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Toxicity of Purified Botulinal Toxin Fed to Mice (38394)

H. SUGIYAMA, B. R. DASGUPTA, AND K. H. YANG

Food Research Institute and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

Toxin in culture fluids of *Clostridium botulinum* type A, B, C, D, E and F behaves as molecules of mol wt several hundred thousand (1). The sedimentation constant of 11.5 found for type E toxin extracted from a botulinogenic food (2) agrees with the above results. These toxic entities are most likely the molecules which are isolated as crystalline type A toxin of mol wt 900,000 (3), type B of mol wt 500,000 (4), and type E of mol wt 350,000 (5).

In slightly acidic solution these isolated preparations can behave as though composed of a single molecular species. However, these units are complexes in which a neurotoxic α component is associated with a nontoxic β component. The complexing is by means other than covalent bonding since chromatographic procedures separate the components. When the β component is removed, the resulting pure type A (6) and E (5) toxins are of mol wt 150,000 and type B toxin is of mol wt 165,000 (7). Both α–β complex and α of type E increase in toxicity when activated with trypsin (5).

Athough Aα and activated Eα lose most of their toxicity when held at 33° for 30 min in buffers, respectively, of pH 3 and 4, the type A and activated E complexes are stable at these conditions (5). To kill mice by feeding Eα requires huge amounts of toxin (8). These observations have led to suggestions that complexing with β stabilizes the biological activity of the α component and that free α would not be toxic when ingested. Accordingly, free α would not contribute to the toxemia of botulinal food poisoning; only the complexed form of toxin would be important (5).

In the experiments to be reported, several botulinal toxin types in their α–β complex and α forms were fed to mice. Comparative toxicities of the two forms are used to consider the food poisoning potential of the uncomplexed, pure toxin.

Materials and Methods. Type A toxic complex was crystalline toxin; Aα was derived from it by chromatography (9). Type B toxic complex was the toxic pool obtained from the Sephadex G-100 run at pH 5.6 while Bα was toxin resulting from the step using DEAE Sephadex A-50 of pH 7.3 (7). Type E complex was the toxic material recovered when the RNAse treated sample was chromatographed on CM Sephadex (10). Eα was the result of chromatographing this toxic complex on QAE Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, NJ) with 0.03 *M* phosphate buffer of pH 6.0. Fractions of the first protein peak which was eluted by a linear NaCl gradient made with the equilibrating buffer were pooled and rechromatographed similarly but at pH 7:55. Eα was the first protein peak eluted by the NaCl gradient. Type F toxic complex was obtained by precipitating the toxin in whole cultures with $(NH_4)_2SO_4$, dissolving the precipitate in 0.07 *M* phosphate buffer of pH 6.0 and chromatographing the clarified solution on DEAE Cellulose (Sigma Chemical, St. Louis, MO). Fα was the result of processing this toxic complex through four other chromatographic steps (11). The α preparations of all toxin types showed essentially only the 150,000 mol wt protein band (except for 167,000 in the case of type B) when they were electrophoresed in sodium dodecylsulfate–polyacrylamide gels (12).

Toxins were stored as precipitates which were developed by adding $(NH_4)_2SO_4$ to final 60% saturation. For use, a desired volume of precipitate suspension was removed and centrifuged. Collected precipitate was then dissolved in *M*/15 phosphate buffer–0.2% gelatin, pH 6.2. Eα stock was the exception; it was the pooled three apex fractions of the toxic protein peak which

was obtained in the last QAE Sephadex step. This pool was used directly since activatability by trypsin is reduced with time when Eα is held as a precipitate formed with $(NH_4)_2SO_4$.

Stock toxin preparations were titrated with the iv test procedure (13). The average time to death of three mice given an iv injection of 0.1 ml sample was converted to ip LD_{50} with a standard curve made with the toxic form being assayed (14). Type E preparations were also assayed after being activated by incubation at 37° for 30 min with final 0.01% crystalline trypsin. Activation increased ip toxicity of both Eα and E complex by 200-fold.

Stock toxins were diluted with 0.2% gelatin in water, pH 6.2, to obtain solutions containing 1×10^6 LD_{50}/0.25 ml. These initial dilutions were at least fivefold dilutions of the stocks and reduced phosphate to levels where buffering action *in vivo* would be minimized. These standardized solutions were diluted serially in 10-fold increments with gelatin solution. The usual range of toxin doses fed to mice was 1×10^3–1×10^6 ip LD_{50}/0.25 ml/mouse with each toxin level being tested in a separate group of 10 mice (HA/IRC strain, body wt about 22 g). Toxin was introduced into the stomach with a feeding needle (Popper & Sons, Inc. New York, NY) connected to a tuberculin syringe. Mice did not have access to food for 1 hr immediately preceding the feeding of toxin. An experiment was the parallel feedings of the complexed and α forms of a given toxin type.

Type E preparations were fed in trypsinized and untrypsinized forms. Maximum challenge doses of Eα were ip LD_{50} of 6×10^6/mouse when activated and 5×10^4 without trypsinization. These doses, which contain the same amount of toxin protein, were stock toxin preparations diluted twofold with gelatin solution.

Test mice were observed for 7 days, although deaths rarely occurred after the third day. The number of ip LD_{50} doses in one intragastric (ig) LD_{50} unit was estimated by the Reed and Muench procedure (15). The highest dose of Eα killed less than half of the 10 mice so that the ig LD_{50} of this toxic preparation was obtained by extrapolating of the dose response line.

Results. Table I shows the lethality of the different botulinal toxin forms when they are fed to mice. When compared as to numbers of ip LD_{50} doses constituting one ig LD_{50}, type B appears to be the most toxic of the complexes tested; E and F are less effective. Untrypsinized E complex apparently undergoes complete activation *in vivo* when it is fed (8). Its ig LD_{50} of 3.5×10^3 ip LD_{50} has a potential toxicity of 7×10^5 ip LD^{50} when activated. This potential value is not significantly different from the 5×10^5 ip LD_{50} units in an ig LD_{50} of activated E complex. With all the toxic complexes, more than several thousand ip LD_{50} are needed to get an ig LD_{50} effect. The α of all toxin types has lethal activity when fed to mice. The α was always less potent than the complex of the corresponding toxin type but the potency difference of the two toxic forms were never more than 15-fold. The greatest difference in ig toxicities was observed when the E complex and Eα were fed without prior trypsinization.

TABLE I. Lethal Potencies of α and α–β complex of Botulinal Toxins Fed to Mice. Intragastric LD_{50} is Expressed as Number of Intraperitoneal LD_{50} Doses.

Toxin type	Intragastric LD_{50} α–β	Intragastric LD_{50} α	Ratio of ig LD_{50} α/α–β
A	5×10^4	2×10^5	4
B	6×10^3	4×10^4	7
F	6×10^5	9×10^5	2
E: activated[a]	5×10^5	6×10^6	12
not activated[b]	3.5×10^3 (7×10^5)	5×10^4 (1×10^7)	14

[a] Activated with trypsin before feeding to mice.

[b] Not trypsinized; figures in parentheses are ip LD_{50} if sample had been trypsinized.

Discussion. Our results agree with other reports that lethality for mice of botulinal toxic complexes administered by the oral route is much less than by the ip route. The 5×10^4 and 6×10^3 ip LD_{50} units in an ig LD_{50} dose of types A and B toxic complexes, respectively, are similar to the 1×10^4 obtained with toxic complexes of presumed similar purity but by titrations based on minimal lethal doses (16).

High multiples of ip LD_{50} doses are needed to produce an ig LD_{50} response when free α is fed to mice. Based solely on these multiples, free α might be considered nontoxic when ingested by man. However, the relative toxicities of α–β complexes (toxic form in foods) administered to mice by the ig and iv routes are also high. This means that the potential toxicity for man of free α should be better assessed by comparing the number of ip LD_{50} doses in one ig LD_{50} of free α

and the complex. In this comparison, α of the different tested toxin types had a potency which ranged from one half to one fifteenth that of the complex of the corresponding toxin type. These results suggest that uncomplexed botulinal toxin should be nearly as toxic as the α–β complex when ingested by man.

Summary. Purified botulinal toxin types A, B, E, and F (α preparations of mol wt 150,000 – 165,000) were fed to mice and their intragastric (ig) LD_{50} calculated in terms of ip LD_{50} units. Similar data were obtained with the α–β complex of the corresponding toxin types. The number of ip LD_{50} doses in an ig LD_{50} of α ranged from nearly equal to about 15 times greater than in the complex of the same toxin type. These results suggest that the α form of botulinal toxins should have only slightly lower toxicity than the complex when they are ingested by man.

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