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Immunological Analysis of Cell-Associated Antigens of *Bacillus anthracis*

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Sera from Hartley guinea pigs vaccinated with a veterinary live spore anthrax vaccine were compared with sera from guinea pigs vaccinated with the human anthrax vaccine, which consists of aluminum hydroxide-adsorbed culture proteins of *Bacillus anthracis* V770-NP-1R. Sera from animals vaccinated with the spore vaccine recognized two major *B. anthracis* vegetative cell-associated proteins that were either not recognized or poorly recognized by sera from animals that received the human vaccine. These proteins, termed extractable antigens 1 (EA1) and 2 (EA2), have molecular masses of 91 and 62 kilodaltons, respectively. The EA1 protein appeared to be coded by chromosomal DNA, whereas the EA2 protein was only detected in strains that possessed the pXO1 toxin plasmid. Both of the extractable antigen proteins were serologically distinct from the components of anthrax edema toxin and lethal toxin. Following vaccination with the live spore vaccine, the EA1 protein was the predominant antigen recognized, as determined by electrophoretic immunotransblots. Vaccine trials with partially purified EA1 demonstrated that it neither elicits protective antibody against anthrax nor delays time to death in guinea pigs challenged intramuscularly with virulent Ames strain spores. In addition, animals vaccinated with sterile gamma-irradiated cell walls had significant antibody titers to the *N*-acetylglucosamine–galactose polysaccharide of *B. anthracis* but were neither protected nor had a delay in time to death following challenge.

Bacillus anthracis, the etiologic agent of anthrax, possesses three virulence factors, a poly-D-glutamic acid capsule, edema toxin, and lethal toxin. Edema toxin is composed of two proteins, edema factor (EF; 89 kilodaltons [kDa]) and protective antigen (PA; 85 KDa), and likewise, lethal toxin is composed of lethal factor (LF; 82 KDa) and PA (1, 5, 18, 26, 27, 30, 34). PA plays a central role in that it is required for the transport of EF and LF into host target cells (6, 17, 22). All three toxin proteins are encoded for by a 110-MDa plasmid, pXO1 (21), whereas the capsule is encoded for by a 60-MDa plasmid, pXO2 (7, 33). Strains that lack either or both of the plasmids are avirulent.

In most countries, livestock at risk are immunized annually with commercial vaccines (i.e., Anvax; Wellcome Animal Research Laboratories, Beckenham, England [Div. Burroughs Wellcome Co.]), which are composed of viable spores of *B. anthracis* Sterne, a nonencapsulated toxigenic variant ($pXO1^+$, $pXO2^-$) which is safe and efficacious in protecting against anthrax (28, 29). In contrast, the human anthrax vaccine licensed in the United States, which is produced by the Michigan Department of Public Health (MDPH), consists of aluminum hydroxide-adsorbed culture filtrates of the nonencapsulated, toxigenic strain V770-NP-1R (2, 8, 24) and is primarily composed of PA.

Because PA plays such a central role, it is a generally held belief that antibody induced to this protein protects the vaccinee by neutralizing toxin activity. Indeed, this may be partially accurate; however, one cannot discount the reports that humoral antibody titers to PA do not correlate well with protection and that protection in animals vaccinated with live spore vaccines is better and more prolonged than is protection in those vaccinated with chemical vaccines (i.e., MDPH, adsorbed PA) (12, 15, 19). Little and Knudson (19) have reported that, although titers to PA were higher in animals vaccinated with the MDPH human vaccine as opposed to those vaccinated with *B. anthracis* Sterne (Anvax), only animals vaccinated with *B. anthracis* Sterne were completely protected against vaccine-resistant *B. anthracis* strains.

In light of the observations presented above, we questioned whether there are proteins other than PA, such as vegetative cell surface antigens, that are antigenic in animals vaccinated with a live spore vaccine but not with the human vaccine. Two such proteins were identified and were designated extractable antigens 1 (EA1) and 2 (EA2). In this report we described the partial purification of EA1 and vaccine studies with this protein, which appears to be a highly antigenic major surface protein. Antibody titers to the cell wall polysaccharide of *B. anthracis* (3, 20) were also quantitated in prechallenged animals to determine whether antibody levels to this *N*-acetylglucosamine–galactose polymer correlated with protection.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. anthracis* strains were obtained from the U.S. Army Medical Research Institute of Infectious Diseases Culture Collection, Fort Detrick, Md. Descriptions of parental strains have been reported previously (19). Strains cured of their pXO1 toxin plasmid (designated with the prefix Δ; see Table 2) have also been described (10). Frozen spore stock suspensions were revived on 5% sheep blood agar by incubating them at 37°C for 18 to 20 h. Growth from blood agar cultures was inoculated into R medium (25) that was buffered with 50 mM Tris hydrochloride (pH 7.5). The cultures, 500 ml in 2-liter sealed screw-top flasks, were incubated in a New Brunswick Scientific incubator shaker at 100 rpm and 37°C for 18 to 20 h.

Cell extraction. Cells were harvested by centrifugation at $10^4 \times g$ for 15 min and washed in 100 ml of R medium. The

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cells were suspended to 0.1 g (wet weight)/ml in sodium dodecyl sulfate (SDS) extraction buffer (13), which consisted of 5 mM Tris hydrochloride, 1% (wt/vol) SDS, and 5 mM 2-mercaptoethanol (pH 9.8), that was heated to 70°C for 30 min, cooled to ambient temperatures, and centrifuged as described above. The supernatant, which contained extracted proteins, was filtered through a 0.22-μm-pore-size, low protein-binding, cellulose acetate filter (Millipore Corp., Bedford, Mass.).

Culture supernatants were filtered, placed in dialysis tubing (3,500-molecular-weight cutoff), concentrated at 4°C in polyethylene glycol, and reconstituted with 0.1 volumes of extraction buffer. The dialysis bags were resealed and boiled for 1 min to release proteins which may have become bound to the tubing. The samples were transferred to a polypropylene vial, boiled for an additional 4 min, and then stored at −20°C.

SDS-polyacrylamide gel electrophoresis. To ensure the saturation of proteins with SDS prior to electrophoresis, the cell extracts and concentrated culture fluids were mixed with 5× sample buffer to give a final concentration of 1% (wt/vol) SDS, 10% (vol/vol) glycerol, and 10 mM 2-mercaptoethanol and placed in a boiling water bath for 5 min. Samples were electrophoresed on polyacrylamide gels (16) containing 10% (wt/vol) acrylamide (acrylamide-bisacrylamide [37.5:1]; pH 8.8) with 5% acrylamide stacking gels (pH 6.8). Electrophoresis was stopped when the bromphenol blue tracking dye had migrated 10 cm.

Electrophoretic immunotransblot. Following electrophoresis, the proteins were electrophoretically transblotted onto nitrocellulose (Nc) (31) in transblot buffer (20 mM Tris, 150 mM glycine, 20% methanol [pH 8.5]; before the addition of methanol) for 16 to 18 h at 150 mA. The 10- by 14-cm Nc sheet was then incubated in 100 ml of quench buffer (0.5% gelatin, 30 mM sodium citrate, 5% [wt/vol] nonfat powdered milk, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 100 mM NaCl [pH 7.5]) for 30 min at 37°C. The treated Nc was cut into 2.5-mm-wide strips and placed into disposable incubation trays (Accutran; Schleicher & Schuell, Inc., Keene, N.H.). The strips were occasionally cut the day before use and then stored at −20°C in quench buffer containing 50% glycerol (2 ml per well). The eight-trough incubation trays were washed in a 96-well microtiter plate washer (Skatron Inc., Sterling, Va.; 12 ports in each trough), with one three-cycle wash, by using 10 mM sodium phosphate-buffered saline (PBS; pH 7.3)–0.3% Tween 20 (PBST). Each strip was incubated in 1 ml of antiserum or monoclonal antibody (MAb) that was empirically diluted in PBST–0.5% gelatin (PBSTG) for 2 h at 37°C and then washed with one four-cycle wash in a microtiter plate washer. For detection of mouse MAb, the strips were incubated for 1 h in rabbit anti-mouse immunoglobulin G (IgG), IgA, and IgM (Calbiochem-Behring, La Jolla, Calif.) diluted 1:400 in PBSTG at 37°C. Detection of rabbit anti-mouse antibody and guinea pig antibody was accomplished by incubating the strips in horseradish peroxidase-protein A conjugate (supplied by Stephen Leppla, U.S. Army Medical Research Institute of Infectious Diseases), diluted 1:5,000 in PBSTG, for 30 min at ambient temperatures. The strips were washed with one five-cycle wash in a microtiter plate washer. Conjugate-antibody complexes were detected by using the chromogenic substrate (4 ml of tetramethylbenzidine [5 mg/ml in methanol], 16 ml of 1% dioctylsodium-sulfosuccinate [Sigma Chemical Co., St. Louis, Mo.], 1 ml of 1 M HEPES [pH 7.5], 59 ml of distilled water, and 20 μl of 3% H_2O_2). The reaction was terminated at 10 min by washing the Nc strips three times in a buffer containing 10 mM HEPES, 0.2% dioctylsodium-sulfosuccinate, and 5% methanol (pH 7.5). The stained strips were transferred to glass plates, realigned, and photographed with film (Polaroid type 55; OPELCO, Washington, D.C.).

Molecular mass determination. Molecular mass protein standards (Bio-Rad Laboratories, Richmond, Calif.) included ovalbumin (45 KDa), bovine serum albumin (66.2 KDa), phosphorylase *b* (92.5 KDa), β-galactosidase (116.5 KDa), and myosin (200 KDa). The standards were treated with 5× sample buffer as described above, electrophoresed in lanes adjacent to those containing cell extracts, and transblotted; and the Nc was stained with AuroDye (Janssen, Piscataway, N.J.) The electrophoretic mobilities of the standards were calculated and plotted, and the molecular masses of the unknown proteins were interpolated.

Partial purification of EA1. *B. anthracis* V770-NP-1R cells ($pXO1^+$, $pXO2^-$) grown in R medium were harvested by centrifugation at $10^4 \times g$ for 15 min and suspended at 0.1 g/ml in 0.1 M acetate buffer–1.25 mM $MgSO_4$ (pH 5.0), and DNase and RNase (Sigma) were added to 1 μg/ml. Following the passage of ice-cold cells through a French pressure cell (SLM Instruments, Inc., Urbana, Ill.) at 32,000 lb/in^2, the disrupted cell suspension was centrifuged at $10^4 \times g$. The pellet was washed 3 times in ice cold 10 mM HEPES–2 mM $MgSO_4$ (pH 7.5) at approximately 0.1 g/ml, followed by three washes with HEPES–$MgSO_4$ buffer containing 0.1 M NaCl (pH 7.5). Microscopic examination of the material that was stained with crystal violet revealed an absence of intact cells. A portion of the particulate matter was suspended at 0.05 g/ml in PBS and sterilized with gamma irradiation (3×10^6 rads) while it was held on ice. Sterility checks were performed by plating 0.1 ml of the suspension on blood agar in triplicate and incubating for 2 days at 37°C. The sterile material was designated as irradiated cell wall and was stored at −20°C for later use in vaccine trials. The remainder of the particulate matter (approximately 100 g [wet weight]) was suspended to 0.1 g/ml in 2 M guanidine hydrochloride (containing 10 mM Tris, 10 mM EDTA, 10 μg of phenylmethylsulfonyl fluoride per ml, 0.02% sodium azide [pH 8.5]) and stirred slowly for 2 h at room temperature. The extracted insoluble particulate matter was removed by centrifugation, and the guanidine-soluble material was precipitated with 10 volumes of ice-cold methanol. The precipitate was dissolved in 2 M guanidine and filter sterilized, and precipitation with methanol was repeated. The precipitate, which was designated guanidine extract, was washed 2 times with 1 volume of distilled water and lyophilized. Electrophoretic analysis revealed that the material was essentially pure EA1 (data not shown).

Polysaccharide-peptidoglycan complex preparation. The insoluble particulate matter described above was suspended to 0.25 g/ml in 1% SDS, heated to 90°C for 5 min, and centrifuged for 10 min at 27,000 × *g*. The supernatant was discarded, and extraction of the upper white portion of the pellet was repeated 2 times with hot 1% SDS and 4 times with distilled water at 90°C, followed by lyophilization. Amino acid and amino sugar composition was determined on samples (1 mg/ml) that were hydrolyzed with 6 N HCl under nitrogen gas for 20 h at 100°C. The hydrolysates were lyophilized to remove the HCl and then analyzed with an amino acid analyzer (model 121 M; Beckman Instruments, Inc., Fullerton, Calif.) equipped with a resin and nanobore flow system (AA-10; Beckman). The instrument was operated according to the specifications of the manufacturer for single-column analysis of physiological fluids. Galactose

oxidase was used to quantitate galactose (Worthington Enzymes Manual, p. 21, 1972; Worthington Diagnostics, Freehold, N.J.) following hydrolysis at 90°C for 18 h with 0.2 M methanesulfonic acid, under nitrogen gas. The samples were diluted 1:10 in 1.0 M sodium phosphate buffer that was adjusted to pH 6.0 with NaOH before the assay was done.

Vaccinations and challenge. Female Hartley guinea pigs (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) received three, 0.5-ml intramuscular (i.m.) injections of the MDPH human vaccine at biweekly intervals. Animals vaccinated with the *B. anthracis* Sterne veterinary vaccine (Anvax; Wellcome Animal Research Laboratories) were given three i.m. injections at biweekly intervals that consisted of 2×10^6, 4×10^6, and 5×10^6 spores, respectively. Vaccinations with guanidine extract (partially purified EA1, 100 μg of PBS per ml) and irradiated cell wall (50 mg of PBS per ml) were administered as three 0.1-ml i.m. injections at biweekly intervals. Subsequent serum collection was performed by intracardiac puncture with a 23-gauge needle following i.m. injection of 30 mg of ketamine (Vetalar; Parke, Davis & Co., Morris Plains, N.J.) per kg and 6 mg of xylazine (Rompun; Haver-Lockhardt, Shawnee, Kans.) per kg. Sera were made to 50% glycerol before they were frozen at −20°C. For vaccine trials, immunized animals were challenged 4 weeks after the last booster with i.m. injection of 1000 Ames strain spores in 0.1 ml of PBS.

Antibody titer determinations. Quantitation of antibody titers to PA and EA1 in sera from guinea pigs was accomplished by using an enzyme-linked immunosorbent assays (ELISA). Purified PA was coated on 96-well, polystyrene, microtiter plates (Linbro) by incubating 100 μl of antigen (1 μg/ml in 50 mM sodium borate [pH 9.0]) in the wells for 2 h at 37°C. For EA1, 20 μl of guanidine extract (50 μg/ml in 2 M guanidine hydrochloride per ml, 10 mM Tris hydrochloride [pH 7.0]) was added to each well. Precipitation onto and binding of EA1 to the polystyrene wells was accomplished by decreasing the guanidine hydrochloride concentration through the addition of 250 μl of 50 mM borate buffer (pH 9.0) and incubation at 4°C for 18 to 20 h. For the polysaccharide ELISA, 100 μl of *o*-stearoyl-polysaccharide (9; prepared by R. J. Doyle), at 5 μg/ml of PBS, was added per well and incubated at 4°C for 18 to 20 h. Unreacted binding sites were blocked with quench buffer (10 mM sodium phosphate, 0.85% NaCl, 0.5% gelatin, 5% powdered milk [pH 7.5]) for 30 min at ambient temperatures. Wells were washed four times with PBST. Throughout the polysaccharide ELISA, Tween 20 was omitted and PBS–0.5% gelatin was used for all washes and dilutions. Antisera were titrated in PBSTG and incubated for 2 h at 37°C, and the wells were washed four times with PBST. Protein A-horseradish peroxidase conjugate, diluted 1:5,000 in PBST, was added (100 μl/ml); and plates were incubated for 30 min at 37°C. The wells were washed four times with PBST. Freshly prepared 2,2′-azinobis (3-ethylbenzthiazoline)sulfonic acid (ABTS; Sigma) substrate (100 μl per well) was added and incubated at ambient temperatures for 20 min. The concentration of ABTS was 1 mg/ml in 0.1 M citrate buffer (pH 4.0). Hydrogen peroxide was added to 0.003% just before use. The reaction was stopped with 50 μl of 10% SDS, and the plates were read at 405 nm in a microplate reader (EL308; Bio-Tek Instruments, Inc., Burlington, Vt.). Titers were taken as the last dilution which had an absorbance of ≥0.1.

Titers to polysaccharide-peptidoglycan complexes were determined by microagglutination. The purified material was suspended in PBS–0.02% sodium azide to 250 μg/ml and sonicated briefly at 4°C to achieve a smooth suspension. The suspension (50 μl) was added to 50 μl of sera that had been previously titrated in PBST. Assays were performed in round-bottom microtiter plates which had been blocked with 300 μl of quench buffer to prevent adsorption of the antibody to the plastic. After brief agitation of the plates to mix the samples, the plates were covered and incubated at ambient temperatures for 18 h. Titers were taken as the last dilution which had a diffuse precipitate.

Indirect fluorescent antibody staining. Whole blood was collected via intracardiac puncture, as described above, from bacteremic, terminally ill guinea pigs. Blood from such animals clots poorly or not at all just before death; therefore, no anticlotting agents (i.e., EDTA, heparin) were added. The blood was diluted in PBS to give 5 to 10 bacilli per ×1,000 oil immersion field following the addition of 5 μl per 5-mm-diameter well on multispot Teflon (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.)-printed microscope spot slides (PGC Scientific). The slides were air dried and fixed in methanol for 5 min. Each spot was washed twice with PBST and blocked with PBSTG for 30 min at 37°C, and 25 μl of sera that was diluted 1:1 in 2× PBSTG was added. After incubation at 37°C for 1 h in an enclosed container containing wet gauze, the spot wells were washed 4 times with PBST. Twenty-five microliters of goat anti-mouse IgG-IgA-IgM-fluorescein isothiocyanate conjugate (Cappel Laboratories, Cochranville, Pa.) diluted 1:200 in PBSTG was added and incubated at 37°C for 30 min, and the wells were washed 4 times with PBST. To reduce fading during fluorescence microscopy, cover slips were added by using a few drops of mountant containing 1,4-diazobicyclo-(2,2,2)-octane (Sigma) at 25 mg/ml in 10% PBS–90% glycerol (pH 7.4) (14).

RESULTS AND DISCUSSION

Comparison of sera from MDPH- and *B. anthracis* Sterne-vaccinated animals. Sera obtained from *B. anthracis* Sterne vaccinees recognized a 91-KDa protein (Fig. 1) in Sterne cell extracts, which is referred to as EA1 (Fig. 2 to 4). In

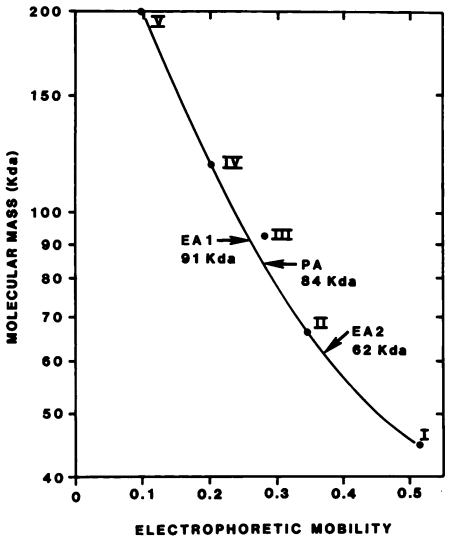


FIG. 1. Approximations of the molecular masses of EA1, EA2, and PA are indicated. Molecular mass protein standards were as follows: ovalbumin (45 KDa; I), bovine serum albumin (66.2 KDa; II), phosphorylase *b* (92.5 KDa; III), β-galactosidase (116.5 KDa; IV), and myosin (200 KDa; V).

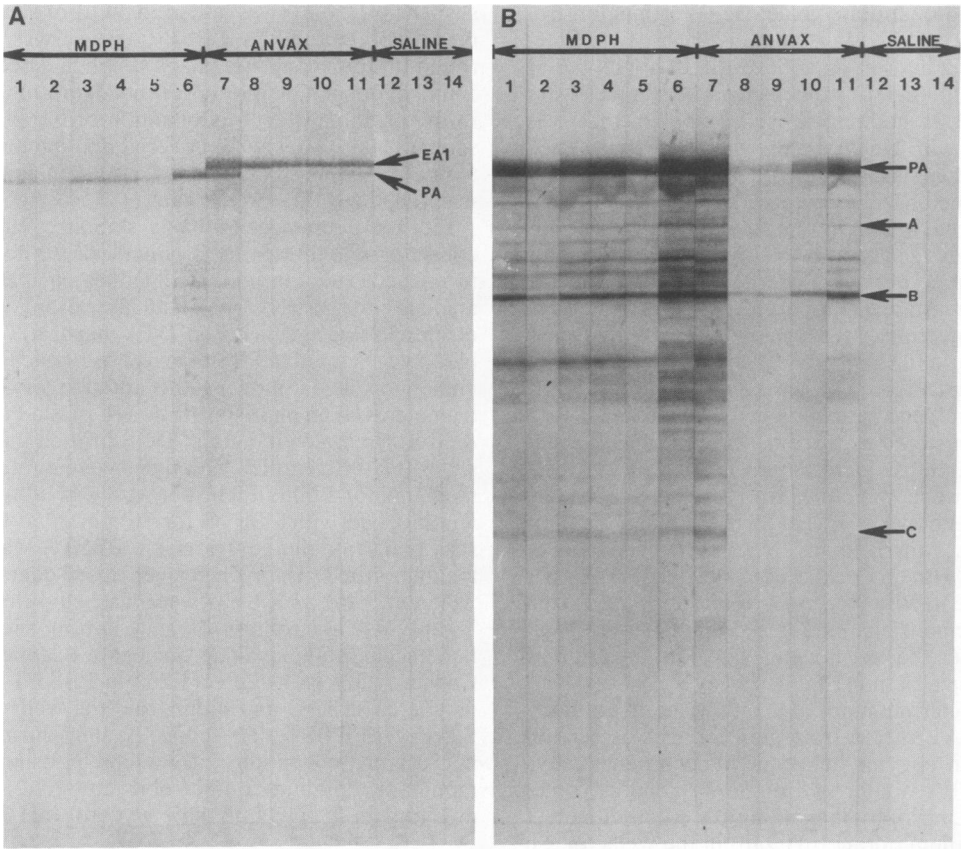


FIG. 2. (A) Comparison of sera from MDPH- and Sterne strain (Anvax)-vaccinated guinea pigs by EITB with SDS-extracted proteins from Sterne strain vegetative cells. (B) Comparison of sera by using culture supernatant proteins from the Sterne strain cells used for panel A. The strips were incubated with sera (diluted 1:100) obtained from animals 2 weeks after they were vaccinated with three injections of either MDPH human vaccine (strips 1 to 6) or the Sterne strain live spore vaccine (strips 7 to 11) at biweekly intervals. Strips 12 to 14 were incubated with sera from control animals injected with saline. Breakdown products of LF (protein A) and PA (proteins B and C) are indicated.

contrast, this protein was not recognized by sera from the MDPH-vaccinated animals. The same observations were made with cell extracts from the virulent Vollum 1B strain (Fig. 3) and *B. anthracis* Ames, New Hampshire, Colorado, and V770-NP-1R (data not shown).

The protective antigen was detected in the cell extracts (Fig. 2 and 3), thereby supporting the observations by Puziss and Howard (23) that PA could be extracted from whole cells. Animals vaccinated with the MDPH human vaccine had two- to fourfold higher titers to PA than did the animals vaccinated with *B. anthracis* Sterne (Anvax) (Table 1). This is consistent with the results of the electrophoretic immunotransblot (EITB) analyses, in that the relative intensities of PA staining by the sera from the Sterne strain-vaccinated animals were diminished as compared with those by the sera from MDPH-vaccinated animals (the identifying numbers for the sera shown in Table 1 correspond to the sera numbers in Fig. 2 and 3). Except where indicated, the results of EITB analyses of sera given in Fig. 2 and 3 were obtained from animals 2 weeks after vaccination. Based on the relative intensities of the bands, it appeared that EA1 rather than PA was initially the principal cell-associated antigen recognized by the Sterne strain-vaccinated animals. However, in guinea pigs that were vaccinated with the MDPH human vaccine, PA was the primary antigen that was recognized, as was also the case with antisera from MDPH-vaccinated humans (Fig. 3, lanes 1 and 2). EA1 did not appear to be a major component of the culture supernatant, but nevertheless, it was clearly present (Fig. 3B, lanes 7 and 8; see also Fig. 2B).

The PA protein in whole-cell extracts gave rise to several

TABLE 1. Antibody titers to PA for sera from MDPH- and Sterne strain-vaccinated guinea pigs

Strip no. in[a]:		Vaccine	Titer[b]
Fig. 2	Fig. 3		
1	16	MDPH	16,384
2	15	MDPH	16,384
3	14	MDPH	32,768
4	13	MDPH	32,768
5	12	MDPH	32,768
6	11	Supernatant[c]	ND[d]
7	10	Sterne strain	ND
8	9	Sterne strain	2,048
9	8	Sterne strain	2,048
10	7	Sterne strain	8,192
11	6	Sterne strain	16,382
12	5	Saline	0
13	4	Saline	0
14	3	Saline	0

[a] The strips are those identified in Fig. 2 and 3.
[b] Reciprocal titer as determined by ELISA.
[c] Animals vaccinated with culture supernatant fluid from Sterne strain cultures.
[d] ND, Not determined.

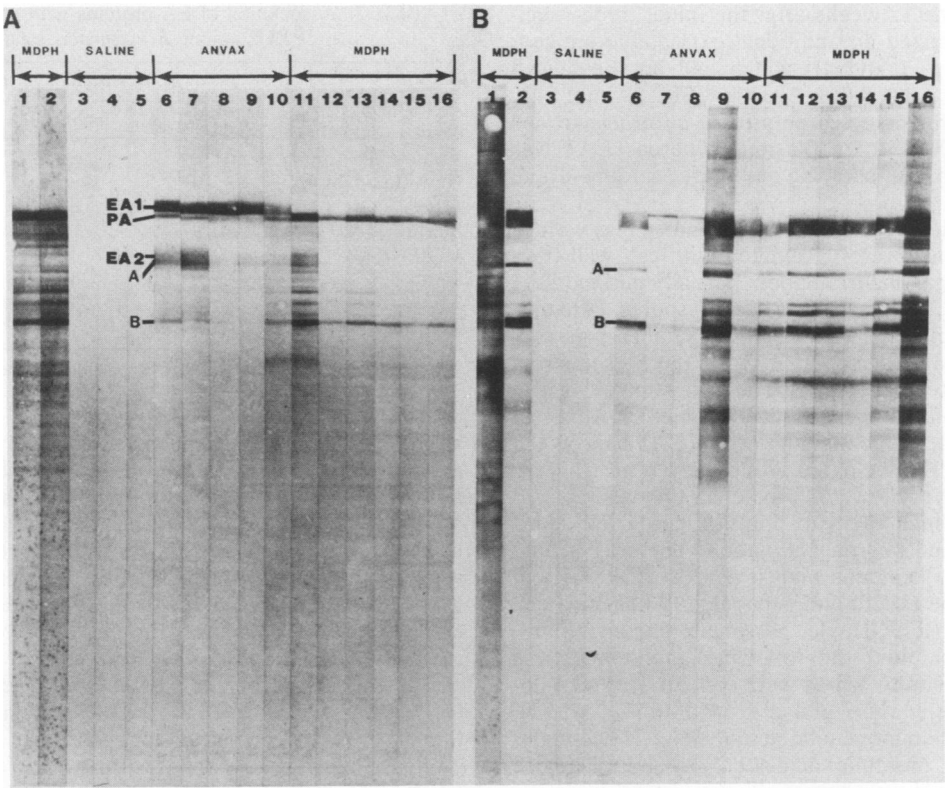
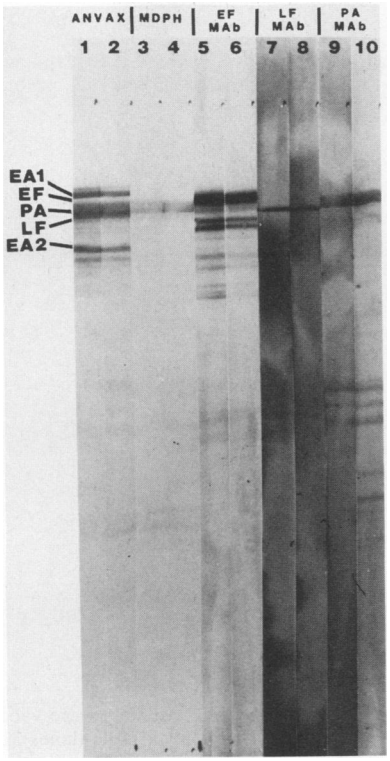


FIG. 3. (A) Comparison of sera from MDPH- and Sterne strain (Anvax)-vaccinated guinea pigs by EITB on SDS-extracted proteins from Vollum 1B strain vegetative cells. (B) Comparison of sera by using culture supernatant proteins from the Vollum 1B cells used for panel A. The protein preparations were treated as described in the legend to Fig. 2. The sera were from animals vaccinated with either the Sterne strain live spore vaccine (strips 6 to 10) or the MDPH human vaccine (strips 11 to 16). Strips 3 to 5 were incubated with sera from control animals that were given saline injections. Strips 1 and 2 were reacted with sera from two human subjects that had been vaccinated with 0.5 ml of the MDPH vaccine (three biweekly injections with booster vaccinations at 6 and 12 months). Breakdown products of LF (protein A) and PA (protein B) are indicated.



lower-molecular-weight entities, including those identified by the letters B and C in Fig. 2B, strips 2 and 4, which may be breakdown products that resulted from protease activity (35). These breakdown products reacted with MAb to PA (MAbs PA3-1F2-1-1 and PA2-2III-8C8-1-1; from S. Little, U.S. Army Medical Research Institute of Infectious Diseases), while another major band, labeled with the letter A (Fig. 2B and 3), was weakly stained by MAbs to LF (LFII-7E-6-2-1) and EF (EFII-SC-10) (data not shown). As shown in Fig. 4, strips 5 and 6, MAbs to EF, diluted 1:5 and 1:50, respectively, stained a protein that was slightly larger than PA but that was distinct from EA1 and EA2. In Fig. 4, strips 7 and 8 were stained with two MAbs to LF and strips 9 and 10 were stained with two MAbs to PA (diluted 1:50). In all cases, the toxin proteins were distinguishable from the EA proteins.

Sera from guinea pigs that had been boosted with the

FIG. 4. EITB analysis of Sterne cell-extracted proteins with sera from guinea pigs vaccinated with the Sterne strain (Anvax) (strips 1 and 2) and MDPH (strips 3 and 4). Strips 5 and 6 were reacted with the MAb to EF (MAb EFIII-9F5-1-1; tissue culture fluid) diluted 1:5 and 1:50, respectively. Two different MAb ascites fluid preparations specific for LF (MAb LFIII-2B2-1-2 and LFII-3E3-3-1) were used to stain strips 7 and 8, respectively. MAb preparations to PA (MAbs PAI-3B6-1-1 and PAI-2D3-3-1) were used to stain strips 9 and 10, respectively. The ascites fluid samples were diluted 1:50.

Sterne strain vaccine 12 weeks after the initial three-vaccination series recognized an additional extractable antigen, EA2 (62 KDa) (Fig. 1 and 4), which had not been well recognized by sera collected at 2 weeks (Fig. 2A and 3A). The EA2 protein was detected only in *B. anthracis* strains that carried the pXO1 toxin plasmid, whereas EA1 was detected in both plasmid-carrying and -cured strains (Table 2).

Vaccine trials with EA1. Five groups of guinea pigs with 10 animals in each group were vaccinated with cell wall fragments sterilized by gamma irradiation, partially purified EA1 (guanidine extract), viable Sterne strain spores (Anvax), MDPH human vaccine, or PBS (control). Guinea pigs vaccinated with cell wall fragments, EA1, or the Sterne strain produced antibody to EA1 (Fig. 5). The highest titers were in the group that received the cell wall (Table 3). Although there appeared to be some staining of the EA1 band when sera from the MDPH-vaccinated group were used (Fig. 5), the mean titer was very low. As predicted, animals injected with cell wall had high titers to polysaccharide, as determined by ELISA and microagglutination. The polysaccharide of *B. anthracis* contains high levels of galactose and *N*-acetylglucosamine (3, 20) and is covalently linked to cell wall peptidoglycan (R. J. Doyle, personal communication). The complexes exhibited the following amino acid and amino sugar compositions which were indicative of peptidoglycan and polysaccharide: glutamic acid (6.9%; wt/wt), alanine (6.8%), *m*-diaminopemilic acid (8.9%), *N*-acetylglucosamine (29.7%), muramic acid (9.1%), and galactose (24.3%). The material was essentially free of other amino acids, thereby indicating an absence of protein. There was a strong correlation between the titers determined by microagglutination and those determined by the polysaccharide ELISA (Table 3). The titer obtained with *Bacillus cereus* ATCC 9680 peptidoglycan material ranged from 1:8 and 1:16 in the animals that received irradiated cell wall, which suggests that the microagglutination titers presented in Table 3 are primarily against the polysaccharide portion of the complexes. Antibodies to PA and LF were very low in animals that received either cell wall or EA1, but were present in a high titer in Sterne strain-vaccinated animals. Guinea pigs that received the MDPH vaccine had the highest titers to PA, but produced relatively low titers to LF. All groups had low or nonexistent titers to EF, as determined by ELISA. To date, we have not demonstrated antibody to EF by EITB in laboratory animals vaccinated with the Sterne

TABLE 2. Association of EA proteins with plasmids pXO1 and pXO2 among *B. anthracis* strains

Strain[a]	Association of the following:			
	pXO1	pXO2	EA1	EA2
Vollum 1B	+	+	+	+
New Hampshire	+	+	+	+
Ames	+	+	+	+
Sterne	+	−	+	+
V770-NP-1R	+	−	+	+
ΔAmes	−	+	+	−
ΔNew Hampshire	−	+	+	−
ΔSterne	−	−	+	−

[a] The Δ indicates strains that have been cured of their pXO1 toxin plasmid.

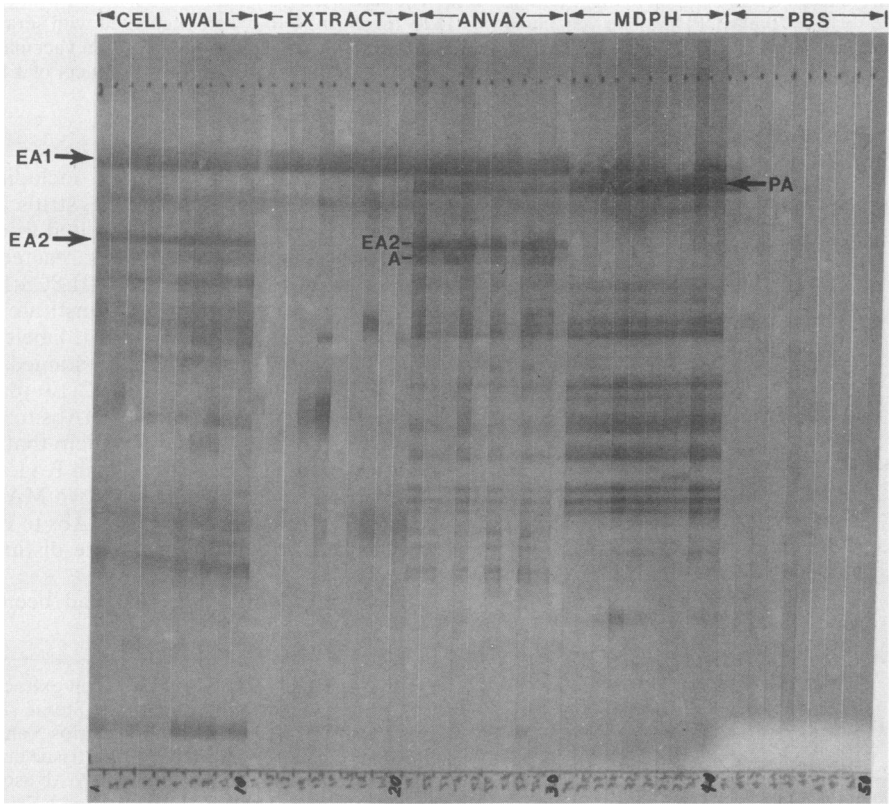


FIG. 5. EITB analysis of Sterne cell-extracted proteins using guinea pig sera from the EA1 vaccine trial. Animals were vaccinated with irradiated cell wall (lanes 1 to 10), guanidine extract (lanes 11 to 20), the Sterne strain (Anvax) (lanes 21 to 30), MDPH (lanes 31 to 40), and PBS (lanes 41 to 50).

TABLE 3. Vaccine trial with irradiated cell wall and quanidine-extracted EA1

Vaccine	Titers (geometric mean [SD]) of the following[a]:						Survival[b]	MTD[c] (days)
	PA	LF	EF	pCHO[d]	CW[e]	EA1		
Cell wall[f]	8 (7.8)	4 (1.9)	7 (5.0)	463 (1.6)	548 (1.8)	31,156 (2.6)	0/10	3.2
Extract[g]	7 (3.2)	1 (1.3)	2 (2.4)	43 (2.4)	9 (1.5)	8,184 (2.2)	0/10	3.4
Sterne strain	6,652 (1.8)	1,023 (1.8)	7 (4.0)	38 (2.4)	14 (4.0)	4,709 (1.9)	10/10	
MDPH	64,463 (1.6)	219 (3.8)	1 (1.3)	7 (8.1)	7 (2.8)	194 (2.5)	10/10	
PBS[h]	3 (4.0)	1 (1.0)	2 (2.3)	1 (1.9)	4 (1.2)	2 (2.8)	0/10	3.3

[a] Geometric mean reciprocal titer with geometric standard deviation.
[b] Survival is indicated by the number that survived/total number examined.
[c] MTD, Mean time to death.
[d] Titer determined by ELISA to *o*-stearoyl–polysaccharide cojugate.
[e] Titer determined by microagglutination of cell wall fragments consisting of peptidoglycan and polysaccharide.
[f] Irradiated cell wall.
[g] Guanidine-extracted antigens (predominantly EA1).
[h] PBS control.

strain or MDPH. The Sterne strain- and MDPH-vaccinated animals were completely protected, whereas the cell wall- and EA1-vaccinated animals were neither protected nor was their mean time to death extended (Table 3).

Indirect fluorescent antibody assays. Sera from animals used in the vaccine trial were tested by the indirect fluorescent antibody test on nonencapsulated Ames strain cells grown on blood agar and compared with the staining characteristics of encapsulated Ames cells obtained from bacteremic, terminally ill guinea pigs. Ames strain cells grown on blood agar were stained with sera from guinea pigs vaccinated with *B. anthracis* Sterne and guanidine extract in a manner characterized as intense patches or uniformly stained (data not shown). However, sera from animals vaccinated with irradiated cell wall, which had high titers not only to EA1 but also to polysaccharide, uniformly stained the vegetative cells. Interestingly, cells which became detached from the slides left EA1 on the slides in such a manner as to produce a ghost image of the cells when they were stained. In current studies with mouse MAb to EA1 (data not shown), we have shown that Ames strain cells from blood agar cultures are stained in a manner identical to that of sera from guinea pigs vaccinated with the guanidine extract. The EA1 MAbs also intensely stained the residue that was left on the slides following cell detachment, thereby providing additional evidence that EA1 is a surface antigen of nonencapsulated cells. Sera from animals vaccinated with irradiated cell wall and guanidine extract stained the ends and septa of Ames strain cells that were obtained from bacteremic guinea pig blood, but did not stain the surface of the capsule (Fig. 6A). This staining pattern was also noted with the MAb to EA1 (data not shown). As stated above, sera from cell wall-vaccinated animals uniformly stained nonencapsulated Ames and Sterne strain cells grown on blood agar. This staining pattern was probably due in part to the high antibody titers to polysaccharide, since MAbs to polysaccharide (currently under study) stain in an identical manner. These data are consistent with reports that the galactose-containing polysaccharide is present on the surface of *B. anthracis* cells grown on blood agar and readily binds galactose-specific lectins (4). As for Sterne strain-vaccinated animals, their sera stained the surface of the capsule and the septa (Fig. 6B; magnification is the same as that for Fig. 6A) of Ames cells that were obtained from infected blood. This suggests that antibody to a noncapsular antigen(s) of the Ames cells, other than EA1 or polysaccharide, is elicited by the Sterne strain cells in the Anvax vaccine. We propose that the lack of protection in animals which possess high titers to EA1 and polysaccharide may in part be a result of the fact that these antigens are masked by the capsule of virulent strains in infected animals.

Demonstration of the fact that high antibody titers to two major surface antigens of the Sterne strain vaccine neither afford protection nor extend the time to death supports the premise that antibody to PA plays a central role in protection

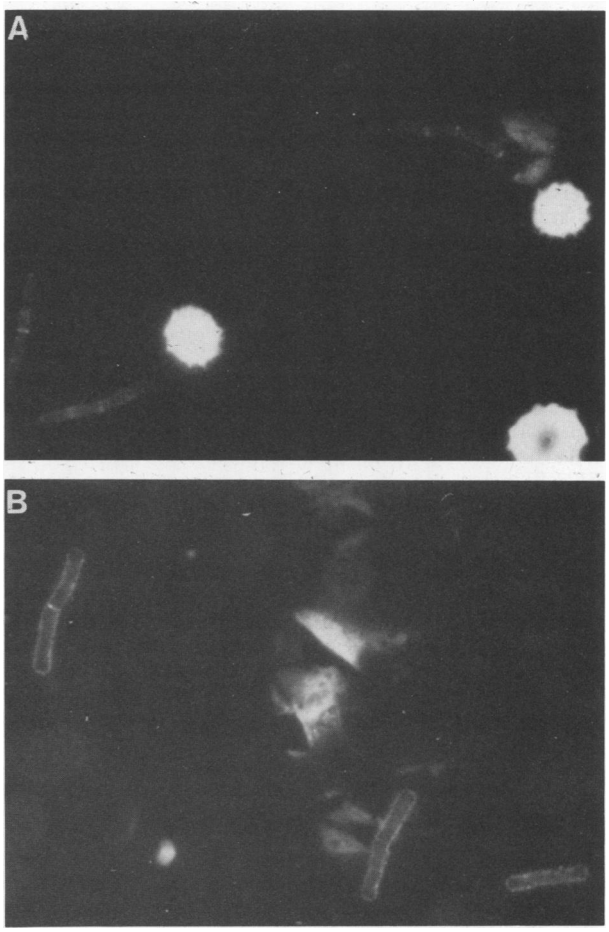


FIG. 6. Indirect fluorescent antibody staining of *B. anthracis* Ames strain cells from infected guinea pig blood. Cells stained with sera from animals vaccinated with guanidine extract (A) and the Sterne strain (B) are shown.

against anthrax and that other antigens may be of little consequence. These results agree well with the observations that live vaccines composed of *B. anthracis* strains which lack either both plasmids or just the pXO1 plasmid, and therefore do not produce PA, are not protective (10, 32). By contrast, *Bacillus subtilis* 1S53 clones that possess the gene encoding PA (11), when used as a live vaccine, provide protection against lethal challenge with virulent *B. anthracis* spores in guinea pigs and with lethal toxin in rats.

Alternative explanations for the differences in protection afforded by the two vaccines must be explored. For example, the presentation of PA to the immune system of the host by live spore vaccines may elicit an immune response (i.e., cell-mediated immunity) that is not obtained with aluminum hydroxide-adsorbed vaccines. Alternatively, crucial epitopes on the PA molecule, which are expressed on the PA released by live vaccines, may be either destroyed or masked when bound to aluminum hydroxide.

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