vaccine effectiveness. This is consistent with the observed efficacies in many field trials, in which the superiority of acellular vaccine could not be confirmed [42].

Since a good protective efficacy has been observed in previous studies by using DTaP containing PT alone, many investigators have raised the argument that a single antigen component (ie, PT) should be sufficient to provide a good protection from disease [27,47]. We also formulated a DTaP that contained only PT or 2 pertussis antigens, and studied its protective effects against intracerebral challenge in mice (Table 3). The values of the calculated ED_{50} and the international unit per dose, except for the DTaP, which included no PT (ie, DTaP/△PT), showed that the DTaP containing monocomponent of PT (ie, DTaP/△pertactin△FHA) provided a poorer protection than that conferred by any 2- or 3-component vaccine (ie, prototype DTaP vaccine). Based on the calculated ED_{50} and effective units, the multicomponent DTaPs would be expected to show better protection than the monocomponent DTaP. Regarding to the superiority of vaccine composed of multicomponent pertussis antigens, the same conclusion has also been made by other investigators [39,43]. It was observed that DTaP lacking the essential protective antigen (ie, PT) also exhibited a basal protection. Understanding of the mechanism of the mutual action between FHA and pertactin is limited. The only available data is that the 2 antigens have been implicated in the adhesion and hence colonization process [44]. Hamstra *et al* [48] had also reported that pertactin can confer protection in the intracerebral challenge test. In this study, when the ED_{50} levels obtained from experiments using DTaP with and without pertactin were compared, we found that the addition of pertactin could enhance the protective effect (ED_{50}, 20.1 and 15.5 for 3-component DTaP and DTaP/△pertactin, respectively). The substitution of FHA with pertactin did not significantly diminish this protective activity (ED_{50}, 15.5 and 14.6 for DTaP/△pertactin and DTaP/△FHA, respectively). Based on the findings that pertactin alone could efficiently protect mice against aerosol challenge with virulent *B. pertussis* [44,45] and

Table 3. Relative potency of vaccine composed of mono-, di- or tri-valent pertussis antigens

Vaccines[a]	ED_{50}[b]	IU/dose
DTaP/△pertactin△FHA	10.1	7.3
DTaP/△pertactin	15.5	11.1
DTaP/△PT	8.0	5.7
DTaP/△FHA	14.6	10.5
DTaP	20.1	14.2
DTaP(J)	11.1	8.0
DTP(F)	18.6	13.4

[a]DTaP lacking certain antigen(s) was indicated by "△". For example, DTaP/△PT represents a formulated vaccine lacking pertussis toxin.

[b]ED_{50} for each tested vaccine was determined by a procedure described by Tiru *et al* [36] from the survival rates in intracerebral challenge using 3- to 5-fold dilutions of sample.

that the protective recombinant pertactin could be overproduced in *Escherichia coli*. [47], we suggest that a 2-component DTaP containing PT and pertactin (not FHA formulated in most 2-component DTaP) may represent another inexpensive and effective vaccine against pertussis.

In conclusion, this study has described several optimal procedures for producing an acellular vaccine DTaP. Studies include the antigens preparation, detoxification, formulation, and assays for toxicity, immunogenicity, and protection activity. The formulated DTaP was shown to be safe and confer a high level of protective immunity in mice. These laboratory data are of particular importance to serve as supporting documents in guiding production or selection of candidate vaccines for pertussis prevention. Results also suggest that clinical trials are needed to determine the safety and efficacy of this vaccine in humans.

Acknowledgments

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