

MAJOR ARTICLE

Determination of Serum Antibody to *Bordetella pertussis* Adenylate Cyclase Toxin in Vaccinated and Unvaccinated Children and in Children and Adults with Pertussis

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Presence of antibody to adenylate cyclase toxin (ACT) has been noted following *Bordetella pertussis* infection. Because ACT is not presently in any acellular pertussis vaccines, it has been considered as a possible antigen to use in *B. pertussis* diagnostic enzyme-linked immunosorbent assay (ELISA) studies. We determined antibody to *B. pertussis* ACT by ELISA and Western blot tests in serum samples obtained from unvaccinated children, from children vaccinated with several diphtheria and tetanus toxoid vaccines (DTP vaccines), from children vaccinated with vaccines containing acellular pertussis components in combination with diphtheria and tetanus toxoids (DTaP vaccines), and from children and adults with pertussis. Primary infections with either *B. pertussis* or *Bordetella parapertussis* stimulated a vigorous antibody response to ACT. In contrast, patients in whom DTP and DTaP vaccines failed had minimal ACT antibody responses. The lack of a significant ACT antibody response in children in whom the vaccine failed is of interest but would seem to preclude the use of ACT in diagnostic tests.

Whooping cough (pertussis) is an infectious disease found in both children and adults and caused by the gram-negative bacterium *Bordetella pertussis* [1, 2]. *B. pertussis* has a number of virulence factors, including fimbriae, pertactin (PRN), pertussis toxin (PT), filamentous hemagglutinin (FHA), lipooligosaccharide, and adenylate cyclase toxin (ACT). ACT is an important virulence factor of *B. pertussis* which disrupts host cyclic 3',5'-adenosine monophosphate (cAMP) metabolism [1–4]. ACT enters a variety of mammalian cells

and can inhibit the microbicidal cytotoxic function of neutrophils, monocytes, and natural killer cells. Its contribution to clinical pertussis may be through impairment of host defenses or through a direct effect on the respiratory mucosa.

The use of *B. pertussis* ACT for diagnosis of pertussis was described by Confer and Eaton [5]. Nasopharyngeal swab specimens carrying as few as 100 *B. pertussis* cfu induced detectable levels of cAMP. Previous immunoblotting studies looked for antibodies to ACT in serum samples obtained from infants, children, and adults [6, 7]. Farfel et al. [7] found ACT antibody in the serum samples of the following subject groups: 15 patients with pertussis, 4 neonates, 4 previously vaccinated children between 2 and 3 years old, and 4 adults who had been vaccinated as children. They did not detect ACT antibody in the serum samples of 4 unvaccinated infants between 4 and 5 months old. Arciniega et al. [6] found ACT antibody in the serum sam-

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ples of 5 patients with pertussis but not in the serum samples of 6 infants who were immunized with vaccine containing whole cell pertussis in combination with diphtheria and tetanus toxoids (DTP vaccine).

In the 1990s, several large vaccine efficacy trials were performed for the evaluation of vaccines with acellular pertussis components in combination with diphtheria and tetanus toxoids (DTaP vaccines) [1]. In these trials, diagnosis was established in many instances by the demonstration of an increase in antibody titer in response to PT and other *B. pertussis* antigens (FHA, PRN, and fimbriae). Because ≥ 1 of these antigens was contained in each of the various DTaP vaccines evaluated, there was concern that this would result in a disproportionate identification of cases among patients in the control group (compared with patients who were vaccinated) and would therefore inflate the reported efficacy of the vaccine.

In consideration of the factors above, it would seem ideal to use an antigen that is *Bordetella* species-specific and not included in a DTaP vaccine for serologic diagnosis. *B. pertussis* ACT has been cloned in *Escherichia coli* by recombinant technology and can therefore be produced in large amounts [3, 4]. In contrast to immunoblotting, ELISA allows the determination of specific quantitative ACT antibody values. To gain a broader understanding of the human immune response to *B. pertussis* ACT, we examined antibodies to ACT by ELISA and immunoblotting in serum samples obtained from DTP or DTaP vaccine recipients; unvaccinated children with and without pertussis; adults with pertussis; DTP or DTaP recipients who subsequently had pertussis; and unvaccinated children with *B. parapertussis* illnesses.

MATERIALS AND METHODS

Serum specimens. Serum samples were obtained from children who were subjects in 4 vaccine efficacy studies and from German children and adults with pertussis [8–13]. Informed consent was obtained from study subjects or their parents or guardians, and the human experimentation guidelines of the US Department of Health and Human Services and those of the specific trial sites in Sweden, Italy, and Germany were followed. Serum samples were specifically collected to determine antibodies to *B. pertussis* antigens.

Antigen and reagents. *B. pertussis* ACT that had been produced in *E. coli* was used (kindly provided by Dr. J. Coote, University of Glasgow) [3]. The possibility of cross-reactions in the serum samples to residual *E. coli* antigens was assessed in a pilot study. The serum samples were incubated with the parent *E. coli* strain ($\sim 10^8$ cells/mL) at 37°C overnight; bacteria were then removed by centrifugation. ELISA was performed on the supernatants for ACT antibody. The ELISA results obtained from samples that underwent this treatment were com-

pared with results obtained from samples that were not incubated with the parent *E. coli* strain, and there was no significant difference between the 2 groups. To our knowledge, there is no reference serum for ACT antibody available, and therefore, for standardization, a freeze-dried preparation of a human serum sample with antibodies to other *B. pertussis* antigens, provided by the National Institute for Biological Standards and Control, United Kingdom (NIBSC, code 89/530), was used as an in-house reference in this study. This preparation has been assigned to have antibody to ACT at a concentration of 100 U/mL. All other reagents used were analytical grade unless otherwise stated.

Determination of titers of ACT antibodies by ELISA. ELISA was used to determine titers of IgG ACT antibodies in each of the serum samples. In brief, wells of microtiter plates were coated with 100 μ L of ACT at a concentration of 2 μ g/mL in carbonate coating buffer (pH 9.6) for 18 h at room temperature. After blocking and washing 3 times with PBS/Tween/10% fetal calf serum (PBSTF), 2-fold dilutions of the reference serum sample and serum samples to be tested in PBSTF were performed on the plate and incubated for 2 h at room temperature. The negative control wells were treated in a similar fashion but did not contain serum samples. After the incubation with anti-human peroxidase conjugate (IgG; Sigma) in PBSTF for 2 h at room temperature, the wells were washed, and 100 μ L of substrate solution containing hydrogen peroxide-tetramethylbenzidine was added to all of the wells. Color was allowed to develop for 15 min at room temperature, and 50 μ L of sulfuric acid at a concentration of 1 mol/L was added to stop the reaction. The plates were then read at an optical density of 450 nm on an Anthos plate reader (Anthos Labtec Instruments, Austria).

The cutoff point for each individual assay was calculated as the mean optical density for the negative control wells plus 2 SDs. On the basis of 92 assays, the detection limit for this assay was established as 2 ELISA units per milliliter (EU/mL) of the standard serum 89/530. Antibody levels in samples giving estimates below the detection limit were regarded as undetectable. The ELISA units were calculated for each of the serum samples against the reference serum 89/530 according to the principles of multiple parallel line bioassay, by comparison of transformed assay response to log concentration with use of ≥ 4 dilutions in the linear range. Assay precision was assessed by 3 additional tests that were performed on 3 different days on 5 serum samples. The coefficient of variation was 3.41%. In all cases, an analysis of variance gave an assessment of linearity and parallelism, which are required as a measure of the statistical validity of the analysis. Ninety-five percent fiducial limits were calculated, and Student's *t* test was used for comparison between groups; $P < .05$ was regarded as statistically significant.

Immunoblotting. The 200-kDa ACT antigen (6.4 μ g) and

a marker (molecular weight, 4–250 kDa) were subjected to 8% acrylamide trisglycine gel (Invitrogen) in the presence of SDS-PAGE and then transferred to nitrocellulose by electrophoresis. After washing and blocking, the individual strips were then reacted with the reference serum or test serum samples (1:500) overnight at room temperature. The strips were washed and then incubated for 2 h with an anti-human peroxidase (IgG) conjugate. The strips were then stained with diaminobenzidine tablets as a peroxidase substrate. The blots were scanned with a scanner and analyzed using Adobe Photoshop software, version 4. The Phoretix 1D quantifier version 5.00 was used to detect the intensity of the bands at 200 kDa for the test and reference samples. For standardization, the ratio of the intensity of a test sample divided by the intensity of a reference sample from the same blot was used to express the results. A ratio ≥ 1.0 was regarded as positive.

RESULTS

The ACT ELISA postimmunization geometric mean titers (GMT) of antibody in the serum samples of healthy infants who received diphtheria-tetanus (DT), DTP, or DTaP vaccines are presented in table 1. Thirteen infants received 2 or 3 doses of German- or Swedish-licensed DT vaccines; 35 infants received 3 or 4 doses of licensed DTP vaccines (10 received US-licensed Connaught vaccine, 20 received US-licensed Lederle vaccine, and 5 received UK-licensed Evans Medical vaccine); and 44 received 3 or 4 doses of investigational DTaP vaccines. Nine infants received a 2-component (PT and FHA) vaccine manufactured by SmithKline Beecham; 10 infants received a 3-component (PT, FHA, PRN) Chiron vaccine; 5 infants received a 3-component (PT, FHA, PRN) vaccine manufactured by SmithKline Beecham; 10 infants received a 4-component

(PT, FHA, PRN, fimbriae type 2) Lederle/Takeda vaccine; and 10 infants received a 5-component (PT, FHA, PRN, fimbriae type 2, and fimbriae type 3) Connaught-Canada vaccine.

As can be seen, all serum samples from these infants and vaccinated children had low values of antibody to ACT (table 1) in comparison with the values for serum samples obtained from unvaccinated children with *B. pertussis* or *B. parapertussis* infections (table 2). The mean values for the serum samples of the DT-, DTP-, and DTaP-recipient subgroups were not statistically different. However, 2 of 5 recipients of the Evans DTP vaccine and 3 of 20 recipients of the Lederle DTP vaccine had ELISA serum values >3 -fold greater than the mean serum value (18 EU/mL) for DT recipients. These values were 68 and 97 EU/mL, for the recipients of the Evans DP vaccine, and 118, 106, and 73 EU/mL, for the recipients of the Lederle DTP vaccine.

The ELISA values of antibody to ACT in convalescent-phase serum samples from previously vaccinated or unvaccinated children with pertussis due to *B. pertussis* or *B. parapertussis* infections are presented in table 2. In unvaccinated children, the serum GMTs after pertussis due to *B. pertussis* or *B. parapertussis* infections were elevated: 872 EU/mL and 512 EU/mL, respectively (48- and 28-fold greater than the GMTs in unvaccinated infants). Only 1 of the subjects, an 8-month-old infant, had a low titer in the convalescent-phase blood sample (titer, 16 EU/mL). In contrast, the GMTs in convalescent-phase serum samples obtained from subjects in whom vaccination had failed were only slightly elevated. The GMT in the serum samples obtained from subjects for whom DTP () vaccine failed was 92 EU/mL, and it was 49 EU/mL in the serum samples of subjects for whom DTaP (Lederle/Takeda) vaccine failed (5- and 3-fold greater than the GMT in serum samples obtained from unvaccinated infants).

Table 1. Geometric mean titers (GMTs) of antibody to adenylate cyclase toxin (ACT) in serum samples from children who received 2–4 doses of diphtheria, tetanus toxoid, and acellular pertussis (DTP), acellular pertussis components in combination with diphtheria and tetanus toxoids (DTaP), or diphtheria-tetanus (DT) vaccines.

Vaccine, manufacturer and/or nation	<i>Bordetella pertussis</i> antigens	Median age, ^a months	No. of doses administered	No. of patients vaccinated	GMT of antibody to ACT (95% CI), EU/mL
DT, German, Swedish	None	7	2–3	13	18 (11–30)
DTP, Connaught, US	Multiple	7	3	10	25 (18–37)
DTP, Lederle, US	Multiple	17	4	20	28 (19–40)
DTP, Evans, UK	Multiple	7	3	5	43 (20–93)
DTaP, SKB	PT, FHA	7	3	9	30 (20–45)
DTaP, Chiron	PT, FHA, PRN	7	3	10	21 (14–32)
DTaP, SKB	PT, FHA, PRN	7	3	5	15 (7–35)
DTaP, Lederle/Takeda	PT, FHA, PRN, Fim-2	21	4	10	15 (10–22)
DTaP, Connaught, Canada	PT, FHA, PRN, Fim-2, Fim-3	7	3	10	24 (17–35)

NOTE. EU, ELISA units; FHA, filamentous hemagglutinin; Fim-2, fimbriae type 2; Fim-3, fimbriae type 3; PT, pertussis toxin; PRN, pertactin.

^a Median age at the time that serum samples were obtained.

Table 2. Geometric mean titers (GMTs) of antibody to adenylate cyclase toxin (ACT) in convalescent-phase serum samples obtained from previously vaccinated or unvaccinated children with pertussis due to *Bordetella pertussis* or *Bordetella parapertussis* infections.

Patient group	No. of children	Median age (range)	GMT of antibody to ACT (95% CI), EU/mL
Unvaccinated with <i>B. pertussis</i> infection	20	8 years (8–16 years)	872 (507–1500)
Unvaccinated with <i>B. parapertussis</i> infection	4	21 months (14–8 years)	512 (186–1412)
Experienced DTP (Lederle) vaccine failure	5	16 months (13–41 months)	92 (49–173)
Experienced DTaP (Lederle/Takeda) vaccine failure	10	25 months (15–36 months)	49 (27–86)

NOTE. DTaP, acellular pertussis components in combination with diphtheria and tetanus toxoids; DTP, diphtheria, tetanus toxoid, and acellular pertussis; EU, ELISA units.

Acute- and convalescent-phase serum specimens were available from the 5 children who experienced DTP vaccine failure. No increase in titer occurred; the acute-phase GMT and the convalescent-phase GMT were both 92 EU/mL. As noted in the findings of vaccine efficacy trials conducted by members of our group [8] and others [9, 13], the clinical illnesses of children who experienced DTP and DTaP vaccine failure were, on average, less severe than the illnesses of unvaccinated children. When illnesses in the 2 vaccine-failure groups are compared, illnesses among the patients who received DTaP vaccine seem less typical of classic pertussis than do illnesses among those who received DTP vaccine. All 5 patients for whom DTP vaccine failed had some combination of paroxysmal cough, whoop, or posttussive vomiting, whereas only 6 of 10 patients for whom DTaP vaccine failed had similar clinical findings.

Immunoblotting was performed on the serum samples obtained from the children in the German vaccine trial [8]. Of the 24 subjects with pertussis due to *B. pertussis* or *B. parapertussis* infection, only 2 subjects with *B. pertussis* infections did not have immunoblotting results positive for ACT antibody (table 2). In contrast, only 1 of the 20 patients who received the Lederle DTP vaccine had a positive immunoblotting result, and none of the 10 Lederle/Takeda DTaP vaccine recipients had immunoblotting results that were positive for ACT antibody (table 1). Of those patients for whom vaccination failed (5 of whom received DTP vaccine and 10 of whom received DTaP vaccine), only 3 recipients of DTaP vaccine had weakly positive immunoblotting results for ACT antibody (table 2).

Over a period of ~2.5 years, we obtained acute-phase and follow-up serum specimens from 11 adults with confirmed *B. pertussis* infections [11]. Using these serum samples, over time we have performed ELISAs to determine antibody values to PT, FHA, PRN, and fimbriae, and we have now determined ACT ELISA antibody values. The ACT GMT increased between 3-fold and 4-fold, and this titer persisted for the 28 months of follow-up. This pattern is dissimilar to that found for PT, but it is somewhat similar to that found for FHA and PRN.

DISCUSSION

ACT is an important toxin that contributes to disease caused by *B. pertussis* and *B. parapertussis* [1–4]. Because the efficacy of the various DTaP vaccines is not optimal, ACT has been a candidate for inclusion in more-complete vaccines. However, at the present time, ACT has not been included as an antigen in any of the available DTaP products. The study by Farfel et al. [7] suggested that DTP vaccines had elicited ACT antibody responses. However, because the serum samples studied were obtained from adults and children between 2 and 3 years old who were vaccinated in infancy, it is likely that the ACT antibodies found were attributable to previously unrecognized *Bordetella* infections [1, 14, 15].

In this investigation, all serum samples obtained from 10 healthy infants (DT recipients) had low values of antibody to ACT, as measured by ELISA. Similarly, the postimmunization GMTs of all vaccine groups (i.e., recipients of the DTP vaccine and the DTaP vaccine) were also low (table 1). However, of all vaccine recipients, only 2 recipients of the Evans DTP vaccine and 3 recipients of the Lederle DTP vaccine had modestly elevated ACT antibody titers (68 and 97 EU/mL and 118, 106, and 73 EU/mL, respectively). Of the 3 Lederle DTP recipients with elevated titers, none had positive Western blot results.

The low titers of ACT antibody in the serum samples of the 13 unvaccinated infants (as well as in the serum samples of almost all of the healthy patients who received vaccine) are of interest. These values could be the result of residual antibodies, acquired transplacentally from the mother. However, this seems unlikely, because there was no significant difference in the GMTs of the groups from whom serum samples were obtained during the second year of life, compared with those of the groups from whom serum samples were obtained at 7 months of age. Further, <5% of infants would be expected to have a measurable titer of residual transplacentally acquired antibodies at 7 months of age [16].

There are 2 more-likely possible explanations for the ACT antibody values present in the serum samples obtained from

healthy infants. *B. pertussis* ACT is a member of the repeat toxin family of cytotoxins, which are also produced by *E. coli* and other gram-negative bacteria [17]. Because there is a similarity between the amino acid sequences of the c-terminal domain of ACT and those of the hemolysins of *E. coli* and other gram-negative bacteria, it is possible that the low titers we noted are attributable to cross-reacting antibodies generated in response to *E. coli* or other organisms in the normal bowel flora [18, 19]. In support of this hypothesis, Arciniega et al. [18] studied serum samples obtained from neonates and their mothers and noted that the samples contained antibodies to ACT and to the α -hemolysin of *E. coli*.

Another possibility relates to the production of ACT during human metabolism. Specifically, ACT catalyzes the synthesis of cAMP in eukaryotic cells, which acts as a messenger in the action of many hormones [20, 21]. It is possible that ACT is released from the cell in certain circumstances, such as at cell death. It is not known, however, if autoantibodies to autologous ACT would cross-react significantly with *B. pertussis* ACT. However, Goyard et al. [21] found that eukaryotic ACT contained an epitope closely related to that specified by a conserved *B. pertussis* sequence.

The high titers in convalescent-phase serum samples obtained from patients with pertussis due to *B. pertussis* or *B. parapertussis* infection suggest the possibility of using ACT antigen detection with ELISA for the diagnosis of pertussis. However, it is apparent that results obtained using ACT antigen are inferior to those obtained using PT antigen. Antibody to ACT is less specific than antibody to PT, and, because ACT is common to *Bordetella* species, detection of antibody to ACT offers no advantage over detection of FHA or PRN antigens. Of particular interest is the lack of a significant ACT antibody response in children for whom the DTP or DTaP vaccines failed. This induced tolerance is intriguing and may be due to the phenomenon called "original antigenic sin" [22]. In this phenomenon, a child responds at initial exposure to all presented epitopes of the infecting agent or vaccine. With repeated exposure when older, the child responds preferentially to those epitopes shared with the original infecting agent or vaccine and can be expected to have responses to new epitopes of the infecting agent that are less marked than normal. Because both vaccines contained multiple antigens (i.e., PT, FHA, PRN, and fimbriae), the patients who had been vaccinated responded to the antigens that they had been primed with and did not respond to the new antigen (i.e., ACT) associated with infection. It is of interest that the GMT was higher among the patients for whom DTP vaccination failed than it was among the patients for whom DTaP vaccination failed. This suggests the possibility that small amounts of ACT were present in the DTP vaccine. This possibility is supported by the finding of slightly elevated ACT postvaccination titers in 5 of 25 recipients of the DTP vaccine.

The data concerning antibody decay after infection in adults is also revealing. The pattern of decay of antibody to PT is different from that of antibody to ACT, FHA, PRN, and fimbriae [23]. The persistence of antibodies to the latter 4 antigens may result from continued stimulation by cross-reacting antigens from other infectious agents [23, 24]. Antibody to PT decreases more acutely because this antigen is *B. pertussis* exclusive.

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