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Relationship of Route of Inoculation and Nature of Toxin Preparation to Bioassay of *Clostridium perfringens* α-Toxin in Mice

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The toxicity of *Clostridium perfringens* was determined in mice utilizing three different routes of inoculation. There was marked variation in the minimum lethal dose with the different routes; the largest amount was required for the intracutaneous route, less for the intravenous route, and least with the intraperitoneal route. The relationship of toxicity to the lecithinase content of different toxin preparations was assayed. It was found that the toxicity of toxin preparations in different states of purification was not correlated with their lecithinase content.

Clostridium perfringens produces a toxin, the α-toxin, which is considered to be the lethal factor in gas-gangrene infections (2, 4, 10). The α-toxin has been identified as an enzyme, a lecithinase (phospholipase-*c*), which acts upon lecithin to yield phosphorylcholine and a diglyceride (3). In the course of experiments to quantitate the α-toxin in mice, it was observed that there was marked variation in the response of mice to the toxin with different parenteral routes of administration and degree of purification of the toxin. In view of these differences, attempts were made to develop standardized procedures for comparing the dose-response relationships of the toxin.

In the usual method of in vivo assay of α-toxin, it is injected intravenously and the LD_{50} is determined. A more precise measurement of toxin is given by the graded dose-effect relationship with average or median survival time as the indicator (5). The minimum lethal dose (MLD) of the toxin was determined, and multiples of these doses were administered by the intracutaneous, intravenous, and intraperitoneal routes to assay the graded dose-survival time effect. The intraperitoneal route was most sensitive, but all three are useful.

Although the in vivo action of the *C. perfringens* toxin is ascribed to its lecithinase content, there has not as yet been a conclusive demonstration of such a relationship. In attempts to demonstrate such a relationship, various toxin preparations were assessed on the basis of lecithinase units per MLD (L/MLD). This procedure permitted the determination of the relationship of enzyme units present in the toxin preparation to toxicity; if the lecithinase is indeed the lethal factor in this toxin, the L/MLD should remain constant regardless of the degree of purity of the toxin preparation. However, this relationship was found to vary with toxin preparations in different stages of purification. The possible reasons for this variation will be discussed.

MATERIALS AND METHODS

Borate-buffered saline (BBS). BBS was used as the diluent in all experiments. It contained 8.50 g of NaCl, 10.94 g of H_3BO_3, and 1.90 g of $Na_2B_4O_7 \cdot 10H_2O$ (Borax) per liter of distilled water; *p*H 7.3 to 7.4. It was sterilized at 121 C for 15 min.

Culture medium. Trypticase Soy Broth, 3% (Baltimore Biological Laboratories), plus 1% Proteose Peptone No. 2 (Difco) at *p*H 6.7 was employed. It was prepared on the day of use, autoclaved at 121 C for 15 min, and allowed to cool to 37 C in an incubator without agitation, so as to exclude dissolved oxygen.

Organism. C. perfringens strain $BP6K_1$ type A was reactivated from the spore stage by transfer into the above medium. It was passed serially four times in tubes after 6 to 8 hr of growth, a procedure which had been found to enhance the toxigenicity, before being used as the inoculum for toxin production.

In vitro lecithinase assay. Assays of lecithinase activity were made by the agar-diffusion method of Sheldon, Moskowitz, and Deverell (9), and the determinations are expressed in terms of lecithinase units.

Production of large batches of toxin. Culture medium (4 liters in 6-liter Ehrlenmeyer flasks) was inoculated with the serially passed organism. The inoculum ratio was 1% (v/v; 10 ml per liter). After 6 hr of growth, the culture fluids were rendered cell free by 30 min of centrifugation, 2,500 rev/min at 4 C in an International PR-1 refrigerated centrifuge.

The clear amber fluid was collected and kept in an ice-salt-methanol bath below 0 C. The lecithinase content varied from 30 to 60 lecithinase units per ml in eight different batches of culture fluids.

Concentration and partial purification of toxin. The toxin was concentrated and partially purified by the method of Roth and Pillemer (6). Their procedure was modified to the extent that BBS was used instead of succinate buffer, and the concentration ratio was 50:1 instead of 180:1. Preparations were tested at two different stages in the purification procedure: procedure A and procedure B. Procedure B constitutes a further purification step of the product obtained in procedure A.

Procedure A. The centrifuged culture fluid was adjusted to *p*H 4.60 ± 0.05 by adding cold 1 N acetic acid slowly while stirring. Approximately 10% by volume was required. Absolute methanol, cooled to −20 C, was added dropwise from a separatory funnel, with constant stirring, to a final concentration of 35% by volume. The temperature was maintained below 0 C with an ice-salt-methanol bath. The mixture then stood at −20 C for 5 to 7 days while precipitation took place.

The clear supernatant fluid was siphoned off and the settlings were centrifuged at 2,500 rev/min for 1 hr at −10 C. The dark-gray, packed precipitate was reconstituted to a 50-fold concentration (relative to the initial culture fluid volume) by resuspending in cold BBS. Samples were tubed, frozen, and stored at −20 C until use. Prior to use, the frozen samples were thawed at room temperature, centrifuged at 0 C to remove insoluble sediment, and diluted to the desired concentration with cold BBS. Three preparations were made and are referred to as AI, AII, and AIII. From 25 to 65% of the lecithinase in the initial culture fluid was recovered, depending upon volumes handled. The difficulties of handling large volumes usually resulted in a lower percentage of recovery from initial culture fluids.

Procedure B. The precipitate obtained in procedure A was dissolved to 25:1 concentration ratio (relative to the volume of initial culture fluids) in cold BBS and adjusted to *p*H 6.0 with 1 N acetic acid. It was centrifuged for 30 min at 0 C and 2,000 rev/min to remove insoluble material. The clear supernatant fluid was adjusted to *p*H 4.6 with cold 1 N acetic acid, and absolute methanol (at −20 C) was added dropwise to 35% concentration while stirring. The temperature of the mixture was maintained below 0 C in an ice-salt-methanol bath. After standing for 18 hr at −20 C, the resulting precipitate was collected by centrifuging at −10 C for 30 min at 2,000 rev/min. The packed precipitate was drained dry by decanting the supernatant fluid; it was then reconstituted to a 50:1 concentration, relative to the volume of the initial culture fluid, with cold BBS. The resulting solution was assayed and then frozen in the same manner as for procedure A. Three preparations were made and are referred to as BI, BII, and BIII. From 90 to 95% of the lecithinase activity present in the product of procedure A was recovered.

Mice used in toxicity studies. White virgin female Swiss-Webster mice, weighing 20 to 25 g, were obtained from Harlan Animal Industries, Inc., Cumberland, Ind. They were maintained in a controlled environment at 22 C and 50% relative humidity.

Injection techniques. Mice were injected with the appropriate lecithinase unit-dose in a 0.25-ml volume, prepared by diluting the particular toxin preparation in BBS. Injections were made with a 26-gauge needle. The toxin was used within 1 hr after dilution and was maintained in an ice bath during this period.

Intraperitoneal (ip) injections were made through the abdominal wall 1 cm above the pubic symphysis, to either side of the midline. Intravenous (iv) administration was given into the caudal veins at the base of the tail. Intracutaneous (ic) injections were given beneath the skin on either side of the abdomen with the needle pointed toward the groin. Uniformity of injection sites and speed of operation were assisted by inserting the mouse into a translucent plastic centrifuge tube so that the hind quarters were left exposed and the ventral side was accessible for any one of the three routes. Ten mice could be treated uniformly in 2 to 3 min. Time of injection and time of death were noted to the nearest minute.

Calculation methods. To estimate median survival time (ST_{50}), individual survival times for each of the mice in a dose-response group were added and divided by the number of mice (usually 10) receiving the same treatment. From this average (or mean) survival time, a standard deviation (SD) was calculated with the usual formula for grouped data:

$$\pm \text{SD} = \sqrt{\frac{\Sigma d^2}{n-1}}$$

where d = deviation in minutes from mean survival time, and n = number responding to the lethal toxin. When individual survival times fell outside 1 SD, they were censored from the final calculation of the mean (7, 8). Values outside ± 1 SD usually occurred either at one or both extremes. After censoring the unreliable values, the remaining survival times were used to compute a new mean as before. In this case, the new mean and median survival times are identical. This is called the ST_{50} because it represents the response time (to death) for 50% of the test animals.

The coefficient of variation (CV) is an index of precision—variations in response to graded doses—calculated by dividing the standard deviation by the mean survival time for each treatment group: ± SD/mean ST. Since both the SD and mean ST are in minutes, the coefficient of variation is expressed as percentage (CV%). Covariation should be random within a given series of graded doses (all the same route), and the mean coefficient of variation (Σ_xCV %/n) expresses the overall precision of the assay.

RESULTS

MLD assay of different preparations of toxin by different routes. The MLD of three preparations of toxin prepared by procedures A and B were assayed by determining the maximal dilution in an 0.25-ml volume that would kill all mice in a

group of 10 within 48 hr. Each preparation was tested with the ip, iv, and ic routes of injection. The results are tabulated on the basis of L/MLD and are presented in Table 1. The preparations AI, AII, and AIII averaged 11.71, 3.75, and 1.73 L/MLD, respectively, by the ip, iv, and ic routes, whereas preparations BI, BII, and BIII, averaged 4.13, 1.69, and 0.78 L/MLD for the same respective routes. With all three routes, the L/MLD obtained with the A preparations were greater than with the B preparations, although the same relationship obtained among the three different routes of inoculation. Also, with all three routes of inoculation, there was less dose variation with the B preparations than with the A preparations.

Correlation of multiple lethal doses with survival time. Groups of 10 mice were injected by each of the three routes at twofold increases in graded dose-levels, ranging from 2 MLD to 64 MLD. These experiments were carried out with preparations AIII and BIII. The survival time data were used to calculate the SD, the CV%, and the ST_{50} for each treatment group. Dose-response curves were plotted on log-log graphic paper relating lecithinase units (dose) to ST_{50} response. The relationship was linear over this range, and the results in Fig. 1 (AIII) compared with Fig. 2 (BIII) show that all three routes of inoculation are useful with both types of preparations. Although the differences in potency of A and B relative to their L/MLD content is not as marked as with the MLD assay, there is a definite decrease in variation of response to preparation BIII, as contrasted with AIII.

DISCUSSION

The marked variation in the MLD, as well as the survival time with a given dosage, obtained with different parenteral routes of administration of the toxin suggests that either the mechanism of toxicity is different for each route, or that there is more interference to the toxin to reach a "critical site" with some routes than with others. The greatest sensitivity observed with the ip route may be due to the bypassing of tissue barriers and delivery of the toxin with minimal dilution to a "critical site." The larger amounts required with the IV route may be due to the dilution of the toxin in the blood and tissue spaces, and to the binding of the lecithinase with substrate on blood

TABLE 1. *Relationship of lecithinase activity to toxicity (L/MLD) of α-toxin prepared by procedures A and B with three different routes of injection*

Toxin prep	L/MLD		
	ip Route	iv Route	ic Route
AI	13.28	4.25	2.13
AII	9.10	3.16	1.15
AIII	12.75	3.83	1.91
Average	11.71	3.75	1.73
BI	4.08	1.70	0.82
BII	4.32	1.73	0.72
BIII	3.99	1.65	0.79
Average	4.13	1.69	0.78

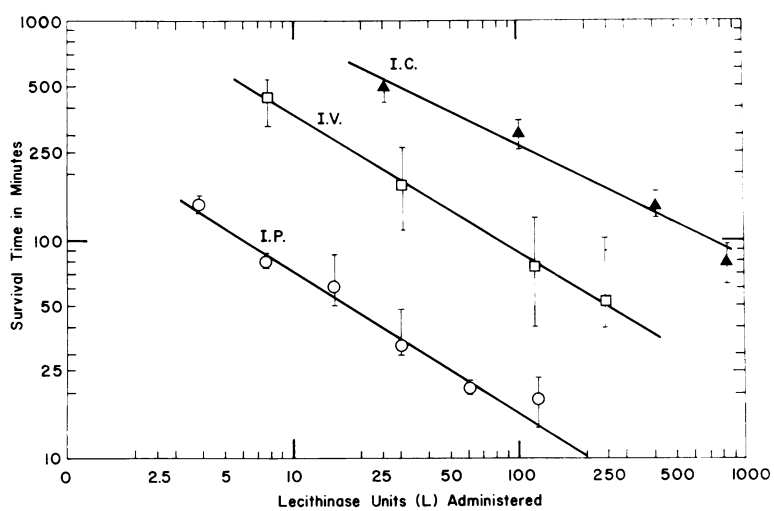

FIG. 1. *Relationship of amount of lecithinase injected to ST_{50}. Toxin preparation AIII; injected ip (○), iv (□), and ic (▲).*

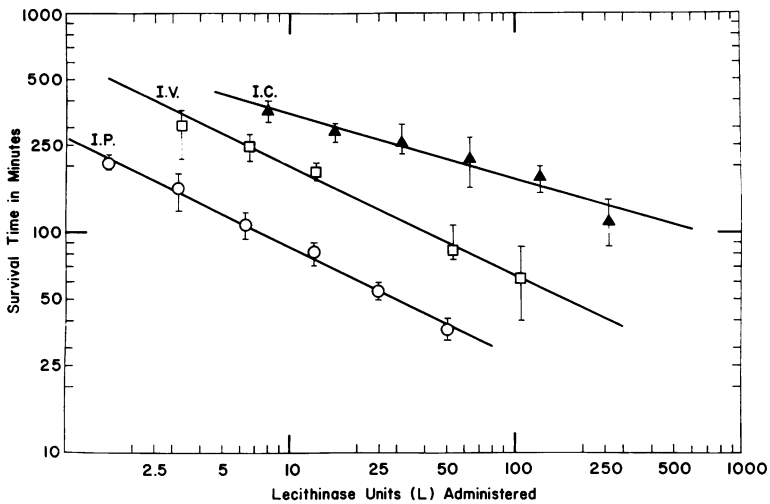

FIG. 2. *Relationship of amount of lecithinase injected to* ST_{50}. *Toxin preparation BIII; injected ip* (○), *iv* (□) *and ic* (▲).

and tissue cells and the lipoproteins of the plasma, which may not be involved in the lethal activity of the toxin. The relatively large amounts of toxin required by the ic route may be due to factors just described, plus the relatively slow absorption from the injection site. Lecithinase is destroyed by proteolytic enzymes, and some of the toxin is probably inactivated by such enzymes in the body; the factors that hinder the rate of delivery of toxin to the assumed "critical site" of action would then also serve to decrease the amount of toxin available there.

The pattern of results obtained with the three routes of injection studied indicate that any of them may be used for the bioassay of the α-toxin; however, from the standpoint of economy of time and materials, the ip route would be the preferred one.

If the lecithinase is indeed the lethal factor, one would expect the L/MLD, and the corresponding relationship in the survival time assay, to be constant. By utilizing two different types of preparation of toxin, variations in this ratio were noted. These results at first glance suggest that the lecithinase is not the lethal factor, or else is not the sole factor contributing to the toxic activity of the preparations. The differences in the pattern of response obtained with the two preparations, however, do not eliminate the lecithinase as the sole lethal factor. It is noted that, although the L/MLD is approximately twice as great with all three routes with the A preparations than with the B, there was much less variation among the different B preparations than with the A. This difference in variation observed with the two preparations was also observed in the assay of survival times. The greater variation obtained with the A preparations may be due to the nonlecithinase impurities interfering in some way with the in vivo activity of the lecithinase.

The manner in which the impurities would interfere with the action of the toxin is unknown, but the possibility of a type of competitive inhibition can be considered. The amount of α-toxin in a culture of *C. perfringens* increases over a period of 6 to 8 hr and then starts to decline; it is thought that the decline in activity is the result of the proteinases in the culture degrading the lecithinase. Such degraded lecithinase may have the ability to combine with substrate even though it lacks enzymatic activity. The various steps in the purification of the toxin may serve to decrease the amount of degraded lecithinase and thus the less purified preparations would have relatively more degraded lecithinase. If such degraded lecithinase competed with the active lecithinase for substrate, more active lecithinase may be required in in vivo situations to exert its toxic activity. Dolby and MacFarlane (1) also noted a variation in the ratio of toxicity to lecithinase units in toxin preparations obtained from different strains of *C. perfringens*. They concluded that the differences in potency were due to small differences in the configuration of α-toxin from different strains. The results obtained in this study, however, suggest that the differences we observed were not due to differ-

ences in configuration; the explanation that was given to explain our results may also apply to those of Dolby and MacFarlane.

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