Botulinal Toxin and Toxoid as Antigens[1] (35008)

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Antitoxins against bacterial exotoxins are conventionally prepared by immunizing animals with antigens which have been freed of specific toxicity by formaldehyde treatment. Such antiserums neutralize the biological activity of the toxin, although the absence of toxicity in the immunizing antigen is evidence that conversion to toxoid involves a modification of the toxin (1).

If toxoiding produces sufficient alteration of the toxin, antiserum prepared with toxoid (antitoxoid) might be distinguishable from that derived with toxin (antitoxin). Evidence for this possibility is given in the comparison of antitoxoids and antitoxins against types A and E toxins (2) of *Clostridium botulinum*. Absorption with toxoid completely removes the mouse protective activity from anti-toxoid but not from antitoxin.

Materials and Methods. Immunizations. Type A immunizing antigens were crystalline toxin (3) and formalinized toxoid (4). These were obtained, respectively, from Drs. E. J. Schantz and G. G. Wright of Ft. Detrick, Frederick, Maryland. Type E antiserums were prepared with the following three antigens : (a) toxoid (5): trypsinized toxin of VH strain toxoided with formalin, absorbed on aluminum phosphate and stored in 0.25% formaldehyde (supplied by Dr. Wright); (b) nonactivated crude toxin: the supernatant fluid of strain Alaska E43 dialysis sac culture precipitated at 60% $(NH_4)_2SO_4$ saturation (6), dissolving the precipitate in 0.02 *M* phosphate buffer, pH 6.0, and dialyzing against the same buffer; (c) activated toxin obtained by incubating the above toxin with crystalline trypsin (7), the enzyme being neutralized immediately thereafter by addition of crystalline soy bean trypsin inhibitor (twice the weight of trypsin used).

Male, albino, New Zealand rabbits were given twice weekly iv injections of antigen. Doses were increased every week if the animal remained healthy. Animals being immunized with toxoids were given initial injections of 0.5 ml of toxoid, im, 3 weeks apart before starting the iv series. Those to be immunized with toxin were conditioned for iv toxin by 2–3 iv administrations of toxin neutralized with an excess of homologous antitoxoid prepared in rabbits. The immunizations were continued until adequate protective titers could be demonstrated by passive mouse protection tests.

Absorption of antiserums. Routine absorptions were performed with type A and E toxoids provided by Dr. Wright. These antigens were the same as those used for immunization except for the absence of adjuvant. The fluid toxoids were freed of formalin by dialysis against 0.04 *M* phosphate buffer, pH 6.0, containing 0.02 *M* NaCl. If needed, toxoids were concentrated by dialysis against 20% (w/v) polyethylene glycol (Carbowax; mol wt, 20,000) in the above buffer. The working solutions of type A toxoid had 0.20 mg and type E 0.48 mg of protein (Folin test, bovine serum albumin standard)/ml.

Antiserums were absorbed serially with small amounts of antigen so that the antigen–antibody ratio would be at or near equivalence during one of the absorptions. Toxoid was added on a volume basis to a 1:5 dilution of antiserums; exception was the type A antitoxin which had an initial 1:10 dilution. The mixtures were incubated at 37°

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for 1 hr and then held 2 days at 4° before centrifugating out the precipitate. The supernatant fluid was collected and the absorption was repeated with another volume of toxoid. Amount of toxoid used is expressed as the cumulative toxoid to antiserum on a volume percentage basis.

Titration of antiserums. Antiserum potency was determined as the reciprocal of the dilution of the original serum which protected half of the mice being challenged with a 5-LD_{50} dose of toxin. Crystalline toxin was used for type A challenges; the type E toxin was the crude, nonactivated toxin similar to the immunizing antigen. Several different lots of type E toxin were used during the couse of the work, but the same preparation was employed throughout a given experiment.

Dilutions of the serum were mixed with an equal volume of toxin having 20 mouse ip LD_{50}/ml. After 30-min incubation at 37° 0.5 ml of each mixture was injected ip into 8–10 mice. Deaths occurring within 4 days were recorded and the dilution of the antiserum which protected half of the mice was calculated (8). Control groups of mice given calculated 0.5, 1, and 5 LD_{50} of toxin were included in every test; the proper distribution of deaths among these controls served as evidence that the antiserum assays were being done with the desired 5-LD_{50} challenge.

Results. The changes in the mouse protective titer of type E antitoxoid and antitoxins during serial absorption with type E toxoid are different (Table I). The activity in the antitoxoid decreases with successive absorptions; presumably, complete removal of titer is possible. In contrast, the titer of the antitoxins reaches a plateau level not reduced by

TABLE I. Changes in Mouse Protective Titers of Type E Antiserums During Serial Absorption with Type E Toxoid.

Absorptions Toxoid to antiserum (%; v/v)	Protective titers[a]		
	Antitoxoid	Antitoxin 1[b]	Antitoxin 2[b]
Original	13,100 (12,000–14,300)	17,500 (15,600–19,700)	7600 (6300–9200)
5	NA[c]	NA	4700 (4000–5800)
10	5300	11,400 (10,100–12,900)	NA
15	NA	NA	4000 (2800–5700)
20	280 (195–400)	12,400 (10,700–14,400)	3500 (3000–4100)
25	175	NA	3500 (2200–5600)
30	NA	11,000 (9300–12,700)	
35	NA	NA	
40	52 (42–58)	12,400 (11,100–13,800)	

[a] Reciprocal of dilution of original serum protecting half of mice challenged with 5 LD_{50} of nonactivated toxin. 95% confidence limits when data permitted statistical calculations are given in parentheses.

[b] Antitoxin 1: immunization with nonactivated toxin; antitoxin 2: immunization with trypsin activated toxin.

[c] NA: not assayed.

TABLE II. Changes in Mouse Protective Titers of Type A Antitoxoid and Antitoxin During Serial Absorption with Type A Toxoid.

Absorptions Toxoid to antiserum (%; v/v)	Residual titers after absorptions	
	Antitoxoid	Antitoxin
Original	11,000 (8500–14,300)	70,000 (62,500–78,400)
5	6000 (4700–7600)	72,000 (58,500–88,500)
10	2400 (2000–2800)	60,000 (46,800–76,800)
15	NA	60,500 (32,400–89,000)
20	740 (670–820)	NA
25	NA	60,000 (43,500–79,200)
30	280 (220–350)	57,000 (42,000–72,000)
35	NA	60,000 (45,500–73,000)
40	120 (75–190)	
50	70 (60–80)	

further additions of toxoid. Essentially the same results are seen when type A toxoid is used to absorb the homologous antitoxoid and antitoxin (Table II).

The type E toxoid used for immunization and absorption is from the VH strain and was prepared some 10 years ago (G. G. Wright, personal communication). The antitoxins were those developed with toxin of the Alaska E43 strain. Although the culture strains from which the antigens were obtained should not be important, the toxoid ages could contribute to the differences observed.

The possibility was tested with a newly prepared toxoid. Toxin equivalent to the immunizing toxin was dialyzed against 0.01 *M* phosphate buffer, pH 6.0, in 0.005 *M* NaCl and chromatographed through a DEAE column equilibrated with the same buffer. Elution with 0.04 *M* phosphate buffer, pH 6.0, in 0.02 *M* NaCl gave a toxic peak having 8×10^4 LD_{50}/mg of N. The pool of toxic fractions was incubated 18 days at 37° in 0.6% formalin and then dialyzed to obtain a formalin-free antigen; it contained 0.1 mg of protein/ml. This toxoid was used to absorb the type E antitoxoid and the antitoxin developed with nontrypsinized toxin. The results (Table III) are essentially those observed with the aged toxoid although the amount of toxoid protein necessary for a given reduction of titer is less for the new toxoid.

Type A antitoxin was serially absorbed with homologous toxoid until the titer reached a plateau not affected by further toxoid additions. Varying amounts of toxin were then added and the mouse protective activities were determined after incubation, refrigeration, and removal of the precipitates. The same experiment was repeated with the type E antitoxin prepared with nontrypsinized antigen. The toxins removed from the respective antitoxins the antitoxic activity which is not precipitated by the corresponding toxiod (Table IV).

TABLE III. Changes in Mouse Protective Titers of Type E Antiserums During Serial Absorptions with Newly Prepared Type E Toxoid.

Absorptions Toxoid to antiserum (%; v/v)	Protective titers[a]	
	Antitoxoid	Antitoxin
Original	15,900 (14,300–17,300)	17,500 (15,600–19,700)
5	NA	12,000 (10,800–13,300)
10	5200 (4200–6500)	NA
15	NA	9800 (8500–11,500)
20	700 (500–900)	10,000 (8500–11,700)
25	NA	10,700 (10,000–11,500)
30	250 (180–330)	
40	90 (70–110)	
50	23 (11–30)	

[a] Against nontrypsinized toxin.

TABLE IV. Reductions in the Mouse Protective Titer When Varying Amounts of Homologous Toxin Are Mixed with Antitoxin Previously Absorbed with Toxoid.

Type E[a]		Type A	
LD_{50} added	Titer	LD_{50} added	Titer
Toxoid[b]	10,900 (10,300–11,600)	Toxoid[b]	60,000 (45,500–79,200)
10,000	8000 (7200–8900)	12,000	50,000 (36,800–68,000)
20,000	6400 (6000–6800)	60,000	15,500 (10,700–22,500)
36,000	1600 (1400–1800)	70,000	5000 (3700–6900)

[a] Antitoxin = against nontrypsinized type E toxin; initially absorbed with trypsinized toxoid.

[b] Plateau titer after serial absorption with the toxoid.

Discussion. The treatment of botulinal toxin with formaldehyde results in a toxoid whose antigenic quality is not identical to the toxin. Absorption with toxoid completely depletes the mouse protective activity from antitoxoid while parallel absorption of antitoxin with toxoid leaves a significant residual titer. The mouse protectivity which cannot be removed with toxoid is reduced with toxin. Thus, toxoid behaves differently from toxin both as an immunizing agent and as an absorbing antigen. These observations are similar to the recent findings (9) that staphylococcal enterotoxoid precipitates less antibody from antienterotoxin than the original toxin and that the amount of antigen needed to reach antigen–antibody equivalence with antienterotoxins is greater with toxoid than with toxin.

The basic specificity of the botulinal toxin is not affected by formaldehyde since both antitoxoid and antitoxin protect against the corresponding neurotoxin. If the same chemical groupings determine both antigenicity and toxigenicity, changes sufficient to destroy toxicity do not affect antigenicity. On the other hand, when type E toxin is trypsinized, the lethality in a given volume may increase a hundred or more fold; yet, the amount of antitoxin needed to protect mice is not much greater than required for the nonactivated toxin (10). One possible explanation would be that determinants of toxigenicity and antigenicity are distinct and that toxin activation is the increase of toxigenic determinants without a concomitant change in the number of antigenic groupings. Reaction of formaldehyde with these two functionally different groups probably would be involved during toxoid formation.

Summary. Antibotulinal serums (types A and E) were prepared by immunizing rabbits with either toxin or toxoid obtained by formaldehyde treatment. Serial absorption of antitoxoid with toxoid removed the mouse protective activity completely. Similar absorption of antitoxin with toxoid left a high plateau of protective titer; this activity not precipitated by toxoid was removable with toxin. Botulinal toxoid differs sufficiently from toxin so that the two can be distinguished as immunizing or antiserum-absorbing antigens.

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