Antibodies Recognizing Protective Pertussis Toxin Epitopes Are Preferentially Elicited by Natural Infection versus Acellular Immunization

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Despite more than 50 years of vaccination, disease caused by the bacterium Bordetella pertussis persists, with rates increasing in industrialized countries over the past decade. This rise may be attributed to several factors, including increased surveillance, emergence of vaccine escape variants, waning immunity in adults, and the introduction of acellular subunit vaccines, which include chemically detoxified pertussis toxin (PTd). Two potently protective epitopes on pertussis toxin (PTx) are recognized by the monoclonal antibodies 1B7 and 11E6, which inhibit catalytic and cell-binding activities, respectively. In order to determine whether the PTx exposure route affects antibody responses to these epitopes, we analyzed sera from 30 adults with confirmed pertussis exposure and from 30 recently vaccinated adults for specific anti-PTx antibody responses and in vitro CHO cell neutralization titers. While overall titers against PTx and the genetically detoxified variant, PTg, containing the R9K and E129G substitutions, were similar in the two groups, titers against specific epitopes depended on the exposure route. Natural infection resulted in significantly higher titers of anti-PTx-subunit I, 1B7-like, and 11E6-like antibodies, while acellular vaccination resulted in significantly higher titers of antibodies recognizing PTd. We also observed a correlation between in vitro protection and the presence of 1B7-like and 11E6-like antibodies. Notably, chemical detoxification, as opposed to genetic inactivation, alters the PTx tertiary and quaternary structure, thereby affecting conformational epitopes and recognition of PTx by 1B7 and 11E6. The lower levels of serum antibodies recognizing clinically relevant epitopes after vaccination with PTd support inclusion of PTg in future vaccines.

Pertussis vaccines, widely introduced as an inactivated whole-cell vaccine in 1950, have been responsible for a dramatic decline in pertussis-related morbidity and mortality but have been unable to eradicate disease despite 95% coverage in many areas. Disturbingly, rates of confirmed pertussis cases in industrialized countries have increased steadily in recent years, coinciding with the introduction of acellular vaccines containing chemically detoxified pertussis toxin (PTd). In the United States, rates increased approximately 5-fold between 1995 and 2005, from 5,158 to 25,616 cases, with local outbreaks occurring during 2010 (1). In addition to increased surveillance and elective undervaccination, it has become clear that the acellular vaccine produces little if any protection against subclinical infection (9). When this is combined with a time-dependent decline in vaccine-induced immunity, adults and adolescents serve as a reservoir for continued circulation of the pathogen, thereby infecting susceptible infants. Epidemiological studies have suggested that pertussis accounts for 12 to 32% of cough illnesses lasting more than 6 days in adolescents and adults, resulting in the recent approval of reduced-dose booster vaccines for this population in 2005 (3, 25, 36).

While Bordetella pertussis produces nearly 20 virulence factors, PTx is clearly a major protective antigen. This AB5 toxin recognizes cell surface glycosides via two to four binding sites on the B subunit, triggering retrograde transport of the toxin and eventual escape of the catalytically active S1 subunit into the cytoplasm. There, the molecule ADP ribosylates the alpha subunit of G protein receptors, altering cellular signaling processes. Experiments have demonstrated the following: (i) reduced virulence of bacteria lacking PTx genes for mice (37, 41–43), (ii) efficacy of the acellular pertussis vaccines (comprised of PTx and 0 to 4 additional virulence factors) in preventing human disease (6, 20, 35, 39, 40), and (iii) protection and even reversal of disease postinfection upon passive administration of anti-PTx antibodies in mice and humans (4, 5, 15, 16, 30, 31, 33). Furthermore, in highly vaccinated populations, circulating strains have emerged with increased virulence, correlating with increased PTx production due to promoter mutations (23).

Antibodies specific to PTx have been analyzed in detail, revealing four or more nonoverlapping immunodominant epitopes on the catalytically active S1 subunit, of which only one is highly protective (2, 21). The Sato group performed a comparison of 32 anti-PTx monoclonal antibodies in several protection assays, including inhibition of catalytic activity, CHO cell clustering, and murine intracerebral and aerosol challenge models (34). One antibody, 11E6, which recognizes an epitope on the S2/S3 subunits of the B oligomer and competitively inhibits receptor binding, performed well in the murine aerosol challenge (23/25 mice survived) but did not pro-
tect against intracerebral challenge (2/30 mice survived). A second antibody, IB7, was the only antibody which conferred significant protection in all assays, including mouse intracerebral challenge. In this study, a greater fraction of IB7-treated animals (25/30) survived than was the case for those treated with polygonal anti-PTx (8/30) or for anti-B-oligomer-treated animals (5/10), with the exception of 7F2 (8/10), which recognizes an S4 epitope that overlaps with the IB7 epitope (32, 34). Posttreatment, the IB7-treated animals carried reduced bacterial and PTx concentrations in the lungs (31, 33) and IB7 was able to protect mice even when administered 9 days postinfection (30). The IB7 antibody appears to bind an epitope spanning the S1 and B subunits, thereby altering toxin intracellular trafficking steps (J. N. Sutherland and J. A. Maynard, unpublished data). Since antibodies recognizing the IB7 and 11E6 epitopes potentially neutralize toxin activity in mice, the ability of vaccines to elicit high titers of high-affinity antibodies recognizing these epitopes may be indicative of protective immunity.

In this study, we compared overall and epitope-specific titers of antibody to PTx in serum samples from 30 convalescent and 30 recently vaccinated adult health care workers in order to determine which route better elicits antibodies binding protective epitopes. While overall anti-PTx titers were similar for both groups, infection resulted in higher titers of not only anti-S1 but also IB7- and 11E6-like antibodies. Since the acellular vaccine includes a chemically inactivated form of PTx, we assessed serum responses to PTd, observing higher anti-PTx than anti-PTd titers in all samples. Chemical detoxification has been previously shown to reduce murine responses to the IB7 epitope while skewing the immune response toward nonneutralizing anti-B-oligomer antibodies (19, 24). The protective epitopes on PTx are conformational and thus particularly sensitive to chemical modification; indeed, we observe significant losses in IB7 and 11E6 binding to PTx after chemical inactivation. In contrast, no loss in binding is observed for the genetically detoxified PTx R9K/E129G variant, PTg (7, 27), and vaccination with PTg is likely to induce stronger responses to these two potently neutralizing anti-B-oligomer epitopes (19, 24). The protective antibodies to PTx are conformational and thus particularly sensitive to chemical modification; indeed, we observe significant losses in IB7 and 11E6 binding to PTx after chemical inactivation. In contrast, no loss in binding is observed for the genetically detoxified PTx R9K/E129G variant, PTg (7, 27), and vaccination with PTg is likely to induce stronger responses to these two potently neutralizing anti-B-oligomer epitopes (19, 24). The protective antibodies to PTx are conformational and thus particularly sensitive to chemical modification; indeed, we observe significant losses in IB7 and 11E6 binding to PTx after chemical inactivation. 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**MATERIALS AND METHODS**

**Human serum samples and control antibodies.** Human sera were obtained from studies conducted at Vanderbilt University Medical Center (VUMC). Patients were excluded if they had received the acellular pertussis vaccine in the previous 5 years or a tetanus booster in the 2 years prior to study enrollment. The sera from exposed adults were previously collected from healthy persons of ages 21 to 64 approximately 3 weeks after identification and confirmation of their pertussis vaccination among health care workers, 070258, Blood bio-banking for establishment/monitoring of immunological assays, and 090806, Samples sharing for characterization of antibodies in vaccinated and convalescent individuals binding a key pertussis toxin neutralizing epitope, and use of those samples for this study was approved by the University of Texas at Austin (IRB 2009-05-0096, neutralizing anti-pertussis toxin response in immunized/convalescent clinical samples). The study was conducted in accordance with the Declaration of Helsinki, and written informed consent was obtained from each participant prior to study entry. The original consent forms allowed for sample use in subsequent studies.

Human intravenous pertussis immunoglobulin (P-IGIV) was obtained from the Massachusetts Public Health Biologic Laboratory (lot IVPIG-2) and was used to ensure consistency between enzyme-linked immunosorbent assay (ELISA) plates. P-IGIV was prepared as a 4% IgG solution from the pooled plasma from donors immunized with tetratnitromethane-inactivated pertussis toxoid vaccine (17). The IgG pertussis toxin antibody concentration of 733 μg/ml is ≾7-fold higher than that contained in conventional intravenous immunoglobulin (IVIG). To determine the role of the S1 and B subunits, thereby altering toxin intracellular trafficking steps (J. N. Sutherland and J. A. Maynard, unpublished data), since antibodies recognizing the IB7 and 11E6 epitopes potentially neutralize toxin activity in mice, the ability of vaccines to elicit high titers of high-affinity antibodies recognizing these epitopes may be indicative of protective immunity.

In this study, we compared overall and epitope-specific titers of antibody to PTx in serum samples from 30 convalescent and 30 recently vaccinated adult health care workers in order to determine which route better elicits antibodies binding protective epitopes. While overall anti-PTx titers were similar for both groups, infection resulted in higher titers of not only anti-S1 but also IB7- and 11E6-like antibodies. Since the acellular vaccine includes a chemically inactivated form of PTx, we assessed serum responses to PTd, observing higher anti-PTx than anti-PTd titers in all samples. Chemical detoxification has been previously shown to reduce murine responses to the IB7 epitope while skewing the immune response toward nonneutralizing anti-B-oligomer antibodies (19, 24). The protective epitopes on PTx are conformational and thus particularly sensitive to chemical modification; indeed, we observe significant losses in IB7 and 11E6 binding to PTx after chemical inactivation. In contrast, no loss in binding is observed for the genetically detoxified PTx R9K/E129G variant, PTg (7, 27), and overall titers of serum samples for PTg are indistinguishable from PTx titers. Thus, we propose that vaccination with PTg is likely to induce stronger responses to these two potently neutralizing epitopes than vaccination with PTd. Moreover, responses to specific protective epitopes may comprise serological correlates of protection for use during development of future pertussis vaccines.
washes with PBS containing 0.05% Tween 20, monoclonal antibodies, test serum samples, and the WHO standard were added at a 42-µg/ml, 1:1.5, 1:3.2, or 1:4.8 dilution, respectively, while P-IGIV was added at a 1:18 dilution in assay buffer containing 4% FBS. All samples were serially diluted 1:10 in assay buffer containing 4% FBS. The plate was incubated at room temperature for 1 h. After an additional three washes, peroxidase-conjugated anti-mouse IgG or anti-human IgG (Sigma-Aldrich) was added at a 1:1,600 or 1:500 dilution in assay buffer, respectively, and the plates were incubated again at room temperature for 1 h. After a final three washes, the plates were developed with tetramethylbenzidine dihydrochloride substrate (Pierce). The reaction was quenched with 1 N HCl, and the absorbance at 405 nm was recorded using the SoftMax Pro v5 software program (Molecular Devices). All plated volumes were 50 µl/well unless otherwise indicated; samples were run in duplicate, and all experiments were repeated at least in duplicate. The same software was used to calculate the serum dilution corresponding to half of the maximum signal, the 50% effective concentration (EC50), using a 4-parameter logistic (4PL) model for each individual curve (28). Reported EC50s are the averages for duplicate runs ± standard deviations; reference and control sera were included on every assay plate.

Measurement of epitope-specific responses. Competition ELISAs were performed similarly to the indirect ELISAs above, with the following modifications. Plates were coated with PTx at 1.0 µg/ml. Test serum samples were added at 50 µl/well, while P-IGIV was added at a 1:18 dilution in assay buffer containing 4% FBS and subsequently serially diluted 1:2 down the column in assay buffer containing 4% FBS. Mouse monoclonal antibody 1B7 or 11E6 was then added to each well at 3.8 µg/ml or 3.0 µg/ml, respectively, in assay buffer containing 4% FBS. After mixing, the plate was incubated at room temperature for 1 h. Peroxidase-conjugated anti-mouse IgG or anti-human IgG (Sigma-Aldrich, St. Louis, MO) was used as the secondary antibody. A 4PL model with parallel line analysis using P-IGIV as the standard curve was used to calculate the serum concentration resulting in half the maximum inhibition, the 50% inhibitory value (IC50) (28). Reported IC50s are the averages for duplicate runs ± standard deviations; reference and control sera were included on every assay plate.

RESULTS

Chemical but not genetic detoxification alters presentation of neutralizing epitopes. The acellular adult booster vaccine used to vaccinate these individuals, Adacel, contains inactive detoxified PTx (PTd) which has been treated with up to 0.07% glutaraldehyde (8). To determine whether the route of PTx exposure, to native PTx during an infection or to PTd during vaccination, could explain the differences in 1B7- and 11E6-like titers in exposed and vaccinated adults, we prepared PTd with procedures similar to those used during vaccine production using formaldehyde. A genetically detoxified variant of PTx, containing two amino acid changes in the S1 subunit (R9K and E129G; PTg) was developed in the late 1980s as an alternative to chemical toxoiding (7, 26) but was not included in the acellular vaccine due to patent issues.

First, we measured binding of the anti-S1 antibody 1B7, anti-S2/3 antibody 11E6, and P-IGIV to PTx, PTd, and PTg using an indirect ELISA. Since the three coating molecules are very similar, they are expected to coat the ELISA wells with similar surface concentrations. These data showed no detectable binding of either 1B7 or 11E6 to PTd and an approximately 100-fold decrease in toxin binding for P-IGIV (Fig. 1). This decrease in antibody binding to PTd suggests that detoxification negatively affects presentation of these epitopes (24). In contrast, binding to PTg was indistinguishable from that to PTx, indicating that the epitopes recognized by 1B7 and 11E6 are preserved in the genetically modified variant.

Exposed and vaccinated groups have similar anti-PTx and anti-PTg titers but not anti-PTd titers. We postulated that loss of the 1B7 and 11E6 epitopes in PTd would negatively impact titers of antibodies recognizing these protective epitopes in adults recently immunized with the acellular vaccine relative to results for adults recently exposed to B. pertussis and native PTx. To assess this, we selected 30 serum samples from adults recently vaccinated against pertussis and 30 samples from adults with confirmed, recent exposure to pertussis from studies conducted at VUMC. Samples with VUMC-detected PTx titers of $>$15 EU/ml were selected to ensure reliable detection of subsets of antibodies recognizing PTx. Anti-PTx antibody titers were confirmed through indirect ELISA using commercial PTx along with individual curve fits. Sera from vaccinated adults had average EC50s of 0.042 ± 0.002, while the sera from exposed adults had average EC50s of 0.048 ± 0.005, respectively (Table 1). There were no significant differences in anti-PTx antibody EC50s between the 30 vaccinated and 30 exposed adults ($P < 0.69$) (Fig. 2), indicating that there was no sample bias for PTx responses between the two groups.

To determine whether antibodies from exposed samples rec-
TABLE 1. PTx responses in exposed and vaccinated sera

| Antibody category | Statistic | Titer for serum group (EC50) | P value* |
|-------------------|-----------|-----------------------------|----------|
|                   |           | Vaccination | Exposure |          |
| Anti-PTx          | Mean      | 0.042       | 0.048    | 0.69     |
|                   | Variance  | 0.002       | 0.005    |          |
|                   | Range     | 0.0009–0.193| 0.006–0.410|         |
|                   | MLD†       | 0.41        |          |          |
| CHO protection    | Mean      | 0.0031      | 0.00161  | 0.17     |
|                   | Variance  | 0.000003    | 0.000013 |          |
|                   | Range     | 0.00048–0.030| 0.00048–0.0065|         |
|                   | MLD       | 0.03        |          |          |
| Anti-PTg          | Mean      | 0.032       | 0.046    | 0.18     |
|                   | Variance  | 0.002       | 0.001    |          |
|                   | Range     | 0.005–0.212 | 0.016–0.159|         |
|                   | MLD       | 0.212       |          |          |
| Anti-PTd          | Mean      | 0.016       | 0.022    | 0.09     |
|                   | Variance  | 0.0001      | 0.0002   |          |
|                   | Range     | 0.004–0.059 | 0.008–0.068|         |
|                   | MLD       | 0.068       |          |          |
| IgG1              | Mean      | 0.033       | 0.061    | 0.14     |
|                   | Variance  | 0.002       | 0.004    |          |
|                   | Range     | 0.005–0.174 | 0.012–0.226|         |
|                   | MLD       | 0.226       |          |          |
| Anti-PTx-S1-220   | Mean      | 0.115       | 0.04     | 0.04     |
|                   | Variance  | 0.027       | 0.002    |          |
|                   | Range     | 0.006–0.590 | 0.011–0.153|         |
|                   | MLD       | 0.59        |          |          |
| 1B7-like antibodies| Mean     | 0.519       | 0.338    | 0.04     |
|                   | Variance  | 0.181       | 0.069    |          |
|                   | Range     | 0.050–1.89  | 0.084–0.895|         |
|                   | MLD       | 1.89        |          |          |
| 11E6-like antibodies| Mean    | 0.229       | 0.081    | 0.0001   |
|                   | Variance  | 0.033       | 0.003    |          |
|                   | Range     | 0.016–0.645 | 0.028–0.296|         |
|                   | MLD       | 0.645       |          |          |

*a MLD, minimum level of detection.

*b P values refer to vaccination versus exposure comparisons.

recognized PTg as efficiently as PTx, we performed another series of indirect ELISAs to measure responses to PTg. We observed no difference in PTg responses between vaccinated and exposed groups, with a strong correlation between PTx and PTg responses (Pearson, 0.64). Moreover, PTx- and PTg-specific responses in vaccinated as well as exposed adults were indistinguishable (EC50 of 0.042 versus 0.032 and of 0.048 versus 0.046, respectively) (Fig. 2; Table 1). Last, recognizing that PTd is immunologically distinct from PTx and PTg, we performed a third set of ELISAs with the serum samples and PTd. As expected, recently vaccinated adults had stronger PTd responses (average EC50 of 0.0160 ± 0.0001) than sera from exposed adults (average EC50 of 0.0220 ± 0.0002) (Table 1). Since exposed adults have also been vaccinated in the past, they retained some, but significantly lower, titers to PTd (P < 0.09) (Fig. 2).

Exposed and vaccinated groups have similar CHO cell neutralizing titers. To analyze overall titers of PTx-neutralizing antibodies, an in vitro CHO cell neutralization assay was employed. Serum samples were diluted in the presence of a fixed concentration of PTx and incubated with CHO cells for 24 h. Wells were scored the following day, with higher protective dilutions indicative of higher titers of toxin-neutralizing antibodies. We observed a strong correlation between CHO cell neutralization titers and PTx EC50 (Fig. 3A; Pearson, 0.78), consistent with a subset of PTx-specific antibodies mediating inhibition of CHO cell clustering. Although the exposed samples provided a greater degree of protection than the vaccinated samples, the difference was not significant (P < 0.17) (Fig. 3B; Table 1).

Exposure route results in different epitope-specific responses. To assess epitope-specific sera responses to PTx, we first performed an ELISA with the isolated S1 subunit, which dominates this highly neutralizing epitope (30). A truncated form of PTx-S1, PTx-S1-220, which can be produced as a recombinant soluble protein in E. coli (38), was used as an antigen. As expected, since S1 represents ~1/5 of the holotoxin, overall anti-PTx-S1-220 titers were lower than anti-PTx titers for both sample populations. However, these did exhibit a moderate correlation with PTx values across all samples (Fig. 4A; Pearson, 0.46). Sera from vaccinated adults resulted in EC50 of 0.115 ± 0.027, while sera from exposed adults were detectable to EC50 of 0.040 ± 0.001 (Table 1). Interestingly, the exposed adults had significantly higher PTx-S1-220 antibody titers than the vaccinated adults (Fig. 5) (P < 0.04). Since both groups had similar anti-PTx EC50, this implies that vaccinated adults have stronger responses to the B oligomer or internal epitopes than exposed adults. This is in agreement with murine studies demonstrating that vaccination with PTd results in a stronger anti-B-oligomer response than vaccination with PTx (24).

To further investigate the antibodies specifically recognizing the 1B7 epitope, we utilized a competition ELISA. Because 1B7 exhibits reduced affinity for the isolated S1 subunit and the epitope appears to span both the S1 and S4 subunits (32, 38), PTx was immobilized and sera diluted in the presence of a fixed concentration of the murine antibody 1B7. Human serum antibodies recognizing the same or an overlapping epitope compete kinetically with 1B7 from PTx binding. At equilib-
rium, both human and 1B7 antibodies will be bound to PTx. By
detecting the amount of 1B7 bound in each well, the titers of
1B7-like antibodies in the serum can be determined. The ex-
posed adults had significantly higher 1B7-like antibody titers
than the vaccinated adults (Fig. 5) \( P \leq 0.04 \). Sera from
vaccinated adults resulted in IC50s of 0.519 ± 0.181, while sera
from exposed adults resulted in IC50s of 0.338 ± 0.069 (Table
1). The presence of 1B7-like antibodies also correlated with
the presence of PTx-specific antibodies (Fig. 4B; Pearson,
0.45).

We then repeated the PTx competition ELISA using the
antibody 11E6, which binds an epitope overlapping with the
cellular receptor-binding site. This epitope is of interest since
it can block the first step in PTx cellular intoxication. PTx was

FIG. 3. Summary of CHO cell neutralization assay. (A) Correlation between PTx antibody titers and protective CHO cell neutralization titers.
(B) CHO cell protection assay, which was not statistically significant as determined by two-tailed \( t \) test for paired samples. ■, exposed samples; □, vaccinated samples.

FIG. 4. Correlation between subsets of PTx-specific antibodies. (A) PTx-S1-220 versus PTx; (B) 1B7-like antibodies versus PTx; (C) 11E6-like antibodies versus PTx; (D) 1B7-like antibodies versus 11E6-like antibodies.
immobilized and sera diluted in the presence of a fixed concentration of the murine antibody 11E6, to allow antibodies binding the same or similar epitopes to compete. Exposure samples demonstrated significantly higher 11E6-like antibody titers than vaccination samples ($P < 0.0001$) (Fig. 3). Sera from vaccinated adults resulted in IC$_{50}$s of 0.229 + 0.033, while sera from exposed adults resulted in IC$_{50}$s of 0.081 + 0.003 (Table 1). The presence of 11E6-like antibodies correlated weakly with the presence of PTx-specific antibodies (Fig. 4C; Pearson, 0.32) and more strongly with the presence of 1B7-like antibodies (Fig. 4D; Pearson, 0.42). A direct correlation was observed between 1B7-like antibodies titers and protection in the CHO assay, indicative of a moderate correlation (Pearson, 0.47; Fig. 6A). The 11E6-like antibody titers had a weaker correlation with CHO cell protection (Pearson, 0.26; Fig. 6B).

**DISCUSSION**

We compared the serum from adults with either confirmed recent exposure to *B. pertussis* or acellular vaccination against pertussis. While there were no significant differences in total anti-PTx or anti-PTg antibody titers or *in vitro* CHO cell neutralization between the groups, there were significant differences for all other assays. Vaccination resulted in higher titers of anti-PTd antibodies, while exposure resulted in higher titers of not only S1 subunit-specific antibodies but also antibodies recognizing two potently neutralizing epitopes, characterized as the epitopes recognized by the 1B7 and 11E6 antibodies. Importantly, chemical toxoiding of PTx alters the structure and/or immunogenicity of these protective epitopes, while the R9K and E129G amino acid substitutions employed during genetic inactivation preserves them.

**Chemical but not genetic PTx detoxification alters recognition by protective antibodies.** In order to reduce the side effects associated with normal toxin function, PTx is detoxified prior to formulation of whole-cell and acellular vaccines by incubation with aldehydes. The process is highly variable, with ~2% of the toxin retaining or reverting to the active form. A milder detoxification process (0.035% formaldehyde for 48 h at 37°C) can stabilize the toxin against degradation during long-term storage without affecting the tertiary structure (33). However, increasing formaldehyde concentrations and incubation times (up to 0.5% for 7 days) results in a gradual cross-linking of the subunits and an increase in observed molecular weight as determined by SDS-PAGE, size exclusion chromatography (SEC), multangle laser light scattering (MALS), and analytical ultracentrifugation (AUC) (28). In contrast, the genetically detoxified PTx 9K/129G retains mitogenic and other B-oligomer-mediated activities eliciting a Th1 response (37) but is uniformly devoid of catalytic activity (8, 36). More critically from a vaccine standpoint, these two key amino acid substitutions do not compromise binding of any tested polyclonal or monoclonal antibodies, including 1B7 and 11E6 (33).

The structural changes associated with aldehyde toxoiding result in the desired loss of *in vitro* CHO cell toxicity (10$^4$ to 10$^6$-fold) but are coupled with altered presentation of PT surface epitopes. We have observed significant losses in binding to PTd relative to that to PTx and PTg of the potently neutralizing 1B7 and 11E6 antibodies and polyclonal human sera (P-IGIV). Production of PTd and the resulting antigenicity are
somewhat variable, which makes it harder to directly compare PTx and PTg with PTd, but three separate PTd preparations consistently demonstrate dramatic losses in 1B7, 11E6, and P-IGIV binding (Fig. 1). These data are consistent with previous reports, in which a 100-fold reduction in reactivity with polyclonal anti-PTx serum and a complete loss of detectable 1B7 binding in a radioimmunoassay were observed (22). The monoclonal antibodies and the P-IGIV are each able to protect mice against infection with B. pertussis, indicating that they each recognize potently protective epitopes. The 11E6 antibody blocks the toxin-cellular receptor interaction, while 1B7 appears to alter toxin intracellular trafficking steps (Sutherland and Maynard, unpublished). Structurally, aldehyde treatment will primarily form cross-links between lysine residues, which appears to be the basis for complexation between the B subunits (10). Since there are no lysines in the native S1 subunit, the structural basis for the loss of 1B7 and 11E6 binding is less clear, but it may result from secondary aldehyde reactions.

These two protective epitopes are not the only ones adversely affected by aldehyde detoxification. Even modest levels of chemical detoxification have been shown to disrupt antibody binding, since PTx treatment with 0.035% or 0.35% formaldehyde impaired binding of 73% and 82% of a panel of 13 neutralizing monoclonal antibodies, respectively (22). A study of 38 PTx-specific monoclonal antibodies, including 13 protective antibodies, showed that formaldehyde treatment weakens binding to some epitopes but strengthens binding to others, especially partially buried epitopes (22). This global change in PTd surface epitope presentation is reflected in a loss of polyclonal antibody recognition of PTd versus that of PTx, about 30-fold in this study (Fig. 1). The P-IGIV contains purified IgG collected from volunteers immunized with hydrogen peroxide-detoxified, as opposed to aldehyde-detoxified, PTx, which explains the loss in reactogenicity against PTd. This product contains an IgG pertussis toxin antibody concentration >7-fold higher than that of conventional intravenous immunoglobulin products and is able to protect mice against B. pertussis infection in the aerosol model even when administered 7 days after infection (5).

After immunization with PTd, guinea pig serum was unable to protect CHO cells against intoxication (33), indicating that not only do neutralizing antibodies lose the ability to bind PTd, but PTd loses the ability to induce strong antibody responses against protective epitopes. Similarly, a study comparing PTg immunization with and without formaldehyde treatment observed similar PTx-specific titers in both groups but significantly lower toxin-neutralizing titers in the formaldehyde-treated group as measured by mouse intracerebral challenge (33). This led us to hypothesize that adults immunized with PTd would show reduced PTx-specific titers compared with adults exposed to native PTx through infection. We compared serum titers to PT variants from 30 adults with recent vaccination and 30 adults with confirmed recent exposure to live B. pertussis. Sera were collected 21 to 25 days after exposure, a sufficient time to boost serum antibody levels. We observed no significant differences between the two groups in PTx- or PTg-specific titers but a significant difference in PTd-specific titers \( (P < 0.05) \) (Fig. 2). Due to the less-dramatic changes in titers for the different PT versions than for the purified antibodies described above, coupled with the inherent variability in PTd preparations and possible variations in ELISA coating efficiencies due to aldehyde-mediated surface modifications, we are unable to directly compare PTx and PTg responses with PTd values.

**Epitope-specific responses induced by vaccination and exposure.** While the overall PTx-specific titers are similar for the vaccinated and exposed groups, the molecular details of which epitopes are being recognized and with what affinity were expected to differ based on altered recognition of different PT preparations by monoclonal antibodies. Here, exposure resulted in significantly higher serum titers recognizing the S1-220 subunit \( (P < 0.04) \), as well as the 1B7 \( (P < 0.04) \) and 11E6-like epitopes \( (P < 0.0001; \) Fig. 5). These titers were determined using a competition assay; thus, antibodies whose epitopes significantly overlap with that of 1B7 or 11E6 but are not necessarily identical and thus will not necessarily confer a similar level of protection against disease will be detected. Since these responses are subsets of the overall PTx responses, sera with higher PTx responses in general also had higher epitope-specific responses (Fig. 4).

To relate epitope specificity to *in vitro* protection against pertussis, we used an *in vitro* CHO cell clustering assay. Although this assay is more physiologically relevant than other *in vitro* assays, such as ADP ribosylation using cell lysates, it is not completely predictive of an antibody’s ability to protect in either mice or humans. This is partly because the clustering morphology is a response to PTx catalytic activities and does not assess mitogenic activities associated with the B oligomer, including leukocytosis and histamine sensitization. Overall, we observed no statistically significant difference in CHO cell neutralization titers between the vaccinated and recently infected populations, although these values did correlate well with the presence of anti-PTx antibodies (Fig. 3). In addition to the inherent constraints of the assay, the health care worker population may exhibit less distinction between exposed and vaccinated groups than other populations. None of the subjects were vaccinated against pertussis in the 5 years preceding study enrollment, and the timing of exposure/vaccination to serum collection was fairly consistent among all participants and was sufficient to observe a rise in serum antibody titers (21 to 25 days). However, through their professional duties, health care workers are exposed to numerous patients, some of whom may be asymptomatic for pertussis, resulting in an undocumented exposure. Similarly, subclinical infection is increasingly recognized as a reservoir for continued circulation of this pathogen. Individuals assigned to the vaccinated group with recent silent boosting through undocumented exposure or subclinical infection would be expected to have serum anti-PTx profiles intermediate to those of the solely vaccinated or exposed group.

We did observe a strong correlation between overall CHO cell neutralization titers and the presence of anti-PTx antibodies (Fig. 3), indicating that a consistent fraction of PTx-specific antibodies are responsible for neutralizing CHO-sensitive activities in both vaccinated and exposed groups. Upon more careful examination of which PTx-specific subsets are responsible, we observed moderate correlations between CHO cell neutralization titers and both the S1-220 and 1B7-like epitope-specific responses (Pearson, 0.46 and 0.45, respectively; Fig. 6). The stronger 1B7-like correlation may reflect the more potent neutralization conferred by antibodies binding this epitope,
which appears to bind PTx with a 1:1 stoichiometry and efficiently block retrograde transport. In contrast, the correlation with 11E6-like antibodies is weaker (Pearson, 0.32). The 11E6-like antibodies bind the receptor binding epitope, which is present in two to four sites on the B subunit (22). Thus, neutralization with 11E6 may require up to four 11E6-like antibody molecules per PTx molecule and a higher dose or affinity in order to effectively neutralize the toxin.

Epitope-specific titers as serological correlates of protection. Serological correlates or cocorrelates of protection facilitate development of specific therapeutics and enhanced vaccines to prevent disease. However, due to the large number and generally immunosuppressive character of virulence factors secreted by Bordetella, identification of strong serological correlates in whooping cough has proven elusive (10). Here we have shown that three antibody preparations which confer potent protection against disease in murine models recognize at least two protective epitopes and react strongly with PTx and PTg but not PTd. We suggest that antibodies recognizing specific protective epitopes with high affinity, as opposed to overall titers against individual virulence factors, may aid in identification of serological correlates of protection. For instance, the failure of P-IGIV passive immunization trials to demonstrate a clear effect on clinical outcome (5, 6, 19) may have been due to low concentrations of clinically relevant antibodies binding key neutralizing epitopes. Here we have presented evidence that serum responses to two protective epitopes are significantly higher following exposure to native PTx than following vaccination with PTd. Further prospective studies would be necessary to determine whether or not 1B7 and/or 11E6 could be used as serological correlates or cocorrelates of protection.

Conclusions. We have documented weak serum responses to two highly neutralizing epitopes on PTx in adults vaccinated with PTd but significantly stronger responses in adults with recent exposure to disease. The dramatic loss of these epitopes by chemical but not genetic detoxification supports inclusion of PTg in future acellular vaccine preparations. Concerns regarding chemical deactivation procedures include lot-to-lot variability, incomplete inactivation, reversion of toxicity, and loss of immunogenicity (14, 29) and may similarly affect protective epitopes on other AB-type toxins. We suggest that development of next-generation vaccines and passive immunization strategies for pertussis should focus on eliciting responses to specific protective epitopes, such as those recognized by the antibodies 1B7 and 11E6.

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