Use of Pertussis Toxin Encoded by \textit{ptx} Genes from \textit{Bordetella bronchiseptica} To Model the Effects of Antigenic Drift of Pertussis Toxin on Antibody Neutralization

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Recently, concern has been voiced about the potential effect that antigenic divergence of circulating strains of \textit{Bordetella pertussis} might have on the efficacy of pertussis vaccines. In order to model antigenic drift of pertussis toxin, a critical component of many pertussis vaccines, and to examine the effects of such drift on antibody neutralization, we engineered a strain of \textit{B. pertussis} to produce a variant pertussis toxin molecule that contains many of the amino acid changes found in the toxin encoded by \textit{Bordetella bronchiseptica} \textit{ptx} genes. This altered form of the toxin, which is efficiently secreted by \textit{B. pertussis} and which displays significant biological activity, was found to be neutralized by antibodies induced by vaccination as readily as toxin produced by wild-type \textit{B. pertussis}. These findings suggest that significant amino acid changes in the pertussis toxin sequence can occur without drastically altering the ability of antibodies to recognize and neutralize the toxin molecule.

Recently, concern has been raised about the possible affects of antigenic drift of \textit{Bordetella pertussis} on the efficacy of pertussis vaccines (16). Others have reported that a gradual change has occurred in the population structure of \textit{B. pertussis} strains in at least two countries, The Netherlands and Finland, as assessed by DNA fingerprinting and examination of the sequence of genes encoding the S1 subunit of pertussis toxin (PT) and pertactin of a number of strains (15, 16). In these studies, clinical isolates from the 1950s and 1960s were found to be predominantly of the type used for vaccine production. In contrast, more recent clinical isolates were found to have alterations in the gene for S1 (resulting in up to three amino acid changes) as well as in a single region of the gene coding for pertactin. This antigenic divergence between vaccine strains and circulating strains of \textit{B. pertussis} has been postulated to have effects on the efficacy of whole-cell pertussis vaccines that have been in use since the 1950s, possibly helping to contribute to the disease burden as well as recent epidemics of pertussis (16). Of course, factors other than antigenic drift might significantly contribute to the reported increase in disease rates in vaccinated populations, including improved surveillance, changes in case definition, changes in vaccines, or waning vaccine-induced immunity.

Recently, concerns about antigenic divergence have become heightened because of the introduction of acellular pertussis vaccines that are replacing whole-cell pertussis vaccines in many countries, including the United States. Acellular pertussis vaccines, unlike the whole-cell vaccine, which is composed of a great number of antigens, consist of only a few, well-defined antigens. Potentially, antigenic drift of any of the antigens contained in acellular pertussis vaccines could have significant effects on the efficacy of these vaccines. Widespread use of acellular pertussis vaccines might even provide a selective advantage for strains with altered antigens that enable the bacteria to better escape a vaccine-induced immune response of the host. Of particular concern is antigenic drift of PT, since an inactivated form of the toxin is a component of all acellular vaccines that are currently available and represents the only antigen in some of these vaccines.

PT, a bacterial toxin with an A-B structure, consists of an enzymatically active S1 subunit that sits atop the B oligomer comprising one copy each of S2, S3, and S5 and two copies of S4 (21, 24). Previous work demonstrated that both the S1 subunit and the subunits of the B oligomer contribute to the protection afforded by the whole molecule (1, 17). Antibodies specific for either the S1 subunit or subunits of the B oligomer have been shown to passively protect mice against an aerosol challenge of \textit{B. pertussis} (20). These studies suggest that changes in any one of the subunits of the toxin due to antigenic drift could potentially affect the efficacy of pertussis vaccines, especially acellular pertussis vaccines. It is of interest to note that antigenic variants of the cholera toxin family, which have an AB\textsubscript{5} structure, are known to be incapable of eliciting cross-neutralizing responses (7).

If vaccine-induced antigenic drift does occur in the era of acellular pertussis vaccines, it may take years to determine the extent to which this drift will have affected vaccine efficacy and ultimately disease burden, since vaccine-driven evolution is likely to be a slow process. In order to help predict the effects that antigenic drift might have on the ability of antibodies induced by acellular pertussis vaccines to recognize and neutralize PT produced by variant strains of \textit{B. pertussis}, we have utilized PT encoded by the \textit{ptx} genes from \textit{Bordetella bronchiseptica} to model such drift. \textit{B. bronchiseptica} does not produce PT, although it contains the genes for the toxin (2, 14). While these genes are transcriptionally silent due to alterations in the promoter region of the \textit{ptx} operon, we have found that the genes encode biologically active toxin (9). The \textit{ptx} genes of \textit{B. bronchiseptica} exhibit differences in the DNA sequence that translate into a number of amino acid differences when compared to the sequence of \textit{B. pertussis} PT. When the crystal structure of PT (21) is examined, it is apparent that most of

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these changes occur on the exterior of the protein that would be accessible to antibodies. Moreover, since PT encoded by the B. bronchiseptica ptx genes is biologically active, these changes occur in regions of the protein that have minimal effects on toxin activity. Since PT is an essential virulence factor (25) and loss of toxicity of this protein would be detrimental for the pathogen, antigenic drift would be expected to occur precisely in those regions that can change without drastically affecting biological activity of the toxin. Thus, B. bronchiseptica PT represents an excellent model with which to study the effects of antigenic drift. Moreover, certain of the differences in amino acid sequence between B. pertussis and B. bronchiseptica PT are known to occur in or near regions that react with neutralizing antibodies (4).

In order to produce the altered form of the toxin encoded by the B. bronchiseptica ptx genes, we replaced the ptx genes of B. pertussis BP536 with the ptx genes from B. bronchiseptica RB50 (see Table 1 for the strains used in this study). We did this by first cloning the EcoRI fragment of B. bronchiseptica RB50 that contains the ptx genes by using standard techniques (19). The appropriately cloned fragment was identified by colony blot analysis with 32P-labeled probe corresponding to the ptx gene encoding the S1 subunit of the toxin. A hybrid ptx region was then constructed that consisted of nucleotides 1 to 629 (EcoRI-BstZ17I fragment) from the B. pertussis BP536 ptx region, nucleotides 630 to 3517 (BstZ17I-MluI fragment) from the B. bronchiseptica RB50 ptx region, and nucleotides 3518–4568 (MluI-BamHI fragment) from the B. pertussis BP536 ptx region (Fig. 1) in the vector pBR322 to generate pSZH60. The ptx region of pSZH60 was then completely sequenced (Lark Sequencing Technologies, Houston, Tex.). This sequence is shown in Fig. 2 (the sequence from B. bronchiseptica RB50 was assigned GenBank accession no. AF242847). The EcoRI-BamHI fragment of pSZH60 (ptx nucleotides 1 to 4569) was then cloned into the suicide vector pSS1129 to generate pSS1129 containing the hybrid ptx region from pSZH60. This plasmid, containing the hybrid ptx sequence, was then introduced into B. pertussis BPH101, a strain with a 2,587-bp deletion in the ptx region from nucleotide 930 to nucleotide 3517 (22), to replace the ptx genes of B. pertussis that encode the transport apparatus necessary for the secretion of PT.

The amino acid sequences for each of the PT subunits encoded by the wild-type and hybrid ptx regions of B. pertussis BP536 and pSZH60, respectively, are shown in Fig. 2. A total of 42 amino acid differences between PT from B. pertussis and the variant PT encoded by pSZH60 are apparent, the majority of which occur in the S5 and S3 subunits. In fact, 12% of the amino acids of S3 differ, and 10% of the amino acids of S5 differ.

We next characterized the variant PT produced and secreted by B. pertussis BPH102. Both BPH102 and the wild-type strain BP536 were grown in Stainer-Scholte medium for 48 h to give an A650 of 0.9. The supernatants were collected after centrifugation for 10 min at 11,000 × g and subsequently sterilized by filtration through Millex-GV filters (0.22 μm pore diameter; 1.4 mm pore size).
Millipore Corporation, Bedford, Mass.). The supernatants were then examined for PT protein content by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblot analysis as previously described (9) and for biological activity. As shown in Fig. 3, approximately the same amount of subunit protein was secreted by both strains. The biological activity contained in the culture supernatant of each strain was assessed by the ability of the supernatant to cluster Chinese hamster ovary (CHO) cells as first described by Hewlett et al. (11), an activity that has been demonstrated to be due to the ability of PT to ADP ribosylate the G proteins of these cells (3). This activity was measured essentially as previously described (3). The culture supernatant from *B. pertussis* BPH102 (producing variant PT) clustered CHO cells at a maximum dilution of 1:8,000, whereas the culture supernatant from *B. pertussis* BP536 (producing wild-type PT) clustered CHO cells at a maximum dilution of 1:32,000. Since immunoblot analysis indicated that the supernatants from both strains contained approximately equal amounts of PT protein and since the supernatant from *B. pertussis* BP536 was able to cluster CHO cells at dilutions fourfold greater than that of *B. pertussis* BPH102, the specific activity (activity/microgram of protein) of wild-type PT produced by BP536 must be approximately fourfold greater than that of the variant PT produced by BPH102.

We next determined the ability of antibodies to neutralize the action of wild-type PT and the variant PT by assessing the ability of antibodies to inhibit PT-induced clustering of CHO cells. The antibodies used in this study were the U.S. Standard Pertussis Antibody (mouse), lot 1, that was generated by immunization of mice three times at 3-week intervals with an inactivated vaccine containing glutaraldehyde-inactivated PT, filamentous hemagglutinin, fimbria types 2 and 3, and pertactin. This vaccine has been reported to be efficacious in protecting children from pertussis (8). This U.S. Standard is a lyophilized preparation of mouse antiserum (pooled from approximately 1,000 mice) that is reconstituted with 500 μl of distilled water and was kindly supplied by Juan Arciniega, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Md. Diluted culture supernatants from either *B. pertussis* BPH102 or *B. pertussis* BP536 in a total volume of 50 μl were mixed with twofold dilutions of antibody (50 μl) for 3 h in 96-well microtiter plates. CHO cells (100 μl) were then
added. The ability of the antibodies to inhibit the ability of the toxin preparations to cluster CHO cells was assessed after 24 h. Samples were run in duplicate. The maximal dilution of anti-serum that neutralized the CHO cell clustering activity of the toxin was determined. The culture supernatants used in this study were diluted such that each well contained equivalent amounts of CHO cell clustering activity (62 U, with a unit of activity defined as the minimal amount of toxin that is capable of clustering CHO cells). Since the specific activity of the variant PT produced by \( B. \) pertussis BPH102 is about four times less than that of the wild-type toxin produced by \( B. \) pertussis BP536, more toxin protein (however, the same amount of activity) was actually present in the wells containing the culture supernatant from \( B. \) pertussis BPH102 than in wells containing culture supernatant from \( B. \) pertussis BP536.

The results from a representative experiment are as follows. Maximal dilutions of antibody (U.S. Standard Pertussis Antibody [mouse], lot 1) capable of neutralizing the variant PT preparation (\( B. \) pertussis BPH102) and the wild-type PT preparation (\( B. \) pertussis BP536) were equivalent (1:64). The fact that more toxin protein was actually present in the culture supernatant of \( B. \) pertussis BPH102 containing the variant PT than that of \( B. \) pertussis BP536 (each supernatant contained 62 U of activity) should, if anything, increase the amount of antibody that was required to neutralize the preparation. On the contrary, the same quantity of antibody neutralized both preparations, indicating that the variant toxin is neutralized as readily as the wild-type toxin. These findings are striking in that they indicate that a significant number of amino acid substitutions can occur in the PT molecule without adversely affecting the ability of antibodies generated by vaccination with acellular pertussis vaccines to neutralize the action of the toxin. These amino acid substitutions are, for the most part, located on exposed regions of the toxin that would be accessible to antibodies. Given the fact that the PT molecule has many constraints on its amino acid sequence because it must retain multiple biological activities, including enzymatic activity (12), the ability to bind to eukaryotic cells (24), the ability to bind the regulatory molecule ATP (10), and the ability to cross the membrane barrier of the eukaryotic cell (26), as well as the ability to properly fold, assemble, and be secreted from \( B. \) pertussis, it seems likely that relatively few amino acid changes could occur without significantly affecting either the biogenesis or the activity of the toxin molecule. The finding that much of the biological activity of the toxin is maintained despite the amino acid alterations that are present in the variant PT molecule indicates that these changes likely occur in regions of the protein where evolutionary changes would be allowed, since they would have relatively little effect on the pathogenicity of \( B. \) pertussis.

The results presented in this report are perhaps surprising, but somewhat reassuring in that they suggest that variations in the amino acid sequence of PT due to antigenic drift may not result in drastic alterations in the ability of neutralizing antibodies to recognize the PT protein. However, amino acid differences other than those present in the variant PT used in this study may occur. Also, it is important to recognize that protection elicited by inactivated PT contained in acellular pertussis vaccines may be due to complex immune mechanisms rather than solely due to the generation of neutralizing antibodies. Therefore, it is important that we remain vigilant to ensure that population shifts of \( B. \) pertussis, if they do occur, do not result in decreased efficacy of pertussis vaccines.

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REFERENCES


FIG. 3. Immunoblot analysis of culture supernatants from \( B. \) pertussis BP536 (wild type), \( B. \) pertussis BPH101 (pts mutant), and \( B. \) pertussis BPH102 (hybrid pts strain). Strains were grown as described in the text. Supernatants (400 \( \mu \)l) were precipitated with an equal volume of 20\% trichloroacetic acid as previously described (9) and were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 15\% polyacrylamide gels followed by immunoblot analysis as described in the text. The S1 subunit was visualized with monoclonal antibody 3CX4 (13), and the S2 subunit was visualized with monoclonal antibody P11B10 (6). Lanes: 1, BP536; 2, BPH101; 3, BPH102.