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Immunoelectrophoretic Analysis, Toxicity, and Kinetics of In Vitro Production of the Protective Antigen and Lethal Factor Components of *Bacillus anthracis* Toxin

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The kinetics of *Bacillus anthracis* toxin production in culture and its lethal activity in rats, mice, and guinea pigs were investigated. Lethal toxin activity was produced in vitro throughout exponential growth at essentially identical rates in both encapsulated virulent and nonencapsulated avirulent strains. The two toxin proteins which produce lethality when in combination, lethal factor (LF) and protective antigen (PA), could be quantitated directly from culture fluids by rocket immunoelectrophoresis. Using purified preparations of these proteins, we determined that a combination of 8 μg of LF and 40 μg of PA was required for a maximal rate of killing (39 to 40 min) in Fischer 344 rats (250 to 300 g). Conversely, a minimum of 0.6 μg of LF and 3 μg of PA was required for lethality. The 50% lethal dose for Hartley guinea pigs was 50 μg of LF and 250 μg of PA, and for Swiss mice it was 2.5 μg of LF and 12.5 μg of PA. Analyses classically reserved for enzyme kinetic studies were used to study the kinetics of lethal activity in the rat model after intravenous injection of LF-PA mixtures. The amounts of LF and PA which were required to give half the rate of killing (i.e., double the minimum time to death) were 1.2 and 5.8 μg, respectively. A theoretical minimum time to death was determined to be 38 min. A third anthrax toxin component, edema factor, was shown to inhibit lethal toxin activity. Edema factor could not be quantitated by rocket immunoelectrophoresis because the protein did not form distinct precipitin bands with available antisera.

Bacillus anthracis has been shown to possess two primary virulence factors: a poly-D-glutamic acid capsule (30) and a tripartite toxin (2, 20) composed of edema factor (EF), protective antigen (PA), and lethal factor (LF). In experimental animals, intradermal injection of a PA-EF mixture produces edema (25), which is probably associated with the adenylate cyclase activity of EF (14). The combination of PA and LF, however, produces lethality in some species (2, 6), apparently through a fluid loss resulting from increased capillary permeability (1). It has been postulated that death is attributable to respiratory distress resulting from fluid loss into the lungs and to circulatory embarrassment due to pressure from fluid loss into the mediastinum (19). Although it is generally accepted that the toxin components are inactive individually, PA alone has been reported to produce transient alterations in neurological and cardiovascular functions in monkeys and chimpanzees (26). It is presently unclear whether EF plays a role in the lethal activity of anthrax toxin (2, 19, 21). Although it is not as potent as diphtheria, botulinum, tetanus, or certain other bacterial toxins (9), the toxin of *B. anthracis* has nevertheless been implicated in the anthrax disease process (15).

It has been reported that anthrax toxin produced in *B. anthracis* culture fluid is found only after the concentration of chains or CFU reaches ca. 10^8/ml (12, 22). At this concentration, *B. anthracis* is in the stationary phase of growth (18). Therefore, due to reports that anthrax toxin is produced only during the stationary phase of growth, it has been considered to be a product of secondary metabolic activity (27, 28).

Little information is available concerning the amounts of the individual toxin components that are produced in vitro (29). Furthermore, in the Fischer 344 rat, which is classically used to determine the potency of anthrax toxin preparations (2), the minimum amount of toxin required for rat lethality and the minimum time to death (MTTD) for rats injected with large quantities of toxin have not been clearly established. The requirement of the simultaneous presence of PA and LF to produce lethal toxin activity was demonstrated by Fish et al. (8). These workers demonstrated that as a constant amount of LF was combined with increasing amounts of PA, the time to death (TTD) reached a minimum and then increased. When increasing amounts of LF were added to a constant amount of PA, the TTD became shorter. However, the unavailability of highly purified PA and LF preparations prevented these and other investigators from determining the optimal proportions of components for lethal activity (2).

The present studies were performed to (i) correlate production of anthrax toxin as measured by rat lethality with production of PA and LF measured immunochemically, (ii) determine the MTTD in a Fischer 344 rat injected with large quantities of PA and LF, (iii) define the minimum amounts of LF and PA required for rat lethality, and (iv) determine the effect of the addition of EF on the lethal toxic activity of mixtures of LF and PA. We additionally sought to demonstrate the value of rocket immunoelectrophoresis (RIE) for the quantitation of anthrax toxin components.

MATERIALS AND METHODS

Bacterial strains and media. *B. anthracis* strains Sterne, Vollum 1B, V770, and V770-NP1-R were obtained from the culture collection of the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md; *B. anthracis* Ames (bovine isolate) was obtained from the U.S. Department of Agriculture, Ames, Iowa; the Colorado strain

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(human isolate) was obtained from A. McChesney, Colorado State University, Fort Collins, Colo.; and the Texas strain (bovine isolate) was obtained from R. D. Welsh, Texas A & M University, College Station, Tex. Plasmid-cured *B. anthracis* ΔVollum 1B, ΔSterne, and ΔV770-NP1-R are derivatives of Vollum 1B, Sterne, and V770-NP1-R, respectively. These strains were cured of their resident plasmid by successive in vitro culture passages at 42.5°C (16). All strains were stored frozen at −70°C in brain heart infusion (Difco Laboratories, Detroit, Mich.) plus 12% glycerol and were revived by culture on sheep blood agar at 37°C.

Preparation of R medium has been previously described (18). RHS medium was R medium supplemented with 5% sterile horse serum (GIBCO Laboratories, Grand Island, N.Y.). All liquid media were sterilized by filtration. Plates of Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) were used for determination of viable counts. Culture of *B. anthracis* and production of small quantities of crude toxin were performed essentially as previously described (18). Briefly, individual colonies from cultures on sheep blood agar were inoculated onto R medium agar plates and incubated for 12 h at 37°C. Several colonies were suspended in 10 ml of R medium to 1×10^6 to 2×10^6 CFU/ml (ca. 0.01 absorbance units at 540 nm, in a 1.0-cm light path on a Coleman Junior spectrophotometer). A portion of this suspension (0.25 ml) was inoculated into 250-ml screw-capped flasks containing 125 ml of R medium. The flasks were sealed, and the cultures were incubated at 37°C with 90 reciprocations per min. Cultures were sampled at various stages of growth for determination of viable count, turbidity, lethal toxicity, and PA and LF concentrations. Filter-sterilized culture supernatants were in some cases placed in dialysis tubing, concentrated 5- to 40-fold with polyethylene glycol, and dialyzed at 4°C against 0.05 M sodium phosphate (pH 7.5) before toxicity and toxin component concentration were assayed.

Determination of viable count and lethal toxicity. Viable counts (CFU per milliliter) were determined by making serial 10-fold dilutions in Dulbecco phosphate-buffered saline containing calcium and magnesium (5) and plating 0.1 ml in triplicate on Trypticase soy agar. After 18 h of incubation at 37°C, CFU per milliliter were determined.

Lethal toxin activity was measured in 250- to 300-g male Fischer 344 rats, in 20-g female Swiss mice, and in 350-g Hartley guinea pigs. In the rats, 1 to 10 ml of culture supernatant, concentrated supernatant, or purified toxin component mixture was injected into the dorsal penile vein, and TTD was recorded (2). Two to three rats were routinely used per determination (18). Lethal toxin activity, expressed as potency or toxic units (TU) per ml, was determined by the method of Haines et al. (11). A plot relating toxin potency units and 1/TTD × 100 was used to quantitate the lethal toxicity of crude supernatants and, in some cases, that of mixtures of purified components (11). In the mice and guinea pigs, 0.125 to 1.0 ml of a 5:1 (wt/wt) purified PA-LF mixture was injected into the tail vein or heart, and deaths occurring within 5 days were recorded. Seven groups of mice (five animals per group) and five groups of guinea pigs (five animals per group) were injected with twofold serial dilutions of the PA-LF mixture, and determinations of the 50% lethal dose (LD_{50}) were performed by the method of Reed and Muench (17). Purified components were diluted into phosphate-buffered saline containing 0.1% bovine serum albumin (BSA) to prevent adsorption to plastic equipment before determination of lethal potency.

Production of toxin components and antisera. The three toxin components were purified from culture supernatant of *B. anthracis* Sterne grown in R medium as previously described (14). Antisera to toxin components were obtained by repeated vaccination of a goat with Anvax (Jensen-Salsbery Laboratories, St. Louis, Mo.), a veterinary vaccine containing 10^6 viable *B. anthracis* Sterne spores per dose. Serum from the Anvax-vaccinated goat, designated as Gt/Anvax, was used to quantitate PA and LF in *B. anthracis* culture supernatant by immunoelectrophoresis.

Analysis of anthrax toxin components by CIE. Culture fluids and purified PA and LF were subjected to crossed-immunoelectrophoresis (CIE) to assess the specificity of immune sera and the purity of toxin component preparations. Samples (10 μl) were electrophoresed in 1% agarose gels prepared on Gel-Bond gel support film and Tris-Barbital buffer (pH 8.8) at 15 V/cm and 4°C. Bromphenol blue was used as a tracking dye to determine the electrophoretic front. Agarose strips containing electrophoresed samples were subjected to electrophoresis into 1% agarose containing serum (10 μl/ml) for 3 h at 7.5 V/cm and 4°C. Operation of electrophoresis was conducted according to the instructions of the manufacturer (Bio-Rad) for the model 1415 electrophoresis cell. All samples and standards were stored in 1% BSA at −20°C.

Quantitation of LF and PA by RIE. Crude culture supernatants were filter sterilized (0.45-μm-pore-size filter) and diluted in 2% BSA to a final concentration of 1% BSA. Typically, 10 μl of sample was electrophoresed overnight into 1% agarose containing either 10 or 20 μl of Gt/Anvax antiserum per ml at 2 V/cm and 4°C. Gels were washed and stained, and the area under the peak was determined with a Bio-Rad rocket peak height estimator. LF standards (0.66 to 3.33 μg/ml) and PA standards (2 to 10 μg/ml) were also prepared with 1% BSA in gel buffer and stored at −20°C in 100-μl portions. Concentrations of PA and LF were determined from standard curves relating rocket peak area to concentration.

RESULTS

Kinetics of lethal toxin production. *B. anthracis* strains grown in R medium were harvested at various points between mid- and late exponential growth (Fig. 1). Lethal toxin activity, as well as PA and LF, was found in all the cultures whose absorbance ranged from 0.007 to 0.27 at 540 nm (1.7×10^6 to 1.0×10^8 CFU/ml). In addition, supernatants from *B. anthracis* Sterne cultures containing as few as 1.4×10^5 CFU/ml were found to possess lethal toxin activity (0.2 TU/ml) and detectable levels of PA and LF.

Identification and quantitation of LF and PA antigens by RIE. As shown in Fig. 2A, LF and PA (rocket 2) were detected in culture fluid with heterologous goat serum, Gt/Anvax. The two toxin components were identified by a tandem well technique in which either purified PA (Fig. 2A, rocket 1) or LF (rocket 3) was added to a well slightly below the sample well. Purified LF and PA formed lines of identity with the inner and outer rockets, respectively. Typically, LF peaks were more tapered, whereas the tops of PA rockets were rounded. It should be noted that a second precipitin line was discernible in the PA rocket when purified PA was used in the tandem well. This second line (indicated by an arrow) diminished as the purified PA concentration was increased, and appears to have been an artifact, as discussed below. Attempts to quantitate EF by RIE were unsuccessful in that purified EF preparations did not form distinct precipitin bands with available antisera. Indications are that EF

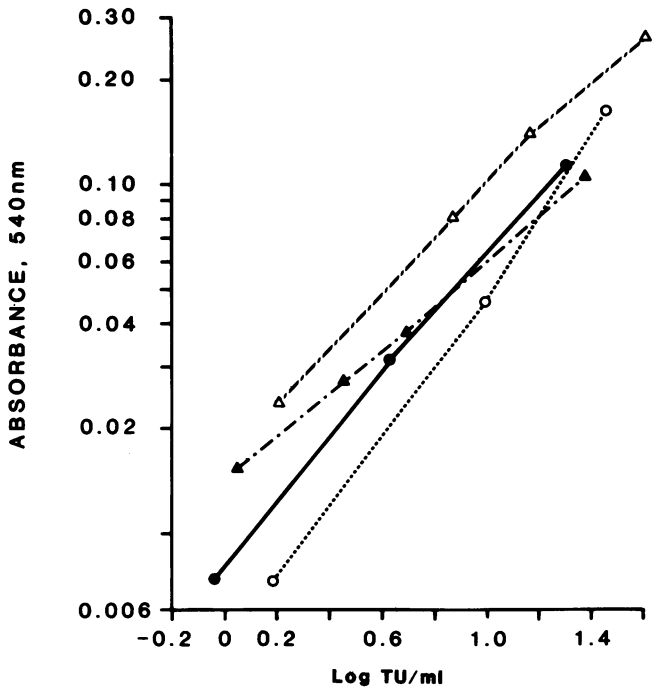

FIG. 1. Relation of *B. anthracis* culture absorbance and lethal toxicity. Shown is the relationship between toxicity (log TU/ml) and culture absorbance at 540 nm and a 1-cm light path for *B. anthracis* Vollum 1B (●), Ames (○), V770-NP1-R (▲), and Sterne (△). Absorbance is plotted logarithmically.

concentrations in culture supernatants were too low to allow quantitation by RIE under the conditions used.

In Fig. 2, gels B and C demonstrate utilization of RIE in quantitation of PA and LF directly from *B. anthracis* Sterne culture supernatant. As in gel A, the outer rocket is PA, and the inner, more tapered rocket is LF. By the use of standard curves relating known concentrations of LF and PA to rocket area, it was possible to quantitate the toxin components. Rockets 1 to 5 (Fig. 2B and C) represent LF and PA detected in 10 μl of culture fluid at various points of growth from exponential through early stationary phase in R and RHS media, respectively. As can be seen, addition of horse serum increased the ratio of LF to PA. In Fig. 2B, the LF rocket peaks were completely enclosed within the PA rocket, whereas the LF rockets from the culture grown in the presence of horse serum protruded through the top of the PA rocket. Although the PA and LF in Fig. 2B and C, samples 1 and 2, could not be quantitated directly, accurate values could be obtained if samples were first concentrated 5- to 10-fold.

CIE analysis of *B. anthracis* culture fluids. CIE analysis of culture fluids with Gt/Anvax heterologous antiserum demonstrated the presence of two predominant precipitin bands (Fig. 3A). These bands were identified as PA and LF by the tandem well technique (Fig. 3B), in which purified PA or LF (data for the latter not shown) was added to a well cut above the sample well in the first dimension. As can be seen, the purified PA formed a line of identity with PA from the culture fluid when the antigens were electrophoresed into the second-dimension gel containing antibody. Except for the plasmid-cured strains, all *B. anthracis* strains used in this study gave similar precipitin bands for PA and LF. When 10-fold-concentrated culture supernatants of strains ΔVollum 1B, ΔSterne, ΔV770-NP1-R, and ΔTexas were tested, no serological evidence for the presence of toxin components was found, indicating that plasmid-cured strains of *B. anthracis* either do not produce toxin components or produce levels which are not detectable by current methods.

Lethal toxin production and LF/PA ratio in R and RHS media. Several *B. anthracis* strains were cultured in R and RHS media to stationary phase (1×10^8 to 2×10^8 CFU/ml). The filter-sterilized culture fluids were assayed for LF and PA concentration by RIE and for lethal activity in rats. Addition of horse serum to the R medium resulted in increases in the supernatant lethal toxicity, the ratio of LF to PA, the LF quantity recovered, and in most cases, the quantity of PA recovered (Table 1). Studies were performed to determine whether other proteins were similar in effect to horse serum. Addition of 0.4% casein, BSA, or gelatin had essentially no effect on the toxicity of *B. anthracis* Sterne culture fluids taken at early stationary phase of growth (Table 2). The concentration of these proteins (i.e., 0.4%) was chosen to be approximately equivalent to the total protein in 5% horse serum.

Quantitative aspects of the lethal activity of PA and LF. In Fischer 344 rats, preliminary tests demonstrated that these animals were not killed in less than 39 min with amounts of

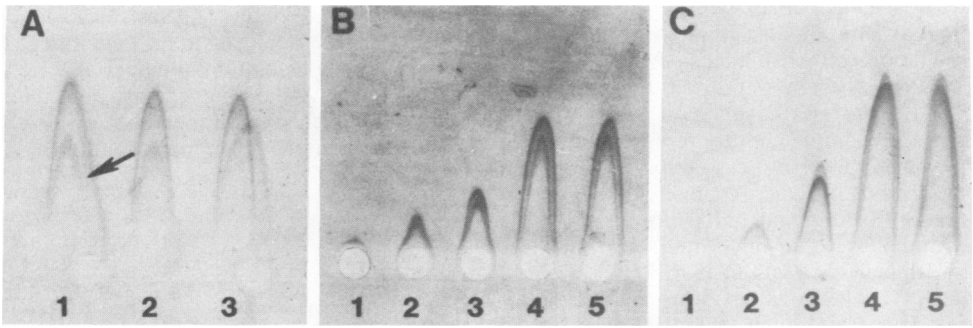

FIG. 2. Identification and quantitation of LF and PA by RIE. Agarose gels containing Gt/Anvax serum (20 μl/ml) were electrophoresed as described in the text. (A) Identification of PA and LF rockets by tandem-well technique. The upper wells of rockets 1 to 3 contained 10 μl of early stationary-phase culture supernatant from *B. anthracis* Sterne, and the lower wells of rockets 1 and 3 contained 10 μl of PA (10 μg/ml) and LF (2.66 μg/ml), respectively. Arrow in rocket 1 indicates a second precipitin line, believed to be an artifact. (B and C) RIE analysis of *B. anthracis* Sterne culture supernatant grown in R medium (B) and RHS medium (C). Wells 1 to 5 in (B) and (C) contained 10 μl of culture supernatant toxin at various points from midexponential to early stationary phases of growth.

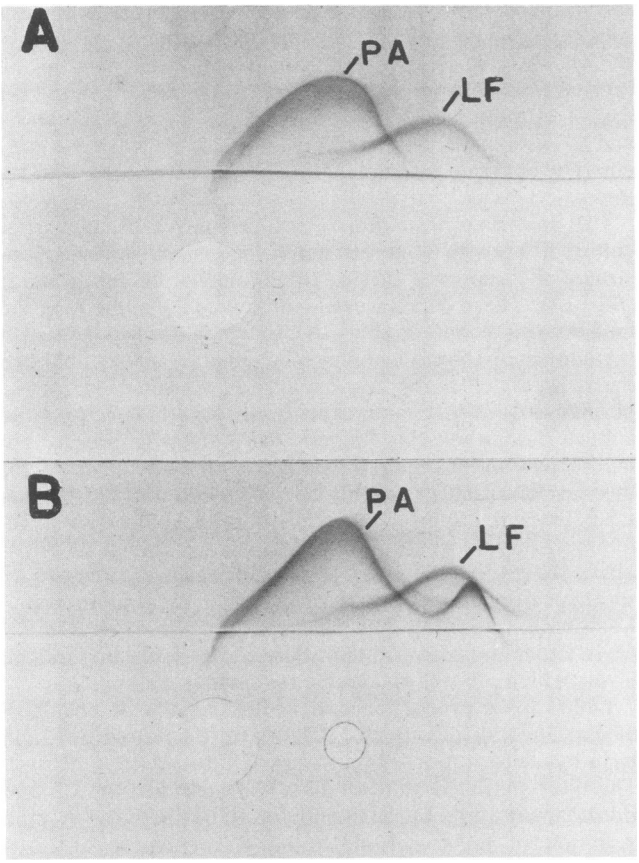

FIG. 3. Analysis of culture supernatant by CIE. Culture supernatant (20 μl) was electrophoresed in the first dimension as described in the text, followed by second dimension electrophoresis into agarose gel containing Gt/Anvax serum (20 μl/ml). (A) CIE analysis of early stationary-phase culture supernatant from *B. anthracis* Sterne. (B) Tandem-well technique for peak identification, in which 10 μl of purified PA (10 μg/ml) was added to an adjacent well in the first dimension. LF peak identification was similarly obtained (data not shown).

PA and LF of up to 160 and 60 μg, respectively. This MTTD was determined by injecting mixtures consisting of 60 μg of either PA or LF and various amounts of the other component (Fig. 4 and 5). The lowest dose of PA which was lethal in the presence of 60 μg of LF was 2 μg (TTD, 120 min). Animals injected with smaller doses became ill but recovered without apparent aftereffects. Similarly, the lowest lethal dose of LF when mixed with excess PA (i.e., 60 μg) was 0.5 μg. These doses thereby define the minimal lethal doses of PA and LF as 8 and 2 μg/kg of body weight, respectively, when the complementary component is in excess. Neither toxin component was lethal when given alone at 600 μg/kg of body weight. As doses of PA or LF were increased above the minimal lethal doses, the TTD decreased to the minimum value of 39 min. The smallest amounts of PA and LF causing death in 38 to 40 min were 40 and 8 μg, respectively. These values were estimated by visual inspection of Fig. 4 and 5, in which PA was varied from 0.5 to 60 μg or LF was varied from 0.25 to 60 μg and the complementary component was held at 60 μg. When both toxin components were decreased concomitantly, the minimum amounts of PA and LF required for lethality were 3 and 0.6 μg, respectively (Table 3). From this data, we have concluded that the LD_{50} for the Fischer 344 rat is 2 to 3 μg of PA and 0.5 to 0.6 μg of LF, since all animals injected with the lower quantities of the two components survived. With the 5:1 PA/LF ratio established for the rat, the LD_{50} for Swiss mice was 12.5 and 2.5 μg of PA and LF, respectively, whereas in Hartley guinea pigs the LD_{50} was 250 and 50 μg, respectively. LD_{50} values for mice and guinea pigs are not given as micrograms per kilogram of body weight, since a correlation between body weight and toxicity was not established. Injection of toxin into guinea pigs via the heart was used after demonstration that there was no difference in TTD of rats injected in either the heart or the penile vein (data not shown).

TABLE 1. Effect of horse serum on toxicity of stationary-phase culture supernatant

Strain[a] and medium	Toxicity (TU/ml)	LF (μg/ml)	PA (μg/ml)	LF/PA Ratio
Sterne (−)				
R	30	4.2	27.8	0.15
RHS	83	5.5	30.7	0.18
V770-NP1-R (−)				
R	98	3.4	22.5	0.15
RHS	135	5.0	28.7	0.17
V770 (+)				
R	120	4.0	26.6	0.15
RHS	143	4.4	18.8	0.24
Vollum 1B (+)				
R	128	3.2	24.7	0.13
RHS	150	4.4	30.7	0.14
Ames (+)				
R	113	3.6	27.4	0.13
RHS	135	4.7	27.2	0.17
Texas (+)				
R	30	2.3	14.6	0.16
RHS	68	5.3	23.4	0.23

[a] +, Virulent; −, avirulent.

It has been proposed that PA, LF, and EF interact at receptors on the surface of cells or tissues (14). If lethality results from toxin binding at well-defined receptors, then the data should fit a simple kinetic model. This possibility was examined with a Michaelis-Menten-type analysis; TTD was considered analogous to enzyme velocity, and toxin was considered analogous to substrate. The constant K_m would then be the amount of toxin component (PA or LF) which gives half the maximum rate of killing (i.e., a TTD double the MTTD) with the other component held in excess. The equation for PA would then be K_m = PA (TTD/MTTD − 1), or as rearranged in a form suitable for plotting, TTD =

TABLE 2. Effect of various proteins on in vitro lethal toxin production by *B. anthracis* Sterne

R medium supplement	TTD (min)	Toxicity (TU/ml)
None	56	52
5% Horse serum	48	113
0.4% Casein	56	52
0.4% BSA (fraction V)	56	52
0.4% Gelatin	55.5	56

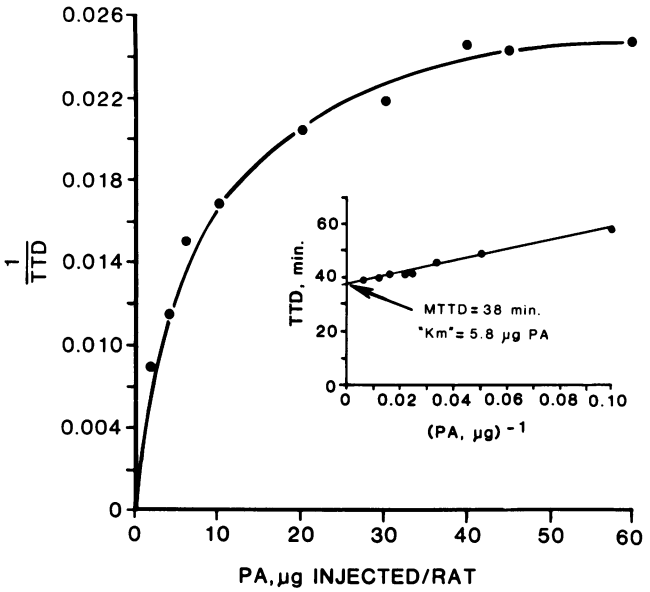

FIG. 4. Reciprocal of TTD versus PA quantities injected into Fischer 344 rats. Reciprocal of TTD of Fischer 344 male rats (300 g) injected with 60 μg of LF plus indicated amounts of PA. A double-reciprocal plot of the data is presented in the insert. The MTTD was 38 min and the K_m ("Km"), or amount of PA required to double the MTTD, was 5.8 μg with LF in excess.

MTTD (K_m/PA + 1). The term PA refers to the amount injected rather than the concentration, since the exact circulatory system volume of each animal is not known. The insets in Fig. 4 and 5 demonstrate that replotting the data for both PA and LF generated straight lines. The replots relating TTD to the reciprocal of the amount of toxin component

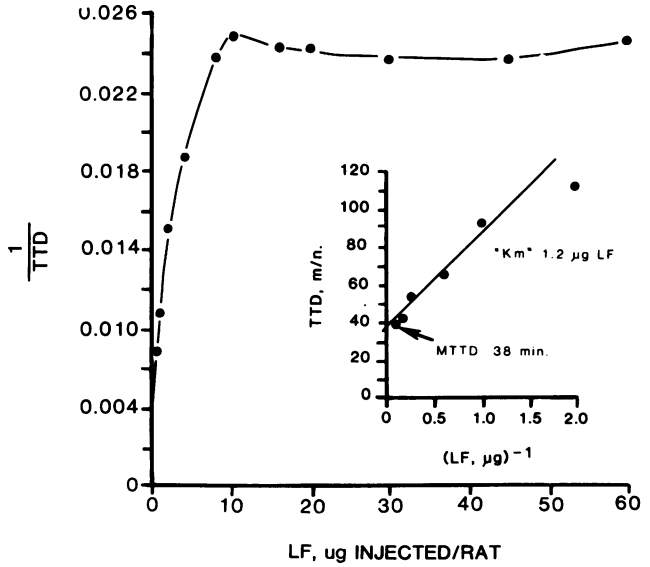

FIG. 5. Reciprocal of TTD versus LF quantities injected into Fischer 344 rats. Reciprocal of TTD of Fischer 344 male rats (300 g) injected with 60 μg of PA plus indicated amounts of LF. A double-reciprocal plot of the data is presented in the insert. The MTTD was 38 min, and the K_m ("Km"), or amount of LF required to double the MTTD, was 1.2 μg with PA in excess.

TABLE 3. Minimum amount of PA and LF required for lethality in Fischer 344 rats

Amount injected (μg)		TTD (min)
PA	LF	
2	0.6	Survived
3	0.5	Survived
3	0.6	160
4	0.8	150

injected yielded K_m values of 5.8 and 1.2 μg for PA and LF, respectively. A maximal theoretical rate of killing was calculated to be 38 min by the ordinate intercept values.

Since previous studies claimed that EF and LF acted competitively (14, 19), the ability of EF to inhibit the lethality of LF was tested. When EF was injected together with LF and PA, inhibition of lethal toxicity was clearly demonstrable (Table 4). This inhibition was manifested when either LF or PA was limiting.

DISCUSSION

B. anthracis was shown to produce LF and PA toxin components from early logarithmic through stationary phases of growth when cultured in the chemically defined R medium. This is in contrast to previous reports (12, 22) which state that anthrax toxin produced in vitro is elaborated only in late logarithmic through stationary phase and is then rapidly destroyed. This contradiction is attributed to (i) our use of improved medium and culture conditions (18) and (ii) our ability to detect low levels of lethal toxicity. The latter required the concentration of logarithmic-phase supernatants up to 40-fold without destroying biological or serological activity and the intravenous injection of up to 10 ml of concentrated dialyzed culture fluid into Fischer 344 rats. The rate of lethal toxin production was linear with respect to culture absorbance (Fig. 1). It should be noted that absorbance rather than CFU per milliliter was used in the differential plot, since *B. anthracis* cells typically form chains, thereby making CFU per milliliter a less accurate measurement of growth. As shown, virulent strains (Vollum 1B and Ames) did not produce toxic activity at rates significantly different from those observed in avirulent strains (Sterne and V770-NP1-R). This might suggest that the virulence of *B. anthracis* strains is not necessarily reflected in the rates of toxin synthesis (23). In addition, the finding that PA and LF are produced during early logarithmic growth indicates that they are not products of *B. anthracis* secondary metabolism (28). In contrast to previous reports that the V770-NP1-R strain produces only PA (13), our data clearly demonstrate that this strain produces lethal toxin activity in the culture

TABLE 4. Inhibition of PA-LF-induced lethal toxicity by EF in Fischer 344 rats

Amount injected (μg)			TTD (min)
PA	LF	EF	
40	1	0	76
40	1	1	93
40	1	5	103
5	8	0	70.5
5	8	8	79
5	8	40	92

conditions described above. As shown in Table 1, PA and LF production by this strain was comparable to that in other strains tested.

Previous studies on PA and LF production in culture (4, 10) were hampered by a lack of convenient methods for quantitating the toxin. Belton and Henderson (3) developed a skin test in rabbits for assaying the immunizing antigen and antibody, based on the capacity of antiserum to neutralize anthrax toxin. Thorne and Belton (24) later described an agar diffusion method for quantitating PA in culture filtrates and for determining antibody titers to this antigen. In this study, RIE was shown to be accurate and sufficiently sensitive for quantitation of PA and LF directly from culture filtrates. Culture fluid typically gave two distinct rocket-shaped precipitate lines with Gt/Anvax serum (Fig. 2). Identities of the rockets were determined by a tandem-well technique in which purified LF or PA was added to a well located partially under the sample well. In both cases, the purified components formed lines of identity with their respective culture fluid components. Identification was also confirmed with affinity-purified antibody to PA and LF (data not shown) and by the absence of precipitin bands for these two proteins when the culture fluids of plasmid-cured strains were analyzed. As indicated by the arrow (Fig. 2A, rocket 1), the culture fluid PA rocket appeared to contain a second precipitin line, or antigen, when purified PA was added to the tandem well. This "second" antigen is proposed to be an artifact, because its presence was dependent on the concentration of PA in either the sample or tandem well. This may suggest that PA interacts with some other culture fluid component at certain concentrations. Also, addition of purified PA directly to the sample increased the PA rocket without leaving behind an otherwise masked rocket (data not shown). Therefore, one may conclude a priori that the PA rocket is the result of a single antigen.

Both toxin components could be quantitated by RIE down to 0.5 μg/ml in unconcentrated samples mixed with 1% BSA. As stated above, PA and LF could also be quantitated in more dilute samples after concentration or by use of increased sample volume or both. Initial studies on EF suggest that this toxin component does not readily lend itself to quantitation by RIE in that the precipitin bands observed were rather diffuse. It should be noted that addition of purified EF to culture samples did not alter the height of either the PA or the LF peak, which suggests a lack of interaction of the toxin components during their quantitation by RIE. This is consistent with the finding that addition of purified PA to culture fluids did not alter the peak height of the LF precipitin band.

In a comparison of gels B and C in Fig. 2, it becomes apparent that the ratio of LF to PA and their absolute amounts produced in vitro were not constant but were influenced by the compositions of the culture media. The basis for stimulation of TU production (shown in Table 1) by horse serum is not understood. Addition of other protein sources to the medium does not influence toxin production (Table 2). This suggests that the influence of horse serum may constitute more than mere protection of toxin components from protease activity or adsorption to glass. It has also been found that incorporation of horse serum into culture medium enhances recovery of EF (S. H. Leppla, unpublished data).

The availability of purified toxin components provided the opportunity to study the kinetics of lethal toxicity in Fischer 344 male rats by the injection of defined combinations of LF and PA toxin components. In preliminary studies (data not shown), it was established that injection of 60 μg of either LF or PA, in the presence of an excess of the other, was sufficient to bring about MTTD. When PA was held constant at 60 μg and LF was varied, the minimum amount of LF needed to produce MTTD was ca. 8 μg. Conversely, when LF was held constant at 60 μg and PA was varied, the lowest amount of PA for MTTD was ca. 40 μg. In both cases, the MTTD was ca. 38 to 40 min (reciprocal value, 0.0263 to 0.025). It was of interest to note the applicability of analytical methods classically applied to enzyme kinetic studies to the studies on the kinetics of anthrax toxin activity (Fig. 4 and 5, inserts). Since the exact volume of the circulation system of the individual rats used is not known, one must utilize absolute amounts of LF and PA injected in such analyses and not their final concentrations in the circulatory system. Therefore, the term K_m refers to the absolute amount, not the concentration, of LF or PA which gives half the reciprocal MTTD with the other component in excess. MTTD is thus analogous to the V_{max} of enzyme activity. Therefore, as stated earlier, the K_m for PA and LF was 5.8 μg and 1.2 μg, respectively, and the MTTD was 38 min. Although we were only able to experimentally obtain an MTTD of 39 min with 60 μg of LF and 160 μg of PA, these analyses predict that with higher amounts, an MTTD of 38 min could ultimately be obtained. This is in contrast to previous reports, in which the MTTD for a 200- to 300-g Fischer 344 male rat was estimated to be 52 to 54 min (7, 11). As stated above, the minimum amounts of PA and LF required for maximal Fischer 344 rat toxicity are 40 and 8 μg, respectively. This apparent 5:1 ratio is also reflected in the K_m values of 5.84 μg of PA and 1.16 μg of LF (i.e., 5.84 μg/1.16 μg = 5.03). One can only speculate at this time as to whether the same ratio is required at the cellular level. However, until the interaction of PA and LF can be studied in vitro, the 5:1 ratio noted in vivo must be viewed with caution.

A model for PA binding to target cell surface receptors and its serving to bind and transport EF and LF has been proposed and is supported by several reports. The inhibition of EF-PA-induced biological activity by LF has been demonstrated in vivo by Stanley and Smith (20) and in vitro by Leppla (14). The data presented in Table 4 demonstrate the converse situation in which EF inhibits PA-LF-induced biological activity. Although the exact mechanism of inhibition is not known, one may speculate that in the above proposed model, EF and LF compete for the same site(s) on PA or complex of PA, or they are allosteric inhibitors of each other. Another model to explain the antagonistic interaction of EF and LF is the possibility that LF functions in some manner mutually antagonistic to EF adenylate cyclase activity or the effects of increased levels of intracellular cyclic AMP. The inhibition of lethal toxin activity by EF and possibly other culture components may explain why culture filtrates which appear similar in their content of LF and PA vary in their toxicity, as shown in Table 1. In addition, the loss of biological activity without loss of serological activity may also explain these disparities.

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