A Recombinant Carboxy-Terminal Domain of the Protective Antigen of *Bacillus anthracis* Protects Mice against Anthrax Infection

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The immunogenicity and protective efficacy of overlapping regions of the protective antigen (PA) polypeptide, cloned and expressed as glutathione S-transferase fusion proteins, have been assessed. Results show that protection can be attributed to individual domains and imply that it is domain 4 which contains the dominant protective epitopes of PA.

Protective antigen (PA) is the dominant antigen in both natural and vaccine-induced immunity to anthrax infection. It is also essential for host cell intoxication in combination with either lethal factor (LF) or edema factor (EF), producing lethal toxin or edema toxin, respectively (6), as it contains the host cell receptor binding site (5) and facilitates the entry of the toxin complex into the host cell. The crystal structure of native PA has been elucidated (15) and shows that PA consists of four distinct and functionally independent domains. Domain 1 is divided into domains 1a, comprising amino acids 1 to 167, and 1b, comprising amino acids 168 to 258; domain 2 comprises amino acids 259 to 487; domain 3 comprises amino acids 488 to 595; and domain 4 comprises amino acids 596 to 735. Cell intoxication is thought to occur when full-length PA (PA83) binds to the cell surface receptor via domain 4, which contains the host cell receptor binding site (10). On binding to the host cell receptor, the N-terminal amino acids (1 to 167, i.e., domain 1a) of domain 1, which contains a furin protease cleavage site (8), are cleaved off, exposing the LF or EF binding site located in domain 1b and the adjacent domain 3 (15). Domains 2 and 3 then form part of a heptameric pore on the cell surface (13, 14), the LF or EF binds to its receptor, and the whole toxin complex undergoes receptor-mediated endocytosis into the cell. After acidification of the endosome, the toxin is translocated into the cell cytosol, where it exerts its cytotoxic effect (7). Therefore, inhibition of the binding and entry of the toxin complex, particularly lethal toxin, into the host cell is clearly important for the prevention of infection.

Anthrax occurs in three main forms: cutaneous, gastrointestinal, and pulmonary (16). If diagnosed early enough, infection can be treated with antibiotics, but symptoms are not always apparent in time for antibiotic treatment to be effective, so vaccination is essential to protect individuals who are at risk of exposure. The current anthrax vaccine licensed in the United Kingdom is an alum-precipitated filtrate of *Bacillus anthracis* strain Sterne cultures grown to maximize the PA content (17). The production of this vaccine requires containment facilities,

and the vaccine itself varies in PA content from batch to batch. There are also problems of transient reactogenicity due to the presence of small amounts of LF and EF and other bacterial components and a limited duration of protection requiring frequent boosts for continued immunity. These problems could be resolved by refining the vaccine components and enriching the vaccine with respect to the key protective component, PA. It is known that antibodies to PA are essential for immunity to anthrax infection (11), but it is not known if the whole PA polypeptide is required to stimulate an effective immune response or whether certain subunits of PA would be equally effective immunogens. To investigate this, a panel of recombinant PA (rPA) domain proteins has been produced and used to immunize mice to assess their immunogenicity and protective efficacy against anthrax spore challenge and also to ascertain if the presence of any one domain is most critical for protection.

Cloning and protein expression. DNA encoding the PA domains, which comprise amino acids 1 to 258, 168 to 487, 1 to 487, 168 to 595, 1 to 595, 259 to 735, 488 to 735, 596 to 735, and 1 to 735 (fusion proteins GST1, GST1b-2, GST1-2, GST1b-3, GST1-3, GST2-4, GST3-4, GST4, and GST1-4, respectively), was PCR amplified from *B. anthracis* strain Sterne DNA and cloned into the *Xho*I and *Bam*HI sites of the expression vector pGEX-6-P3 (Amersham-Pharmacia) downstream of and in frame with the *lac* promoter. Proteins produced by this system were expressed as fusion proteins with an N-terminal glutathione S-transferase (GST) protein. Initial extraction of the fusion proteins indicated that they were produced as inclusion bodies. These were solubilized by using 8 M urea and renatured by dialysis against an arginine buffer to stabilize the protein upon refolding. Successful refolding of the fusion proteins allowed them to be purified on a glutathione Sepharose CL-4B affinity column (Amersham-Pharmacia). However, fusion protein GST1b-2 (amino acid residues 168 to 487) could not be eluted from this column and was therefore purified by ion-exchange chromatography. The yields of the fusion proteins varied between 1 and 43 mg liter of culture⁻¹. To check that the fusion proteins were of the expected molecular weights and that they could be recognized by antibodies to PA, the fusion proteins were run on sodium dodecyl sulfate (SDS)–10 to 15% polyacrylamide gels (PhastGel; Amersham-

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FIG. 1. SDS-PAGE analysis of GST fusion proteins. Lane 1, GST1; lane 2, GST1b-2; lane 3, GST1-2; lane 4, GST1b-3; lane 5, GST1-3; lane 6, rPA; lane 7, GST1-4; lane 8, GST2-4; lane 9, GST3-4; lane 10, GST4; lane 11, rPA.

Pharmacia). Protein bands were detected either by staining with PhastGel Blue R or, after electrophoretic transfer onto polyvinylidene difluoride membranes (Millipore), by using mouse anti-rPA sera. Analysis of the fusion proteins by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 1) and Western blotting (Fig. 2) showed protein bands of the expected sizes that specifically bound anti-rPA antisera, showing that the GST protein tag did not interfere with PA epitope recognition in vitro. Some degradation was apparent in all of the fusion proteins investigated, showing similarity with rPA expressed in *Bacillus subtilis*. The rPA-truncated proteins GST1, GST1b-2, and GST1-2 were particularly susceptible to degradation in the absence of domain 3. This has similarly been reported for rPA constructs that contained mutations in domain 3 and that could not be purified from *B. anthracis* culture supernatants (3), suggesting that domain 3 may stabilize domains 1 and 2.

Immunization and challenge. Female A/J mice (Harlan Olac, Blackthorn, United Kingdom) were immunized intra-

muscularly (i.m.) with 10 μ g of protein adsorbed to a 20% (vol/vol) solution of 1.3% Alhydrogel (HCl Biosector, Fredrikssund, Denmark) on days 1 and 28 of the study. We also included groups of mice that were immunized with rPA (expressed and purified from *B. subtilis* [12]), with recombinant GST control protein, or with fusion proteins comprising domains 1, 4, and 1 to 4 which had the GST tag removed by incubation with PreScission Protease (16 h at 4°C; Amersham-Pharmacia) and removal of the GST on a glutathione Sepharose column. Blood samples from mice were collected 37 days after primary immunization for serum antibody analysis by enzyme-linked immunosorbent assay. Mice were challenged intraperitoneally with either 10^5 or 10^6 spores of the *B. anthracis* STI strain (equivalent to 10^2 or 10^3 minimum lethal doses [MLDs] [1], respectively) on day 70 of the immunization regimen and were monitored for 14 days postchallenge to determine their protected status. Humane treatment end points were strictly observed so that any animal displaying a predetermined set of clinical signs, which together indicated that it had a lethal infection, was culled.

All of the fusion proteins were immunogenic and stimulated mean serum anti-rPA immunoglobulin G (IgG) concentrations in the A/J mice ranging from 6 μ g ml $^{-1}$ for the GST1b-2 fusion protein-immunized group to 1,488 μ g ml $^{-1}$ for the GST1-4 fusion protein-immunized group (Fig. 3). The control mice, immunized with GST only, had no detectable antibodies to rPA. The predominant subclass of IgG stimulated by immunization with any of the fusion proteins was IgG1, followed by IgG2a and IgG2b in lesser amounts and by IgG3, of which there was no detectable amount. The IgG subclass data show a strong bias to a Th2 type of response after immunization with either the fusion proteins or rPA in the presence of Alhydrogel. This has previously been noted after rPA immunization in the presence of Alhydrogel (20) and shows that the qualitative immune response stimulated after immunization with complete or partial domains of rPA is the same as that stimulated by the whole rPA, irrespective of whether protection is conferred.

Mice were challenged 42 days after booster immunization, and the numbers of mice which survived 14 days postchallenge are shown in Table 1. At the lower challenge level of 10^2 MLDs, mice in the GST1-2-, GST4-, and cleaved 4-immunized groups were all fully protected, but some breakthrough in protection for those in the groups immunized with GST1, cleaved 1, GST1b-2, GST1b-3, and GST1-3 was observed despite their having functionally significant anti-rPA titers (with the exception of the GST1b-2-immunized group). The mice in these groups that died had a mean time to death (MTTD) of 4.5 ± 0.2 days, which was not significantly different from that of the GST control-immunized group, in which all mice died with an MTTD of 4 ± 0.4 days. This suggests that the immune response had not been appropriately primed by these proteins to achieve resistance to the infection. As has been shown in other studies, for mice and guinea pigs (9, 18), there is no precise correlation between antibody titer to PA and protection against challenge, although a certain threshold of antibody titer may be required for protection (4), suggesting that the stimulation of cell-mediated components of the immune response is also necessary. GST1, GST1b-2, and GST1-2 were the least stable fusion proteins produced, as shown by the

FIG. 2. Western blot analysis of fusion proteins. Proteins were detected by using mouse anti-rPA antisera. Lane 1, GST1; lane 2, GST1b-2; lane 3, GST1-2; lane 4, GST1b-3; lane 5, GST1-3; lane 6, rPA; lane 7, GST1-4; lane 8, GST2-4; lane 9, GST3-4; lane 10, GST4; lane 11, rPA.

FIG. 3. Anti-rPA IgG concentrations 37 days after primary immunization in A/J mice immunized i.m. on days 1 and 28 with either 10 µg of fusion protein or 10 µg of domain protein cleaved from GST. Results shown are means $\pm$ standard errors of the mean for samples taken from five mice per treatment group.

SDS-PAGE and Western blotting results. This instability was possibly due to the proteins' greater susceptibility to degradation in the absence of domain 3 and may have resulted in the loss of protective epitopes. The structural conformation of the proteins may also be important for stimulating a protective

immune response. The removal of domain 1a from the fusion proteins yielded both reduced antibody titers and lower levels of protection against challenge than were found for the intact counterparts GST1-2 and GST1-3. Similarly, mice immunized with GST1 alone were partially protected against challenge, but when this immunization was combined with that involving domain 2, as in the GST1-2 fusion protein, full protection was seen at the 10^2 -MLD challenge level. However, the immune response stimulated by immunization with the GST1-2 fusion protein was insufficient to provide full protection against the higher, 10^3 -MLD challenge level, which again could be due to the loss of protective epitopes caused by degradation of the protein.

The mice in the groups challenged with 10^3 MLDs of STI spores were all fully protected, except for those in the GST1-, GST1-2-, and cleaved 1-immunized groups, for which there was some breakthrough in protection, and those in the control group immunized with GST only, which succumbed to infection with an MTTD of 2.4 ± 0.2 days. All mice in the groups immunized with fusion proteins containing domain 4 were fully protected against challenge with 10^3 MLDs of STI spores (Table 1). Brossier et al. showed a decrease in protection for mice immunized with a mutated strain of *B. anthracis* that expressed PA without domain 4 (2), and this was confirmed in this study, where immunization with GST1-3 resulted in a breakthrough in protection despite high antibody titers.

These data indicate that domain 4 contains the dominant

TABLE 1. Survival of A/J mice^a

Fusion protein	No. of survivors/no. challenged (%) at:	
	10^2 MLDs ^b	10^3 MLDs
GST1	3/5 (60)	1/5 (20)
GST1b-2	1/5 (20)	ND ^c
GST1-2	5/5 (100)	3/5 (60)
GST1b-3	3/5 (60)	ND
GST1-3	4/5 (80)	ND
GST1-4	ND	5/5 (100)
GST2-4	ND	5/5 (100)
GST3-4	ND	5/5 (100)
GST4	5/5 (100)	5/5 (100)
GST1 + GST4	ND	5/5 (100)
Cleaved 1	1/5 (20)	2/5 (40)
Cleaved 4	5/5 (100)	5/5 (100)
Cleaved 1-4	ND	5/5 (100)
rPA	ND	4/4 (100)
Control	0/5 (0)	0/5 (0)

^a Mice were immunized i.m. on days 1 and 28 with either 10 µg of fusion protein or 10 µg of domain protein cleaved from GST and observed for 14 days postchallenge with STI spores.

^b 1 MLD = approximately 10^3 STI spores (1).

^c ND, not done.

protective epitopes of PA. Domain 4 represents the 139 amino acids of the carboxy terminus of the PA polypeptide. It contains the host cell receptor binding region (10), which has been identified as being in and near a small loop located between amino acid residues 679 and 693 (19), and it is therefore essential for host cell intoxication, as previous studies have demonstrated that expressed forms of PA containing mutations (19) or deletions (3) in the region of domain 4 are non-toxic. The crystal structure of PA shows domain 4, and in particular a 19-amino-acid loop of the domain (703 to 722), to be more exposed than the other three domains, which are closely associated with each other (15). This structural arrangement may make the epitopes in domain 4 the most prominent for recognition by immune effector cells.

This investigation has further elucidated the role of PA in the stimulation of a protective immune response, demonstrating that protection against anthrax infection can be attributed to individual domains of PA. Work is continuing to explore the immune mechanisms stimulated by immunization with rPA domains, to investigate immunoreactivity to the domains as surrogate markers of efficacy, and to examine the utility of these domains as novel vaccine candidates.

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