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CLOSTRIDIUM BOTULINUM TYPE B TOXOIDS[1]

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Abstract

Fluid and alum-precipitated *C. botulinum* Type B toxoids were prepared by methods very similar to those used in the production of Type A toxoid, as described in a preceding paper. These Type B toxoids had little protective effect in mice but induced a moderately high degree of immunity in guinea-pigs as shown by their resistance to multiple lethal doses of Type B toxin and the development of Type B antitoxin. A relationship was observed between the Type B antitoxic titre and resistance to Type B toxin.

Methods of preparing *Clostridium botulinum* Type A toxoid have been outlined and their properties described in a previous publication from this laboratory (1). The present paper discusses the results obtained when these methods were applied with minor modifications in the production of *C. botulinum* Type B toxoid. The Type B strain, No. 7949, used in toxin production, has been maintained in our collection for a number of years since it was received from the National Type Culture Collection. It came originally from the National Canner's Association collection where it was listed as No. 213B.

Experimental Methods

The Type B cultures were grown on the casein digest medium mentioned above for four to five days, a somewhat shorter period than the six to seven days used in Type A toxin production (1). Maximum lysis of the culture was usually apparent at this time; if incubated for a longer period a decline in toxicity and flocculation titre occurred. After filtration through paper pulp and Mandler candles, the Type B culture filtrates were tested for sterility and for toxicity in mice and guinea-pigs. The sterile filtrates were treated with varying percentages of formalin, usually 0.6% and incubated until detoxified for guinea-pigs. The antigenicity of the detoxified material was tested in mice and guinea-pigs. Fluid toxoid was precipitated with alum by the procedure previously described for Type A toxoid.

Results

Relative Toxicity of Type B Toxin for Mice and Guinea-pigs

As shown by Stevenson, Helson, and Reed (2) there is a marked difference in the sensitivity of guinea-pigs and mice to Type B toxin. In the case of a representative sample of Type B filtrate, 0.00002 ml. killed a 480 gm. guinea-pig within four days, while the killing dose for a 20 gm. mouse in the

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same interval was 0.0017 ml., that is, 1.0 ml. of this toxin represented 50,000 m.l.d. for the guinea-pig but only 588 m.l.d. for the mouse. Six other Type B filtrates contained 600 to 1400 mouse m.l.d. per ml. In contrast, Type A culture filtrates had approximately the same degree of toxicity per kilogram of body weight in mice and guinea-pigs. Even more striking differences in the relative toxicity for mice and guinea-pigs were noted with the dried acid-precipitated Type B toxin, No. 135, selected as 'standard' for testing the tolerance of immunized animals and in determining the antitoxic content of their sera. The minimum lethal dose of this toxin for a 20 gm. mouse was approximately 2.5 μgm., for a 250 to 300 gm. guinea-pig approximately 0.005 μgm. A previously described (2) Type A precipitated toxin was some 2500 times more toxic for mice than this Type B precipitated toxin but the Type A and Type B toxins had approximately the same toxicity for guinea-pigs.

Combining Activity of Toxin with Antitoxin

Since a standardized univalent Type B antitoxin was not available, a divalent botulinus antitoxin containing a specified unit titre of Type A and Type B antitoxin (Lederle purified globulin preparation No. 144*H*163*K*) was used in determining the combining activity of Type B toxin with antitoxin. This procedure seemed justifiable in that Type A and Type B botulinus toxins appeared to be unrelated antigenically as shown, both by the fact that univalent Type A antitoxin had no significant neutralizing activity for Type B toxin and that mice and guinea-pigs immunized with Type A or Type B toxoids exhibited no reciprocal immunity.

In the *in vitro* tests of combining activity with antitoxin, Lf values ranging from 2.3 to 7.3 were obtained for seven lots of toxoid; double zones of flocculation were encountered with two of these lots. No correlation was noted between Lf values and toxicity for mice.

The combining activity of the Type B filtrate per unit of antitoxin as determined in mice appeared very similar to that of the Type A toxin. The L+/10 dose of the first lot of Type B filtrate was 0.125 ml., as compared with the range of 0.056 to 0.125 ml. noted for six Type A toxins. It should be noted, however, that 0.125 ml. of the Type A culture filtrates represented 25,000 to 50,000 mouse m.l.d., whereas 0.125 ml. of the Type B filtrate corresponded to only 75 to 100 m.l.d. for the mouse. The L+/10 dose of the 'standard' Type B toxin No. 135, was 0.55 mgm. or approximately 220 mouse m.l.d.; that of the standard Type A toxin, No. 29, was 0.0156 mgm., approximately 15,600 mouse m.l.d.

Detoxification with Formalin

Treatment with 0.4 to 0.8% formalin at 37° C. rendered 500 ml. amounts of the first lot of Type B culture filtrate non-toxic for mice in seven days but at 10 days it was still toxic for guinea-pigs. This result emphasized the need for using guinea-pigs rather than mice in testing for residual toxicity in formalinized Type B filtrates. Formalin was added to six other larger lots

of Type B filtrate to give a concentration of 0.6%. After three weeks' incubation at 37° C., two of the six lots were still toxic for guinea-pigs. At the end of four weeks' incubation all lots were non-toxic; 200 to 300 gm. guinea-pigs given 5.0 ml. doses remained without symptoms of botulinus toxemia during a six week period of observation. The pH range of the seven Type B toxoids, 5.4 to 5.7, compared closely with that of the Type A toxoids tested, pH 5.4 to 6.0.

Antigenicity of Fluid and Alum Toxoids

Unlike the Type A toxoids, which were excellent antigens in both mice and guinea-pigs, the same amount of Type B toxoid had little immunizing effect in mice, although it was relatively efficient in guinea-pigs.

In Mice

Table I presents the results obtained in mice with five different fluid toxoids given in three 1.0 ml. doses at weekly intervals. Seven days after the third dose the animals were tested for resistance to 5, 10, 50, and 100 m.l.d. of 'standard' Type B toxin, No. 135. Only 13% survived 5 m.l.d., 7% 10 m.l.d., none withstood 50 or 100 m.l.d. By contrast, three 1.0 ml. doses of Type A fluid toxoid protected over 60% of mice against 100,000 LD_{50} of Type A toxin, over 85% against 10,000 LD_{50} of the same.

TABLE I

RESISTANCE TO TYPE B TOXIN IN MICE IMMUNIZED WITH THREE DOSES OF FLUID OR TWO DOSES OF ALUM-PRECIPITATED TYPE B TOXOID

Number Type B toxoids tested	State	Tolerance for 'standard' Type B toxin No. 135		
		Dosage m.l.d.	No. of mice injected	Survival, %
5	Fluid	5	31	13
		10	28	7
		50	17	0
		100	10	0
5	Alum-precipitated	5	17	41
		10	13	31
		50	12	8
		100	10	10

Two doses of alum-precipitated Type B toxoid conferred on mice a somewhat higher resistance to Type B toxin than three doses of Type B fluid toxoid.

In Guinea-pigs

In testing the toxicity of the various formalin-treated Type B filtrates, 5.0 ml. doses were given to guinea-pigs weighing 200 to 300 gm.; heavier guinea-pigs received proportionately larger doses. Six weeks after injection the animals were bled and two days later tested for resistance to 100 to

10,000 m.l.d. of the 'standard' Type B toxin. A significant proportion of guinea-pigs withstood 10,000 m.l.d., which represents a much higher degree of immunity than that produced in mice (Table II). The level of resistance to homologous toxin was, however, lower than that shown in guinea-pigs receiving the same amount of Type A toxoid.

TABLE II

RESISTANCE TO TYPE B TOXIN IN GUINEA-PIGS IMMUNIZED WITH ONE DOSE OF FLUID OR ALUM-PRECIPITATED TYPE B TOXOID

Number of toxoids tested	State	Tolerance for 'standard' Type B toxin No. 135		
		Dosage	No. of guinea-pigs injected	Survival, %
5	Fluid	100	20	75
		500	10	70
		1000	42	70
		5000	12	50
		10,000	25	44
5	Alum	100	9	100
		500	2	100
		1000	16	100
		10,000	12	8

The serum of these guinea-pigs was tested for the presence of Type B antitoxin by injecting mice with mixtures of serum and varying amounts of the 'standard' Type B toxin, No. 135. Although mice were obviously less satisfactory for such titrations than the more sensitive guinea-pig, scarcity of the latter limited their use. As shown in Table III, the antitoxic titres of these guinea-pig sera ranged from <0.002 to 10 units per ml.; the majority, 63 and 66%, had titres between 0.002 and 0.1 units per ml. Of the Type-A-toxoid guinea-pigs, the majority had Type A antitoxic titres of 0.01 to 1.0 units per ml.

TABLE III

ANTITOXIN TITRES OF SERA COLLECTED FROM GUINEA-PIGS SIX WEEKS AFTER A SINGLE IMMUNIZING DOSE OF FLUID OR ALUM-PRECIPITATED TYPE B TOXOID

Number of toxoids tested	State	Number of sera tested	Percentage of guinea-pigs with antitoxic titres of units/ml.				
			<0.002	0.002 to 0.01	>0.01 to 0.1	>0.1 to 1.0	>1.0 to 10
5	Fluid	98	12	28	35	20	5
5	Alum	32	22	13	53	6	6

When a comparison was made between the antitoxic titres of these guinea-pigs and their resistance to Type B toxin, a fairly close relationship was observed (Table IV). The proportion of guinea-pigs protected against the

TABLE IV

RELATIONSHIP BETWEEN THE ANTITOXIC TITRE OF GUINEA-PIG SERA AND THEIR RESISTANCE TO TYPE B TOXIN

Antitoxic titre units/ml.	Challenging dose of Type B toxin No. 135							
	100 m.l.d.		1000 m.l.d.		5000 m.l.d.		10,000 m.l.d.	
	No. tested	Survival, %	No. tested	Survival, %	No. tested	Survival, %	No. tested	Survival, %
0.002	8	88	7	100	0	—	1	0
0.002 to 0.01	6	100	11	64	0	—	12	25
0.01 to 0.1	9	89	15	74	5	80	26	35
0.1 to 1.0	8	100	7	100	3	100	2	100
1.0 to 10	1	100	2	100	1	100	1	100
	32	94	42	81	9	89	42	31

larger amounts of toxin, 5000 and 10,000 m.l.d., was greater in the groups having higher antitoxic titres, >0.1 to 10 units. However, some of the guinea-pigs with low antitoxic titre, <0.002 unit per ml., were able to tolerate 100 to 1000 m.l.d. of Type B toxins without symptoms of toxemia.

Local Necrosis Produced by Types A and B Toxoid

Certain lots of Types A and B toxoid, both fluid and alum-precipitated, produced a slight to extensive, 6 to 10 mm. in diameter, necrotic reaction in guinea-pigs; this tendency appeared to be somewhat more marked with Type B than with Type A toxoids. Alum precipitation definitely reduced, and in some lots eliminated, these necrotizing properties. Guinea-pigs on an inadequate diet, low in vitamin C and possibly also in other essential nutritional elements, appeared to be more sensitive than normal animals, such animals injected with Type B toxoids developed much larger necrotic areas.

Discussion

From a purely immunologic standpoint, these differences in the relative toxicity and antigenicity of *C. botulinum* Type B toxins and toxoids in mice and guinea-pigs seem particularly interesting. In the mouse, the Type B culture filtrates displayed relatively low toxicity, at least as compared with that shown by Type A toxins, and the Type B toxoids derived from them were poor immunizing agents. In the guinea-pig, Type B culture filtrates were highly toxic and Type B toxoids had a very high protective potency. By contrast, the botulinus Type A culture filtrates appeared about equally toxic for mice and guinea-pigs, the Type A toxoids were highly effective in inducing resistance to Type A toxin in both animal species.

A somewhat similar situation is encountered with diphtheria and tetanus toxins in these two animal species. In the mouse, diphtheria culture filtrates exhibit only a low degree of toxicity while tetanus cultures are highly toxic;

diphtheria toxoids induce little specific immunity, tetanus toxoids are highly effective in inducing resistance to homologous toxin. In the guinea-pig, on the other hand, both diphtheria and tetanus culture filtrates are highly toxic; both toxoids induce relatively high levels of immunity.

These two instances suggest that there may be some fundamental relationship between the potential degree of reactivity of the tissues of a given animal species to bacterial toxins and the antibody response of homologous toxoids. Our knowledge of the mechanism of toxin action in the animal body is too limited to permit elaboration of these possibilities.

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