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Comparative safety and efficacy against *Bacillus anthracis* of protective antigen and live vaccines in mice

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The efficacy and mechanisms of protection of two live vaccines and of a protective antigen (PA) vaccine against *Bacillus anthracis* were studied in inbred mice. Mice that differed in their natural resistance to killing by Sterne, a non-encapsulated, toxigenic vaccine strain of *B. anthracis*, were used. Vaccination with live Sterne spores protected Sterne-resistant mice against challenge with the virulent Vollum 1B (V1B) strain of *B. anthracis*, but only at doses of Sterne $\geqslant 0.1$ 50% lethal dose. The live *B. subtilis* recombinant strain PA2, which produces the PA component of anthrax toxin, fully protected (CBA/J) or partially protected (BALB/cJ) Sterne-resistant mice against V1B. Neither immunization with the cell-free PA vaccine nor passive administration of anti-PA antiserum protected Sterne-resistant mice against V1B. Sterne-susceptible A/J mice were not protected against V1B by either live vaccine or by the PA vaccine. However, immunization with strain PA2 induced anti-PA antibody and protected A/J mice against Sterne. A/J mice passively treated with antitoxin antibodies also survived Sterne, and survivors were then partially protected against V1B. Thus, immunity to Sterne correlated with an effective anti-PA response. Immunity to fully virulent V1B also required PA but may involve mechanisms in addition to humoral immunity.

Key words: Anthrax; Sterne; *Bacillus subtilis* recombinant; protective antigen; immunization; 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 two toxins

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.

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required for virulence.[5,6] The lethal toxin consists of the proteins protective antigen (PA) and lethal factor (LF), while the edema toxin is composed of PA together with a third protein, 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 vaccine licensed in the United States is produced by the Michigan Department of Public Health and will be designated MDPH-PA. 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.[9,11]

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.[5,6,12–19] 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.*[14] 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.*[14] The efficacy of this and other candidate vaccines against diverse strains of *B. anthracis* needs to be investigated in well-characterized animal systems.

As reviewed elsewhere, animal species differ in their natural resistance to anthrax, and also in their ability to be immunized against the disease.[7,11,20,21] 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.[11,21] Inbred mouse strains differ in their susceptibilities to lethal infection by both encapsulated and nonencapsulated, toxigenic strains of *B. anthracis.*[11,21,22] 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 and of MDPH-PA.

Results and Discussion

In this study, we compared the protective efficacy and safety in inbred mice of currently licensed human and veterinary vaccines and of a new prototype live vaccine.[14] We used three strains of mice that differed in innate susceptibility to lethal anthrax infection to examine the influence of host factors on protective efficacy. As shown in Table 1, all strains of mice were susceptible to low doses of the fully virulent Vollum 1B (V1B) challenge strain of *B. anthracis*, although CBA/J and BALB/cJ had a significantly longer time to death than did A/J mice.[11] In contrast, there was a $\geqslant 10^3$-fold difference in susceptibility to lethal infection by Sterne, the live vaccine strain of *B. anthracis*.

Table 1 Susceptibility of inbred mice to vaccine and challenge strains of *B. anthracis* and *B. subtilis*

Mouse strain	LD_{50} (CFU)[a]		
	Vollum 1B[b]	Sterne[c]	*B. subtilis* PA2[d]
A/J	5.5	2.5×10^3	$>10^8$
CBA/J	25	2×10^7	$>10^8$
BALB/cJ	6.6	7×10^7	$>10^8$

[a] Data from Welkos *et al.*[11] and Welkos and Friedlander.[21]
[b] Virulent strain of *B. anthracis*, containing toxin-encoding (pX01) and capsule-encoding (pX02) plasmids.
[c] Nonencapsulated vaccine strain of *B. anthracis* (contains pX01 only).
[d] Recombinant strain of *B. subtilis* that contains the PA gene from pX01 cloned into pUB110.[14]

The PA-producing, recombinant *B. subtilis* strain PA2 was avirulent in all three strains of mice.

Mice were immunized three times at two-week intervals with MDPH-PA, Sterne spores, or PA2 bacilli. Animals that survived immunization were challenged with 10–20 LD_{50} *B. anthracis* strain V1B 4 weeks after the last vaccine dose.

As shown in Table 2, immunization of the Sterne-resistant mouse strains with Sterne induced complete (CBA/J) or partial (BALB/cJ) protection against lethal challenge with V1B. Protection only occurred after immunization with high doses of Sterne spores, from 0.1 to 1 50% lethal dose (LD_{50}).[11] BALB/cJ mice required doses of more

Table 2 Lethality and protective efficacy of the Sterne spore vaccine for mice

Strain	Immunization				Response to challenge		
	Vaccine (CFU)[a]	No. doses	Vaccine mortality (%)	Anti-PA titer[b]	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	1334 (20.6)	12/12	(100)	—
	2×10^7	1	33	10000 (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	10000 (2.6)	6/10	(60)	16.8
	7.6×10^7	1	0	3162 (0)	7/8	(88)	—[f]

[a] Control mice were vaccinated sc with phosphate-gelatin diluent and the remaining mice were immunized with the spore vaccine as described in the text. 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 parenthesis).
[c] Surviving mice were challenged with *B. anthracis* strain Vollum 1B as described in the text. 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.

than 10^7 spores for significant protection (> 60%). Likewise, CBA/J mice were only protected when inoculated with at least 2×10^6 spores, and multiple doses of the latter were required. Inoculation with a dose of 2×10^7 spores killed a third of the animals in this experiment. The survivors were completely protected against challenge with V1B. 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 resistant mouse strains.[21]

The Sterne-susceptible A/J strain was not protected against challenge with V1B by immunization with 2×10^2 or 3×10^3 Sterne spores. The latter dose approximates the LD_{50} (Table 1), and, as expected, half of the vaccinated A/J mice died.

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*.[14,19] Both recombinant host strains produced biologically active PA, and the *B. subtilis* strain induced protective immunity in lethally challenged guinea pigs. A dose of at least 10^6 bacilli was required to obtain a detectable anti-PA titer in mice. Doses of 10^8 CFU of the PA-producing *B. subtilis* strain PA2 were nontoxic to mice (Table 1).

Strain PA2 elicited uniformly very high anti-PA titers in A/J mice but failed to protect them against challenge with V1B. However, it completely protected the CBA/J mice and partially protected BALB/cJ (Table 3). In contrast, *B. subtilis* control strain BST1, which is devoid of the PA gene, and *B. anthracis* strain ΔSterne-1, which is cured of the toxin-encoding plasmid pXO1, failed to protect (Table 3 and data not shown). These data demonstrate the protective efficacy of bacilli synthesizing the PA antigen alone in the CBA/J mice.

The mouse strains varied in the extent of protection afforded by live vaccines against fully virulent strains of *B. anthracis*. The protection induced in BALB/cJ mice by immunization with Sterne and with *B. subtilis* PA2 was less than that obtained in

Table 3 Protection against *B. anthracis* after immunization with a PA-producing recombinant *B. subtilis*

Mouse strain	Immunization			Response to challenge		
	Vaccine	No. CFU[a]	Anti-PA titer[b]	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	13335 (4.2)	0/10	(0)	2.4
		1×10^8	316230 (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	23714 (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	5623 (1.9)	0/8	(0)	5.3
		1×10^8	13335 (1.8)	3/8	(37.5)	9.0

[a] Mice were vaccinated with either PBS (controls) or preparations of live bacilli (see text). The latter included the PA-producing recombinant *B. subtilis* strain PA2 and control strain BST1 (contains puB110 vector alone).

[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] Surviving mice were challenged with *B. anthracis* strain Vollum 1B as described in the text. Data shown as no. survivors/total no. challenged, with the percentage survival in parentheses.

[d] TTD values are given as harmonic means.

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 V1B strain.[11,21] Also, they were about equally sensitive to toxin and the *in vitro* cytotoxicity assay (A. Friedlander, unpublished data).[12] Although we did not study growth *in vivo* of the *B. subtilis* recombinant, the BALB/cJ mice were very refractory to proliferation of Sterne.[21] 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.[24]

Vaccines that protect against anthrax induce serum antibodies to the components of anthrax toxin, especially PA; however, the role of these antibodies in the protection afforded by PA is not clear.[5–10] All Sterne-vaccinated groups with 88–100% survival against a V1B challenge (one BALB/cJ and two CBA/J groups) had mean reciprocal anti-PA titers of at least 1334 (Table 2). Groups with few or no survivors had mean titers $\leqslant 133$. However, several immunized groups were partially protected while having low titers of anti-PA antibody. For example, CBA/J mice given one dose of 2×10^6 Sterne lacked a detectable anti-PA titer but had an increased time-to-death (TTD) after challenge. BALB/cJ mice vaccinated three times with 5×10^6 spores had very low anti-PA titers but a 50% survival rate. To assess further the role of PA and of anti-PA antibodies in protection, mice were actively immunized with MDPH–PA or passively treated with anti-PA antibodies. MDPH–PA, a cell-free vaccine composed primarily of PA, contains aluminum hydroxide, an adjuvant that appears to increase humoral immunity with little or no increase in cell-mediated immunity.[23] Immunization of guinea pigs with MDPH–PA protects them against challenge with some strains of virulent *B. anthracis*, including Vollum 1B.[7–9] MDPH–PA failed to protect A/J or CBA/J mice against Vollum 1B, and only slightly extended the time to death (TTD), despite induction of very high titers of anti-PA antibody (Table 4). The anti-PA titer induced by the non-protective MDPH–PA in CBA/J mice was, in fact, higher than that induced by the PA2 vaccine, which was protective (see Tables 3 and 4). Thus, the titer of anti-PA antibody measured by ELISA did not correlate with protection.

In another experiment, antisera were collected from MDPH–PA- and PA2-immunized mice, and passively transferred to CBA/J mice before or after absorption to remove anti-PA antibodies. None of the antisera from A/J or CBA/J mice protected or

Table 4 Protective efficacy of MDPH–PA for mice

	Immunization		Response to challenge	
Strain	Vaccine[a]	Anti-PA titer[b]	Survival[c]	TTD[d]
A/J	PBS	< 10	0/10 (0)	3.1
	MDPH–PA	758578 (1.8)	0/10 (0)	4.2
CBA/J	PBS	< 10	0/5 (0)	4.5
	MDPH–PA	100000 (2.1)	1/10 (10)	6.2

[a] Mice were vaccinated sc three times with PBS or with 0.2 ml of MDPH–PA at 2-week intervals.

[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] Surviving mice were challenged with *B. anthracis* strain Vollum 1B as described in the text. Data shown as no. survivors/total no. challenged, with the percentage survival in parentheses.

[d] TTD values are given as harmonic means.

extended the TTD of CBA/J mice challenged with V1B (Table 5). Sera from the passively immunized mice were not assayed for anti-PA titers; however, the titers in the unabsorbed inocula were comparable to those in monoclonal anti-PA preparations, which yielded high titers of serum anti-PA in passively immunized A/J mice (described below). These results suggest three possibilities: (1) that humoral immunity to PA may not be the only mechanism stimulated by immunization with live vaccines in mice, and perhaps additional antigens or other mechanisms, i.e. cell-mediated immunity, contribute to protection; (2) that anti-PA antibody is the main mechanism of protection, but immunity was overwhelmed by the challenge dose (10 LD_{50} s); or (3) that anti-PA antibodies produced in mice cannot neutralize toxin.

To test the third possiblity, antisera from A/J and CBA/J mice immunized with MDPH–PA or PA2 were tested for their ability to inhibit *in vitro* killing of macrophages by toxin.[12] As shown in Fig. 1, all four immune sera were able to neutralize the macrophage cytotoxicity of PA and LF. Thus, PA induced neutralizing antibodies in mice, but the role of humoral immunity in *in vivo* protection elicited by PA requires further study.

A/J mice could not be protected against V1B by any of the vaccines. Although the mice immunized with PA2 and MDPH–PA produced high titers of anti-PA antibody, critical humoral responses may have been deficient. Anti-PA antisera from A/J mice neutralized toxin *in vitro* (Fig. 1). However, these animals are deficient in complement component C5 (C5),[25,26] and normal complement levels might be required for antitoxic antibodies to be protective *in vivo*. To test this, sera obtained from a C5-positive strain of mice, B10.D2/nSnJ, or from the congenic C5-deficient strain, B10.D2/oSnJ, were

Table 5 Lack of protection of CBA/J mice by passive transfer of serum

Antiserum preparations			Passive serum treatments		Challenge	
Preparation[a]	Group	Anti-PA titer[b]	Dilution[c]	Total protein (mg)[d]	No. dead total[e]	TTD[f]
A/J anti-MDPH:						
unabsorbed	1	1 000 000	1/2.5	16.8	6/6	5.0
absorbed	2	1000	None	14.0	5/5	4.3
CBA/J anti-MDPH:						
unabsorbed	3	1 000 000	1/3	18	6/6	3.9
absorbed	4	316	None	20	5/5	5.3
A/J anti-PA2:						
unabsorbed	5	1 000 000	1/2.5	21.6	6/6	3.6
absorbed	6	31.6	None	17	5/5	3.3
CBA/J anti-PA2						
unabsorbed	7	100 000	1/2	25	6/6	3.6
absorbed	—	not done	—	—	—	—
PBS	8	0	—	0	5/5	4.8

[a] Sera were collected from A/J or CBA/J mice that had been immunized with three biweekly doses of MDPH–PA vaccine or *B. subtilis* strain PA2. The anti-PA antibody titer was determined for each antiserum pool. Half of each pool was absorbed on a PA affinity column, and removal of anti-PA antibody from the eluted material was confirmed by ELISA assay.

[b] Geometric mean of the reciprocal titer.

[c] Serum preparations for passive transfer to mice were diluted in PBS (or used undiluted) to yield comparable doses of total protein.

[d] Serum treatments were divided into two, 0.5-ml doses given intraperitoneally as described in the text. The total protein content (in mg) is shown.

[e] Mice (five–six/group) were challenged sc with 10 LD_{50} doses of Vollum 1B.

[f] TTD-harmonic mean.

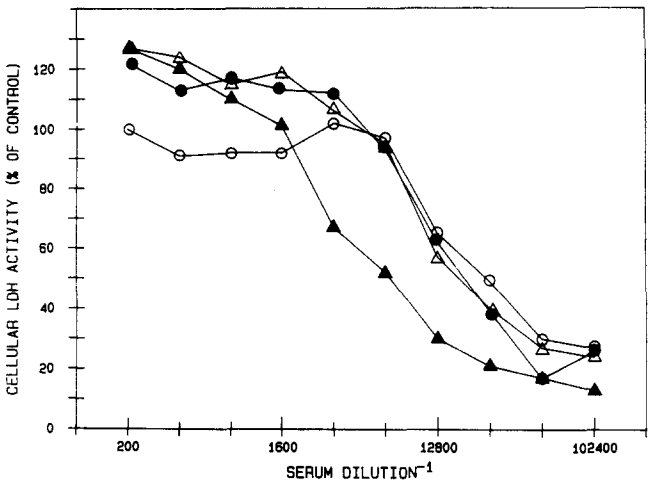

Fig. 1. PA-neutralizing titers of sera from mice immunized with MDPH–PA or PA2. Sera were pooled from A/J mice immunized with MDPH–PA (○) or PA2 (●) and from CBA/J mice immunized with MDPH–PA (△) or PA2 (▲), diluted, and pre-incubated with PA for 6 h at 4°C. The mixture was then added to macrophage cultures in the presence of excess LF and cytotoxicity determined as the release of cellular LDH activity.[12] PA and LF in the absence of antibody reduced cellular LDH activity to 28% of that in controls incubated without toxin.

administered passively to both unimmunized and PA2-immunized A/J mice. The normal serum failed to protect either group of mice against lethal V1B (Table 6). The complement deficiency of A/J mice does not appear to be directly responsible for the failure to express acquired immunity in these mice. The lack of protection against Vollum 1B induced by immunization with strain PA2 or with MDPH–PA (Table 4) may be due to an overwhelming challenge dose (as discussed above for CBA/J mice). Alternatively, serum antibody response to PA might be less important than acquired cell-mediated immunity, which might be defective in A/J mice.

In contrast to the results of Vollum 1B challenge, both passive treatment with normal serum alone and immunization with PA2 protected A/J mice against Sterne infection (Table 6). The protective effects of normal serum or PA2 immunization afforded A/J mice against Sterne (Table 6) are probably due to separate mechanisms. The presence in mice of normal levels of C5, a leukocyte chemoattractant,[27,28] correlated with the ability to mobilize a large inflammatory response, and to clear Sterne from the site of inoculation in unimmunized mice (S. Welkos *et al.*, manuscript in preparation).

On the other hand, protection of A/J mice against Sterne by active PA2 immunization could be due to stimulation of anti-PA antibody. A/J and other C5-deficient strains of mice are unusually susceptible to killing by Sterne spores (Tables 1 and 2).[11,21] The pathogenesis of a lethal Sterne infection in A/J mice resembles that of infection by strain V1B, with local replication, invasion, and toxemia.[21] *In vivo* production of toxin by Sterne is important in the protection induced against anthrax in Sterne-resistant mice (Tables 2 and 3) and other animals.[8–10] The mortality observed in some vaccinated animals probably results from the replication and release of lethal quantities of toxin by the bacteria. The LD_{50} of Sterne for A/J mice may be too low to allow replication and production of toxin (and possibly other antigens) at levels sufficient for immunization.

To test this hypothesis, A/J mice were treated with monoclonal antibodies (MAbs)

Table 6 Protection of A/J mice against lethal *B. anthracis* infection by active immunization with PA2 and passive complement administration

Immunization			Challenge[d]			
			Survival		TTD	
Vaccine[a]	Anti-PA titer[b]	Serum treatment[c]	Sterne	Vollum 1B	Sterne	Vollum 1B
PA2	100000 (2.1)	C5-positive	nd[e]	0/9	nd	4.0
		C5-negative	nd	0/6	nd	3.8
		PBS	8/8	0/9	—	3.9
PBS	< 10	C5-positive	4/6[f]	0/9	20.6	4.1
		C5-negative	0/5[f]	nd	3.6	nd
		PBS	1/9[f]	0/5	3.4	3.5

[a] Mice were immunized with *B. subtilis* PA2 (1×10^8 CFU) or PBS four times at 2 week intervals.
[b] Serum anti-PA titers were determined as described in text and shown as the geometric mean reciprocal (SD).
[c] Mice were given three 0.5-ml intraperitoneal doses of PBS or of undiluted sera from either C5-negative or -positive mice, as described in the text. The doses were administered 24 h before, 1 h before, and 24 h after sc challenge.
[d] Mice were challenged 4 weeks after the last vaccine dose with 10 LD_{50} doses of Vollum 1B or 20–40 LD_{50} doses of Sterne. Survival data are no. survivors/total no., and TTD data are harmonic means.
[e] nd—not done.
[f] Data from S. Welkos and A. Friedlander.[21]

directed against the three toxin components PA, LF, or EF. The animals were challenged with Sterne spores at intervals up to 21 days after treatment with the antibody preparations. As shown in Table 7, high serum antibody titers in the anti-PA- and anti-LF-treated mice were maintained for at least a week. Mice treated with anti-PA or anti-LF MAbs were significantly protected from lethal infection in comparison to untreated controls, as shown by the increased number of survivors and longer TTD values (Table 7). Also, in the anti-PA-treated groups, protection correlated with the serum antibody titer. The lack of protection with the anti-EF ascites was probably related to its low antibody content and weakly positive neutralizing activity. These results suggest that anti-PA (or -LF) antibody protects A/J mice when they are challenged with a toxigenic, nonencapsulated strain but not when challenged with a fully virulent strain of *B. anthracis*. Sterne infection of A/J mice could be used as a model to study the *in vivo* interactions of toxin and the immune system.

The survivors of Sterne infection (Table 7) were used to determine whether a dose of Sterne greater than the LD_{50} could immunize A/J mice against virulent challenge with V1B, as hypothesized above. Although mice treated with Mabs alone did not survive V1B challenge, the anti-PA- or anti-LF-treated and Sterne-challenged mice were significantly protected against lethal infection (Table 8). Serum antibodies to both PA and LF were detected in mice treated with either anti-PA or anti-LF monoclonal ascites and inoculated with Sterne (Table 8). Sublethal doses of Sterne alone did not elicit significant anti-PA titers (Table 1). The results demonstrated that A/J can be immunized against challenge with a fully virulent strain if an adequate dose of Sterne is given. Protection of the mice by the antibodies may have allowed Sterne to replicate more extensively and release immunizing amounts of toxin components and possibly other antigens.

Although MDPH–PA was ineffective, the protection provided by live vaccines for the Sterne-resistant CBA/J mice was similar to that observed in other laboratory animals, such as guinea pigs and rabbits.[8–10,29–31] In earlier studies, outbred mice were

Table 7 Passive protection of A/J mice against Sterne with MAbs to toxin components

A. Characteristics of MAbs

Monoclonal Ascitic fluids				
Number	Specificity	IgG conc. (mg/ml)	Antitoxin titer[a]	Tissue culture neutralization titer[b]
3B6	anti-PA	11.07	1.2×10^6 (1.9)	10000
2E7-1-1	anti-LF	7.67	1.0×10^5 (0.0)	31623
SC10	anti-EF	0.80	1.0×10^3 (0.0)	positive

B. Treatment of mice and challenge with Sterne

Treatment[c]		Sterne challenge[d]					
		Antitoxin titer[e]	Day	No. LD_{50} doses	No. survivors		TTD[f]
Antibody	Group				Total no.	(%)	
Anti-PA:							
1/2	1–A	3.2×10^6 (2.0)	1	nd[g]	—		—
1/2	1–B	1.0×10^6 (0.0)	7	nd	—		—
1/2	1–C	4.3×10^5 (1.5)	21	20	2/6	(33.3)	10.1
1/10	2–A	1.4×10^5 (3.6)	1	10	4/10	(40)	10.7
1/10	2–B	1.4×10^5 (3.6)	1	20	4/20	(20)	10.3
1/10	2–C	1.9×10^6 (3.2)	7	nd	—		—
1/10	2–D	3.2×10^4 (0.0)	21	20	0/6	(0)	5.0
1/10	2–E	5.6×10^3 (7.3)	28	nd	—		—
1/100	3–A	8.3×10^4 (3.3)	1	nd	—		—
1/100	3–B	5.6×10^4 (1.8)	7	nd	—		—
1/100	3–C	3.2×10^3 (0.0)	21	20	0/5	(0)	3.4
Anti-LF:							
1/10	4–A	6.3×10^3 (2.1)	1	20	3/10	(30)	7.5
1/10	4–B	1.8×10^4 (1.8)	7	20	0/10	(0)	5.7
1/10	4–C	1.8×10^4 (7.3)	28	nd	—		—
Anti-EF:							
1/5	5–A	1.3×10^2 (1.8)[h]	1	20	0/10	(0)	3.7
1/5	5–B	2.0×10^2 (1.9)	7	20	0/10	(0)	3.2
PBS:	6–A	< 10	1	10	0/10	(0)	3.5
	6–B	< 10	1	20	0/10	(0)	3.2
	6–C	< 10	7	20	0/10	(0)	3.2
	6–D	< 10	28	nd	—		—

[a] Titers of antibody to toxin components PA, EF, or LF were determined by ELISA and shown as the reciprocal of the geometric mean (SD).

[b] Neutralizing activities were determined by inhibition of macrophage cytotoxicity (anti-PA and anti-LF MAbs) and of cAMP production by CHO cells (anti-EF monoclonal antibody). The latter was weakly positive; the titer was not determined.

[c] Mice were given one 0.5-ml intraperitoneal dose of ascites diluted 1/2, 1/10, or 1/100 in sterile isotonic saline.

[d] Mice were challenged with Sterne spores 1, 7, or 21 days after treatment with ascites or PBS. They were inoculated sc with 10 or 20 LD_{50} doses in 0.2-ml volumes.

[e] Sera were collected from mice that had been treated with ascites but were not challenged with spores. The sera were collected from five mice/group on days 1, 7, 21 or 28 after treatment.

[f] TTD—time to death (harmonic mean).

[g] nd—not done.

[h] One of the five mice sampled had no detectable anti-EF titer (< 10).

Table 8 Challenge of passively—and actively—immunized A/J mice with *B. anthracis* strain Vollum

Immunization		Vollum 1B challenge[a]				
		Titers[c]		No. survivors		
Treatment	Group[b]	Anti-PA	Anti-LF	Total no.	(%)	TTD[d]
Antitoxin and Sterne:[e]						
anti-PA (1/2)	1–C	3.2×10^4 (0.0)	1.0×10^4 (0.0)	2/2	(100)	—
anti-PA (1/10)	2–A	1.0×10^4 (0.0)	6.8×10^2 (1.9)[f]	1/4	(25)	5.4
anti-PA (1/10)	2–B	1.3×10^4 (1.8)	7.5×10^2 (1.8)	3/4	(75)	20
anti-LF (1/10)	4–A	4.6×10^2 (50)	4.7×10^3 (1.9)	2/3	(67)	12
Antitoxin alone:[g]						
anti-PA (1/10)	2–E	5.6×10^3 (7.3)	nd[h]	0/10	(0)	2.7
anti-LF (1/10)	4–C	nd	1.8×10^4 (7.3)	0/10	(0)	3.3
PBS[i]	6–D	< 10	< 10	0/20	(0)	3.4

[a] Mice that survived treatment with antitoxin ascites and inoculation with Sterne, or PBS controls (Table 7), were challenged sc with 10 LD_{50} doses of Vollum 1B spores. Challenge was 4 weeks after Sterne inoculation.

[b] As indicated on Table 7.

[c] Sera were collected 2 days prior to challenge and were assayed for antibody to PA or LF by ELISA. Geometric mean (SD) given.

[d] TTD—time to death (harmonic mean).

[e] Mice treated with antitoxin ascites that survived subsequent inoculation with Sterne spores, as described in Table 7.

[f] Mean titer of sera from three mice; one additional serum sample had an anti-LF titer of < 10.

[g] Mice treated with antitoxin ascites but not Sterne spores, and challenged as described in [a].

[h] nd—not done.

[i] Control mice that were neither pretreated with ascites nor inoculated with Sterne prior to challenge.

not protected against anthrax by live vaccines or filtered culture preparations, and the investigators concluded that these animals could not be immunized.[29–31] However, this conclusion is questionable, given the uncharacterized antigens and immunization schedules used. For example, the cell-free vaccines were crude filtrate preparations of bacterial cultures grown in rich media or body fluids; the PA content and purity were unknown.[29,31] In one study, the spore vaccine was administered only once.[30] The interval between vaccination and challenge was usually shorter (1–2 weeks)[29–31] 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.

The present studies on the protective efficacy for inbred mice of MDPH–PA and 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 *B. anthracis* strain V1B, although the vaccine was only effective at doses of Sterne $\geqslant 0.1$ LD_{50}. The Sterne-susceptible A/J mice were not protected against V1B by Sterne unless treated with antitoxin prior to immunization; (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. However, PA2 protected A/J mice against a lethal Sterne infection. The protection of A/J mice appeared to be due to antitoxin antibodies; (4) Protective antigen has a major role in protection against nonencapsulated and encapsulated toxigenic strains of *B. anthracis* and may be sufficient alone in mice (Tables 3 and 7) as well as other animals.[10,14] Although immunity to Sterne correlated

with an effective anti-PA response, protection against fully virulent strains may involve mechanisms in addition to humoral immunity (this paper).[8,9] Neither active immunization with MDPA–PA nor passive administration of anti-PA antiserum protected mice against V1B. The inbred mice are useful models for testing the safety and efficacy of anthrax vaccines. Also, the differences in immune responses of the three mouse strains might provide helpful markers for identifying antigens and host responses involved in active immunity against anthrax.

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 he U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), Frederick, MD. These strains included a toxigenic, encapsulated strain (V1B); a toxigenic, nonencapsulated strain (Sterne); and a strain deficient in both capsule and toxin production (ΔSterne-1).[10] *Bacillus subtilis* BST1 was derived by transformation of the asporogenic strain *B. subtilis* 1S53 with the plasmid vector pUB110 as described.[14] The PA component of anthrax toxin was cloned into this plasmid to generate *B. subtilis* strain PA2.[14,19] This strain produces full-length, biologically active PA.[14]

Immunization and challenge studies. Spores for immunization or challenge were prepared and frozen as previously described.[11] Prior to inoculation, spores were thawed and diluted in 0.4% Na_2HPO_4, pH 7.0, with 0.2% gelatin (PG). The dilutions were plated on trypticase soy agar plates for viable counts. Vegetative bacteria for immunization were prepared as previously described.[14] Mice (5 to 12 per group) were immunized subcutaneously (sc), except where indicated, with 0.2 ml volumes of diluent, spores, vegetative bacteria, or MDPH–PA. 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 V1B. 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 TTD values were calculated.[29,32]

Serological assay. Antibody titers to PA, LF, or EF in sera from actively or passively immunized mice were determined by an enzyme-linked immunosorbent assay (ELISA). Sera from four to six 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 h 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.

Passive protection with normal and immune sera. Blood was collected from normal C5-positive B10.D2/nSnJ and congenic C5-negative B10.D2/oSnJ mice. Two pools of C5-positive and -negative sera were made, and the sera transferred to A/J mice as described previously.[21] Sera were obtained from individual A/J and CBA/J mice immunized as described above with either MDPH–PA or PA2. The sera were combined into four pools, and half of each pool was absorbed onto a column of purified PA conjugated to CNB-activated Sepharose CL–6B, kindly provided by S. Leppla (unpublished data). Serum proteins were eluted with phosphate-buffered saline (PBS), and removal of anti-PA antibodies from the eluted material was confirmed by ELISA assay. The absorbed and unabsorbed serum preparations were diluted in PBS to yield comparable doses of total protein. Protein concentrations were determined by using bicinchoninic acid (Pierce Chemical Co., Rockford, IL).[33] CBA/J mice were injected intraperitoneally with 0.5 ml of serum 24 h before and 24 h after challenge. Mice were challenged sc with 10 LD_{50} doses of *B. anthracis* strain V1B, and observed daily for deaths.

Passive protection with monoclonal antitoxin antibodies. Ascitic fluids containing neutralizing monoclonal antibodies against PA, LF, or EF were kindly provided by S. Little (manuscript submitted). Toxin-neutralizing activities were determined by inhibition of cyclic AMP production in Chinese hamster ovary cells (CHO, anti-EF monoclonal antibody),[34] and of macrophage cytotoxicity (anti-PA and anti-LF MAbs).[12,35,36] The macrophage assays included a lactic dehydrogenase release assay[12] and the colorimetric tetrazolium reduction procedure of Mosmann,[35] modified as described by Green *et al.*[36] The CHO cell assay to detect neutralization of EF activity was kindly performed by S. Little.[34] The MAbs were diluted in sterile isotonic saline and mice were injected intraperitoneally with one 0.5-ml dose. Mice were inoculated sc with 10 or 20 LD_{50} doses of Sterne spores 1, 7, or 21 days after treatment. On each of these 3 days, sera were collected from mice that had been treated with ascitic fluids (five mice/group) but were not challenged with spores. Titers of anti-PA, anti-LF, and anti-EF antibody were determined as described above. Four weeks after inoculation with Sterne, mice that survived were challenged with 10 LD_{50} of V1B and observed daily for deaths.

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