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The safety and protective efficacy of the Sterne vaccine strain of Bacillus anthracis and of a recombinant Bacillus subtilis strain were investigated in mice. Strains of mice which vary in their natural resistance to killing by the Sterne strain were used. Vaccination with Sterne spores protected Sterne-resistant CBA/J mice against challenge with a fully virulent strain of B. anthracis, but only at vaccine doses within a magnitude of the 50% lethal dose (LD_{50}). The Sterne-susceptible A/J mice were not protected. Bacillus subtilis

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Protective efficacy and safety of live anthrax vaccines for mice

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Short title: Live anthrax vaccines

In conducting the research described in this report, we adhered to the *Guide for the Care and Use of Laboratory Animals*, as promulgated by the Committee on Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the author do not purport to reflect the positions of the Department of the Army or the Department of Defense.

Abstract

The safety and protective efficacy of the Sterne vaccine strain of *Bacillus anthracis* and of a recombinant *Bacillus subtilis* strain were investigated in mice. Strains of mice which vary in their natural resistance to killing by the Sterne strain were used. Vaccination with Sterne spores protected Sterne-resistant CBA/J mice against challenge with a fully virulent strain of *B. anthracis*, but only at vaccine doses within a magnitude of the 50% lethal dose (LD_{50}). The Sterne-susceptible A/J mice were not protected. *Bacillus subtilis* recombinant strain PA2, which produces the protective antigen component of anthrax toxin, protected CBA/J but not A/J mice. Both strains of mice developed high antibody titers to protective antigen. BALB/cJ and CBA/J mice were similarly resistant to lethal Sterne infection, but BALB/cJ mice were more difficult to protect by immunization with either live vaccine. The inbred mouse model for anthrax is recommended for testing the efficacy and safety of new vaccines and characterizing the mechanisms of immunity to anthrax.

Key words: anthrax; Sterne; *Bacillus subtilis* recombinant; vaccination; mice

Introduction

Vaccines designed to protect humans and animals against lethal infection by *Bacillus anthracis* have been available for more than 50 years. Livestock have been protected against anthrax by live-spore vaccines since 1935.[1-4] The most commonly used veterinary vaccine consists of spores of the Sterne strain of *Bacillus anthracis*.[1] This strain lacks the capsule synthesized by virulent isolates, but produces the other factor required for virulence, a three-component exotoxin.[5,6] This tripartite toxin consists of the proteins: protective antigen (PA), lethal factor (LF), and edema factor (EF).[5,6] The vaccines currently licensed for human use in the United States and United Kingdom are partially purified, cell-free, culture filtrates composed primarily of PA (U.S.) or PA and LF (U.K.). The attributes of these vaccines have been reviewed in detail elsewhere.[5-10]

Vaccines that protect animals against toxin or spore challenge must contain or synthesize PA, either alone or in combination with LF or EF.[8-10] Recently the protective efficacies of the Sterne spore vaccine and of cell-free toxin component preparations were compared in guinea pigs. Immunization with the Sterne vaccine protected the animals to a greater extent and for a longer time against intramuscular or

aerosol challenge with virulent *B. anthracis* spores than did immunization with the toxin protein products[8,9] (B. Ivins, personal communication). In addition, the live vaccines protected the animals against numerous challenge strains, even though they often induced lower antibody titers to PA than did the cell-free preparations.[8,9] These data suggest that antigens in addition to PA, or arrangements of PA epitopes that are different from those in the present human vaccines, play an important role in active immunity. A drawback of capsule-negative vaccine strains is their virulence for some animals at doses required to immunize.[9,19]

Recently, aspects of the molecular biology of toxin and capsule production have been elucidated, and improved methods for purification and assay of toxin have been developed.[5,6,11-18] These findings are stimulating research to develop toxin component preparations and live vaccines that are more broadly effective and less reactogenic than current vaccines.

Toward this goal, the gene which encodes PA was recently transferred into *B. subtilis*.[13] Preliminary experiments indicated that the live recombinant strains were avirulent in guinea pigs, induced significant anti-PA titers, and protected against challenge with spores of a virulent strain of *B. anthracis*.[13] The efficacy of this and other candidate vaccines against diverse strains of *B. anthracis* needs to be investigated in well-characterized animal systems.

A mouse model for anthrax was recently developed and is

being used to investigate the pathogenesis of the disease and the genetics of susceptibility to *B. anthracis* infection[19] (S. Welkos, manuscript submitted). Inbred mouse strains differ in their susceptibilities to lethal infection by both encapsulated and nonencapsulated, toxigenic strains of *B. anthracis*. The purpose of this study was to characterize the safety and protective efficacy for mice of the Sterne spore and recombinant *B. subtilis* anthrax vaccine strains.

Results and discussion

The protective efficacy of the Sterne spore vaccine was studied in strains of mice differing in their natural resistance to lethal Sterne infection. As shown in Table 1, immunization of the mice with Sterne spores induced partial (BALB/cJ) or complete (CBA/J) protection in Sterne-resistant strains against lethal challenge with Vollum-1B. Protection only occurred after immunization with high doses of Sterne spores, from 0.1 to 1 LD_{50}.[19] BALB/cJ mice required doses of more than 10^7 spores for significant protection (>60%). Likewise, CBA/J mice were only protected when inoculated with at least 2 x 10^6 spores, and multiple doses of the latter were required. Inoculation with a dose of 2 x 10^7 spores killed a third of the animals. The survivors were completely protected against challenge with Vollum-1B. The mortality after Sterne immunization probably resulted from replication and toxin production by the vaccine

strain. These events are detectable only after high doses in the resistant mouse strains (S. Welkos, manuscript submitted).

The Sterne-susceptible A/J strain was not protected against challenge with Vollum-1B by immunization with 2 x 10^2 or 3 x 10^3 Sterne spores. The latter dose approximated the Sterne LD_{50} for A/J[19] (S. Welkos, manuscript submitted), as confirmed by death of half of the vaccinated A/J mice.

Vaccines that protect against anthrax induce serum antibodies to the protein components of anthrax toxin, especially protective antigen.[5-10] All Sterne-vaccinated groups with 88 - 100% survival against a Vollum-1B challenge (one BALB/cJ and two CBA/J groups) had mean reciprocal anti-PA titers of at least 1334 (Table 1). Groups with few or no survivors had mean titers ≤133. However, the data suggest that antigens in addition to intact toxin components may contribute to immunity, as several immunized groups were partially protected while having low titers of anti-PA antibody. For example, CBA/J mice given one dose of 2 x 10^6 Sterne lacked a detectable anti-PA titer but had an increased time-to-death (TTD) after challenge. BALB/cJ mice vaccinated three times with 5 x 10^6 spores had very low anti-PA titers but a 50% survival rate.

The pathogenesis of lethal Sterne infection in the susceptible A/J strain resembles that of infection by *B. anthracis* strain Vollum-1B (S. Welkos, manuscript submitted). The organisms germinated, multiplied, and produced toxin at the site of inoculation. Subsequently, they invaded systemically,

replicated, and produced toxin. The production of toxin by Sterne is probably important in the protection induced against anthrax, as demonstrated by the association between complete protection and high anti-PA titers in the relatively Sterne-resistant CBA/J mice. The mortality observed in some vaccinated animals might result from the replication and release of lethal quantities of toxin by the bacteria. The LD_{50} of Sterne for A/J mice may have been too low to allow replication and toxin production at levels sufficient for immunization.

Although Sterne was an ineffective live vaccine for Sterne-susceptible mice, it was effective within a restricted dose range for resistant strains. Thus, the protection provided by Sterne spores for these resistant mice was similar to that observed in other laboratory animals such as guinea pigs and rabbits.[8-10,21-23] In earlier studies, outbred mice were not protected against anthrax by live vaccines or filtered culture preparations, and the investigators concluded that these animals could not be immunized.[21-23] However, this conclusion is questionable, given the uncharacterized antigens and immunization schedules used. For example, the acellular vaccines were crude filtrate preparations of bacterial cultures grown in rich media or body fluids; the PA content and purity were unknown.[21,23] In one study, the spore vaccine was administered only once.[22] The interval between vaccination and challenge was usually shorter (1 - 2 weeks)[21-23] than the 2 to several weeks that several workers have used recently (this study).[7-10] The serological responses

were not reported, and thus the level of specific immunity present at time of challenge was unknown. Finally, the susceptibilities of the mouse strains to lethal infection by the live vaccine were not characterized.

In order to analyze the role of PA in immunity against anthrax, the gene encoding PA was initially cloned into *Escherichia coli* and then transferred into *B. subtilis*.[13,18] Both recombinant host strains produced biologically active PA, and the *B. subtilis* strain induced protective immunity in lethally challenged guinea pigs. Preliminary vaccination with the *B. subtilis* recombinant of the three strains of inbred mice described above showed that the subcutaneous (sc) route yielded higher anti-PA titers than the intraperitoneal (ip) route. Also, at least 10^6 bacilli were required to obtain a detectable titer. Doses of 10^8 CFU of the PA-producing *B. subtilis* strain PA2 were nontoxic to the mice; equivalent doses of Sterne represent approximately 10^5 LD_{50}s for A/J mice.

Strain PA2 elicited uniformly very high anti-PA titers and completely protected the CBA/J mice (Table 2). In contrast, *B. subtilis* control strain BST1 and *B. anthracis* strain ΔSterne-1, which is cured of the toxin-encoding plasmid pXO1, failed to protect (Table 2 and data not shown). These data demonstrate the protective efficacy of protective antigen alone in the CBA/J mice. The recombinant *B. subtilis* strain failed to protect A/J mice, and the causes of strain variation in the degree of protection afforded by live vaccines require further

investigation. Although the A/J mice immunized with PA2 produced high titers of an anti-PA antibody detectable by ELISA, critical humoral responses may have been deficient. For example, these animals are deficient in complement component 5 (C5), and C5 has been reported to be involved in induction of specific antibody.[24-27] Perhaps A/J mice are unable to produce antibodies which can neutralize toxin *in vivo*. Alternatively, serum antibody response to PA might be less important than other acquired immune responses, such as cell-mediated immunity, which might be defective in A/J mice.

The degree of protection induced in BALB/cJ mice by immunization with Sterne and with *B. subtilis* PA2 was less than that obtained in CBA/J mice. The basis for this difference in immune protection is unknown. These two strains of mice are both innately more resistant than A/J to lethal infection by the fully virulent Vollum-1B strain.[19] Also, they are about equally sensitive to toxin in the *in vitro* cytotoxicity assay (A. Friedlander, personal communication).[11] Although I did not study growth *in vivo* of the *B. subtilis* recombinant, the BALB/cJ mice were very refractory to proliferation of Sterne (S. Welkos, manuscript submitted). If replication of the live vaccine is required to induce immunity, host resistance to growth might slow the rate at which acquired immunity develops. A similar mechanism to explain strain differences in acquisition of immunity was recently described for mycobacterial infections in mice.[28] Such differences might provide markers useful in

elucidating the critical antigens and host responses involved in active immunity against anthrax.

My studies on the protective efficacy for inbred mice of live anthrax vaccines can be summarized as follows. (1) Inbred mouse strains varied in their abilities to be immunized with live vaccines against anthrax. (2) Sterne spores protected relatively Sterne-resistant mice against challenge with the Vollum-1B strain of *B. anthracis*, although the vaccine was only effective at doses within a magnitude of the LD_{50}. The Sterne-susceptible A/J mice were not protected. (3) The PA-producing, *B. subtilis* recombinant induced high titers of anti-PA antibody in the three strains of mice tested, but only fully protected the CBA/J strain against Vollum-1B. (4) Protective antigen has a major role in protection against anthrax and may be sufficient alone (Table 2).[10,13] However, additional antigens present in live *B. anthracis* vaccines might contribute to immunity (this paper).[8,9] Inbred mice provide useful models for testing vaccine safety and efficacy and for characterizing the mechanisms required for an effective host response to *B. anthracis*.

Materials and methods

Mice

Female mice were purchased from Jackson Laboratories, Bar Harbor, ME and were used when 6 to 8 weeks old.

Bacterial strains

Strains of *B. anthracis* were obtained from the culture collection of the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), Frederick, MD. These strains included a toxigenic, encapsulated strain (Vollum-1B), a toxigenic, nonencapsulated strain (Sterne), and a strain deficient in both capsule and toxin production (ΔSterne-1). The ΔSterne-1 strain is a derivative of Sterne that was cured of the toxin-encoding plasmid pX01 by growth at elevated temperature.[10] *Bacillus subtilis* BST1 was derived by transformation of the asporogenic strain *B. subtilis* 1S53 with the plasmid vector pUB110 as described.[13] The PA component of anthrax toxin was cloned into this plasmid to generate *B. subtilis* strain PA2.[13,18] This strain produces full-length, biologically active PA.[13]

Immunization and challenge studies

Spores for immunization or challenge were prepared and frozen as previously described.[19] Prior to inoculation, spores were thawed and diluted in 0.4% Na_2HPO_4, pH 7.0, with 0.2% gelatin (PG) and the dilutions plated on trypticase soy agar plates for viable counts. Vegetative bacteria for immunization were prepared as previously described.[13] Mice (5 to 12 per group) were immunized sc, except where indicated, with 0.2 ml volumes of diluent, spores, or vegetative bacteria. The mice were vaccinated with either one or three equal doses given at 2-week intervals. Two

days prior to challenge, mice were bled from the retro-orbital plexus to obtain serum samples. The mice were challenged 4 weeks after the last vaccine dose with *B. anthracis* strain Vollum-1B. A subcutaneous dose of spores equal to 10 - 20 times the LD_{50} was given. The number of deaths in each group and the TTD, in days, of each mouse were recorded. The harmonic mean TTDs were calculated.[20,21]

Serological assay

Antibody titers to PA in postimmunization sera from mice were determined by enzyme-linked immunosorbent assay (ELISA). Sera from four individual mice per immunization group were assayed, and the samples were tested in duplicate. The microtiter ELISA method of Little and Knudson[8] was used with the following modification. Mouse antibodies were detected by adding 100 μl of rabbit antiserum to mouse immunoglobulins (IgG, IgA, IgM - Behring Diagnostics, La Jolla, CA) at a final dilution of 1/400. The plates were incubated for 2 hours at 37 °C prior to addition of horseradish peroxidase-protein A conjugate and substrate. For each mouse group, the geometric mean titer and standard deviation were calculated from the individual reciprocal titers of antibody to PA.

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Table 1 Lethality and protective efficacy of the Sterne spore vaccine for mice

Strain	Immunization: Vaccine (CFU)[a]	No. doses	Vaccine Mortality, %	Anti-PA titer[b]	Response to Challenge: Survival[c]	TTD[d]
A/J	diluent	3	0	<10	0/6 (0)	2.8
	Sterne:					
	3×10^2	1	0	32 (0)	0/8 (0)	2.3
	3×10^2	3	0	<10	0/8 (0)	2.6
	3×10^3	1	50	56(15.5)	0/4 (0)	2.3
CBA/J	diluent	3	0	<10	1/7 (14)	6.4
	Sterne:					
	6×10^5	1	0	nd[e]	0/2 (0)	2.5
	2×10^6	1	0	<10	4/12 (33)	10.1
	2×10^6	3	0	1,334(20.6)	12/12(100)	-
	2×10^7	1	33	10,000(2.6)	8/8 (100)	-
BALB/cJ	diluent	3	0	<10	0/6 (0)	5.0
	Sterne:					
	7.6×10^6	1	0	133 (7.1)	2/8 (25)	7.1
	5.2×10^6	3	0	18 (1.9)	4/8 (50)	7.3
	1.2×10^7	3	0	10,000 (2.6)	6/10(60)	16.8
	7.6×10^7	1	0	3,162 (0)	7/8 (88)	-[f]

[a] Control mice were vaccinated sc with phosphate-gelatin diluent and the remaining mice were immunized at 2-week intervals with live spores of *B. anthracis* strain Sterne. The inoculum size is given in CFU.

[b] Serum anti-PA antibody titers were determined by ELISA on serum collected 2 days prior to challenge from four mice/group. The titers are shown as the geometric mean reciprocal (SD in parentheses).

[c] Mice which survived vaccination were challenged 4 weeks after the last vaccine dose with 10-20 LD_{50} *B. anthracis* strain Vollum-1B. Data shown as no. survivors/total no. challenged with the percentage survival in parentheses.

[d] TTD values are given as harmonic means.

[e] nd - not done

[f] One mouse died 4 days after challenge.

Table 2 Protection against *B. anthracis* after immunization with PA$^+$ recombinant *B. subtilis*

Mouse Strain	Immunization: Vaccine, No. CFU[a]	Anti-PA titer[b]	Response to Challenge: Survival[c]	TTD[d]
A/J	PBS	<10	0/10 (0)	2.1
	BST1 1×10^6	<10	0/7 (0)	2.4
	1×10^8	24(3)	0/10 (0)	2.9
	PA2 1×10^6	13,335(4.2)	0/10 (0)	2.4
	1×10^8	316,230(0.0)	0/12 (0)	3.3
CBA/J	PBS	<10	0/7 (0)	4.2
	BST1 1×10^8	<10	0/9 (0)	4.8
	PA2 1×10^8	23,714(1.8)	9/9(100)	-
BALB/cJ	PBS	<10	0/11 (0)	5.1
	BST1 1×10^8	10	0/7 (0)	5.1
	PA2 $10^7 - 10^8$	5,623(1.9)	0/8 (0)	5.3
	1×10^8	13,335(1.8)	3/8 (37.5)	9.0

[a]Mice were vaccinated three times at 2-week intervals with either phosphate-buffered saline (controls) or preparations of live bacilli. The latter included the PA-producing recombinant *B. subtilis* strain PA2 and control strain BST1 (contains puB110 vector alone).

[b]See footnote b of Table 1.

[c]Vaccinated mice were challenged with the Vollum-1B strain of *B. anthracis* as described in footnote c of Table 1.

[d]Same as footnote d of Table 1.