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Functional Analysis of Pneumolysin by Use of Monoclonal Antibodies

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We have produced a panel of monoclonal antibodies to pneumolysin, the membrane-damaging toxin from *Streptococcus pneumoniae*. We have used these antibodies to identify three regions of the toxin sequence that are involved in the lytic mechanism of this toxin. Two of these sites probably form the cell binding site of this toxin. Antibodies to the third site inhibit the lytic action of this toxin but not the binding of this toxin to cells. This site is engaged in the oligomerization process involved in the formation of pores in cell membranes. Two of these epitopes are also present in the related toxin perfringolysin O.

Pneumolysin belongs to a family of membrane-damaging toxins produced by four genera of gram-positive bacteria. These toxins can lyse all cells with cholesterol in their plasma membranes (18). At sublytic concentrations, pneumolysin is known to have several effects on cells of the immune system, such as the ability to inhibit the respiratory burst of neutrophils and antibody synthesis by B cells (3).

The anticellular activity of this group of toxins is believed to be linked to the ability of these proteins to form pores in eukaryotic cell membranes. Analysis of the relationship between the structure and function of pneumolysin by site-directed mutagenesis and random mutagenesis has revealed several regions of the molecule involved in pore formation by this toxin (3). However, functionally related regions of the molecule still remain to be identified.

Pneumolysin has been shown to play a role in the virulence of *Streptococcus pneumoniae* in animal models of infection (14). The immunization of mice with genetically altered versions of this toxin (toxoid) conferred protection against challenge with virulent pneumococci of a range of capsular serotypes (1). The construction of isogenic pneumolysin-negative mutants of this pneumococcus caused the virulence of the organism to be reduced in animal models of infection (2). The demonstration of pneumolysin as a protective immunogen and of its role in virulence has led to studies of the possibility of including this protein in a new pneumococcal vaccine.

By using a mouse model of pneumococcal infection, it has also been shown that animals presenting with high-level bacteremia die rapidly after treatment with penicillin even though the organisms are eliminated from the blood (5). The role of the pneumococcal cell wall in inflammation and the pathology of pneumococcal infection has been well-defined (19). By using an isogenic pneumolysin-negative mutant of this pneumococcus in an animal model of pneumococcal pneumonia, it has been shown that this toxin is involved in the mortality of the disease (2). Using the same mutant, we have shown that pneumolysin mediates acute pulmonary injury and inflammation during experimental pneumonia (4, 16). Such results suggest that the release of toxin from pneumococci during penicillin therapy could have an adverse effect on the host.

With these facts to mind, we have raised monoclonal antibodies to pneumolysin. Several of these antibodies neutralize this toxin in in vitro assays. We have identified the regions of this toxin recognized by these antibodies. These antibodies have also allowed further studies of the relationship between the structure and mode of action of pneumolysin.

MATERIALS AND METHODS

Antigens. Recombinant pneumolysin was expressed in *Escherichia coli* and purified from cell extracts as previously described (9). Protein homogeneity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Perfringolysin O (PFO) was kindly provided by Y. Ohno-Iwashita (Tokyo Institute of Gerontology, Tokyo, Japan)

Antibody production. BALB/c mice were purchased from Letica (Barcelona, Spain). Ten males and ten females between 3 and 6 weeks of age were immunized as follows. All mice received an initial dose of 10 micrograms of toxin in 200 μl of complete Freund's adjuvant by the intraperitoneal route. On days 7 and 14 from the initial injection, animals received 10 and 20 μg of toxin, respectively, in 200 μl Freund's incomplete adjuvant by the intraperitoneal route. On day 21, all mice were given 20 μg of pneumolysin in sterile saline via the intravenous route.

One week after the final booster, all mice were bled from the tail vein, and antibody production was measured by enzyme-linked immunosorbent assay (ELISA) with preimmune serum as a control. All animals had titers of between 1/2,000 and 1/20,000. The mice with the highest titers were selected for monoclonal antibody production. Three days before the fusion experiment, animals received a final boost of 10 μg of pneumolysin in saline via the intravenous route. When animals were sacrificed, blood samples were kept for analysis of antibody levels.

Splenocytes from immunized animals were fused to Sp2/0 cells at a ratio of 1:1 by standard methods (8). Hybrids were selected in hypoxanthine-aminopterin-thymidine medium. To improve hybridoma growth, a 1:1 mixture of RPMI 1640 and Hybridoma Medium High Protein (Gibco) supplemented with 10% (vol/vol) fetal calf serum was used. Hybridomas of interest were subcloned twice by limiting dilution and finally adapted for growth in serum-free medium by use of DCCM-2 (Biological Industries) or PFHM-II (Gibco) medium.

Monoclonal antibodies from positive hybridomas were isotyped by direct ELISA, as described below, on pneumolysin-coated plates by using a kit supplied by Boehringer Mannheim.

Immunoglobulins G (IgGs) were prepared from supernatants by using either AFFI-T columns (Kem-En-Tec) or affinity chromatography on protein A fast-flow columns (Pharmacia). The purity and reactivity of fractions were assessed by SDS-PAGE and ELISA, respectively.

Anti-keyhole limpet hemocyanin monoclonal antibody [IgG1(κ)] prepared by us and MOPC-141 [IgG2b(κ)] from Sigma were used as negative controls in all assays.

Direct ELISA. An ELISA was used for the screening of mouse blood samples, hybridoma supernatants, and final antibody preparations. Flat-bottomed 96-well polystyrene enzyme immunoassay plates (Costar) were coated with 100 μl of a 10-μg/ml solution of the appropriate antigen in 50 mM carbonate buffer (pH 9.6) and incubated at 37°C for 6 h. Plates were left for 1 h at 37°C and overnight at

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4°C with 200 μl of blocking solution (1% bovine serum albumin [BSA] in phosphate-buffered saline [PBS]). Plates were washed in PBS–0.01% Tween 20 (PBS-T), 100 μl of test sample was added, and plates were incubated at 37°C for 3 h. Plates were washed again, class-specific goat anti-mouse immunoglobulin conjugated with horseradish peroxidase (Sigma) (100 μl at a dilution of 1/2,000 for IgM and IgA and IgA and 1/5,000 for IgG in PBS-T) was added, and plates were incubated for 3 h at 37°C. After being washed, 100 μl of substrate (3.4 mg of *O*-phenylenediamine [Sigma] with 0.02% [wt/vol] hydrogen peroxide in 10 ml of 50 mM phosphate-citrate buffer [pH 5.0]) was added and plates were incubated at 37°C for 30 min in the dark. Plates were read at 492nm on an SLT 340 ATC plate reader (SLT Instruments, Linz, Austria). Wells with isotype-matched controls were used as blanks. All determinations were made in triplicate.

Estimation of relative antibody affinity. The method of Rath et al. (15) was used to compare the relative affinities of the binding of monoclonal antibodies. Briefly, wells of an ELISA plate were coated with 1 μg of toxin, and the titer of each monoclonal antibody was determined to find the dilution giving half the maximal optical density value. This amount of antibody was mixed with free pneumolysin at 10^{-6} to 10^{-14} M concentrations, and the mixture was added to the well of an ELISA plate previously coated with pneumolysin and blocked as described above. Plates were incubated for 3 h at 37°C, and the amount of antibody bound was determined with secondary conjugated antibody as described above.

The concentration of pneumolysin required to inhibit the binding of each monoclonal antibody by 50% was calculated by using a polynomic fitted curve produced by CRVPLOT version 5.22 (Simply Software, Firfield, Ohio). This value was considered the relative affinity of that antibody.

Cross-inhibition ELISA. Monoclonal antibodies were grouped on the basis of cross-inhibition ELISA studies.

Monoclonal antibodies were biotinylated by standard procedures (12), and titers against pneumolysin were determined as described above.

Plates were coated with pneumolysin (1 μg per well) and blocked with BSA as described above. One hundred microliters of 1/10 serial dilutions of inhibitor monoclonal antibody, starting at 10 μg per well, was added, and plates were incubated at 37°C for 2 h. Then 100 μl of biotinylated monoclonal antibody was incubated for another 2 h at 37°C. The binding of the labelled antibody to pneumolysin was later estimated by incubation with horseradish peroxidase conjugated to streptavidin (Pierce) at a dilution of 1/10,000 for 2 h at 37°C. Washings and color development were carried out as described above for direct ELISA.

Monoclonal antibodies were challenged against each other both ways, except for PLY-3 and PLY-10, whose biotinylated derivatives did not perform well.

The absorbances stated correspond to those measured after the subtraction of blank mean optical density. For each antibody, the inhibition-positive control was provided by the biotinylated antibody challenged by 10 μg of the same unlabelled preparation. Wells into which no inhibitory antibody was added were considered inhibition-negative controls; thus, their mean optical density corresponds to 100% binding of the labelled antibody. The percent inhibition afforded by any biotinylated antibody was calculated with reference to this absorbance.

Antibodies were considered to bind to the same region of pneumolysin and therefore to be members of the same complementation group if the inhibition was more than 50%.

Western blotting (immunoblotting). Purified pneumolysin (500 ng per track) was run on SDS-PAGE gels and transferred to nitrocellulose by standard techniques. Strips of nitrocellulose were cut and blocked by incubation at room temperature in 3% BSA in PBS for 1 h. After being washed, blots were successively incubated with 5 μg of antibody, 3.75 U of sheep anti-mouse immunoglobulin-alkaline phosphatase Fab fragments (Boehringer Mannheim), and bromochloroindolyl phosphate (BCIP)-nitroblue tetrazolium substrate in alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris-HCl [pH 9.6]). Plates were washed three times with PBS-T between incubations. A triple final wash with 50 mM Tris-HCl (pH 9.6) buffer was made just before the addition of substrate solution. Color development was stopped by washing in tap water, and all tests were done in triplicate.

Inhibition of hemolytic activity. Toxin neutralization assays were done on standard U-bottomed microtiter plates. The same assay procedure was used for both pneumolysin and PFO. The hemolytic activities of toxin batches were assessed immediately before the neutralization test (9). The end point of this assay was taken as the well in which 50% of erythrocytes were lysed. A stock toxin solution in PBS, corresponding to twice this end point, was prepared. For neutralization tests, 25 μl of serial twofold dilutions of antibodies were added across 12 wells. The first well contained 1 μg of antibody. Twenty-five microliters of stock toxin solution (equivalent to 1 hemolytic unit) was added to each well, and plates were incubated at 37°C for 30 min. Fifty microliters of 1.6% sheep erythrocytes in PBS was added, and plates were incubated at 37°C for 30 min. The titer of antibody was taken as the highest dilution of antibody which completely inhibited hemolysis. All tests were done in triplicate.

Inhibition of binding of toxin to erythrocytes. A similar assay procedure was used to measure the binding of both pneumolysin and PFO to erythrocyte membranes. Dilutions of toxin, from 260 to 3 ng/ml, were made in 1.5 ml of Hanks buffered saline (GibcoBRL). Monoclonal antibody (1 μg) was added, and tubes were incubated at 37°C for 1 h and then cooled to 4°C when 1.5 ml of 2% sheep erythrocytes at 4°C was added. After a further incubation at 4°C for 30 min, cells were washed three times in ice-cold Hanks buffered saline and then lysed in water. Membranes were harvested, washed twice in water by centrifugation at 10,000 × *g* in a microcentrifuge, and then resuspended in SDS-PAGE loading buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated in 5% (wt/vol) skim milk powder in PBS for 60 min, washed in PBS, and incubated with 1/1,000 dilution of rabbit anti-pneumolysin antiserum (9) in PBS for 90 min (this polyclonal antiserum also recognizes PFO). Membranes were washed, incubated for 60 min in 1/2,000 goat anti-rabbit antiserum (Sigma), and then extensively washed in PBS. The proteins recognized by anti-pneumolysin antiserum were visualized with enhanced chemiluminescence reagents (Amersham) according to the manufacturer's instructions.

Inhibition of pore formation in liposomes. Toxin was preincubated with antibodies as described above for the neutralization of binding. After incubation, toxin was interacted with liposomes, and pores were observed by transmission electron microscopy as previously described (10).

Analysis of toxin binding by flow cytometry. Flow cytometry was used to determine how the binding of pneumolysin to erythrocytes affected reactivity with monoclonal antibodies.

In all assays, 1 μg of monoclonal antibody was reacted with 16 hemolytic units of pneumolysin. Mouse polyclonal antibody to pneumolysin was used as a positive control. Six duplicate wells were prepared as described above for the neutralization of hemolytic activity. The cells from all six wells were collected, washed in PBS–5% BSA, and resuspended in 50 μl of PBS–5% BSA. Five micrograms of fluorescein isothiocyanate (FITC)-conjugated anti-mouse γ chain (Sigma) was added, and the mixture was incubated for 1 h on ice. Cells were washed at 4°C in PBS–5% BSA and finally fixed in 150 μl of 1% paraformaldehyde in PBS. Cells were stored at 4°C prior to analysis by flow cytometry on a FACScan (Becton Dickinson). Cells to which no toxin had been added were used to establish a fluorescence background. For each sample, 50,000 events were recorded, and data were processed by using LYSIS II version 1.1 software (Becton Dickinson).

In a second series of experiments, erythrocytes were incubated on ice with this toxin for 1 h prior to the addition of monoclonal antibody. Hemolysis and fluorescence-activated cell sorter (FACS) analysis were done after interaction with monoclonal antibody for 1 h on ice.

Reaction of monoclonal antibodies with truncated versions of pneumolysin. To locate regions of the pneumolysin molecule recognized by selected monoclonal antibodies, such antibodies were tested for the ability to recognize pneumolysin molecules in which increasing amounts of the C terminus of the protein had been deleted by manipulation of the pneumolysin gene (13). Crude extracts of *E. coli* cells expressing shortened versions of the pneumolysin molecule fused to glutathione *S*-transferase (GST) were prepared as previously described. These extracts were subjected to SDS-PAGE and subsequent Western blotting as described above for binding experiments. Then monoclonal antibodies were tested for the ability to recognize truncates by using the full-length pneumolysin molecule expressed as a GST fusion as a positive control.

Reaction of monoclonal antibodies with proteolytically nicked toxin. Samples of pneumolysin monomer (1 mg/ml) were digested with proteinase K at 25°C. The toxin/enzyme ratio was 20:1 (wt/wt). The enzyme was inactivated with phenylmethanesulfonyl fluoride after 30 min. Fragments from the digest were purified by anion-exchange chromatography (MonoQ; Pharmacia). These fragments and intact toxin were probed with monoclonal antibodies by Western blotting, as described above, to determine which fragments were recognized. Fragments were also N-terminally sequenced to determine the exact point of cleavage.

RESULTS

Antibody production. We have obtained a panel of purified monoclonal antibodies which react with pneumolysin in both ELISAs and Western blot assays. The majority of these antibodies are IgG, although one is IgM and another is IgA. Subsequent discussions of results concentrate on those for IgG monoclonal antibodies (Table 1).

Characterization of monoclonal antibodies. All of the IgG antibodies produced reacted with pneumolysin in Western blot assays, suggesting that they recognize linear epitopes. Antibodies PLY-4 and PLY-5 also recognize PFO on Western blots.

By means of a cross-inhibition ELISA, we have established that these antibodies fall into five groups (Table 2). The inhibition percentages given are those obtained with 10 μg of inhibitor antibody, at which the binding of biotinylated antibody was completely inhibited by unlabelled antibody. Antibodies PLY-5 and PLY-6 recognize the same epitope, as they inhibit each other and also behave similarly in relation to the rest of the monoclonal antibodies. Antibody PLY-8 blocks the binding of PLY-4, PLY-5, and PLY-6, but we did not observe the reverse effect, possibly because of the different affinities of

TABLE 1. Characteristics of monoclonal antibodies to pneumolysin

Antibody	Subisotype	Neutralizing ability[a]
Control	IgG1(κ)	—
PLY-1	IgG1(κ)	>1,000
PLY-2	IgG1(κ)	250
PLY-3	IgG1(κ)	31.2
PLY-4	IgG1(κ)	<0.5
PLY-5	IgG1(κ)	1
PLY-6	IgG1(κ)	0.5
PLY-7	IgG1(κ)	31.2
PLY-8	IgG1(κ)	—
PLY-9	IgG2b(κ)	—

[a] Neutralizing ability is expressed in the nanograms of antibody required to neutralize 1 hemolytic unit of pneumolysin. Data are the means of three experiments. —, nonneutralizing.

these antibodies. As PLY-8 has no neutralizing activity (Table 1) compared with those of PLY-4 and PLY-5, the blocking effect of preincubation with PLY-8 on the latter antibodies may be due to steric hindrance. The preincubation of pneumolysin with antibody PLY-7 induces a large positive modulation on the subsequent binding of PLY-5, although the reverse reaction does not occur.

Reactions of monoclonal antibodies with truncated versions of pneumolysin. In an attempt to further characterize these monoclonal antibodies, the reactions of these five groups were tested by using a series of truncated versions of the pneumolysin molecule. Truncated versions of the pneumolysin molecule had been constructed as part of a previous study in which shortened versions of this toxin were expressed as fusion proteins with GST to facilitate purification. In the present study, the reactions of monoclonal antibodies with crude extracts of *E. coli* cells expressing these constructs were tested (Table 3). As expected, all the antibodies tested recognized the full-length molecule (471 amino acids) fused to GST. There was no reaction with *E. coli* expressing GST alone. Antibody PLY-5 (group II) appears to recognize an epitope on the extreme C-terminal end of pneumolysin. This antibody is also highly neutralizing, indicating that this region of the molecule is involved in the action of this toxin. Further support for the role of this region in toxin activity came from studies using truncated versions of this toxin. A version of this toxin lacking the 6 C-terminal amino acids had greatly reduced hemolytic activity and reduced ability to bind to cells (13). PLY-7 (group III) recognizes an epitope N terminal to amino acid 419. Since the reaction with the pneumolysin molecule truncated at amino acid 419 is much weaker than those with longer constructs, it is possible that the epitope concerned is at or around this amino acid. The group IV antibody PLY-8 is similar to PLY-5 in that it recognizes only the 6 C-terminal amino acids of pneumolysin. However, it has a different specificity on the basis of cross-inhibition ELISA results and is also nonneutralizing.

Reactions of monoclonal antibodies with proteolytically-nicked pneumolysin. Proteinase K digestion of pneumolysin resulted in two major fragments which were found to coelute on an anion-exchange chromatography column, suggesting that the toxin had been nicked. These fragments were separated by SDS-PAGE and found to have apparent molecular masses of 37 and 15 kDa. The two fragments and intact toxin were probed with monoclonal antibodies to determine which fragments were recognized. All of the antibodies, except for PLY-4, recognized the 37-kDa fragment (Table 3). N-terminal amino acid sequencing of this fragment gave the sequence Asn-Val-Pro-Ala. This sequence corresponds to amino acids 143 to 146; therefore, the 15-kDa fragment is the 142 N-terminal amino acids of pneumolysin. No monoclonal antibody recognized the N-terminal 15-kDa fragment. Antibody PLY-4 recognized only the intact toxin. It did not recognize either the 15- or 37-kDa fragment.

Effects of monoclonal antibodies on toxin binding to erythrocytes. Monoclonal antibodies were tested for the ability to block the binding of pneumolysin to erythrocytes in a semiquantitative binding assay. Two antibodies, PLY-5 and PLY-7, were found to inhibit this binding.

Effects of monoclonal antibodies on toxin oligomerization. We have previously shown that toxin pores can be visualized on liposomes by electron microscopy (10). The preincubation of this toxin with PLY-4 prevented the formation of these pores. While numerous pores were seen with control toxin, only a single structure was observed by electron microscopy for liposomes treated with toxin that had been preincubated with PLY-4. An analysis of pore formation by toxin neutralized with PLY-5 or PLY-7 was not done, as these antibodies prevent this toxin from binding to the membrane.

Analysis of toxin interaction with erythrocytes by using flow cytometry and monoclonal antibodies. After mixing pneumolysin with anti-pneumolysin monoclonal antibodies, erythrocytes were added and cell-associated antibody was detected with FITC-labelled anti-mouse antibodies. The binding of the FITC conjugate was detected by FACS analysis. The results of these studies are shown in Fig. 1.

When pneumolysin was preincubated with PLY-5 or PLY-7, no fluorescent labelling of cells was observed. This is consistent with PLY-5 and PLY-7 inhibition of the binding of this toxin to erythrocytes. When pneumolysin was bound to cells prior to the addition of monoclonal antibodies PLY-5 and PLY-7, again no binding of the FITC conjugate was detected by FACS. This implies that the epitopes recognized by PLY-5 and PLY-7 become hidden when the toxin is associated with cells. The epitope recognized by PLY-8 also appears to become hidden after this toxin binds to cells. Similar experiments with PLY-4 and PLY-9 showed that these antibodies recognize this toxin in its cell-associated form, indicating that the epitopes recognized by these antibodies remain exposed. When pneumolysin was preincubated with a mixture of antibodies PLY-9 and PLY-5 or PLY-7, no labelling of cells was observed. This observation is consistent with PLY-5 and PLY-7 inhibition of cell binding by pneumolysin. In contrast, cells treated with pneumolysin preincubated with a mixture of PLY-9 and PLY-4 were labelled. In summary, PLY-5 and PLY-7 block cell binding by this toxin and the epitopes recognized by these antibodies become hidden after the interaction of this toxin with cells.

TABLE 2. Cross-inhibition ELISA[a]

Inhibitor antibody	% Binding with antibody (group)				
	PLY-4 (I)	PLY-5 (II)	PLY-7 (III)	PLY-8 (IV)	PLY-9 (V)
PLY-3	0	134	107	100	89
PLY-4	0	112	109	96	65
PLY-5	66	0	81	76	80
PLY-6	75	0	82	86	78
PLY-7	91	1,242	0	79	145
PLY-8	30	0	87	0	70
PLY-9	30	69	103	108	0
PLY-10	104	112	106	99	76
None	100	100	100	100	100

[a] Plates were coated with pneumolysin and incubated with the inhibitor antibody. The binding of a second biotinylated antibody was then measured.

TABLE 3. Reactions of monoclonal antibodies with truncated versions of pneumolysin and PFO

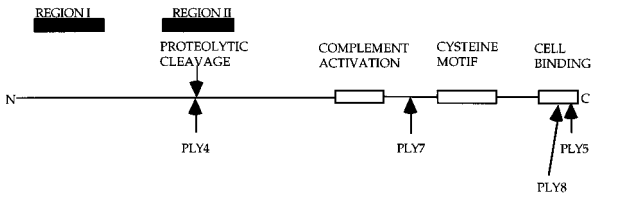
Antibody	Group	Neutralizing ability[a]	Reaction with[b]:							
			Pneumolysin residues							PFO
			1–142[c]	1–419	1–430	1–441	1–450	1–465	1–471	
PLY-4	I	+++	−	+	+	+	+	+	+	+
PLY-5	II	+++	−	−	−	−	−	±	+	+
PLY-7	III	+	−	±	+	+	+	+	+	−
PLY-8	IV	−	−	−	−	−	−	±	+	−
PLY-9	V	−	−	+	+	+	+	+	+	−
Polyclonal mouse serum		+++	+	+	+	+	+	+	+	+

[a] Scale is from highest neutralizing ability (+++) to nonneutralizing (−).
[b] +, reaction; ±, weak reaction; −, no reaction.
[c] Measured by reaction with the N-terminal fragment generated by proteolytic cleavage.

DISCUSSION

We have produced a panel of monoclonal antibodies to the membrane-damaging toxin pneumolysin. We have characterized the IgG monoclonal antibodies produced (Fig. 1). All the antibodies tested reacted with linear epitopes within the toxin sequence, as judged by the ability to react with denatured antigen in Western blot assays. Two antibodies (PLY-4 and PLY-5) also recognized PFO in Western blot assays. We detected no cross-reaction with listeriolysin O in Western blot assays (data not shown). Monoclonal antibodies to listeriolysin O have also been produced (11). In this study, antibodies which recognized both listeriolysin O and pneumolysin were produced, indicating that these two proteins share some epitopes. Antibodies to these cross-reacting epitopes were not detected in our study.

Antibodies were tested by cross-inhibition ELISA to determine whether they were derived from the same clone or recognized the same epitope on this toxin. On the basis of these studies, antibodies were divided into five groups. When representative members of each of the five groups were tested for the ability to react with truncated versions of the pneumolysin molecule, several interesting features emerged. Antibody PLY-4 (group I) is highly neutralizing, and results from the reaction of this antibody with proteolytically nicked toxin suggest that the point of interaction is around residue 142 or 143 of pneumolysin, as discussed below. Antibody PLY-5 (group II) is also highly neutralizing, recognizing the full-length molecule but interacting weakly with the molecule lacking the 6 C-terminal amino acids. Thus, a second neutralizing epitope must exist within the last 6 C-terminal amino acids of this protein. Both group I and group II antibodies also recognize PFO. On the basis of observations with PLY-7, a third neutralizing epitope around residue 419 may exist. Alternatively, on the basis of the positive modulation effect of PLY-7 on the binding of PLY-5 discussed above, it may be that PLY-7 alters the conformation of the native molecule in such a way that the orientation of another important epitope (namely, the one recognized by PLY-5) is altered. The epitope recognized by group IV antibody PLY-8 also appears to be in the last 6 amino acids of the pneumolysin sequence. However, this antibody is not neutralizing and does not recognize PFO. It is therefore different from PLY-5, which also recognizes this region of the molecule. The group V antibody PLY-9 recognizes an epitope between residues 142 and 419 and is nonneutralizing.



Antibody	Neutralising	Inhibition of binding	Inhibition of oligomerisation	Epitope exposed after binding
PLY-4	+	-	+	+
PLY-5	+	+	ND	-
PLY-7	+	+	ND	-
PLY-8	-	-	-	-

FIG. 1. Summary of the functional effects of monoclonal antibodies. Open boxes indicate functional regions of this toxin (14). Filled boxes indicate the two major epitopes identified by epitope mapping (17). ND, not done (these antibodies block binding to the cell membrane).

Analysis of the binding of antibodies to proteolytic fragments of pneumolysin shows that all of the antibodies except for PLY-4 recognize the C-terminal 37-kDa region of this toxin. Antibody PLY-4 recognized only the intact toxin (it recognized neither cleavage product). The epitope for this antibody is therefore most likely to span this site in this toxin. This site has been identified by N-terminal sequencing (Asn-Val-Pro-Ala) and shown to be between two asparagine residues at positions 142 and 143; thus, the N-terminal fragment consists of amino acids 1 to 142. The epitope recognized by PLY-4 is a neutralizing epitope and common to PFO. A previous study that mapped immunogenic epitopes of pneumolysin by using synthetic peptides (17) identified two immunoreactive sites at the amino-terminal end of the molecule. That study predicted two major antigenic sites in the N-terminal region of pneumolysin. One of these antigenic sites runs from amino acids 131 to 152. The proteolytic cleavage site is therefore in the center of this epitope. Therefore, we propose that PLY-4 recognizes the epitope predicted from studies with synthetic peptides (17). PLY-4 does not inhibit the binding of this toxin to cells and must therefore inhibit some other stage in the action of this toxin. Analysis of the ability of this toxin to oligomerize to form pores in liposomes showed that neutralization with PLY-4 blocked the assembly of these pores. Therefore, a functional site at around residue 142 is involved in the oligomerization process. A similar situation has been described for monoclonal antibodies to streptolysin O. Some antibodies were shown to block the lytic step but not toxin binding, which was inhibited by another type of antibodies (6).

Assays of the effects of monoclonal antibodies on the ability of this toxin to bind to erythrocytes showed that preincubation with PLY-5 or PLY-7 abolished the ability of this toxin to bind to cells. The inhibition of toxin binding by PLY-5 suggests that the epitope recognized by this antibody in the extreme C ter-

minus of the molecule is involved in cell binding. This confirms our previous finding, in which we showed that the pneumolysin molecule truncated at residue 465 was unable to bind to cells (13). However, antibody PLY-8 also recognizes this region of the molecule but does not neutralize or prevent the binding of this toxin to cells. Therefore, we propose that there are two epitopes very close to the C terminus of the pneumolysin molecule. The 7 C-terminal amino acids of pneumolysin are EDKVEND. This region is highly charged (five of seven residues) and predicted by Jameson and Wolf (7) to be antigenic. As the results from interactions with truncates demonstrated that the linear epitopes recognized PLY-5 and PLY-7 are both different and well separated, this finding raises several possibilities. First, this toxin may have two cell membrane binding sites. Secondly, the linear epitopes recognized by PLY-5 and PLY-7 may form a single binding site when pneumolysin is in its native, folded conformation. If these two sites are adjacent in the native conformation, it is possible that the binding of one antibody affects the binding of the second. When PLY-7 was bound to this toxin first, a large positive modulation of the binding of PLY-5 was observed. Positive modulation was not seen when PLY-5 was added before PLY-7. If the sites recognized by PLY-5 and PLY-7 constitute the cell binding site of this toxin, we can postulate that the binding of PLY-7, which recognizes a region around amino acid residue 419, alters the conformation of this site in such a way that the exposure of the site for PLY-5 (the 6 C-terminal amino acids) is increased. This may be possible because the epitope for PLY-5 is on the extreme C terminus of the protein, which may be more mobile. However, it is interesting that the binding of PLY-7 has little effect on the subsequent binding of PLY-8, which also recognizes the C-terminal end of this protein. FACS analysis also provides support that the PLY-5 and PLY-7 epitopes are involved in the cell binding site of this toxin. This analysis showed that when antibody was added to this toxin before cells, binding was inhibited. However, when toxin was bound to cells before PLY-5 or PLY-7 was added, antibody was unable to bind to this toxin, showing that this region of the toxin had become hidden. The epitope for PLY-8 also becomes hidden, which may be expected if it too is on the C-terminal end of this protein, close to the epitope for PLY-5. This toxin was detected on cells with other monoclonal antibodies, such as PLY-4 and PLY-9. It would not be surprising if antibodies directed at the cell binding site could no longer bind to this toxin after it had interacted with its target.

As antibody PLY-5 also recognizes a linear epitope on PFO, a similar epitope should be present in this protein. These proteins have about 45% sequence homology at the amino acid level. A comparison of the C-terminal regions of these toxins shows little primary sequence homology in this region. This region is associated with cell binding in the case of pneumolysin. Antibody PLY-5 inhibits the binding of PFO to erythrocytes, indicating that the similar epitope in PFO is involved in the binding of this toxin to cells.

To summarize, we have determined that there are at least three epitopes which elicit antibodies capable of neutralizing the lytic action of pneumolysin. Two of these, one in the 6 C-terminal amino acids and the other around residue 419, appear to be in the cell binding site of this toxin. A third site not involved in cell binding is destroyed by proteolytic nicking of this toxin between amino acids 142 and 143. This site is involved in the ability of this toxin to oligomerize in cell membranes.

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