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EVALUATION OF ACELLULAR DPT VACCINES IN INFANTS

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ABSTRACT: Two acellular DPT vaccines containing, as pertussis components, the genetically detoxified pertussis toxin mutant PT-9K/129G, either alone or combined with FHA and 69K, were evaluated for safety and immunogenicity in infants 8-14 months old. Both vaccines induced very mild local reactions which were consistent with the presence of alum and the previous administration of two doses of whole-cell DPT vaccine. A marked increase in specific antibodies to each pertussis component and in pertussis toxin neutralizing antibodies was observed after one dose of either acellular vaccines. All vaccinees also acquired an excellent protective immunity against diphtheria and tetanus, as assessed *in vitro* and *in vivo*.

INTRODUCTION

The genetically detoxified pertussis toxin (PT) mutant PT-9K/129G is naturally devoid of the toxic properties of PT and maintains the physicochemical and the immunological properties of the wild type toxin[1,2].

We have developed acellular pertussis vaccines containing the PT-9K/129G mutant alone or together with the filamentous haemagglutinin (FHA)[3] and an outer membrane protein named 69K or pertactin[4]. These proteins are involved in the adhesion of *B. pertussis* to mammalian cells[5] and therefore, if included in a vaccine, are expected to prevent bacterial colonization. Both FHA and 69K are purified from cultures of the recombinant strain *B. pertussis* W28-9K/129G which produces the non toxic PT mutant PT-9K/

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129G and therefore these products cannot be contaminated with activate pertussis toxin. Since no chemical treatment is necessary to inactivate the toxin, these molecules can be safely used in pertussis vaccines without fear of potential reversion to toxicity, loss of immunogenicity, and batch-to-batch variations[6,7].

In previous studies we have shown that acellular pertussis vaccines containing PT-9K/129G alone[8] or combined with FHA and 69K[9] are extremely safe in adult volunteers and that are able to induce high levels of humoral and cellular immunity. These results are confirmed by phase II clinical trials in infants and children which are now in progress. Since it is likely that one of these pertussis formulations will be used for mass immunization and administered within the DPT vaccination schedule, we have prepared two trivalent vaccines, in which diphtheria and tetanus toxoids have been combined with only PT-9K/129G (DPT3/P/AH) or with PT-9K/129G, FHA and 69K (DPT7/PFK/AH).

In this paper we report the safety and the immunogenicity in infants of these new acellular DPT vaccines.

MATERIALS AND METHODS

Study design. Forty-five healthy infants of both sexes, 8 to 14 months of age, were recruited at the Health Local Unit of Ozieri, Sardinia, Italy. Among these, twenty-one and twenty-four subjects received intramuscularly one dose of DPT3/P/AH or DPT7/PFK/AH vaccine, respectively. All infants had previously received at least two doses of conventional whole-cell DPT vaccine.

Safety assessment and serology. Rectal temperature was monitored at 3, 6 and 24 hours after vaccination. Drowsiness, fussiness, appetite, vomiting, redness, swelling and pain were monitored 3 and 6 hr after vaccination and then at bed-time throughout the first week and on the 14th evening.

For redness and swelling we have reported in table 1 only values which were ≥ 1 cm; for fussiness and appetite only values monitored throughout the first three days after vaccination.

Parents were instructed to record the rectal temperature of the infant and to evaluate any local and systemic reactions. All infants were home-visited 24 hs after the vaccine ad-

TABLE I

Adverse reactions after vaccination

Reaction	DPT3/P/AH	DPT7/PFK/AH
Fever	2/21	3/24
Drowsiness	2/21	3/24
Fussiness	5/21	6/24
Appetite	4/21	4/24
Vomiting	2/21	2/24
Redness	4/21	1/24
Swelling	4/21	3/24
Pain	2/21	6/24

ministration by a follow-up nurse who monitored parents by phone throughout 14 days.

Venous blood samples for *in vivo* and *in vitro* evaluation of specific IgG and toxin-neutralizing antibodies were obtained before and 4 weeks after vaccination from 7 and 11 infants receiving, respectively, the DPT3/P/AH or the DPT7/PFK/AH vaccines.

Vaccines. Both the acellular DPT vaccines were prepared at Sclavo Laboratories (Siena, Italy). Each 0.5 ml single dose vial of DPT3/P/AH contained 15 Lf of diphtheria toxoid, 10 Lf of tetanus toxoid, 10 µg of PT-9K/129G, 0.05 mg of thimerosal and 2 mg of aluminium hydroxide. Each 0.5 ml single dose vial of DPT7/PFK/AH contained 20 Lf of diphtheria toxoid, 12.5 Lf of tetanus toxoid, 5 µg of PT-9K/129G, 5 µg of FHA, 3 µg of 69K, 0.05 mg of thimerosal, and 1 mg of aluminium hydroxide.

Antigens. The non toxic mutant PT-9K/129G and FHA were purified from the culture supernatants of the recombinant strain *B. pertussis* W28-9K/129G[1] according to a modified version of the method described by Cowell et al.[10]. The 69K protein was extracted from cell paste of *B. pertussis* W28-9K/129G[1] by heat treatment and purified to the homogeneity by anionic exchange chromatography and gel filtration (Manetti & Rappuoli, manuscript in preparation). Before use, each antigen underwent a mild stabilization with formaldehyde.

Diphtheria and tetanus toxoids were obtained as previously described[11].

CHO cell toxin neutralization assay. Pertussis toxin-neutralizing antibodies induced by vaccination were using, as standard, the U.S. Reference Human Pertussis Antiserum (lot # 3), containing 640 neutralizing units, kindly provided by the Center for Drugs and Biologics, Bethesda, MD. U.S.A. Briefly, sera from volunteers obtained after administration of one or two doses of PFK/2 vaccine were diluted directly in the wells of flat-bottomed microplates (Costar, Cambridge, MA, U.S.A.) in 25 µl of Dulbecco's Modified Eagle Medium (DMEM, Flow Laboratories, Mc Lean, VA, U.S.A.). Purified wild type PT (120 pg) in 25 µl of Coulter medium DMEM was added to each well and the plates were incubated for 3 h at 37°C. After the incubation period, 0.2 ml of DMEM containing $1x10^4$ CHO cells, previously treated with 1 mg/ml of trypsin, were added to each well and incubated for 48 h at 37°C in atmosphere of 5% CO_2. As positive control, the clustering effect of PT alone was titered in each plate. Neutralizing titers were expressed as the reciprocal of the highest serum dilution causing complete inhibition of the clustering activity induced by the native toxin.

Vero cell toxin neutralization assay. Diphtheria toxin-neutralizing antibodies induced by vaccination were tested by the VERO cell assay using as standard the NIH Reference Antiserum (lot # A50), containing 3,200 neutralizing units. The test was performed in the wells of flat-bottomed microplates (Costar, Cambridge, MA, U.S.A.). Antisera from vaccinated infants and the NIH reference serum were diluted in culture medium M199, containing 10% FCS, 2 mM glutamine, 25 mM Hepes, 50 µg/ml gentamicin, directly in the wells by twofold serial dilutions. The volume of antisera added to each well is 20 µl. After that, the diphtheria toxin (80 LF/ml diluted $1:10^5$) was added to each well in a volume of 20 µl/ml. The plates are then incubated for 3 h at 37°C. After the incubation time, 200 µl of culture medium containing 10^9 VERO cells are added to each well. The microtiter plates were then incubated at 37°C and, 48-72 h later, cells were strained with crystal violet and the stain was solubilized with 50% solution in water (vol/vol) of ethanol. Absorbance values at 560

nm of samples were measured against blank on a Titertek Multiskan (Flow Laboratories, Inc., McLean, VA, U.S.A.). Controls for each plate included wells with cells and diphtheria toxin (positive control), and wells with cells but no toxin (negative control). Each serum sample was tested in duplicate and absorbance values were averaged. The neutralizing titer is expressed as the reciprocal of the highest dilution of serum samples giving absorbance values comparable to those of the negative control (100% of protection).

Elisa

The ELISA method was performed as previously described[2,8]. Wells of flat-bottomed polystyrene microtest plates (Dynatech Laboratories, Inc., Alexandria, VA, U.S.A.) were coated with 100µl of PBS pH 7.4 containing 1 µg of purified PT or FHA or 69K, diphtheria toxoid or tetanus toxoid. The coating was performed for 2 h at 37°C and overnight at 4°C in a humidified chamber. The coating buffer was aspirated, and wells were washed with 200 µl of PBS containing 0.05% Tween 20 and 0.02% sodium azide (PTA). To minimize non-specific adsorption of serum proteins to the plastic, wells were coated with 200 µl of a blocking solution consisting of 1% bovine serum albumin (BSA) in PBS, and then incubated for 2 h at 37°C. Plates were then washed three times in PTA and 200 µl of fivefold diluted test serum were added to the wells. The U.S. Reference Human Pertussis Antiserum (lot # 3) containing 200 ELISA Units (EU) per ml of IgG anti-PT and 200 EU/ml anti-FHA was used as reference standard. In the case of the 69K protein, we used an "in-house standard" immune serum to which we assigned a value of 20 EU/ml of IgG anti-69K, as well as 10 EU/ml of IgG anti-diphtheria and anti-tetanus toxoids. Following incubation at 37°C for 2 h, plates were washed three times with PTA and a conjugate of anti human IgG-alkaline phosphatase was added. Plates were then incubated at 37°C for 2 h and washed three times with PTA. Finally, 100µl of p-nitrophenyl phosphate substrate (Sigma Chemical Co., St. Louis, MO, U.S.A.) 1mg/ml in 1 M diethanolamine, pH 9.8, containing 1 mM $MgCl_2$, was added to each well. The enzyme-substrate reaction, which developed at room temperature, was stopped after 30 min, and the absorbance values of the samples was measured at 405 nm against blank (substrate in diethanolamine, pH 9.8) on a Titertek Multiskan (Flow Laboratories, Inc., McLean, VA, U.S.A.). Controls for each plate included wells with serum samples but no antigen, and wells with antigen but no serum samples. Each serum sample was tested in duplicate and absorbance values were averaged. Total IgG antibodies to PT, FHA and 69K in the test samples were expressed as geometric mean of EU/ml, determined according to the parallel line bioassay procedure described by Manclark et al.[14].

Diphtheria and tetanus antitoxin tritation in vivo. The assays were performed according to W.H.O. recommendations using rabbits for diphtheria and mice for tetanus antitoxin tritation. The results, expressed in International Units (IU) per ml, were obtained according to the N.I.H. reference diphtheria antiserum (Lot # A50, containing 6 IU/ml) and the N.I.H. reference tetanus antiserum (Lot # A50, containing 6 IU/ml), used as standards.

RESULTS

Safety. The follow-up performed after the administration of DPT3/P/AH (table 1) showed that no infants had fever over 38°C with two exceptions (38.1°C and 38.2°C). However, in both cases, the temperature was over 38°C for only one measurement

and, in no cases, the administration of anti-pyretic drugs was necessary. Unusual drowsiness was only noticed in two infants. Five out of twenty-one infants were more irritable than usual throughout 72 hr following vaccination, but all of them maintained a normal activity. Three infants had loss of appetite (two of them after the first three days), while two infants had an isolate episode of vomiting on day 2 and 4, respectively. Four out of twenty-one subjects had redness and swelling at the site of injection and finally, two infants had local pain that in one case was very mild.

As far as vaccination with DPT7/PFK/AH is concerned, the follow-up of adverse reactions (table 1) showed that only one out of twenty-four infants had redness and three had swelling. Local pain was reported in six infants. Three infants had fever over 38°C. Drowsiness in the first twenty-four hours after vaccination was noticed in three infants. Mild fussiness occurred in six subjects and for four infants, parents reported loss of appetite throughout 72 hr after vaccination while two infants had isolate episodes of vomiting.

Serology. The humoral response of infants to the DPT3/P/AH vaccine is reported in figure 1 and table 2 and that one to DPT7/PFK/AH vaccine is reported in figure 2 and table 2.

As expected, all the infants, after two doses of conventional DPT cellular vaccine, showed low titers of anti-PT, anti-FHA and anti-69K IgG antibodies, assayed by ELISA (Figures 1, 2), but a good immune response against diphtheria and tetanus, either evaluated as passive protection *in vivo* (Table 2) or as specific antibodies *in vitro* (Figures 1, 2).

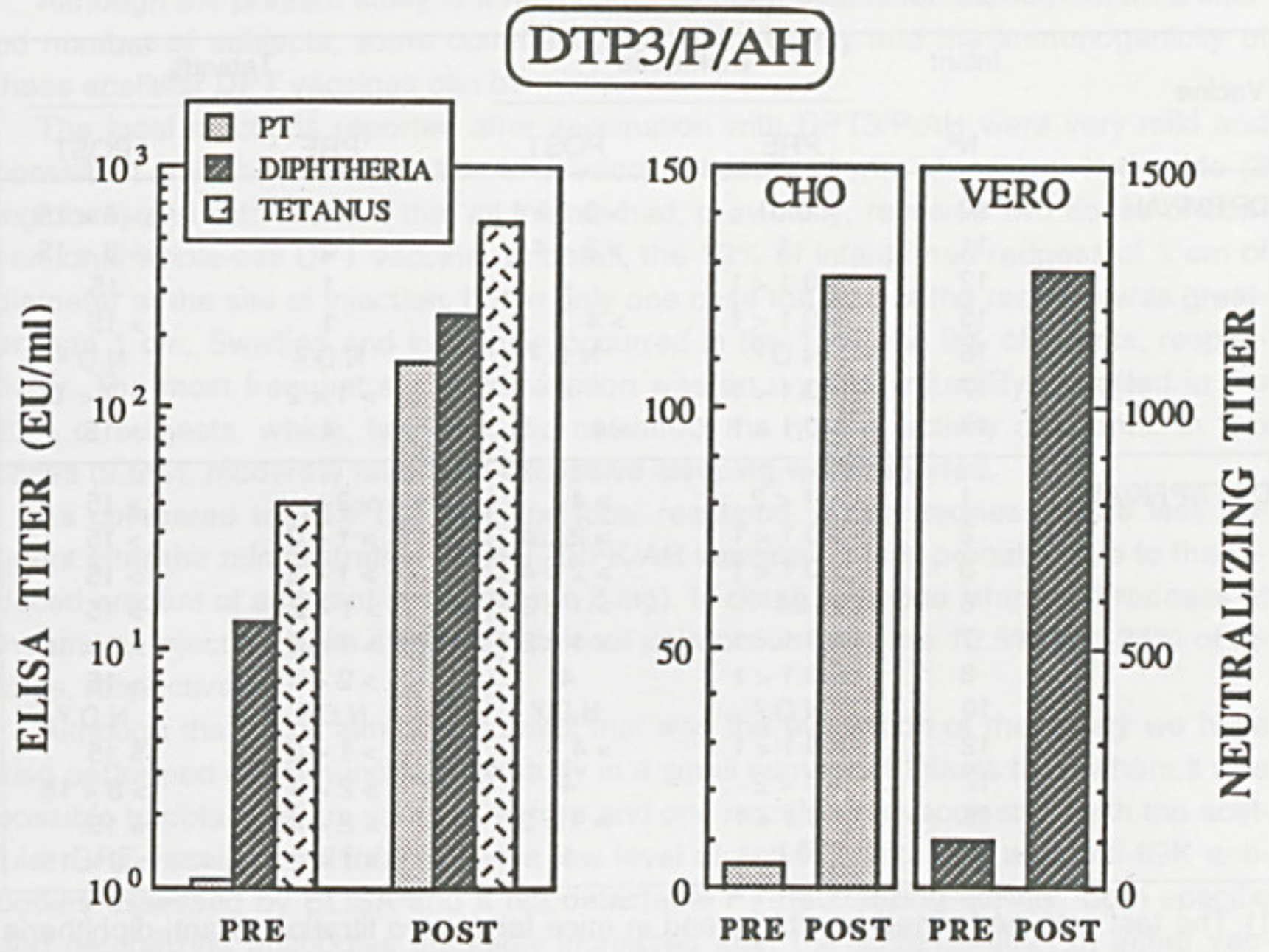

Figure 1 — Specific IgG antibodies (ELISA) as well as pertussis (CHO) and diphtheria (VERO) toxin neutralizing antibodies in infants receiving DPT3/P/AH vaccine.

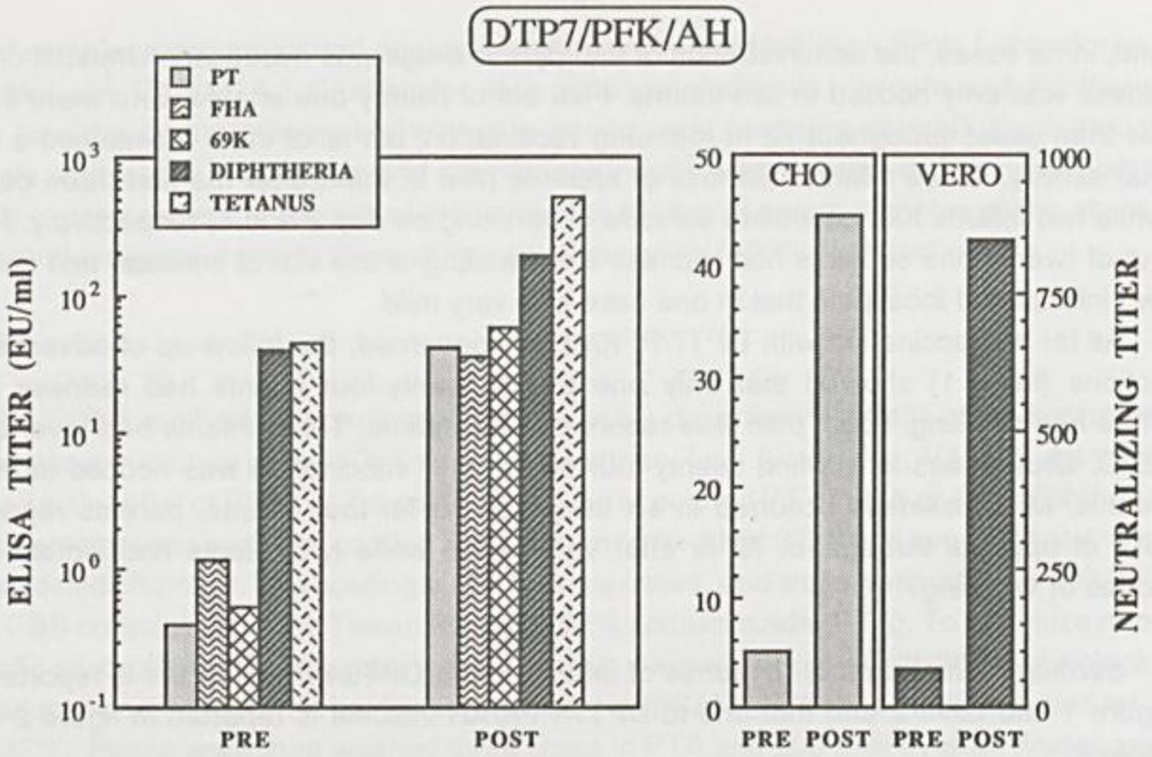

Figure 2 — Specific IgG antibodies (ELISA) as well as pertussis (CHO) and diphtheria (VERO) toxin neutralizing antibodies in infants receiving DPT7/PFK/AH vaccine.

TABLE II

Diphtheria and tetanus antitoxin titration *in vivo*[1]

Vacine	Infant	Diphtheria		Tetanus	
	Nº	PRE	POST	PRE	POST
DPT3/P/AH	10	< 0.01	> 0.1 < 1	1	> 8 < 15
	11	1	> 2 < 4	2	> 8 < 15
	12	> 0.1 < 1	4	1	15
	15	> 0.1 < 1	> 4	1	> 15
	16	N.D.[2]	N.D.[2]	N.D.[2]	N.D.[2]
	17	> 0.1 < 1	4	> 1 < 2	> 8 < 15
	19	> 0.1 < 1	> 4	4	> 15
DPT7/PFK/AH	1	> 1 < 2	> 4	> 2	> 15
	2	> 0.1 < 1	> 2 < 4	> 1 < 2	> 15
	3	> 0.1 < 1	> 2 < 4	> 1 < 2	> 15
	6	0.1	> 1 < 2	> 1 < 2	> 15
	7	> 0.1 < 1	> 1 < 2	> 1 < 2	> 8 < 15
	8	> 0.1 < 1	4	> 2 < 4	> 15
	10	N.D.[2]	N.D.[2]	N.D.[2]	N.D.[2]
	12	> 0.1 < 1	> 4	> 1 < 2	> 15
	17	> 1 < 2	4	> 2 < 4	> 8 < 15
	18	> 0.1 < 1	> 1 < 2	> 2 < 4	> 15
	19	0.1	1	> 1 < 2	> 8 < 15

1) The test was performed in rabbits and in mice for *in vivo* titration of anti-diphitheria and anti-tetanus neutralizing antibodies, respectively. Results are expressed as International Units (IU)/ml.
2) N.D. Not Determined.

Total IgG antibodies to each vaccine components increased after the administration of the acellular DPT vaccines. A similar rise was noted for pertussis toxin-neutralizing antibodies, evaluated by the CHO cell assay, and for diphtheria toxin-neutralizing antibodies, assayed by the VERO cell assay (Figure 1, 2). A high serum activity against diphtheria and tetanus toxins was also observed in animals, using the *in vivo* assay recommended by WHO. In fact, sera from infants receiving one dose of either DPT acellular vaccine showed a marked increase of passive protection against diphtheria and even more pronounced protection against tetanus. In most cases the *in vivo* diphtheria antitoxin titers were over 4 IU/ml and the tetanus antitoxin titers were over 15 IU/ml (Table 2).

DISCUSSION

In a previous phase I[8,9] and a phase II (Podda et al., manuscript in preparation) clinical study, we had carefully tested the safety and the immunogenicity of two monovalent acellular pertussis vaccines, one containing the genetically detoxified pertussis toxin mutant PT-9K/129G[1] and the other containing PT-9K/129G combined with FHA and 69K. Both vaccines proved to be safe and immunogenic in adults and children. Since the final formulation of pertussis vaccine, to be introduced in the vaccination schedule of children, is expected to contain also diphtheria and tetanus toxoids, we have prepared two acellular DPT vaccines (DPT3/P/AH and DPT7/PFK/AH) which pass the test of the American and European pharmacopea and we have tested them in infants.

Although the present study is a not controlled open evaluation carried out on a limited number of subjects, some comments about the safety and the immunogenicity of these acellular DPT vaccines can be made.

The local reactions reported after vaccination with DPT3/P/AH were very mild and consistent with the administration of a vaccine adsorbed with aluminium hydroxide (2 mg/dose) and with the fact that all infants had, previously, received two doses of conventional whole-cell DPT vaccine. In detail, the 19% of infants had redness of 1 cm of diameter at the site of injection, but in only one case the size of the reaction was greater than 1 cm. Swelling and local pain occurred in the 19% and 9% of infants, respectively. The most frequent systemic reaction was an unusual irritability, reported in the 23% of subjects, which, however, did not affect the normal activity of infants. In two cases (9.5%), moderate fever and excessive sleeping were reported.

As compared with DPT3/P/AH, the local reactions, mainly redness, were less frequent after the administration of DPT7/PFK/AH vaccine. This is probably due to the reduced amount of adjuvant (1 mg versus 2 mg). In detail, only one infant had redness at the site of injection while swelling and local pain occurred in the 12.5% and 25% of infants, respectively.

Although the major aim of this small trial was the evaluation of the safety we have also performed an immunogenicity study in a small number of infants from whom it was possible to obtain serum samples before and one month after vaccination with the acellular DPT vaccines. All infants had a low level of anti-PT, anti-FHA and anti-69K antibodies assessed by ELISA and a not detectable PT-neutralizing activity. Both specific and neutralizing antibodies markedly increased after the administration of either vaccines but the enhancement of the humoral immunity, probably due to the higher amount of adjuvant, was more pronounced after vaccination with DPT3/P/AH. Also the

serum antibody responses to diphtheria and tetanus toxoids, which was already good before vaccination, augmented more than ten times after one dose of either acellular DPT vaccines as evaluated *in vitro* in terms of ELISA and VERO titers. The enhanced serum activity against diphtheria and tetanus was confirmed by *in vivo* antitoxin antibody titrations. In fact, sera from all vaccinees were able to confer a high passive protective immunity to the animals reaching in most cases, after one dose of acellular DPT vaccine, values of more than 4 IU/ml against diphtheria and even more than 15 IU/ml against tetanus.

In conclusion, the careful follow-up of 21 infants receiving DPT3/P/AH and of 24 infants receiving DPT7/PFK/AH in addition to the immune response evaluation performed in some of them proved the substantial safety and the excellent immunogenicity of these acellular DPT vaccines and encourages their further evaluation in enlarged clinical trials.

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