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Epitopes on the S1 Subunit of Pertussis Toxin Recognized by Monoclonal Antibodies

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To identify the neutralizing epitopes on the S1 subunit (A promoter) of pertussis toxin, we characterized anti-S1 monoclonal antibodies (MAbs) X2X5, 3CX4, and 6FX1. We confirmed by immunoblot analysis that these MAbs bind to the S1 subunit and not to the B oligomer of pertussis toxin and that they recognize different epitopes by a competitive binding enzyme-linked immunosorbent assay. These MAbs had differential abilities to neutralize the lymphocytosis-promoting factor activity of pertussis toxin in mice: 3CX4 and 6FX1 had partial neutralizing abilities, while MAb X2X5 had none. With these MAbs, the epitopes on the S1 subunit were examined by using trypsinized S1 peptides, recombinant truncated S1 molecules, and synthetic peptides. The non-neutralizing MAb X2X5 bound in immunoblots to tryptic peptides of various sizes as small as 1.5 kilodaltons; the neutralizing MAbs 3CX4 and 6FX1 bound only to a 24-kilodalton tryptic peptide band. Immunoblot studies with recombinant truncated S1 molecules demonstrated that amino acid residues 7 to 14 and 15 to 26 play an important role in the binding of neutralizing MAbs and the non-neutralizing MAb, respectively. The binding of these MAbs was not dependent upon the presence of C-terminal amino acid residues 188 to 234. To further define B-cell epitopes, the binding of the MAbs we tested to synthetic peptides representing the entire S1 subunit were examined. Neutralizing MAbs 3CX4 and 6FX1 bound to none of these peptides, further suggesting that these MAbs recognize conformational epitopes. The non-neutralizing MAb X2X5 bound to peptides 11 to 26 and 16 to 30, demonstrating that the major antigenic determinant recognized by this MAb is a linear epitope located within residues 16 to 26.

Pertussis toxin (PT) is an important component in the development of acellular pertussis vaccines. The hexameric toxin is composed of five different subunits (31), designated S1 (molecular weight, 26,026), S2 (molecular weight, 21,925), S3 (molecular weight, 21,873), S4 (two copies; molecular weight, 12,059), and S5 (molecular weight, 11,013). The S1 subunit exerts the ADP-ribosyltransferase activity of the toxin (4, 14, 31), while the B oligomer (subunits S2 through S5) possesses eucaryotic cell receptor-binding sites (14, 32). It is well documented that the humoral response against PT plays an important role in the protection of experimental animals against bacterial challenge (23, 26, 35). The molecular structure of the PT operon has been determined recently (17, 18, 22), and the toxin subunits have been expressed in various recombinant expression systems (7, 21). Thus, in the future it will likely be possible to produce recombinant or synthetic vaccines which retain immunogenicity without the undesirable toxic side effects. To design these products rationally, it is important to define the epitopes required for protection. In our present study, we investigated B-cell epitopes on the S1 subunit of PT recognized by monoclonal antibodies (MAbs), utilizing peptides generated by enzymatic digestion, recombinant truncated S1 molecules expressed in *Escherichia coli* (10), and synthetic peptides.

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MATERIALS AND METHODS

Mice. Female BALB/c mice (5 to 6 weeks old) and outbred N:NIH(S) mice (2 weeks old) were obtained from the animal unit of the National Institutes of Health, Frederick, Md.

PT. PT was purified from culture supernatants of *Bordetella pertussis* 114 (29). S1 subunit was dissociated from B oligomer as described previously (9, 29) and dialyzed in 50 mM sodium phosphate buffer, pH 7, containing 2 M urea. The purity of the preparations was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16). One can routinely get 95% purity. Protein concentration was measured by the method of Bradford (5), using ovalbumin as the standard. Purified S1 subunit was stored at 4°C until use.

MAbs. We have used three MAbs (X2X5, 3CX4, and 6FX1) of the immunoglobulin G subclass 1 isotype which have been reported to bind to the S1 subunit of PT (J. Kenimer et al., submitted for publication). Briefly, these hybridoma cell lines were obtained by hybridization of SP2/0-Ag14 myeloma cells with the spleen cells of BALB/c mice hyperimmunized with inactivated PT. MAb 21.18.1 (15), specific for tetanus toxin and of the same immunoglobulin isotype (immunoglobulin G subclass) as the others, was used as a negative control. Ascitic fluids containing these MAbs were prepared in BALB/c mice and partially purified by precipitation with 50% ammonium sulfate. Purified MAbs were conjugated to biotin as described before (30).

ELISA. To assess whether the MAbs recognize the same epitopes, a competitive binding assay was performed, using an enzyme-linked immunosorbent assay (ELISA) essentially

as described by Engvall and Perlmann (12). Briefly, the wells of polystyrene microdilution plates were coated with 50 μl of PT (4 μg/ml) per well overnight. Plates were washed three times with phosphate-buffered saline (PBS) containing 0.1% Brij 35 (Sigma Chemical Co., St. Louis, Mo.). Nonspecific binding sites were then blocked with PBS containing 2% bovine serum albumin (BSA) for 2 h. After the plates were washed, 25 μl of partially purified MAb (20 to 100 μg/ml) was added to each well. The MAb was allowed to bind to PT for 30 min at room temperature; 25 μl of biotinylated MAb (1 μg/ml) per well was then added. Plates were incubated for 4 to 24 h at room temperature. The amount of biotinylated MAb bound was measured by reaction with 60 μl of avidin-conjugated peroxidase (1.2 μg/ml) per well for 1 h. After plates were washed, the color reaction was developed by the addition of 100 μl of 2,2'-azino-di(3-ethylbenzthiazoline-sulfonic acid) (0.54 mg/ml) per well prepared in 0.1 M citrate buffer (pH 4.2) containing 0.03% hydrogen peroxide. The reaction was quenched with the addition of 2 mM sodium azide, and the optical density was measured at 550 nm with an ELISA reader.

The binding of MAbs to synthetic peptides was performed essentially as described above, with minor changes. The microtiter wells (Costar EIA plate no. 3050) were coated with 50 μl of peptides (5 μg/ml) per well overnight. The nonspecific binding sites were blocked with either 2% BSA or 0.1% Brij 35 for 2 h. Antibodies were allowed to bind for 1 h, and the amount of antibodies bound was measured by reaction with alkaline phosphatase-conjugated anti-mouse immunoglobulin (Sigma). The reaction was stopped by the addition of equal amounts of 2 M NaOH. The A_{405} was measured.

Immunoblot analysis. Antigen preparations (approximately 1 μg of protein per lane) were separated by SDS-PAGE (27) in "minigels" of 16% polyacrylamide. Proteins were electrophoretically transferred to nitrocellulose (NC) (6, 33). In the study with recombinant S1 molecules, we used the carbonate buffer system (10 mM $NaHCO_3$, 3 mM Na_2CO_3, 20% methanol, pH 9.9) described by Dunn (11) instead of Tris-glycine-methanol (33) to electrophoretically transfer these proteins to NC paper. Strips of the NC paper were stained with 0.01% amido black in 45% methanol–10% acetic acid (6). For antibody binding, nonspecific binding sites on the NC paper were blocked by incubation for 1 h in 2% BSA–PBS, and the strips were reacted with MAbs overnight at room temperature. Antibodies bound to electroblotted proteins were detected with peroxidase-conjugated goat anti-mouse immunoglobulin, using 4-chloro-1-naphthol as chromagen (0.5 mg of chromagen per ml of distilled water containing 17% methanol and 0.02% H_2O_2).

In vivo neutralization of the lymphocytosis-promoting factor (LPF) activity of PT by MAbs. The assay for the lymphocytosis-promoting activity of PT was carried out as described previously (25). Partially purified MAbs serially diluted in PBS containing 0.2% gelatin were mixed with an equal volume of PT. These mixtures were incubated for 30 min at 37°C, and 0.2 ml of each mixture was injected into the tail vein of an individual N:NIH(S) mouse (13 to 15 g). Three to 4 days later, mice were bled from the tail vein and the number of leukocytes was counted. Experiments were carried out in quadruplicate.

Tryptic digestion of the S1 subunit. A preparation of S1 subunit (10 μg/ml) was boiled for 3 min to inactivate endogenous protease activity. The preparation was mixed at a ratio of 50:1 with freshly prepared tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (Sigma) prepared in distilled water and incubated at 37°C for 30 min. At the end of the incubation, the reaction was quenched by the addition of sample buffer containing 2% SDS, 8% glycerol, and 5% 2-mercaptoethanol, followed by boiling for 3 min. These trypsin-digested S1 peptides were then subjected to SDS-PAGE and immunoblotting.

Preparation of peptides. The selection of specific peptides to be synthesized was based on a hydrophilicity profile (13) of the amino acid sequence of the S1 subunit (18). In addition, 45 overlapping 15-mers (residues 1 to 15, 6 to 20, 11 to 26, 16 to 30, 21 to 35, etc.) covering the entire S1 subunit, using both published sequences (18, 22), were also prepared. The peptides were synthesized by using solid-phase chemistry as described previously (19). Peptides were synthesized by either the Laboratory of Biochemistry and Biophysics, Food and Drug Administration, Bethesda, Md., or the Johnson & Johnson Biotechnology Center, Inc., La Jolla, Calif. Polyclonal antisera to several of these peptides (8; a generous gift from D. Burns and S. Hausman, Food and Drug Administration) were raised by hyperimmunization of N:NIH(S) mice with each peptide conjugated to BSA.

Peptides 65 to 79 and 168 to 182 were conjugated to keyhole limpet hemocyanin to ensure that they bound to the ELISA plate. Briefly, keyhole limpet hemocyanin was dialyzed in PBS and mixed with each peptide (peptide:keyhole limpet hemocyanin, 5:20) at room temperature for 30 min. A 0.1-ml portion of fresh glutaraldehyde (0.5%) was added, and the reaction was allowed to proceed for 3 h at room temperature. The mixture was then dialyzed extensively against PBS before use.

Recombinant S1 subunit and truncated S1 species. The recombinant expression of truncated S1 proteins in *E. coli* and the preparation of these proteins from the cell lysates have been described previously (10).

RESULTS

Binding specificity of 3CX4, 6FX1, and X2X5. MAbs 3CX4, 6FX1, and X2X5 have been shown previously to bind to the S1 subunit of PT (J. Kenimer, submitted for publication). When the binding specificity of the MAbs was reexamined by Western blot, using a purified S1 subunit which had been stored for a few weeks at 4°C, two protein bands (27.5 and 24 kilodaltons [kDa]) in addition to the expected S1 subunit band (28 kDa) were detected (Fig. 1, left panel). We were concerned that these MAbs might be detecting some contaminating S2 subunit (23 kDa); however, the MAbs bound only to the upper band corresponding to the S1 subunit of the denatured holotoxin (Fig. 1, right panel). When the gel was loaded with a high concentration of PT (7 μg per lane) to visualize all subunits by the amido black staining, there were a few faint bands in the immunostaining which did not correspond to the S1 subunit. However, when the smaller amount of protein was used, such nonspecific faint bands disappeared. Since it had been shown previously that the C-terminal portion of the S1 subunit is susceptible to proteolysis once it is separated from the B oligomer (8), it is possible that the 24-kDa band represents the S1 protein whose C terminus was proteolytically cleaved. These results demonstrate that the MAbs are indeed specific for the S1 subunit and that the amino acid residues in the C terminus of the S1 molecule may be unimportant for the binding of these MAbs.

MAbs X2X5, 3CX4, and 6FX1 recognize different epitopes on PT. To confirm that MAbs X2X5, 3CX4, and 6FX1 recognize different epitopes, we performed a competitive

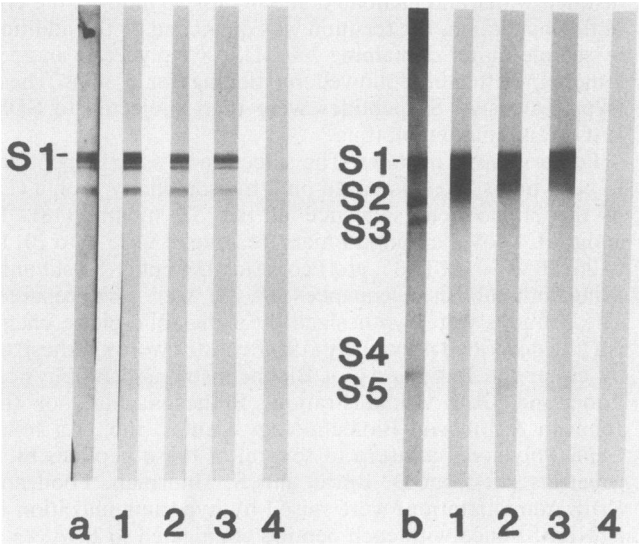

FIG. 1. Binding specificity of MAbs to the subunits of PT. NC strips in the left panel were blotted from gels containing purified S1 subunit which had been refrigerated for 3 to 4 weeks prior to use; the NC strips in the right panel were blotted from gels of holotoxin. Strips a and b were stained with amido black; strips, 1, 2, 3, and 4 were immunostained with MAbs X2X5, 3CX4, 6FX1, and 21.18.1. MAb 21.18.1 (a negative control) has antigenic specificity to tetanus toxin.

binding assay, using ELISA. Plates coated with PT were first incubated with individual unlabeled MAbs and then reacted with biotin-conjugated MAb; binding of the biotinylated MAb was visualized with avidin-conjugated peroxidase. The competitive experiment was carried out in the linear portion of the binding curve of each labeled MAb. Results of this experiment are summarized in Fig. 2. Data are expressed as percent binding of biotin-conjugated MAb in the presence of an unlabeled MAb; binding in the presence of the negative control MAb (21.18.1) was taken as 100%. Another

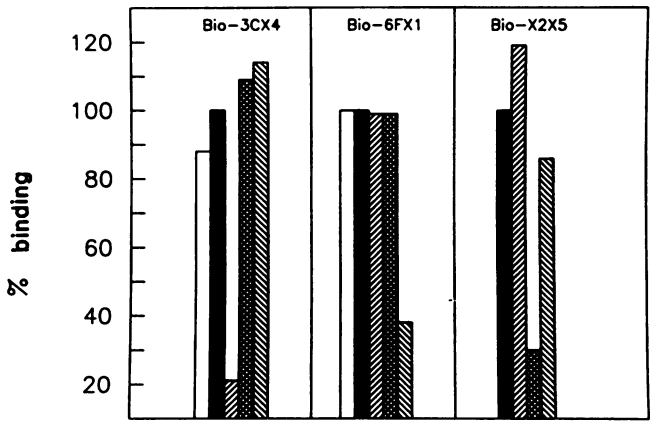

FIG. 2. Competitive inhibition of the binding of biotin-conjugated MAb to PT by various unlabeled MAbs. The ratio of biotinylated MAb to unlabeled MAb was 1:40. Percent binding was calculated relative to the degree of binding of biotin-conjugated MAb in the presence of control MAb 21.18.1. Normal mouse serum (1:10 in PBS) was also used as an additional control in some cases. Symbols: ▭, normal mouse serum; ■, 21.18.1; ▨, 3CX4; ▩, X2X5; ▧, 6FX1.

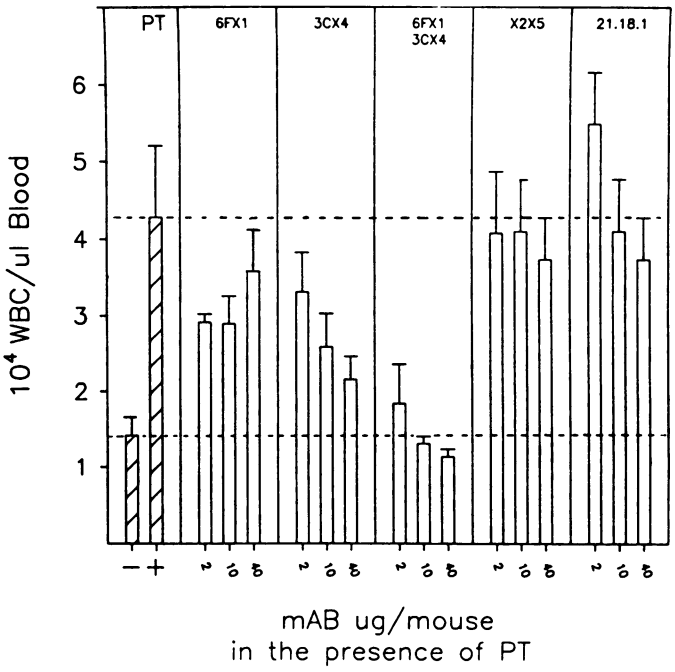

FIG. 3. Neutralization of LPF activity of PT by MAbs. Mice were injected with a mixture of PT (80 ng per mouse) and various amounts (2 to 40 μg) of individual MAbs. The experiment was carried out in quadruplicate. Striped bars show the negative control (PBS alone) and the positive control (PT alone). The combination of 6FX1 and 3CX4 was used at a ratio of 1:1; the value given for the amount of antibodies per mouse represents the total amount of the two MAbs. WBC, Leukocytes.

negative control, normal mouse serum, gave values similar to those obtained with MAb 21.18.1. In each case, the binding of the anti-S1 MAb was inhibited by the homologous MAb and not by heterologous MAbs: the percent binding of biotin-conjugated MAbs 3CX4, X2X5, and 6FX1 in the presence of homologous, underivatized MAbs was decreased to 21, 39, and 32%, respectively. The binding of all three MAbs in the presence of heterologous MAbs was in the range of 87 to 120%. Thus, we concluded that 3CX4, X2X5, and 6FX1 recognized different epitopes on PT. The same conclusion was also reached after we tested a higher ratio of unlabeled MAbs to biotin-conjugated MAbs (up to 200:1).

In vivo neutralization of PT LPF activity. PT has been shown to manifest many systemic effects in vivo, such as LPF activity (20), histamine sensitization (34, 36), and hypoglycemia (24). In the present study, we examined whether the anti-S1 MAbs can block the in vivo LPF activity of PT. Three days after mice were given mixtures of PT (80 ng per mouse) and various amounts of MAbs (2 to 40 μg per mouse), the number of leukocytes in the blood of each animal was counted (Fig. 3). In animals treated with PT alone, there was approximately a threefold increase in the number of leukocytes. There was a significant inhibition of the in vivo LPF activity of PT by preincubation of PT with MAbs 3CX4 and 6FX1. The degree of PT-mediated lymphocytosis in the presence of MAbs 3CX4 and 6FX1 was in the range of 21 to 68 and 50 to 89%, respectively, of the lymphocytosis response shown with PT only. As little as 2 μg of the combination of these two MAbs gave 85% inhibition of LPF activity of PT. Thus, it appears that these two MAbs could partially neutralize PT by themselves and

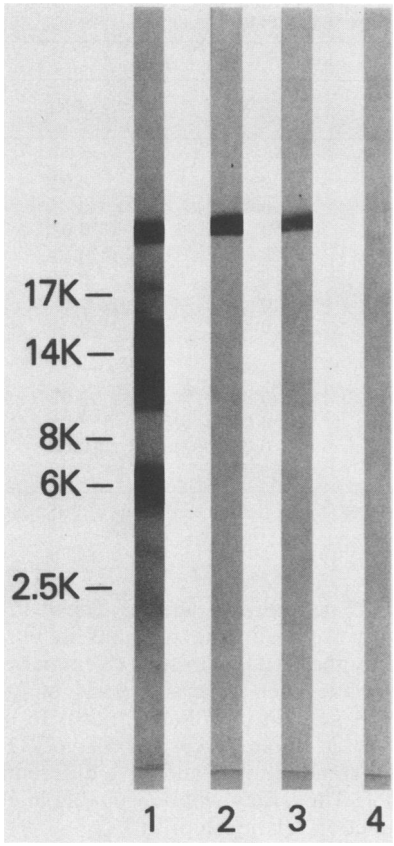

FIG. 4. Reactivity of MAbs to a partial tryptic digest of S1. Purified S1 subunit was digested with tolylsulfonylphenylalanyl chloromethyl ketone-trypsin at a ratio of 1:50 (wt/wt) for 2 h at 37°C, separated by SDS-PAGE, blotted onto NC paper, and reacted with MAbs (4 μg per strip): lane 1, X2X5; lane 2, 3CX4; lane 3, 6FX1; lane 4, control 21.18.1.

cooperated with each other in the neutralization of PT. In this particular experiment the neutralizing ability of MAb 6FX1 did not titrate well. However, in other experiments it gave a good titration effect. The LPF activity of PT in the presence of X2X5 and 21.18.1 (a negative control) was 82 to 97 and 97 to 117%, respectively, relative to the lymphocytosis response exhibited with PT alone. Since the LPF activity was measured at a suboptimal concentration of PT, the inability of X2X5 to neutralize LPF activity of PT was not due to the insufficient amount of X2X5 MAb used. Thus, we conclude that X2X5 MAb has no toxin-neutralizing ability by this assay.

Binding of MAbs to tryptic S1 peptides. To identify regions of the S1 subunit containing neutralizing epitopes, we examined the ability of our MAbs to bind to S1 tryptic peptides. Purified S1 protein was partially digested with trypsin, and peptides were separated by SDS-PAGE (27). Binding of the MAbs to the tryptic peptides was examined by Western blot analysis (Fig. 4). X2X5 bound to tryptic peptides of approximately 24, 18, 13, 11, 9, 5, 2.5, and 1.5 kDa; 3CX4 and 6FX1 bound only to the 24-kDa peptide and not to other low-molecular-weight peptides. Fragments smaller than 24 kDa appear to lack the specific conformation recognized by 3CX4 and 6FX1. These results suggest that X2X5 recognizes a linear epitope, while 3CX4 and 6FX1 may recognize conformational epitopes. However, we cannot exclude the possibility that 3CX4 and 6FX1 recognize a small peptide fragment which could not be resolved by SDS-PAGE or that the neutralizing epitope contains trypsin-hypersensitive arginine residues.

Binding of MAbs to truncated recombinant S1 molecules. To further identify the epitopes recognized by these MAbs, we analyzed their abilities to bind in Western blots to several recombinant truncated S1 molecules which were produced by recombinant expression in *E. coli* (10). Results of such an experiment are shown in Fig. 5. It should be pointed out that, since we used partially purified S1 recombinant products, there was some bacterial protein contamination which showed as faint bands. Furthermore, when MAb X2X5 was used for the immunostaining of recombinant S1 proteins, this MAb gave more than one distinct band with some recombinant proteins. This was thought to be due to the aggregation as well as the degradation of the particular recombinant S1 protein. Neutralizing MAb 3CX4 bound to truncated recombinant S1 molecule possessing residues 7 to 234 of the native sequence and not to the recombinant molecule possessing residues 15 to 234. These results suggest that residues 7 to 14 play an important role in the binding of the neutralizing MAb 3CX4. On the other hand, the binding of non-neutralizing MAb X2X5 was dependent on the presence of residues 17 to 26, as illustrated by its ability to bind to the truncated

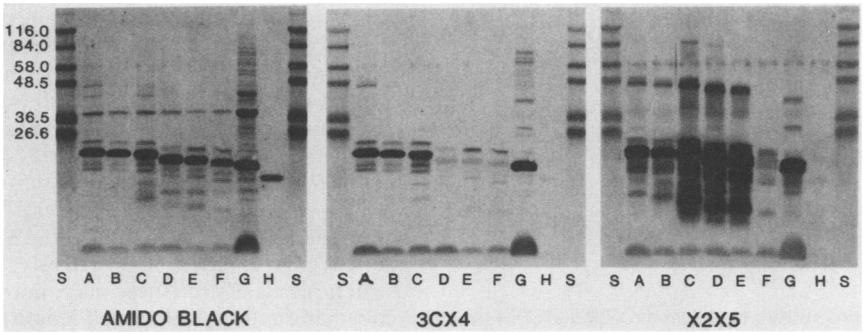

FIG. 5. Reactivity of MAbs to recombinant truncated S1 proteins. (Left) Amido black staining pattern of the truncated S1 molecules; (middle) immunoblot staining with 2 μg of MAb 3CX4; (right) immunoblot staining with 2 μg of MAb X2X5. Lane S, Prestained molecular weight markers; lane A, truncated S1 containing residues 3 to 234; lane B, residues 5 to 234; lane C, residues 7 to 234; lane D, residues 15 to 234; lane E, residues 17 to 234; lane F, residues 27 to 234; lane G, residues 1 to 187; lane H, residues 69 to 234. It should be noted that each truncated protein, by virtue of the expression vector construction (10), possesses a methionylvaline at the amino terminus; the truncated protein in lane H (residues 69 to 234) contains methionylalanylthreonine at its amino terminus.

TABLE 1. Determination of the binding ability of MAbs to peptides

Peptides[a]	Relative A_{405}				
	Polyclonal antibodies	21.18.1	3CX4	6FX1	X2X5
Expt 1					
6–17 (TVYRYDSRPPED)	0.79	0	0	0.02	0
27–39 (GNNDNVLDHLTGR)	1.64	0	0	0	0
44–58 (GSSNSAFVSTSSSRR)	1.47	0	0	0.02	0
65–79 (EHRMQEAVEAERAGR)	0.03	0	0	0	0
102–117 (SSYFEYVDTYGDNAGR)	1.46	0	0	0	0
168–182 (SQQTRANPNPYTSRR)	0.07	0.01	0	0	0.01
201–210 (MARQAESSEA)	1.43	0.01	0	0	0.01
Expt 2					
6–17 (TVYRYDSRPPED)	ND	0.03	0.04	0.07	0.02
6–20 (TVYRYDSRPPEDVF)	ND	0.03	0.04	0.06	0.14
11–26 (DSRPPEDVFQNGFTAW)	ND	0.02	0.02	0.06	0.84
16–30 (EDVFQNGFTAWGNND)	ND	0.01	0.03	0.06	0.8

[a] Peptides 65 to 79 and 168 to 182 were conjugated to keyhole limpet hemocyamin to coat plastic wells for ELISA. In experiment 1, PBS containing 0.15% Brij was used to block the nonspecific binding; in experiment 2, PBS containing 2% BSA was used.

S1 molecule composed of residues 17 to 234 but not to the recombinant molecule containing only residues 27 to 234. All three MAbs were able to bind to a truncated S1 recombinant protein composed of residues 1 to 187; this result confirmed that C-terminal amino acid residues 188 to 234 are not important for the binding of these MAbs in Western blots.

Binding of synthetic S1 peptides by MAbs. To localize more precisely epitopes recognized by these MAbs, peptides were synthesized based on their potential to possess antigenic determinants as predicted by hydrophilicity profiles (13). The binding of our MAbs to these peptides (amino acid residues 6 to 17, 6 to 20, 11 to 26, 16 to 30, 27 to 39, 44 to 58, 102 to 117, 168 to 182, and 201 to 210) was examined by ELISA. As positive controls, we included murine polyclonal antisera to each peptide raised by immunization of mice with peptide-BSA conjugates. Nonspecific binding sites were blocked with 0.1% Brij 35–PBS instead of BSA, since the positive control antisera would contain antibodies to BSA. Polyclonal antisera to each peptide bound significantly to the corresponding peptides, except peptides 65 to 79 and 168 to 182 (Table 1). The low binding of polyclonal antibodies to these two peptides was not due to the inability of the peptides to bind to the plastic wells since these peptides were conjugated to keyhole limpet hemocyanin. The non-neutralizing MAb X2X5 bound to peptides 11 to 26 and 16 to 30, while neutralizing MAbs 3CX4 and 6FX1 bound to neither these peptides nor any of the 45 overlapping 15-mers (1 to 15, 6 to 20, 11 to 25, 16 to 30, 21 to 35, etc.) covering the entire S1 subunit sequence (data not shown). From this experiment, we concluded that MAb X2X5 recognized a linear epitope within amino acid residues 11 to 30, the major portion of which is localized within residues 16 to 26. MAbs 3CX4 and 6FX1 did not react with any of the peptides.

DISCUSSION

In this study, we have shown that MAbs X2X5, 3CX4, and 6FX1, which are specific to the S1 subunit of PT, recognize different epitopes. MAbs 3CX4 and 6FX1 weakly neutralize the in vivo LPF activity of PT; however, when used together, they neutralized PT very effectively. A 10-μg amount of a mixture of the two neutralizing MAbs was sufficient to completely inhibit the LPF activity of PT. In contrast, MAb X2X5 had no neutralizing ability. These in vivo results correlate very well with the previous in vitro findings as determined by the CHO cell assay (Kenimer et al., submitted). There have been several reports of MAbs reactive against the S1 subunit of PT (1, 25, 28). In our study, we have shown that three MAbs, 3CX4, 6FX1, and X2X5, recognize different epitopes and have differential abilities to neutralize PT. Thus, they will be valuable in elucidating the structure-function relationships in PT.

Using these MAbs, we investigated the neutralizing epitopes on the S1 subunit utilizing tryptic peptides of the S1 protein, recombinant truncated S1 species, and synthetic S1 peptides. The non-neutralizing MAb X2X5 was shown to bind peptides as small as 1.5 kDa, suggesting that it recognizes a linear epitope; studies with S1 truncated molecules and synthetic S1 peptides confirmed the linearity of this antigenic determinant and localized the major portion of its epitope to residues 16 to 26 in the S1 protein.

The 24-kDa degradation product of the S1 subunit was recognized by each of the three MAbs we studied. Since the C-terminal portion of the S1 subunit has been reported to be the most susceptible to proteolysis (8), the 24-kDa molecule most likely represents an amino-terminal polypeptide of the S1 subunit missing approximately 4 kDa of its carboxyl terminus. These results imply that the C-terminal amino acids are not critical for the binding of these MAbs; this was further confirmed by the results obtained with the S1 truncated molecules (Fig. 5). This does not rule out the possibility that portions of the carboxyl teminus contribute to other discontinuous epitopes, all of which may be needed to elicit the neutralizing antibody response.

It has been shown previously that S1 protein blotted onto NC paper retains enzymatic activity (8), suggesting that even after boiling and SDS treatment the S1 subunit may regain its active conformation; alternatively, secondary or tertiary structural characteristics may not be necessary for the enzymatic activity. When full-length S1 subunit was digested to fragments of <24 kDa, neutralizing MAbs 3CX4 and 6FX1 no longer bound to such fragments. Thus, tryptic digestion may have destroyed the epitopes recognized by our neutralizing antibodies. Supporting data were obtained from the experiments performed with recombinant truncated S1 molecules and with synthetic peptides. By using truncated S1

molecules, residues 7 to 14 were shown to be necessary for binding of neutralizing MAb 3CX4. The importance of this region for the enzymatic activity of PT and the maintenance of an immunoprotective epitope has been reported recently by Cieplak et al. (10). However, neither MAb 3CX4 nor 6FX1 bound to any of the S1 synthetic peptides, including peptides 1 to 15, 6 to 17, 6 to 20, and 11 to 26, suggesting that these neutralizing MAbs recognize conformational epitopes in the regions including residues 7 to 14. A similar conclusion was reached by Bartoloni et al., who used recombinant fusion S1 proteins (2). The recognition of conformational epitopes by MAbs has been amply described in the literature (3); thus, our findings are not surprising.

It is anticipated that these studies will lead to a better understanding of the antigenic structure of PT. Such knowledge will permit the rational design and development of acellular vaccines for protection against whooping cough.

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