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The components of the Bacillus anthracis exotoxins, protective antigen (PA), lethal factor (LF), and edema factor (EF), from 24 isolates were separated by isoelectric focusing gel electrophoresis and detected by Western blot with monoclonal antibodies. Only two isoforms each were observed for PA and EF. Four isoforms were identified for LF. The biological activities of both lethal toxin and edema toxin were measured by using in vitro cell-based assays. This study provides another method of characterizing various isolates of B. anthracis by determining the isoelectric points of the exotoxin components and may be useful in the development of protective vaccines against B. anthracis infection.

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Western blot analysis of the exotoxin components from *Bacillus anthracis* separated by isoelectric focusing gel electrophoresis☆

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Abstract

The components of the *Bacillus anthracis* exotoxins, protective antigen (PA), lethal factor (LF), and edema factor (EF), from 24 isolates were separated by isoelectric focusing gel electrophoresis and detected by Western blot with monoclonal antibodies. Only two isoforms each were observed for PA and EF. Four isoforms were identified for LF. The biological activities of both lethal toxin and edema toxin were measured by using in vitro cell-based assays. This study provides another method of characterizing various isolates of *B. anthracis* by determining the isoelectric points of the exotoxin components and may be useful in the development of protective vaccines against *B. anthracis* infection.
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Keywords: *Bacillus anthracis*; Anthrax; Exotoxin; Isoelectric focusing gel electrophoresis

Two major virulence factors of *Bacillus anthracis*, a poly-γ-D-glutamic acid capsule and two binary exotoxins, lethal toxin (LeTx), and edema toxin (EdTx), are considered to be the major pathogenic factors of anthrax. The capsule is expressed by strains containing plasmid pXO2 (95 kb) [1] while the components of the exotoxins, protective antigen (PA; M_r 82.7 kDa), lethal factor (LF; M_r 90.2 kDa), and edema factor (EF; M_r 88.8 kDa) [2], are expressed by strains that contain plasmid pXO1 (184 kb) [3]. LeTx, composed of PA and LF, is cytotoxic for certain macrophage cell lines [4] and is lethal for most mammals. The target of LF is identified as mitogen-activated protein-kinase-kinase (MAPKK) [5,6]. EdTx is composed of PA and EF and causes edema due to the adenylate cyclase activity of EF when injected subcutaneously in various animals [7]. PA plays a pivotal role in the assembly of, and intoxication by, both toxins. Proteolysis of PA, before or after binding to a cell receptor [8–10], results in the loss of a 20-kDa amino-terminal fragment from PA, and exposes a site on the 63-kDa carboxyl-terminal fragment of PA (PA63) to which LF and EF competitively bind [11]. PA63 forms heptameric oligomers on the cell surface [12] and promotes internalization of the toxin complex [13] into the cell by receptor-mediated endocytosis [4,14]. In the acidic environment of the endosome, PA63 oligomers form a pore in the endosomal membrane through which LF and EF are transferred into the cytoplasm [15,16] to interact with their respective intracellular target(s).

Numerous isolates of *B. anthracis* have been described from environmental sources and infections of humans or animals. Strains that are $pXO1^+$, $pXO2^-$ are used as live spore vaccines for livestock (Sterne, Weybridge, or $34F_2$) [17] and humans (ST-I) [18,19]. Differences in the virulence of *B. anthracis* isolates exist, as demonstrated in survival studies with vaccinated guinea pigs after challenge and LD_{50} tests [20–24]. Heterogeneity of isolates of *B. anthracis* was revealed by multiple-locus variable-number tandem repeat analysis (MLVA) and genotyping of the PA gene (*pag*). MLVA was used to determine the copy number of variable-number tandem repeats (VNTR) at six chromosomal and one for each plasmid genetic loci [25]. Genotyping of *pag* identified five different point mutations from 26 different

☆ Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the US Army.

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isolates examined that resulted in three different amino acid sequences for PA [26]. In this report, the exotoxins, containing PA, LF, and EF proteins, from 24 isolates of *B. anthracis*, were separated by isoelectric focusing (IEF) gel electrophoresis followed by Western blot analysis with monoclonal antibodies specific for the individual components. Additionally, the in vitro biological activities of LeTx and EdTx from the isolates were determined.

Materials and methods

Purification of anthrax toxin. Anthrax exotoxin was purified from various isolates of *B. anthracis* from RM-medium culture supernatant fluids as described previously [27]. Bacterial colonies, obtained from 18 to 20 h growth on blood agar plates, were suspended in 150 ml RM-medium in 250-ml Erlenmeyer flasks and cultures were grown at 37 °C until confluent, usually 18–20 h. Bacteria were removed from the cultures by centrifugation at 10,000*g* and the supernatant fluid was passed through 0.2 μm cellulose acetate low-protein binding filters. The supernatant fluids were adjusted to pH 7.0 and supplemented with 0.2 mM 1,10-phenanthroline, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mM mercaptoethanol. Polyethylene glycol (PEG) 8000 (10% w/v; Fisher Scientific, Fairlawn, NJ) was dissolved in the supernatant fluids before adding hydroxylapatite (0.5% w/v; Calbiochem fast flow; Calbiochem, San Diego, CA). The suspensions were shaken on ice for 1–2 h, then incubated overnight at 4–6 °C. The hydroxylapatite resin was collected onto an econo-column (Bio-Rad Laboratories, Hercules, CA) and washed with 10 mM Tris–HCl, pH 7.5. The toxin components were eluted with 0.66 M potassium phosphate, pH 7.0, 0.01 M EDTA, concentrated using a Centricon 30 device (Amicon, Beverly, MA), dialyzed against 0.01 M Tris–HCl, pH 8.0, 2 mM EDTA, and maintained at −70 °C. Protein concentrations were determined from triplicate readings using the Bio-Rad microplate protein assay (Bio-Rad Laboratories).

Isoelectric focusing gel electrophoresis and Western blots. IEF gel electrophoresis was performed in 5.5% polyacrylamide gels by using the Hoefer SE-600 [28]. Gels (140 mm × 140 mm × 0.5 mm) were prepared to contain final concentrations of 5.5% acrylamide (29.2% C, 0.8% T), 10% glycerol, and either 2.0% pH 3–10 ampholines, 0.5% pH 5–7 ampholines for native IEF, or 2.4% pH 3–10 ampholines for denatured IEF (BioLyte ampholines; Bio-Rad Laboratories). After the polyacrylamide gel solution was degassed for 10 min, polymerization was achieved by adding 0.2% Temed (Bio-Rad Laboratories) and 0.04% ammonium persulfate (Bio-Rad Laboratories). For denatured IEF gels, Triton X-100 (1.5%), prepared from a 30% stock containing AG 501-X8 resin (Bio-Rad Laboratories), was added after degassing. Gels were polymerized at room temperature overnight. Gels were prefocused for 100 V h and were run for 3000 V h under 3000 $V_{max,}$ 80 mA_{max}, and 100 W_{max} at 15–20 °C. The anode buffer was 10 mM glacial acetic acid and 20 mM NaOH was used as the cathode buffer. Samples were diluted with 10 mM Tris–HCl, 2 mM EDTA, pH 8.0, buffer plus an equal volume of sample buffer, which consisted of 30% glycerol, 2.4% ampholines, pH 3–10, to yield a protein concentration of about 200 μg/ml. Focused proteins were electrophoretically transferred to 0.45-μm nitrocellulose membranes in 0.7% acetic acid and reversing the leads to the power supply which was set at 100 V for 1 h at 15–20 °C. After the transfer, nitrocellulose membranes were blocked with 5% milk and 0.1% Tween 20 in PBS before incubating with monoclonal antibodies (mAbs). To detect PA, membranes were incubated in mAb PA 14B7 [29]. To simultaneously detect LF and EF, the membranes were incubated in both mAbs LF 3F6 and EF 9F5 [30,31]. The membranes were rinsed three times in PBST for 5 min each and then incubated with HRP-labeled goat anti-mouse IgG (Kirkegaard & Perry, Gaithersburg, MD) for 1 h. Reactive bands were detected by enhanced chemiluminescence (ECL; Amersham–Pharmacia Biotech, Piscataway, NJ) as directed by the manufacturer. The isoelectric points (p*I*s) of the test proteins were determined by linear regression analysis (StandardCurve!Plus, ChemSW Software, Fairfield, CA) using prestained IEF standards (Bio-Rad Laboratories).

Cytotoxicity assay. The in vitro cytotoxicity of the toxin preparations was determined by measuring the viability of J774A.1 cells, a murine macrophage-like cell line, after exposure to toxin preparations. On the day before the experiment, J774A.1 cells were plated in 96-well microtiter plates at 1×10^5 cells/well in Dulbecco's minimal essential medium with high glucose, 5% heat-inactivated fetal bovine serum, 25 mM Hepes, and 100 U penicillin and 100 μg streptomycin/ml. Dilutions of each toxin preparation were incubated with the cells for 4 h at 37 °C and then for an additional 2 h after adding 25 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) at 5 mg/ml PBS to each well. After incubating for 2 h, the cells were lysed and the reduced product was solubilized by adding 20% SDS in 50% dimethylformamide [32]. Absorbance values were obtained using a Bio-Tek model 312a microplate reader (BioTek Instruments, Winooski, VT) at $A_{570-690\,nm}$. The protein concentration that resulted in 50% cytotoxicity was determined by four-parameter logistic regression analysis (SoftMax Pro, Molecular Devices, Sunnyvale, CA) where $y = (A - D)/(1 + (x/C_B)) + D$ and '*C*' was the midpoint of the curve. Results represent averages from two assays with triplicate samples.

Cyclic adenosine monophosphate assay. EdTx activity was measured by determining the expression of cyclic adenosine monophosphate (cAMP) in Chinese hamster ovary (CHO) cells exposed to the adenylate cyclase activity of EF [7]. CHO cells were cultured in 96-well cell culture plates at 1×10^5 cells/well in Eagle's minimal essential medium with 4 mM glutamine, 5% heat-inactivated fetal bovine serum, and 25 mM Hepes 20–24 h before testing. The next morning, CHO cells were exposed to toxin preparations for 2 h in 200-μl volumes. Total extra- and intracellular cAMP was measured by a Biotrack cAMP enzyme immunoassay (EIA) kit (Amersham–Pharmacia Biotech) as directed. Absorbance readings were obtained using a BioTek EL312a microplate reader (BioTek Instruments) and concentrations were calculated from the dilutions by four-parameter logistic regression analysis of the standard curve (KC4 software; BioTek Instruments). Results, from a single assay with duplicate samples, are expressed as picomoles of cAMP per microgram of protein. Background cAMP levels were 0.159 ± 0.0993 pmol cAMP in five assays.

Results and discussion

Multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA) of six chromosomal (*vrr*A, *vrr*B_1, *vrr*B_2, *vrr*C_1, *vrr*C_2, and CG3) and two plasmid (pXO1-aat and pXO2-at) open reading frames (ORFs) can be used to differentiate between *B. anthracis* isolates [25]. Additionally, five point mutations have been identified in the gene encoding for PA (*pag*) among the 26 isolates examined that translated into three different amino acid sequences for PA [26]. We report here on differences in the p*I*s of the components PA, LF, and EF from the exotoxins of 24 isolates of *B. anthracis* obtained from different geographical regions by using Western blot analysis of proteins separated by vertical IEF gel electrophoresis.

The 24 *B. anthracis* isolates examined in this study are listed in Table 1. Cultures were grown in sealed flasks

Table 1
Description of *B. anthracis* isolates used in this study

Isolate	Description	Reference
Sterne ($34F_2$)	US veterinary vaccine and UK chemical vaccine strain, 1936	[17]
ST-I	Russian live vaccine strain, 1940	[18,19]
V770-NP1-R	US chemical vaccine strain; non-proteolytic rough mutant of V770, 1954	[40]
Texas	Cow, Texas, 1980	R.D. Welsh, Texas A&M
Goldberg	1958	G.G. Wright
Kansas 7322	Cow; Kansas, 1954	[20]
17T5	Kudu: South Africa, 1957	[22]
Vollum 1B	Derived from Vollum, 1957	[41]
V770	Cow; Florida, 1951	[20]
Buffalo	Buffalo; Dickinson county, Iowa, 1979	[22]
Ames	Cow; Texas, 1981	[22][a]
NH	Human; New Hampshire, 1957 (non-vaccinated mill worker)	[42]
Zimb-1 BA1086	Human; Zimbabwe, 1982	M. Hugh-Jones, LSU
Namibia 93/60B	Wildebeest; Etosha National Park, 1993	M. Hugh-Jones, LSU
Norway B6273/93	Cow; Norway, 1993	M. Hugh-Jones, LSU
Ohio ACB	Human; Ohio, 1952	[20]
Colorado	Human; Colorado, 1980	A. McChesney, CSU
Turkey #6/6	Goat; Turkey, 1995	M. Hugh-Jones, LSU
SK31	Wildebeest; S. Africa, 1974	CDC, [22]
SK61	Human inhalation; California, 1976 from contaminated Pakistan yarn	CDC, [22]
SK102	Pakistan yarn; New Jersey, 1976 (related to SK61)	CDC, [22]
SK128	Ireland yarn; Massachusetts, 1976	CDC, [22]
SK162	Goat skin rug from Haiti, 1976	CDC, [22]
SK465	Buffalo meat; Clay county, Iowa, 1979	CDC, [22]

[a] Although the geographic origin of the Ames isolate was originally identified as Iowa, it has subsequently been identified as Texas.

containing RM-medium [27] for 18–20 h, which coincided with stationary phase growth [33]. Two isolates, 17T5 and Zimbabwe, required longer growth times (24–26 h) or a heavier inoculum to achieve sufficient growth within 18–20 h (data not shown). The exotoxins were isolated from the culture supernatants according to published procedures [27] and subjected to vertical IEF gel electrophoresis, either without Triton X-100 to focus PA or with Triton X-100 for separation of LF and EF. Proteins were detected by enhanced chemiluminescence (ECL) Western blot analysis with mAbs specific for the individual components, PA, LF, or EF (Figs. 1 and 2). These mAbs were previously reported to neutralize the biological activity of the respective exotoxin [29–31] and were used to ensure the presence of functional epitopes on each protein.

PA was detected by using mAb PA 14B7 which recognizes an epitope within the carboxy-terminal portion of PA that is involved in binding of PA to the cell receptor [29]. All the isolates of *B. anthracis* that were examined had an immunoreactive band to PA with a p*I* of ca. 5.4 (Fig. 1). An exception was 17T5, which had a major immunoreactive band at p*I* of 5.6 (Fig. 1). Most of the isolates also had minor immunoreactive bands at a p*I* of ca. 5.2 (Sterne, ST-I, Texas, Goldberg, Vollum 1B, Buffalo, Ames, NH, Zimbabwe, Namibia, Ohio ACB, SK31 SK 128, and SK 465) and/or 5.6 (Sterne, NH and Namibia). 17T5 had a minor immunoreactive band at a p*I* of 5.4. The reason why the p*I* of PA from 17T5 was greater than those from the other isolates examined in this study is not known at this time. The p*I* of PA from Sterne was reported previously as 5.5 from protein-stained horizontal IEF gels [34].

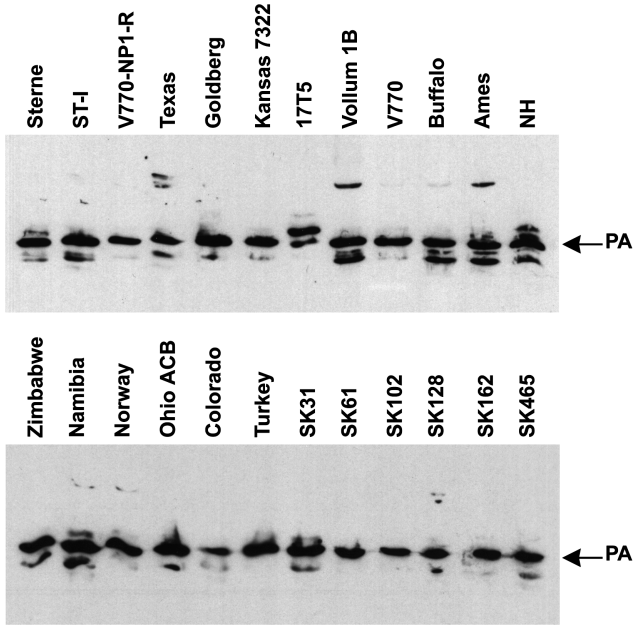

Fig. 1. Western blot analysis of anthrax exotoxins separated by native IEF and probed with mAb PA 2II 14B7.

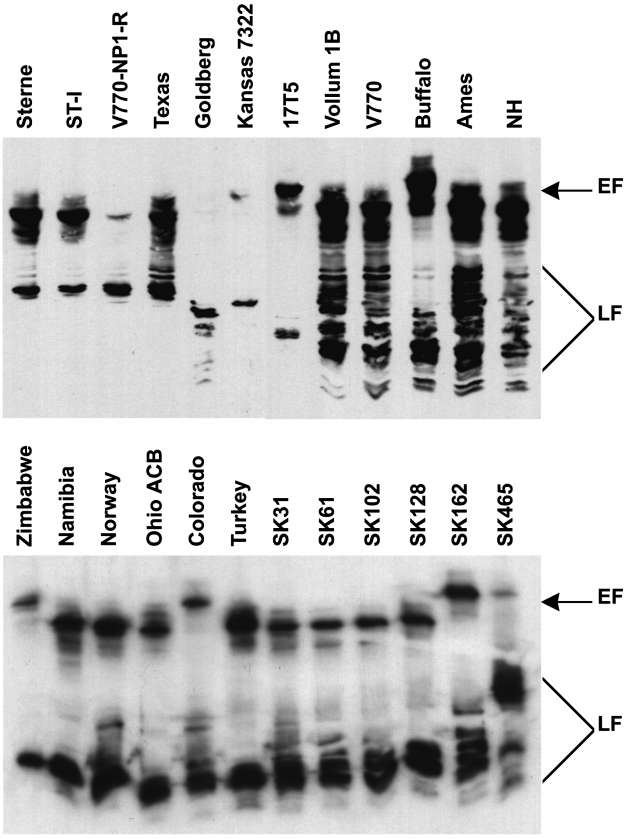

Fig. 2. Western blot analysis of anthrax exotoxins separated by denatured IEF and probed with mAbs LF I 3F6 and EF III 9F5.

Western blots for LF were performed with mAb LF 3F6. This mAb neutralizes LeTx cytotoxicity in vivo but not in vitro [30]. The epitope for this hybridoma has not been mapped. All the isolates of *B. anthracis* that were examined contained LF reactive bands (Fig. 2). Four LF isoforms were identified. The first, represented by Sterne, ST-I, V770-NP1-R, Texas, and SK465, consisted of immunoreactive band(s) at a p*I* of ca. 5.9. The second, represented by Goldberg and Kansas 7322, had immunoreactive bands that displayed a p*I* of ca. 5.5. LF from the third isoform pattern, displayed by 17T5 and Zimbabwe, had a p*I* of ca. 5.4. The fourth isoform pattern, represented by the majority of the isolates (15/24, 62.5%; Vollum 1B, V770, Buffalo, Ames, NH, Namibia, Norway, Ohio ACB, Colorado, Turkey, SK31, SK61, SK102, SK128, and SK162) had a major immunoreactive band at a p*I* of ca. 5.3. Some of these isolates (Vollum 1B, V770, Buffalo, Ames, NH, and SK162) also had more broadly focused immunoreactive bands that may have been a result of protease activity in the cultures of these isolates before the exotoxins were collected. The p*I* of LF from Sterne was reported previously as 5.8 from protein-stained horizontal IEF gels [34].

MAb EF 9F5, which recognizes an epitope within the amino-terminus of EF and neutralizes EdTx activity by blocking the binding of EF to cell-bound PA [31], was used to detect EF (Fig. 2). The majority of isolates (16/24, 66.7%; Sterne, ST-I, V770-NP1-R, Texas, Vollum 1B, V770, Ames, NH, Namibia, Norway, Ohio ACB, Turkey, SK31, SK61, SK102, and SK128) had a major immunoreactive band at a p*I* of ca. 6.6. The immunoreactive band of V770-NP-1-R was not as prominent as that from the other isolates. Five isolates, 17T5, Buffalo, Zimbabwe, Colorado, SK162, had an immunoreactive band at a p*I* of ca. 6.8 while Kansas 7322 and SK465 had a weakly immunoreactive band at this p*I*. One isolate examined, Goldberg, was characterized by an absence of an EF immunoreactive band. The p*I* of EF from Sterne was reported previously as 5.9 and 6.4 from protein-stained horizontal IEF gels [34].

The cytotoxicity assay and the cAMP ELISA were performed to determine if the components necessary for LeTx and EdTx were present in a biologically active form in each isolate preparation. LeTx cytotoxic activity from the various preparations was measured by determining the protein concentration (ng/ml) that resulted in 50% cell death as measured by the cellular reduction of MTT by viable J774A.1 cells (Table 2). All isolates examined expressed biologically active PA and LF as determined by cytotoxicity to J774A.1 cells. The LeTx activity ranged from 21.2 to 112.6 ng/ml. The relative activity of EdTx was evaluated by an ELISA that measured the level of cAMP in CHO cells after being exposed to the adenylate cyclase activity of EF (Table 2). Seven isolates expressed low levels of cAMP (i.e., <1 pmol/μg protein; V770-NP1-R, Texas, Goldberg, Kansas 7322, Colorado, SK31 and SK465). Of those seven isolates, three had very weak immunoreactive bands by Western blot (V770-NP1-R, Kansas 7322, and SK465) and one lacked an immunoreactive EF band (Goldberg). Because mAb EF 9F5 recognizes an epitope involved in binding of EF to PA63, the absence of a reactive band in the Western blot suggests an absence of that epitope in these isolates. The absence of an epitope on EF involved in binding to PA may result in a decrease in or lack of adenylate cyclase activity in vitro. However, three other isolates (Texas, Colorado, and SK31) had immunoreactive bands in the Western blot that were comparable with the others, but their adenylate cyclase activity in vitro was between 0.27 and 0.80 pmol/μg protein. These results suggest a decrease in the enzymatic activity of EF from these isolates. For the remaining 17 isolates, the amount of cAMP ranged from 2.87 to 17.70 pmol/μg protein.

A plot depicting LeTx cytotoxicity on the abscissa (ng protein/ml at 50% viability) and EdTx adenylate cyclase activity on the ordinate (pmol cAMP/μg protein) suggests that there is no correlation between the biological activities of the two exotoxins (R(Spearman) $= -0.190$), $p = .371$ (SigmaStat, Systat Software, Point Richmond, CA) (Fig. 3). Additionally, there does not appear to be a

Table 2
Approximate p*I* and relative biological activity of LeTx and EdTx from various *B. anthracis* isolates

Isolate	Approximate p*I*			Cytotoxicity (ng/ml)[a]	cAMP (pmol/μg protein)[b]
	PA	LF	EF		
Sterne	5.4	5.9	6.6	49.7	9.62
ST-I	5.4	5.9	6.6	28.1	7.48
V770-NP1-R	5.4	5.9	6.6[c]	40.7	0.36
Texas	5.4	5.9	6.6	92.1	0.27
Goldberg	5.4	5.5	neg	42.5	0.09
Kansas 7322	5.4	5.55	6.8[c]	44.5	0.19
17T5	5.6	5.4	6.8	63.5	2.87
Vollum 1B	5.4	5.3	6.6	21.6	4.83
V770	5.4	5.3	6.6	44.0	10.00
Buffalo	5.4	5.3	6.8	78.8	17.70
Ames	5.4	5.3	6.6	21.2	8.87
NH	5.4	5.3	6.6	30.2	10.01
Zimbabwe	5.4	5.4	6.8	112.6	5.71
Namibia	5.4	5.3	6.6	32.6	6.99
Norway	5.4	5.3	6.6	45.1	12.76
Ohio ACB	5.4	5.3	6.6	37.4	4.15
Colorado	5.4	5.3	6.8	45.1	0.39
Turkey	5.4	5.3	6.6	40.5	8.20
SK31	5.4	5.3	6.6	36.9	0.80
SK61	5.4	5.3	6.6	51.8	4.62
SK102	5.4	5.3	6.6	49.9	4.43
SK128	5.4	5.3	6.6	35.7	4.53
SK162	5.4	5.3	6.8	26.3	16.08
SK465	5.4	5.9	6.8[c]	43.5	0.18

[a] Amount of LeTx (ng protein/ml) that demonstrated half-maximal cytotoxicity (50%) of J774A.1 cells in vitro. Average of two assays, each performed in triplicate ($r^2 > 0.9500$).

[b] Amount of cAMP (pmol/μg protein) stimulated by EdTx on CHO cells. Results are from a single assay performed in duplicate.

[c] Weakly reactive band at given p*I*.

relationship between LeTx cytotoxicity or EdTx adenylate cyclase activity with the ability of certain *B. anthracis* isolates to kill guinea pigs after vaccination [20–24]. For example, Zimbabwe and Ames, both identified as isolates that can override immunity in guinea pigs, had comparable EdTx adenylate cyclase activity levels (5.71 and 8.87 pmol/μg protein, respectively) but differed in their levels of LeTx cytotoxicity (112.6 and 21.2 ng/ml, respectively). Similarly, both 17T5 and Buffalo isolates, which also can override immunity in guinea pigs, had somewhat similar LeTx cytotoxicity levels (63.5 and 78.8 ng/ml, respectively) but differed in their levels of EdTx adenylate cyclase activity (2.87 and 17.70 pmol/μg protein, respectively). Finally, Namibia, an isolate that can overcome preexisting immunity in guinea pigs, and ST-I, a live vaccine isolate, had comparable LeTx cytotoxicity levels (32.6 and 28.1 ng/ml, respectively) and EdTx adenylate cyclase activity (6.99 and 7.48 pmol/μg protein, respectively). Thus, the ability of some isolates to override immunity in guinea pigs may be dependent upon other *B. anthracis* virulence factors, capsule production [35], surface composition of the spores as well as physiological characteristics of the animal itself.

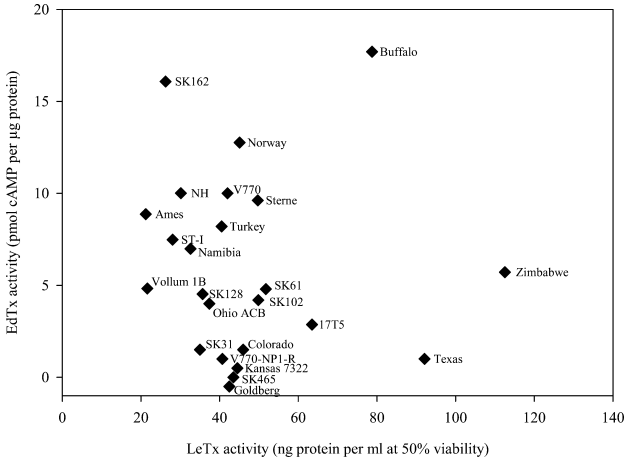

Fig. 3. Relationship between LeTx cytotoxicity and EdTx adenyl cyclase activity of various isolates of *B. anthracis*. To allow visualization of overlapping data points, data from some isolates were changed slightly.

The human anthrax vaccine used in the United States, AVA Biothrax (previously known as MDPH-PA or AVA), is prepared by adsorbing filtered culture supernatants of V770-NP1-R to aluminum hydroxide gel (Alhydrogel). The major immunogen in AVA Biothrax is PA [36] and protection against infection or intoxication requires PA [37,38]. The results of this study support the use of a PA-based vaccine in that there was very little variation in the p*I* of PA and that the epitope recognized by mAb PA 14B7 was present in all the isolates examined. The United Kingdom prepares their human anthrax vaccine, anthrax vaccine precipitated (AVP), from cultures of the Sterne isolate which are adsorbed to potassium aluminum phosphate (alum). ELISA titers of guinea pigs injected with MDPH-PA showed no immunological activity to EF, whereas after vaccination with AVP, antibodies against EF were

Table 3
Separation of *B. anthracis* isolates based upon the p*I* of PA, LF, and EF

PA	EF	LF	Isolate
5.6	6.8	5.4	17T5
		5.9	SK465
	6.8	5.5	Kansas 7322
		5.4	Zimbabwe
		5.3	Buffalo, Colorado, SK162
5.4		5.9	Sterne, ST-I, V770-NP1-R, Texas
		5.5	Goldberg[a]
		5.4	
	6.6	5.3	Vollum 1B, V770, Ames, NH, Namibia, Norway, Ohio ACB, Turkey, SK31, SK61, SK102, SK128

[a] Goldberg arbitrarily assigned to group with greatest number of isolates, although no EF was readily detected by Western blot.

measured [39]. The absence of EF from the AVA Biothrax may have occurred during the preparation of the vaccine (i.e., during filtration of the culture supernatant fluids) or EF was not present in the culture. Our results suggest that the absence of anti-EF antibody titers resulted from the very low levels of EF expressed by the V770-NP1-R strain. V770-NP1-R is a non-proteolytic, noncapsular mutant strain derived from V770 [40]. PA and EF antigens from V770 and V770-NP1-R each had similar p*I*s. However, the isoelectric pattern of LF from V770-NP1-R had a pI of 5.9 and V770 at had a p*I* of 5.3 with noticeable proteolysis evident. V770-NP1-R and V770 isolates had similar LeTx cytotoxic activity (40.7 and 44.0 ng/ml, respectively) but differed in the cAMP activity (0.36 and 10.0 pmol/μg protein, respectively).

Separation of the isolates based upon the p*I* of the individual components is shown in Table 3. The table divides the isolates, first on the p*I* of PA (either 5.6 or 5.4) then EF (either 6.8 or 6.6). Goldberg was arbitrarily assigned a pI of 6.6 for EF, the largest group, although no EF was readily detected by Western blot. Further studies examining the basis for differences in virulence among isolates and characterizing the components of the exotoxins are anticipated.

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