INFECTION AND IMMUNITY, Feb. 2002, p. 661–664 Vol. 70, No. 2
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.2.661–664.2002
Copyright © 2002, American Society for Microbiology. All Rights Reserved.

Anthrax Spores Make an Essential Contribution to Vaccine Efficacy

Fabien Brossier, Martine Levy, and Michèle Mock*

Unité des Toxines et Pathogénie Bactérienne (CNRS URA 2172), Institut Pasteur, 75725 Paris Cedex 15, France

Received 6 August 2001/Returned for modification 28 September 2001/Accepted 9 November 2001

Anthrax is caused by *Bacillus anthracis*, a gram-positive spore-forming bacterium. Septicemia and toxemia rapidly lead to death in infected mammal hosts. Currently used acellular vaccines against anthrax consist of protective antigen (PA), one of the anthrax toxin components. However, in experimental animals such vaccines are less protective than live attenuated strains. Here we demonstrate that the addition of formaldehyde-inactivated spores (FIS) of *B. anthracis* to PA elicits total protection against challenge with virulent *B. anthracis* strains in mice and guinea pigs. The toxin-neutralizing activities of sera from mice immunized with PA alone or PA plus FIS were similar, suggesting that the protection conferred by PA plus FIS was not only a consequence of the humoral response to PA. A PA-deficient challenge strain was constructed, and its virulence was due solely to its multiplication. Immunization with FIS alone was sufficient to protect mice partially, and guinea pigs totally, against infection with this strain. This suggests that spore antigens contribute to protection. Guinea pigs and mice had very different susceptibilities to infection with the nontoxigenic strain, highlighting the importance of verifying the pertinence of animal models for evaluating anthrax vaccines.

The disease anthrax is caused by *Bacillus anthracis*, a gram-positive spore-forming bacterium. It affects mammals including humans. After entry into the host, the spores germinate and yield toxin-producing, capsulated bacilli. Toxemia and septicemia rapidly lead to death. The bacilli secrete three proteins, protective antigen (PA), lethal factor (LF), and edema factor (EF), and these proteins combine to form the lethal (PA plus LF) and edema (PA plus EF) toxins. PA is the common cell binding component and is required for toxin activity (14). Two large virulence plasmids, pXO1 and pXO2, encode toxin production and the formation of a poly-γ-D-glutamic acid capsule, respectively (20, 30). Curing *B. anthracis* wild-type strains of either plasmid attenuates virulence. However, the residual virulence of pXO1$^-$ strains appears to be strongly influenced by the backgrounds of both pXO2 and the chromosome (32, 35). A pXO2$^+$ pXO1$^-$ derivative of the Ames strain, a strain often described as particularly virulent (4, 13), has been shown to be as virulent for mice as the parental strain, due exclusively to bacterial multiplication and associated septicemia (35). Curing *B. anthracis* wild-type strains of pXO2 yields toxigenic, noncapsulated, attenuated strains with vaccinal properties. One such strain, the Sterne strain, is used in the form of a live-spore vaccine for the immunization of animals. Although it performs satisfactorily (7, 26), it has side effects in some species. The recent development of a genetically detoxified Sterne strain derivative, RPLC2, may provide a valuable alternative to the residual virulence of the Sterne strain (2). The live vaccine is not considered suitable for human use, but PA-based cell-free vaccines, prepared from culture supernatants of the Sterne strain, have been licensed (7, 5, 27). Multiple immunizations are required to confer protection, and there are cases of reactogenicity. Recombinant PA can now be produced from various heterologous organisms including *Bacillus subtilis* (11, 17, 34), and the safety and consistency of PA preparations have been improved. Highly purified PA preparations and recombinant PA have been tested in various animal models, including mice, guinea pigs, rabbits, and monkeys (4, 10, 33, 36), in combination with various adjuvants (9, 12). These studies have yielded varied results. They also indicate that PA-based vaccines are less protective than live-spore vaccines against virulent isolates of *B. anthracis* (16, 33). Thus, some strains, for example, the Ames strain, have been termed "vaccine resistant" because full protection in guinea pigs immunized with PA is possible only with adjuvants unsuitable for human vaccines (4, 12, 16). Moreover, several studies illustrate the difficulty of evaluating PA vaccines and establishing a direct correlation between PA-specific antibody titers and protection (9, 12, 26, 28, 29).

In recent years, efforts have been made to improve acellular vaccines. There is evidence that spore antigens present in live-spore vaccines make a contribution to protection (3). Immunization with PA targets toxemia but not septicemia, and it is possible that an immune response to spore antigens would enhance protective efficacy by targeting the early steps of infection. We thus tested the efficacy of a vaccine composed of PA and formaldehyde-inactivated spores (FIS) of a genetically detoxified Sterne strain (RPLC2). Virulent *B. anthracis* strains and two animal models were used: (i) guinea pigs, the animals most commonly used for testing anthrax vaccines (13, 16, 26, 29), and (ii) mice, which are very sensitive to anthrax and particularly difficult to protect (12, 33). Inclusion of FIS in an acellular vaccine resulted in total protection against virulent strains in both animal models, under conditions where PA alone failed. However, the findings for the two animal species differed, indicating the importance of assessing the pertinence of animal models for evaluating anthrax vaccines.

* Corresponding author. Mailing address: Unité des Toxines et Pathogénie Bactérienne (CNRS URA 2172), Institut Pasteur, 28 rue du Docteur Roux, 75725 Paris Cedex 15, France. Phone: (33) 145688312. Fax: (33) 145688954. E-mail: mmock@pasteur.fr.

MATERIALS AND METHODS

Bacterial strains. *B. anthracis* strains were grown in brain heart infusion medium or on CAP agar plates (25). Spectinomycin (60 μg/ml) was added as

TABLE 1. Protective immunity induced in guinea pigs by the combination PA plus FIS

Immunization	Protection[a]	Serological response[b] (titer)	
		Anti-PA	Anti-spores
PA	2/9 (22)	9,600	ND
PA + 10^5 FIS	2/4 (50)	17,700	1,000
PA + 10^6 FIS	3/5 (60)	7,900	2,000
PA + 10^7 FIS	5/5 (100)	12,800	2,000
PA + 10^8 FIS	9/9 (100)	11,200	15,000
10^8 FIS	1/4 (25)	<100	11,000
Control	0/10 (0)	<10	<100

[a] Number surviving/number challenged (percent). Animals were challenged with 300 LD_{50}s.
[b] Titers of antibodies against PA and spores were determined by ELISA. Values given are reciprocal geometric mean titers. ND, not determined.

appropriate. The following *B. anthracis* strains were used: the Sterne strain 7702 ($pXO1^+$), its derivatives RPLC2 (carrying point mutations affecting the catalytic sites of EF and LF) (2) and SM11 (with the genes encoding the S layer deleted) (19), and the virulent strains ($pXO1^+$ $pXO2^+$) 17JB (30) and 9602 (a strain isolated in a fatal human case of anthrax in France [1]). Spores of *B. anthracis* strains were prepared as previously described (21). When necessary, spores were inactivated with formaldehyde (4%) after overnight incubation at 37°C. Spores were conserved in sterile water at 4°C.

Construction of strain 9602P. The *pagA* gene carried by pACP41 (22) was cleaved with *Nco*I, blunted, and ligated to the nonpolar spectinomycin resistance cassette (Spc-H+1) (18). The inactivated gene was inserted into pAT113, and the construct was transferred into strain 9602 by "heterogramic" mating as previously described (21). The mutant strain carrying the *pagA* gene deletion (9602P) in place of the wild-type copy was selected on CAP plates containing bicarbonate and spectinomycin. The plates were incubated at 37°C in a 5% CO_2 incubator to allow transcription of the spectinomycin resistance cassette from the *pagA* gene promoter. The construct in 9602P was verified by PCR, and the absence of PA production was checked by immunoblotting using antibodies specific for PA.

Immunization and challenge of experimental animals. Seven-week-old female Swiss outbred mice (six per group) (Iffa Credo, l'Arbresle, France) and female Hartley guinea pigs weighing 200 to 300 g (Charles River, Saint-Aubin les Elbeuf, France) were used for virulence and immunization experiments. The components used for immunization were purified PA (10 μg/mouse and 40 μg/guinea pig) and/or FIS of RPLC2. Aluminum hydroxide (0.3%), which is the adjuvant licensed for human vaccines (5, 27), was used for all immunizations. Mice were injected subcutaneously (200 μl), and guinea pigs were injected intradermally (100 μl). Animals were immunized twice, on days 0 and 15. Serum samples were taken from the retro-orbital plexus of mice and by cardiac puncture from guinea pigs on day 33. Animals were challenged subcutaneously on day 35. The 50% lethal dose (LD_{50}; the dose of spores killing half the animals) of 17JB was 500 spores for mice; the LD_{50} of both 9602 and 9602P was 50 spores for mice. The LD_{50} of 9602 was 100 spores for guinea pigs. Mice were challenged with a dose equivalent to 30 times the LD_{50} of 17JB, 9602, or 9602P, and guinea pigs were challenged with 300 times the LD_{50} of 9602. Surviving animals were sacrificed 2 weeks after challenge.

Serological tests. Enzyme-linked immunosorbent assays (ELISAs) were used to determine titers of antibodies (total immunoglobulin) specific for the purified PA as previously described (23). Titers of antibodies to spore surface proteins were also determined by ELISA. Wells of 96-well microtiter plates (Nunc) were coated with formaldehyde-treated SM11 spores (10^7/well) overnight at 37°C. Spores were then fixed with paraformaldehyde (3.4%). Anti-species antibodies coupled to peroxidase were used at a dilution of 1/1,000. An arbitrary A_{492} value of 0.5 was used to calculate the endpoint titers.

Neutralizing assays on macrophages. Murine macrophages (RAW264.7) were seeded in a 96-well microtiter plate (Nunc) (2×10^4 cells/well) and incubated for 16 h at 37°C under a 5% CO_2 atmosphere. PA (50 ng/ml, the 100% effective concentration) was preincubated for 1 h at 37°C under a 5% CO_2 atmosphere with dilutions of sera from mice immunized with PA alone or with PA plus FIS. The complexes were then incubated with LF (1 μg/ml) and the macrophages for 3 h at 37°C under a 5% CO_2 atmosphere. Cell viability was quantified by a colorimetric assay (8).

Statistics. The χ^2 test was used for statistical analysis.

RESULTS

Protective efficacy of PA plus FIS in guinea pigs. The contribution of FIS to PA vaccine efficacy was first evaluated in guinea pigs (Table 1). The highly virulent strain 9602, which has an LD_{50} similar to that of the Ames strain (13), was used for challenge. Animals receiving PA alone were poorly protected (22%), in agreement with the findings of other immunoprotection studies. In contrast, inclusion of FIS in the vaccine enhanced protection and did so in a dose-dependent manner. Indeed, protection was 100% when 10^7 spores or more were included. Immunization with spores alone, even at the highest dose, provided only partial protection (25%). The difference in survival between guinea pigs immunized with PA alone and those immunized with PA plus 10^8 FIS was statistically significant ($P < 0.05$). The difference in survival between guinea pigs immunized with 10^8 FIS alone and those immunized with PA plus 10^8 FIS was also statistically significant ($P < 0.02$). Thus, the combination PA plus FIS provided full protection under conditions where PA alone or FIS alone failed to do so.

Protective efficacy of PA plus FIS in mice. Two challenge strains, the laboratory strain 17JB and 9602, were used with mice. Their LD_{50}s were 500 and 50 spores per mouse, respectively. Fifty percent and 33% of mice immunized with preparations of PA or FIS alone, respectively, were protected against 17JB (Table 2). In contrast, 100% of those immunized with the combination PA plus FIS survived the lethal challenge, although small numbers of animals were used. In the experiment with strain 9602, using larger number of animals, none of the animals immunized with either PA or FIS alone survived the lethal challenge, confirming the virulence of strain 9602 and the difficulty of protecting mice. Nevertheless, all animals immunized with the combination PA plus FIS were protected against 9602 ($P < 0.01$).

Characterization of the humoral immune response. In both animal models, the immunization procedure elicited significant titers of antibody against PA (>5,000) and spore antigens (>1,000) (Tables 1 and 2). Sera from immunized animals were used to probe blots of spore surface antigen preparations. Few protein bands were recognized (data not shown). The most strongly labeled antigen was a polypeptide with a molecular size above 250 kDa.

TABLE 2. Protective immunity induced in mice by the combination PA plus FIS

Immunization	Protection[a]		Serological response[b] (titer)		
	17JB	9602	Anti-PA	Neutralizing titer[c] ± SD	Anti-spores
PA	3/6 (50)	0/18 (0)	8,600	1,060 ± 300	ND
10^8 FIS	2/6 (33)	0/12 (0)	40	ND	18,000
PA + 10^8 FIS	6/6 (100)	24/24 (100)	12,000	580 ± 120	4,400
Control	0/6 (0)	0/18 (0)	<10	ND	<100

[a] Number surviving/number challenged (percent). Animals were challenged with 30 LD_{50}s of one of the two virulent strains.
[b] Titers of antibodies were determined by ELISA. Values given are reciprocal geometric mean titers. ND, not determined.
[c] Reciprocal of the dilution of the pooled sera sufficient to neutralize half of the activity against macrophages of the lethal toxin. Experiments were performed in triplicate.

TABLE 3. Protective efficacy of FIS against challenge with strain 9602P

Immunization	Protection[a]	
	Guinea pigs	Mice
PA	0/5 (0)	0/6 (0)
10^8 FIS	5/5 (100)	3/6 (50)
Control	0/5 (0)	0/6 (0)

[a] Number surviving/number challenged (percent). Mice were challenged with 30 LD_{50}s, and guinea pigs were challenged with a lethal dose (4×10^8 spores).

To determine if the 100% protection observed with the combination PA plus FIS was the result of higher PA-neutralizing antibody titers, we determined the neutralizing activities of pools of sera from mice immunized with PA alone or with PA plus FIS (Table 2). The toxin-neutralizing activity of the PA-plus-FIS pool was no higher than that of the PA pool. Therefore, the differences in protection observed could not be due to differences in toxin inactivation.

Protective efficacy of FIS against a capsulated PA-deficient strain. Infection involves both spore germination and subsequent vegetative-cell multiplication. The protection conferred by immunization with inactivated spores may act on the first of these processes. To test this, we constructed a challenge strain the virulence of which is entirely due to its multiplication properties. To avoid differences in the virulence background of the strain, we constructed an isogenic nontoxigenic derivative of 9602 (9602P). To make as small a change as possible, we introduced a nonpolar deletion into the *pagA* gene, which encodes PA.

Strain 9602P was highly attenuated for virulence in guinea pigs; 10^6 to 10^7 spores were required to kill half of the animals. As expected, immunization with PA was unable to protect against lethal challenge with 9602P, and no survival was observed (Table 3). In contrast, immunization with FIS gave 100% protection. The difference in survival between the two groups was statistically significant ($P < 0.01$). These data strongly suggest that in guinea pigs, the immune response against FIS is sufficient to protect against infection with 9602P.

Unlike guinea pigs, mice were highly sensitive to 9602P, and the LD_{50} of this strain was similar to that of the wild-type parental strain, 9602 (50 spores per mouse). Thus, although 9602P did not cause toxemia, its overall virulence in mice was not substantially affected. Immunization with PA did not protect mice against challenge with 9602P (Table 3), whereas immunization with FIS protected 50% of the mice against such a challenge. These data point to the higher sensitivity of mice to infection.

DISCUSSION

Anthrax involves both toxemia and septicemia leading to death of the infected host. PA-based vaccines only target toxemia, whereas live-spore vaccines may protect against both the effects. Numerous studies evidence the substantial contribution of PA as a vaccine component, but optimal protection is nevertheless best achieved by live vaccines. Here, we report the successful immunization of guinea pigs and mice with a combination of PA and FIS. In both animal models, protection reached 100%. In the sensitive mouse model, the PA-neutralizing antibody titers, which have been considered a marker of protective immunity (24), were not affected upon addition of FIS. However, protection was 0% after immunization with PA and 100% after immunization with PA plus FIS in mice infected with strain 9602. The effect of spores on protective immunity is therefore not a consequence of greater neutralization of toxin activity. Animals also developed an antibody response to spore antigens, as determined by ELISA. The improved protection may be a consequence of the response to these antigens, as has been proposed for live-spore vaccines (3). The contribution of FIS to protection against infection was demonstrated by constructing a PA-deficient strain, 9602P, and using it for challenge. Immunization with FIS was sufficient to protect guinea pigs totally, and mice partially, against infection with the capsulated 9602P strain. Interestingly, the virulence of 9602P in mice and guinea pigs was very different: guinea pigs were relatively resistant to 9602P, and thus the toxin is likely to be the main virulence factor in anthrax-infected guinea pigs. This probably also explains why guinea pigs are easier to protect with PA-based vaccines than mice. Moreover, successful passive protection with anti-PA sera has been reported in guinea pigs (15). In contrast, mice were as sensitive to 9602P as to the wild-type parental strain, 9602, which supports observations made with $pXO2^+$ derivatives of virulent strains (32, 35). Therefore, control of septicemia might differ greatly between host species. Protection of hosts highly susceptible to infection probably requires more than PA-mediated toxin neutralization.

In summary, we present evidence that inclusion of killed spores greatly enhances the protective efficacy of a PA-based vaccine. Immunization with FIS plus PA provides a synergistic protective immunity acting on both toxemia and infection. The immune response induced by FIS may act early by blocking germination, a critical step at the onset of pathogen multiplication (6, 31). The molecular mechanisms of the protective immunity induced by FIS and the putative role of spore antigens need to be further investigated. It is clear that full protection against anthrax requires a multifactorial immune response. The results presented here may serve as the basis for the first design, for human use, of a subunit vaccine as protective as the current live veterinary vaccine.

ACKNOWLEDGMENTS

We thank A. Fouet and P. Goossens for critical reading of the manuscript, E. Duflot for technical assistance, and P. Sylvestre, F. Ramisse, and A. Labarre at the Centre d'Étude du Bouchet for assistance with animal experiments.

F. Brossier was supported by the Direction Générale des Armées (grant 9934030).

REFERENCES

1. **Berthier, M., J. L. Fauchère, J. Perrin, B. Grignon, and D. Oriot.** 1996. Fulminant meningitis due to *Bacillus anthracis* in 11-year-old girl during Ramadan. Lancet **347:**828.
2. **Brossier, F., M. Weber-Levy, M. Mock, and J.-C. Sirard.** 2000. Role of toxin functional domains in anthrax pathogenesis. Infect. Immun. **68:**1781–1786.
3. **Cohen, S., I. Mendelson, Z. Altboum, D. Kobiler, E. Elhanany, T. Bino, M. Leitner, I. Inbar, H. Rosenberg, Y. Gozes, R. Barak, M. Fisher, C. Kronman, B. Velan, and A. Shafferman.** 2000. Attenuated nontoxinogenic and nonencapsulated recombinant *Bacillus anthracis* spore vaccines protect against anthrax. Infect. Immun. **68:**4549–4558.
4. **Fellows, P. F., M. K. Linscott, B. E. Ivins, M. L. Pitt, C. A. Rossi, P. H. Gibbs, and A. M. Friedlander.** 2001. Efficacy of a human anthrax vaccine in guinea pigs, rabbits, and rhesus macaques against challenge by *Bacillus anthracis* isolates of diverse geographical origin. Vaccine **19:**3241–3247.

5. **Friedlander, A. M., P. R. Pittman, and G. W. Parker.** 1999. Anthrax vaccine: evidence for safety and efficacy against inhalational anthrax. JAMA **282:** 2104–2106.
6. **Guidi-Rontani, C., M. Weber-Levy, E. Labruyère, and M. Mock.** 1999. Germination of *Bacillus anthracis* spores within alveolar macrophages. Mol. Microbiol. **31:**9–17.
7. **Hambleton, P., J. A. Carman, and J. Melling.** 1984. Anthrax: the disease in relation to vaccines. Vaccine **2:**125–132.
8. **Hansen, M. B., S. E. Nielsen, and K. Berg.** 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods **119:**203–210.
9. **Ivins, B., P. Fellows, L. Pitt, J. Estep, J. Farchaus, A. Friedlander, and P. Gibbs.** 1995. Experimental anthrax vaccines: efficacy of adjuvants combined with protective antigen against an aerosol *Bacillus anthracis* spore challenge in guinea pigs. Vaccine **13:**1779–1784.
10. **Ivins, B. E., M. L. Pitt, P. F. Fellows, J. W. Farchaus, G. E. Benner, D. M. Waag, S. F. Little, G. W. Anderson, Jr., P. H. Gibbs, and A. M. Friedlander.** 1998. Comparative efficacy of experimental anthrax vaccine candidates against inhalation anthrax in rhesus macaques. Vaccine **16:**1141–1148.
11. **Ivins, B. E., S. L. Welkos, G. B. Knudson, and S. F. Little.** 1990. Immunization against anthrax with aromatic compound-dependent (Aro−) mutants of *Bacillus anthracis* and with recombinant strains of *Bacillus subtilis* that produce anthrax protective antigen. Infect. Immun. **58:**303–308.
12. **Ivins, B. E., S. L. Welkos, S. F. Little, M. H. Crumrine, and G. O. Nelson.** 1992. Immunization against anthrax with *Bacillus anthracis* protective antigen combined with adjuvants. Infect. Immun. **60:**662–668.
13. **Ivins, B. E., P. F. Fellows, and G. O. Nelson.** 1994. Efficacy of a standard human anthrax vaccine against *Bacillus anthracis* spore challenge in guinea-pigs. Vaccine **12:**872–874.
14. **Leppla, S.** 1995. Anthrax toxins, p. 543–572. *In* J. Moss, B. Iglewski, M. Vaughan, and A. T. Tu (ed.) Bacterial toxins and virulence factors in disease. New York, N.Y.
15. **Little, S. F., B. E. Ivins, M. F. Fellows, and A. M. Friedlander.** 1997. Passive protection by polyclonal antibodies against *Bacillus anthracis* infection in guinea pigs. Infect. Immun. **65:**5171–5175.
16. **Little, S. F., and G. B. Knudson.** 1986. Comparative efficacy of *Bacillus anthracis* live spore vaccine and protective antigen vaccine against anthrax in the guinea pig. Infect. Immun. **52:**509–512.
17. **McBride, B., A. Mogg, J. L. Telfer, M. S. Lever, J. Miller, P. C. B. Turnbull, and L. Baillie.** 1998. Protective efficacy of a recombinant protective antigen against *Bacillus anthracis* challenge and assessment of immunological markers. Vaccine **16:**810–817.
18. **Mesnage, S., T. Fontaine, T. Mignot, M. Delepierre, M. Mock, and A. Fouet.** 2000. Bacterial SLH domain proteins are non-covalently anchored to the cell surface via a conserved mechanism involving wall polysaccharide pyruvylation. EMBO J. **19:**4473–4484.
19. **Mesnage, S., E. Tosi-Couture, M. Mock, P. Gounon, and A. Fouet.** 1997. Molecular characterization of the *Bacillus anthracis* main S-layer component: evidence that it is the major cell-associated antigen. Mol. Microbiol. **23:**1147–1155.
20. **Mikesell, P., B. E. Ivins, J. D. Ristroph, and T. M. Dreier.** 1983. Evidence for plasmid-mediated toxin production in *Bacillus anthracis*. Infect. Immun. **39:** 371–376.
21. **Pezard, C., P. Berche, and M. Mock.** 1991. Contribution of individual toxin components to virulence of *Bacillus anthracis*. Infect. Immun. **59:**3472–3477.
22. **Pezard, C., E. Duflot, and M. Mock.** 1993. Construction of *Bacillus anthracis* mutant strains producing a single toxin component. J. Gen. Microbiol. **139:** 2459–2463.
23. **Pezard, C., M. Weber, J.-C. Sirard, P. Berche, and M. Mock.** 1995. Protective immunity induced by *Bacillus anthracis* toxin-deficient strains. Infect. Immun. **63:**1369–1372.
24. **Reuveny, S., M. D. White, Y. Y. Adar, Y. Kafri, Z. Altboum, Y. Gozes, D. Kobiler, A. Shafferman, and B. Velan.** 2001. Search for correlates of protective immunity conferred by anthrax vaccine. Infect. Immun. **69:**2888–2893.
25. **Sirard, J. C., M. Mock, and A. Fouet.** 1995. Molecular tools for the study of transcriptional regulation in *Bacillus anthracis*. Res. Microbiol. **146:**729–737.
26. **Turnbull, P. C. B.** 1991. Anthrax vaccines: past, present and future. Vaccine **9:**533–539.
27. **Turnbull, P. C. B.** 2000. Current status of immunization against anthrax: old vaccines may be here to stay for a while. Curr. Opin. Infect. Dis. **13:**113–120.
28. **Turnbull, P. C. B., M. G. Broster, J. A. Carman, R. J. Manchee, and J. Melling.** 1986. Development of antibodies to protective antigen and lethal factor components of anthrax toxin in humans and guinea pigs and their relevance to protective immunity. Infect. Immun. **52:**356–363.
29. **Turnbull, P. C. B., S. H. Leppla, M. G. Broster, C. P. Quinn, and J. Melling.** 1988. Antibodies to anthrax toxin in humans and guinea pigs and their relevance to protective immunity. Med. Microbiol. Immunol. **177:**293–303.
30. **Uchida, I., T. Sekizaki, K. Hashimoto, and N. Terakado.** 1985. Association of the encapsulation of *Bacillus anthracis* with a 60-megadalton plasmid. J. Gen. Microbiol. **131:**363–367.
31. **Welkos, S., S. Little, A. Friedlander, D. Fritz, and P. Fellows.** 2001. The role of antibodies to *Bacillus anthracis* and anthrax toxin components in inhibiting the early stages of infection by anthrax spores. Microbiology **147:**1677–1685.
32. **Welkos, S. L.** 1991. Plasmid-associated virulence factors of non-toxigenic ($pXO1^{-}$) *Bacillus anthracis*. Microb. Pathog. **10:**183–198.
33. **Welkos, S. L., and A. M. Friedlander.** 1988. Comparative safety and efficacy against *Bacillus anthracis* of protective antigen and live vaccines in mice. Microb. Pathog. **5:**127–139.
34. **Welkos, S. L., T. J. Keener, and P. H. Gibbs.** 1986. Differences in susceptibility of inbred mice to *Bacillus anthracis*. Infect. Immun. **51:**795–800.
35. **Welkos, S. L., N. J. Vietri, and P. H. Gibbs.** 1993. Non-toxigenic derivatives of the Ames strain of *Bacillus anthracis* are fully virulent for mice: role of plasmid pXO2 and chromosome in strain-dependent virulence. Microb. Pathog. **14:**381–388.
36. **Zaucha, G. M., L. M. Pitt, J. Estep, B. E. Ivins, and A. M. Friedlander.** 1998. The pathology of experimental anthrax in rabbits exposed by inhalation and subcutaneous inoculation. Arch. Pathol. Lab. Med. **122:**982–992.

Editor: J. T. Barbieri