Basis of Type A and F Toxicities of *Clostridium botulinum* Strain 84 (36933)

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Cultures of *Clostridium botulinum* are classified as types which are based on serologically distinguishable toxins. The typing has been possible and practical because individual cultures generally produce only one type of toxin and neutralization of that toxin with heterologous types of antitoxin is absent or minimal (1).

C. botulinum strain 84, isolated by Giménez and Ciccarelli (3) from an Argentine soil sample, is an exception to these generalizations. To protect mice against its toxin required a mixture of type A and F antitoxins when the challenge dose was more than 20 LD_{50}/mouse. The culture was not a mixture of type A and F organisms since more than 300 subcultures started with isolated colonies and 6 other subcultures, each originated from a different single cell, produced toxic fluids of dual type specificities.

Whether strain 84 produced a single species of toxin with each molecule having both A and F specificities or whether the culture, in spite of its purity, produced a mixture of type A and F toxins was not resolved. The present communication describes experiments concerned with the two alternatives.

The study was based on the principle used to identify different botulinal toxin types; specifically, type A toxin combines with (and is neutralized by) type A antitoxin but not with type F antitoxin while type F toxin combines with type F but not with type A antitoxin [(2, 3), unpublished observations]. If the dual type toxicities of strain 84 are due to a single species of toxic molecules possessing both A and F antigenic determinants, precipitation of the molecule with type A antitoxin should result in the simultaneous reduction of toxicity of both specificities. Similarly, absorption of the toxic solution with type F antitoxin should remove toxicity of both specificities. On the other hand, if the A and F antigenic determinants are located on different molecules, absorption of the toxic fluids with either types A or F antitoxins should remove only the toxicity of specificity corresponding to the antitoxin.

Materials and Methods. C. botulinum strain 84 was obtained from Dr. Giménez (Universidad Nacional de Cuyo, Mendoza, Argentina). The subculture used in the present work was one of six started in our laboratory with different isolated colonies and was maintained in Difco cooked meat medium reconstituted to contain final 0.5% glucose and 0.2% soluble starch. Culture fluids of all six subcultures killed mice with signs of botulism; their lethality for mice (0.5 ml of undiluted culture fluid injected ip) could be neutralized only a mixture of type A and F antitoxins.

Strain 84 toxin was a concentrate of toxin produced in N-Z amine broth (w/v, 1% N-Z amine type B, an enzymatic digest of casein, Sheffield Chemical, Norwich, NY; 2% Difco proteose–peptone; 1% Difco yeast extract; 1.0% glucose; 0.45% NaCl; 0.05% sodium thioglycollate, pH 7.3). The medium, given a 10% (v/v) inoculum of an actively growing culture, was incubated 4 days at 30°. The supernatant fluid prepared by centrifugation was made 60% saturated with $(NH_4)_2SO_4$, held overnight at 4°, and the precipitate collected by centrifugation. The precipitate was then extracted with a volume of 0.05 *M* phosphate buffer (pH 6.8) equal to 1/40 the originally recovered culture supernatant fluid. After insoluble material was removed by centrifugation, the toxic extract was di-

alyzed against the phosphate buffer, sterilized by filtration (Millipore, 0.45 μm pores), and stored at 4°. The concentrated toxin had 3.6 × 10^6 LD_{50}/ml.

Antitoxins for absorbing strain 84 toxin were prepared in rabbits. Type A antitoxin was developed with crystalline toxin (from Dr. E. J. Schantz, Fort Detrick, Frederick, MD) by the method described previously (4) and had 200 IU/ml. Type F antitoxin was obtained with toxoid derived from toxin of the Langeland strain that had been obtained from Dr. M. W. Eklund, National Marine Fisheries, Seattle, WA. The toxin, produced and then concentrated with $(NH_4)_2SO_4$ as described for strain 84 toxin, was partially purified by a DEAE-cellulose chromatographic step (unpublished data). The pool of the toxic fractions was then toxoided (final 0.3% formalin, 3 wk incubation at 37°). The antiserum obtained had 24 CDC units/ml (titration according to the Center for Disease Control, Atlanta, GA).

Anti-84 serum was the sheep antiserum prepared by Giménez and Ciccarelli (3). Equine type A antitoxin (Porton antiserum, obtained from Dr. J. Keppie, Microbiological Research Establishment, England) and type F antitoxin (obtained from CDC) were used in some of the mouse protection experiments. As might be expected, the results with these sera were the same as those obtained with antitoxins produced in rabbits.

Toxoids were used to absorb anti-84 serum. Type A toxoid was a liquid preparation obtained from Dr. G. G. Wright, Fort Detrick, and was concentrated 4-fold (volume reduction) by dialysis against 25% (w/v) Carbowax (polyethylene glycol, MW 20,000) in 0.05 *M* Tris–HCl buffer of pH 7.2. Formalin in the toxoid was removed during the dialysis and a subsequent dialysis against 0.85% NaCl. Type F toxoid, obtained from CDC, was dialyzed in the same manner as the type A toxoid.

Similar procedures were used in the absorptions of toxin with antitoxins and of antitoxin with toxoids. In the former instances, antitoxin was added to toxin and the mixture was incubated at 25° for 2 hr before holding overnight at 4°. Any precipitate that formed was removed by centrifugation. The absorption was then repeated by adding the same or lesser amount of antitoxin to the supernatant fluid. The serial, stepwise absorptions were continued until there was no detectable precipitate formation. One of the absorption steps should be a mixture at, or near, toxin–antitoxin equivalence (4). Controls were portions of toxin treated with 0.85% saline or phosphate buffer instead of antitoxin.

Absorption of antitoxin with toxoids differed only in incubation of 30 min at 37° before transferring to 4°. Since toxoids were in short supply, significant, but not necessarily complete, reductions in antitoxic titers were expected.

Toxic titers were determined by the ip mouse assay procedure. Serial twofold dilutions of samples were made in 0.2% gelatin–0.4% Na_2HPO_4, pH 6.4. Each of the successive dilutions expected to cause 100 to 0% mortality was injected into groups of 6 mice (0.5 ml/mouse). LD_{50}/ml was calculated by the Reed and Muench method (7) from deaths occurring within 4 days.

Types A and F titers in strain 84 toxin were assayed by neutralizing with the appropriate type of antitoxin the toxicity of specificity other than the one being studied. Thus, to determine type F titer, an equal volume of type A antitoxin containing 20 or 40 IU/ml was added to the twofold serial dilutions of the 84 toxin. After incubation of 30 min at 37°, 0.5 ml of the mixtures was injected into each mouse (6 mice/toxin dilution). The procedure resulted in each mouse being protected with an amount (5 or 10 IU) of antitoxin which would neutralize all toxicity of type A specificity. Consequently, any death would be due to toxicity of type F specificity. Type A toxicity in 84 toxin was determined similarly by using mice protected with 2 or 5 CDC units of type F antitoxin.

The anti-84 serum type A and F antitoxic titers were based on the survival of mice being challenged ip with 5 LD_{50} of the appropriate toxin type (crystalline type A or DEAE-cellulose treated type F). Serial twofold serum dilutions were mixed with an equal volume of toxin containing 20 LD_{50}/ml. After incubation of 30 min at

TABLE I. Residual Toxicities in Strain 84 Toxic Fluids After Absorption with Type A or F Antitoxin.

Expt.	Absorbed with antitoxin, type	Toxicity	
		Type[a]	LD_{50}/ml
1	Nil (control)[b]	A + F	4.3×10^5
	A	A	3.8×10^5
		F	3.6×10^3
		A + F	4.4×10^3
		A	3.0×10^1
		F	4.1×10^3
2	Nil (control)	A + F	3.4×10^5
	F	F	3.2×10^3
		A + F	3.2×10^5
		A	2.9×10^5
		F	6.0×10^1

[a] A + F: determined with mice not protected with antitoxin so measures toxin type having the higher titer; A: determined with mice protected with 2–5 CDC units of anti-F; F: determined with mice protected with 5–10 IU of type A (Porton) antitoxin.

[b] Toxin treated with 0.05 *M* phosphate buffer (pH 6.8). Titers of controls in the 2 experiments vary because final dilutions differed.

37°, each of the mixtures was injected into groups of six mice (0.5 ml mixture/mouse). Deaths within 4 days were used in the Reed and Muench procedure (7) to calculate the antitoxin dilution which would protect half of the mice being challenged. The reciprocal of the calculated dilution was multiplied by two so that the values cited represent the theoretical use of 1 ml of the antitoxin dilution.

Results. Toxin of strain 84, diluted with 0.05 *M* phosphate buffer (pH 6.8) to contain a calculated 4×10^6 LD_{50} in 8 ml, was absorbed serially by additions of 200, 200, 20, 20, 20, and 20 IU of rabbit anti-A serum. The absorbed and control preparations, now diluted 1:1.3, were then assayed for type A, type F and total toxicities (Expt. 1, Table I).

Nonspecific inactivation of toxin by the absorption manipulations was negligible since the titer of the control toxin preparation, as determined with mice not protected with antitoxin, was within the range of assay values that could be expected of the theoretical 3.8 $\times$ 10^5 LD_{50}/ml (starting 5×10^5 LD_{50}/ml corrected for 1.3-fold dilution). As verified by the control, type A LD_{50} value, this titer was expected to be of type A toxicity. This was the consequence of the type F titer being 10% that of type A (3); because of the higher A toxicity, toxin diluted to contain threshold lethal levels of type A toxicity would have less than lethal levels of type F toxicity.

Approximately 1% of the starting toxicity remained after absorption with type A antitoxin; this toxicity was of type F specificity. The absorption reversed the relative titers of A and F toxicities so that the absorbed toxic solution now had a type A titer which was less than that of type F. The absorption did not influence significantly the type F titer.

Only a trace amount of type F toxicity remained after absorbing strain 84 toxin with type F antitoxin (Expt. 2, Table I). The maximum titers, as determined in mice not protected with any antitoxin, of the control and absorbed toxin preparations were comparable, the type A titer not being significantly affected by the treatment with F antitoxin.

In both absorption trials complete removal of toxicity homologous to the type of antitoxin being added was not achieved. Howev-

TABLE II. Antitoxic Titers of Sheep Anti-84 Serum Following Absorptions with Type A or F Toxoid.

Expt.	Absorbed with toxoid, type	Antitoxic activity	
		Type	Titer[a]
1	Nil (control)[b]	A	8000
	A	F	7100
		A	840
		F	7200
2	Nil (control)	A	12,000
	F	F	11,000
		A	11,000
		F	1000

[a] Reciprocal of dilution (administered in 1 ml volumes) which would protect 50% of mice being challenged with 5 LD_{50} of toxin. Controls in two experiments have different titers because of unequal dilution during absorptions.

[b] Antitoxin treated with 0.85% saline.

er, the absorbed toxins had no lethal activity when tested in mice protected with both antitoxin types (0.5 antitoxin unit of type used for absorption and 1 unit of the other type). The incomplete removal of homologous toxicity probably resulted because insufficient amounts of antitoxins were used during the absorption procedures.

Sheep anti-84 serum was absorbed with concentrated type A toxoid (Table II). The procedure removed about 90% of anti-A activity without affecting anti-F titer. As expected from this observation, absorption of the serum with type F toxoid reduced anti-F titer without affecting significantly the serum anti-A titer.

Data summarized in Table I indicated that the crude concentrate of 84 toxin had a type F titer which was about 1% that of type A. The possibility was examined that the toxin concentration procedure might have caused the toxicity ratio different from the 10% suggested by Giménez and Ciccarelli (3).

A portion of the original culture fluid, not treated with $(NH_4)_2SO_4$, was assayed for type A and F toxicities. Type A titer of 2.6×10^5 LD_{50}/ml and type F titer of 3.3×10^3 LD_{50}/ml confirmed the approximate 1% F to A toxicity ratio. A ratio of about 0.5% was calculated from the toxicities produced by a different subculture that was grown in cooked medium for 4 days: type A = 4.3×10^4 LD_{50}/ml and type F = 2.2×10^2 LD_{50}/ml. This approximate 1% ratio was obtained whether the antitoxins used in the assays were rabbit or horse sera.

Discussion. The dual toxic specificities in culture fluids of strain 84 could result from a homogeneous population of toxic molecules, each with a single "hybrid" antigenic determinant that reacts with both type A and F antitoxin. In such a case, the antitoxic property of anti-84 serum would be due to a single species of antibody molecules. Absorbing such a serum with either type A or F toxoid would simultaneously remove antitoxic activity of both specificities. The experimental results did not support this possibility; only antitoxic activity of the homologous type was reduced (precipitated). The observations are those expected with an antiserum obtained from an animal that has been immunized with two distinct antigens.

The separate type A and F determinants could be on the same toxic protein. However, when such molecules are precipitated from solution by either type A or F antibody toxicity of both specificities would be reduced simultaneously. The absorption data showed that such did not occur; absorption with anti-A or anti-F serum removed only the toxicity of the type corresponding to the antitoxin used to treat the toxic solution. This specific precipitation of only the homologous type of toxicity must mean that strain 84 produces a mixture of two different toxic proteins, one with type A and the other with type F specificity.

The present results indicate that strain 84 produces toxic titer of type F specificity that is about 1% that of type A; these results contrast with the 10% ratio estimated earlier (3). The lower percentage would explain why in the original investigation mice were not killed even when challenged with an amount of toxin, which on the basis of 10% ratio, was calculated to contain 2 LD_{50} of type F toxicity.

Lethal property of *C. botulinum* type C_β culture filtrates is neutralized by anti-C_α serum but that of C_α cultures is not neutralized by anti-C_β serum (6). The explanation is offered by the recent observations (5) that C_α cultures produce C_1, C_2, and D toxins while C_β cultures produce only C_2 toxin. Thus, C_2 toxin (antigen) common to C_α and C_β cultures would result in anti-C_α serum having antibody that can completely neutralize the toxicity of C_β cultures. However, anti-C_β serum would neutralize only the C_2 component of C_α culture fluids; toxicity of C_1 and D would not be affected. The strain 84 toxicity of dual type specificities is a related phenomenon concerned with type A and F toxic components and the culture is not unique among *C. botulinum* in producing a mixture of antigenically distinct toxic molecules.

Summary. *Clostridium botulinum* strain 84 culture fluid concentrate, whose toxicity requires for neutralization a mixture of type A and F antitoxins, was absorbed separately

with the two types of antitoxin. Toxic titer of the type that corresponded to the antitoxin used for absorption was reduced to a low level without a significant decrease in the toxicity titer of the heterologous type. Absorption of anti-84 serum with type A or F toxoid reduced only the homologous type of antitoxic activity. Culture strain 84 produces a mixture of type A and F toxin molecules and not a single toxic molecular species which reacts with A and F antitoxins.

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1. Bowmer, E. J., Bull. W. H. O. **29**, 701 (1963).

2. Dolman, C. E., and Murakami, L., J. Infec. Dis. **109**, 107 (1961).

3. Giménez, D. F., and Ciccarelli, A. S., Zentralbl. Bakteriol., Parasitenk. Infektionskr. Hyg., Abst. 1: Orig. **215**, 212 (1970).

4. Heimsch, R. C., Champion, L. A., and Sugiyama, H., Proc. Soc. Exp. Biol. Med. **135**, 151 (1970).

5. Jansen, B. C., Onderstepoort J. Vet. Res. **38**, 93 (1971).

6. Pfenninger, W., J. Infec. Dis. **35**, 347 (1924).

7. Reed, L. J., and Muench, H., Amer. J. Hyg. **27**, 493 (1938).

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