Comparative Efficacy of *Bacillus anthracis* Live Spore Vaccine and Protective Antigen Vaccine against Anthrax in the Guinea Pig

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Several strains of *Bacillus anthracis* have been reported previously to cause fatal infection in immunized guinea pigs. In this study, guinea pigs were immunized with either a protective antigen vaccine or a live Sterne strain spore vaccine, then challenged with virulent *B. anthracis* strains isolated from various host species from the United States and foreign sources. Confirmation of previously reported studies (which used only protective antigen vaccines) was made with the identification of 9 of the 27 challenge isolates as being vaccine resistant. However, guinea pigs immunized with the live Sterne strain spore vaccine were fully protected against these nine isolates. In experiments designed to determine the basis of vaccine resistance, guinea pigs which were immunized with individual toxin components and which demonstrated enzyme-linked immunosorbent assay antibody titers comparable to those induced by Sterne strain vaccine were not protected when challenged with a vaccine-resistant isolate. We concluded that antibodies to toxin components may not be sufficient to provide protection against all strains of *B. anthracis* and that other antigens may play a role in active immunity. As a practical matter, it follows that the efficacy of anthrax vaccines must be tested by using vaccine-resistant isolates if protection against all possible challenge strains is to be assured.

Two virulence factors have been described for *Bacillus anthracis*, each of which is associated with a separate plasmid (11, 17, 28). The capsule, composed of poly-D-glutamic acid, inhibits phagocytosis (14, 32) and is nonimmunogenic (22). Anthrax toxin, readily obtained from culture supernatants of toxin-producing strains of *B. anthracis* (20), is composed of edema factor (EF), protective antigen (PA), and lethal factor (LF) (2, 23, 25). The individual toxin components are not toxic. A PA-LF combination produces lethality after intravenous injection in some species (2, 25), and a PA-EF mixture causes edema when injected subcutaneously (8, 25). The production of both capsule and toxin is required for the bacterium to be fully virulent.

An excellent review of the various anthrax vaccines and immunogen preparations has been published by Hambleton et al. (12). Live attenuated spore vaccines are licensed currently only for veterinary use in the United States and have been shown for some time to be effective in protecting livestock (10, 13, 18, 26). At least three different cell-free anthrax vaccine preparations have been used in human trials (4, 6, 19, 30). The predominant component in these cell-free filtrates is PA. The commercial product licensed for human use in the United States it is supplied by the Michigan Department of Public Health and will be referred to as PA vaccine. This vaccine is not a highly purified product and has been reactogenic in some recipients (4).

Although various antigen preparations appear to provide a substantial degree of protection when immunized animals are challenged with the standard Vollum strain, early studies by Auerbach and Wright (1) and Ward et al. (29) demonstrated that certain *B. anthracis* isolates were able to override this immunity in guinea pigs. This current study, undertaken as part of an overall effort to evaluate and improve the PA vaccine presently used for humans, seeks to confirm and expand upon those early studies. Furthermore, a better understanding of the virulence of *B. anthracis* should result from the elucidation of vaccine resistance among strains of the bacillus.

MATERIALS AND METHODS

Animals. Female Hartley guinea pigs, weighing 300 to 350 g at the beginning of the immunizations, were used for this study.

Protection studies. The cell-free anthrax vaccine is prepared commercially by the Michigan Department of Public Health by adsorbing a V770-NP1-R culture filtrate to aluminum hydroxide gel. Filtration of the culture through sintered glass filters removes most of the LF and EF toxin components. The major toxin component of the PA vaccine is, therefore, PA. The vaccine was administered intramuscularly (i.m.) in three 0.5-ml doses at 2-week intervals. The commercial, live, veterinary, Sterne strain spore vaccine (Burroughs Wellcome Co.) was administered in three doses: 0.2, 0.3, and 0.5 ml i.m. at 2-week intervals. The stock spore vaccine contained 5×10^6 to 6×10^6 spores per ml. According to the manufacturer's recommendations, either 1.0 ml (cattle, horses, mules) or 0.5 ml (sheep, swine, goats), as a single dose or two doses, is

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protective. Controls for each experiment received physiological saline according to the corresponding immunization protocol.

We prepared purified toxin components LF and EF (16) for use as immunogens by adsorbing each to aluminum hydroxide gel (Alhydrogel; Accurate Chemical and Scientific Corp., Westbury, N.Y.), following a procedure similar to that used for the preparation of the PA vaccine (19). Briefly, toxins were adsorbed to a 1% suspension of aluminum hydroxide gel at pH 5.9 with stirring for 3 days at 4°C. Adsorbance values at 280 nm indicated that 93, 87, and 57% of PA, LF, and EF toxin components, respectively, had been adsorbed to the gel. The final product was suspended in phosphate-buffered saline (PBS) (2.3 mM sodium phosphate, monobasic; 7.5 mM sodium phosphate, dibasic; 0.15 M sodium chloride; pH 7.3) to yield 40 µg of protein per ml. Guinea pigs were immunized i.m. at 2-week intervals with three 0.5-ml doses of LF- or EF-adsorbed antigens either alone or concomitantly with 0.5 ml of PA vaccine given in the opposite flank.

TABLE 1. Histories of isolates used this study

Isolates	Source and date of isolation
Vollum	Cow; ca. 1944
Vollum 1B	Derived from Vollum
Ames	Cow; Iowa, 1980
Buffalo	Buffalo; Iowa, 1979
17T5	Kudu; South Africa, 1957
NH	Human; New Hampshire, 1957
SK31	Wildebeest; South Africa, 1974.
ACB	Human; Ohio, 1952
SK61	Human; California, 1976
SK162	Human; Florida, 1976
VH	Human; South Africa, 1952
V770	Cow; Florida, 1951
G28	South Africa, 1939
107	Human; Haiti, 1943
205	Goat; South Africa, 1942
Nebr	Cow; Nebraska, 1978
57	Goat; South Africa, 1946
Albia	Iowa, 1963
1928	Cow; Iowa, 1925
3515	1963
SK102	Pakistan wool; New Jersey, 1976
SK128	Ireland wool; Massachusetts, 1976
SK465	Buffalo; Iowa, 1979
C4880C	Mill; North Carolina, 1978
M36	Derived from Vollum
Vollum 1	South Africa, 1948
TX368203	Cow; Texas, 1983

Two weeks after the last immunization dose, serum was collected for titers and the animals were challenged.

Challenge isolates. Strains of *B. anthracis* used for challenges were isolated from various host species, animal products or handling facilities, and from various geographical areas of the United States and foreign sources between 1925 and 1983 (Table 1). Spore suspensions used for challenges were obtained from cultures grown at 37°C for 3 days on blood agar plates. Spores were washed from the culture with phenolized gel phosphate buffer (28 mM sodium phosphate, dibasic; 0.2% gelatin; 1% phenol; pH 7.2). Spore suspensions were heat shocked at 60°C for 30 min, washed, resuspended in phenolized gel phosphate, and held at 4°C until diluted for challenge. A standard challenge dose of 1,000 spores in 0.5 ml, administered i.m. 14 days after the last immunization dose, was used throughout unless noted.

ELISA. For enzyme-linked immunosorbent assay (ELISA), purified PA, LF, or EF toxin components (16) were diluted to 1µg/ml with 0.05 M sodium borate buffer (pH 9.5), and 100 µl added to each well of 96-well microtiter plates (Linbro) and incubated overnight at 4°C. Plates were incubated for 30 min at room temperature with 200 µ1 of PBS-0.5% gelatin (PBS-gelatin), washed two times with PBS, and frozen at -70°C in freezer bags. Before use, plates were washed three times with PBS-0.05% Tween 20 (PBS-Tween), and 100-µl sample volumes were added per well. Dilutions were made with PBS-gelatin. After incubation either overnight at 4°C or for 2 h at 37°C, plates were washed three times with PBS-Tween and incubated for 2 h at room temperature with horseradish peroxidase conjugated to staphylococcal protein A (Sigma) at a 1/5,000 dilution. For color development, plates were washed five times with PBS-Tween and incubated for 20 min at room temperature with 2.2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) (Sigma) at 1 mg/ml in 0.1 M sodium citrate buffer (pH 4.0)-0.003% hydrogen peroxide. The reaction was stopped by adding 100 µl of 10% sodium dodecyl sulfate to each well. Plates were read on a Dynatech Microelisa Auto Reader MR580 (Dynatech Instruments, Inc.) at a wavelength of 405 nm. Positives were scored as those wells giving a reading of >0.300 in duplicate assays.

RESULTS AND DISCUSSION

Protection of animals immunized against *B.*

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anthracis is usually demonstrated by challenging with the Vollum strain, the proposed neotype culture of *B. anthracis* (24), or with one of its derivatives, i.e., Vollum 1B, M36, or V1b-189. It is apparent from the literature that protection against challenge with the Vollum strain or one of its derivatives can be achieved by using any one of several different cell-free preparations (3, 12, 15, 19, 31). However, Table 2 (experiment 1) lists, with Vollum and Vollum 1B, those strains of *B. anthracis* that killed 50% or more of the PA-immunized animals. The data confirmed the findings of Auerbach and Wright (1) and Ward et al. (29) that, although guinea pigs were immunized effectively against a Vollum challenge, they were not protected against challenge with some isolates of *B. anthracis.* The immunogen preparation used for guinea pigs by Auerbach and Wright (1) was a cell-free lyophilized antigen preparation of the Vollum R1-NP strain. We tested 4 of the 10 strains they used to challenge their guinea pigs (Vollum, M36, 107, and V770), two of which (107 and V770) were shown to kill immunized guinea pigs (1). However, we could not duplicate their results showing vaccine-resistance of these two isolates (107 and V770) in guinea pigs immunized with our commercial PA vaccine. This discrepancy might be explained by the different antigen preparations used for immunizations. Although we found 9 of 27 isolates tested to be resistant to immunization with the PA vaccine, we cannot say that this reflects the percentage or proportion of vaccine-resistant isolates found in nature. A larger number of isolates will have to be assayed before a percentage or proportion of vaccine-resistant isolates can be determined.

Vaccination of guinea pigs with Sterne strain spores appears to provide broad protection against i.m. challenge with various anthrax isolates (Table 2, experiment 2). Three graded, immunizing doses of spores were administered to immunize each guinea pig and, at the same time, to preclude the occasional death in guinea pigs resulting from a dose of $>10^6$ Sterne spores. A dose-response curve of the Sterne spore vaccine was obtained by injecting guinea pigs with 0.5 ml i.m. in a single dose or as two doses 14 days apart. The animals were then challenged i.m. with 2,500 spores of Vollum 1B 2 weeks after immunization (Table 3). The data indicates that excellent protection and antibody response to PA antigen can be achieved with two immunization doses of 10^6 Sterne spores. The dose of spores administered, the strain of avirulent spores used, and the presence of adjuvants (5, 9, 10, 18, 27) are all important factors influencing the ability of a spore vaccine to protect against challenge.

TABLE 2. Survival of guinea pigs after immunization with PA vaccine or Sterne spore vaccine and i.m. challenge with 1,000 spores of various *B. anthracis* isolates

Challenge isolate[a]	Vaccine efficacy (survivors/total)			
	Expt 1		Expt 2	
	Saline	PA vaccine	Saline	Sterne spore vaccine
Vollum	0/6[b]	6/6	ND[b]	ND
Vollum 1B	1/8	10/10	2/5	8/8
Ames	1/6	0/6	0/6	6/6
Buffalo	1/6	1/6	0/6	5/6
17T5	0/6	1/6	0/6	7/8
NH	0/6	3/9	0/6	8/8
SK31	0/6	3/6	0/6	6/6
ACB	0/6	1/6	0/6	6/6
SK61	0/6	2/6	0/6	6/6
SK162	1/6	2/6	0/6	6/6
VH	0/6	3/6	1/6	6/6

[a] Challenge dose, 10^6 spores i.m.
[b] ND, Not done.

TABLE 3. Survival and average reciprocal ELISA titer to PA antigen of guinea pigs immunized i.m. with 0.5 ml of a suspension of Sterne spore vaccine either as a single dose or as two doses 14 days apart.

No. of spores injected per dose[a]	Survivors/ total	ELISA titer[b]
Single dose		
10^4	5/10	14
10^5	5/10	29
10^6	7/10	260
Two doses		
10^4	4/9	36
10^5	8/10	230
10^6	9/10	5,600
Controls (none)	5/10	10

[a] Guinea pigs were challenged i.m. with 2,500 spores of Vollum 1B 14 days after immunization.
[b] Average reciprocal ELISA titer to PA antigen.

Antibody responses of guinea pigs immunized with PA vaccine or Sterne spore vaccine are shown in Table 4. Antibody titers demonstrated that PA was present and immunogenic in the PA vaccine. The titers against PA, LF, and EF antigens of sera obtained from guinea pigs immu nized with Sterne

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spore vaccine suggested that protection with the PA vaccine might be enhanced by addition of LF or EF toxin components to yield a similar antibody response. When such an experiment was performed (Table 5), protection against Vollum 1B challenge was observed whenever the PA toxin component was part of the vaccine. However, no component vaccine was capable of eliciting protection against a vaccine-resistant isolate (NH), even when antibody titers approached those of Sterne spore-vaccinated animals. Neither LF nor EF alone provide protection against either challenge strain. Evaluation of protection afforded by immunization against anthrax has been made by either survival tests or measurement of the serological titer to the antigen used as an immunogen. Our results indicate that, although a high ELISA titer was obtained after immunization, as demonstrated by immunization with Sterne strain spores or PA vaccine + LF, it did not reflect the level of expected protection. This was demonstrated after challenge with a vaccine-resistant isolate. Ward et al. (29), who used an antigen preparation very similar to ours, also recorded deaths of guinea pigs with high antibody titers which were challenged with a vaccine-resistant isolate.

TABLE 4. Average reciprocal ELISA titer to PA, LF, and EF antigens and percentage of animals responding after immunization with either PA or Sterne spore vaccine

Immunization	No. of Animals	Reciprocal ELISA titer[a]		
		PA	LF	EF
PA vaccine	74	10,000 (100)	10 (49)	5 (26)
Sterne spore vaccine	35	3,000 (100)	1,000 (100)	100 (97)

[a] Parentheses indicate percent of animals having the indicated titer.

The apparently greater virulence among the vaccine-resistant isolates might be reflected by their 50% lethal dose (LD_{50}). Results from only two of the vaccine-resistant isolates, NH and Ames, showed that they have LD_{50}s eightfold less than that of Vollum 1B (Vollum 1B, 395 CFU; NH, 51 CFU; Ames, 49 CFU). However, these two isolates have LD_{50}s approximately equal to that of the Vollum isolate (33 CFU). Although there was an apparent decrease in virulence of our Vollum 1B strain compared to previously reported LD_{50}s for this strain (subcutaneous LD_{50}, <10 spores; 21), it did kill our control guinea pigs. The Vollum 1B strain also has been studied recently for quantitation of lethal toxin activity (7, 20) and plasmid isolation (11, 17). Comparison of the vaccine-resistant isolates with the Vollum cultures suggested that it is not the difference in hte LD_{50}s of the isolates that determines vaccine resistance but some other factor(s) (1, 29).

TABLE 5. Protective efficacy of PA, LF, and EF combinations measured by average reciprocal ELISA titer to PA, LF, or EF antigens or by survival against i.m. challenge with 1,000 spores of Vollum 1B or NH isolates

Vaccine	Antibody response (reciprocal ELISA titer)			Survivors/total after challenge with:	
	PA	LF	EF	Vollum 1B	NH
Sterne	7,800	480	10	7/7	12/12
PA	14,200	25	4	5/6	4/12
LF	10	2,500	4	1/6	1/6
EF	250	10	2,500	0/6	0/6
PA + LF	12,600	1,000	10	6/6	2/6
PA + EF	10,000	18	750	6/6	1/6
None	0	0	0	1/6	0/6

This study, which compares protection of guinea pigs after immunization with either the PA vaccine or spore vaccine against challenge by various *B. anthracis* strains, indicates the superior protective efficacy of the latter. Immunization with cell-free preparations which contained components of the anthrax toxin did not provide an adequate protective response against some challenge isolates of *B. anthracis.* The fact that the spore vaccine provided protection against all isolates tested suggests that other antigens may play a role in active immunity. Since this vaccine is a live immunogen, safety factors must be considered before its use. In evaluations of anthrax vaccines, it is important to test protection by using a wide variety of challenge isolates.

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