Neutralizing Monoclonal Antibodies Directed against Defined Linear Epitopes on Domain 4 of Anthrax Protective Antigen[▽]

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The anthrax protective antigen (PA) is the receptor-binding subunit common to lethal toxin (LT) and edema toxin (ET), which are responsible for the high mortality rates associated with inhalational *Bacillus anthracis* infection. Although recombinant PA (rPA) is likely to be an important constituent of any future anthrax vaccine, evaluation of the efficacies of the various candidate rPA vaccines is currently difficult, because the specific B-cell epitopes involved in toxin neutralization have not been completely defined. In this study, we describe the identification and characterization of two murine monoclonal immunoglobulin G1 antibodies (MAbs), 1-F1 and 2-B12, which recognize distinct linear neutralizing epitopes on domain 4 of PA. 1-F1 recognized a 12-mer peptide corresponding to residues 692 to 703; this epitope maps to a region of domain 4 known to interact with the anthrax toxin receptor CMG-2 and within a conformation-dependent epitope recognized by the well-characterized neutralizing MAb 14B7. As expected, 1-F1 blocked PA's ability to associate with CMG-2 in an in vitro solid-phase binding assay, and it protected murine macrophage cells from intoxication with LT. 2-B12 recognized a 12-mer peptide corresponding to residues 716 to 727, an epitope located immediately adjacent to the core 14B7 binding site and a stretch of amino acids not previously identified as a target of neutralizing antibodies. 2-B12 was as effective as 1-F1 in neutralizing LT in vitro, although it only partially inhibited PA binding to its receptor. Mice passively administered 1-F1 or 2-B12 were partially protected against a lethal challenge with LT. These results advance our fundamental understanding of the mechanisms by which antibodies neutralize anthrax toxin and may have future application in the evaluation of candidate rPA vaccines.

Bacillus anthracis has long been recognized as a serious public health threat, given the ease with which *B. anthracis* spores can be disseminated via aerosol and due to the high mortality rate that accompanies spore inhalation. These fears were realized in the fall of 2001, when *B. anthracis* spores were circulated through the U.S. postal system, resulting in five deaths, 22 known cases of infection, and the possible exposure of more than 30,000 people (7). Although routine vaccination of civilians against anthrax is neither necessary nor desirable, certain segments of the population, notably emergency first responders and research laboratory personnel, remain at risk of exposure and are in need of an effective preexposure vaccine. In the United States, the only licensed anthrax vaccine, Anthrax Vaccine Adsorbed (AVA) or Biothrax, has been subject to controversy for years, due to safety concerns (3), and is not considered an ideal vaccine due to its protracted vaccination schedule (six injections over 18 months). AVA, which consists of formalin-treated culture filtrate from an attenuated strain of *B. anthracis* adsorbed to aluminum hydroxide, is also inherently difficult to manufacture and to standardize. A recombinant anthrax vaccine, manufactured by VaxGen, was considered to be a leading candidate to replace AVA and to supply the Strategic National Stockpile with 75 million doses, but it was recently discontinued because of concerns regarding

vaccine production and stability. For these reasons, there is now a concerted effort to develop a new anthrax vaccine based on a defined antigenic composition.

Current anthrax vaccines are focused on protective antigen (PA), the primary determinant in AVA that is involved in immunity to anthrax infection (21). PA is the receptor-binding subunit common to both lethal factor (LF), and edema factor (EF), which, upon PA binding form lethal toxin (LT) and edema toxin (ET), respectively. The anthrax toxins are responsible for the high mortality rates associated with inhalational *B. anthracis* infection. PA is secreted by *B. anthracis* as an 83-kDa protein (PA₈₃) that binds two known anthrax toxin receptors (ATRs), namely, tumor endothelium marker 8 (TEM-8) and capillary morphogenesis protein 2 (CMG-2) (5, 6, 19). Following receptor engagement, the amino-terminal 20-kDa region of the PA (PA₂₀) is proteolytically cleaved by a furinlike protease, exposing a region of PA that allows for the formation of the holotoxin. The membrane-bound 63-kDa PA (PA₆₃) spontaneously oligomerizes into heptamers and then associates either with LF, a zinc-dependent protease specific for certain members of the mitogen-activated protein kinase kinase family (12, 13), or EF, a calmodulin-, Ca²⁺-dependent adenylate cyclase (20), to form LT and ET.

It is well established that animals immunized with recombinant PA, or passively administered anti-PA antiserum, are protected against LT/ET exposure or spore challenge (reviewed in references 16 and 7). For example, we recently reported that LT-challenged mice treated with goat anti-PA antiserum (either alone or in combination with antibiotics) demonstrated increased survival rates compared to untreated

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mice (18). However, the antibody response to PA is complicated: total anti-PA titers, as measured by enzyme-linked immunosorbent assays (ELISAs), do not correlate well with protection (35). This is likely due to the fact that a large number of anti-PA antibodies are directed against non-neutralizing (or possibly even toxin-enhancing) epitopes (29, 41). Pioneering work by Little et al. (23) suggests that at least 20 antigenic determinants exist on PA. At present, only a few of these epitopes have been described in detail; several have been shown to be targets of monoclonal antibodies (MAbs) capable of effective neutralization in vivo or in vitro (1, 8, 10, 17, 44, 45). The fact that key neutralizing epitopes on PA remain widely unidentified poses a significant barrier to the evaluation of the efficacy of vaccines based on full-length and truncated PA subunits.

PAs can be divided into four functional domains. Domain 1 (residues 1 to 258) contains the furin recognition site (¹⁶⁴RKKR¹⁶⁷), as well as the region of PA that is recognized by LF and EF (30). Domain 2 (residues 259 to 487) and part of domain 3 (residues 488 to 595) are implicated in heptamerization, pore formation, and translocation of EF/LF across endosomal membranes (4, 27, 28, 34). Domain 4 (residues 596 to 735) constitutes the region of the PA involved in receptor recognition and attachment (39). Antibodies against domain 4 are postulated to be the most effective in neutralizing LT and ET (1), since they are proposed to interfere with PA binding to ATR. However, the only neutralizing epitope that has been characterized in detail is the one recognized by MAb 14B7 (24, 37, 40). This MAb contacts the face of domain 4 that is involved in receptor recognition, and has been proposed to encompass residues 671 to 721. Certainly, additional neutralizing epitopes exist on domain 4 (1, 46). In particular, Abboud and Casadevall (1) suggested a linear epitope, immediately adjacent to or possibly overlapping the 14B7 binding site, as the target of neutralizing antibodies. The characterization of antibodies that are both linear epitope dependent and neutralizing will be useful in the evaluation specific correlates of immunity; such MAbs can be incorporated into functional screening assays of potential vaccine candidates.

The goal of our study was to identify additional B-cell epitopes, within domain 4 of PA, that constitute the targets of neutralizing MAbs. Toward this end, we screened a collection of B-cell hybridomas, produced from PA₈₃-immunized mice, for MAbs capable of binding to a domain 4-specific peptide array. We identified two domain 4-reactive MAbs, 1-F1 and 2-B12. 1-F1 recognized a peptide spanning residues 692 to 703; this epitope maps to a region of domain 4 that overlaps with the binding site recognized by the well-characterized neutralizing antibody 14B7 (residues 671 to 721) (23, 40). 2-B12, on the other hand, recognized a peptide spanning residues 716 to 727; this epitope maps adjacent to the core 14B7 binding site and at a location not previously identified as being a target of neutralizing antibodies. Both MAbs neutralized LT in vitro and partially protected mice against LT challenge. These results advance our fundamental understanding of the mechanisms by which antibodies neutralize anthrax toxin and may have future application in the evaluation of candidate rPA vaccines.

MATERIALS AND METHODS

Cell lines. The J774A.1 murine macrophagelike cell line was obtained from the American Type Culture Collection. Cells were cultured in Dulbecco modified Eagle medium 1× high glucose with GlutaMAX (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37°C with 5% CO₂ (18).

Recombinant anthrax toxin proteins. Histidine-tagged, recombinant PA₈₃ and PA₃₂ were obtained from the Wadsworth Center Protein Expression Core (18). PA₃₂ is a truncated form of PA₈₃ containing only domains 3 and 4 and was previously shown to be properly folded and capable of binding to ATR (11). Recombinant LF (rLF) was obtained from BEI Resources (Manassas, VA; catalog no. NR-4367). Functional LT was produced by mixing rLF and rPA₈₃ at a 1:1 (wt/wt) ratio in sterile phosphate-buffered saline (PBS). Toxin stocks were qualified by dose-response titrations, as previously described (18), and used at 250% tissue culture effective concentration(s) (TCEC₅₀) for in vitro toxin neutralization assays. For in vivo studies, mice were challenged with 200 µg of LT by intraperitoneal injection, as described below. Recombinant proteins were stored at -20°C as single-use aliquots at a concentration of 1 mg/ml in PBS, except for the rLF obtained from BEI Resources, which was frozen at a concentration of 500 µg/ml in 1% bovine serum albumin. PA₈₃ was biotinylated by using EZ-Link Sulfo-NHS-LC Biotin (Pierce Biotechnology, Inc., Rockford, IL).

Hybridoma production and screening. Female BALB/c mice (Taconic Laboratories, Hudson, NY) ~8 weeks of age were immunized with PA₈₃-MIS (50 µg) administered by intraperitoneal injection on days 0, 14, and 24 and boosted with a single injection of PA₈₃ (100 µg) without adjuvant 3 days prior to B-cell fusion. PA₈₃-MIS consists of rPA (PA₈₃) covalently coupled to a nontoxic muramyl dipeptide derivative, as described previously (18). The mice were sacrificed 3 days after the final boost, and total splenocytes were harvested at the Wadsworth Center Immunology Core Laboratory for MAb production using a ClonA-Cell-HY hybridoma cloning kit (Stem Cell Technologies, Vancouver, British Columbia, Canada).

Hybridoma supernatants were screened by ELISA for reactivity against PA₈₃ and PA₃₂. Nunc Maxisorb 96-well microtiter plates were coated with PA₈₃ or PA₃₂ (0.1 µg/well) overnight at 4°C. Prior to the addition of hybridoma supernatant (0.1 ml/well), the plates were blocked with Blocker Casein (Pierce Biotechnology, Inc.) and washed three times with PBS containing 0.05% Tween 20. The plates were probed with horseradish peroxidase (HRP)-labeled goat anti-mouse immunoglobulin G (IgG) (Invitrogen Life Technologies) and developed with SureBlue TMB 1-Component microwell peroxidase substrate (KPL, Inc., Gaithersburg, MD). Microtiter plates were analyzed by using a SpectraMax 250 microtiter plate reader (Molecular Devices, Union City, CA). Antibodies that reacted with PA₃₂ were considered to be specific for domains 3 and 4 and were further evaluated for LT-neutralizing activity (see below) and for reactivity with a PA peptide array library (see below).

PA peptide array ELISAs were performed essentially as described above. A collection of 31 overlapping peptides, 12 amino acids (aa) in length and each overlapping by 4 aa, spanning PA₈₃ (GenBank accession no. AAA22637) were custom synthesized by New England Peptide (Gardner, MA). Lyophilized peptides were solubilized in dimethyl sulfoxide (DMSO) at 10 mg/ml and then diluted to 1:5 (vol/vol) into PBS and frozen at -20°C as single-use aliquots. The sequences of the individual domains 3 and 4 peptides are shown in Table 1. For ELISAs, Nunc Maxisorb 96-well microtiter plates were coated with peptides overnight at 4°C, washed with PBS, and then blocked with Blocker Casein before being treated with hybridoma supernatants or human AVA serum diluted 1:100 into PBS. As negative controls, wells were coated with peptides spanning the signal sequence region of PA or amine-only derivatives only.

Selected hybridomas were produced in ascites by the Wadsworth Center Immunology Core Laboratory, and MAbs were purified by standard endotoxin-free protein A purification by the Wadsworth Center Protein Expression Core. The subclass of each MAb was determined by ELISA. Nunc Maxisorb plates were coated with PA₈₃ and then reacted with each MAb for 1 h. The plates were then washed and incubated with HRP-conjugated goat anti-mouse immunoglobulin specific (IgG1, IgG2a, IgG2b, and IgG3) antibodies (Southern Biotechnology Associates, Birmingham, AL).

MAb affinity determination and competitive binding assays by SPR. The affinities of individual MAbs for PA₈₃ were determined by surface plasmon resonance (SPR) by using a Biacore 3000 instrument (GE Healthcare, Troy, NY). PA₈₃ was immobilized on a CM5 chip using NHS/EDC amine coupling and with a ligand density of ~4,000 response units (RU). Chips were equilibrated for 3 min in running buffer (HBS-EP; 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20) prior to injection of individual MAbs at a constant flow rate of 30 µl/min. Dissociation was monitored for 10 min. Association (K_a) and

TABLE 1. Domain 3 and 4 peptide sequences

Peptide	Range (aa)	Sequence (aa)
65	484–495	IQETTARIIFNG
66	492–503	IFNGKDLNLVER
67	500–511	LVERRIAAVNPS
68	508–519	VNPSOPLETTKPD
69	516–527	TTKPDMTLKEAL
70	524–535	KEALKIAFGFNE
71	532–543	GFNEPNGNLQYQ
72	540–551	LQYQGDITFEFD
73	548–559	TEFDFNFDDQOTS
74	556–567	QOTSQNIKNQLA
75	564–575	NQLAELNATNIY
76	572–583	TNIYTVLDKIKL
77	580–591	KIKLNAKMNIIL
78	588–599	NILIRDKRFHYD
79	596–607	FHYDRNNIAVGA
80	604–615	AVGADESWKEA
81	612–623	VKEAHREVINSS
82	620–631	INSSTEGLLNI
83	628–639	LLNIDKDIRKIL
84	636–647	RKILSGYIVEIE
85	644–655	VEIEDTEGLKEV
86	652–663	LKEVINDRYDML
87	660–671	YDMLNISSLRQD
88	668–679	LRQDGKTFIDFK
89	676–687	IDFKKYNDKLPL
90	684–695	KLPLYISPNPYK
91	692–703	PNYKVNVAVTK
92	700–711	AVTKENTINPS
93	708–719	INPSENGDTSTN
94	716–727	TSTNGIKKILIF
95	724–735	ILIFSCKGYEIG

dissociation (K_d) rate constants were calculated as previously described (33). For competitive binding studies, individual MABs (50 μ g/ml) were injected four to five times (10 μ l/injection) into the microfluidics cartridge to saturate ligand bound to the sensor surface. Subsequently, individual MABs (50 μ g/ml) were injected (20 μ l/injection) in series. All MABs were diluted in HBS-EP (pH 7.4).

In vitro toxin neutralization assay. PA₈₃ reactive antibodies were evaluated for their ability to neutralize LT in vitro, with an MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide]-based cytotoxicity assay, as described previously (18). Neutralization activity was defined as amount of MAB (μ g/ml) required to protect 50% of the cells in culture from the effects of LT, a value commonly referred to as the TCEC₅₀. J774A.1 cells were seeded in 96-well plates ($\sim 6 \times 10^4$ cells/well) and incubated until they achieved 70 to 80% confluence. LT (60 ng; ~ 2 TCEC₅₀) was mixed with various dilutions of MAB and then incubated for 4 h at 37°C before being applied to the J774.1 cells. Cell viability was determined with a cell growth determination kit (Sigma-Aldrich). As controls, cells were treated with PBS (cell viability control) or LT (cell death control). The percent protection was determined as follows: $\{1 - [(PBS - MAB)/(PBS - LT)]\} \times 100$.

Receptor CMG-2 solid-phase binding assay. Nunc Maxisorb plates (96 well) were coated overnight with soluble recombinant CMG-2 receptor (1 μ g/well in PBS containing 2 mM MgCl₂), kindly provided by Robert Liddington (Burnham College). Biotinylated PA₈₃ (1 μ g) was mixed with MAB supernatants for 1 h at room temperature and then applied to CMG-2-coated wells. The plates were incubated for 1 h at room temperature, washed to remove unbound biotinylated PA₈₃, and then incubated with avidin-HRP, as described above for ELISAs.

Mouse passive protection studies. For in vivo passive protection studies, female BALB/c mice (Taconic Laboratories), 6 to 8 weeks of age, were challenged by intraperitoneal injection with LT (100 μ g in 200 μ l of PBS) administered to the lower left quadrant of the abdomen. Approximately 5 min later, the animals

were injected in the lower right quadrant with saline only (controls) or with MABs diluted into saline. After toxin challenge, mice were scored for signs of distress and illness (e.g., muscle weakness, difficulty breathing, and reduced responsiveness to stimuli) every 2 h for the first 24 h and thereafter every 8 h. Mice were euthanized when they reached a score of 9 on a behavioral scale, with 9 representing moribund (adapted from Swearengen [42]). Death was used as an endpoint in the present study, and all mice were monitored for a total of 15 days. All animals used in the present study were housed under conventional, specific-pathogen-free conditions and were treated in strict compliance with the guidelines established by the National Institutes of Health and the Institutional Animal Care and Use Committee at the Wadsworth Center.

RESULTS

More than two decades ago, Little et al. (23) proposed that at least 20 distinct antigenic regions exist on PA. Since that original report, only a few of these antigenic regions have been characterized in any detail. Identification of the exact epitopes on PA that are recognized by neutralizing MABs has proven challenging since several of the best-characterized MABs (e.g., 14B7) are directed against conformation-dependent epitopes. However, there is evidence that linear B-cell epitopes on PA, specifically within domain 4, are the target of neutralizing antibodies (1). Identification of these epitopes is critical to our understanding of the key residues on PA that are involved in protective immunity to anthrax toxin and may have important implications for the evaluation of the efficacies of future anthrax vaccines.

Identification of MABs that recognize linear epitopes on domain 4. We produced a collection of B-cell hybridomas from the spleens of BALB/c mice immunized with an adjuvanted derivative of rPA (see Materials and Methods). Hybridoma supernatants were screened simultaneously by ELISA for reactivity with PA₈₃ and PA₃₂. PA₃₂ is a recombinant protein consisting only of PA domains 3 and 4 (11). PA₃₂ has been previously shown to be properly folded and capable of binding to ATRs on cell surfaces. From a total of 384 cloned hybridomas that were screened by this method, 39 ($\sim 10\%$) produced MABs that reacted with PA₈₃. Six of these recognized PA₃₂, suggesting that they were specific for domain 3 or 4. These six MABs were further subjected to a peptide array ELISA consisting of 12-aa peptides with 4-aa overlaps, spanning domains 3 and 4 of PA₈₃ (Table 1). Two MABs were specific for peptides from domain 3, whereas three MABs were reactive with peptides from domain 4 (Fig. 1). One MAB did not react with the peptide array library and was not studied further. One domain 3-reactive MAB, 2-A7, and two domain 4-reactive MABs, 1-F1 and 2-B12, were further evaluated.

MABs 1-F1 and 2-B12 recognize distinct epitopes within domain 4. A number of key characteristics of the three MABs chosen for further study are outlined in Table 2. MAB 2-A7 was determined to be an IgG2a that recognized a peptide spanning residues 532 to 543 within domain 3 of PA₈₃, an area not expected to contribute to the neutralization of the toxins (28). Biacore analysis revealed that 2-A7 recognizes rPA with a dissociation constant (K_d) of approximately 1.6×10^{-9} M. 1-F1 was determined to be an IgG1 that recognized a peptide spanning residues 692 to 703, a region of domain 4 known to be an important target of neutralizing antibodies (1, 23, 40). 1-F1 bound PA₈₃ with an affinity similar to that of 2-A7. 2-B12, also an IgG1, recognized a peptide spanning residues 716 to 727.

FIG. 1. Reactivity of 1-F1, 2-A7, and 2-B12 with peptides spanning domains 3 and 4 of PA. MAbs 2-A7 (A), 1-F1 (B), and 2-B12 (C) were applied to 96-well microtiter plates coated with overlapping 12-mer peptide library spanning domains 3 (A) and 4 (B and C) of PA. The solid black bars represent the reactivity of the MAbs with the indicated peptides, expressed as the percent increase over background, as described in Materials and Methods. Each panel represents the data from a single representative experiment. The amino acid sequence of each peptide is given in Table 1.

BIAcore analysis revealed that 2-B12 recognized rPA with a K_d of approximately 1.2×10^{-10} M.

We used PyMOL to model the locations of the putative linear epitopes recognized by MAbs 2-A7, 1-F1, and 2-B12 on the three-dimensional structure of PA₈₃ in association with the cell surface receptor CMG-2 (Fig. 2A). This modeling confirmed that all three putative epitopes mapped to the surface PA₈₃ and were not buried within the tertiary structure of the protein. The epitopes recognized by 1-F1 (red) and 2-B12 (blue) were situated within close proximity to the interface between PA₈₃ (yellow) and CMG-2 (purple). The putative epitope recognized by 1-F1 (residues 692 to 703) is located between the small and large loops of domain 4 and is nested within the region of PA₈₃ that is recognized by the neutralizing MAb 14B7 (Fig. 2B). The putative epitope recognized by 2-B12 (residues 716 to 727) is only partially located within the large loop (residues 704 to 723) and yet overlaps by 6 aa with the region of domain 4 recognized by 14B7 (Fig. 2B) (21, 36). Based on this information, we predicted that 14B7 would competitively inhibit (completely or partially) 1-F1 and 2-B12 from binding to PA₈₃.

We initially used competitive ELISAs to test these predictions. However, these assays were confounded by the need to use secondary-enzyme-linked reagents whose activities potentially complicated interpretation of relative amounts of MAb binding (data not shown). BIAcore analysis, on the other hand, provides a means to measure MAb-target interactions in real time without the need for secondary labels or detection reagents (2). Indeed, Marvaud et al. utilized this technology to determine whether murine MAbs bound to similar regions on *Clostridium perfringens* Iota toxin (25). BIAcore sensor chips were coated with PA₈₃ and then exposed to MAbs individually or in series. When tested individually, MAbs 14B7, 1-F1, 2-B12, and 2-A7 were each capable of associating with PA₈₃ immobilized on the sensor chips, as evidenced by measurable changes in SPR. The changes, expressed as RU, were as follows; 14B7, 434 RU; 1-F1, 254 RU; 2-B12, 220 RU; and 2-A7, 300 RU. When tested in series, 14B7 blocked the ability of 1-F1 and 2-B12 to bind to PA₈₃, since there was no measurable increase in RU upon subsequent addition of these MAbs (Fig. 3). On the other hand, the binding of the domain 3-specific MAb, 2-A7, was not affected by 14B7, as indicated by an increase in RU of >300 (Fig. 3). Identical results were obtained when 1-F1 and 2-B12 were applied to the chip in reverse order (i.e., 14B7 followed by 2-B12 and then 1-F1). These data demonstrate that 1-F1 and 2-B12 (but not 2-A7) recognize epitopes within or adjacent to the region of PA₈₃ bound by 14B7.

MAbs 1-F1 and 2-B12 neutralize LT in vitro. We used a well-established murine macrophage cell-based toxicity assay to determine the capacity of 1-F1, 2-B12, and 2-A7 to neutralize LT in vitro (18). In this assay, the TCEC₅₀ for 1-F1 was ~ 0.002 $\mu\text{g/ml}$ (Fig. 4). At concentrations of ≥ 0.007 $\mu\text{g/ml}$, 1-F1 completely protected J774 cells from the effects of LT. This is virtually identical to the protection profile observed for 14B7 (data not shown). The TCEC₅₀ for 2-B12 was determined to be ~ 0.004 $\mu\text{g/ml}$. However, maximal protection conferred by 2-B12 never exceeded 90%, suggesting that it is slightly less effective than 14B7 and 1-F1 at neutralizing LT in vitro. 2-A7

TABLE 2. Physical characteristics of MABs specific for PA domains 3 and 4

MAB	Epitope ^a	Range (aa)	Domain	Constant ^b		Isotype
				K_a (1/M)	K_d (M)	
2-A7	GFNEPNGNLQYQ	532–543	3	6.1×10^8	1.7×10^{-9}	IgG2a
1-F1	PNYKVNYYAVTK	692–703	4	5.0×10^8	2.0×10^{-9}	IgG1
2-B12	TSTNGIKKILIF	716–727	4	8.4×10^9	1.2×10^{-10}	IgG1
14B7	Conformation dependent	671–721	4	NA	4.3×10^{-9c}	IgG1

^a Reactive linear peptides (12-aa length, 4-aa overlap) spanning PA₈₃ domains 3 and 4.

^b The association (K_a) and dissociation (K_d) constants were determined by SPR analysis. NA, not available.

^c Previously described by Maynard et al. (26).

was completely ineffective at neutralizing LT, confirming that domain 3 antibodies are generally not protective.

Domain 4-specific MABs disrupt PA binding to CMG-2. Antibodies directed against domain 4 are proposed to neutralize LT and ET by preventing the association of PA₈₃ with ATRs, such as CMG-2, on the surfaces of host cells (40, 43). 14B7, for example, inhibits PA₈₃'s binding to host cells by >95% (23). We used an in vitro binding assay to determine the effects of 1-F1 and 2-B12 on the association of soluble rPA with plate-bound CMG-2. Biotin-labeled rPA₈₃ was incubated with individual MABs (14B7, 1-F1, 2-B12, or 2-A7) and then applied to microtiter plates coated with CMG-2. 14B7 was used as a positive control for the studies, and all data were normalized to these values. 1-F1 was as effective as 14B7 in preventing the interaction between rPA₈₃ and CMG-2. 2-B12, on the other hand, was only 73% as effective as 14B7 in disrupting rPA-receptor engagement (Fig. 5). The inability of 2-B12 to completely block PA binding to CMG-2 was not due to limiting amounts of MAB, as evidenced by the fact that a 20-fold dilution of 2-B12 was sufficient to neutralize LT in vitro (Fig. 5). As expected, 2-A7, which is directed against domain 3, demonstrated little capacity to disrupt PA binding to CMG-2.

MABs 1-F1 and 2-B12 protect mice against LT challenge. We used a previously established LT challenge model (18, 41) to examine whether 1-F1 and 2-B12 are protective in vivo. In this model, BALB/c mice were administered LT (100 µg) by intraperitoneal injection and 5 min later were treated with individual MABs (550 µg/animal) by the same route. Using this model, we have previously shown that control animals injected with LT plus saline die within 48 h, whereas animals treated with anti-PA₈₃ polyclonal IgG survive beyond 15 days (18). Consistent with our previous findings, all five LT-challenged, saline-treated control animals died within 24 h (Fig. 6). Mice treated with 2-A7 died at a rate indistinguishable from that for the control animals, confirming, at least for the present, that MABs against domain 3 do not afford any protection against LT in vivo. In contrast, 1-F1 and 2-B12 each conferred partial protection against LT. 1-F1 conferred 60% protection by 20 h and 40% protection by 80 h. 2-B12 conferred 80% protection by 20 h and 60% protection by 80 h. Although we were unable to establish the level of protection for 14B7 in this model due to limited reagent availability, we expect that individual administration of 14B7 should result in levels of protection similar to those reported here (8, 24). Our results confirm that 1-F1 and 2-B12 are effective in protecting mice against lethal LT challenge.

Reactivity of human anti-AVA serum with linear epitopes on domain 4. We wanted to determine whether the linear B-cell epitopes on domain 4 of PA identified in the present study are in fact recognized by humans. To test this, serum from a single individual immunized repeatedly with AVA was subjected to domain 4 peptide array ELISA. This serum had been previously established as having high LT neutralizing activity in vitro (data not shown). The ELISA revealed that the serum antibodies reacted with five distinct peptides, although reactivity against only three (peptides 83, 91, and 92) of the five peptides was >5% above background (Fig. 7). The most reactive peptides, 91 and 92, span residues 692 to 711 (PNYKVNYYAVT ENTIINPS) of PA₈₃, which defines the linear epitope recognized by 1-F1 (Fig. 2). These data suggest the linear epitope defined by MAB 1-F1 is in fact a target of neutralizing antibodies in humans and that serum antibody reactivity with this peptide may possibly serve as an indicator of immune status of individuals immunized with AVA and/or candidate rPA vaccines.

DISCUSSION

Domain 4 (residues 596 to 735) constitutes the region of PA involved in receptor recognition and attachment (39). The region from aa 679 to 693 is especially important in binding to the host cell surfaces because these residues form a small loop that directly interacts with TEM-8 and CMG-2. Although it has been appreciated for decades that domain 4 in general, and the small loop in particular, is the target of neutralizing antibodies (23), the specific B-cell epitopes within this region of PA that are responsible for eliciting protective immunity have not been fully defined. One exception is the conformation-dependent epitope recognized by 14B7 (24, 37, 40). We have now identified and characterized two additional neutralizing MABs directed against domain 4. MAB 1-F1 binds to a linear epitope immediately adjacent to the region of domain 4 that is recognized by 14B7. 2-B12, on the other hand, binds a linear epitope within the large loop of domain 4, a region not previously known to be the target of neutralizing antibodies. Identification of these epitopes is significant because it furthers our understanding of the key residues on PA that are involved in protective immunity to anthrax toxin and may have important implications for the evaluation of the efficacies of future anthrax vaccines.

14B7 and 1-F1 are similar in a number of key respects, including IgG subclass, affinity for PA, and capacity to block

FIG. 2. Modeling the location of the epitopes on PA recognized by 2-A7, 1-F1, and 2-B12. (A) The location of the linear epitopes recognized by MAbs 1F1, 2A7, and 2B12, modeled on the PA₈₃ structure by using PyMOL (<http://www.pymol.org/>) and PDB accession 1T6B (representing PA₈₃ bound to CMG-2) (38). The image on the right is rotated 180° with respect to the image on the left. The solid horizontal line on the bottom of the figure represents the cell surface. The ATR CMG-2 is depicted in purple. The four domains of PA are colored as follows: domains 1 and 2, gray; domain 3, orange; and domain 4, yellow. The epitopes recognized by the individual MAbs are colored as follows: 1-F1, red; 2-A7, green; 2-B12, blue. (B) Linear depiction of the epitopes recognized by 1-F1 (top; residues 692 to 703) and 2-B12 (bottom; residues 716 to 727) aligned with the residues recognized by 14B7 (middle; residues 671 to 721). The alignment reveals that the 1-F1 epitope is nested within the 14B7 recognition region, whereas the 2-B12 overlaps partially with the C-terminal region.

PA-ATR interactions, as well as efficacy in LT neutralization. 1-F1 differs from 14B7 in that it recognizes a linear epitope (residues 692 to 703) adjacent to the small loop on domain 4 (residues 679 to 692), whereas 14B7 recognizes a conformation-dependent epitope, which has been difficult to map precisely. Early analysis indicated that 14B7 binds to a region encompassing residues 671 to 721 (24). Rosovitz et al. (37) significantly refined that model by revealing that the critical residues of domain 4 recognized by 14B7 overlap, but are distinct from, those that contact the ATRs. For example, mutagenesis of Asp683 reduced PA binding to cell surfaces by more than 1,000-fold but had little effect on the ability of 14B7 to bind to PA. Detailed computer modeling of 14B7 docked to PA (40), along with other recent studies, has further narrowed

the putative epitope to ~14 aa (residues 680 to 692) (22, 24, 37, 40). In light of this information, it is interesting that the epitope recognized by 1-F1 lies just outside of the region of domain 4 thought to make direct contact with cell surfaces. Nonetheless, we propose that 1-F1, like 14B7, neutralizes LT by physically interfering with the ability of PA to associate with CMG-2. This conclusion is based on our observation that 1-F1 was as effective as 14B7 in blocking the association of PA with CMG-2, in an *in vitro* binding assay. The identification of 1-F1 underscores the importance of this face of domain 4 as a target of neutralizing antibodies. 14C7 and 3B6, two other IgG1 MAbs that compete with 14B7 for binding to PA, were described by Little et al. (23). However, the exact epitopes recognized by the MAbs were never identified. Given domain 4's

FIG. 3. 14B7 blocks 1-F1 and 2-B12 from associating with PA₈₃. A representative sensorgram generated by BIAcore analysis demonstrates that 14B7 prevents 1-F1 and 2-B12 from binding to PA₈₃. PA₈₃ was immobilized on a CM5 chip by using NHS/EDC amine-coupling, as detailed in Materials and Methods. 14B7 (50 μ g/ml) was injected four to five times (10 μ l/injection) into the microfluidics cartridge to saturate ligand bound to the sensor surface. Subsequently, individual MABs 1-F1, 2-B12, and 2-A7 (50 μ g/ml) were injected (20 μ l/injection) once each in series. The gray trace indicates the relative RU measured over time.

small size, it is likely that 14C7 and 3B6 bind to residues closely adjacent to, if not identical, to those recognized by 1-F1, 2-B12, or 14B7 (1, 8, 40).

The epitope recognized by 2-B12, as determined by peptide array reactivity, spans residues 716 to 727 and is situated within the large loop of domain 4 (residues 704 to 722) immediately adjacent to (and overlapping with) the region recognized by 14B7. Indeed, the proximity of the two epitopes was confirmed by competitive binding assays, in which 14B7 was able to completely block 2-B12's ability to associate with PA₈₃. Although the large loop of domain 4 is not postulated to be involved in direct physical association with ATRs (43), the observation that 2-B12 partially inhibited ($\sim$ 70%) PA₈₃ from binding to CMG-2 suggests that this MAB neutralizes LT by preventing toxin-receptor interactions. This mechanism is not implausible, if we consider that IgG's mass is almost 10 times greater than that of domain 4. It is unclear, however, whether steric hindrance alone accounts for 2-B12's capacity to neutralize LT in vitro and in vivo. In a mouse model, for example, 2-B12 was as effective as 1-F1 in preventing LT-induced death. 2-B12 is

FIG. 4. 1-F1 and 2-B12, but not 2A7, neutralize LT in vitro. LT (60 ng; $\sim$ 2 TCEC₅₀) was mixed with serial twofold dilutions of 1-F1 ($\square$), 2-B12 ($\circ$), and 2-A7 ($\bullet$) at the indicated concentrations and then applied to J774.1 cells grown in 96-well microtiter plates. The percent protection afforded by each MAB (y coordinate) was calculated as described in Materials and Methods. Each data point represents the average (with the standard error of the mean) of four assays.

FIG. 5. MAB-mediated disruption of PA binding to ATR. Biotinylated PA₈₃ was mixed with indicated MABs (100 μ g each) for 1 h before being applied to wells of a 96-well microtiter plate coated with recombinant CMG-2. The plates were then probed with avidin-HRP and developed as described in Materials and Methods. The degree to which each MAB interfered with PA binding to CMG-2 ($\blacksquare$) was normalized to the value obtained for 14B7. For comparative purposes, the MABs (5 μ g) were assayed for LT neutralizing activity ($\blacksquare$) as described in Fig. 4. Each data point represents the average (with the standard error of the mean) of four to eight replicates.

somewhat unusual, in that its affinity for PA is $\sim$ 10-fold greater than the affinities measured for 14B7 and 1-F1. It is possible, therefore, that the greater affinity of 2-B12 for its target, compared to the affinities of 14B7 and 1-F1, compensates for the fact that 2-B12 binds to a region of PA outside the receptor recognition domain. Several groups have recently documented the importance of antibody affinity in the neutralization of anthrax toxins (1, 46).

In the present study, we also identified and characterized a domain 3-specific MAB, 2-A7. 2-A7 recognizes a linear epitope (residues 532 to 543) that lies outside the region of domain 3 that is involved in PA oligomerization and heptamer formation (28). 2-A7 had no discernible capacity to neutralize LT in vitro or in vivo. However, the affinity of 2-A7 for PA was comparable to that the affinities observed for 14B7 and 1-F1. These data underscore the importance of epitope specificity as a primary determinant of antibody-mediated immunity to anthrax toxins. At least in the challenge model used in the present study, Fc-mediated clearance of toxin-antibody com-

FIG. 6. 1-F1 and 2-B12 protect mice against LT. BALB/c mice ($n = 3$ to 5/group) were administered LT (100 μ g) by intraperitoneal injection and 5 min later treated with individual MABs (550 μ g/animal) as described in Materials and Methods. Animals were treated with saline ($\blacksquare$), 2-A7 ($\bullet$), 1-F1 ($\square$), or 2-B12 ($\circ$). An asterisk indicates statistical significance ($P < 0.05$) using the log-rank test and a Bonferroni-corrected threshold compared to the saline control group.

FIG. 7. Human anti-AVA serum reacts with the linear epitope on PA recognized by 1-F1. Serum from a single AVA-vaccinated individual was tested for reactivity against the PA₈₃ domain 4 peptide array, as described in Fig. 1. The solid black bars represent the reactivity of the serum with the indicated peptides, expressed as the percent increase over background, as described in Materials and Methods. The amino acid sequence of each peptide is given in Table 1. The data presented are the averages of two independent experiments.

plexes does not contribute substantially to protection. This fact may account in part for the repeated observations that anti-PA antibody titers, as determined by ELISA, often fail to correlate with immunity to anthrax. On the other hand, serum reactivity with specific antigenic determinants or linear epitopes, such as those defined by 1-F1 and 2B12, could be useful as predictors of protection. In this regard, it was very interesting that serum antibodies from an AVA-immunized individual reacted with linear peptides corresponding to the epitope on domain 4 recognized by the neutralizing MAb 1-F1. Peptide arrays are increasingly being applied to infectious diseases (31, 32) and will likely have important applications for biodefense vaccines as an additional means to assess neutralizing antibody titers in the sera of vaccinated individuals.

Considering that rPA will most certainly be a constituent of the next-generation anthrax vaccine (9, 14, 15, 15, 18), much remains to be done in terms of identifying the specific regions of PA that elicit neutralizing (and non-neutralizing) antibodies, and to better understand how these antibodies interfere (or not) with toxin attachment, oligomerization, and translocation (17, 36, 45). For example, in our screen to identify domain 4 MABs, we identified 33 MABs that reacted with domains 1 or 2. Preliminary characterization of those MABs has indicated that roughly 60% of them either fail to neutralize LT or actually enhance LT-induced cell death; the remainder demonstrates various levels of protection in vitro (C. Kelly-Cirino, unpublished data). In our opinion, a complete and systematic identification of both conformation-dependent and conformation-independent epitopes is necessary if protective immunity to anthrax toxins is to be fully understood and if the efficacy of candidate human rPA vaccines is to be properly evaluated. Although beyond the scope of the present study, MABs 1-F1

and 2-B12 could be further investigated as possible therapeutics for the treatment of anthrax intoxication.

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