Characterization of lethal factor binding and cell receptor binding domains of protective antigen of *Bacillus anthracis* using monoclonal antibodies

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Lethal toxin from *Bacillus anthracis* is composed of protective antigen (PA) and lethal factor (LF). Anti-PA mAbs that neutralized lethal toxin activity, either *in vivo* or *in vitro*, identified three non-overlapping antigenic regions on PA. Two distinct antigenic regions were recognized by the four mAbs that neutralized lethal toxin activity by inhibiting the binding of ¹²⁵I-LF to cell-bound PA. Mapping showed that one mAb, 1G3_{PA63}, recognized an epitope on a 17 kDa fragment located between amino acid residues Ser-168 and Phe-314. The three other mAbs, 2D3_{PA}, 2D5_{PA} and 10D2_{PA}, recognized an epitope between amino acids Ile-581 and Asn-601. A single antigenic region was recognized by the three mAbs, 3B6_{PA}, 14B7_{PA} and 10E10_{PA63}, that inhibited binding of ¹²⁵I-PA to cells. This region was located between amino acids Asp-671 and Ile-721. These results confirm previously defined functional domains of PA and suggest that LF may interact with two different sites on PA to form lethal toxin.

Keywords: *Bacillus anthracis*, protective antigen, epitope mapping

INTRODUCTION

Bacillus anthracis produces two protein exotoxins. Protective antigen (PA), combined with either lethal factor (LF) or oedema factor (EF), constitutes lethal toxin or oedema toxin, respectively (Beall *et al.*, 1962; Stanley & Smith, 1961). After PA (83 kDa) binds to a cellular receptor (Escuyer & Collier, 1991), cleavage occurs at a trypsin-sensitive site localized to amino acid residues 164-Arg-Lys-Lys-Arg-167 (Leppla *et al.*, 1987; Singh *et al.*, 1989). Proteolysis releases a 20 kDa, N-terminal fragment (PA20), exposing a binding site for either LF or EF on the

receptor-bound, 63 kDa, C-terminal fragment, PA63 (Leppla *et al.*, 1987, 1990; Novak *et al.*, 1992). The toxin complex enters the cell by receptor-mediated endocytosis, and LF and EF are thought to be transported into the cytosol (Friedlander, 1986; Gordon *et al.*, 1988). Recently, Milne *et al.* (1994) showed that PA63 forms heptameric oligomers, postulated as functioning in the translocation event. Lethal toxin causes severe pulmonary oedema and death in susceptible animals (Beall *et al.*, 1962; Fish *et al.*, 1968; Stanley & Smith, 1961), and is rapidly cytolytic for macrophages *in vitro* (Friedlander, 1986). LF has recently been reported to exhibit homology to zinc metalloendopeptidases (Klimpel *et al.*, 1994; Kochi *et al.*, 1994). Oedema toxin causes oedematous lesions (Fish *et al.*, 1968; Stanley & Smith, 1961) attributed to the calmodulin-dependent adenylate cyclase activity of EF (Leppla, 1982). Based upon the model, PA thus contains domains involved in binding to cell receptors, binding of either catalytic moiety LF or EF, and translocating the toxin effector molecule into the cytoplasm (Leppla, 1991).

This work compares the ability of mAbs produced against PA to neutralize lethal toxin activity with their ability to inhibit binding of PA to cells and of LF to cell-bound PA.

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Abbreviations: EF, oedema factor; HBSS, Hanks' balanced salt solution; LF, lethal factor; PA, protective antigen; T-PA, trypsin-treated PA; Ch-PA, chymotrypsin-treated PA; Ch/T-PA, chymotrypsin/trypsin-treated PA.

mAbs which effected these two binding interactions were mapped to three different regions on PA.

METHODS

B. anthracis antigens. PA and LF were purified as previously described (Leppla, 1988).

Generation and purification of proteolytic fragments of PA. PA63 and PA20 (Fig. 1) were prepared by limited digestion of PA with trypsin and separation of fragments by FPLC on MonoQ resin (Pharmacia-LKB) (Bhatnagar *et al.*, 1989) using 20 mM ethanolamine, pH 9.5. Fragments were dialysed against 10 mM CHES buffer, pH 9.5, and stored at -70°C .

PA fragments of 47 kDa (PA47) and 37 kDa (PA37) (Fig. 1; Leppla, 1991) were prepared by limited digestion of PA ($3\text{--}5\text{ mg ml}^{-1}$) with chymotrypsin, type VII ($N\alpha$ -*p*-tosyl-L-lysine chloromethyl ketone (TLCK)-treated, 125 μg), in 2.3 mM NaH_2PO_4 , 7.5 mM Na_2HPO_4 , 0.15 M NaCl, pH 7.3 (PBS), for 10 min at room temperature. The reaction was stopped by adding PMSF and the sample was adjusted to 5 M urea and 50 mM HEPES. Chromatography was then performed on MonoS resin (Pharmacia-LKB) using 50 mM HEPES, 5 M urea buffer, pH 7.55. Fractions were eluted with a linear gradient of 0–0.5 M NaCl in 50 mM HEPES, 5 M urea buffer, pH 7.55. Fragments were identified by SDS-PAGE. Urea present in the PA47 and PA37 fragments was lowered to $<0.5\text{ M}$ by buffer exchange to 20 mM HEPES, 50 mM NaCl, pH 7.55, by using 30 kDa cutoff membranes. Fragments were stored at -70°C .

To generate PA17 (Fig. 1), PA ($3\text{--}5\text{ mg ml}^{-1}$) was subjected to limited digestion with chymotrypsin, type VII (125 μg) for 5 min followed by trypsin, type XIII (12.5 μg) for 5 min on ice. The sample was adjusted to 5 M urea and 50 mM MES after adding PMSF. The digest was then chromatographed over MonoS resin using 50 mM MES, 5 M urea buffer, pH 6.15. Fractions were eluted with a linear gradient of 0–1 M NaCl in 50 mM MES, 5 M urea, pH 6.15. PA17 was identified by SDS-PAGE. To ensure the removal of PA47 reactive fragments, the PA17 preparation was diluted fourfold in 5 mM NaH_2PO_4 , 15 mM Na_2HPO_4 , 0.5 M NaCl, pH 7.0, and passed four times over an immunoaffinity chromatography column containing rabbit anti-PA47 sera bound to hydrazide resin according to the manufacturer's instructions (Bio-Rad). The unbound PA17 pool was adjusted to 4 M urea and the volume reduced by using a 10 kDa cutoff membrane. Fragments were stored at -70°C .

Trypsin-treated PA (T-PA), chymotrypsin-treated PA (Ch-PA), chymotrypsin- and trypsin-treated PA (Ch/T-PA; Novak *et al.*, 1992), and chymotrypsin-treated PA63 (Ch-PA63) were also prepared but without subsequent chromatography.

Hybridomas. Female BALB/c mice were immunized subcutaneously (0.4 ml) and intraperitoneally (0.2 ml) with purified PA63 (50 μg) in Freund's complete adjuvant (1:1; day 0) or Freund's incomplete adjuvant (1:1; day 14). Subcutaneous injections were administered in two sites. On days 33 and 34, mice were injected both intravenously (0.1 ml) and intraperitoneally (0.1 ml) with 100–200 μg of PA63 in saline. Hybrid cell lines, prepared by fusing spleen cells with SP2/O-Ag14 myeloma cells (Earley & Osterling, 1985), were screened by ELISA for PA63 reactivity. The preparation of ascites, quantification of Ig, and determination of antibody subclass were as described previously (Little *et al.*, 1988).

Previously described mAbs produced against PA (Little *et al.*, 1988) were also included in the analysis. Therefore, for

identification purposes, the mAbs are designated with a subscript of the immunizing antigen: PA63 or PA.

Polyclonal antiserum. Fragments of PA, generated by limited proteolysis with chymotrypsin and trypsin (Novak *et al.*, 1992), were separated on 20 cm SDS-PAGE gels (12%, w/v, acrylamide). The resolved protein bands were identified by staining with Pro-Green (Integrated Separation Systems) according to the manufacturer's instructions, excised, destained, rinsed in distilled water, and frozen at -20°C . Polyclonal rabbit antiserum against PA47 and PA17 was prepared by injecting the rabbits intramuscularly and subcutaneously with polyacrylamide gel slices that contained the respective fragment homogenized with adjuvant (1:1). The primary injection included Freund's complete adjuvant; all subsequent injections included Freund's incomplete adjuvant. Polyclonal antiserum against PA was produced in rabbits injected intramuscularly and subcutaneously with purified PA (100 μg protein) and Freund's complete or incomplete adjuvant as above.

ELISA and ELISA competition assay. The ELISA was as described previously (Little *et al.*, 1990). Competition among mAbs for a single antigenic site was measured by using a competitive-binding ELISA (Little *et al.*, 1988) with the following changes. Purified mAbs were biotinylated by an *N*-hydroxysuccinimide derivative of biotin with an amino-hexanoate spacer arm (Vector Laboratories). A 1:100 dilution of ascites mAb was used to determine the percentage of biotinylated mAb bound in the presence of competing, unlabelled mAbs. The percentage binding was calculated from the following formula: percentage binding = $(A_{405}\text{ test well}/A_{405}\text{ control well}) \times 100$, where test wells contained biotinylated mAb plus competitor, and control wells contained diluent instead of competitor. Absorbance readings were obtained using a Bio-Tek model EL-310 autoreader.

Neutralization assay in rats. Neutralization of lethal toxin activity *in vivo* was assayed in male Fischer 344 rats (Little *et al.*, 1988), which were injected intravenously with mixtures of 40 μg PA plus 8 μg LF (approximately 13 LD₅₀; Ezzell *et al.*, 1984), and ascitic fluid after preincubation for 1 h at 37°C . Ascitic fluids that protected or delayed the death of rats were tested at lower concentrations until no protection was observed. All mAbs were tested for neutralizing ability *in vivo*.

Cellular neutralization assays. An *in vitro* colorimetric assay, developed to assess cell growth and viability (Green *et al.*, 1984; Mosmann, 1983), was used as described previously (Little *et al.*, 1990) to study the interactions between mAbs, PA, LF and J774A.1 murine macrophage cells. Briefly, the three different *in vitro* neutralization protocols were as follows. (i) (PA + LF + mAb); ascitic fluid containing mAb was preincubated in lethal toxin (400 ng PA ml⁻¹ plus 40 ng LF ml⁻¹) for 1 h at 37°C before adding to J774A.1 cells. (ii) (PA + cell); J774A.1 cells were preincubated with PA (400 ng ml⁻¹) at 4°C for 2 h. In separate 96-well plates, ascitic fluids containing mAb were preincubated in LF (40 ng ml⁻¹) at 37°C for 1 h. After aspirating the PA from the cells, mAb dilutions in LF were added. (iii) (PA + LF + cell); J774A.1 cells were washed twice in cold incubation medium and then were preincubated with PA (800 ng ml⁻¹) and LF (80 ng ml⁻¹) at 4°C for 2 h. After washing the cells twice with cold incubation medium, dilutions of ascitic fluids containing mAb were added. Only those mAbs that neutralized lethal toxin in the first protocol were tested in the last two protocols. Cell viability was calculated by using the average A_{540} of pairs of wells receiving either toxin plus mAb or mAb alone. We chose the concentration of ascites Ig ($\mu\text{g ml}^{-1}$) that inhibited 80% of cell death as a quantitative measure of the

Fig. 1. Schematic representation of major protease-sensitive sites on PA and PA63 and fragments generated by limited proteolysis by trypsin (T-PA) or chymotrypsin (Ch-PA and Ch-PA63). Amino acid residues are numbered from the N-terminal glutamic acid of mature PA.

Table 1. Description of recombinant clones used in this study

PA deletion*	Mutant plasmid	Expression host	Source
PA-735	pYS2	<i>E. coli</i> BL-21	Singh <i>et al.</i> (1989)
PA-735	pYS5	<i>B. subtilis</i> WB600	Singh <i>et al.</i> (1989)
PA-728	pYD17	<i>B. subtilis</i> DB104	Singh <i>et al.</i> (1991)
PA-721	pYS10	<i>E. coli</i> BL-21	Singh <i>et al.</i> (1991)
PA-670	pYD2	<i>B. subtilis</i> WB600	This study
PA-601	pYD3	<i>B. subtilis</i> WB600	This study
PA-580	pBKPPAΔ65	<i>E. coli</i> JM109	Little & Lowe (1991)

* PA deletions are designated according to the number of amino acid residues remaining of the original 735 amino acids.

in vitro neutralizing ability of the mAb. Absorbance readings were obtained using a Bio-Tek model EL-310 autoreader.

Binding studies with ^{125}I -PA. PA was iodinated by using the ^{125}I -Bolton-Hunter reagent (Amersham) (specific activity 2.0×10^6 c.p.m. μg^{-1} to 5.0×10^6 c.p.m. μg^{-1}). mAbs ($100 \mu\text{g}$ ascites Ig ml^{-1}) were preincubated with ^{125}I -PA ($0.075 \mu\text{g ml}^{-1}$) for 2 h at 4°C and added to L6 rat skeletal muscle myoblast cells in 24-well plates (2×10^5 to $4 \times 10^5 \text{ ml}^{-1}$) previously washed with cold Hanks' balanced salt solution (HBSS). After incubating for 6 h at 4°C on ice, the monolayers were washed three times with cold HBSS, solubilized by adding $0.75 \text{ ml } 0.1 \text{ M NaOH}$, and the radioactivity measured. Specific binding was determined by subtracting nonspecific background binding from total binding. Nonspecific binding, determined by adding 250-fold excess of unlabelled PA in addition to ^{125}I -PA, was less than 10% of total binding. Data are reported as percentage inhibition of the specific binding occurring in control wells incubated without mAb. Assays were done in triplicate.

Binding studies with ^{125}I -LF. Binding studies, with LF iodinated by the chloramine-T method (specific activity 2.0×10^7 c.p.m. μg^{-1} to 3.0×10^7 c.p.m. μg^{-1}), were based upon previously described methods (Little *et al.*, 1990). L6 cells (2×10^5 to $4 \times 10^5 \text{ ml}^{-1}$) were preincubated with $400 \mu\text{l}$ PA ($0.075 \mu\text{g ml}^{-1}$) for 18 h at 4°C on ice. Cells were washed with cold HBSS and then incubated with $400 \mu\text{l}$ ascitic fluid containing mAb ($100 \mu\text{g Ig ml}^{-1}$) for 1 h at 4°C , followed by the addition of $20 \mu\text{l}$ per well of ^{125}I -LF (final concentration 50 ng ml^{-1}). After incubating for 2 h at 4°C on ice, monolayers were washed three times with cold HBSS, solubilized with $0.75 \text{ ml } 0.1 \text{ M NaOH}$, and the mean count recorded from triplicate samples. Specific binding was determined by subtracting nonspecific binding from total binding. Nonspecific

binding, determined by adding ^{125}I -LF to cells in the absence of PA, was less than 15% of total binding. Data were reported as percentage inhibition of the specific binding occurring in control wells incubated without mAb.

Immunoblot analysis of PA fragments. Fragments of PA, prepared by limited proteolysis with chymotrypsin ($125 \mu\text{g ml}^{-1}$) or chymotrypsin ($125 \mu\text{g ml}^{-1}$) and trypsin ($12.5 \mu\text{g ml}^{-1}$) (Novak *et al.*, 1992), were separated by SDS-PAGE under reducing conditions on 10% (w/v) Tricine gels (Novex). Proteins were electrophoretically transferred to $0.2 \mu\text{m}$ pore nitrocellulose (Towbin *et al.*, 1979). The membranes were blocked with 5% (w/v) nonfat milk in 20 mM Tris/HCl , 0.5 M NaCl , pH 7.5 (TBS) before incubation with antibodies diluted in TBS containing 5% milk and 0.05% Tween 20. Membranes were washed with TBS containing 0.05% Tween 20 and then incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (Kirkegaard & Perry). An enhanced chemiluminescence method (ECL; Amersham) was used to detect reactive bands. Rabbit polyclonal antibodies were detected by using goat anti-rabbit IgG conjugated to horseradish peroxidase (Kirkegaard & Perry).

Immunoblot analysis of PA expressed by recombinant clones.

Recombinant clones used in this study are listed in Table 1. PA deletions are designated according to the number of amino acid residues remaining of the original 735 amino acids. Construction of *Escherichia coli* JM109 cells containing pBKPPAΔ65, a recombinant plasmid containing a deletion of 155 amino acids from the C-terminal coding region of the PA gene, *pag*, in pBluescript (KS+), was described previously (Little & Lowe, 1991). Cells, harvested from cultures grown as described by Little & Lowe (1991), were suspended in 25 mM Tris/HCl (pH 8.5), 100 mM NaCl , 30% (w/v) sucrose, 1 mM PMSF ,

1 mM EDTA and 1 mM 1,10-phenanthroline. Lysozyme (300 µg in 50 µl 0.1 M EDTA) was added and the cells were incubated on ice for 30 min. The suspension was then centrifuged at 10000 *g* for 10 min. The supernatant fluid was collected and analysed by SDS-PAGE on 10% Tricine gels (Novex). Separated proteins were electrophoretically transferred to 0.45 µm nitrocellulose (Towbin *et al.*, 1979), and membranes were developed as an immunoblot as described above for the PA fragments, using PBS buffers instead.

Construction of recombinant clones pYS2, pYD17 and pYS10, which express full-length *pag*, a 7-amino-acid deletion and a 14-amino-acid deletion from the C-terminus of PA, respectively, was described previously (Singh *et al.*, 1989, 1991). Protein from pYD17 was purified as described by Singh *et al.* (1991). Cells from 100 ml cultures of pYS2 or pYS10, induced with IPTG, were suspended in 5 ml 0.1 M HEPES, pH 7.4, 10 mM EDTA, 5% (v/v) heat-inactivated foetal bovine serum, 2% (v/v) glycerol and 1 mM 1,10-phenanthroline and sonicated in four 30 s intervals. Supernatants, collected after centrifugation for 15 min at 10000 *g*, were purified on a mAb 10G4_{PA} column. Protein was eluted with 2 M sodium thiocyanate in 20 mM HEPES, pH 7.4, after the column was washed with 40 ml PBS. Eluted protein was subjected to SDS-PAGE after buffer exchange to 10 mM HEPES, pH 7.4, electrophoretically transferred to nitrocellulose, and analysed in an immunoblot as above.

Recombinant plasmids pYD2 and pYD3, which express a 65- and a 134-amino-acid deletion, respectively, from the C-terminus of PA, were generated by PCR amplification of the native PA gene from pYS5 template (Singh *et al.*, 1989). A non-mutagenic oligonucleotide primer, corresponding exactly to nucleotides 2842–2864 of *pag* (numbering system of Welkos *et al.*, 1988), a region containing a *Pst*I recognition site unique to *pag* and pYS5, was used in all amplification reactions (Singh *et al.*, 1991). Mutagenic oligonucleotide primers were designed to be complementary to the intended new 3' end of the PA gene and to introduce two in-frame stop codons and a unique *Bam*HI site. For pYD2 the primer for deleting nucleotides 3901–4095 was 5' CATTATATTTTAAATCTATAAATGGATCC-TATTATTGCCGTAACTAGAAATATTC 3' and for pYD3 the primer for deleting nucleotides 3694–4095 was 5' CTACTGACTCATCCGCCCCAGGATCCTATTAATTTC-TATCATAATGAAAACG 3'. Mutant constructs were confirmed by sequence analysis. Plasmids pYD2, pYD3 and pYS5 were transformed into *Bacillus subtilis* WB600 (Wu *et al.*, 1991), which lacks six major extracellular proteases. Cultures were grown in brain heart infusion medium containing 5 µg kanamycin sulfate ml⁻¹ at 37 °C for 6–8 h until the OD₆₀₀ read 2.2–2.5 using a Shimadzu UV160U spectrophotometer. The cultures were centrifuged at 10000 *g* and the supernatants were removed and supplemented to 1 mM PMSF and 10 mM EDTA. Supernatants were concentrated threefold with a Centriprep 30 device (Amicon) and analysed by immunoblot analysis after SDS-PAGE, as described above.

RESULTS

In vivo neutralization of lethal toxin

Two mAbs against PA neutralized lethal toxin activity (13 LD₅₀) *in vivo*. mAbs 1G3_{PA63} and 10E10_{PA63} protected 100% of animals (three of three) with 100 µg and 1000 µg ascites Ig, respectively.

The *in vivo* neutralizing ability of selected PA mAbs from an earlier study (Little *et al.*, 1988) were recalculated as

concentration of ascites Ig instead of ascites protein. mAbs 3B6_{PA} and 14B7_{PA} protected rats with 926 µg and 23 µg of ascites Ig, respectively. mAbs 2D3_{PA} and 2D5_{PA} delayed death by about 18 h with 1700 µg and 1300 µg of ascites Ig, respectively. No survival or delay of death was observed with mAbs 10D2_{PA} and 10G4_{PA}, even at the highest concentrations tested, 1100 µg and 923 µg ascites Ig, respectively.

In vitro neutralization of lethal toxin

To measure *in vitro* neutralization of lethal toxin cytolytic activity, mAbs developed in this study and those previously reported (Little *et al.*, 1988) were tested by preincubating them with PA (400 ng) and LF (40 ng) before adding them to J774A.1 cells [experiment (i), PA + LF + mAb]. Seven mAbs, at concentrations of 0.02–40 µg Ig ml⁻¹, neutralized *in vitro* lethal toxin cytolytic activity under these conditions (Table 2). One other mAb, 10E10_{PA63}, showed only weak neutralizing activity at 400 µg Ig ml⁻¹. Only these eight mAbs were tested further.

Preincubating J774A.1 cells with PA, before adding mAb dilutions in LF [experiment (ii), PA + cells], demonstrated neutralization by five mAbs, 1G3_{PA63}, 2D3_{PA}, 2D5_{PA}, 10D2_{PA} and 10G4_{PA} (Table 2). mAbs 3B6_{PA}, 14B7_{PA} and 10E10_{PA63} did not neutralize lethal toxin activity under these conditions (Table 2). When J774A.1 cells were preincubated with PA (800 ng ml⁻¹) and LF (80 ng ml⁻¹) before addition of mAbs [experiment (iii), PA + LF + cells], five mAbs demonstrated only very weak neutralizing ability (1G3_{PA63}, 2D3_{PA}, 2D5_{PA}, 10D2_{PA} and 10G4_{PA}) (Table 2).

As shown in Table 2, two major groups of neutralizing activities were identified. The first group (1G3_{PA63}, 2D3_{PA}, 2D5_{PA}, 10D2_{PA} and 10G4_{PA}) neutralized lethal toxin cytolytic activity whether they were added before or after PA bound to cells. However, only mAb 1G3_{PA63} was highly potent before and after PA bound to cells. The second group (3B6_{PA}, 14B7_{PA} and 10E10_{PA63}) neutralized lethal toxin cytolytic activity only when combined with PA before being added to cells, and was inactive when added after PA had bound.

Seven of the mAbs were IgG₁ and one was IgG_{2b} subtype (Table 2).

Inhibition of toxin formation on target cells

Binding studies demonstrated that of the eight mAbs that neutralized lethal toxin cytolytic activity in the macrophage cytotoxicity assay, four, 1G3_{PA63}, 2D3_{PA}, 2D5_{PA} and 10D2_{PA}, inhibited binding of ¹²⁵I-LF to cell-bound PA by 78–102% (Table 3). mAbs 3B6_{PA}, 14B7_{PA} and 10E10_{PA63} inhibited binding of ¹²⁵I-PA to the receptor by 90–98% (Table 3). mAb 10G4_{PA}, which neutralized lethal toxin cytolytic activity only at high concentrations (Table 2), had only a modest effect on inhibition of binding of ¹²⁵I-PA to the receptor and had no effect on binding of ¹²⁵I-LF to cell-bound PA (Table 3).

Table 2. Neutralizing activity of mAbs in cell-protection assays

The results are given as the concentration of ascites IgG ($\mu\text{g ml}^{-1}$) required to neutralize lethal toxin as measured at 80% of control wells. See Methods for description of each assay.

mAb	Antibody subtype	Cell-protection assay		
		Expt (i) (PA + LF + mAb)	Expt (ii) (PA + cells)	Expt (iii) (PA + LF + cells)
1G3 _{PA63}	G ₁	0.02	0.04	250
2D3 _{PA}	G ₁ *	0.1	5	250
2D5 _{PA}	G ₁ *	0.03	0.6	250
10D2 _{PA}	G ₁ *	0.1	10	900
10G4 _{PA}	G ₁ *	40	70	800
3B6 _{PA}	G ₁ *	12	> 1000	> 1000
14B7 _{PA}	G ₁ *	2	> 1000	> 1000
10E10 _{PA63}	G _{2b}	400	> 1000	> 1000

* Antibody subtypes were reported previously (Little *et al.*, 1988).

Table 3. Percentage binding inhibition for mAbs that neutralized lethal toxin activity

mAb	Percentage binding inhibition	
	¹²⁵ I-PA to cell*	¹²⁵ I-LF to PA†
1G3 _{PA63}	7	102
2D3 _{PA}	39	80
2D5 _{PA}	38	78
10D2 _{PA}	21	88
10G4 _{PA}	54	3
3B6 _{PA}	98‡	1
14B7 _{PA}	95‡	—1
10E10 _{PA63}	90	20

* Inhibition of binding of ¹²⁵I-PA to L6 cells.

† Inhibition of binding of ¹²⁵I-LF to PA bound on L6 cells.

‡ These data were reported previously (Little *et al.*, 1988).

Competition between mAbs for antigenic sites

A competitive-binding ELISA against PA₆₃ antigen identified three separate antigenic groups among the neutralizing mAbs (Table 4). Two separate antigenic regions were recognized by mAbs that inhibited binding of ¹²⁵I-LF to cell-bound PA, while mAbs that inhibited binding of ¹²⁵I-PA to cells recognized a single antigenic region. mAb 10G4_{PA} recognized the same antigenic region as identified by mAbs 2D3_{PA}, 2D5_{PA} and 10D2_{PA}.

Location of antigenic region recognized by mAb 1G3_{PA63}

ELISA titration curves of mAb 1G3_{PA63} ascites against protease-treated preparations and selected fragments of PA demonstrated that this mAb reacted most strongly

with PA₆₃ and Ch-PA₆₃, with lesser reactivity against PA and T-PA (Fig. 2). No other PA fragments were recognized by mAb 1G3_{PA63} ascites by ELISA. Immunoblots with mAb 1G3_{PA63} demonstrated reactivity against proteolytic fragments PA₃₇ and PA₁₇ but not PA₄₇ (Fig. 3, lanes 1 and 2). Major fragments in these preparations were identified by rabbit polyclonal antiserum against PA (Fig. 3, lanes 3 and 4). The higher than expected apparent mass of the PA₁₇ fragment was noted previously (Novak *et al.*, 1992).

Location of the antigenic region recognized by mAbs 2D3_{PA}, 2D5_{PA}, 10D2_{PA} and 10G4_{PA}

ELISA titration curves of mAb 2D3_{PA} against selected fragments of PA showed that this mAb recognized an antigenic region on the PA₄₇ fragment (Fig. 4). Similar results were observed for mAbs 2D5_{PA}, 10D2_{PA} and 10G4_{PA} (data not shown). Immunoblot analysis with mAb 2D3_{PA} of fragments resulting from chymotrypsin-treated or chymotrypsin- and trypsin-treated PA preparations separated by SDS-PAGE also demonstrated recognition of an epitope within the PA₄₇ fragment (Fig. 5, lanes 1 and 2). Similar results were observed for mAbs 2D5_{PA}, 10D2_{PA} and 10G4_{PA} (data not shown). Immunoblots developed with rabbit anti-PA₁₇ antibody showed reactivity with the PA₃₇ and PA₁₇ fragments, which confirmed their presence in these preparations (Fig. 5, lanes 3 and 4).

Immunoblots of recombinant proteins expressing deletions of *pag* generated into the C-terminal coding region of PA showed that mAb 2D3_{PA} recognized full-length PA produced by the parent clone pYS5 and deletions of 65 and 134 amino acids, PA-670 and PA-601, respectively (Fig. 6, lanes 1–3). However, mAb 2D3_{PA} did not recognize a deletion of 155 amino acids (PA-580; Fig. 6, lane 4). Similar results were observed with mAbs 2D5_{PA}, 10D2_{PA} and 10G4_{PA} (data not shown). Immunoblots with

Table 4. Competitive antibody-binding ELISA between biotinylated and unlabelled neutralizing mAbs for PA₆₃ antigen

The results are shown as the percentage of biotinylated mAb bound in the presence of a saturating concentration of unlabelled mAb. Determinant groups are identified by boxes. Subscript suffixes have been omitted.

Unlabelled mAb	Biotinylated mAb*							
	1G3	2D3	2D5	10D2	10G4	3B6	14B7	10E10
1G3	4	58	67	60	58	78	88	73
2D3	22	0	3	0	0	103	84	66
2D5	37	0	3	0	4	116	93	42
10D2	52	2	5	0	10	89	84	70
10G4	57	24	8	0	0	58	34	79
3B6	111	110	97	100	35	0	5	7
14B7	85	92	95	89	34	0	1	6
10E10	100	68	49	56	44	0	1	2

Fig. 2. ELISA titration curves of mAb 1G3_{PA63}. Serially diluted mAb was added to 96-well plates coated with PA (▲), PA63 (●), PA47 (▼), PA37 (◆), PA17 (■), T-PA (□), Ch-PA (△) or Ch-PA63 (○).

rabbit polyclonal antiserum against PA identified PA produced by the deletion clones (Fig. 6, lanes 5–8). These results suggest that mAbs 2D3_{PA}, 2D5_{PA}, 10D2_{PA} and 10G4_{PA} recognized an epitope between amino acids Ile-581 and Asn-601.

Location of antigenic region recognized by mAb 3B6_{PA}, 14B7_{PA} and 10E10_{PA63}

MAbs 3B6_{PA}, 14B7_{PA} and 10E10_{PA63} also recognized an antigenic region on the PA47 fragment, as demonstrated by similar titration curves and immunoblot results against proteolytic fragments of PA as 2D3_{PA} (data not shown).

Fig. 3. Immunoblot analysis of fragments generated by limited proteolysis of PA with chymotrypsin or chymotrypsin and trypsin using mAb 1G3_{PA63} (lanes 1 and 2) or rabbit polyclonal antiserum against PA (lanes 3 and 4). Samples were loaded as follows: Ch-PA, lanes 1 and 3; Ch/T-PA, lanes 2 and 4. Molecular mass markers are indicated on the left and the major fragments of PA are indicated on the right.

mAb 10E10_{PA63} did not recognize the recombinant protein expressed by deletion clone pBKPPAΔ65, PA-580 (data not shown), findings similar to mAbs 3B6_{PA} and 14B7_{PA} (Little & Lowe, 1991) and 2D3_{PA} (Fig. 6). Immunoblots showed that mAb 3B6_{PA} recognized PA-735 (pYS2) and deletion clones PA-728 and PA-721 (Fig.

Fig. 4. ELISA titration curves of mAb 2D3_{PA}. Serially diluted mAb was added to 96-well plates coated with PA (▲), PA63 (●), PA47 (▼) or PA37 (◆).

Fig. 5. Immunoblot analysis of fragments generated by limited proteolysis of PA with chymotrypsin or chymotrypsin and trypsin using mAb 2D3_{PA} (lanes 1 and 2) or rabbit polyclonal serum against PA17 (lanes 3 and 4). Samples were loaded as follows: Ch/T-PA, lanes 1 and 3; Ch-PA, lanes 2 and 4. Molecular mass markers are indicated on the left and the major fragments of PA are indicated on the right.

7, lanes 1–3). mAb 3B6_{PA} did not recognize deletion clone PA-670 (Fig. 7, lane 4). Similar results were observed with mAbs 14B7_{PA} and 10E10_{PA63} (data not shown). These results suggest that these mAbs map between Asp-671 and Ile-721. Immunoblots with rabbit polyclonal anti-serum against PA identified PA produced by the deletion clones (Fig. 7, lanes 5–8).

Fig. 6. Immunoblot analysis of PA recombinant proteins using mAb 2D3_{PA} (lanes 1–4) or rabbit anti-PA polyclonal serum (lanes 5–8). Lanes 1 and 5, PA-735 (pY55); lanes 2 and 6, PA-670; lanes 3 and 7, PA-601; lanes 4 and 8, PA-580. Molecular mass markers are indicated on the left.

Fig. 7. Immunoblot analysis of PA recombinant proteins using mAb 3B6_{PA} (lanes 1–4) or rabbit anti-PA polyclonal serum (lanes 5–8). Lanes 1 and 5, PA-735 (pY52); lanes 2 and 6, PA-728; lanes 3 and 7, PA-721; lanes 4 and 8, PA-670. Molecular mass markers are indicated on the left.

DISCUSSION

Identification of antigenic region recognized by mAbs 3B6_{PA}, 14B7_{PA} and 10E10_{PA63}

The proposed model for the formation of lethal or oedema toxin from *B. anthracis* identifies regions of PA that interact with cell receptors, bind LF or EF, and translocate the toxin into the cytosol (Leppla, 1991). The extreme C-terminus of PA takes part in binding to cell receptors (Singh *et al.*, 1991). We previously reported that mAbs 3B6_{PA} and 14B7_{PA} blocked binding of PA to cells

(Little *et al.*, 1988) and identified an antigenic region within the 155 C-terminal amino acids of PA (Little & Lowe, 1991). The activities of mAbs 3B6_{PA}, 14B7_{PA} and 10E10_{PA63} in the *in vitro* macrophage cytotoxicity assay provides additional indirect evidence that PA binding is required for toxin formation. This report further maps the antigenic region recognized by mAbs that block the binding of PA to cells between amino acids Asp-671 and Ile-721. In contrast to an earlier report (Singh *et al.*, 1991), the immunoblot reactivity we observed between these mAbs and the PA deletion proteins may have been due to a partial restoration of native conformations of the proteins or epitopes during electrophoretic transfer to nitrocellulose in the presence of Towbin buffer (Towbin *et al.*, 1979), which lacks SDS.

Identification of antigenic region recognized by mAbs 2D3_{PA}, 2D5_{PA} and 10D2_{PA}

Two different, non-competitive antigenic regions were defined with respect to location on PA63 by the competitive-binding ELISA and immunoblot analysis by mAbs that neutralized lethal toxin activity by inhibiting the binding of LF to cell-bound PA. One region, recognized by mAbs 2D3_{PA}, 2D5_{PA} and 10D2_{PA}, resides within the PA47 fragment. Immunoblots of recombinant proteins expressing deletions of *pag* generated into the C-terminal coding region of PA demonstrated that these mAbs recognized an epitope between amino acids Ile-581 and Asn-601. mAb 10G4_{PA}, which also mapped to the same region, was able to neutralize lethal toxin activity *in vitro*, probably due to steric hindrance effects, as it did not inhibit significantly the binding of ¹²⁵I-PA to cells or ¹²⁵I-LF to cell-bound PA. The absence of any interference between these mAbs and mAbs that block binding to cells, such as 3B6_{PA}, 14B7_{PA} and 10E10_{PA63}, in the competition ELISA, their inability to significantly block ¹²⁵I-PA binding to the cell receptor, and their ability to neutralize lethal toxin activity in the macrophage cytotoxicity assay after PA was bound to cells suggest that the C-terminal portion of PA plays a role in interaction with cell receptors as well as with LF. Although a successive loss of C-terminal amino acids demonstrated a corresponding decreased ability to bind to cell receptors, recombinant proteins with deletions of up to seven amino acids were able to bind LF or EF, similar to full-length PA (Singh *et al.*, 1991). We have not yet evaluated the ability of recombinant proteins with deletions longer than 14 amino acids to interact with either LF or EF.

Identification of antigenic region recognized by mAb 1G3_{PA63}

The second antigenic region recognized by mAbs that inhibited binding of ¹²⁵I-LF to PA was defined by mAb 1G3_{PA63}. This mAb appeared to be more directly involved in LF binding than the other non-competitive region represented by 2D3_{PA}, 2D5_{PA} and 10D2_{PA}. mAb 1G3_{PA63} was at least 10-fold more active in neutralizing toxin activity when added after PA bound to cells and completely blocked binding of ¹²⁵I-LF to PA. The epitope recognized by mAb 1G3_{PA63} was identified on PA17 by

immunoblot analysis. Reactivity of mAb 1G3_{PA63} with PA37 and PA17, however, was not confirmed by ELISA. The different results observed between the two assays may be explained by differences in the preparation of the fragments rather than in differences in immobilization interactions of the antigen on plastic or nitrocellulose (Brennand *et al.*, 1986). The PA37 and PA17 fragments used in the ELISA were prepared in the presence of 5 M urea; upon removal of urea by buffer exchange or dilution, complete or proper refolding of the molecule or epitope may not have occurred. We also did not observe reactivity of mAb 1G3_{PA63} to PA17 prepared in the presence of 5 M urea and passively bound to nitrocellulose or polyvinylidene fluoride membranes which were analysed by immunoblotting (data not shown). The antigenic region recognized by 1G3_{PA63} on PA37 and PA17 may have been restored on immunoblots after SDS-PAGE during electrophoretic transfer in Towbin buffer, which lacks SDS.

ELISA reactivity of mAb 1G3_{PA63} against PA63 was 2.5 orders of magnitude greater than against PA or T-PA, suggesting that proteolysis and subsequent removal of the N-terminal 20 kDa fragment is required to expose the epitope recognized by this mAb. mAb 1G3_{PA63} retained its reactivity against Ch-PA63, suggesting that the chymotrypsin site is not part of its epitope. Any conformational changes resulting from the binding of antigen to plastic (Hollander & Katchalski-Katzir, 1986) apparently did not expose the antigenic site(s) after proteolytic nicking of PA.

Whether these mAbs directly inhibit binding of PA to cells or of LF to cell-bound PA, or whether they do so indirectly, by steric hindrance or by causing a change in conformation of PA upon binding, is not known. If the inhibition is effected indirectly, the antigenic regions identified by these mAbs in this study may not accurately reflect the actual contact regions with the cell receptor or effector molecule, LF or EF. Identifying mAbs that neutralize lethal toxin activity will enable us to clarify the role of the toxins in pathogenesis as well as the role of antibodies in immunity, and to test their efficacy in protection against anthrax infection. Furthermore, specific knowledge of the binding site on PA for LF and EF suggests that PA fragments or peptides containing this site may be effective as vaccines against infection. Studies are presently under way to characterize further the antigenic regions recognized by these mAbs.

Putative translocation region on PA not identified by mAbs

In addition to binding to cells and binding LF or EF, PA is also postulated to contain a region required for translocation of the toxin (Leppla, 1991). The putative region for this activity has been associated with the chymotrypsin site, Phe-313 to Asp-315 (Novak *et al.*, 1992, Singh *et al.*, 1994). Lethal toxin constructed with PA previously treated with chymotrypsin (Novak *et al.*, 1992) or mutated by altering the residues in this region (Singh *et al.*, 1994), was nontoxic to cells. The ability of PA to bind to cell receptors and LF was not affected. In screening

mAbs for this study, we did not identify any mAb that neutralized the cytolytic activity of lethal toxin without inhibiting binding of 125 I-PA to cells or 125 I-LF to cell-bound PA.

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