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Detection of Antibodies Inhibiting the ADP-Ribosyltransferase Activity of Pertussis Toxin in Human Serum

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Bordetella pertussis **produces a protein virulence factor termed pertussis toxin. Many candidate pertussis vaccines are based on the rationale that an immune response that neutralizes the virulence activities of this toxin, which are thought to arise from its catalytic ADP-ribosyltransferase activity, would be beneficial. The report describes two methods that quantify the inhibition of this activity by human serum. One, termed a direct assay, involves an initial incubation of toxin with serum, a second incubation that activates the toxin, and a third incubation that measures the ADP-ribosyltransferase activity of the mixture. The other assay, termed a plate assay, involves immobilization of the toxin, exposure of the immobilized toxin to serum and washing of the plate, and then activation and assay of the toxin's ADP-ribosyltransferase activity. The plate assay may be more selective than the direct assay in terms of identifying antibodies that neutralize the toxin in vivo. Sera from controls, selected patients presenting with cough, and vaccinated infants were first analyzed by the direct assay. In contrast to sera from controls, sera from several of the patients and vaccinated infants strongly inhibited activity. Dose-response curves of inhibition were determined for samples from three vaccinated infants by both the direct and plate assays. One of the samples had a dose-response curve of a different shape and thus differed not only in titer but also in functional characteristics. A comparison of inhibition of ADP-ribosyltransferase activity and neutralization in a CHO cell assay indicated that there was incomplete agreement between the two assays. Taken together, these results indicate that measurement of inhibition of ADP-ribosyltransferase activity by human serum is practical and may be useful in the evaluation of responses to pertussis vaccines.**

The bacterium *Bordetella pertussis* is the causative agent of the disease whooping cough (for a review, see reference 52). A vaccine consisting of killed, whole-cell *B. pertussis* is used in the United States and effectively protects vaccinated individuals against disease (9). However, unpleasant side reactions and concerns arising from reports (regardless of their validity) of an association between vaccination and permanent neurologic damage threaten acceptance of the whole-cell vaccine (11, 33). As a result, there is a need for information that will allow for the rational selection and use of antigens for alternative acellular vaccines. Of obvious benefit would be the identification of the serological correlates of human protection; however, to our knowledge, a definitive serological correlate has not been established.

The observation that antibodies capable of agglutinating *B. pertussis* have correlated with postvaccination protection (32, 34) has prompted the testing of vaccines containing specific surface proteins. Vaccines that contain the surface proteins FHA and pertactin have protected mice, and monoclonal antibodies that recognize pertactin have conferred passive protection (40, 49). In addition, because it is a virulence factor, attention has also focused on a protein toxin released by *B. pertussis*, termed pertussis toxin. Monoclonal antibodies that recognize pertussis toxin can also confer passive protection to mice from *B. pertussis* (41, 42, 44). Despite these promising studies in animals, in a large clinical trial in Sweden, acellular vaccines containing formalin-treated pertussis toxin, with or without FHA, failed to produce levels of protection considered equivalent to the protection provided by whole-cell vaccines, and serological measurements did not correlate with protection (1). Thus, questions remain concerning the choice of antigens, the formulation of the vaccine, and methods of vaccine evaluation.

Because of the concern that the biological activities of pertussis toxin may contribute to undesired effects and because many of the actions of the toxin are thought to arise from its catalytic ADP-ribosyltransferase activity (24, 25), considerable effort has been devoted toward developing methods that produce immunoprotective analogs that lack this activity (6, 10, 17, 45, 48). It has become clear that simple enzyme-linked immunosorbent assays (ELISAs) or Western blot (immunoblot) assays fail to selectively identify antibodies that neutralize pertussis toxin (for a discussion, see reference 20). Thus, development of other assays needs attention. One candidate assay involves measuring the neutralization of the ability of the toxin to cause CHO cells to clump (12, 16), an effect that is thought to arise from the toxin's ADP-ribosyltransferase activity (7). However, in the clinical vaccine trial held in Sweden, the results of CHO cell neutralization assays did not correlate with protection (1).

Another candidate assay involves a measurement of inhibition of the pertussis toxin's ADP-ribosyltransferase activity in an enzymatic assay (19, 30). This report describes methods for measuring this inhibition on the basis of current knowledge of the relationship between the structure of the toxin and the regulation of this activity. In short, pertussis toxin comprises an A promoter, consisting of a single catalytic S1 subunit, and a B oligomer, containing one S2,

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one S3, two S4, and one S5 subunits. The B oligomer binds to receptors on target cells, delivering the S1 subunit (51). The sequence of the pertussis toxin gene predicts that the mature, proteolytically processed S1 subunit contains two cysteine residues (28, 39). These cysteines form an intrasubunit disulfide bond which apparently must be reduced for the subunit to express enzymatic activity (22–24, 37, 47). Nucleotides and phospholipids promote the dissociation of the S1 subunit from the B oligomer, facilitate the reduction of the disulfide bond within the S1 subunit, and increase its ADP-ribosyltransferase activity in vitro. It is likely that similar events activate the toxin in vivo (8, 17, 18, 27, 38).

In addition to describing practical assay methods, this report also discusses the potential advantages and disadvantages of the different ADP-ribosyltransferase and CHO cell assays and why different assays may yield different results.

MATERIALS AND METHODS

Materials. Pertussis toxin was obtained from List Biological Laboratories or the state of Michigan Department of Public Health. Transducin was purified as described previously (21, 53) and was stored at ≤−70°C. High-pressure liquid chromatography-purified monoclonal antibody 3CX4 (26) was a generous gift from James Kenimer, U.S. Food and Drug Administration. Other reagents for the ADP-ribosyltransferase assay were from common commercial sources. Acellular and whole-cell vaccines were provided by Lederle Laboratories (Pearl River, N.Y.) and were part of a previously described study (5). The whole-cell vaccine had a potency of 6 units per dose of the Lederle pertussis component (9). Each dose of the acellular vaccine contained 300 hemagglutinating units of the Takeda acellular pertussis component (Takeda Chemical Industries, Osaka, Japan), which included approximately 3.0 μg of pertussis toxin treated with formaldehyde and heat.

Serum samples. Serum samples were taken from the following essentially cough-free subjects: two males (subject X, age, 41 years; subject Y, age, 50 years) and one female (subject Z; age, 45 years). Sera from these subjects were used as controls. Samples were selected from patients in a study involving an evaluation for pertussis in patients presenting with cough (see Table 1) (35, 36). Evaluation included culture and direct fluorescent-antibody assays of nasopharyngeal swab specimens. For direct fluorescent-antibody assay, smears of swab specimens were stained with fluorescence-labeled polyclonal antisera to *B. pertussis* and *Bordetella parapertussis* (Difco). A positive score was given upon detection of three organisms with *B. pertussis* staining but not *B. parapertussis* staining. For cultures, swabs were inoculated immediately onto Bordet-Gengou media with and without methicillin and were incubated in a humidified 5% CO_2 atmosphere for 7 days. Positive cultures were confirmed as *B. pertussis* on the basis of specific polyclonal antibody fluorescence. Sera from subjects C1 to C5 were culture and direct fluorescent-antibody assay negative. Subject C1 had a cough but was not diagnosed with pertussis, whereas subjects C2 to C5 were diagnosed with pertussis on the basis of serological criteria (35). Subjects C6 and C8 to C10 were culture and/or direct fluorescent-antibody assay positive for *B. pertussis*; subject C7 was clinically diagnosed with pertussis and was epidemilogically linked to a culture-proven case of pertussis (36). Serum samples were also obtained from children who were participating in a vaccine study (see Table 2) (5).

Protein determinations. Protein determination assays were conducted as described previously (4, 29).

CHO cell assay. The titers of antibodies that neutralized the clumping of CHO cells caused by pertussis toxin were determined as described previously (12).

ELISA. An ELISA was used to determine the titers of immunoglobulin G (IgG) and IgA antibodies that recognized pertussis toxin adsorbed directly onto a plastic 96-well plate (31).

Direct ADP-ribosyltransferase assay. For the direct ADP-ribosyltransferase assay, diluted serum samples were added to pertussis toxin, the mixture was incubated, and the entire contents of the mixture were assayed. Samples of serum were diluted into 0.1% Lubrol–1 mg of bovine serum albumin per ml–50 mM Na_xPO_4 (pH 7.5) (L/B/PO_4). A total of 20 μl of this buffer, with and without added serum or monoclonal antibody 3CX4, was added to 10 ng of pertussis toxin in 30 μl of the same buffer, and the mixture was incubated for 60 min at room temperature. To the resulting 50 μl was then added 25 μl of a [^{32}P]NAD reaction mixture. The components of the reaction mixture were adjusted so that the concentrations achieved by all additions in the final 100 μl of the assay were 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1% Lubrol, 3 mM dithiothreitol, 25 μM [^{32}P]NAD (0.125 μCi), 1 mM ATP, 100 μM GDP, and 50 mM NaCl. The mixture was incubated at 30°C for 5 min in order to activate the toxin (8, 17, 18, 27, 38). A total of 25 μl of a buffer containing 20 μg of purified transducin was then added. After another 5 min at 30°C, the reaction was terminated by the addition of 50 μl of a solution of 0.03% sodium deoxycholate and 5 mM NAD. After 15 min on ice, 50 μl of 40% trichloroacetic acid was added, and the tubes were then vortexed and incubated on ice for another 15 min. Two milliliters of water was then added, the tubes were vortexed, and then the tubes were centrifuged at 3,000 × *g* for 45 min. The supernatant was then aspirated and the pellets were resuspended in 100 μl of 50 mM Tris base–192 mM glycine–1% sodium dodecyl sulfate, incubated at 90 to 95°C for 3 min, and vortexed. The sample was then spotted onto a piece of Whatman 3MM filter paper and washed for 15 min with 50% isopropanol–5% acetic acid–5% trichloroacetic acid. The filter papers were then washed twice (15 min each wash) with 50% isopropanol–5% acetic acid. The filter papers were then rinsed with 50% isopropanol and allowed to partially dry, and the radioactive sample remaining on the filter paper was counted with a water-tolerant scintillation fluid. A standard curve of inhibition of ADP-ribosyltransferase activity was run for each assay by using the monoclonal antibody 3CX4, which inhibits ADP-ribosyltransferase activity by binding to the S1 subunit of the toxin (20, 26). The standard curves indicated that the differences between ADP-ribosyltransferase assays were inconsequential (data not shown). In the direct assay, monoclonal antibody 3CX4 essentially completely inhibited the ADP-ribosyltransferase activity of the toxin (Fig. 1). Radioactivity values were normalized by first subtracting a blank (i.e., the result from assay tubes containing no toxin; typically, 50 to 75 cpm) and then dividing by the values for tubes to which no serum or monoclonal antibody 3CX4 was added after subtracting the blank (typically, 2,000 cpm).

Plate ADP-ribosyltransferase assay. In the plate ADP-ribosyltransferase assay, the toxin is immobilized and exposed to diluted serum samples, the serum is removed, and then the immobilized toxin is assayed for ADP-ribosyltransferase activity. A total of 1.5 μg of fetuin in 50 μl of 50 mM Na_x PO_4 (pH 7.5) was added to all wells of a 96-well enzyme

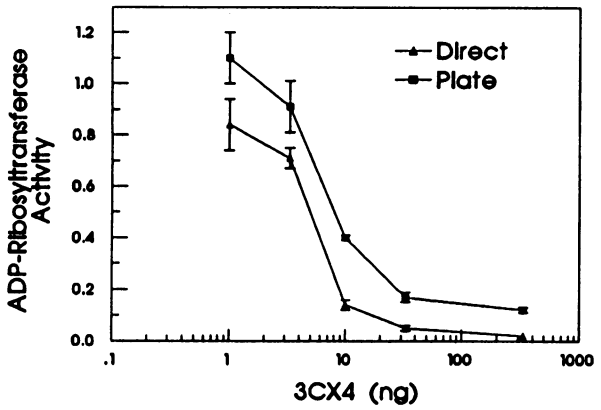

FIG. 1. Inhibition of ADP-ribosyltransferase activity by monoclonal antibody 3CX4. Various amounts of 3CX4 were incubated with pertussis toxin in either a direct or plate ADP-ribosyltransferase assay, and the ADP-ribosyltransferase activity of the mixture was determined (for details, see text). In both experiments, values were normalized by subtracting a blank without toxin and dividing by the results from tubes containing no 3CX4 or serum, also without the blank.

immunoassay-radioimmunoassay plate (no. 3590; Costar) for 2 h at room temperature. The wells were washed with 50 mM Na_xPO_4 (pH 7.5; five times; 200 μl per well), and the remaining binding sites were blocked with 10 mg of bovine serum albumin per ml in 50 mM Na_xPO_4 (pH 7.5; 200 μl per well) for 2 h at room temperature. A total of 7.5 ng of pertussis toxin in 70 μl of L/B/PO_4 was then added to all wells (except the wells used for no toxin blank determinations), and the wells were allowed to stand overnight at 4°C. The wells were then aspirated and washed five times (200 μl per well) with L/B/PO_4. A total of 70 μl L/B/PO_4 with or without monoclonal antibody 3CX4 or serum was then added to the washed wells and allowed to stand for 60 min at room temperature. Wells were washed as described above and were then incubated with 70 μl of 1 mM ATP in L/B/PO_4 for 30 min at 37°C to release the S1 subunit. A total of 50 μl of supernatant from each well was then collected and assayed for ADP-ribosyltransferase activity as described above for the direct ADP-ribosyltransferase assay. In the plate ADP-ribosyltransferase assay, monoclonal antibody 3CX4 failed to completely inhibit the ADP-ribosyltransferase activity of the toxin (Fig. 1), perhaps because the binding system introduces steric hindrances which reduce efficient antibody-toxin interactions. Radioactivity values were normalized by first subtracting a blank (i.e., the result from assay tubes containing no toxin; typically 50 to 75 cpm) and then dividing by the value for tubes to which no serum or monoclonal antibody 3CX4 was added after subtracting the blank (typically, 1,200 cpm).

RESULTS

To test the practicality of measuring the inhibition of ADP-ribosyltransferase activity by human serum, we used the direct ADP-ribosyltransferase assay to survey three serum samples from controls and 10 serum samples from patients presenting with cough enrolled in study in which they were evaluated for infection with *B. pertussis* (Table 1). As a control, one sample from a patient who was not diagnosed with pertussis was chosen. As an additional control, in the first survey the samples were handled blindly in terms of clinical and serological data. In order to conserve material, the samples were assayed singly at amounts of 1, 2, and 4 μl. Results of the survey indicated that titers of antibodies that inhibit the ADP-ribosyltransferase activity of pertussis toxin are elevated in some of the patients compared with the titers in controls (Fig. 2).

We then performed a similar survey of sera from 10 infants, 5 of whom were vaccinated with whole-cell (Lederle) and 5 of whom were vaccinated with acellular (Lederle-Takeda) pertussis vaccines. From each individual, the first sample was obtained at the time of initial vaccination (age, 2 months ± 2 weeks). The infants were vaccinated again at 4 months of age and then again at 6 months of age, at which time the second sample was taken. A third sample was taken 1 month after the third vaccination (age, 7 months). The results of this survey indicated that some of the infants responded to vaccination and produced titers of antibodies that inhibited ADP-ribosyltransferase activity to levels similar to or greater than those seen for the patients presenting with cough (Fig. 3).

TABLE 1. Characteristics of patients presenting with cough

Patient no.	Age (yr), sex[a]	Duration of cough (days)[b]	Direct fluorescence assay/culture	ELISA[c]		CHO titer	ADP-R assay[d]
				IgG	IgA		
C1	34, F	14	−/−	0	11	<40	—[e]
C2	19, F	123	−/−	444	40	1,280	0.28
C3	21, M	6	−/−	52	7	160	—
C4	22, F	22	−/−	149	35	640	0.45
C5	21, F	54	−/−	385	33	2,560	0.46
C6	19, F	60	+/+	273	34	640	0.53
C7	61, F	13	ND[f]/ND	554	52	1,920	0.13
C8	33, F	50	+/−	164	40	640	0.72
C9	17, M	39	+/+	321	25	3,840	0.27
C10	13, F	44	+/+	572	15	2,560	0.21

[a] F, female; M, male.
[b] Duration of cough at the time that samples were taken for evaluation.
[c] Results expressed in ELISA units, as described previously (31).
[d] ADP-R assay, ADP-ribosyltransferase activity in the presence of 1 μl of serum relative to that without serum (see text). The determination was done in triplicate, and the standard errors were all ≤0.02. These data are also given in Fig. 4. The initial screen of these samples is shown in Fig. 2.
[e] —, the sample was not assayed in triplicate; results of singlet determinations are given in Fig. 2.
[f] ND, not determined.

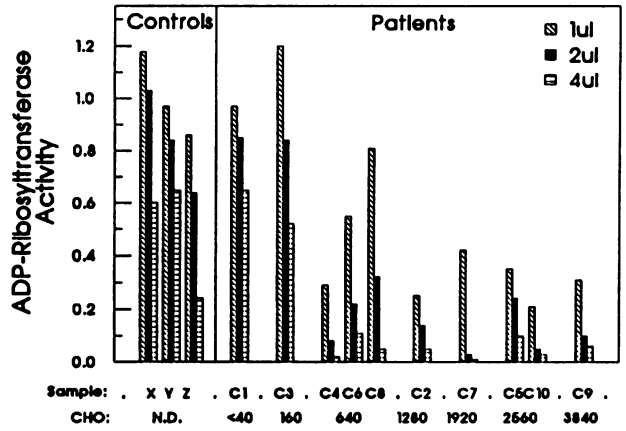

FIG. 2. Inhibition of ADP-ribosyltransferase activity by sera from patients presenting with cough. A total of 1, 2, and 4 μl of serum from patients (C1 to C10) and controls (subjects X, Y, and Z) were incubated with 10 ng of pertussis toxin, and the ADP-ribosyltransferase activity of the mixture was determined by the direct method. Values were normalized to the activity of 10 ng of toxin without serum. Single determinations were made. Samples are grouped according to the titers obtained from a CHO cell assay. N.D., not determined.

In order to confirm the differences between samples indicated by the surveys, we made in one experiment triplicate determinations of the inhibition of ADP-ribosyltransferase activity by 1 μl of selected samples from the sets described above using the direct assay. The results confirmed that the differences were real (Tables 1 and 2).

A comparison of the extent of inhibition of ADP-ribosyltransferase activity in the surveys with neutralization in the CHO cell assay prompts the notion that there is incomplete agreement between the two assays (Fig. 2 and 3; Tables 1 and 2). In order to better examine this relationship, the inhibition of ADP-ribosyltransferase activity data in Fig. 4 were plotted against CHO cell data by using logarithmic scales (Fig. 4). It should be noted that there is no theoretical

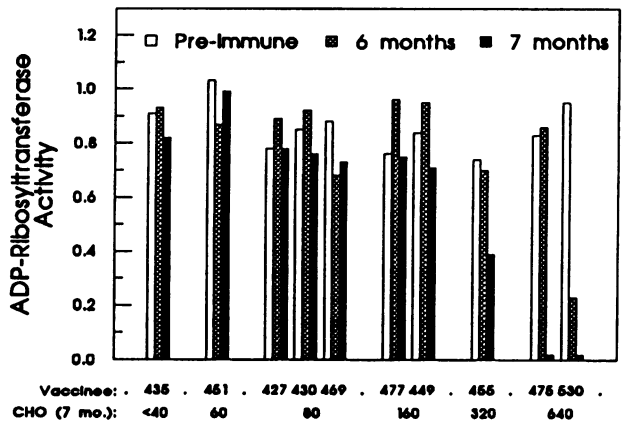

FIG. 3. Inhibition of ADP-ribosyltransferase activity by sera from vaccinated infants. A total of 1 μl of serum from vaccinated infants was incubated with 10 ng of pertussis toxin, and the ADP-ribosyltransferase activity of the mixture was determined by the direct method. Values were normalized to the activity of 10 ng of toxin without serum. Single determinations were made. The CHO values are from the sample taken from an infant at 7 months of age.

TABLE 2. Serological characterization of vaccinated infants

Vaccine and subject no.	Age (mo)	ELISA IgG[a]	CHO titer	ADPR[b]
Whole cell				
449	2	0	40	—[c]
	6	27	80	—
	7	107	160	0.52
455	2	3	40	—
	6	0	<40	—
	7	97	320	0.47
469	2	3	60	—
	6	2	<40	—
	7	37	80	—
475	2	0	60	—
	6	7	80	—
	7	191	640	0.07
530	2	0	40	—
	6	101	160	0.29
	7	311	640	0.03
Acellular				
427	2	2	40	—
	6	24	80	—
	7	33	80	—
430	2	0	<40	—
	6	21	80	—
	7	22	80	0.71
435	2	6	80	—
	6	0	<40	—
	7	2	<40	—
451	2	0	<40	—
	6	9	80	0.85
	7	3	60	1.07
477	2	15	160	—
	6	4	40	—
	7	16	160	—

[a] Results are expressed in ELISA units, as described previously (31).
[b] ADP-R, ADP-ribosyltransferase activity in the presence of 1 μl of serum relative to that without serum (see text). The determination was done in triplicate, and the standard errors were all <0.02. These data are also given in Fig. 4. The initial screen of these samples is shown in Figure 3.
[c] —, sample was not assayed in triplicate; results of singlet determinations are given in Fig. 3.

basis for assuming that this type of plot should yield a linear relationship. Nonetheless, we found it helpful to draw a line through the points to help determine whether there was any apparent relationship. Examination of this plot supports the notion stated above; although, in general, there is a correlation between the two assays, some samples inhibited well in one assay but not in the other.

Because the assay that measured inhibition of ADP-ribosyltransferase activity allows for quantification of inhibition over a wide range of added amounts of serum, it allows for further characterization of samples by using kinetic analysis. For example, different slopes of the dose-response curves can indicate different mixtures of antibodies with various affinities, interactions, or degrees of inhibition. To further define the differences among serum samples, we conducted dose-response studies with samples from three vaccinees (Fig. 5). By the direct assay, the data indicate that the two samples with greatest inhibition (from vaccinees 475 and 530 [Table 2]) had similar sigmoidal curves, with the inhibition occurring over a range of about 2 log units. These results are consistent with the inhibition that arises from a population of antibodies with essentially equivalent affinities and extents of inhibition, both within a sample and between the two samples. [The effect of adding a reversible noncom-

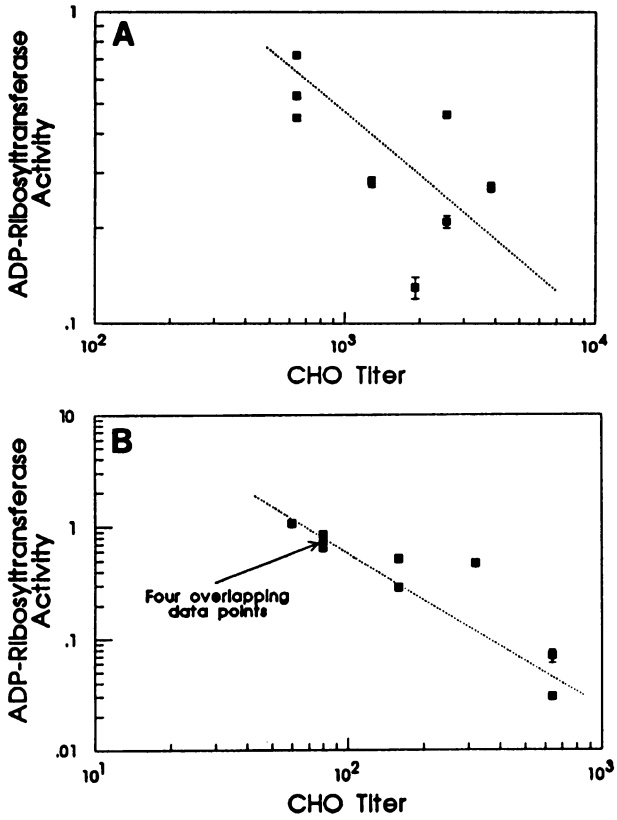

FIG. 4. Relationship between inhibition of ADP-ribosylation activity and CHO cell titers. (A) Patients; (B) vaccinated infants. The data in Tables 1 and 2 for inhibition of ADP-ribosyltransferase activity are plotted versus CHO cell titer. The CHO cell data between the panels are not strictly comparable; the assays were performed at different times with different sets of reagents.

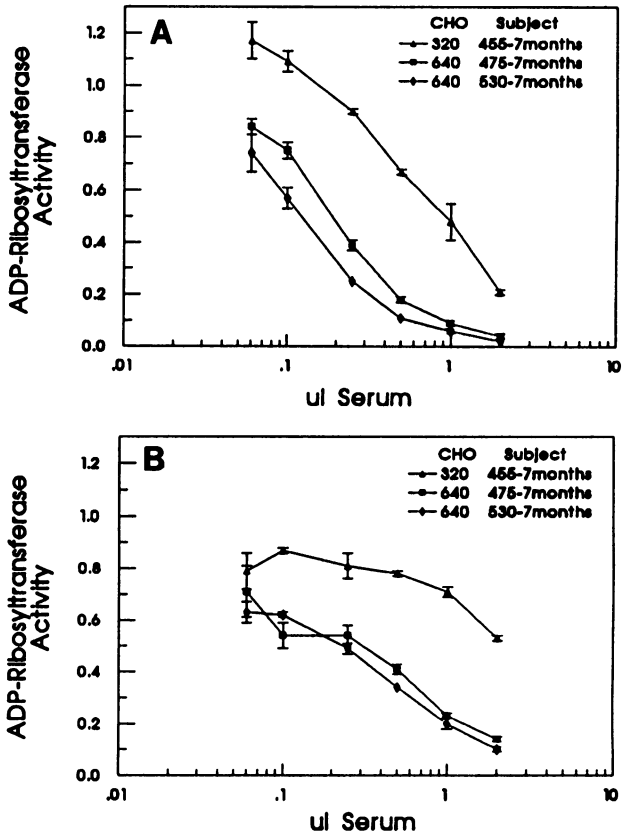

FIG. 5. Dose-response curves of inhibition of ADP-ribosyltransferase activity by sera. A total of 0.65 to 2 μl of serum from infants vaccinated with the whole-cell vaccine were analyzed by both the direct (A) and plate (B) ADP-ribosyltransferase assays. The standard curves of inhibition by the monoclonal antibody 3CX4 for these two assays are given in Fig. 1. Values were normalized as described in the text. The means and standard errors of triplicate determinations are given. The titers of the samples determined in a CHO cell assay are given inside the figure.

petitive inhibitor (I) to an enzyme assayed under linear conditions is $V = V_{max}/(1 + [I]/K_i)$ at the given $[I]$ (46). Plotting this relationship on a semilogarithmic plot yields a sigmoidal curve, with approximately 90% of the inhibition occurring over a 2-log-unit range of inhibitor concentrations (data not shown).] However, the curve that arose from the sample from the third subject (subject 455) displayed a different shape, indicating that the sample differs in some functional characteristic. One possibility is that the serum sample from subject 455 may contain a more heterogeneous mixture of inhibiting antibodies.

Theoretically, the measurement of inhibition of ADP-ribosyltransferase activity by the direct assay could yield false-positive results in terms of identification of a protective antibody. In the direct assay, an inhibiting antibody might fail to recognize holotoxin, but it might inhibit activity by binding to the S1 subunit after it has dissociated from the B oligomer. Because it would not bind to holotoxin, such an antibody would probably have little effect in vivo, and thus, the assay would yield a false-positive result. In order to avoid this potential problem, we developed a plate assay designed to allow the antibody to interact only with holotoxin (19). In the plate assay, the toxin was bound to fetuin that was previously immobilized in a well of a 96-well plate, exposed to serum, and washed; and then the toxin was activated and assayed. Using this assay, we reexamined the three samples from the direct assay described above (Fig. 5B). The samples from patients 475 and 530 remained essentially equipotent, with curves of basically the same shape. The fact that the curves were basically the same is consistent with the notion that the two samples contained similar titers of functionally equivalent (in terms of the plate assay) inhibiting antibodies. These results indicate that the antibodies in the samples from subjects 475 and 530 bind to and neutralize holotoxin. The sample from subject 455 inhibited the toxin to a lesser extent than did the other two samples and had a curve of a different shape. At least two explanations can be offered as causes for the reduction in potency of the sample from subject 455 in the plate assay compared with the potency in the direct assay. First, some of the antibodies from subject 455 might recognize epitopes that are not found on nonactivated holotoxin. Alternatively, immobilization of the toxin to fetuin may have introduced steric hindrances to the binding of antibodies, even though the relevant epitope is present on the holotoxin. The introduction of such steric hindrances may be an advantage of the plate assay if, in fact, the toxin is largely bound in a similar fashion in vivo. If so, then antibodies that bind to and neutralize the toxin in such a bound state might better neutralize the toxin in vivo.

DISCUSSION

Controversies concerning the whole-cell pertussis vaccine have prompted the development of alternative vaccines. These efforts have increased the need for the identification of serological correlates of protection suitable for use in human clinical trials. Because of the assumed contribution of pertussis toxin to the virulence of *B. pertussis*, considerable attention has been devoted to developing vaccines that induce antibodies that neutralize this toxin.

Because simple ELISA and Western blot assays present pertussis toxin in a heterogeneous, denatured form, these methods theoretically fail to provide a correlate of protection (20). An alternative is to devise immunoprecipitation (20) or ELISA methods in which the toxin is bound to immobilized fetuin (26). These methods should preserve the structural features of the toxin. However, a monoclonal antibody that recognizes the toxin in both of these assays fails to neutralize the toxin's actions on cultured cells (20, 26). Thus, assays that simply detect the presence of antibodies that recognize nondenatured toxin are theoretically not sufficient to selectively identify protective antibodies. These observations suggest that assays that measure neutralization of the function(s) of the toxin that contribute to virulence would more likely correlate with protection.

The potential contribution of pertussis toxin to the virulence of *B. pertussis* may arise exclusively or in large part from its catalysis of the ADP-ribosylation of G proteins. This notion prompts the hypothesis that antibodies that bind to the toxin and that interfere with catalysis could contribute to protection. However, several issues are worth considering before implementing an enzymatic assay in a vaccine trial. For example, would measurement of inhibition of catalytic activity merely duplicate the results of other assays already widely used, such as the CHO cell assay? Alternatively, whether or not the results would be duplicative, would measurement of inhibition of catalytic activity hold any practical or analytical advantages over other assays?

Duplication. An analysis of current knowledge concerning pertussis toxin suggests reasons why assays that measure the neutralization of the effects of pertussis toxin on CHO cells or inhibition of ADP-ribosyltransferase activity measured by an enzymatic assay might give different results. Neutralization of toxin in both assays can arise from antibodies that bind to the S1 subunit of the native holotoxin and that directly inhibit ADP-ribosyltransferase activity (26, 43, 44). However, antibodies that bind to the B oligomer can both protect mice from *B. pertussis* and neutralize the effects of the toxin on CHO cells (2, 14, 15, 43, 50), but they fail to inhibit the toxin's activity in an ADP-ribosyltransferase assay (43, 44). In our hands, both monoclonal and polyclonal antibodies against the B oligomer have failed to inhibit activity in the ADP-ribosyltransferase assay performed as described here (data not shown). This observation suggests that these antibodies prevent intoxication by interfering with binding to CHO cells, its subsequent internalization, or if it is internalized, the activation of the toxin promoted by cellular substances (8, 17, 18, 27, 38). Thus, antibodies that neutralize the toxin in the CHO cell assay may or may not inhibit activity in the ADP-ribosyltransferase assay. Similarly, one can devise scenarios in which antibodies that inhibit toxin activity in an ADP-ribosyltransferase assay fail to inhibit activity in the CHO cell assay, particularly if a direct assay for ADP-ribosyltransferase activity is used. Thus, it is not surprising that incomplete agreement between the CHO and ADP-ribosyltransferase assays has been observed (19, 30, 44).

Because the CHO and ADP-ribosyltransferase assays are different, one question is whether one of the two assays better correlates with protection. For example, if some antibodies blocked entry of the toxin into CHO cells but not the virulence target (as might be the case if a virulence target is an immune system cell), then the CHO cell assay might fail to correlate with protection. On the other hand, the assay based on inhibition of catalytic activity could yield false-negative results if antibodies that recognize the B oligomer but that do not inhibit catalytic activity are an important component of the protection arising from vaccination. Immunization of mice with purified B oligomer can elicit a protective response (50). Thus, establishment of the relative importance of antibodies directed against the B oligomer in humans vaccinated with holotoxin preparations would indicate whether assays that measure inhibition of ADP-ribosyltransferase activity are likely to yield false-negative results.

Practical and analytical issues. The assay that measures inhibition of ADP-ribosyltransferase activity may hold some advantages over the currently used CHO cell assay, as described previously (12). First, the ADP-ribosyltransferase assay is based on unbiased quantitative determinations made by a scintillation counter, whereas the CHO cell assay relies on a subjective interpretation of clumping patterns of cultured cells. Second, the reagents for the ADP-ribosyltransferase assay can be stored frozen for indefinite intervals; and then they can be thawed and used immediately, and the results can be obtained before the next day. From frozen stocks, it takes several days to perform the CHO cell assay. Third, the addition of various amounts of serum to the ADP-ribosyltransferase assay yields a broad dose-response curve. In contrast, CHO cell assays yield a titer that is based on the breakpoint of a sharp dose-response curve (12). The broader dose-response curve of the results in the ADP-ribosyltransferase assay may allow for easier kinetic analysis of polyclonal antisera. This type of analysis can reveal qualitative differences between sera that are not revealed by single-point titer measurements (Fig. 5). Finally, the components of the ADP-ribosyltransferase assay are easily purified biochemical reagents. In contrast, the CHO cell assay requires the unique CHO cell line. The ease with which the reagents of the ADP-ribosyltransferase assay can be produced and defined suggests that the assay should allow different laboratories to establish close agreement concerning absolute, and not just relative, titers.

For example, it has been proposed that inhibition of ADP-ribosyltransferase activity by antibodies that bind to S1 subunit arises from recognition of a single, immunodominant region of the polypeptide (3). This type of antibody essentially completely inhibits ADP-ribosyltransferase activity in the direct assay used in the study described here (Fig. 1) (20). If anti-S1 antibodies of this type are largely responsible for the inhibition quantified by the direct assay, then it is possible to calculate the amount of inhibiting antibody in human serum samples. One mole of antibodies completely inhibits the activity of 2 mol of toxin (20). If IgG antibodies are the predominant inhibiting moiety, with a mass roughly 50% greater than that of the toxin (150,000 versus 100,000 daltons), then the inhibition of 10 ng of toxin would arise from 7.5 ng of antibody. If 1 μl of serum was sufficient to inhibit 10 ng of toxin, then the concentration of inhibiting antibodies would be 7.5 μg/ml. Such a titer could arise from less than 0.1% of the IgG in serum, assuming that the concentration of serum IgG is 10 mg/ml (13).

Conclusions. The data reported here and elsewhere (19, 30) demonstrate that it is practical to quantitate inhibition of ADP-ribosyltransferase activity by small samples of serum from humans or mice. Although there are considerable technical differences that distinguish the methods used in the study described here and elsewhere (30), both this and previous studies of sera (19, 30) demonstrate that results from ADP-ribosyltransferase assays do not duplicate the results obtained by the CHO cell assay. In addition, monoclonal antibodies that also neutralize pertussis toxin in one but not both of these assays have been identified (43, 44). Thus, an important question arises. Does protection correlate with a positive result in both of these assays, perhaps coupled with detection of antibodies that recognize other antigens or that block other actions of pertussis toxin? Analysis of serum from vaccine failures in human clinical trials should help answer this question. Finally, it is important to recognize that the number of vaccinees that we studied was small, and the serum samples may not accurately represent the subject groups from which they were drawn. Thus, a larger study will be required in order to establish whether the different vaccine formulations lead to different responses in terms of the antibodies that inhibit the ADP-ribosyltransferase activity of pertussis toxin.

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