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A Monoclonal Antibody to *Bacillus anthracis* Protective Antigen Defines a Neutralizing Epitope in Domain 1

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Antibody (Ab) responses to *Bacillus anthracis* toxins are protective, but relatively few protective monoclonal antibodies (MAbs) have been reported. Protective antigen (PA) is essential for the action of *B. anthracis* lethal toxin (LeTx) and edema toxin. In this study, we generated two MAbs to PA, MAbs 7.5G and 10F4. These MAbs did not compete for binding to PA, consistent with specificities for different epitopes. The MAbs were tested for their ability to protect a monolayer of cultured macrophages against toxin-mediated cytotoxicity. MAb 7.5G, the most-neutralizing MAb, bound to domain 1 of PA and reduced LeTx toxicity in BALB/c mice. Remarkably, MAb 7.5G provided protection without blocking the binding of PA or lethal factor or the formation of the PA heptamer complex. However, MAb 7.5G slowed the proteolytic digestion of PA by furin in vitro, suggesting a potential mechanism for Ab-mediated protection. These observations indicate that some Abs to domain 1 can contribute to host protection.

Bacillus anthracis causes anthrax, a disease that primarily affects grazing animals. However, the fact that *B. anthracis* spores can be made into potent biological weapons has made this microbe a major focus of defense-related research. The primary *B. anthracis* virulence factors, toxin production and capsule formation, are encoded by two large plasmids, pXO1 and pXO2, respectively. *B. anthracis* toxins are made up of three proteins known as protective antigen (PA), lethal factor (LF), and edema factor (EF), which interact in a binary fashion to produce edema toxin (PA plus EF) and lethal toxin (PA plus LF; LeTx) (4). The three-dimensional structure of 83-kDa PA (PA_{83}) consists of four folding domains (20, 23). PA_{83} binds via domain 4 to anthrax toxin receptors in host cells. A cell-associated furin-like protease cleaves PA_{83} domain 1, yielding 63-kDa and 20-kDa fragments known as PA_{63} and PA_{20}. The PA_{63} fragment then polymerizes into a heptameric structure that binds EF or LF and promotes its entry into the cell.

A role for antibody (Ab) in protection against *B. anthracis* toxins is strongly supported by experimental evidence (15, 25). However, experiments with monoclonal Abs (MAbs) have produced mixed results. Several MAbs were tested in a guinea pig model, but only one was partially protective (12). Recently, Brossier et al. generated two neutralizing MAbs which bound domains 2 and 4 of PA_{83} (2). The relative inefficacy of MAbs in comparison with immune sera may reflect the need for Abs to bind at multiple sites for optimal neutralization or to bind to nonneutralizing epitopes. The importance of understanding the relationship between specificity and neutralizing activity is further highlighted by the observation that some Abs can enhance LeTx toxicity (18). To this end, our group has generated two MAbs to PA_{83} with one neutralizing MAb binding to domain 1, a location that would not be predicted to translate into protection, defining a new neutralizing epitope for this toxin component.

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MATERIALS AND METHODS

***B. anthracis* PA_{83} and LF.** Recombinant PA_{83} and LF were expressed and isolated from *Escherichia coli* as previously described (1) or obtained from Wadsworth Laboratories, NYS Department of Health (Albany, NY).

Mice. Female BALB/c mice, 6 to 8 weeks old (NCI, Bethesda, MD), were immunized with 10 μg of PA_{83} in complete Freund's adjuvant (Sigma, St. Louis, MO). Two weeks later, the mice were boosted with 10 μg of PA_{83} in incomplete Freund's adjuvant. The mice were bled and the sera stored at −20°C for analysis of titers by enzyme-linked immunosorbent assay (ELISA).

Hybridomas. Hybridomas were generated by fusing splenocytes to the NSO myeloma fusion partner (8). The MAb isotype was established by ELISA using isotype-specific reagents.

ELISA. Ab binding to PA_{83} and expressed PA domains was measured by ELISA. Briefly, polystyrene plates were coated with 1 μg/ml (12.05 μM) PA_{83} or expressed PA domains in phosphate-buffered saline (PBS) and blocked with 200 μl of 1% bovine serum albumin in PBS. Primary Ab binding was detected using alkaline-phosphatase-labeled goat anti-mouse Ab reagents. Competition assays to evaluate MAb specificity were done as previously described (3). Briefly, a variable amount of a MAb was mixed with a constant amount of a second MAb, and relative binding to PA_{83} was assayed by ELISA. Binding of the Abs was detected by isotype-specific alkaline-phosphatase-conjugated goat anti-mouse reagent. For all steps, incubations were done at 37°C for 1 h, and absorbances were measured with a microtiter plate reader at 405 nm (Labsystems Multiskan, Franklin, MA).

MAb V_H and V_L sequences. Hybridoma RNA was isolated using TRIzol reagent (Gibco BRL, Gaithersburg, MD) per the manufacturer's instructions. cDNA was prepared with oligo(dT) primer and superscript II reverse transcriptase (QIAGEN, Valencia, CA). MAb variable (V) domains were generated by PCR with universal 5′-end (sense) V region and specific 3′-end (antisense) constant region primers as described previously(21).

Enzymatic digestion of PA. PA_{83} was digested with furin (Sigma, St. Louis, MO) or trypsin (Promega, Madison, WI). For trypsin digestions, 10 μg of PA_{83} in 150 mM $NaCl_2$, 20 mM Tris (pH 8.2) was mixed with trypsin (1 μg/ml) for 30 min at room temperature (RT) in a volume of 20 μl. For furin digestions, 10 μg of PA_{83} was incubated for 30 s to 15 min at 30°C in 20 μl of 1 mM $CaCl_2$, 1 mM

β-mercaptoethanol, 0.5% Triton X-100, 100 mM HEPES (pH 7.5) and mixed with 0.02 U to 10 U of furin. Digested products were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (10). Proteins were visualized by staining overnight with GelCode blue stain (Pierce, Rockford, IL), and bands were analyzed using the Image J software (National Institutes of Health, Bethesda, MD).

Immunoblot analysis. PA_{83} and its products were solubilized in Laemmli sample buffer containing β-mercaptoethanol, boiled for 10 min, and then separated by SDS-PAGE (10). Proteins were transferred to nitrocellulose membranes (0.20-μm pore size) by electroelution. The membranes were washed with PBS, blocked with 5% dry milk in PBS, incubated with MAb 7.5G or 10F4, washed, and incubated with isotype-matched goat anti-mouse secondary Ab conjugated to horseradish peroxidase. Proteins were visualized by developing them with an ECL chemiluminescence kit (Pierce, Rockford, IL).

MTT cell assay. An MTT [3,(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide] assay was used to determine toxin toxicity to mouse macrophage cell lines. MTT (Sigma, St. Louis, MO) was dissolved at 5 mg/ml in sterile PBS at RT, sterilized by passage through a 0.22-μm filter, and stored in the dark at 4°C. This assay relies on the oxidation of MTT to an insoluble pigment by live cells. MHS alveolar cells and J774 macrophage-like cells (6×10^4) were incubated in a 96-well plate with 100 ng each of PA and LF and/or 10 μg/ml of MAb for 4 h at 37°C. A 25-μl volume of a 5-mg/ml stock solution of MTT was added to each well, and after 2 h of incubation at 37°C, 100 μl of the extraction buffer (12.5% SDS, 45%*N*, *N*-dimethylformamide) was added and the cells were then incubated overnight at 37°C. Optical densities were measured at 570 nm (Labsystem Multiskan, Franklin, MA).

Generation of PA_{83} domains. PA_{83} domains were generated using plasmid pET22bPA as the template, as described previously (1). The forward primer 5′TTAAGTCTAGACGAAGTTAAACAGGAGAACCG3′ was used to generate all domain combinations, with domain-specific reverse primers as follows: for domain 1, 5′TTAATGTCGACTAGCTGCCACAAGGGGGTG3′; for domains 1and 2, 5′TTAATGTCGACTTGTTTCTTGAATTTGCGGTAAC3′; for domains 1 to 3, 5′TTAATGTCGACTTGCTATGTTATTTCTATC3′; for domains 1 to 4, 5′TTAATGTCGACTTTATCCTATCTCATAGCCTTT3′. Products were cloned into a glutathione *S*-transferase expression vector, pGEX-KG (Pharmacia Biotech, Piscataway, NJ), which contains the thrombin coding sequence, and then transformed into DH5α cells (Invitrogen, Carlsbad, CA). DNA was isolated and sequenced to confirm the PA sequence (25).

Radiolabeling of toxins with ^{188}Re and binding of labeled toxins to macrophages. PA_{83} and LF were labeled with ^{188}Re via generation of SH groups on the proteins as described previously (6). For binding experiments, 2.8×10^6 J774 macrophage cells were incubated with increasing amounts of ^{188}Re-labeled PA_{83} (0.28 to 1.92 nM). Alternatively, MAb 7.5G (0.28 to 1.92 nM) was added to the tubes with the macrophages, followed immediately by the addition of equimolar (1:1) concentrations of ^{188}Re-labeled PA_{83}. The cells were incubated for 1 h at 4°C and collected by centrifugation at 1,200 rpm at 4°C for 6 min, and radioactivity was measured using a gamma counter (Wallac, Wallac Oy., Turku, Finland). The binding of ^{188}Re-labeled PA_{83} to macrophages was calculated as the ratio of activity in the pellet to the activity in the tube before the cells were collected and is expressed as a percentage. For binding experiments involving LF, macrophages were incubated at 4°C for 1 h with radiolabeled LF (0.28 to 1.92 nM). For assessing the binding of LF to PA_{83}, cells were first incubated with increasing concentrations (0.28 to 0.84 nM) of unlabeled PA_{83} for 1 h at 4°C, followed by the addition of either equimolar concentrations of radiolabeled LF (1:1) or equimolar concentrations of radiolabeled LF and unlabeled MAb 7.5G (1:1:1), with an additional incubation for 1 h at 4°C. Radioactivity was measured and percentages of binding were calculated as described above.

Passive-protection studies. BALB/c mice were injected intravenously (i.v.) as previously described (19). An amount of 100 μg each of PA and LF in 100 μl of PBS was injected into the tail veins of BALB/c mice. Various concentrations of MAbs were administered intraperitoneally 24 h prior to toxin administration. The mice were monitored daily for mortality. All animal work was done in accordance with institutional regulations.

Pulmonary-function analysis. Whole-body plethysmography (WBP; Buxco Electronics, Inc., Wilmington, NC) was used to measure pulmonary function in unrestrained, nonanesthetized BALB/c mice. Mice were placed in an enclosed chamber, and baseline readings were taken over a period of 5 min before i.v. injections of LeTx were given. Additional lung function measurements were taken at 1.5 h and 24 h after the LeTx injections. Parameters measured by WBP included inspiratory time (in seconds), which is the time from the start of an inspiration to the end of the inspiration, and expiratory time (in seconds), which is the time from the end of an inspiration to the start of the next inspiration. We also measured peak inspiratory flow (in milliliters/second), which is the maximal negative box pressure occurring in one breath; peak expiratory flow (in milliliters/second), which is the maximal positive box pressure occurring in one breath; and the tidal volume (in seconds) (7).

Isolation of PA oligomer from cells. CHO-K1 cells were plated at 2×10^5 cells/well in 24-well plates 24 h prior to the experiment. PA_{83} (2.5 μg/ml) was added and incubated at 37°C with the cells for various time intervals (15 to 120 min), and unbound toxin was then removed by washing with PBS and treatment with 0.5 mg/ml trypsin (Gibco, Rockville, MD). Cells were then lysed in 100 μl of modified radioimmunoprecipitation assay lysis buffer (50 mM Tris Cl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 μg/ml protease inhibitors). Cell lysates were solubilized in Laemmli sample buffer containing β-mercaptoethanol, boiled for 10 min, and then separated on a 12% SDS-PAGE gel (10). Proteins were transferred to nitrocellulose membranes (0.20-μm pore size) by electroelution. The membranes were washed with PBS, blocked for 1 h with 5% dry milk in PBS, and then incubated with MAb 10F4 (immunoglobulin G1 [IgG1]) for 1 h at RT. After three washes with PBS, the membranes were incubated with isotype-matched goat anti-mouse secondary Ab conjugated to horseradish peroxidase. Proteins were visualized by developing them with an ECL chemiluminescence kit (Pierce, Rockford, IL).

Statistics. Data were analyzed by the Student *t* test and by log rank analysis (Sigmastat, Chicago, IL).

RESULTS

Generation of MAbs. PA_{83} injected into BALB/c mice with Freund's adjuvant elicited Ab responses (Fig. 1A). Mice labeled C and D received 2 μg and 10 μg of PA_{83}, respectively (three mice per group). There was considerable mouse-to-mouse variation in the response to vaccination, but several mice produced significant levels of IgM (data not shown) and IgG (Fig. 1A). We recovered two PA_{83}-binding MAbs from mouse D3: 10F4 (IgG1) and 7.5G (IgG2b). Competition assays revealed that MAb 7.5G binding did not inhibit binding of MAb 10F4, implying that they bound to different epitopes on PA_{83} (data not shown). Consequently, we investigated the binding of MAbs 7.5G and 10F4 to PA fragments by ELISA. MAb 7.5G bound to PA_{20}, whereas MAb 10F4 bound to PA_{63} (Fig. 2A). To better delineate the specificity of these MAbs, we studied their reactivities with expressed PA domains. The combinations of expressed PA domains generated included (i) domains 1 and 2; (ii) domains 1, 2, and 3; (iii) domains 1, 2, 3, and 4; and (iv) domain 1. We confirmed that MAb 7.5G bound primarily to domain 1 (Fig. 2B, upper panel), whereas MAb 10F4 bound primarily to domain 4, with some binding to domains 2 and 3 (Fig. 2B, lower panel). In addition, binding of MAb 7.5G to PA prevented trypsin but not furin digestion of PA (Fig. 2C), mapping the binding site of this MAb to the first 157 amino acids of domain 1.

Ig gene utilization. V region sequence analysis revealed that MAbs 7.5G and 10F4 (GenBank nucleotide sequence accession numbers DQ355823 and DQ355824, respectively) used the same germ line, V_H7183 (11), and the J_H4 gene elements (22). However, MAbs 7.5G and 10F4 (DQ355833 and DQ355834, respectively) utilized different IgG(κ) light-chain gene elements, V_kKK4 (24) and BA9 (16) gene elements and J_H4 (24) and J_H2 (16) gene elements, respectively. Hence, the differences in specificity may be a consequence of light chain contributions.

Effect of MAb on LeTx toxicity to murine macrophages. The addition of LeTx to J774 macrophages reduced their viability as measured by the MTT assay ($P < 0.05$) (Fig. 1B). Unlike an irrelevant Ab (MOPC21), both MAb 7.5G and 10F4 significantly inhibited the cytotoxic activity of the LeTx (Fig. 1B). MAb 10F4 was significantly less efficient than MAb 7.5G in

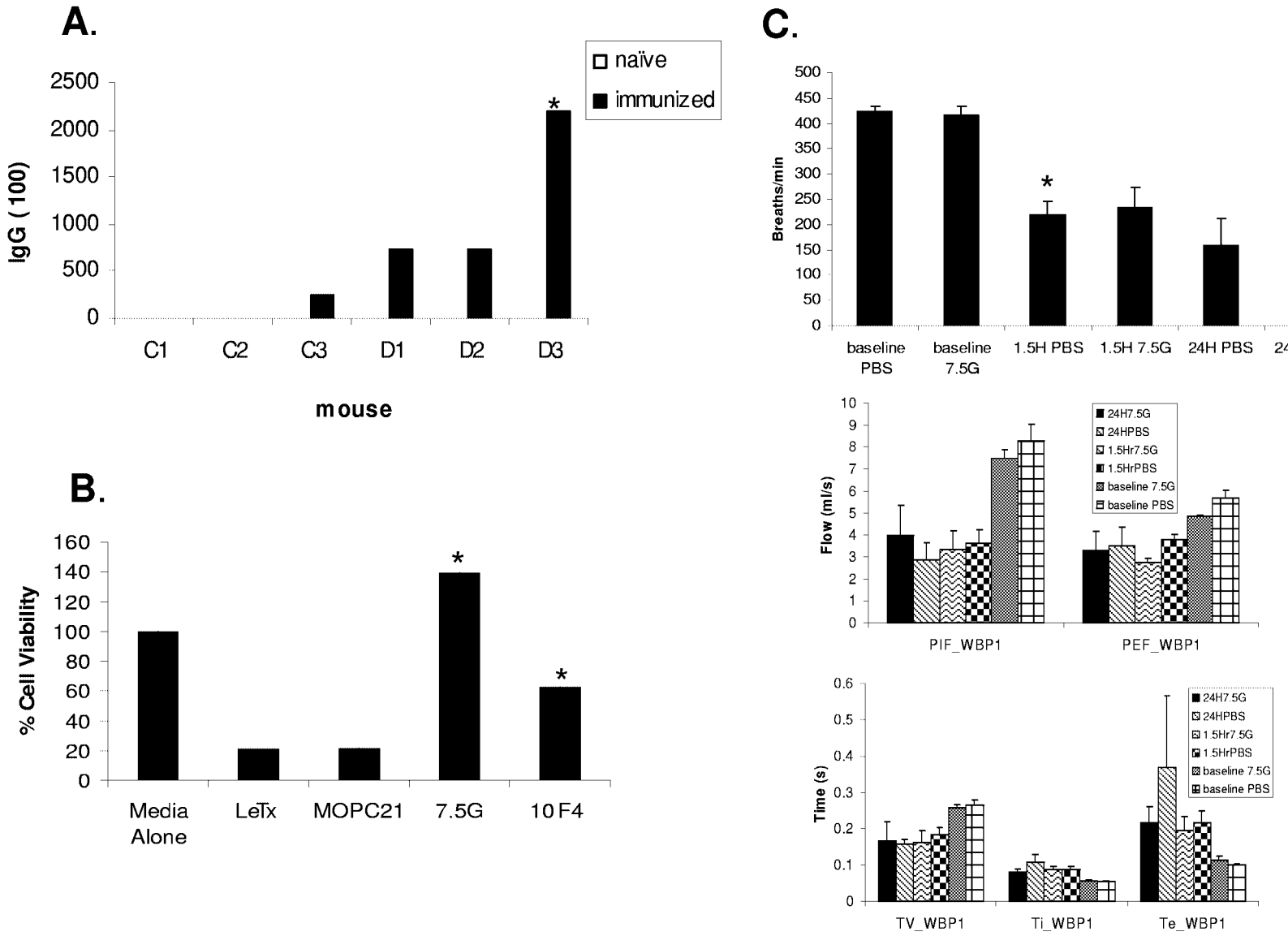

FIG. 1. (A) Inverse Ab titers of BALB/c mice immunized with PA_{83} protein. Mice were immunized with 2 μg (C mice) or 10 μg (D mice) of PA_{83} protein. Mouse D3 was chosen for hybridoma generation using standard hybridoma technology. (Naïve mice, no responses.) (B) Analysis of cellular toxicity in the presence of PA_{83} MAbs by the MTT assay. MAbs 7.5G and 10F4 can protect MSH alveolar and J774 macrophage monolayers against LeTx-mediated toxicity as compared to medium alone and LeTx alone. Bars denote the average results for four wells, and error bars (too small to be visible) denote standard deviations. (C) Lung function as measured by WBP in mice treated with MAb before administration of LeTx. MAbs 7.5G and PBS were administered intraperitoneally 24 h prior to LeTx injection. Bars denote the average results for three mice, and error bars indicate standard deviations. TV, tidal volume; Ti, inspiratory time; Te, expiratory time; PIF, peak inspiratory flow; PEF, peak expiratory flow; *, $P < 0.05$.

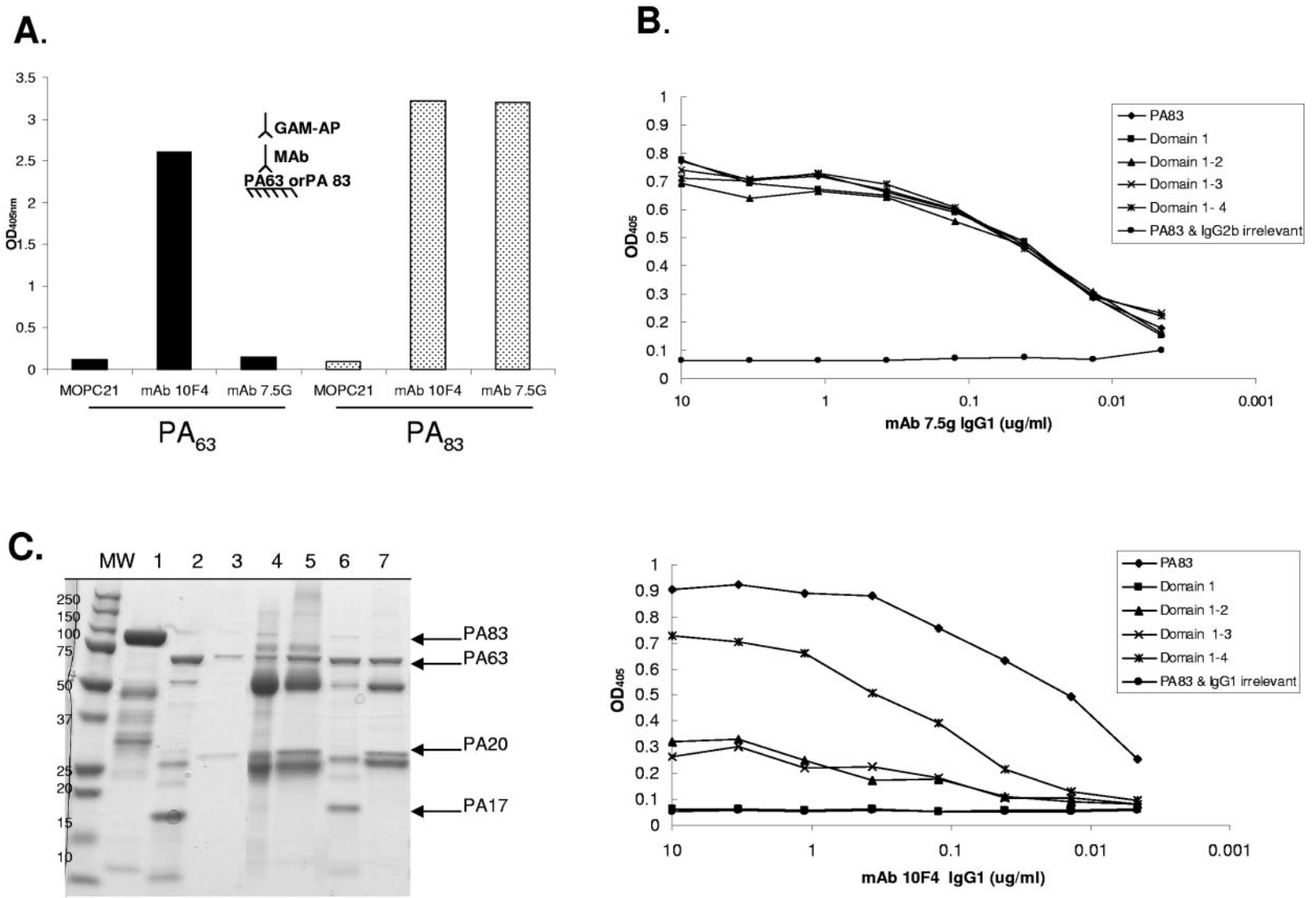

FIG. 2. (A) Analysis of Ab binding to PA_{63} and PA_{83} by ELISA. MOPC21 IgG1 was used as a negative control for the ELISA. Insert, schematic of ELISA. GAM-AP, alkaline phosphatase-labeled goat anti-mouse Ab reagent. (B) Analysis of Ab binding to expressed PA domains (12.05 μM). MAb 7.5G (upper panel) bound to all expressed domains: (i) domains 1 and 2 (1–2); (ii) domains 1, 2, and 3 (1–3); (iii) domains 1, 2, 3, and 4 (1–4); and (iv) domain 1. MAb 10F4 (lower panel) bound primarily to domains 1 to 4 with minimal binding to domains 1and 2 and 1 to 3. ELISAs were done twice with similar results. PA_{83} was used as a positive control. OD_{405}, optical density at 405 nm. (C) SDS-PAGE analysis of MAb 7.5G epitope mapping. MW, molecular weight markers (in kDa); lane 1, PA_{83}; lane 2, PA_{83} digested with trypsin; lane 3, PA_{83} digested with furin; lane 4, PA_{83} digested with trypsin plus MAb 7.5G; lane 5, PA_{83} digested with furin plus MAb 7.5G; lane 6, PA_{83} digested with trypsin plus MOPC21; lane 7, PA_{83} digested with furin plus MOPC21.

TABLE 1. Survival analysis of BALB/c mice treated with MAb prior to i.v. administration of LeTx

Expt[a]	Amt of LeTx (μg)/mouse	Amt of MAb 7.5G	Median survival (days) of mice		*P* value
			Treated	Control	
I[b]	100	100 μg	4	2 (NSO)	0.2573
II[c]	100	500 μg	1	1 (PBS)	0.241
III[c,d]	100	1 mg	2	1 (PBS)	0.0059
IV[c,d]	90 + 37.5	1 mg	4	1 (PBS)	0.0241

[a] Ten mice per group were used.
[b] In this experiment, Ab was administered as ascites fluid. NSO refers to the control group which received ascites fluid generated from the NSO myeloma partner which produces no immunoglobulins.
[c] For these experiments, Ab was purified as described by the manufacturer (Pierce, Rockford, IL).
[d] Statistically significant for MAb-treated mice compared to control mice within the same experiment.

protecting against LeTx toxicity. We evaluated the protective efficacy of MAb 7.5G in a mouse model of toxin injury. Administration of MAb 7.5G prolonged the survival of LeTx-injected BALB/c mice (median survival of 4 days) relative to PBS-treated, LeTx-injected BALB/c mice (median survival of 1 day) ($P = 0.02$) (Table 1). This experiment was done twice with similar results. Given the relatively modest effects on prolongation of survival, we sought validation of protective efficacy by an alternative method. Pulmonary-function measurements were taken 5 min before LeTx i.v. injections and 1.5 h and 24 h after LeTx injections. Administration of LeTx resulted in a profound depression of respiratory function as evidenced by reduced breathing rate (Fig. 1C, upper panel) and tidal volume (Fig. 1C, lower panel). To our knowledge, this phenomenon has not been described previously and presumably reflects pulmonary toxicity accompanying the systemic effects of shock and hemorrhage (5). Administration of MAb 7.5G prior to toxin injection was associated with a small, yet significant, amelioration of the respiratory distress as measured relative to control mice (Fig. 1C).

Analysis of mechanism of action of MAb. To understand the mechanism of action of MAb 7.5G, we investigated its effect on the several steps involved in LeTx-mediated macrophage cytotoxicity. We examined whether the binding of PA_{83} to its receptor on macrophages was affected by MAb 7.5G by measuring specific binding of ^{188}Re-PA_{83} in the presence and absence of MAb 7.5G. Equimolar concentrations of MAb 7.5G did not reduce the binding of PA_{83} to macrophages (Fig. 3A). In addition, a 50-fold molar excess of Ab (12 nM) did not prevent binding of PA_{83} (0.24 nM) to macrophages. We then investigated the possibility that MAb 7.5G binding may interfere with the subsequent binding of LF. The binding of LF was significantly enhanced in the presence of PA_{83}, and MAb 7.5G did not affect the subsequent binding of LF to PA_{83} (Fig. 3B). We speculated that one of the possibilities for the observed MAb-mediated protection was the interaction with Fc receptors on the cell surface. To test this possibility, we blocked the Fc receptors on macrophage cell lines with MAb 2.4G2 (anti-FcγRII/FcγRIII), a rat MAb that binds mouse Fc receptors (BD Pharmingen, Bedford, MA). Cells were then incubated with ^{188}Re-PA_{83} and MAb 7.5G, and again, the presence of Ab to Fc receptors did not block PA_{83} binding to macrophage receptors, suggesting that LeTx Ab protection is not Fc receptor mediated (Fig. 3C). We evaluated whether removal of Ab glycosylation affected MAb 7.5G efficacy but found no effect (data not shown). Since Ab glycosylation is essential for interaction with Fc receptors, this experiment provided additional evidence that Fc receptors were not involved in the protective effects. In addition, we measured toxin-mediated cell death in the presence or absence of MAb 2.4G2 using the MTT assay. Again, we noted no differences in MAb 7.5G protection in vitro (data not shown), confirming the above-described result. Lastly, we investigated the possibility that the binding of MAb 7.5G prevented the formation of the PA_{63} oligomer. For these experiments, we used CHO cells, an epithelial cell line which is susceptible to LeTx (13, 14, 17). MAb 7.5G did not impede the formation of oligomer (Fig. 3D). Hence, we conclude that the reduction in toxicity associated with the presence of MAb 7.5G was not a result of Ab-mediated interference with PA or LF binding, engagement of Fc receptors, or PA_{63} oligomerization.

Cleavage of PA_{83} and dissociation of PA_{20}. To examine whether Ab binding affects the dissociation of PA_{20} from PA_{63}, PA_{83} was digested with 2 U of furin for intervals of 30 s and 1, 2, 3, 4, and 5 min in the presence and absence of MAb 7.5G (Fig. 4). The amounts of the digested products PA_{20} and PA_{63} increased while the amount of undigested PA_{83} decreased over time. The amounts of PA_{83}, PA_{20}, and PA_{63} were similar at the 30-s and 1-min intervals, irrespective of Ab treatment. At the 2-, 3-, and 4-min intervals there was less PA_{20} and PA_{63} and more PA_{83} when MAb 7.5G was present, suggesting that MAb 7.5G may slow the proteolytic cleavage of PA_{83} to PA_{20} and PA_{63}.

DISCUSSION

MAbs 7.5G and 10F4 mapped to domains 1 and 4, respectively. The ability of MAb 7.5G to bind domain 1 was confirmed by two independent methodologies: binding studies on protease-digested PA_{83} and *E. coli*-expressed domains of PA. For MAb 10F4, the activity against LeTx is consistent with reports that most protective PA_{83}-binding MAbs have mapped to domain 4 (2, 13). Similar reports have been generated for other toxins, such as *Clostridium perfringens* iota-toxin (15). However, the finding that MAb 7.5G bound to the first 157 amino acids of domain 1 of PA_{83} was unexpected, because none of the protective MAbs described bind to this domain. Furthermore, domain 1 maps to the PA_{20} subunit that is cleaved from PA_{83}, which is not believed to play a critical role in LeTx toxicity (4). Analysis of the mechanism of action of MAb 7.5G revealed that this MAb had no effect on the binding of PA_{83} to macrophage-like cells, did not interfere with oligomerization of PA_{63} to form the channel heptamer, and did not require the presence of Fc receptors. However, furin-mediated proteolysis for PA_{83} was slower in the presence of MAb 7.5G. Although the relevance of this finding to the protective efficacy of MAb 7.5G has not been unequivocally proven, we propose that MAb 7.5G mediates partial protection against LeTx by slowing the conversion of PA_{83} to PA_{63}, thus delaying the toxic effects of LeTx. This mechanism could explain why MAb 7.5G administration results in only a modest prolongation in survival. We note that this effect was measured only in the initial stages of PA_{83} digestion with furin and that our

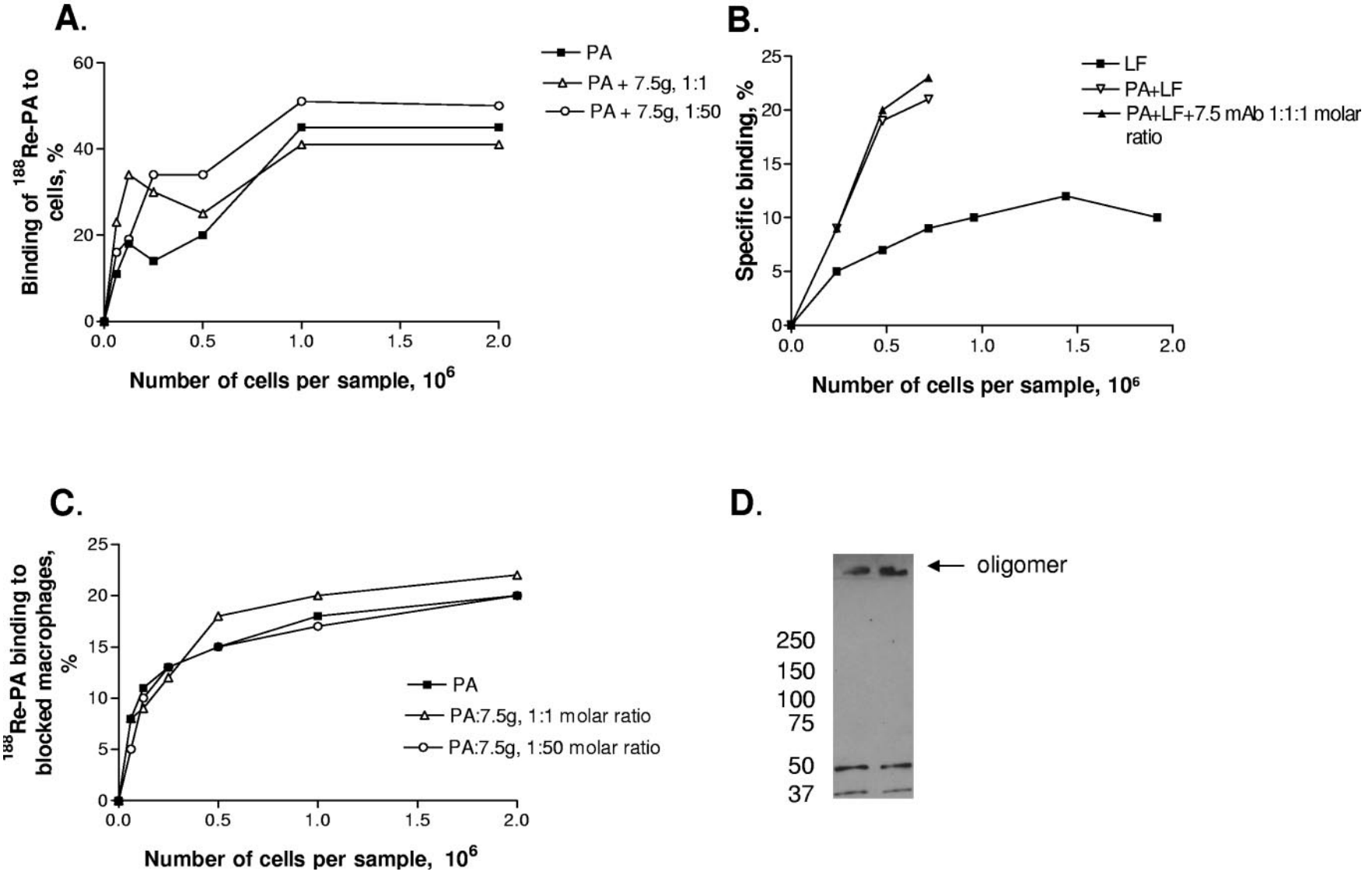

FIG. 3. (A) Binding of ^{188}Re-PA_{83} to J774 macrophages at 4°C in the absence and presence of MAb 7.5G. Molar ratios of PA_{83}: MAb were 1:1 and 1:50. (B) Binding of ^{188}Re-PA_{83} and LF to macrophages in the absence and presence of MAb 7.5G. (C) Binding of ^{188}Re-PA_{83} to J774 macrophages with blocked Fc receptors. These experiments were done for 15 and 60 min at 4°C with similar results. (D) PA_{63} oligomer formation in the presence and absence of MAb 7.5G. Lane 1, PA_{63}; lane 2, PA_{63} with MAb 7.5G.

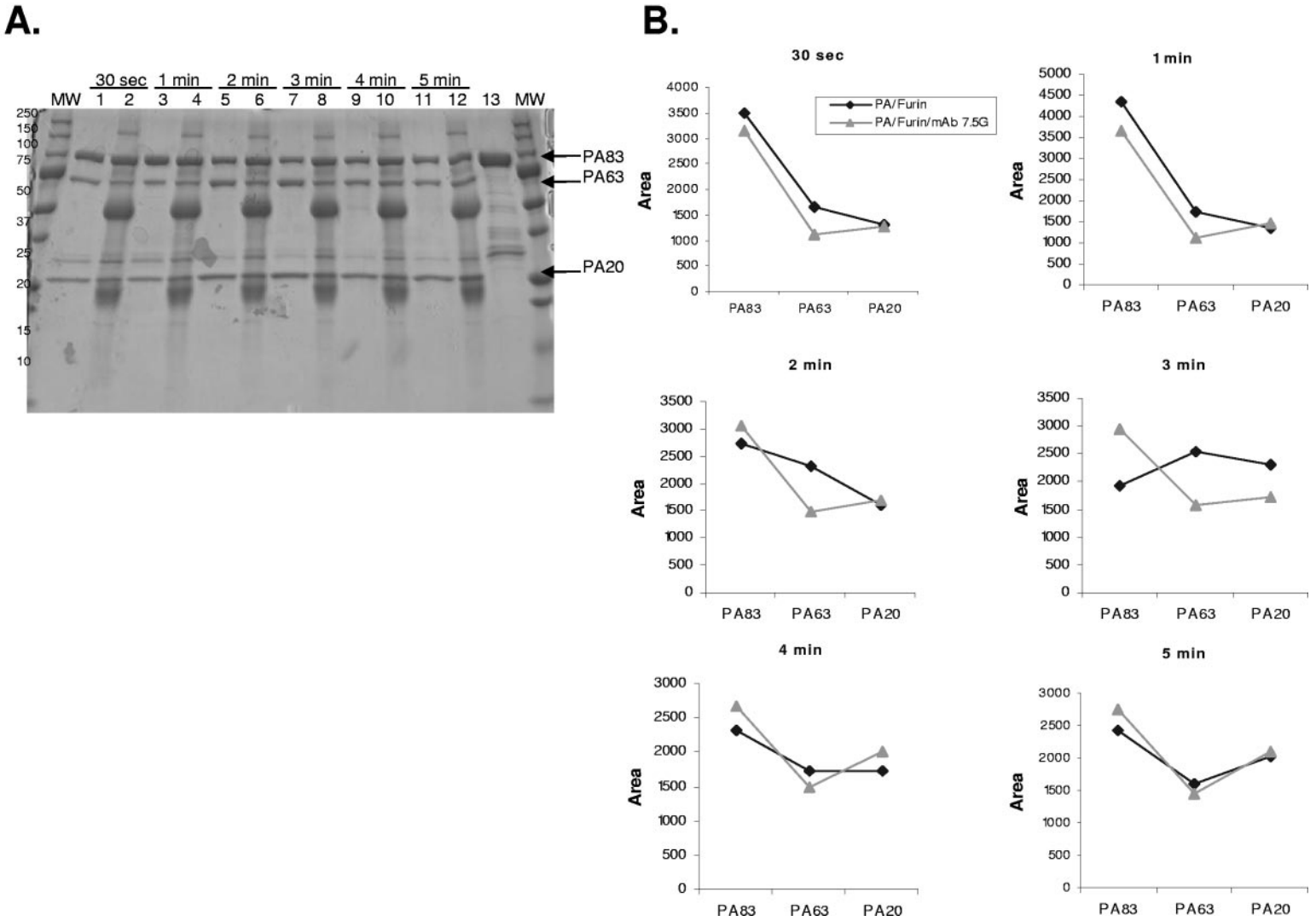

FIG. 4. (A) Cleavage of PA_{83} with furin in the absence and presence of MAb 7.5G over several time intervals. MW, molecular weight markers (in kilodaltons); lanes 1, 3, 5, 7, 9, and 11, PA_{83} with furin; lanes 2, 4, 6, 8, 10, and 12, PA_{83} with furin plus MAb; lane 13, undigested PA_{83}. Aliquots of PA_{83} were removed at 30-s and 1-, 2-, 3-, 4-, and 5-min time intervals for SDS-PAGE analysis. (B) ImageQuant analysis of bands. Key in 30-s graph applies to all panels.

inability to observe an effect on PA_{63} heptamer formation almost certainly reflects timing effects, as the latter experiment was done after a significantly longer incubation. Alternatively, our data can be interpreted as indicating that the neutralizing effect of MAb 7.5G reflects activity other than interference with receptor binding, proteolytic cleavage, PA_{63} oligomerization, or LF binding. In this scenario, MAb 7.5G could be a novel tool for dissecting activities that may provide new insights into the mechanism of LeTx. Nevertheless, our observation that MAb 7.5G can mediate protection against LeTx is significant because it establishes that Ab to the PA_{20} fragment could conceivably contribute to host defense. Consistent with this notion, analysis of the relative efficacies of various PA domain immunizations revealed partial protection for domain 1-immunized mice challenged with LeTx (9).

In summary, our results reaffirm the value of using MAbs to empirically determine the capacity of different domains to elicit protective Abs. We note that our MAbs had modest protective effects in vivo, consistent with the observation that other MAbs to PA_{83} have failed to mediate significant protection in vitro (13) and implying that Ab titers to LeTx in PA_{83}-vaccinated individuals may not correlate with Ab efficacy. Given the proposed mechanism of action for MAb 7.5, involving a slowing of furin digestion, one might anticipate that MAbs with higher affinity to domain 1 or MAbs that bind to epitopes closer to the cleavage site may confer significant protection. Targeting epitopes near the furin cleavage site has the added attraction of interfering with the first step in the complex choreography of LeTx action. A detailed mapping of PA_{83} structural regions that elicit useful, useless, or potentially deleterious Ab responses may lead to later-generation PA_{83}-derived vaccines that elicit only useful Ab responses.

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