



Use of a Toxin Neutralization Assay To Characterize the Serologic Response to Adenylate Cyclase Toxin after Infection with *Bordetella pertussis*

Joshua C. Eby,^a Mary C. Gray,^a Jason M. Warfel,^{b*} Tod J. Merkel,^b Erik L. Hewlett^a

Division of Infectious Diseases, University of Virginia, Charlottesville, Virginia, USA^a; Division of Bacterial, Parasitic and Allergenic Products, Center for Biologics Evaluation and Research, FDA, Silver Spring, Maryland, USA^b

ABSTRACT Adenylate cyclase toxin (ACT) is an essential virulence factor of *Bordetella pertussis*, and antibodies to ACT protect against *B. pertussis* infection in mice. The toxin is therefore a strong candidate antigen for addition to future acellular pertussis vaccines. In order to characterize the functionality of the immunologic response to ACT after infection, we developed an assay for testing the ability of serum samples from subjects infected with *B. pertussis* to neutralize ACT-induced cytotoxicity in J774 macrophage cells. Baboons develop neutralizing anti-ACT antibodies following infection with *B. pertussis*, and all sera from baboons with positive anti-ACT IgG enzyme-linked immunosorbent assay (ELISA) results neutralized ACT cytotoxicity. The toxin neutralization assay (TNA) was positive in some baboon sera in which ELISA remained negative. Of serum samples obtained from humans diagnosed with pertussis by PCR, anti-ACT IgG ELISA was positive in 72%, and TNA was positive in 83%. All samples positive for anti-ACT IgG ELISA were positive by TNA, and none of the samples from humans without pertussis neutralized toxin activity. These findings indicate that antibodies to ACT generated following infection with *B. pertussis* consistently neutralize toxin-induced cytotoxicity and that TNA can be used to improve understanding of the immunologic response to ACT after infection or vaccination.

KEYWORDS *Bordetella pertussis*, nonhuman primate, adenylate cyclase, assay development, bacterial toxin, neutralization

In 1976, only 1,010 cases of pertussis were reported to the CDC (1). Since that time, the number of cases has increased, reaching a peak of 48,277 reported cases in 2012 in the United States, despite a high rate of vaccination (2). The reemergence of pertussis has been attributed to a combination of improved detection, vaccine refusal, and failure of the current acellular pertussis vaccine (aP) (3). Formulations of aP licensed in the United States contain between three and five *Bordetella pertussis* antigens (pertussis toxin, filamentous hemagglutinin, pertactin, fimbriae 2, and fimbriae 3), and the addition of antigens to aP has been suggested as a possible remedy to the limited vaccine efficacy and duration of protection (4).

Adenylate cyclase toxin (ACT) is a strong candidate for addition to future acellular vaccines (5) because it is a protective antigen and essential virulence factor (6). ACT is critical for the infection of adult mice and for causing death in infant mice infected with *B. pertussis* (7, 8). Passive transfer of serum from mice infected with *B. pertussis* to naive mice protects from infection with *B. pertussis* (9). In addition, the duration of *B. pertussis* infection has been found to be shorter in mice vaccinated with aP containing ACT than in those vaccinated with aP alone (10–12). While infection of humans by *B. pertussis* results in the generation of anti-ACT antibodies (13, 14), the ability of these antibodies

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Address correspondence to Joshua C. Eby, jce4u@virginia.edu.

* Present address: Jason M. Warfel, Bristol-Myers Squibb, Syracuse, New York, USA.

to neutralize ACT function is incompletely characterized (15, 16), and the role of the antibodies in protection is not known.

ACT is a single polypeptide consisting of a catalytic domain that converts ATP to cyclic AMP (cAMP) and a cell-binding domain that associates with CD11b/CD18, the β_2 -integrin present on phagocytic leukocytes. After binding, ACT translocates its catalytic domain directly across the plasma membrane into the cytoplasm and generates supraphysiologic levels of intracellular cAMP, resulting in cell dysfunction and, under some conditions, death (17–20). For example, 30 ng/ml ACT kills 30% of J774 cells, a macrophage-like cell line, in 2 h.

We have taken advantage of the potent and rapid cytotoxic effects of ACT, developing an assay for measuring serum neutralization of cytotoxicity. With this easy-to-perform, sensitive, and specific assay, we have found that the anti-ACT antibody response to infection is neutralizing. The assay will be a valuable method for evaluating the immunologic response to *B. pertussis* infection and vaccines in future studies.

RESULTS

The nonhuman primate model of pertussis allows for study at specific intervals postimmunization and frequent sampling of serum after challenge to characterize the host antibody response. Following primary infection of unvaccinated baboons with *B. pertussis*, serum anti-ACT IgG, measured by enzyme-linked immunosorbent assay (ELISA), was detected in each subject by day 24 to 33 (Fig. 1A). While previously infected baboons were negative for anti-ACT IgG 30 to 60 days after their primary infection, IgG response to ACT was present on days 13 to 21 following secondary infection. Of the samples from previously infected baboons, those from subject b showed a weak positive response (3.77 ± 0.13 SD U/ml on day 21) following secondary infection. Interestingly, no anti-ACT IgG response was detected in serum from baboons that had been vaccinated with a whole-cell pertussis vaccine (wP) or aP and then infected with *B. pertussis*.

To further characterize the anti-ACT response, we measured neutralization by serum of ACT-induced cytotoxicity toward J774 cells. As described in Materials and Methods, recombinant purified ACT was combined with serum (at 1:50 final dilution) and added to J774 cells. Cell viability was measured after 2 h, and the results were expressed as percent toxin neutralization.

ACT-neutralizing activity was detected at days 17 to 19 following primary infection of unvaccinated baboons, at least 7 days earlier than anti-ACT IgG was detected using ELISA (Fig. 1). The toxin neutralization assay (TNA) was positive on day 0 in two of the three previously infected baboons, and the magnitude of inhibition increased after secondary challenge, whereas the ELISA was negative for these samples on day 0. TNA remained negative throughout the testing period on serum of baboons vaccinated with wP or aP prior to challenge with *B. pertussis*.

In summary, all of the baboon sera with anti-ACT IgG response possessed neutralizing activity. The TNA was positive earlier in the time course of infection than was ELISA for both unvaccinated primarily infected baboons and previously infected baboons infected a second time. In animals vaccinated with wP and aP, there was no evidence of an anti-ACT antibody response, by ELISA or TNA, during the 28 to 35 days following challenge.

We used the same assays to study sera from humans recently infected with *B. pertussis*. All human subjects tested positive for pertussis by PCR and reported symptoms consistent with pertussis. Most subjects were identified during a multicounty outbreak of pertussis in central Virginia in 2015, and all case subjects whom we tested had been vaccinated (Table 1). Of the 18 serum samples tested within 4 to 9 weeks after reported symptom onset, 13 samples were positive by anti-ACT IgG ELISA. When serum samples were tested by TNA, 14/18 samples were positive. Sample V014 was negative by anti-ACT IgG ELISA but was positive by TNA. All serum samples that were positive by anti-ACT IgG ELISA possessed neutralizing activity against ACT.

In addition to using anti-ACT ELISA and TNA, we tested all sera for anti-pertussis

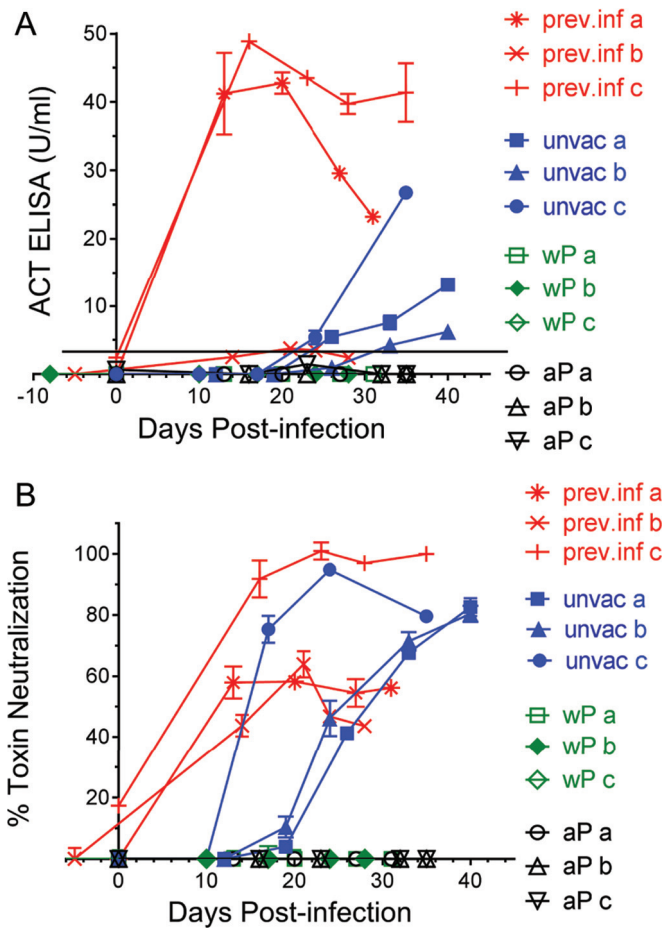


FIG 1 Use of ELISA and toxin neutralization assay to evaluate serial serum samples from baboons inoculated with *B. pertussis*. Sera from baboons previously infected (prev. inf) and given a secondary challenge with pertussis 1 to 2 months after clearing primary infection, unvaccinated (unvac) and given a primary challenge, wP vaccinated prior to primary challenge, and aP vaccinated prior to primary challenge were tested for anti-ACT response following inoculation, as described in Materials and Methods. Serum samples from 3 different subjects (a, b, and c) were tested within each group. (A) Shown are serum anti-ACT IgG ELISA titers calculated as described in Materials and Methods. Values of ≥ 3.32 U/ml (represented by a black horizontal line) are considered positive. (B) Shown are results from the TNA using a 1:50 final dilution of serum. Data represent the mean and SD of the results from each sample tested in duplicate (ELISA) or triplicate (TNA).

toxin (PT) IgG by ELISA, since this is the only serologic test currently used as a single-sample assay for the diagnosis of pertussis (21, 22). Serum samples V018, V020, and V021 all showed negative TNA and anti-ACT ELISA results while having positive PCR results and positive anti-PT IgG ELISA results, consistent with a diagnosis of pertussis without anti-ACT antibody response, 4 to 7 weeks after symptom onset. Sample V006 was negative by anti-ACT ELISA, TNA, and anti-PT ELISA, findings that likely represent a false-positive PCR result. V010 showed positive anti-ACT ELISA and TNA results but was negative by anti-PT ELISA, consistent with pertussis with negative PT antibody response at 5 weeks. Overall, anti-PT ELISA was positive in 16/18 subjects.

Because V018, V020, and V021 had positive anti-PT ELISA results but were negative by both ELISA and TNA for ACT, we tested these 3 samples at higher concentrations (dilutions of $< 1:50$) to determine if there were lower levels of antibodies that could neutralize the cytotoxicity of ACT. As controls, pertussis-positive sera (V008, Fig. 2) and pertussis-negative sera (N030 and N038 are shown) were also tested at higher concentrations. When a 1:20 dilution of serum was used, instead of 1:50, serum sample V018 neutralized the cytotoxicity of ACT, V020 weakly neutralized ACT, and V021 exhibited no neutralizing activity (Fig. 2). The pertussis-negative control sera had no neutralizing

TABLE 1 Serum from PCR-positive cases 4 to 9 weeks after symptom onset^a

Sample	Sex/age (yr) ^b	C _T value	Primary vaccine series	aP boost	Date (mo/day/yr) of:			ACT ELISA (U/ml)	TNA (%) ^d	PT ELISA (U/ml)
					Last vaccination	Approx symptom onset ^c	Time from symptom onset to sample (wk)			
V002	F/14	26.3	5 aP	+	03/14/11	08/25/14	8	47.3	100	300
V003	M/17	HC ^e	5 aP	+	08/14/08	08/11/14	4	44.8	100	640
V006	F/18	28.1	5 ap	+	07/08/08	05/15/15	6	4.8	0	24.1
V007	M/16	14.0	5 aP	+	07/20/10	05/28/15	4	63.9	100	464
V008	F/17	27.8	5 aP	+	08/08/11	05/28/15	5	30.5	85.4	184
V009	M/12	20.6	5 aP		08/06/08	05/05/15	8	93.7	100	215
V010	M/13	23.2	5 aP	+	10/14/12	06/01/15	5	156	100	73.8
V011	M/22	34.5	3 wP, 2 aP	+	04/27/07	07/26/15	4	30.6	95.5	413
V012	M/15	29.0	5 aP	+	08/30/10	05/07/15	9	45.5	85.4	803
V013	F/18	19.5	5 ap	+	07/10/08	06/17/15	9	23.6	69.8	329
V014	M/13	32.9	5 aP	+	08/23/13	07/01/15	7	9.8	19.3	94.1
V015	F/15	32.7	5 aP	+	08/07/12	09/21/15	5	21.7	60.4	276
V016	F/14	36.2	5 aP	+	01/18/12	09/20/15	6	54.3	97.4	170
V017	F/15	17.5	5 aP	+	01/14/11	10/23/15	6	44.4	98.0	290
V018	M/15	31.9	5 aP	+	06/28/11	10/22/15	7	0.71	0	1,404
V019	F/17	29.8	5 aP	+	08/18/09	11/01/15	5	30.0	81.1	563
V020	M/17	27.5	5 aP	+	04/23/10	11/14/15	4	3.28	0	293
V021	M/14	32.1	5 aP	+	02/13/13	10/12/15	7	0.75	0	1,040

^aBolded values are considered positive (see Table 2 for data from normal controls). Data represent the mean of the results from each sample tested in duplicate (ELISA) or triplicate (TNA).

^bF, female; M, male.

^cAt onset of clinical illness, all subjects reported symptoms consistent with pertussis.

^dPercent toxin neutralization using a 1:50 final serum dilution, as described in Materials and Methods.

^eHC, sample was from a household contact of subject V002 with clinical symptoms of pertussis.

activity, even at these higher serum concentrations (lower dilutions). Thus, dilutions of serum lower than 1:50 (i.e., higher concentrations) can be used to detect low-level ACT-neutralizing antibodies in serum samples without compromising the performance of the neutralization assay.

Serum samples from 22 control subjects with no symptoms or reported epidemiologic linkage to pertussis were tested by TNA, and none possessed neutralizing activity

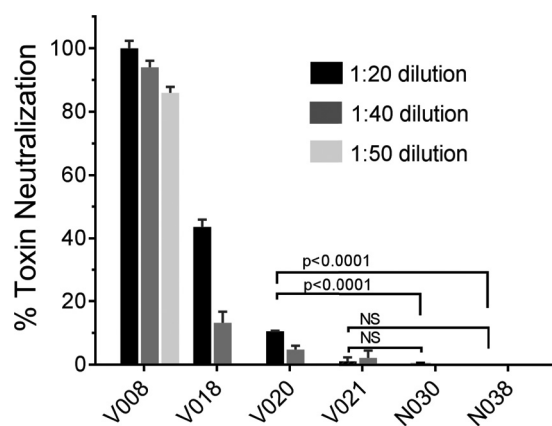


FIG 2 Use of more-concentrated serum in the TNA results in positive tests from convalescent-phase samples, while control samples remain negative. Serum from samples V018, V020, and V021 was tested for neutralization of ACT activity using a higher concentration of serum. Serum samples from a pertussis-positive subject (V008 and others not shown) and pertussis-negative control subjects (N030, N038, and others not shown) were also tested. The 1:20 dilution of serum sample V020 is significantly different from 1:20 dilutions of serum from pertussis-negative control samples N030 and N038. The 1:20 dilution of sample V021 is not significantly different (NS) from 1:20 dilutions of N030 or N038. Shown are *P* values from multiple comparison testing using ANOVA with Tukey's *post hoc* test to determine statistical significance ($P \leq 0.05$). Data represent the mean and SD of the results from each sample tested in triplicate.

TABLE 2 Serum samples from control subjects^a

Sample	Sex/age (yr) ^b	Primary vaccine series	aP boost	Date (mo/day/yr) of:		ACT ELISA (U/ml) ^c	TNA (%) ^d	PT ELISA (U/ml) ^e
				Last vaccination	Serum sample			
N030	M/17	5 aP	+	06/02/10	12/22/15	3.7	0	4.1
N032	M/16	5 aP	+	09/27/10	12/22/15	3.3	0	21.1
N033	M/13	5 aP	+	07/29/13	12/22/15	4.1	0	37.3
N036	F/17	5 aP	+	08/04/09	12/22/15	9.0	0	6.4
N037	F/17	5 DTP	+	08/26/10	12/22/15	1.6	0	6.0
N038	F/20	5 DTP		05/22/99	12/22/15	2.3	0	3.0
N039	F/16	5 aP	+	05/25/10	01/07/16	2.5	0	5.1
N040	F/25	5wP	+	04/19/94	07/23/15	14.1	0	7.1
N041	F/24	5wP	+	08/20/13	07/23/15	12.5	0	19.0
N042	F/25	5wP	+	02/20/08	07/23/15	58.8 , 10.3 ^f	0	5.7
N043	F/25	5wP	+	01/10/15	03/30/15	8.0	0	75.3
N044	F/25	5wP	+	04/20/10	07/23/15	7.7	0	23.3
N045	F/28	5wP	+	04/02/14	07/23/15	9.7	0	20.1
N046	F/39	5wP			01/27/85	16.9	0	7.9
N047	F/57	5wP	+	01/2012	02/02/12	3.0	0	37.0
N048	F/26	5wP	+	08/20/13	01/28/14	15.3	0	21.9
N049	F/27	5wP	+	01/22/10	01/29/15	10.2	0	59.9
N050	F/28	5wP	+	02/15/15	03/30/15	12.8	0	106
N051	F/24	5wP	+	04/24/11	02/26/14	7.4	0	66.0
N052	F/25	5wP	+	01/10/15	01/29/15	9.8	0	72.9
N053	M/39	5wP			01/27/85	12.9	0	11.0
N054	M/40	5wP	+	07/24/06	01/10/12	17.6	0	3.6

^aBolded values are considered positive (see titers below). Data represent the mean of the results from each sample tested in duplicate (ELISA) or triplicate (TNA).

^bM, male; F, female.

^cACT titer mean ± SD, 8.85 ± 4.95 U/ml; normal, ≤18.8 U/ml (2 SD).

^dPercent toxin neutralization using a 1:50 final serum dilution, as described in Materials and Methods.

^ePT titer mean ± SD, 28.17 ± 29.4 IU/ml; normal, ≤87.0 IU/ml (2 SD).

^fTiter obtained using catalytic domain as antigen.

(Table 2). One of 22 control samples was positive by anti-ACT ELISA, and 1/22 samples was positive by anti-PT ELISA (N042 and N050, respectively, Table 2).

ACT is a member of the repeats-in-toxin (RTX) family of bacterial toxins, which includes HlyA of *Escherichia coli* and other bacterial proteins. Serum testing by anti-ACT IgG ELISA using the ACT holotoxin molecule as an antigen can detect antibodies to other RTX toxins (23, 24), because the C-terminal 1,700-amino-acid hemolysin component of ACT is homologous to other RTX toxins (25, 26). The ELISA used for our studies is based on the detection of ACT holotoxin, and control sample N042 was positive by ELISA. It was, however, negative when retested using the N-terminal catalytic domain of ACT as the ELISA antigen (Table 2), suggesting that the positive result was attributable to reactivity to the RTX domain of ACT.

Serial serum samples were obtained from several subjects with pertussis (Table 3). The TNA showed a positive result as early as 1 week after reported symptoms for V015, when anti-ACT ELISA was negative. Serum sample V002 was positive at 16 months by anti-ACT ELISA and TNA. At 17 months, TNA was weakly positive in serum from subject V003.

All results of the anti-ACT ELISA, TNA, and anti-PT ELISA are summarized in Fig. 3, which illustrates two important points: first, that serum samples with positive anti-ACT ELISA results uniformly exhibit neutralizing activity, and second, that there is neutralizing activity in some sera that are negative by anti-ACT ELISA. The sample with a positive TNA and negative anti-PT ELISA was acquired 16 months following symptom onset. In the 4- to 9-week period, the sensitivity of TNA for *B. pertussis* infection was 83.3%, specificity was 100%, and the positive predictive value (PPV) was 100%. The sensitivity of anti-ACT ELISA in the 4- to 9-week period was 72.2%, specificity was 95.5%, and the PPV was 92.9%. The sensitivity of anti-PT ELISA in the 4- to 9-week period was 88.9%, specificity was 95.5%, and the PPV was 94.1%.

TABLE 3 Serial testing of serum from PCR-positive cases^a

Sample	Sex/age (yr) ^b	C _T value	Primary vaccine series	aP boost	Date (mo/day/yr) of:		Time from symptom onset to sample	ACT ELISA (U/ml)	TNA (%) ^d	PT ELISA (U/ml)
					Last vaccination	Approx symptom onset ^c				
V002A	F/14	26.3	5 aP	+	03/14/11	08/25/14	2 wk	31.5	84.5	357
V002B	F/14						8 wk	47.3	100	300
V002C	F/15						16 mo	30.4	97.1	60.2
V003A	M/17	HC ^e	5 aP	+	08/14/08	09/15/14	4 wk	44.8	100	640
V003B	M/17						10 wk	24.8	92.7	512
V003C	M/18						17 mo	3.59	1.3	110
V011A	M/22	34.5	3 wP, 2 aP	+	04/27/07	07/31/15	5 days	4.2	0	7.4
V011B	M/22						4 wk	30.6	95.5	413
V011C	M/22						4.5 mo	22.3	92.1	182
V015A	F/15	32.7	5 aP	+	08/07/12	09/28/15	1 wk	13.3	61.3	86.7
V015B	F/15						5 wk	21.7	60.4	276

^aBolded values are considered positive (data for normal controls are given in Table 2). Data represent the mean of the results from each sample tested in duplicate (ELISA) or triplicate (TNA).

^bF, female; M, male.

^cAt onset of clinical illness, all subjects reported symptoms consistent with pertussis.

^dPercent toxin neutralization using a 1:50 final serum dilution, as described in Materials and Methods.

^eHC, sample was from a household contact of subject V002 with clinical symptoms of pertussis.

DISCUSSION

In this study, all serum samples from humans and baboons with anti-ACT IgG response after infection with *B. pertussis* neutralized ACT activity. ACT inhibits multiple functions of phagocytic leukocytes *in vitro*: neutrophil extracellular trap (NET) formation, the oxidative burst, and phagocytosis (15, 27). However, it is unknown whether blocking these functions *in vivo* promotes clearance of the organism. Because ACT inhibits opsonin-mediated phagocytosis by leukocytes, neutralizing this activity would be expected to promote antibody-mediated bacterial clearance (28). Vaccination of mice with the acellular pertussis vaccine (aP) that includes ACT results in improved clearance of *B. pertussis* in comparison to vaccination with aP alone (11); this finding may be due to the generation of anti-ACT neutralizing antibodies or other effects of ACT. The implications of generating neutralizing antibodies must be taken in the context of the entire immune response. For example, blocking inhibition of the oxidative burst may result in more reactive oxygen species and NET formation, eliciting greater local inflammation in response to *B. pertussis*. Further testing of these hypotheses in animal models is required in order to understand the role of neutralizing antibodies in clearance of or protection from pertussis.

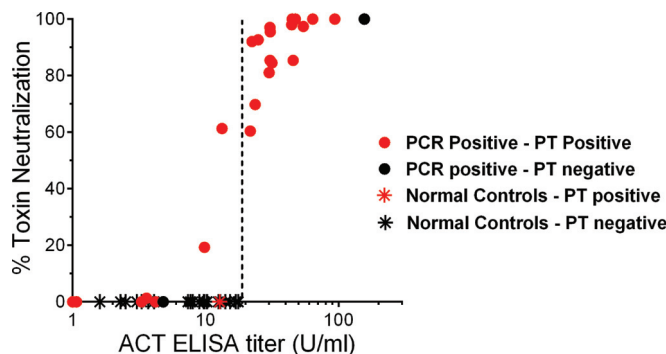


FIG 3 Summary of anti-ACT IgG ELISA, toxin neutralization assay, and anti-PT IgG ELISA results. Shown are the results of the anti-ACT IgG ELISA (x axis) and TNA (y axis) for all human serum samples. Red symbols indicate anti-PT ELISA-positive samples, and black symbols represent anti-PT IgG ELISA-negative samples. Closed circles represent serum from pertussis-diagnosed subjects (PCR positive with symptoms), and stars represent serum samples from control subjects. The dashed line indicates the cutoff for a positive anti-ACT IgG ELISA value. Data represent the mean of the results from each sample tested in duplicate (ELISA) or triplicate (TNA).

Mobberley-Schuman et al. studied serum from patients with pertussis and found that only 1/24 serum samples neutralized ACT function. This low percentage of ACT-neutralizing antisera (compared with our findings) may be attributable to the assay that they used, which measured serum neutralization of the effect of ACT on phagocytosis of live *B. pertussis* (15). In their experiments, antibodies to ACT may have been adsorbed by ACT on the surface of *B. pertussis*, whereas active ACT is released (29). In addition, because active secretion and release of ACT from bacteria are required to develop concentrations high enough to inhibit neutrophil function (30), a functionally low concentration of ACT was likely present in the media at the time that bacteria were combined with neutrophils. Furthermore, multiple *B. pertussis* virulence factors influence phagocytosis (31). Farfel et al. reported that sera from patients with pertussis did not neutralize ACT-induced intracellular cAMP generation (16); however, crude extracts of ACT were used rather than purified ACT, and the primary data were not shown in the paper. Based on the findings of our study, we suggest that neutralizing antibodies would have been identified in most serum samples tested by Mobberley-Schuman et al. (15) and Farfel et al. (16) if the TNA had been used for testing serum.

In comparison to the serum anti-ACT ELISA responses measured by Cherry et al. in unvaccinated study subjects with pertussis (14), serum anti-ACT ELISA responses of subjects with pertussis in the present study are low in magnitude at 4 to 9 weeks after symptom onset. Consistent with the observations in our study, Cherry et al. found that subjects that had been vaccinated with wP or aP prior to infection with *B. pertussis* responded to infection with an increase in anti-ACT IgG ELISA results that was small in comparison to that of unvaccinated subjects (14). The diminished anti-ACT responses in vaccinated baboons compared to unvaccinated baboons is reminiscent of these findings, suggesting that vaccination with aP or wP prior to infection results in suppression of the anti-ACT response to pertussis. Cherry et al. (14) hypothesized that the immunologic phenomenon of original antigenic sin (OAS) accounted for the reduced antibody response to infection in previously vaccinated hosts. Based on this hypothesis, the response to a secondary exposure to an infecting agent is dominated by memory cells generated by the primary exposure (32, 33). It is unclear how OAS would explain the suppressed anti-ACT response to infection in subjects that received wP, since wP contains ACT. Nonetheless, the observation that the anti-ACT response following infection is reduced by prior vaccination is intriguing and deserves additional investigation.

While the anti-ACT response following infection is reduced in previously vaccinated humans relative to unvaccinated humans, the response is absent in previously vaccinated baboons, and this difference is one of several between human pertussis and the baboon model of pertussis. Interestingly, the anti-ACT IgG ELISA is negative on day 0 in previously infected baboons, 30 to 60 days following their primary infection (Fig. 1). This finding is remarkable because Warfel et al. found that antibody responses to other *B. pertussis* antigens are detectable in previously infected baboons on day 0 (34), and serial serum samples from humans show anti-ACT IgG ELISA positivity beyond 9 weeks in several cases (Table 3). In addition to serologic differences between baboons and humans, vaccinated and previously infected baboons that are secondarily infected remain asymptomatic (34), whereas human samples in this study were obtained from symptomatic subjects. The human subjects in this study were adolescents and adults, whereas baboons were weanlings. The humans acquired the infection by natural transmission, whereas the baboons were inoculated by intratracheal and intranasal challenge with a large single dose (10^9 to 10^{10} CFU) of bacteria. Finally, the *B. pertussis* strain used to infect baboons is a clinical isolate now used for laboratory experiments (35). Altogether, the baboon model of pertussis is invaluable for a controlled study of *B. pertussis* infection and vaccination, but one should be aware of the differences between the baboon model of pertussis and human pertussis when interpreting findings.

As demonstrated by this study of human and baboon sera, the same TNA can be used to study sera from different genera. Unlike ELISA, TNA does not require a

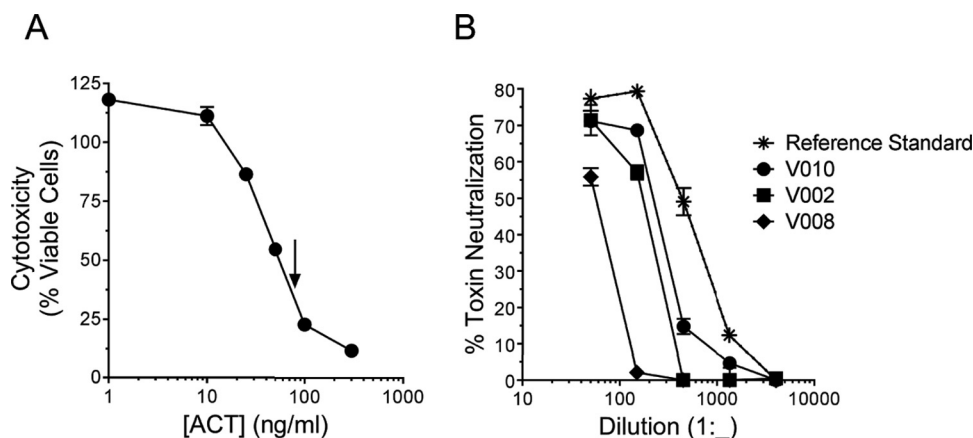


FIG 4 Neutralization of ACT cytotoxicity by serial dilutions of convalescent human serum. (A) The concentration of ACT to be used for the toxin neutralization assay was chosen based on cytotoxicity of ACT toward J774 cells. ACT was added to 30,000 J774 cells grown overnight on a 96-well plate. After 2 h, cytotoxicity (percent viable cells) was measured by a CCK-8 assay. The arrow indicates the concentration of ACT (80 ng/ml) chosen for use in the toxin neutralization assay. Data represent the mean and SD of the results from 3 experiments performed in triplicate. (B) ACT was mixed with serum at the indicated dilution and added to J774 cells as described in Materials and Methods. The final dilution was 1:x, where x is the value on the x axis. Percent toxin neutralization was calculated as described in Materials and Methods. The reference standard for human convalescent anti-pertussis serum was provided by NIBSC (89/530). Data represent the mean and SD of the results from 2 experiments performed in triplicate.

secondary antibody, so there is no species-specific adjustment of the assay required. The lack of secondary antibody and the design of the TNA eliminate the need for blocking steps, washing steps, and serial dilutions. There is no need for serial dilutions because of the dynamic concentration dependence of ACT-induced cytotoxicity and the concentration of purified ACT used (80 ng/ml), such that any inhibition of ACT results in a substantial reduction in cytotoxicity (Fig. 4). In this study, despite the low anti-ACT IgG ELISA values of infected subjects, the TNA was positive when anti-ACT ELISA was positive and showed a positive result when ELISA was negative in two human cases. The TNA also exhibited positive results at earlier time points after challenge than ELISA in several baboon serum samples. The assay is thus very sensitive.

Neutralization requires that an antibody not only bind to ACT but also block its function. Not all anti-ACT monoclonal antibodies block ACT function, as in the case of N042 (Table 2) (36). The positive anti-ACT ELISA result for N042 reflected binding to the RTX component of ACT, the immunodominant component of the toxin in mice (37), which is common to other bacterial toxins. Thus, the finding of neutralization of ACT is more specific than just binding, as is detected by ELISA. In addition, a high concentration (low dilution) of antiserum can be used in the TNA without affecting the test performance, whereas high concentrations of serum in the ELISA can result in a high background signal, requiring greater dilution before use in the assay. While the serum dilution used for most TNA tests was 1:50, the results shown in Fig. 2 indicate that the assay results may be more sensitive without compromising specificity by using a dilution of 1:20. In sum, TNA is a sensitive, specific, and easily performed assay that measures functional activity of the anti-ACT response.

With this novel assay, we have found that *B. pertussis* infection results in the generation of neutralizing antibodies to ACT in vaccinated humans. This serum neutralization assay will be useful for evaluating the anti-ACT response to infection and vaccines in future studies.

MATERIALS AND METHODS

Bacterial growth and tissue culture. *B. pertussis* strain D420 was grown as described previously (38). J774A.1 (J774) murine macrophage cells were cultured in Dulbecco's modified Eagle's medium with 25 mM glucose (Gibco) plus 10% heat-inactivated fetal bovine serum (FBS-HI; Gibco) at 37°C in 5% CO₂.

To produce recombinant ACT, *Escherichia coli* XL1-Blue cells (Stratagene, La Jolla, CA) containing pT7CACT1 plasmid with wild-type *cyaA* and *cyaC* were grown as described previously (36, 39). Recombinant ACT was extracted in 8 M urea, purified, and stored as described previously (40).

Vaccination, infection, and evaluation of baboons. All animal procedures were performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International in accordance with protocols approved by the CBER Animal Care and Use Committee and principles outlined in the *Guide for the Care and Use of Laboratory Animals* by the Institute for Laboratory Animal Resources, National Research Council (46). Baboons obtained from the Oklahoma Baboon Research Resource at the University of Oklahoma Health Sciences Center were inoculated with human doses of a diphtheria-tetanus-acellular pertussis vaccine (DTaP) or a diphtheria-tetanus-whole-cell pertussis vaccine (DTwP) administered intramuscularly (i.m.) at 2, 4, and 6 months of age, as described previously (34). For DTaP, three animals were vaccinated with the U.S.-licensed vaccine Daptacel (Sanofi Pasteur Ltd., Toronto, Canada), which contains diphtheria toxoid, tetanus toxoid, and five pertussis antigens (detoxified pertussis toxin, filamentous hemagglutinin, pertactin, fimbriae 2, and fimbriae 3). For DTwP, three animals were vaccinated with triple antigen (Serum Institute of India Ltd., Pune, India), which meets WHO recommendations for potency, as documented previously (41). Unvaccinated animals were matched for age. Baboons that had previously been infected with *B. pertussis* were tested by twice-weekly cultures of nasopharyngeal aspirates and found to be clear of infection for 1 to 2 months prior to subsequent infection (34). Direct challenge studies were performed as described previously (38, 42). The inoculum for each direct challenge was between 10^9 and 10^{10} CFU, as determined by measurement of optical density and confirmed by serial dilution and plating to determine the number of CFU per milliliter of inoculum. Peripheral blood was collected for serum separation, as previously described (38).

Evaluation of human subjects. All human subject research was performed according to a protocol reviewed and approved by the Institutional Review Board for Health Sciences Research at the University of Virginia (UVA). Subjects were identified on the basis of a positive *B. pertussis* diagnostic PCR result from the UVA Health System clinical microbiology laboratory. The PCR test was developed at UVA using commercially available reagents in compliance with laboratory standards and detects the IS481 element of *bordetellae* (43, 44). Primers, TaqMan fluorescent probes, and master mix were manufactured by BioGX, and analysis was performed on the BD Max instrument. Determination of positive PCR tests was based on the following criteria. A sample was considered positive for pertussis if the pertussis-specific total fluorescence was ≥ 220 fluorescence units and there was amplification within 40 cycles. A sample processing control (SPC), which does not react with IS481, was incorporated into each specimen and carried through each step of the procedure. The SPC was considered positive if total fluorescence was ≥ 220 fluorescence units and the threshold cycle (C_T) was ≤ 30.5 . A sample was considered negative if the SPC was positive and there was no amplification of pertussis-specific DNA within 40 cycles.

Once identified as positive by PCR, subjects were contacted by phone, as per study protocol, and informed consent was obtained from the subject or his/her parent or guardian. Clinical history was collected, and subjects were asked if they would donate a sample of blood for these studies. All subjects with positive PCR tests (Table 1) reported symptoms consistent with pertussis. Thus, they fulfilled the CDC criteria for a positive pertussis case. Control serum samples were obtained from subjects who reported no known exposure to pertussis, no recent respiratory symptoms, and no history of pertussis (Table 2). Immunization records of PCR-positive and control subjects were obtained using the Virginia Immunization Information System, which provides a record of vaccines administered in the state of Virginia (Tables 1 and 2).

Enzyme-linked immunosorbent assay. ELISA was used to quantify titers of anti-ACT IgG and anti-PT IgG antibodies in serum samples. For anti-ACT IgG and anti-PT IgG assays, 96-well flat-bottom ImmunoPlate MaxiSorp plates (Thermo Scientific) were coated with 100 μ l of ACT at 1 μ g/ml or 100 μ l PT at 1 μ g/ml in 50 mM carbonate-bicarbonate coating buffer (pH 9.6) overnight at room temperature (RT). Plates were washed 3 times with phosphate-buffered saline (PBS) (pH 7.4) plus 0.05% Tween 20 (PBST) and blocked with 100 μ l of Blocker Blotto in Tris-buffered saline (TBS; Thermo Scientific) for 1 h at room temperature. Wells were washed, and 3-fold dilutions (starting at 1:100) of reference serum standard (National Institute for Biological Standards and Control [NIBSC] code 89/530 for ACT or 06/140 for PT) and serum samples to be tested were made in PBST plus 10% Blocker Blotto (PBSTB), added to the ELISA plate, and incubated for 1 h, with mixing at room temperature (RT). Negative-control wells were treated similarly but did not contain subject or reference serum. Plates were washed, 100 μ l of 1:10,000 goat anti-monkey IgG(H+L)-horseradish peroxidase (HRP) (AbD Serotec, for baboon studies) or goat anti-human IgG(H+L) F(ab')₂-HRP (Novex, Life Technologies for human studies) was added, and plates were incubated for 1 h, with mixing at RT. After washing, 100 μ l of SureBlue 3,3',5,5'-tetramethylbenzidine (TMB) microwell peroxidase substrate was added and plates were incubated in the dark, with mixing at RT. The reaction was stopped by the addition of 100 μ l of 1 N HCl, and absorbance at 450 nm was read in a μ Quant plate reader (Biotech Instruments). The ACT ELISA results were calculated for each serum sample against reference serum 89/530, which has been assigned an anti-ACT IgG titer of 100 U/ml, using linear regression with ≥ 3 dilutions in the linear range (14). The PT ELISA results were calculated using reference standard 06/140, which has an anti-PT IgG content of 335 IU/ml (45).

The cutoff values for a positive result were calculated as described previously (14). For serum samples from humans, positive was defined as the mean \pm 2 SD of ELISA titers of pertussis-negative control subject samples (positive cutoff for anti-ACT IgG ELISA, ≥ 18.8 U/ml; positive cutoff for anti-PT IgG ELISA, ≥ 87.0 IU/ml). For baboon serum samples taken at serial time points (Fig. 1A), a positive value for anti-ACT IgG was defined as the mean \pm 2 SD of ELISA titers of initial samples (day 0) from naive, aP-treated, and wP-treated baboons (positive cutoff, ≥ 3.32 U/ml).

Toxin neutralization assay. The toxin neutralization assay (TNA) is based on the cytotoxicity of ACT toward J774 macrophage-like cells. The J774 cells (30,000 cells in 90 μ l) were seeded in each well of a 96-well plate and allowed to attach overnight at 37°C with 5% CO₂. ACT was incubated with serum

samples for 10 min, and the samples were mixed at 4°C and added to the cells at a final ACT concentration of 80 ng/ml. Cells were incubated at 37°C for 2 h, and the number of viable cells was determined using the CCK-8 assay (Dojindo Laboratories), which measures the reduction of WST-8, a water-soluble tetrazolium salt, by dehydrogenases in live cells. The percentage of viable cells was determined for ACT alone and for ACT with serum compared to control cells by the following equation: $[(\text{experimental} - \text{blank})/(\text{control cells} - \text{blank})] \times 100$. The results are reported as percent toxin neutralization, determined as follows: $[(\% \text{ viable cells treated with ACT and serum}) - (\% \text{ viable cells treated with ACT alone})]/[(\text{control cells (set as 100\%)} - (\% \text{ viable cells treated with ACT alone})] \times 100$. A blank is a well on the 96-well plate containing medium and CCK-8 reagent but no J774 cells. Control cells are J774 cells in the 96-well plate that are not treated with ACT or serum.

The concentration of ACT used in the TNA was chosen based on the highly reproducible concentration-response curve shown in Fig. 4A; with a concentration of ACT of 80 ng/ml, subtle inhibition of toxin activity results in a substantial change in cytotoxicity detected by the CCK-8 assay.

The final dilution of serum used for the TNA was 1:50, unless otherwise specified, and this dilution was chosen based upon characteristics of the assay. Testing of human serum samples and the pooled human reference standard (NIBSC 89/530, which exhibited positive anti-ACT ELISA results ranging from 30.5 to 156 U/ml) showed that inhibition of toxin activity by serum is concentration dependent (Fig. 4B). Due to a high signal-to-noise ratio and no increase in nonspecific signal in wells containing concentrated serum samples from control subjects, a concentrated serum specimen could be examined by TNA, and we selected a dilution of 1:50. In ELISA, serum more concentrated (lower dilution) than 1:100 resulted in signal in the control subject wells. Because none of the 22 serum samples from control subjects neutralized ACT, a cutoff value for positivity of >0% toxin neutralization was used.

Statistics. Multiple comparisons were made using analysis of variance (ANOVA) with Tukey's *post hoc* test. A *P* value of ≤ 0.05 was considered significant. Statistical analysis was performed by using GraphPad Prism software and Microsoft Excel.

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