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Stochastic humoral immunity to *Bacillus anthracis* Protective Antigen: Identification of anti-peptide IgG correlating with seroconversion to Lethal Toxin neutralization

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Abstract

A substantial fraction of individuals vaccinated against anthrax have low to immeasurable levels of serum Lethal Toxin (LeTx)-neutralizing activity. The only known correlate of protection against *Bacillus anthracis* in the currently licensed vaccine is magnitude of the IgG response to Protective Antigen (PA); however, some individuals producing high serum levels of anti-PA IgG fail to neutralize LeTx *in vitro*. This suggests that non-protective humoral responses to PA may be immunodominant in some individuals. Therefore, to better understand why anthrax vaccination elicits heterogeneous levels of protection, this study was designed to elucidate the relationship between anti-PA fine specificity and LeTx neutralization in response to PA vaccination. Inbred mice immunized with recombinant PA produced high levels of anti-PA IgG and neutralized LeTx *in vitro* and *in vivo*. Decapeptide binding studies using pooled sera reproducibly identified the same 9 epitopes. Unexpectedly, sera from individual mice revealed substantial heterogeneity in the anti-PA IgG and LeTx neutralization responses, despite relative genetic homogeneity, shared environment and exposure to the same immunogen. This heterogeneity permitted the identification of specificities that correlate with LeTx-neutralizing activity. IgG binding to six decapeptides comprising two PA epitopes, located in domains I and IV, significantly correlate with seroconversion to LeTx neutralization. These results indicate that stochastic variation in humoral immunity is likely to be a major contributor to the general problem of heterogeneity in vaccine responsiveness and suggest that vaccine effectiveness could be improved by approaches that focus the humoral response toward protective epitopes in a greater fraction of vaccinees.

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Keywords

Bacillus anthracis; Protective antigen; Vaccine; B cell epitope; Mice

INTRODUCTION

Bioterrorism is a real and ongoing threat that was heightened by the malicious release of *Bacillus anthracis* spores through the U.S. postal system in 2001. Consequently, adequate protection from anthrax infection through vaccination remains an important concern for military forces. These issues have prompted careful review of the safety and efficacy of currently licensed anthrax vaccines over the last decade.

Anthrax infection has three main forms: cutaneous, gastrointestinal, and inhalational, each with its own route of entry and associated mortality [1]. The primary route associated with bioterrorism is inhalational anthrax, which has a projected mortality rate of at least 40% even with modern standard medical care [2]. The major virulence factors of this highly pathogenic, spore-forming, Gram-positive rod are a poly-D-glutamic acid capsule and a tripartite toxin [1]. The tripartite toxin consists of three proteins: Protective Antigen (PA), Lethal Factor (LF), and Edema Factor (EF) [1]. After binding the widely-expressed anthrax receptors on host cells, PA forms pores that enable entry of the EF and LF toxin components into the cells [3, 4]. LF is a zinc-dependent metalloprotease that cleaves mitogen-activated protein kinase kinases [5], and EF is a calmodulin-dependent adenylate cyclase [6]. While none of these proteins are toxic individually, the combination of PA and LF makes Lethal Toxin (LeTx), and the combination of PA and EF makes Edema Toxin (EdTx) [1]. These toxins subvert the host immune system, which helps to establish *B. anthracis* infection and permit excessive bacterial growth.

Experimental anthrax vaccines that generate neutralizing antibodies to anthrax LeTx are sufficient for protection from challenge with virulent *Bacillus anthracis* in animal models [7]. The most widely used human vaccines, Anthrax Vaccine Precipitated (AVP; [8, 9]) and Anthrax Vaccine Adsorbed (AVA; [9, 10]) are alum-based preparations of culture filtrates from toxigenic, non-encapsulated strains of *B. anthracis*. IgG antibodies directed to PA are the only reported correlates of the toxin-neutralizing antibody response to these vaccines [11, 12]. The only human anthrax vaccine approved in the United States is AVA, or Biothrax®. The AVA vaccination schedule consists of intramuscular injections at 0, 1, 6, 12, and 18 months and requires yearly boosters [12-15]. We previously observed significant variation in the human response to the AVA vaccine, with only half of vaccinees producing measurable levels of neutralizing antibodies [12, 16]. Sera from 7.5% of vaccinees produced high titer anti-PA IgG antibodies but failed to measurably neutralize LeTx *in vitro*, suggesting that some individuals may produce immunodominant responses to non-protective PA epitopes [12, 16]. Additional disadvantages include the requirement for large containment and production facilities, high incidence of adverse reactogenicity at the injection site, onerous immunization schedule and possible batch-to-batch variation.

The ideal anthrax vaccine would generate a rapid, long-lasting protective response in all immunized individuals with infrequent boosters and little reactogenicity. Approaches to improve the anthrax vaccine have included the use of better-defined recombinant protective antigen [17, 18], inclusion of experimental adjuvants [19, 20] and reduction in the number of priming vaccinations [21, 22]. A better understanding of the biological factors that influence vaccine responsiveness would additionally bolster vaccine development. For example, identification of protective and non-protective epitopes may permit refinement of the PA vaccine to target the most protective epitopes and eliminate highly immunogenic but non-

protective epitopes. Genetic polymorphisms that affect vaccine responsiveness may contribute to variation in the generation of protective humoral immunity following vaccination. Genetic associations have been reported to influence the humoral responses to smallpox [23] and measles [24, 25] vaccination in humans and production of protective levels of neutralizing anti-retroviral antibodies in mice [26]. Studies measuring the heritability of vaccine responses in monozygotic twins also reveal a significant contribution of non-genetic factors to variation in humoral vaccine responsiveness [27]. However, the extent to which such non-heritable variation is governed by the environment or stochastic features of adaptive immunity is unknown.

2. Materials and Methods

2.1 Mice

Six-8 week-old A/J strain mice were purchased from Jackson Laboratories (Bar Harbor, ME), and housed in specific pathogen-free conditions at the Oklahoma Medical Research Foundation Laboratory Animal Resources Facility. The Oklahoma Medical Research Foundation Institutional Animal Care and Use Committee approved all mouse experiments.

2.2 Production of recombinant (r)PA protein

rPA was produced as an amino-terminal His₆-tagged protein [28] using methods previously described [28, 29].

2.3 Immunization and blood sampling

Mice were immunized subcutaneously with rPA (50 µg/ 0.1 mL/injection) emulsified 1:1 in complete Freund's adjuvant (CFA; Difco, Lawrence, KS) on day 0, then boosted with rPA (50 µg rPA/0.1 mL/injection) emulsified 1:1 in incomplete Freund's adjuvant on either days 10, 24 and 38, or 14, 24 and 42. Control mice were immunized with PBS/adjuvant only on identical schedules. Blood samples were collected 4 days after each boost.

2.4 Enzyme-linked immunosorbent assay (ELISA)

ELISAs were performed as described previously except that plates were coated with 1 µg/well rPA instead of rLF [29]. Positive signals were defined as OD values exceeding 3 standard deviations (SD) above the mean OD for 1:100-diluted control samples from mice immunized with adjuvant alone. Anti-PA titer was defined as the inverse of the last serum dilution giving a positive signal.

2.5 In vitro LeTx neutralization assay

Inhibition of LeTx activity was determined as previously described except assays used either a 1:1 ratio of rPA:rLF or a 3:1 ratio of rPA:rLF (1:1 ratio = 50 ng/well rPA + 50 ng/well rLF; 3:1 ratio = 75 ng/well rPA + 25 ng/well rLF; final well volume, 100 µL) [29, 30] as indicated in the Figure legends. Neutralization titer was defined as the inverse of the last serum dilution giving a positive signal. For assays using 1:1 rPA:rLF, a positive signal was defined as 50% cell viability. For assays using 3:1 rPA:rLF, a positive signal was defined as OD values exceeding 4 SD above the mean OD of diluted samples from PBS/adjuvant-immunized mice. This corresponded to cell viability values 37%.

2.6 In vivo LeTx challenge

rPA and control mice were challenged on day 120 with 3X LD₅₀ dose of LeTx for A/J mice (300 µg PA and 125 µg LF [29]). Mice were monitored for 5 days and mortality recorded. Survival curves were compared using the Mantel-Cox test.

2.7 Solid-phase humoral epitope mapping

Decamer peptides overlapping by 8 amino acids and spanning the entire length of the PA protein (GenBank accession number AAA22637) were covalently synthesized onto polyethylene solid phase supports using a 96-well format as previously described [31]. Peptides were incubated with serum dilutions of 1:200. Detection of bound antibodies utilized peroxidase-labeled goat anti-mouse IgG and SureBlue Reserve TMB substrate (both from KPL, Gaithersburg, MD) in a modified ELISA protocol [31]. An epitope was defined as a region of high antibody binding in which two or more consecutive solid-phase peptides exhibited ELISA OD₄₅₀ values greater than or equal to the average OD₄₅₀ plus 5 standard deviations (SD) of a known low response region (PA amino acids (aa) 97-126 or aa 497-526).

2.8 Data analysis

Differences in antibody binding to particular decapeptides were determined by 2-tailed *t*-test, with P values of 0.05 considered statistically significant. Increases of peptide binding activity 0.5 OD were considered biologically significant.

3. Results

3.1 rPA immunization results in high titers of neutralizing anti-PA antibodies and protection from LeTx challenge

To generate antisera that contained PA-specific antibodies, neutralized LeTx and was suitable for epitope mapping studies, groups of A/J mice were immunized on day 0 with rPA in complete Freund's adjuvant and boosted on days 10, 24, and 38 with rPA in incomplete Freund's adjuvant. Sera were collected 4 days after each boost and again on day 119 and were tested for anti-PA antibodies by ELISA. As expected, sera from PBS/adjuvant-immunized mice failed to bind rPA. In rPA-immunized mice, high anti-PA IgG titers were observed 2 weeks after the initial immunization, and IgG titers reached the highest levels by day 28 (Figure 1A).

To assess whether these antibodies conferred protection against LeTx, the capacity of sera to neutralize LeTx *in vitro* was evaluated using a RAW 264.7 mouse macrophage cell death assay. LeTx neutralization was detected at day 14 and peaked by day 28 (Figure 1B). Sera from control mice lacked detectable LeTx neutralization activity. To confirm that the anti-PA responses were protective *in vivo*, the mice were challenged on day 120 with 3X the LD₅₀ dose of LeTx for A/J mice [29]. As shown in Figure 1C, 9 of 10 rPA-immunized mice survived the challenge, while only 1 of 7 PBS/adjuvant-immunized mice survived (*p*=0.0025). These results indicate that immunization of A/J mice with rPA elicits a high titer of anti-PA IgG that neutralizes LeTx *in vitro* and *in vivo*.

3.2 Sequential B-cell epitopes of PA are reproducibly identified using pooled serum samples

Sequential B cell epitopes of rLF and rEF were highly reproducible in previous independent experiments that used pools of sera from immunized A/J mice [29, 30]. Therefore, we used pooled sera to identify sequential B cell epitopes of PA. Mice were immunized with rPA or PBS/adjuvant alone, and serum samples from each group (*n*=18-20) were collected and pooled 14, 28, and 42 days after the initial immunization. The pooled samples were analyzed by solid-phase peptide mapping using a modified ELISA to detect IgG binding to PA decapeptides overlapping by eight amino acids and spanning the entire PA protein. This process was performed with three independent groups of mice; the averaged results are shown in Figure 2. Multiple decamer peptides of PA were bound by anti-PA IgG in the pooled sera of rPA-immunized mice, with the responses becoming stronger and more

diverse with each immunization. As expected, pooled sera from the PBS/adjuvant alone mice showed no significant binding to PA decapeptides. An epitope was defined as reactivity to two or more consecutive decapeptides with threshold OD₄₅₀ the average OD₄₅₀ plus 5 standard deviations (SD) of a defined, common low response region. A total of 15 antigenic regions were thus identified; 9 of these were detected in all three independent experiments, and the remaining 6 were identified in two of the three independent experiments (Table 1). Epitopes 3 and 7 were in regions previously determined to elicit neutralizing antibodies in mice and humans [12, 32-34]. These pooled samples showed a relatively reproducible hierarchy of peptide binding.

3.3 The response to rPA vaccination is heterogeneous across individual serum samples

Although the sequential B cell epitopes identified using pooled serum samples were highly reproducible, the anti-PA titer and LeTx neutralization capacity showed notable variability between individuals (Figure 1). To confirm and further investigate this observation, two separate groups of 10 A/J mice each (Group 1 and Group 2) were immunized with rPA, and the anti-PA titer, LeTx neutralization capacity, and recognized sequential B cell epitopes were determined for individual mice. Similar to earlier results, substantial variation in the endpoint titers of anti-PA IgG (ranging from 1.5 to 2 logs difference between mice) and LeTx-neutralizing activity was observed (Supplementary Figure 1). Moreover, no significant correlation between serum titers of anti-PA IgG and LeTx neutralization were detected at any time point (Spearman's test; data not shown), suggesting that only a subset of PA antibody specificities contribute to LeTx neutralization.

To assess heterogeneity of the fine specificity of the IgG response to rPA, serum samples from these twenty mice were analyzed by solid-phase epitope mapping. Unlike the reproducibility of results obtained using pooled serum samples, patterns of fine specificity of the rPA response in individual mice showed remarkable variation (representative decapeptide binding patterns of two individual mice from each group of 10 that were immunized are shown in Figure 3). We next identified specific epitopes defined in the same manner as those identified using pooled sera. A total of 38 antigenic regions were identified in the individual samples. Common antigenic regions were further defined as those regions bound by IgG in at least 40% (8 of 20) of the samples. Using this definition, 14 common antigenic regions were identified (Table 2). Thus, 24 of 38 (63%) antigenic regions of PA were bound by IgG from only six or fewer (30%) of the 20 individual mice. Nearly all (10 of 14) of the common antigenic regions were also detected in the experiments using pooled sera. Together, these results show that the magnitude, neutralization capacity, and specificity of the B-cell response elicited by rPA vaccination are heterogeneous among genetically identical animals.

3.4 IgG binding to six PA decapeptides comprising two epitopes correlates with seroconversion to neutralization

Lack of correspondence between anti-PA IgG antibody titers and increases in LeTx neutralization capacity suggested that changes in fine specificity rather than magnitude of the anti-PA response accounted for improved LeTx neutralization over the course of vaccination. In support of this, only 4 of 10 rPA-immunized mice in Group 1 exhibited measurable LeTx neutralization at Bleed 1 (day 18), a time point when all 10 Group 1 mice had anti-PA IgG titers above 6400. By Bleed 2 (day 32), the remaining 6 serum samples, which contained anti-PA IgG but failed to neutralize LeTx at day 18, all acquired LeTx neutralizing activity. Similar results were observed in Group 2. In total, paired samples from 13 individual mice (6 of 10 Group 1 mice and 7 of 10 Group 2 mice) were identified in which one early sample contained IgG anti-PA antibodies but no detectable Lethal Toxin

neutralization activity and one subsequent sample from the same mouse contained both IgG anti-PA antibodies and Lethal Toxin neutralization activity (Figure 4A and B).

To test whether differences in fine specificity may be responsible for heterogeneity in the LeTx neutralization response, decapeptide binding patterns were compared between the paired samples from the 13 informative individual mice in which seroconversion to Lethal Toxin neutralization occurred in a manner that was largely independent of the magnitude of the anti-PA antibody response. This analysis identified six PA decapeptides that showed statistically significant increases in IgG binding concurrent with the appearance of serum LeTx neutralizing activity (Figure 4C-H). Three of these decapeptides were overlapping (Figure 4C-E) and constituted Epitope 1/A, which includes PA amino acids 71-84 in Domain 1 and was recognized by 85% of the 20 rPA-immunized mice. The other three decapeptides associating with seroconversion to neutralization were also overlapping and constituted Epitope 9/B, which includes PA amino acids 637-650 in Domain 4. This epitope was targeted by 75% of the rPA-immunized mice. Thus, antibody responses targeted against these two epitopes may be important for LeTx neutralization.

4. Discussion

A significant problem with vaccination is variation in responsiveness among individuals, which can impact the level of protection induced. For example, some individuals develop immunodominant responses to non-protective epitopes. Indeed, 7.5% of AVA vaccine recipients who were within 2 years post vaccination had anti-PA IgG titers 1:1000, yet had LeTx neutralization activity indistinguishable from unvaccinated controls [12], suggesting that this is an important problem in anthrax vaccination. Therefore, our work has focused on identifying the fine specificity of antibodies that confer protection to anthrax toxin components [12, 16, 29, 30, 32]; this knowledge will permit development of vaccines that target selected domains, regions or peptides to elicit protective responses.

In the present study we mapped 19 sequential B cell epitopes of PA in mice using sera containing neutralizing and protective anti-PA antibodies. We identified six decapeptides located within two of these antigenic regions, or epitopes that demonstrated statistically significant correlation with seroconversion to LeTx neutralization: PA amino acids 71-84 and 637-650. These results are consistent with previous studies showing that peptides overlapping these epitopes 6 and 2 aa, respectively, are recognized by antibodies from PA-immunized mice [35, 36]. Additionally, other studies have shown that a LeTx-neutralizing mAb reacts with a peptide overlapping 2 aa in epitope 9/B [36] and that mice immunized with a 19-aa peptide including epitope 9/B (AVGADESVVKEAHREVINS; amino acids 633-651) are protected against LeTx challenge [37]. While the appearance of antibodies binding to these six decapeptides correlated with seroconversion to neutralization, some serum samples neutralized LeTx without these specificities, indicating that these antibodies were not the only determinants of LeTx neutralization in rPA-immunized A/J mice. This is expected since this analysis was necessarily restricted to epitopes common among multiple individual mice and thus lacked power to identify neutralizing specificities present in a minority of the mice examined. Two other epitopes we identified were previously reported to be neutralizing but were not significant correlates of seroconversion to neutralization in this study: the furin cleavage site (Epitope 7/L; [12, 32, 34, 38]) and the 2 β ₂-2 β ₃ loop in Domain II (Epitope 3/I; [39, 40]). 50% of individual mice in our study recognized each of these epitopes. Thus, multiple antibody specificities have neutralizing capabilities, and combinations of these antibodies are likely to have additive neutralizing effects.

Previous studies mapping common epitopes of PA or LF in human vaccinees showed that common protective epitopes are recognized by at most 75% of vaccinees [12, 16]. In

addition, sera from 40 to 50% of AVA vaccinees fail to effectively neutralize LeTx *in vitro* [12, 16]. Multiple studies clearly indicate that some variations in vaccine responsiveness are caused by genetic polymorphisms of the host [23-25]. This conclusion is further supported by studies in murine models demonstrating that responsiveness to PA depends on genetic background [35]. Thus, it was surprising that the analysis of individual mice revealed a high degree of variation in the fine specificity of the humoral response to rPA, particularly given that the mice were genetically identical and shared the same immunogen and environment. This study, the first to map sequential B cell epitopes of an anthrax toxin component in individual mice, demonstrates that a high degree of individual variation in the fine specificity of the humoral response to a recombinant protein is due to stochastic factors.

One mechanism mediating the stochastic nature of the vaccine response may relate to the natural precursor frequency of antigen-specific lymphocytes, which could be influenced by the random nature of antigen receptor generation as well as previous exposure to cross-reactive antigens. In support of this idea, Kwok, et al. described a correlation between PA-specific Th cell precursor frequency and the concentration of anti-PA IgG induced after vaccination of humans with AVA [41]. A second mechanism may involve temporal influence of the earliest B cell clones responding to particular epitopes, wherein early responses become favored. Other unidentified factors could involve minor changes in anatomical location of injections with respect to the proximity of secondary lymphoid tissue; variation in immune status of individual animals, including stress levels or nutritional status at the time of injection; or environmental littermate effects.

We conclude that stochastic variation is likely to be an underestimated but significant contributor to the fine specificity of vaccine responses. Development of strategies to elicit protective responses in greater fractions of vaccinees is desperately needed. We suggest that this might be accomplished by selectively reducing the number of available B cell epitopes in a way that directs the humoral immune response to desired, protective specificities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Sequential IgG epitopes of *Bacillus anthracis* Protective Antigen (PA) in A/J mice
- Stochastic humoral immune response to PA vaccination in inbred mice
- IgG binding to six PA decapeptides correlates with Lethal Toxin neutralization

Figure 1. Immunization of A/J mice results in high-titer anti-PA antibodies that are neutralizing *in vitro* and protective *in vivo*

A/J mice were immunized with rPA and CFA on day 0, then boosted with rPA and IFA on days 10, 24 and 38, and bled on days 14, 28, 42 and 119. (A) Anti-PA IgG antibody titers of sera from A/J mice immunized with rPA were assessed at specified time points. Antibody levels were measured by using a standard ELISA. (B) Sera collected were subjected to an *in vitro* neutralization assay using a 1:1 ratio of PA to LF (LeTx). Sera exhibit neutralization as early as Day 14, peak at Day 28, and remain undiminished at Day 119. (C) A/J mice immunized with rPA are protected from an *in vivo* LeTx challenge using 3 times the A/J LD₅₀ (300 µg PA plus 125 µg LF) for LeTx ($P = 0.0025$). Each symbol in A and B represents the response of an individual mouse. Error bars in A and B indicate the mean $\pm$ SEM.

Figure 2. Fine specificity of the humoral anti-PA response following A/J immunization
IgG antibody binding to decapeptides of PA overlapping by eight amino acids was measured using a solid-phase ELISA. The data shown are the antibody responses in pooled sera from rPA immunized mice on days 14, 28 and 42 and in pooled day 42 sera from mice immunized with adjuvant alone. The data are the averages of the results of three independent immunization and mapping experiments. Mapping within each experiment was performed using sera pooled from 18 to 20 mice. Epitopes were numbered in order of reproducibility and reactivity at day 42, as shown in Table 1.

Figure 3. Fine specificity of the humoral anti-PA response of individual A/J mice following rPA immunization

Serum samples were collected from rPA-immunized A/J mice on days 18, 32 and 46 (Group 1) or on days 14, 28 and 43 (Group 2) and reported as Bleed 1, 2 and 3 respectively. Individual samples were diluted 1:200, and solid-phase ELISA of individual samples was used to measure IgG antibody binding to overlapping PA decapeptides, similar to Figure 2. Results of two representative Group 1 mice are shown in the upper two panels, and results of two representative Group 2 mice are shown in the lower two panels. Toxin neutralization assay (TNA) titer, determined using a 3:1 ratio of PA to LF, is listed at the upper left of each graph for each individual sample (Neg = negative). Epitope numbers and letters correspond to those shown in Tables 1 and 2, respectively.

Figure 4. Identification of six decapeptides that correlate with seroconversion to neutralization
 Sera from 13 individual A/J mice were found to have positive anti-PA IgG titers and undetectable LeTx neutralization at an early bleed, then develop LeTx neutralization activity in the subsequent blood sample. (A) Anti-PA IgG titers changed very little from the non-neutralizing to neutralizing bleeds in the 13 mice, but (B) all 13 mice acquired LeTx neutralization titers in the subsequent blood sample. (C-H) PA decapeptide binding of non-neutralizing and neutralizing bleeds of these 13 A/J mice were compared, and six decapeptides were identified that bound significantly more IgG upon seroconversion to LeTx neutralization (Peptide was determined to be significant if the average increase in OD was greater than 0.5).

Table 1

PA epitopes in pooled sera of rPA-immunized mice

Epitope Number	Decapeptide Number	Amino Acid Number	Epitope Reproducibility ^a	Average OD $\pm$ SEM ^b
1	33-42	65-92	3/3	2.283 $\pm$ 0.401
2	129-135	257-278	3/3	1.453 $\pm$ 0.228
3	168-174	335-356	3/3	1.316 $\pm$ 0.240
4	213-215	425-438	3/3	0.860 $\pm$ 0.082
5	147-151	293-310	3/3	0.831 $\pm$ 0.150
6	287-290	573-588	3/3	0.761 $\pm$ 0.123
7	90-103	179-214	3/3	0.734 $\pm$ 0.164
8	293-298	585-604	3/3	0.614 $\pm$ 0.181
9	317-321	633-650	3/3	0.538 $\pm$ 0.077
10	263-267	525-542	2/3	0.689 $\pm$ 0.212
11	313-314	625-636	2/3	0.665 $\pm$ 0.077
12	283-284	565-576	2/3	0.564 $\pm$ 0.202
13	357-359	713-726	2/3	0.367 $\pm$ 0.068
14	365-367	729-742	2/3	0.234 $\pm$ 0.033
15	196-199	391-406	2/3	0.230 $\pm$ 0.051

^a Number of independent experiments in which region was identified as an epitope^b Average OD and standard error of the mean for three independent mapping studies

Table 2

Common PA epitopes defined in individual sera of rPA-immunized mice

Epitope Letter	Decapeptide Number	Amino Acid Number	Epitope Incidence ^a	Average OD $\pm$ SEM ^b
A (1)	32-40	63-88	17/20	1.151 $\pm$ 0.088
B (9)	319-322	637-652	15/20	1.651 $\pm$ 0.181
C (6,8)	287-296	573-600	15/20	1.035 $\pm$ 0.127
D (13)	350-360	699-728	15/20	0.705 $\pm$ 0.069
E (11)	300-314	599-636	12/20	1.375 $\pm$ 0.134
F	325-334	649-676	12/20	1.039 $\pm$ 0.155
G	275-280	549-568	11/20	1.177 $\pm$ 0.190
H (2)	129-136	257-280	11/20	1.030 $\pm$ 0.174
I (3)	168-171	335-350	10/20	0.548 $\pm$ 0.087
J (14)	365-367	729-742	10/20	0.460 $\pm$ 0.055
K	156-162	311-332	10/20	0.386 $\pm$ 0.053
L (7)	90-103	179-214	10/20	0.369 $\pm$ 0.068
M (5)	141-150	281-308	8/20	0.973 $\pm$ 0.227
N	338-348	675-704	8/20	0.503 $\pm$ 0.047

^aNumber of independent samples in which region was identified as an epitope^bAverage OD and standard error of the mean for positively responding mice Numbers in () correspond to epitopes in Table 1