Protective Immunity Induced by *Bacillus anthracis* Toxin-Deficient Strains

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The two toxins secreted by *Bacillus anthracis* are composed of binary combinations of three proteins: protective antigen (PA), lethal factor (LF), and edema factor (EF). Six mutant strains that are deficient in the production of one or two of these toxin components have been previously constructed and characterized (C. Pezard, E. Duflot, and M. Mock, J. Gen. Microbiol. 139:2459–2463, 1993). In this work, we examined the antibody response to the in vivo production of PA, LF, and EF in mice immunized with spores of strains producing these proteins. High titers of antibody to PA were observed after immunization with all strains producing PA, while titers of antibodies to EF and LF were weak in animals immunized with strains producing only EF or LF. In contrast, immunization with strains producing either PA and EF or PA and LF resulted in an increased antibody response to EF or LF, respectively. The differing levels of protection from a lethal anthrax challenge afforded to mice immunized with spores of the mutant strains not only confirm the role of PA as the major protective antigen in the humoral response but also indicate a significant contribution of LF and EF to immunoprotection. We observed, however, that PA-deficient strains were also able to provide some protection, thereby suggesting that immune mechanisms other than the humoral response may be involved in immunity to anthrax. Finally, a control strain lacking the toxin-encoding plasmid was unable to provide protection or elicit an antibody response against bacterial antigens, indicating a possible role for pXO1 in the survival of *B. anthracis* in a host.

Bacillus anthracis is the etiological agent of anthrax, a disease often fatal to humans and many animals species. *B. anthracis* possesses two virulence-associated plasmids, pXO1 and pXO2. pXO2 encodes an antiphagocytic poly-γ-D-glutamic acid capsule (4, 15), while pXO1 encodes two exotoxins (16). The two toxins are composed of three different proteins: edema factor (EF; 89 kDa), lethal factor (LF; 83 kDa), and protective antigen (PA; 85 kDa) (for a review, see reference 12). PA is the common receptor binding domain of the toxins and can interact with the two different effector domains, EF and LF, to mediate their entry into target cells (12). The combination of PA and EF forms the edema toxin. EF is a calmodulin-dependent adenylate cyclase (11) which induces an increase in intracellular levels of cyclic AMP in eucaryotic cells (12, 17). This effect is ultimately responsible for the intense edema seen at the site of inoculation of edema toxin in experimental animals (3). The lethal toxin is composed of PA and LF. Recently, it has been proposed that LF is a Zn^{2+}-binding metalloprotease (10). However, the intracellular target of LF and the molecular mechanisms leading to death after intravenous injection of lethal toxin remain unknown (1).

The role of PA as a protective antigen against anthrax was established soon after discovery of the toxin (14, 22). Livestock is commonly vaccinated with a suspension of viable, virulence-attenuated spores of the Sterne strain, which lacks pXO2 (5, 23). This unencapsulated strain still carries pXO1 and there-

fore produces the three toxin factors (7). The efficacy and duration of the protection conferred by the Sterne strain in experimentally vaccinated animals are much greater than those of that obtained with cell-free PA vaccines, despite the fact that antibody titers against PA induced by live vaccines are often lower than those induced by cell-free vaccines (13, 23, 26). Extensive studies on the role of adjuvants in immunoprotection have also led to the conclusion that there is no direct correlation between anti-PA titers in serum and protection (9, 24). Providing full protection against anthrax, therefore, appears to be a complex problem.

Extensive work based on recent advances in the understanding of the genetics of *B. anthracis* has been devoted to the design of a new anthrax vaccine with either live strains or cell-free toxin components. Attempts have been made to express PA in heterologous systems; however, these new vaccine strains differ from the Sterne strain in providing protection, suggesting that other antigens, in addition to PA, may play an important role in active immunity (8, 26). We have previously constructed Sterne strain-derived *B. anthracis* mutants that are deficient in the production of one or two toxin components (2, 19, 20). In the present work, we used these strains to study the contribution of in vivo-produced PA, EF, and LF to the immune response. Antibody response to each toxin component and its respective role in immunoprotection were analyzed. We found that simultaneous production of PA with EF or LF potentiated the antibody response to the latter two components. In addition, the protection afforded by the various mutants to a lethal challenge with *B. anthracis* Sterne suggested a significant contribution of pXO1 to the survival of *B. anthracis* in a host.

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MATERIALS AND METHODS

Bacterial strains. Construction and characterization of *B. anthracis* strains used in this work have been previously described (19, 20). 7702 is the parental Sterne strain. Single mutant strains RP8, RP9, and RP10 produce EF-LF, PA-LF, and PA-EF, respectively. Only RP9 is still lethal, with a 50% lethal dose of 10^7 spores, which is 10-fold greater than that of the Sterne strain (19). The double mutant strains RP42, RP4, and RP31 produce PA, LF, and EF, respectively. Plasmid-free strain 7700 was derived by curing 7702 of pXO1 as previously described (2).

Immunization and challenge of mice. Spore suspensions used for immunization and challenge were prepared as previously described (19). Swiss mice (10 or 20 per group) were immunized subcutaneously with a single dose (0.5 ml) of spores at different concentrations (2×10^6 to 2×10^8/ml) of the appropriate *B. anthracis* strain. A group of 50 control mice received 0.5 ml of physiological saline. Six weeks after immunization, mice were challenged subcutaneously with 10^9 spores of *B. anthracis* Sterne. This represents a dose 1,000 times the 50% lethal dose for Swiss mice (19). Statistical analysis of protection was performed by the χ^2 test. For each mutant strain, the cumulative number of survivors was compared to those of nonimmunized controls and mice immunized with the other strains.

Preparation of antigens used in ELISA. PA, LF, and EF were purified from the culture supernatants of *B. anthracis* mutant strains as previously described (20). The extracellular antigen preparation represents crude material released by strain 7700 into the medium. Bacteria were grown overnight in brain heart infusion broth, and the culture supernatant was brought to 70% ammonium sulfate saturation. The precipitate was dialyzed against 10 mM Tris buffer (pH 7.5), and the crude protein preparation (approximately 700 μg of protein per ml) was used in an enzyme-linked immunosorbent assay (ELISA).

Serological studies. For serological studies, mice (10 per group or 20 to 30 per group for RP9) were immunized with approximately 5×10^7 to 5×10^8 spores of the appropriate *B. anthracis* strain. Six weeks after immunization, mice were bled from the retro-orbital plexus to obtain serum samples.

Titers of antibody to PA, LF, EF, or extracellular antigens were determined by an ELISA. Ninety-six-well polystyrene plates (Nunc) were coated with 100 ng of the appropriate antigen in phosphate-buffered saline (PBS) per well. Plates were incubated for 1 h at 37°C and then overnight at 4°C. Unoccupied binding sites were saturated by addition of 150 μl of PBS-containing 2% (wt/vol) bovine serum albumin per well and incubation for 1 h at 37°C. Thereafter, plates were washed with PBS containing 0.1% (vol/vol) Tween 20 and serum samples diluted twofold from 1/10 to 1/50,000 were then added to the appropriate wells. Plates were incubated at 37°C for 1 h and washed with PBS–0.1% Tween 20. Horseradish peroxidase-conjugated goat anti-mouse serum (1:1,000; Amersham) was then added to each well, and incubation was continued for 1 h at 37°C. Plates were washed with PBS–0.1% Tween 20, and 100 μl of an *o*-phenylenediamine (Abbott Laboratories) substrate solution was added to each well and allowed to react for 4 min at room temperature. The reaction was stopped by addition of 2N H_2SO_4, and plates were read at 492 nm on a microplate reader (LP200 Diagnostics Pasteur). A plot of A_{492} versus antibody dilution resulted in a response that was linear for serum dilutions ranging from 1/100 to 1/50,000. Antibody titers were defined as the serum dilution at $A_{492} = 0.5$. For statistical analysis, Student's *t* test was used.

RESULTS AND DISCUSSION

Antibody response to toxin components after infection with *B. anthracis* mutants. The contribution of each toxin component to antibody response and immunoprotection in live vaccine has always been difficult to evaluate because of the lethality of the Sterne spore vaccine for experimental animals. The mutants used in this study were avirulent or had greatly reduced virulence (RP9), therefore allowing new insights into the respective roles of PA, LF, and EF.

The antibody response of mice to each anthrax toxin component produced in vivo by the various *B. anthracis* mutant strains was studied. Sera from all groups of mice were analyzed by ELISA 6 weeks after a single immunization dose. The antibody titers are reported in Fig. 1. All PA-producing strains (RP42, RP10, and RP9) elicited high levels of specific antibodies against PA. In mice immunized with mutants producing EF (RP31), LF (RP4), or EF and LF (RP8), the response to these proteins was weak and low specific antibody titers were found (Fig. 1). In contrast, when LF or EF was produced by strains which also produce PA (RP9 and RP10), a significant increase in the response against LF or EF was observed. As shown in Fig. 1 and Table 1, sera obtained after RP9 immunization
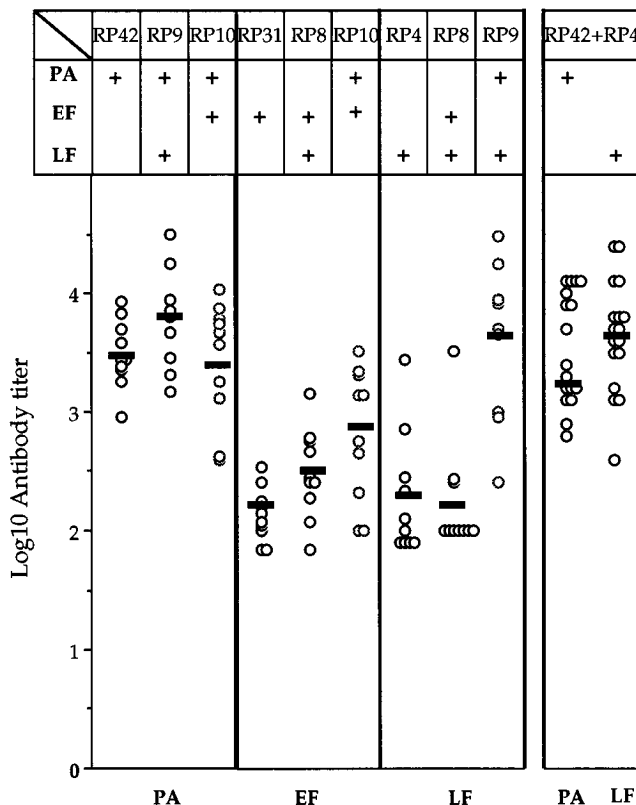

FIG. 1. ELISA-detected antibodies to PA, EF, and LF in mice immunized with *B. anthracis* mutant strains. Circles represent individual animal titers, and the black bar represents the arithmetic mean log titer of the group. Strains used for immunization and the respective toxin components produced are indicated at the top. At the bottom are indicated the toxin components against which the antibody titers were determined. The right panel represents the titers of antibodies to PA and LF in animals immunized with a mixture of strains RP42 and RP4.

exhibited high titers of antibody to LF, whereas, the titers were significantly lower ($P < 0.009$) after RP4 immunization. A similar effect was found for EF when titers obtained with strains RP31 and RP10 were compared ($P < 0.04$). The fact that titers of antibody to EF were always lower than those of antibodies to PA and LF might be a consequence of the weaker expression of the *cya* gene with respect to that of *pag* or *lef*, as previously demonstrated by the use of transcriptional gene fusions (21). This intriguing observation that titers of antibodies to EF and LF were significantly higher in animals immunized with bacteria also producing PA is probably not a consequence of differences in toxin expression by the mutant strains. The six mutant strains used in this study have been characterized in a previous work, and we determined that PA, LF, and EF were synthesized in vitro in amounts similar to those obtained from the parental Sterne strain (20). However, to rule out possible differences in in vivo toxin production, mice were immunized with a mixture of RP42 and RP4 spores and the resulting titers were compared to those of antibodies from mice receiving RP9 (Fig. 1). A lethal effect was observed, as 4 mice in a group of 20 died after immunization. From these experiments, it appears that RP42 and RP4 spores are able to reconstitute in vivo the biological effects of an active lethal toxin through independent production of PA and LF. Analysis of sera showed that titers of antibody to LF in animals immunized with RP42 and RP4 were significantly higher than in

TABLE 1. Serum titers of antibodies to toxin components
and extracellular antigens after immunization

Strain	Serum antibody titer[a] in response to:			
	PA	EF	LF	Extracellular antigens
7700[b]	<10	<10	<10	50
RP42	3,100	<100	<100	1,805
RP31	<100	137	<100	1,031
RP4	<100	<100	204	2,770
RP8	<100	302	172	3,586
RP9	5,947	<100	3,919	1,270
RP10	2,691	662	<100	759

[a] Reciprocal geometric mean ELISA-determined titers of antibodies to toxin components (from data presented in Fig. 1) and to extracellular antigens are shown.
[b] The data from animals immunized with plasmid-free strain 7700 are added.

TABLE 2. Protective efficacy of *B. anthracis* mutant strains

Immunizing strain[a]	Toxin factor(s) produced	No. of survivors/no. challenged (%) at immunizing spore dose of:		
		10^6	10^7	10^8
7700		0/10 (0)	0/10 (0)	0/10 (0)
7702	PA, EF, LF	0/8 (0)		
RP31	EF	4/20 (20)	5/20 (25)	5/20 (25)
RP42	PA	3/20 (15)	9/20 (45)	14/20 (70)
RP4	LF	6/20 (30)	7/20 (35)	15/20 (75)
RP8	EF, LF	0/10 (0)	1/10 (10)	8/10 (80)
RP10	PA, EF	9/20 (45)	11/20 (55)	15/20 (75)
RP9[b]	PA, LF	6/10 (60)	12/18 (67)	10/10 (100)

[a] Two (4%) of 50 control mice survived the challenge (see Materials and Methods).
[b] Groups of 20 (10^6) or 30 (10^7 and 10^8) mice were immunized with strain RP9, and survivors were challenged.

those receiving only RP4 ($P < 0.003$) and were similar to those found in RP9-immunized animals. This result suggests that the increased immunogenicity of LF originates from a protein complementation mechanism. Thus, the ability of PA to bind to cell surface receptors, and facilitate the internalization and intracellular processing of EF or LF, may be a prerequisite for the higher antibody response to these two proteins. In contrast, when EF and LF are produced from strains deficient in PA production, these two toxin components may not have the opportunity to interact with the appropriate effector cells. An alternative explanation may be that the biologically active edema or lethal toxins act on immune cells, interfering with the immune response (6, 18). This seems less likely, because in such a case one would also expect a difference in the antibody response to PA (RP42). Further experiments including mutants with catalytically inactive EF or LF proteins are needed to elucidate the potentiation of the antibody response induced by PA.

To define a common marker for all of the strains tested, we also evaluated the humoral response against other bacterial antigens unrelated to the toxins. For this purpose, titers of antibodies against extracellular antigens present in the culture medium of plasmid-free strain 7700 were determined by ELISA. The results are shown in Table 1 and compared with the titers obtained against the toxin components. It appeared that the six mutant strains induced a similar antibody response to extracellular antigens. This supports the notion that the mutants are able to develop similarly in a host. In contrast, spores of plasmid-free strain 7700 did not induce an antibody response to extracellular antigens, indicating that they may not have germinated or that the resulting bacteria did not multiply or did so only poorly. These results point out for the first time a possible role of pXO1 in bacterial survival in a host. In addition to the toxin structural and regulatory genes (12, 17, 25), this large virulence plasmid may also carry other determinants involved in spore germination and/or bacterial multiplication in vivo. Parental Sterne strain 7702 merits a specific comment. We previously reported that the 50% lethal dose of this strain is at least 10-fold lower than that of mutant strain RP9, which produces lethal toxin only. Because of its virulence, it was difficult to estimate the antibody response to toxin components elicited by the Sterne strain. Immunization with sublethal spore doses ($\leq10^6$) induced only weak and highly variable responses to toxin components and no significant response to extracellular antigens (data not shown).

Protection of mice with *B. anthracis* mutants. To compare the antibody response with the protective efficacy of the strains, protection experiments were conducted. The protection provided to mice by the various *B. anthracis* mutants against a lethal challenge with the Sterne strain was tested 6 weeks after a single immunization. Four spore doses, ranging from 10^5 to 10^8, were studied. Since no protection was observed at a dose of 10^5 spores of any of the strains, only data for the 10^6- to 10^8-spore doses are presented in Table 2. The six mutant strains were capable of protecting to a greater or lesser extent. Immunization with larger doses ($\geq10^7$ spores) of any of the three strains producing PA, as either a single component or in combination with LF or EF, provided protection and confirmed the role of PA as a major protective antigen involved in the humoral response. This study also suggests that both EF and LF contribute to immunoprotection, since strains RP10 and RP9 were significantly more effective than the other strains ($P < 0.01$) and provided protection even in smaller doses. This protection may be the result of potentiation of the antibody response to EF and LF in the presence of PA observed with these strains. Although lethal toxin-producing strain RP9 still exhibits lethality for mice at higher doses, animals surviving lethal doses of this strain were efficiently protected. In contrast, no protection was observed in animals surviving immunization with the Sterne strain. However, as noted above, bacterial multiplication and humoral response to toxins cannot be significantly estimated with the Sterne strain. It should be pointed out that strains RP9 and Sterne are isogenic and differ only in the inactivation of the *cya* gene encoding EF (RP9). Therefore, the differences in virulence, immunoprotection, and antibody response observed between these two strains highlight the role of adenylate cyclase (EF) in the pathogenesis of anthrax.

The control strain, 7700, never protected. This observation is in agreement with the serological data suggesting that this strain, lacking pXO1, may not be able to develop normally in vivo. Moreover, this hypothesis is supported by the fact that, in contrast to strain 7700, the three mutant strains deficient in PA production are still able to provide protection to some extent ($P < 0.001$). It is unlikely that the weak antibody response to toxin components elicited by these strains could account for their protective effect. It seems, rather, that this protection results from some cellular or humoral responses to *B. anthracis* unrelated to toxin production.

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