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Immunization against Anthrax with *Bacillus anthracis* Protective Antigen Combined with Adjuvants

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The protective efficacy of immunization against anthrax with *Bacillus anthracis* protective antigen (PA) combined with different adjuvants was tested in Hartley guinea pigs and CBA/J and A/J mice. Adjuvant components derived from microbial products that were tested included threonyl-muramyl dipeptide (threonyl-MDP); monophosphoryl lipid A (MPL); trehalose dimycolate (TDM); and the delipidated, deproteinized, cell wall skeleton (CWS) from either *Mycobacterium phlei* or the BCG strain of *Mycobacterium bovis*. Non-microbially derived adjuvants tested included aluminum hydroxide and the lipid amine CP-20,961. In guinea pigs, all adjuvants and adjuvant mixtures enhanced antibody titers to PA as well as survival after a parenteral challenge of virulent *B. anthracis* Ames spores. In contrast, PA alone or combined with either aluminum hydroxide or CP-20,961 failed to protect mice. Vaccines containing PA combined with threonyl-MDP or MPL-TDM-CWS protected a majority of female CBA/J mice. Statistical analysis of survival data in the guinea pigs indicated that PA-MPL-CWS and PA-MPL-TDM-CWS were more efficacious than the currently licensed human anthrax vaccine.

Recent studies on anthrax and *Bacillus anthracis* have focused on the development and testing of new, prototype anthrax vaccines (15–21, 35, 38, 40). In the United States, the currently licensed human anthrax vaccine (MDPH-PA) consists of aluminum hydroxide-adsorbed supernatant material, primarily protective antigen (PA), from fermentor cultures of a toxigenic, nonencapsulated strain of *B. anthracis*, V770-NP1-R (26, 27, 44). This vaccine is available for persons at risk for anthrax, specifically those working with animals and unprocessed animal products, such as hides, wool, meat, and bones (4, 5, 25). Subcutaneous (s.c.) injection of the vaccine occasionally causes locally painful reactions (5), and six immunizations within 18 months are initially required, with yearly boosters thereafter (5). Although epidemiological studies indicate that MDPH-PA offers some protection to humans (4, 5), recent reports (23, 33) suggest that this vaccine may provide only partial protection against some strains of *B. anthracis*. Although human anthrax is relatively rare in the United States (43), with only four documented cases from 1979 through 1988, it remains a significant problem in numerous countries throughout Africa, the Middle East, Europe, and Asia (11).

The guinea pig has been used frequently both in anthrax vaccination research (17–19, 21, 23, 27, 33–35, 44) and in investigations of *B. anthracis* pathogenesis. In addition, a mouse model has been developed for studying specific and nonspecific resistance to anthrax and the mechanisms of *B. anthracis* pathogenesis (38–42). We recently developed and tested in both guinea pigs and mice several prototype live vaccines against anthrax (16, 18, 20, 21), including (i) recombinant strains of *Bacillus subtilis* that contain the cloned *B. anthracis* PA gene, (ii) vaccinia virus constructs containing the cloned PA gene, and (iii) aromatic-compound-dependent (Aro^-) mutants of the toxinogenic, nonencapsulated *B. anthracis* strain UM23-1. Several new adjuvants which enhance the host immune response to a number of antigens have recently been described (2, 8, 28). In this study, therefore, we decided to examine in Hartley guinea pigs and CBA/J and A/J mice the protective efficacy of PA combined with certain of these adjuvants against parenteral challenge with spores from the *B. anthracis* Ames strain. This strain is highly virulent for both mice and guinea pigs. In previous immunization studies (23, 33), *B. anthracis* Ames has either partially or fully overcome vaccination by MDPH-PA. Our goal is to replace the current human anthrax vaccine with a new vaccine exhibiting improved efficacy, safety, and ease of administration.

MATERIALS AND METHODS

Experimental animals. Female Hartley guinea pigs weighing 350 to 375 g at the start of immunization regimens were obtained from Charles River Laboratories, Wilmington, Mass. Male and female CBA/J and female A/J mice weighing 25 g at the time of the first immunizations were purchased from Jackson Laboratories, Bar Harbor, Maine.

In conducting the research described in this report, we adhered to the *Guide for the Care and Use of Laboratory Animals* (24a) as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

***B. anthracis* spore challenge.** *B. anthracis* Ames was originally obtained from the U.S. Department of Agriculture, Ames, Iowa. Spores of this reportedly "vaccine-resistant" strain (23, 33) were prepared and stored as previously described (21). Guinea pigs were challenged intramuscularly (i.m.) with 4,300, 7,300, or 200,000 spores in 0.2 ml of phosphate-buffered saline (PBS) (10) containing 0.1% gelatin. CBA/J mice received a 0.2-ml s.c. challenge with 700

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spores, whereas A/J mice were challenged s.c. with 50 spores. The 50% lethal doses (LD_{50}) of Ames spores administered i.m. to guinea pigs and s.c. to CBA/J mice and A/J mice have been reported as 100, 35, and <1 spore, respectively (21).

PA. *B. anthracis* PA, which was produced and purified in the Bacteriology Division of the U.S. Army Medical Research Institute of Infectious Diseases according to published methods (22), was a gift from S. H. Leppla. The PA was administered in arbitrarily selected doses (with or without adjuvant) of 70 μg to guinea pigs and 28 μg to mice.

Adjuvants. Alhydrogel, an aluminum hydroxide gel suspension, was purchased from Accurate Chemical and Scientific Corp., Westbury, N.Y. PA was adsorbed to a 1% suspension of the aluminum hydroxide as previously described (23) and then administered i.m. to guinea pigs in 0.5-ml doses.

Lipid amine CP-20,961 was the gift of I. Otterness, Pfizer Central Research, Groton, Conn. As described in published methods (2), the material (10 mg) was dissolved in 0.2 ml of ethanol and then mixed with 0.3 ml of Tween 80, 48.9 ml of soybean lipid emulsion (Intralipid; Cutter Laboratories, Berkeley, Calif.), and 7 mg (0.6 ml) of antigen. Guinea pigs were injected i.m. with 100 μg of CP-20,961 and 70 μg of PA per 0.5-ml dose. Mice received s.c. 40 μg of CP-20,961 and 28 μg of PA in 0.2 ml.

N-Acetylmuramyl-L-threonyl-D-isoglutamine (8; threonylmuramyl dipeptide, or threonyl-MDP) was the gift of N. Byars, Syntex Research, Palo Alto, Calif. Threonyl-MDP was combined with PA according to the manufacturer's recommendations. For guinea pigs, 1.2 mg of threonyl-MDP powder was mixed with 2.4 ml of emulsion vehicle (supplied with the adjuvant) and then added to 2.4 ml of PBS containing 1.68 mg of PA. Fifty micrograms of threonyl-MDP was delivered in each 0.2-ml i.m. dose. For mice, 0.4 mg of adjuvant, 1.0 ml of emulsion vehicle, and 1.0 ml of PBS containing 0.56 mg of PA were mixed together. Each 0.1-ml s.c. dose contained 20 μg of threonyl-MDP.

The following products were purchased from Ribi Immunochem Research, Inc., Hamilton, Mont.: (i) monophosphoryl lipid A (MPL), a derivative of diphosphoryl lipid A from *Salmonella minnesota* R595 that had been detoxified by removal of the phosphate group at the reducing end of the molecule; (ii) trehalose dimycolate (TDM), the purified cord factor from *Mycobacterium phlei*; and (iii) the delipidated, deproteinized cell wall skeleton (CWS) from either *M. phlei* or the BCG strain of *Mycobacterium bovis*. MDP has been reported to be the immunomodulatory component of CWS (28). MPL (100 μg), MPL (25 μg)-CWS (250 μg, from *M. phlei*), or MPL (125 μg)-TDM (125 μg)-CWS (125 μg, from the BCG strain of *M. bovis*) were mixed with PA (70 μg) and PBS (0.5 ml). Animals were given mixtures of adjuvant and antigen according to the manufacturer's recommendations. Mice received 0.2 ml s.c. in one site. Guinea pigs received 0.2 ml s.c. in each of two different sites and 0.1 ml intraperitoneally, for a total of 0.5 ml.

Serological studies. Two days before challenge, guinea pigs were bled by cardiac puncture, and mice were bled from the retroorbital plexus. Sera were assayed for antibody to PA by enzyme-linked immunosorbent assay (ELISA) as described previously (16, 23, 39).

MDPH-PA. MDPH-PA, the licensed human anthrax vaccine (production lot no. 18), was purchased from the Michigan Department of Public Health, Lansing.

Statistical analysis. Logistic regression analysis (1, 14) of the cumulative guinea pig survival data was used to estimate the odds of survival, adjusted for challenge dosage, of animals treated with each vaccine preparation. The ratios of survival odds for MDPH-PA were used as a measure of relative efficacy of the preparations. Fisher's exact tests (6, 31) were done to compare various vaccine preparations with a standard preparation (MDPH-PA or PBS) within a number of experimental groups with respect to the number of mice or guinea pigs surviving challenge after immunization with one of the vaccine preparations (16). The Bonferroni correction (31) was applied to comparisons within each group to maintain the overall alpha level (probability of incorrectly finding two preparations significantly different due to random sampling effects) at $P = 0.05$. The Bonferroni correction was implemented by dividing the desired pairwise comparison at this level. For instance, if five vaccine preparations were tested against a standard such as MDPH-PA, a pairwise alpha level of $0.05/5 = 0.01$ would be used as the level of significance for each of the comparisons within that group. Thus, the significance level used to test individual comparisons varies between groups depending on the number of within-group comparisons made, but the overall alpha level for every group is 0.05.

RESULTS AND DISCUSSION

Immunization of guinea pigs. Several adjuvant preparations combined with PA were compared with each other and with MDPH-PA with respect to protection and elicitation of anti-PA antibody. In the first experiment (Table 1), guinea pigs received one or more injections of each preparation and then were challenged i.m. 10 weeks after the first immunization with 7,300 (73 LD_{50}) of *B. anthracis* Ames spores. Although all the preparations protected some of the animals, only PA combined with MPL-TDM-CWS protected all challenged animals with either one or two immunizations. PA combined with MPL-CWS was also very efficacious, protecting 100 and 83% of the guinea pigs immunized with one or two doses, respectively. MDPH-PA was fully protective only after three immunizations. Although the level of protection in experimental animals cannot be determined by anti-PA titers, note that MPL-TDM-CWS-PA elicited the highest antibody titers to PA.

An optimal anthrax vaccine must engender maximal, long-lasting immunity with a minimum number of injections. Thus, in the second experiment (Table 2), we compared the adjuvants combined with PA with each other, giving the guinea pigs a single immunization and then measuring protection either 6 or 24 weeks later after an i.m. challenge of 4,300 (43 LD_{50}) of *B. anthracis* Ames spores. The serological response was examined at 4, 15, and 24 weeks. All adjuvants increased the protective efficacy of PA; however, only MPL-TDM-CWS-PA and MPL-CWS-PA protected animals completely from either the 6- or 24-week challenge.

The experiments described above demonstrated that some of the vaccine preparations (MPL-CWS-PA and MPL-TDM-CWS-PA) were fully protective against a challenge dose of 4,300 spores for up to 24 weeks after immunization. However, after 24 weeks, the 4,300-spore challenge dose was insufficient to kill all of the control animals given PBS, possibly because of the increased resistance of the older, heavier guinea pigs to spore challenge. Therefore, to define more accurately the level of efficacy of the candidate vaccines, in the final guinea pig experiment, 8- and 24-week Ames spore challenge doses were increased to 200,000 (2,000 LD_{50}) spores. The data presented in Table 3 again demonstrate that the two most protective vaccines were PA

TABLE 1. Immunization of guinea pigs: challenge at 10 weeks with 7,300 spores

Immunization regimen[a]	No. surviving/no. challenged[b] % survival)	Serological response[c] at:	
		4 wk	10 wk
PBS	0/8 (0)	<10	<10
PA (no adjuvant)			
1 dose	5/11 (45)	3,162	1,001
2 doses	7/10[d] (70)	3,831	28,183
MDPH-PA			
1 dose	7/11 (64)	20,706	1,233
2 doses	8/11[d] (73)	21,544	11,103
3 doses	8/8[d] (100)	86,597	64,938
$Al(OH)_3$-PA			
1 dose	4/11 (36)	2,136	431
2 doses	8/12[d] (67)	10,000	19,573
CP-20,961–PA			
1 dose	4/11 (36)	2,873	826
2 doses	8/10[d] (80)	1,100	12,588
Threonyl-MDP–PA			
1 dose	12/18[d] (67)	ND	3,981
2 doses	13/20[d] (65)	ND	47,310
MPL-PA			
1 dose	6/10 (60)	6,189	3,981
2 doses	8/11[d] (73)	10,000	23,713
MPL-CWS-PA			
1 dose	9/9[d] (100)	11,007	6,309
2 doses	10/12[d] (83)	17,782	14,678
MPL-TDM-CWS-PA			
1 dose	8/8[d] (100)	27,384	20,535
2 doses	8/8[d] (100)	42,170	1,154,782

[a] Guinea pigs were immunized at 0 weeks (one dose), 0 and 4 weeks (two doses), or 0, 2, and 4 weeks (three doses).
[b] Animals were challenged i.m. with 73 LD_{50} of *B. anthracis* Ames.
[c] Reciprocal geometric mean anti-PA ELISA titers of sera from guinea pigs bled 4 and 10 weeks after the first immunization. ND, not done.
[d] Compared with PBS controls, significant at the *P* level (0.0063) determined by the Bonferroni group correction. None of the groups was significantly better than MDPH-PA when either one-immunization or two-immunization regimens were compared.

combined with MPL-CWS and MPL-TDM-CWS. In another experiment (data not shown), a single 0.5-ml i.m. dose of MPL-TDM-CWS-PA protected 19 of 20 guinea pigs from an i.m. challenge of 500,000 *B. anthracis* Ames spores (5,000 LD_{50}) 8 weeks after immunization. Although PA combined with MPL, MPL-CWS, and MPL-TDM-CWS was examined in this study, it would be interesting in future studies to investigate the immunizing efficacy of PA combined with TDM, CWS, TDM-CWS, and MPL-TDM. Indeed, we have recently demonstrated (data not shown) that a single dose of PA (70 μg)-MPL (125 μg)-TDM (125 μg) completely protected guinea pigs from an i.m. challenge of 7,000 *B. anthracis* Ames spores 8 weeks after immunization.

Immunization of mice. Guinea pigs are frequently used to characterize anthrax vaccine efficacy and disease pathogenicity (17–19, 21, 23, 27, 33–35, 44). Nevertheless, animal species vary greatly in their susceptibilities to anthrax and their immune responses. Because the pathogenesis of the disease and the nature of protective immunity in humans are not well understood, anthrax vaccine efficacy should be determined in more than one animal model or bioassay (19). CBA/J and other strains of inbred mice are useful and sensitive animal models for evaluating the efficacy and safety of anthrax vaccines (38–42). Mice and guinea pigs respond differently to some anthrax vaccines. Although live vaccines have demonstrated efficacy in both species (16, 17, 19, 21, 23, 33–35, 38, 40), nonliving vaccines containing PA have proven far more efficacious in guinea pigs than in mice (19, 38, 40). Furthermore, inbred strains of mice vary in their abilities to be immunized. Among strains previously tested, CBA/J mice are the best protected (38, 40), whereas A/J mice are extraordinarily sensitive to infection and are not well protected by immunization.

As observed previously (38, 40), MDPH-PA failed to protect mice against anthrax challenge despite the stimulation of high anti-PA ELISA titers (Table 4). In earlier studies, PA adsorbed to aluminum hydroxide gel was similarly ineffective (38). Also, PA combined with CP-20,961 did not protect the mice (Table 4). In contrast, PA combined with either threonyl-MDP or MPL-TDM-CWS protected female CBA/J mice against *B. anthracis* Ames spore challenge. Two doses of the former and three doses of the latter provided 80 and 77% protection, respectively (Table 4). In a previous study (40), PA combined with MPL-TDM-CWS protected 89% of the immunized female mice.

Anti-PA titers were not a reliable indicator of protection in CBA/J mice. Although groups of mice exhibiting ≥50% protection had anti-PA titers of ≥8,900, the relationship between humoral antibody and survival was unclear. Several

TABLE 2. Immunization of guinea pigs: challenge at 6 and 24 weeks with 4,300 spores

Immunization[a]	No. surviving/no. challenged[b] (% survival)		Serological responses[c] at:		
	6 wk	24 wk	6 wk	15 wk	24 wk
PBS	0/15 (0)	3/15 (20)	<10	<10	<10
$Al(OH)_3$	4/15 (27)	ND	<10	ND	ND
Threonyl-MDP	1/19 (5)	ND	22	ND	ND
MPL-TDM-CWS	2/15 (13)	ND	<10	ND	ND
PA (no adjuvant)	9/20[d] (45)	3/18 (17)	274	224	298
$Al(OH)_3$-PA	9/15[d] (60)	16/18[e] (89)	4,640	4,084	2,021
CP-20,961–PA	11/18[d] (61)	11/18 (61)	830	938	1,000
Threonyl-MDP–PA	17/20[d] (85)	15/19[e] (79)	2,387	2,113	2,198
MPL-PA	14/19[d] (74)	13/17[e] (76)	1,194	681	1,000
MPL-CWS-PA	15/15[d] (100)	18/18[e] (100)	9,310	7,627	2,738
MPL-TDM-CWS-PA	15/15[d] (100)	16/16[e] (100)	3,900	9,380	3,162

[a] Guinea pigs were immunized at 0 weeks.
[b] Animals were challenged with *B. anthracis* Ames spores. ND, not done.
[c] Reciprocal geometric mean anti-PA ELISA titers of sera from guinea pigs bled at 6, 15, or 24 weeks after immunization.
[d] Compared with PBS controls, significant at the *P* level (0.0050) determined by the Bonferroni group correction.
[e] Compared with PBS controls, significant at the *P* level (0.0071) determined by the Bonferroni group correction.

TABLE 3. Immunization of guinea pigs: challenge at 8 and 24 weeks with 200,000 spores

Immunization[a]	No. surviving/no. challenged[b] (% survival)		Serological response[c] at:	
	8 wk	24 wk	8 wk	24 wk
PBS	0/19[d] (0)	0/20 (0)	<10	<10
MPL	0/19 (0)	ND	<10	ND
PA (no adjuvant)	1/19[d] (5)	ND	379	ND
$Al(OH)_3$-PA	5/20 (25)	ND	2,985	ND
CP-20,961–PA	3/20 (15)	7/19[e] (37)	707	398
Threonyl-MDP–PA	9/20[f] (45)	10/20[e] (50)	3,980	3,758
MDPH-PA	9/18[f] (50)	5/19 (26)	1,045	1,679
MPL–MDPH-PA	12/20[f] (60)	ND	3,350	ND
MPL-PA	9/19[f] (47)	ND	2,371	ND
MPL-CWS-PA	13/20[f] (65)	19/19[e,g] (100)	1,679	1,995
MPL-TDM-CWS-PA	18/19[d,f] (95)	19/20[e,g] (95)	7,943	3,539

[a] Guinea pigs were immunized at 0 weeks.
[b] Animals were challenged with *B. anthracis* Ames spores. ND, not done.
[c] Reciprocal geometric mean anti-PA ELISA titers of sera from guinea pigs bled 8 or 24 weeks after immunization.
[d] Compared with MDPH-PA (8-week challenge), significant at the P level (0.0055) determined by the Bonferroni group correction.
[e] Compared with PBS controls (24-week challenge), significant at the P level (0.0100) determined by the Bonferroni group correction.
[f] Compared with PBS controls (8-week challenge), significant at the P level (0.0050) determined by the Bonferroni group correction.
[g] Compared with MDPH-PA (24-week challenge), significant at the P level (0.0125) determined by the Bonferroni group correction.

of the vaccine regimens elicited serum anti-PA titers of ≥100,000 yet failed to induce protective immunity (Table 4). In other immunization studies (16, 18–21, 23, 33–35, 38, 40), researchers similarly noted that anti-PA titers do not strictly correlate with levels of protection. It is apparent from these and previous investigations that although PA is an essential protective immunogen, immune mechanisms in addition to the anti-PA antibody measured by ELISA appear to be involved in specific immunity to anthrax.

In contrast to the high levels of protection afforded female CBA/J mice by some of the PA vaccines, no more than one-third of the immunized male CBA/J mice in any group survived. Although three doses of PA combined with MPL-TDM-CWS protected 77% of female mice, the same regimen protected only 31% of male mice (Table 4). The s.c. LD_{50} of spores of the toxigenic, encapsulated *B. anthracis* Ames strain was the same (35 spores) for both male and female CBA/J mice. Also, the susceptibility of mice to toxigenic, nonencapsulated strains of *B. anthracis* is not sex associated but is linked to the autosomal *Hc* locus (38, 39). The influence of sex on resistance to disease has been reported in numerous model systems; both genetic and nongenetic, e.g., hormone-dependent, mechanisms are implicated (7, 13, 32, 37). Our studies suggest a possible sex-associated difference in response to immunization with various vaccine preparations. The role of gender in both innate and acquired resistance to anthrax requires further evaluation.

A/J mice are extraordinarily sensitive to infection by *B. anthracis* strains (39, 41, 42), even by those less-virulent, nonencapsulated strains that lack the capsule plasmid pXO2 (12). In past studies, the only vaccines protecting A/J mice against *B. anthracis* Ames spore challenge were the Tn*916*-generated Aro^- mutants of a toxigenic, nonencapsulated *B. anthracis* strain, UM23-1 (20, 21). Here, we tested PA combined with several adjuvants for protective efficacy against a 100-LD_{50} spore challenge in A/J mice (Table 5). Partial protection of female A/J mice was seen only in those animals given two doses of MPL-TDM-CWS-PA. The level of protection (36%) approximated that (45%) afforded against a similar challenge of 100 LD_{50} of *B. anthracis* Ames spores by immunization with Aro^- mutants of *B. anthracis* (21). In the latter study, immunization with the Aro^- mutant protected 70% of A/J mice from a spore challenge dose of 60 LD_{50}.

Logistic regression statistical analysis (1, 14) of the cumulative guinea pig survival data indicated that the most protective vaccine preparations were MPL-TDM-CWS-PA and MPL-CWS-PA. Both preparations were more efficacious than MDPH-PA, increasing survival odds by factors of 42.2 and 8.6, respectively. PA alone (without adjuvant) was less efficacious than MDPH-PA by a factor of 3.9. The other preparations had estimated survival odds with relative efficacies of less than 2.0 and were not significantly different from MDPH-PA ($P > 0.05$) by the chi-square goodness-of-fit test. The mouse survival data agreed with these conclusions, indicating the superiority of PA vaccines containing the adjuvants derived from microbial products. In another recent study, Iacono-Connors et al. (16) noted that MPL-TDM-CWS combined with either *B. anthracis* Sterne PA or PA produced in a recombinant baculovirus clone was highly protective in guinea pigs and partially protective in mice. Indeed, efficacy in experimental anthrax vaccine preparations depends on the presence of PA in a chemical vaccine or the production of PA in a living vaccine (17–19). As demonstrated in these studies and in those by Turnbull et al. (35), injecting purified PA by itself without adjuvant at least partially protects guinea pigs from virulent *B. anthracis* Ames spore challenge. However, it is clear from several previous investigations (16, 19, 23, 33–35, 38, 40) that the elicitation of high anti-PA titers by itself does not directly correlate with protection against virulent spore challenge in either guinea pigs or mice. These and other data (34, 35) clearly demonstrate that the addition of Freund's adjuvant, killed *Corynebacterium ovis* or *Bordetella pertussis*, or purified compounds derived from microbes greatly enhances the protective immune response to PA in experimental animals. Others noted previously (19, 23, 33, 38, 40) that immunization with the live *B. anthracis* Sterne spore veterinary vaccine protected experimental animals better than immunization with MDPH-PA, although the anti-PA titers elicited were invariably lower. Possible reasons suggested (19) for this phenomenon included (i) that ELISA titers reflect the polyclonal antibody response to the entire PA molecule rather than to individual, immunogenic domains,

TABLE 4. Immunization of male and female CBA/J mice

Sex and immunization regimen[a]	No. surviving/no. challenged[b] (% survival)	Serological response[c]
Male		
PBS, 2 doses	0/12 (0)	<10
MPL-TDM-CWS, 2 doses	0/10 (0)	11
PA (no adjuvant)		
1 dose	0/12 (0)	51
2 doses	0/12 (0)	5,109
MDPH-PA		
1 dose	1/12 (8)	6,813
2 doses	0/12 (0)	12,328
CP-20,961–PA		
1 dose	0/12 (0)	121
2 doses	2/12 (17)	14,678
Threonyl-MDP–PA		
1 dose	0/12 (0)	1,101
2 doses	3/12 (25)	237,138
MPL-CWS-PA		
1 dose	0/12 (0)	1,101
2 doses	1/12 (8)	34,807
MPL-TDM-CWS-PA		
1 dose	4/12 (33)[d]	1,211
2 doses	3/12 (25)	287,299
3 doses	4/13 (31)	185,880
Female		
PBS, 2 doses	0/10 (0)	<10
MDPH-PA		
1 dose	1/10 (10)	501,187
2 doses	1/10 (10)	707,946
3 doses[e]	1/10 (10)	100,000
CP-20,961–PA		
1 dose	0/10 (0)	19,953
2 doses	1/10 (10)	1,000,000
3 doses	0/9 (0)	464,515
Threonyl-MDP–PA		
1 dose	6/10 (60)[f]	8,913
2 doses	8/10 (80)[g]	630,957
3 doses	5/10 (50)[h]	1,412,538
MPL-TDM-CWS-PA		
1 dose	1/10 (10)	8,414
2 doses	6/10 (60)[f]	446,684
3 doses	10/13 (77)[g]	242,447

[a] Male or female mice were immunized at 0 weeks (one dose), 0 and 4 weeks (two doses), or 0, 2, and 4 weeks (three doses).
[b] Animals were challenged at 8 weeks with 700 *B. anthracis* Ames spores (20 LD_{50}).
[c] Reciprocal geometric mean anti-PA ELISA titers of sera from mice bled 8 weeks after the first immunization.
[d] $P = 0.047$ compared singly with PBS controls and MDPH-PA groups. However, none of the groups of male mice differed significantly from the controls or MDPH-PA-immunized animals at the P levels (0.007 and 0.0125, respectively) determined by the Bonferroni group corrections.
[e] Data reported previously by Welkos and Friedlander (40).
[f] Compared with PBS controls, significant at the P level (0.0125) determined by the Bonferroni group correction; not significantly different from the MDPH-PA-immunized animals.
[g] Compared with PBS controls, significant at the P level (0.0125) determined by the Bonferroni group correction. Compared with MDPH-PA, significant at the P level (0.017) determined by the Bonferroni group correction.
[h] $P = 0.032$ compared with PBS controls but not significantly different at the P level (0.0125) determined by the Bonferroni group correction.

TABLE 5. Immunization of A/J mice

Immunization regimen[a]	No. surviving/no. challenged[b] (% survival)	Serological response[c]
PBS, 2 doses	0/11 (0)	<10
MPL-CWS, 2 doses	0/10 (0)	26
MPL-TDM-CWS, 2 doses	0/11 (0)	75
MDPH-PA		
1 dose	0/11 (0)	48,696
2 doses	0/11 (0)	81,125
CP-20,961–PA, 2 doses	0/12 (0)	56,234
Threonyl-MDP-PA, 2 doses	0/15 (0)	87,332
MPL-CWS-PA		
1 dose	1/11 (9)	7,197
2 doses	0/10 (0)	38,311
MPL-TDM-CWS-PA		
1 dose	0/11 (0)	1,778
2 doses	4/11 (36)[d]	100,000

[a] Mice were immunized at 0 weeks (one dose) or 0 and 4 weeks (two doses).
[b] Animals were challenged at 8 weeks with 50 *B. anthracis* Ames spores (100 LD_{50}).
[c] Reciprocal geometric mean anti-PA ELISA titers of sera from mice bled 8 weeks after the first immunization.
[d] $P = 0.045$ compared with PBS controls in a single-comparison Fisher's exact test.

and the Sterne spore vaccine may elicit lower anti-PA titers overall but higher titers to specific, protective domains; (ii) that the PA produced and secreted directly into the host by *B. anthracis* Sterne may differ in its three-dimensional antigenic structure from the PA in MDPH-PA, which is produced and purified; and (iii) that cell-mediated immunity may be critical in specific resistance to anthrax, and immunization with live Sterne spores may better stimulate cell-mediated immune mechanisms than immunization with MDPH-PA.

The present investigations and those of Turnbull et al. (34, 35) demonstrate that the protective efficacy of MDPH-PA was substantially augmented by adjuvants known to enhance cell-mediated immune responses (8, 28, 29). These studies suggest that there is nothing immunogenically inferior about the PA in MDPH-PA. Rather, it is likely that the diminished protective efficacy of MDPH-PA is due to its inability to stimulate sufficiently the full complement of immune mechanisms responsible for protection against anthrax.

In the United States and many other countries, only aluminum phosphate and aluminum hydroxide are approved as adjuvants for human use (30). Prior to use as a licensed human vaccine, any new adjuvant in combination with an antigen must undergo extensive human safety studies to determine local or systemic reactogenicity. MPL-CWS was recently used as an adjuvant for a candidate malarial vaccine in five human volunteers (30). The MPL-CWS adjuvant in combination with antigen stimulated specific antibody formation in the volunteers. The experimental vaccine also elicited moderate local reactogenicity. One subject exhibited a mild systemic reaction to the vaccine. MPL and CWS have also been used with mild to moderate side effects to boost immunity in human cancer patients (24, 36, 45). The threonyl-MDP adjuvant is currently being tested in human clinical trials (9). In the animal studies reported here, the only reactogenicity occasionally seen was a small, nonnecrotic granuloma in mice after s.c. injection of PA combined with MPL-TDM-CWS.

The development of new adjuvants with potential for human use has greatly increased the possibility of new-generation vaccines that are both safe and highly efficacious. Several new adjuvants combined with PA in these studies provided high, long-lasting levels of protection to guinea pigs with no mortality or obvious morbidity. Previously, only immunization with viable Sterne spores conferred strong protection on experimental animals (19, 23, 33), but the use

of the Sterne spore vaccine occasionally killed the host animal (19, 21, 23, 33, 40). Immunization with certain adjuvants combined with PA may eliminate the risks to safety associated with the live Sterne spore anthrax vaccine yet retain a high level of protection similar to that afforded by live-strain immunization.

Although the mechanisms of specific and nonspecific resistance to anthrax are not clearly defined, the effects of the adjuvants used in these studies on host immune systems have been extensively investigated in a number of studies. Lipid amine CP-20,961 stimulates interferon production and, with specific antigen, proliferation of B-cell clones (3). MDP and its derivatives, such as threonyl-MDP, induce interleukin-1 production in macrophages and, in combination with antigen, elicit cell-mediated immune responses such as T-cell proliferation and interleukin-2 production (8, 28, 29). MPL (28, 29) is a stimulator of interleukin-1 and interferon production and B-cell proliferation. TDM (28, 29) activates macrophages and may act as an antigen "anchor" or carrier in antigen-adjuvant emulsions, aiding in bringing the immunogen to antigen-presenting cells.

In this study, the most efficacious adjuvants in guinea pigs were MPL-TDM-CWS and MPL-CWS; in female CBA/J mice, threonyl-MDP and MPL-TDM-CWS were the most efficacious. The relative efficacy of these and other adjuvants in immunization protocols may vary because of differences in (i) the immunization route, (ii) the number of immunizations, (iii) the immunogen used, (iv) the microbial agent being immunized against, (v) the challenge route, and (vi) those modalities of the host immune system that must be stimulated to engender specific resistance. We are continuing studies comparing living and nonliving prototype anthrax vaccines, and we are examining the mechanisms of specific and nonspecific resistance to anthrax in experimental animal models. After completion of parenteral challenge studies, selected vaccine candidates will be tested for efficacy against aerosol challenges with virulent spores.

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