Generation and Characterization of Human Monoclonal Antibodies Targeting Anthrax Protective Antigen following Vaccination with a Recombinant Protective Antigen Vaccine

Xiangyang Chi, Jianmin Li, Weicen Liu, Xiaolin Wang, Kexin Yin, Ju Liu, Xiaodong Zai, Liangliang Li, Xiaohong Song, Jun Zhang, Xiaopeng Zhang, Ying Yin, Ling Fu, Junjie Xu, Changming Yu, Wei Chen

Laboratory of Vaccine and Antibody Engineering, Beijing Institute of Biotechnology, Beijing, China

The anthrax protective antigen (PA) is the central component of the three-part anthrax toxin, and it is the primary immunogenic component in the approved AVA anthrax vaccine and the "next-generation" recombinant PA (rPA) anthrax vaccines. Animal models have indicated that PA-specific antibodies (AB) are sufficient to protect against infection with *Bacillus anthracis*. In this study, we investigated the PA domain specificity, affinity, mechanisms of neutralization, and synergistic effects of PA-specific antibodies from a single donor following vaccination with the rPA vaccine. Antibody-secreting cells were isolated 7 days after the donor received a boost vaccination, and 34 fully human monoclonal antibodies (hMAb) were identified. Clones 8H6, 4A3, and 22F1 were able to neutralize lethal toxin (LeTx) both *in vitro* and *in vivo*. Clone 8H6 neutralized LeTx by preventing furin cleavage of PA in a dose-dependent manner. Clone 4A3 enhanced degradation of nicked PA, thereby interfering with PA oligomerization. The mechanism of 22F1 is still unclear. A fourth clone, 2A6, that was protective only *in vitro* was found to be neutralizing *in vivo* in combination with a toxin-enhancing antibody, 8A7, which binds to domain 3 of PA and PA oligomers. These results provide novel insights into the antibody response elicited by the rPA vaccine and may be useful for PA-based vaccine and immunotherapeutic cocktail design.

*B*acillus anthracis, the causative agent of anthrax, has long been considered a serious public health threat, particularly after the bioterrorism attacks that occurred after 11 September 2001. *B. anthracis* secretes a tripartite toxin that consists of protective antigen (PA; 83 kDa), lethal factor (LF; 85 kDa), and edema factor (EF; 89 kDa). LF is a Zn^{2+}-dependent metalloprotease (1) that combines with PA to form lethal toxin (LeTx), which causes death when injected into animals intravenously. EF is a Ca^{2+}-dependent adenylyl cyclase enzyme (2) that combines with PA to form edema toxin (EdTx), which causes edema at the site of inoculation. PA by itself is not known to have pathogenic effects but is considered the central component of the anthrax toxin. It primarily functions as a vehicle mediating the cellular uptake of EF and LF. PA binds to cell surface receptors (CMG-2/TEM-8) on target host cells (3, 4). Following binding, PA is cleaved by furin-like protease produced by the target cells to PA20, a 20-kDa peptide that is released into the surroundings. The remaining fragment, PA63 (also known as nicked/activated PA63), remains bound to the receptor and forms a heptamer or octamer that can translocate as many as three molecules of EF or LF from the cell surface into the cytosol via endocytosis, leading to the biological effects of EF and LF (5).

Vaccination against PA is sufficient to elicit immune protection. PA is the primary immunogenic component in the anthrax vaccine adsorbed (AVA) formulation that is currently licensed in the United States for human use. Although effective, AVA requires a much longer time (6 months) to produce protective immunity than that required for any effective postexposure anthrax prophylaxis, and the exact antigenic composition of the vaccine remains unknown and varies between batches (6, 7). The only immunogenic component in the "next-generation" anthrax vaccine is recombinant PA (rPA). The rPA vaccine can potentially elicit a faster protective immune response to be effective in postexposure cases and be produced in more homogenous standard formula-

tions (7). Clinical trials have demonstrated the safety and immunogenicity of the rPA vaccine (8, 9). Vaccination with rPA is known to elicit a polyclonal antibody (Ab) response. Most of the studies that have been conducted characterize the antibody responses induced by the AVA vaccine in humans (10). To date, there have not been very many studies to characterize the human antibody responses to the rPA vaccine.

The conventional treatment following potential exposure to aerosolized *B. anthracis* spores was antibiotics combined with PA-based anthrax vaccine. While antibiotics are effective in killing bacteria, they are unable to clear toxin components from the bloodstream (11). Passive immunization with monoclonal antibodies (MAbs) against toxin components has been shown to be highly protective in postexposure cases, particularly when combined with antibiotics (12, 13). Characterizing the protective rPA vaccine-induced antibody response in humans may identify naturally occurring neutralizing antibodies that could be used therapeutically.

Most of the monoclonal antibodies neutralizing PA-mediated toxicity have been produced in murine sources ([14], [15]), although some protective monoclonal antibodies against PA have been developed in chimpanzees, transgenic mice, and humans ([16]–[18]). The first Food and Drug Administration-approved monoclonal antibody against PA is fully human ([19]). Identification and characterization of novel protective anti-PA antibodies could contribute to the development of additional therapeutics for treating anthrax in patients.

In this study, we assessed the neutralizing potential of a large panel of monoclonal antibodies generated from an individual donor immunized with the rPA vaccine. We further characterized the domain specificities, affinities, mechanisms of neutralization, and potential synergistic effects of the protective human monoclonal antibodies (hMAbs). Overall, our results provide novel insights into the donor's antibody responses to the rPA vaccine and information about the design of PA-based vaccines and immunotherapeutic cocktails of hMAbs.

MATERIALS AND METHODS

Recombinant anthrax toxins. Recombinant protective antigen (rPA) and lethal factor (rLF) were expressed in *Escherichia coli* and purified as described previously ([20], [21]). The four domains of PA—domain 1 (residues 1 to 258), domain 2 (residues 258 to 487), domain 3 (residues 488 to 595), and domain 4 (residues 596 to 735)—were expressed as previously described ([22], [23]). To obtain PA63, PA83 was treated with trypsin at a final trypsin/PA weight ratio of 1:1,000 for 30 min at room temperature. The reaction was stopped by adding a 10-fold excess of soybean trypsin inhibitor (Sigma), and PA63 was purified using a HiTrap Q HP column (GE Healthcare Life Sciences).

Human subjects. After informed consent was obtained, a male volunteer was immunized with rPA vaccine (an investigational anthrax vaccine developed by Beijing Institute of Biotechnology, which is composed of purified rPA absorbed by aluminum hydroxide adjuvant) and received his second rPA vaccination 28 days later. Peripheral blood (35 ml) was drawn 7 days after the second (boost) vaccination (day 35). Peripheral blood mononuclear cells (PBMCs) were isolated from the blood by a standard Ficoll-Hypaque (Dakewe Biotech) density gradient method ([24]). The presence of PA-specific antibody-secreting cells (ASCs) was confirmed by linked immunosorbent spot assay (ELISpot) as described previously ([25]). Briefly, filter plates with hydrophilic mixed cellulose ester (MCE) membrane (Millipore) were coated with 10 µg/ml of PA or anti-human IgG antibody (BD Pharmingen). After washing and blocking, 1×10^6 cells from PBMC suspensions were added to each well. Anti-human IgG conjugated to horseradish peroxidase (HRP) antibody (Sigma) was then added after appropriate washing. The individual ASCs were visualized by addition of the 3-amino-9-ethylcarbazole substrate solution (Dakewe Biotech), and the spots were counted with a BioReader 4000 (Bio-Sys GmbH). The presence of toxin-neutralizing antibodies in the blood serum was confirmed by a cell viability assay ([26]).

Fluorescent cell sorting and single-cell PCR. PBMCs were stained with anti-CD3-fluorescein isothiocyanate (FITC), anti-CD20-FITC, anti-CD19-phycoerythrin (PE)-Alexa 610, and anti-CD27-PE (Molecular Probes) and anti-CD38-PE-Cy5 (Beckman Coulter). ASCs were defined as CD19$^+$ CD3$^-$ CD20$^-$ CD27^{high} CD38^{high}. Single ASCs were sorted using a MoFlo XDP cell sorter (Beckman Coulter) into 96-well plates. PCR amplification of paired VH and VL genes was performed as described previously ([24]), with the following modifications. Briefly, single cells were sorted into 96-well plates containing 20 µl of RNase-free water (Qiagen) and 0.25 µl of RNasin (Promega). Double-stranded cDNA was produced using the OneStep reverse transcription-PCR (RT-PCR) kit (Qiagen). The nested PCR was carried out using TransStart *Taq* DNA polymerase (TransGen Biotech). The PCR products of positive clones were purified

and sequenced by Sangon Biotech. Sequences were analyzed using IMGT/V-QUEST (http://www.imgt.org/IMGT_vquest/share/textes/).

Expression of recombinant hMAbs from linear expression cassettes. The cytomegalovirus (CMV) promoter, Ig leader sequence, VH/VL gene, Ig constant egion (IgG1), and poly(A) sequence fragments were assembled by overlapping PCR into full-length Ig heavy- and light-chain linear expression cassettes as previously described ([27]). The purified PCR products of paired full-length Ig H and L genes were cotransfected into a HEK293T (human embryonic kidney cell line; ATCC CRL-11268) cell expression system using TurboFect transfection reagent (Thermo Scientific). The culture supernatants were collected by aspiration and stored at $-20°C$ until further use.

Enzyme-linked immunosorbent assay (ELISA). Nunc plates (Thermo Scientific) were coated overnight with 2 µg/ml of PA, PA domain 1, PA domain 2, PA domain 3, or PA domain 4. After a washing with phosphate-buffered saline (PBS) plus 0.1% Tween 20, serial dilutions of culture supernatants of HEK293T cells starting at the desired dilution were added to each well and incubated for 1 h at 37°C. HRP-conjugated anti-human IgG antibody (Sigma) and then 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Merck KGaA) were added after appropriate washing. The reaction was stopped with 2 M H_2SO_4. The optical density (OD) was detected at 450 nm.

BLI. The kinetics of binding of the hMAbs to PA was studied using a ForteBio Octet instrument (Pall Life Science) based on biolayer interferometry (BLI) measurements by following the manufacturer's instructions. Briefly, anti-hIgG Fc capture Biosensors (Pall Life Science) were loaded with hMAbs at 20 µg/ml diluted in HEPES buffer (GE Healthcare Life Sciences). After capture, a 1-min wash in loading buffer removed excess unbound hMAbs to establish a new baseline signal. The sensors were dipped into wells containing PA at several diluted concentrations of from 300 to 50 nM in loading buffer. The sensors were then dipped into wells containing loading buffer to measure the (off-rate) dissociation rate. Equilibrium dissociation constant (K_D) values were calculated as the ratio of off-rate values to on-rate values. Data analysis was performed using ForteBio Data Analysis software package 7.0.

Cell viability assay. J774A.1 cells (murine macrophage cell line; ATCC TIB-67) were seeded in 96-well plates (Costar) at a density of 3×10^4/well and cultured overnight at 37°C. Twofold serial dilutions of hMAbs were preincubated with LeTx (0.1 µg/ml of PA and 0.1 µg/ml of LF) for 1 h at 37°C. The cell culture medium was replaced with the hMAb-toxin mixture and incubated with the cells for 1.5 h or 4 h at 37°C. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Invitrogen) was added to each well to a final concentration of 1 mg/ml, and the cells were incubated at 37°C for an additional 4 h. The hMAb-toxin mixture was replaced with the extraction buffer (0.5% [wt/vol] SDS, 25 mM HCl in 90% isopropyl alcohol). The OD of each well was measured at 570 nm, and the percent viability was calculated as $(OD_{measured} - OD_{dead\ control})/(OD_{live\ control} - OD_{dead\ control})$. The live and dead cell controls were treated with media only or a 10-fold excess of LeTx, respectively. The experiments were performed in duplicate; at least three independent experiments were performed.

Animal experiments. Male Fischer 344 rats (Vital River Laboratories) weighing 160 to 180 g were injected intravenously with a mixture of antibody and LeTx (10 µg of PA and 10 µg of LF per rat) at the desired molar ratios in 200 µl of PBS, or with LeTx only. Animals were observed for signs of malaise and mortality over 48 h. All animal experiments were performed according to protocols approved by the Animal Care and Use Committee of the Beijing Institute of Biotechnology.

Inhibition of furin cleavage. PA83 (36 pmol, 3 µg) was incubated with 0.25 µl of furin (New England BioLabs) in the absence or presence of antibody (33 pmol, 5 µg) for 1 h at 37°C. The reaction was performed in 20 mM Tris-HCl (pH 8.5) to preserve monomeric PA63 after furin cleavage. Samples were resolved on a 7.5% nonreducing SDS-PAGE gel (Bio-Rad Laboratories) and visualized using Coomassie brilliant blue (Amresco).
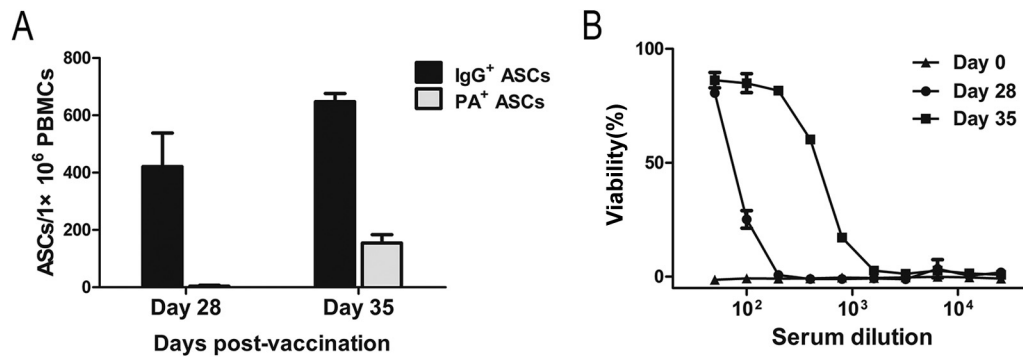
FIG 1 Characterization of the serum antibody response to recombinant anthrax protective antigen (rPA) vaccination. (A) ELISpot was used to determine the number of PA-specific and total IgG$^+$ ASCs per million PBMCs at day 28 and day 35 postvaccination. Error bars indicate the standard deviations of duplicates. (B) The ability of the donor serum, collected at day 0, day 28, and day 35, to protect J774A.1 cells from LeTx-mediated cell death was assessed using the MTT assay. Data are means ± SDs from at least three independent experiments, each conducted in duplicate.

Inhibition of oligomerization. PA63 (48 pmol, 3 μg) was incubated with antibody (33 pmol, 5 μg) for 30 min at room temperature. Oligomerization was induced using 50 mM morpholineethanesulfonic acid (MES) buffer (pH 5.5). Samples were resolved on a 4% to 20% nonreducing SDS-PAGE gel (Bio-Rad Laboratories) and visualized using Coomassie brilliant blue (Amresco). Subsequently, Western blot assay was performed with a rabbit anti-PA polyclonal antibodies (previously prepared in our laboratory) and a goat anti-rabbit HRP-conjugated IgG antibody (Santa Cruz Biotechnology).

Statistical analysis. Data from the *in vitro* assays were analyzed using the Student *t* test. Animal survival curves were generated and analyzed using the log rank test. All statistical analyses were performed using GraphPad Prism 5. A *P* value of <0.05 was considered statistically significant.

RESULTS

Human antibody response to immunization with the rPA vaccine. A male donor was immunized twice with the next-generation rPA vaccine. A blood sample was collected 7 days after the second (boost) vaccination (day 35). The frequencies of total IgG$^+$ and PA-specific IgG$^+$ ASCs were determined as a percentage of total PBMCs using a standard ELISpot (25). The number of total IgG$^+$ spots increased from 421 per million at day 28 to 647.5 per million at day 35 (Fig. 1A). Similarly, the number of PA-specific spots increased from 3.5 per million at day 28 to 154 spots per million at day 35 (Fig. 1A). The percentage of PA-specific IgG$^+$ ASCs as a fraction of total IgG$^+$ ASCs increased from 0.8% to 23.8% 7 days after the boost vaccination. The protective immune response after vaccination was confirmed using the MTT assay as previously described (22). Prior to vaccination, the serum isolated from the vaccinated patient did not mitigate LeTx-induced cell death. However, at day 28 the 50% inhibitory concentration (IC$_{50}$) in the serum against LeTx was 87.4 and increased at day 35 to 514.1 (Fig. 1B). Taken together, the results indicated that PA-specific and neutralizing polyclonal Ab responses were effectively induced by the boost rPA vaccination.

Rapid generation and characterization of PA-specific hMAbs following rPA vaccination. Seven days after the donor received the boost vaccination, ASCs defined as CD19$^+$ CD3$^-$ CD20$^-$ CD27^{high} CD38^{high} were sorted from total PBMCs as single cells using fluorescent cell sorting. A total of 529 pairs of VH and VL genes were amplified from single ASCs. To rapidly express the MAb genes, linear expression cassettes (27) were produced by PCR. Full-length Ig H and L genes were cotransfected into a HEK293T cell expression system. IgG concentrations of approximately 1 μg/ml were obtained from the transfected culture supernatants. The recovery was sufficient for screening clones in ELISAs and cell viability assays. ELISA was used to screen 529 hMAbs produced from single ASCs for reactivity to PA. Of the hMAbs screened, 34 clones (6.4%) were PA specific (Table 1). Domain specificity of the PA-specific hMAbs was determined by ELISA. Of the 34 PA-specific clones, 14 hMAbs did not clearly bind to any of the PA domains, and only one hMAb bound to domain 2. Surprisingly, 12 of the 34 hMAbs specifically bound to domain 4 of PA (Table 1).

To determine the neutralizing capabilities of the PA-specific hMAbs, a cell viability assay was performed using J774A.1 cells. The 34 hMAbs were categorized as protective (11.8%), toxin enhancing (55.9%), or toxin indifferent (32.3%) depending on their ability to prevent cell death after a 1.5-h incubation with the cells (Fig. 2A; Table 1). The viabilities of cells treated with LeTx only were used as references to determine hMAbs to be protective, indifferent, or enhancing (28). We selected five of the clones for in-depth characterization (Table 2).

Among the protective hMAbs in a 4-h incubation, 22F1 (IC$_{50}$ = 0.0047 μg/ml, 0.03 nM) conferred the highest level of protection and 4A3 (IC$_{50}$ = 0.029 μg/ml, 0.19 nM) and 8H6 (IC$_{50}$ = 0.03 μg/ml, 0.2 nM) showed similar neutralization capacities, while the remaining hMAb, 2A6 (IC$_{50}$ = 0.2 μg/ml, 1.33 nM), required a much higher concentration to achieve measurable protection (Fig. 2B). Clone 8A7 is a representative toxin-enhancing antibody that augmented LeTx toxicity *in vitro* (Fig. 2A). Fischer 344 rats (*n* = 5) were challenged with LeTx to evaluate the *in vivo* neutralization potential of the hMAbs, as previously described (29). Consistent with the *in vitro* results, 22F1 showed the highest level of protection and resulted in 100% survival even when mixed with PA at the molar ratio of 1:3 (data not shown). However, 2A6 was not able to offer any protection *in vivo*. Similarly, 8A7 provided no *in vivo* protection (Fig. 2C).

The binding affinities of all of the hMAbs were determined using the Fortebio Octet system. Interestingly, 22F1, which had the highest neutralizing capacity both *in vitro* and *in vivo*, had an extremely low binding affinity for PA83 but bound to PA63 with moderate affinity (*K*$_D$ = 4.21 nM). The four protective antibodies did not clearly bind to a specific domain of PA.

TABLE 1 Characteristics of the 34 PA-specific human monoclonal antibodies generated in this study

Function[a]	Clone	VH[b]	DH[c]	JH[d]	HCDR3[e]	VL[f]	JL[g]	Specificity[h]
Protective	2A6	HV4-31	HD2-15	HJ4	ATGDGGVAGTFDF	KV3-15	KJ4	Whole PA
	4A3	HV4-39	HD3-9	HJ6	ASLPLVYDSLTGHYFQYYGLNV	LV1-40	LJ2	Whole PA
	8H6	HV4-39	HD6-13	HJ4	ASGYSTSFDY	KV1-12	KJ2	Whole PA
	22F1	HV4-31	HD4-17	HJ5	ARASLRGIL	KV3-11	KJ4	PA63
Enhancing	1H2	HV3-30	HD1-26	HJ4	AKDGREALLAS	LV3-25	LJ2	D1
	1H5	HV3-23	HD4-23	HJ5	AKGGGNSGWFDT	KV4-1	KJ1	D4
	1D10	HV3-74	HD3-10	HJ4	AGDPGYYGSGSRAGFDS	KV3-11	KJ5	Whole PA
	2A2	HV4-61	HD3-3	HJ6	ATGKYYDFWTGPKFGMDV	KV1-9	KJ4	D3
	3C4	HV1-3	HD2-8	HJ4	ARQGGPSRTGGYYFDY	KV4-1	KJ4	D4
	8A7	HV3-23	HD3-10	HJ1	AKFSGHYGSGSFHLPEYFQH	KV1-5	KJ1	D3
	9F11	HV3-48	HD5-18	HJ4	ARDRDTNIWYPDGFDS	KV1-5	KJ1	D3
	11G6	HV3-23	HD4-23	HJ5	ARGGGNSGWFDP	KV4-1	KJ1	D4
	11B10	HV3-23	HD4-23	HJ5	AKGGGNSGWFDS	KV4-1	KJ1	D4
	11E12	HV3-23	HD3-10	HJ6	AKVRFGDDGMDV	KV1-5	KJ2	D1
	12B8	HV3-23	HD4-23	HJ5	AKGGGNSGWFDS	KV4-1	KJ1	D4
	12C9	HV4-39	HD4-17	HJ4	ARMLGYGDYHCDY	KV1-12	KJ4	Whole PA
	14A4	HV3-23	HD4-23	HJ5	AKGGGNSGWFDP	KV4-1	KJ1	D4
	15F8	HV5-10-1	HD3-3	HJ4	TRHSDFWSGYHDY	KV1-5	KJ1	Whole PA
	15H10	HV3-7	HD1-26	HJ4	ARGATWTDF	KV3-15	KJ1	Whole PA
	18C11	HV3-30	HD5-18	HJ2	AKEFGHDHWYFDF	KV3-15	KJ4	Whole PA
	19D2	HV3-23	HD4-23	HJ5	ARGGGNSGWFDS	KV4-1	KJ1	D4
	19H4	HV3-23	HD6-19	HJ2	AKRQGAALHKWHFDR	KV4-1	KJ1	Whole PA
	19F8	HV4-59	HD5-12	HJ4	ARGGDGDYLQF	KV1-NL1	KJ2	Whole PA
Indifferent	2G6	HV3-23	HD3-10	HJ3	AKDVSMIREFDAFDI	LV4-69	LJ3	D4
	3D6	HV5-51	HD3-3	HJ4	ARTNVGVPGFDF	LV6-57	LJ3	Whole PA
	3C8	HV3-53	HD5-18	HJ6	ASLVDTTMGYYGVDV	KV1-33	KJ2	Whole PA
	3A11	HV3-30	HD6-6	HJ4	AKLYTSSTSLDF	LV3-25	LJ2	D4
	7D5	HV3-23	HD4-23	HJ5	AKGGGNSGWFDP	KV4-1	KJ2	D4
	8D4	HV3-30	HD5-18	HJ6	AKDLRERYTYGYWVSLYYDGMDV	LV1-44	LJ3	Whole PA
	11D6	HV3-30	HD6-19	HJ4	ARISLASSSGWFDY	KV3-11	KJ4	Whole PA
	11F11	HV3-23	HD4-23	HJ5	AKGGGNSGWFDS	KV4-1	KJ1	D4
	12H3	HV3-23	HD3-10	HJ3	AKDVSMIREFDAFDI	LV4-69	LJ3	D4
	15H8	HV1-2	HD5-18	HJ6	ASHLRMEV	KV1-27	KJ1	D3
	16D2	HV3-30	HD3-10	HJ4	AKDQLYFGSQSPGHY	KV4-1	KJ4	D2

[a] Function categorized based on the cell viability assays.
[b] H-chain V-region gene usage.
[c] H-chain D-region gene usage.
[d] H-chain J-region gene usage.
[e] Amino acid sequence of the heavy-chain third complementarity-determining region.
[f] L-chain V-region gene usage.
[g] L-chain J-region gene usage.
[h] PA domain specificity of the hMAbs based on subunit ELISA.

Protective hMAb 8H6 inhibited furin cleavage of PA. *In vivo*, after PA83 binds to its cell surface receptors, PA20 is proteolytically removed by furin or furin-like proteases. *In vitro*, PA63 and PA20 remain associated with each other after furin cleavage, but the two fragments can be dissociated by SDS-PAGE. To determine whether any of the hMAbs inhibited the furin cleavage of PA, the antibodies were preincubated with PA83 and then furin was added. Furin cleavage was inhibited by 8H6 but none of the other hMAbs (Fig. 3A). 8H6 inhibited furin-mediated PA83 cleavage in a dose-dependent manner; 16.5 pmol (2.5 μg) of antibody completely inhibited cleavage of 36 pmol (3 μg) of PA83 (Fig. 3B). 8H6 provided an excellent level of protection both *in vitro* and *in vivo* and had a very high binding affinity with PA83, yet it did not show clear domain specificity.

Protective hMAb 4A3 enhanced the degradation of nicked PA. Following the furin-mediated cleavage of PA83, the remain-

ing PA63 fragment oligomerizes, facilitating the binding of LF/EF. *In vitro*, PA63 remains associated with PA20 after cleavage, preventing the oligomerization of PA63. However, the electrostatic interactions between PA63 and PA20 can be disrupted by anion-exchange chromatography, and low pH (pH 5.5 to 6.0) can favor the oligomerization of PA63. To determine whether any of the hMAbs inhibited PA oligomerization, each antibody was preincubated with purified PA63 before the pH was reduced to 5.5. Then the samples were resolved by nonreducing SDS-PAGE. No oligomers were observed in the presence of 4A3, indicating that 4A3 inhibited oligomerization. Interestingly, the presence of 4A3 also resulted in the appearance of two distinct bands (approximately 17 kDa and 30 kDa), several faint bands between 63 kDa and 15 kDa, and a weaker PA63 band (Fig. 4A, lanes 7 and 8). To confirm that these bands were PA fragments, the membranes were probed with rabbit anti-PA polyclonal antibodies (Fig. 4B). Taken to-
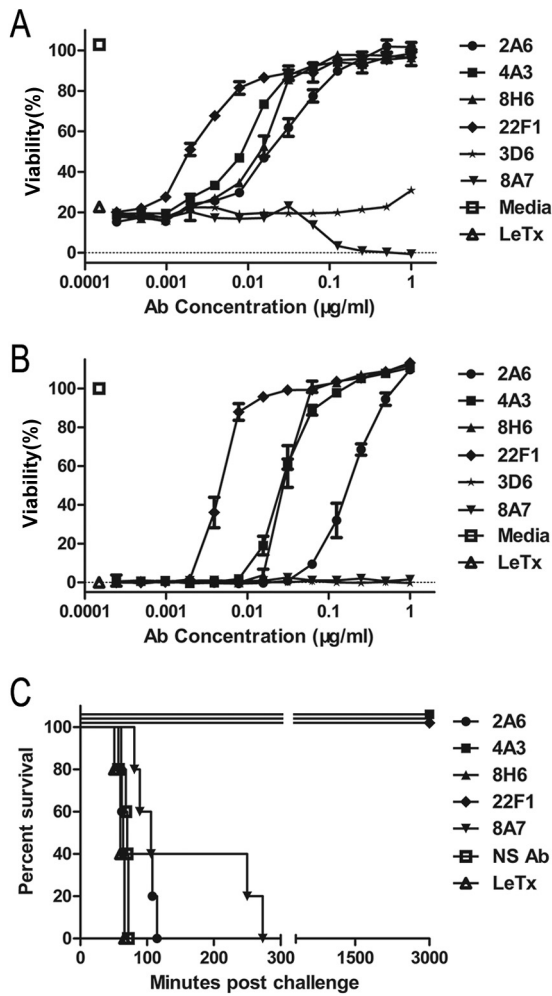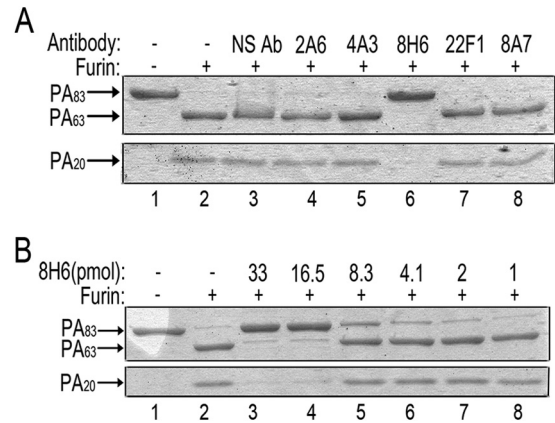
FIG 3 Clone 8H6 neutralized PA by inhibiting furin cleavage. (A) PA was incubated with furin in the presence or absence of each hMAb. Furin cleavage was determined by the absence of the full-length PA83 band and the presence of the PA63 and PA20 bands. NS Ab, nonspecific antibody. (B) 8H6 inhibited furin cleavage in a dose-dependent manner. *In vitro* proteolysis of 36 pmol of PA83 was completely inhibited by 16.5 pmol of antibody. Results shown were obtained by nonreducing SDS-PAGE.

0.198 nM), suggesting that 4A3 specially interacted with PA63 after PA20 was cleaved off.

Toxin-enhancing hMAb 8A7 bound PA63 oligomers and synergized with hMAb 2A6 to provide better protection. We also assessed the effect of clone 8A7 (toxin enhancing) on the formation of PA63 oligomers by nonreducing SDS-PAGE. 8A7 was preincubated with purified PA63 before the pH was reduced to 5.5 to induce oligomerization. In the presence of 8A7, the bands indicating antibody and PA63 oligomers became much fainter, while a high-molecular-mass band larger than the oligomers appeared (data not shown). These results suggested that 8A7 bound the oligomers and formed a high-molecular-mass supercomplex that could not be resolved by SDS-PAGE (Fig. 4A, lanes 13 and 14). Although binding capacity of 8A7 toward oligomers and its high affinity for PA domain 3 do not favor its individual neutralization efficacy against LeTx, these bindings contribute to the synergistic interactions of 8A7 and 2A6 (Fig. 5).

We combined the five clones characterized in detail to determine whether there were synergistic interactions. Most of the antibody combinations did not show significant changes in cell neutralization levels (data not shown), with an exception. While neither 2A6 (protective *in vitro*) nor 8A7 (toxin-enhancing) provided *in vivo* protection, the combination resulted in robust toxin

FIG 2 Protective efficacy of PA-specific human monoclonal antibodies (hMAbs) *in vitro* and *in vivo*. Serial 2-fold dilutions of the indicated hMAbs mixed with LeTx were incubated with J774A.1 cells for 1.5 h (A) or 4 h (B). The viability of the J774A.1 cells was determined using the MTT assay. Data are means ± SDs from at least three independent experiments, each conducted in duplicate. (C) Fischer 344 rats (*n* = 5/group) were challenged with LeTx (10 μg of PA and 10 μg of LF per rat) to determine whether the hMAbs provided *in vivo* protection (the molar ratio of antibodies to PA was 1:1). NS Ab, nonspecific antibody.

gether, the results suggested that 4A3 enhanced the degradation of PA63, thereby possibly interfering with PA oligomerization and providing protection *in vitro* and *in vivo*. While 4A3 bound to PA83 with low affinity, it bound to PA63 with high affinity (K_D =

TABLE 2 Human monoclonal antibody clones characterized in detail

Clone	K_D (nM)[a]	*In vitro* IC$_{50}$ (nM)[b]	*In vivo* survival rate (%)[c]	Domain specificity[d]	Function[e]
2A6	3.05 ± 1.36	1.33	0	Whole PA	Unknown
4A3	179 ± 54.4	0.19	100	Whole PA	Interference with oligomerization
8H6	0.636 ± 0.838	0.2	100	Whole PA	Inhibition of furin cleavage
22F1	Very low affinity	0.03	100	PA63	Unknown
8A7	0.869 ± 2.48			Domain 3	Binding to oligomers

[a] Determined by the Fortebio Octet system.
[b] Determined using cell viability assays.
[c] Determined using animal experiments.
[d] Based on subunit ELISA.
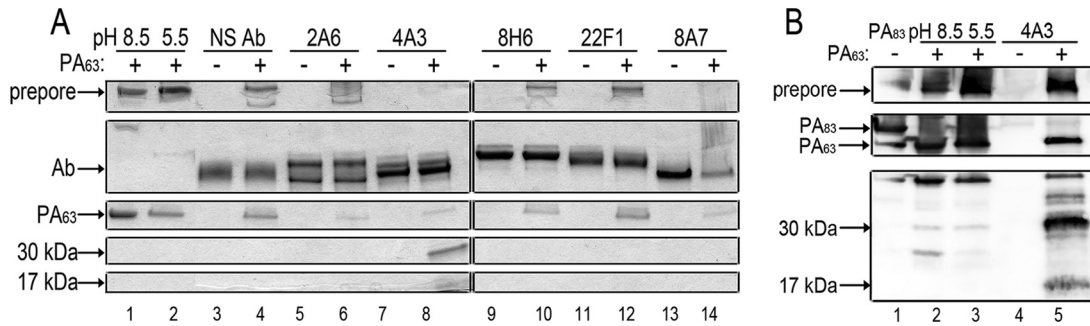[e] Based on furin cleavage and oligomerization assays.

FIG 4 Clone 4A3 inhibited PA63 oligomerization and clone 8A7 bound PA63 oligomers. (A) PA63 was allowed to oligomerize in the presence (+) or absence (−) of each hMAb. Samples where antibody was present were preincubated to allow binding. Samples were resolved by nonreducing SDS-PAGE gel. NS Ab, nonspecific antibody. (B) Clone 4A3 enhanced the degradation of PA63 and thereby interfered with PA oligomerization. Samples were subjected to nonreducing SDS-PAGE and Western blotting using rabbit anti-PA polyclonal antibodies.
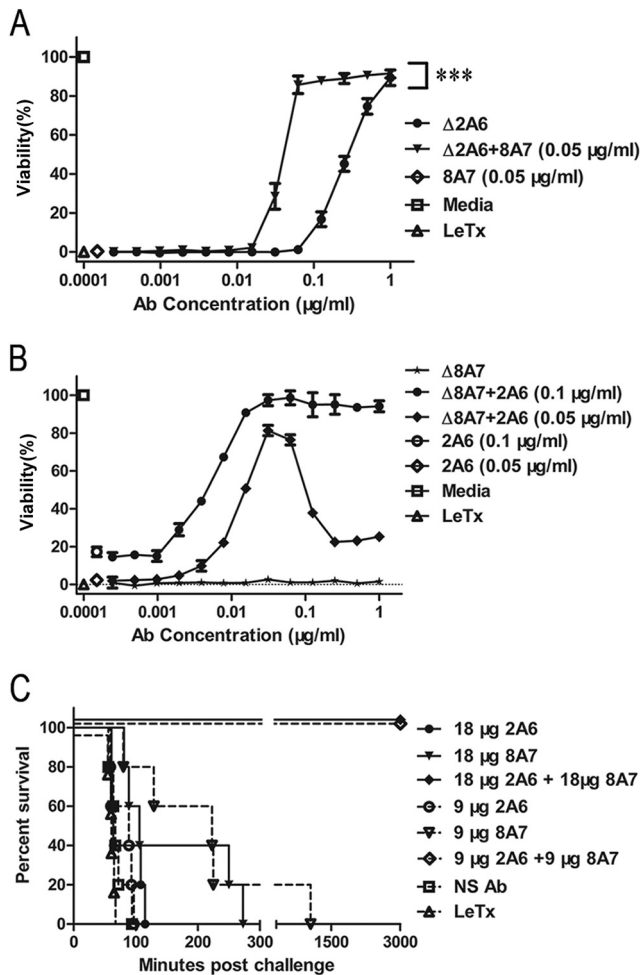

FIG 5 The combination of 2A6 and 8A7 enhanced protection against LeTx. (A) The viability of J774A.1 cells was measured using the MTT assay in the presence of 2A6 alone or in combination with 8A7. The concentration of 8A7 was held constant at 0.05 μg/ml, and 2A6 was titrated from 1 μg/ml. ***, $P <$ 0.05 for absence of 8A7 versus corresponding presence of 8A7, by t test. (B) The viability of J774A.1 cells was measured using the MTT assay in the presence of 2A6 alone or in combination with 8A7. The concentration of 2A6 was held constant at 0.1 or 0.05 μg/ml, and 8A7 was titrated from 1 μg/ml. Data shown are means ± SDs from at least three independent experiments, each conducted in duplicate. (C) Fischer 344 rats ($n = 5$/group) were challenged with LeTx (10 μg of PA and 10 μg of LF per rat) and simultaneously injected with 2A6 alone, 8A7 alone, or the combination (the molar ratio of antibodies to PA was 1:1 or 0.5:1). Survival time is shown. NS Ab, nonspecific antibody.

neutralization *in vitro* (Fig. 5A). To determine the optimal ratio of 2A6 and 8A7 that protected against cell death, an MTT assay was performed titrating both 2A6 and 8A7 (Fig. 5B). An excessive concentration (higher than 0.1 μg/ml) of 8A7 decreased the synergistic interactions between 2A6 and 8A7. Similarly, the augmented protection was also dependent on the concentration of 2A6. The synergistic protections given by 2A6 and 8A7 peaked at optimal molar ratio of 1:1 (Fig. 5B). The synergistic effects of 2A6 and 8A7 were then tested *in vivo* (Fig. 5C). The combination of 2A6 and 8A7 at the optimal molar ratio achieved 100% survival when the molar ratio of antibody to PA was 1:1 or 0.5:1.

DISCUSSION

The antibody responses induced by the AVA vaccine in humans are well understood; however, there has been very little systematic research characterizing the human antibody responses to the next-generation anthrax vaccine that is based solely on PA. To our knowledge, we are the first to characterize the domain specificities, affinities, mechanisms of neutralization, and synergistic effects of hMAbs from an individual donor immunized with the rPA vaccine. We were able to identify the specificities, function, and Ig composition of the endogenous antibody repertoire by using single-cell PCR to preserve the natural Ig VH and VL pairing from individual B cells. Despite the fact that we have characterized the response of only a single subject, the specificities and functions of the PA response in humans may be more important than the quantity of the anti-PA (10).

Of the clones we isolated from the donor, 6.4% were PA reactive, and approximately one quarter of the IgG+ ASCs was PA reactive 7 days after the donor received a boost vaccination. The total number of PA-specific clones obtained after boost vaccination was less than expected. This is likely due to low endogenous expression levels for some PA-specific clones. An alternative explanation is that the most prevalent subclasses IgG1 were used to produce linear expression cassettes, but immunoglobulin isotype has been shown to influence the V region structure and antibody affinity and/or specificity, so if there was a mismatch, we may have reduced PA-specific binding (30). However, the majority of the circulating monoclonal antibodies were not PA reactive, despite the blood sample being collected at the peak of the immune response after the boost rPA vaccination. In addition, while most of the PA-reactive hMAbs did not clearly bind to any of the recom-

binant PA domains that we expressed, of the antibodies that did bind, domain 4 was the most frequent target.

Based on the biological activity of PA in anthrax infection, antibody-based neutralization could occur at several different steps, including (i) receptor binding, (ii) enzymatic cleavage to PA63, (iii) PA63 oligomerization, (iv) LF or EF binding to PA63 oligomers, and (v) disruption of preformed PA oligomers through formation of a supercomplex (11). The four neutralizing antibodies obtained in this study (2A6, 4A3, 8H6, and 22F1) were characterized in detail to determine if they function through any of the mechanisms described above. Inhibition of receptor binding of both PA83 to the target cell and LF/EF to PA63 has been proposed as the primary mechanism of action for neutralizing antibodies. Vaccines based solely on PA domain 4 (cell receptor binding domain) have been reported to effectively protect mice from LeTx challenge (31, 32). However, despite the fact that domain 4 was the most frequently targeted domain among the hMAbs where domain specificity could be established, none of the neutralizing hMAbs from this donor bound domain 4 or blocked receptor binding. In fact, we could not establish clear domain specificity for any of the four neutralizing antibodies. One possibility is that these antibodies recognize conformational epitopes in PA and therefore have low affinity for individual subunits. Clone 22F1 provided the highest level of protection both in vitro and in vivo, but we were not able to establish its mechanism of action. Interestingly, 22F1 has extremely low binding affinity for PA83 but binds to PA63 with moderate affinity. Antibodies 8H6 and 4A3 were found to be strongly neutralizing both in vitro and in vivo. 8H6 inhibited furin-mediated PA cleavage with very high affinity. 4A3 acted in a previously unreported manner to interfere with PA oligomerization by promoting the degradation of PA63. Despite having low affinity for PA83, 4A3 bound to PA63 with a high affinity, indicating that 4A3 specifically interacted with PA63 after PA20 was cleaved from PA83. Finally, 2A6 required a high concentration to neutralize toxins in vitro and was not protective in vivo. No mechanism of action was identified for 2A6.

While 2A6 alone was not protective in vivo, the combination of 2A6 and 8A7, a toxin-enhancing antibody, was strongly neutralizing in vitro and in vivo. Synergism required an optimal mix of 2A6 and 8A7. This prozone-like effect has been reported previously (28, 33). 8A7 bound to PA domain 3, which is involved in oligomerization. Despite the fact 8A7 that bound to the PA63 oligomers, it did not inhibit PA-mediated cellular toxicity. Interestingly, MAbs specific to PA domain 3 derived from animals or humans generally do not provide any protection against LeTx in vivo (10, 14). Together with our findings, these results suggest that while MAbs specific for domain 3 of PA are not neutralizing when used alone, they may provide some benefit to the host in combination with other MAbs.

In summary, our study demonstrated the production of novel hMAbs following vaccination with the next-generation rPA anthrax vaccine. Although the epitope specificity could not be determined for the neutralizing hMAbs, three were able to neutralize PA-meditated toxicity in vitro and in vivo using different mechanisms. The surprising synergistic protection provided by 8A7, a toxin-enhancing antibody, paired with a mildly neutralizing antibody, 2A6, suggests that many potentially neutralizing antibodies (or combinations) may be discarded during initial screening for practical reasons. Our results provide novel insights into the humoral response to the rPA vaccine in an individual donor. Fur-

ther, they illustrate the relationship between antibody binding specificity and function and suggest that a targeted monoclonal antibody cocktail might be an effective anthrax therapeutic.

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