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A PRELIMINARY STUDY OF THE ANTIGENIC ACTIVITY OF MIXTURES OF *CLOSTRIDIUM BOTULINUM* TOXOID TYPES A AND B[1]

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Abstract

When Types A and B *Clostridium botulinum* toxoids were mixed the resultant divalent toxoid was found to have a very high protective potency against both Type A and Type B botulinus toxin. The resistance to Type A toxin induced in guinea-pigs and mice by the divalent toxoid mixture was only slightly less than that conferred by a similar volume of univalent Type A toxoid. Guinea-pigs given the divalent toxoid developed a comparable or slightly higher degree of immunity to Type B toxin than those injected with univalent Type B toxoid.

Although no evidence of reciprocal protection had been observed between Type A and Type B toxoids, admixture with Type A toxoid appreciably increased the antigenicity of the Type B toxoid for mice. Mice immunized with the divalent, A + B, toxoid exhibited a higher level of resistance to Type B toxin than those given the same number of doses of Type B toxoid alone. No adjuvant effect was noted on mixing Type A or Type B botulinus toxoids with tetanus or diphtheria toxoids.

In two preceding papers in this series (1, 2), a comparison was made of the relative antigenicity of *C. botulinum* Type A and B toxoids. This analysis showed that the botulinus Type A toxoids induced a very high level of immunity in both mice and guinea-pigs. The Type B toxoids, on the other hand, while eliciting a moderately high degree of immunity in guinea-pigs, had only a slight protective effect in mice. These differences were observed with both the fluid and alum-precipitated toxoids. No cross protection was conferred by the toxoids of either type; mice and guinea-pigs resistant to multiple lethal doses of Type A toxin died with typical symptoms of botulinus toxemia on receiving a dose of Type B toxin just sufficient to kill a normal animal of the same species. Similarly mice and guinea-pigs immunized with Type B toxoid exhibited no greater tolerance for Type A toxin than normal animals of the same species. The present paper evaluates the immunizing activities in guinea-pigs and mice of mixtures of Types A and B toxoid and compares the protection obtained with that produced by the univalent toxoids.

Relative Immunizing Activity of Type B Toxoid Alone and in the Presence of Type A Toxoid

Three lots of Type A and four lots of Type B fluid toxoids and the alum-precipitated toxoids derived from them were used in the preparation of fluid and alum-precipitated divalent, A + B, toxoids. The two types of toxoid, A and B, were mixed in equal proportions. The protective properties of the

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divalent preparations against Type A and Type B toxin were determined in mice and guinea-pigs.

In Guinea-pigs.

Guinea-pigs weighing 250 to 300 gm. were injected subcutaneously with single 5.0 ml. doses of univalent, Type A or Type B, or divalent, A + B, fluid and alum-precipitated botulinus toxoids. Heavier animals received proportionately larger doses. It should be noted that in the 5.0 ml. dose of divalent toxoid, the animals were receiving only 2.5 ml. of each type of toxoid, that is only half the amount given as a univalent toxoid. Six weeks after injection, the guinea-pigs were bled from the heart. Two days later they were challenged with multiple lethal doses of Type A or Type B toxins.

As had been observed with previous lots of toxoid, these univalent Type A and Type B toxoids protected guinea-pigs against several thousand times the lethal dose of homologous toxin; the resistance developed to Type A toxin was somewhat greater than to Type B toxin. The divalent, A and B, toxoids conferred a high degree of protection in these animals against both Type A and Type B toxin, Table I. The Type A antitoxic content of sera of guinea-pigs given divalent fluid toxoids, although appreciable, was on the average

TABLE I

RELATIVE RESISTANCE TO TYPE A AND TYPE B *C. botulinum* TOXIN IN GUINEA-PIGS IMMUNIZED WITH UNIVALENT TYPE A, UNIVALENT TYPE B, AND DIVALENT, A + B, TOXOIDS

Toxoid	Challenging dose of toxin					
	Type A toxin No. 29			Type B toxin No. 135		
	Dose, m.l.d.	No. of guinea-pigs injected	Survival, %	Dose, m.l.d.	No. of guinea-pigs injected	Survival, %
Fluid toxoid						
A, univalent	16,000	28	61			
	32,000	17	53			
B, univalent				100	14	79
				1000	33	67
				10,000	21	43
A + B, divalent				100	6	100
				1000	3	100
				10,000	6	100
Alum-ppt. toxoid						
A, univalent	160,000	10	70			
	320,000	8	63			
B, univalent				100	9	100
				1000	13	100
				10,000	9	11
A + B, divalent	160,000	10	80	100	14	100
	320,000	4	75	1000	19	84
				10,000	14	86

somewhat lower than that in animals injected at the same time with univalent Type A fluid toxoid. This was probably traceable, as indicated above, to the lower dosage of Type A toxoid administered in the divalent preparation. The Type A response in guinea-pigs given alum-precipitated Type A and divalent, A + B, toxoids was relatively similar.

On the other hand, although the series of guinea-pigs used was relatively smaller, the results suggested that admixture with Type A fluid toxoid slightly increased the antigenicity of the Type B toxoids for guinea-pigs. This was indicated by a somewhat higher percentage survival to multiple lethal doses of Type B toxin and by a slightly higher mean level of Type B antitoxin in guinea-pigs immunized with divalent toxoid (Table II). However, in view of the degree of variation in the antitoxic response of individual animals and the relatively small number used, these differences are of questionable significance.

TABLE II

RELATIVE ANTITOXIC TITRE OF SERA OF GUINEA-PIGS GIVEN A SINGLE DOSE OF UNIVALENT TYPE A, UNIVALENT TYPE B, OR DIVALENT A + B TOXOIDS

Toxoid	Type A antitoxic titre						Type B antitoxic titre					
	No. of sera tested	Percentage of sera with titres of (units/ml.)					No. of sera tested	Percentage of sera with titres of (units/ml.)				
		<0.001	0.001 to 0.01	>0.01 to 0.1	>0.1 to 1.0	>1.0 to 10		<0.001	0.001 to 0.01	>0.01 to 0.1	>0.1 to 1.0	>1.0 to 10
Fluid												
A, univalent	23	48	17	22	13	0						
B, univalent							89	13	21	49	14	2
A + B, divalent	14	57	43	0	0	0	15	20	40	13	0	27
Alum												
A, univalent	19	21	26	26	16	11						
B, univalent							27	26	15	44	7	7
A + B, divalent	52	38	21	15	21	4	67	4	5	40	39	13

In Mice

Three lots of mice were inoculated at seven day intervals with three 1.0 ml. doses of univalent or divalent fluid toxoid. Three other lots of mice received two 1.0 ml. doses at 10-day intervals of the alum-precipitated univalent or divalent toxoids. Seven days after the last dose of toxoid the Type-A-immunized animals were challenged with 10,000 to 100,000 m.l.d. of 'standard' Type A toxin No. 29; the Type-B-immunized mice with 5, 50, or 100 m.l.d. of 'standard' Type B toxin, No. 135. The mice given the divalent A + B toxoids were divided into two groups; one group was injected with 10,000 or 100,000 m.l.d. of Type A toxin, the other with 5, 50, or 100 m.l.d. of Type B toxin.

TABLE III

RELATIVE RESISTANCE TO TYPE A AND TYPE B BOTULINUS TOXIN IN MICE IMMUNIZED WITH UNIVALENT TYPE A OR TYPE B OR DIVALENT FLUID AND ALUM-PRECIPITATED TOXOIDS

Toxoid	Challenging dose of toxin					
	Type A toxin No. 29			Type B toxin No. 135		
	Dose, m.l.d.	No. of mice injected	Survival, %	Dose, m.l.d.	No. of mice injected	Survival, %
Fluid toxoids						
A, univalent	10,000	26	81			
	100,000	29	62			
B, univalent				5	53	13
				50	16	0
				100	8	0
A + B, divalent	10,000	6	67	5	23	83
	100,000	8	38	50	12	58
				100	7	71
Alum-ppt. toxoids						
A, univalent	10,000	17	100			
	100,000	16	94			
B, univalent				5	24	46
				50	11	9
				100	8	12
A + B, divalent	10,000	26	69	5	34	82
	100,000	26	62	50	40	65
				500	46	81

The results shown in Table III are in agreement with the previous observations that mice immunized with the fluid Type A toxoid are highly resistant to Type A toxin, while those injected with Type B fluid toxoid develop very little tolerance for Type B toxin (1, 2). Mice given the divalent toxoid also survived multiple lethal doses of Type A toxin although the degree of resistance was usually not as high as that following a series of doses of the univalent Type A toxoid. This again was probably referable to the fact that the mice were receiving in a 1.0 ml. dose of divalent toxoid only half as much Type A toxoid as in 1.0 ml. of the univalent preparation. By contrast, the level of immunity to Type B toxin, was higher in the mice given the divalent toxoid than in those injected with the Type B univalent toxoid alone. These differences were noted with both fluid and alum-precipitated materials. The protective potency of the alum-precipitated Type B toxoid, which was considerably greater than that of fluid preparations of the same type, was further increased by mixing with alum-precipitated Type A toxoid.

In view of the lack of reciprocal immunity observed in experimental animals injected with univalent Type A or Type B toxoids, this improvement in the antigenicity of Type B toxoid on admixture with Type A toxoid did not appear to be due to an antigenic factor common to the two types of toxoid.

Rather, it seemed as if the adjuvant property of the Type A toxoid was 'non-specific' in nature, related perhaps to some sensitizing or preparatory stimulative effect exerted by this toxoid on the antibody mechanism of the injected animal that enabled it to respond more readily to the inferior antigen, Type B toxoid. If such were the case, mixing Type B toxoid with other good immunizing agents might also increase its immunizing activity. Conversely, Type A toxoid might display a similar adjuvant effect on admixture with other less active antigens. The very limited series of experiments described below failed, however, to support either of these suggestions.

Effect of Mixing Type A or Type B Botulinus Toxoids with Other Antigens

Fluid and alum-precipitated Type A or Type B toxoids were mixed with two other bacterial toxoids, tetanus and diphtheria, and with Type 2 pneumococcus suspensions and type specific carbohydrate.

Tetanus toxoid was selected because it resembles the Type A botulinus toxoid in being prepared from a toxin highly lethal for the mouse and guinea-pig and in having good immunizing potency in both animal species.

Diphtheria toxoid resembles Type B botulinus toxoid, in being derived from culture filtrates with low toxicity for mice and high toxicity for guinea-pigs, and in inducing low immunity in mice and high immunity in guinea-pigs. The Type 2 pneumococcus suspensions and carbohydrates were chosen as examples of particulate and soluble preparations having the same type-specific antigen.

Mice were used as the principal test animals because the augmentative effect of combining Types A and B toxoids on the Type B immunity response had been more marked in these animals. They were injected at seven-day intervals with two or three 0.5 ml. doses of each of the univalent toxoids alone or with the same number of 1.0 ml. doses of the divalent mixtures. Ten days after the last injection the animals were divided into groups and tested for resistance to multiple lethal doses of homologous toxin or for protection against infection with moderately large doses of pneumococcus culture.

As shown in Table IV, the fluid tetanus toxoid used in these experiments was a poor immunizing agent in mice; its protective properties were not improved by mixing with botulinus Type A, Type B, or diphtheria fluid toxoid. Alum-precipitation greatly increased the effectiveness of this toxoid. Mixing with alum-precipitated botulinus Types A or B or diphtheria toxoids did not, however, result in any further increase in antigenic potency.

Mice injected with fluid or alum-precipitated diphtheria toxoid developed little resistance to diphtheria toxin (Table V). No improvement in their protective effect was noted when they were mixed with equal parts of Type A or Type B botulinus toxoid or with tetanus toxoid, at least in so far as could be judged from the small number of mice used.

TABLE IV

NUMBER OF MICE SURVIVING MULTIPLE LETHAL DOSES OF TETANUS TOXIN FOLLOWING IMMUNIZATION WITH TETANUS TOXOID ALONE OR IN MIXTURE WITH OTHER TOXOIDS

Toxoid	State	Dose of tetanus toxin		
		20 m.l.d.	200 m.l.d.	2000 m.l.d.
Tetanus	Fluid	1/4	0/2	—
+ botulinus Type A	Fluid	0/3	0/2	0/2
+ botulinus Type B	Fluid	0/4	0/3	—
+ diphtheria	Fluid	2/6	0/2	—
Tetanus	Alum	3/4	1/2	1/1
+ botulinus Type A	Alum	2/8	2/2	1/2
+ botulinus Type B	Alum	3/4	2/2	0/2
+ diphtheria	Alum	2/3	2/3	1/2

TABLE V

NUMBER OF MICE SURVIVING MULTIPLE LETHAL DOSES OF DIPHTHERIA TOXIN FOLLOWING IMMUNIZATION WITH DIPHTHERIA TOXOID ALONE OR IN MIXTURE WITH OTHER TOXOIDS

Toxoid	State	Dose of diphtheria toxin	
		4 m.l.d.	20 m.l.d.
Diphtheria	Fluid	2/3	2/3
+ botulinus Type A	Fluid	3/3	0/2
+ botulinus Type B	Fluid	3/5	0/5
+ tetanus	Fluid	3/3	2/2
Diphtheria	Alum	1/2	1/3
+ botulinus Type A	Alum	1/2	0/2
+ botulinus Type B	Alum	1/2	0/1
+ tetanus	Alum	1/2	0/2

It appeared from these preliminary experiments therefore that the presence of botulinus Type A toxoid had no effect on the immunizing activity of diphtheria and tetanus toxoids, nor could it be shown that the addition of either of these preparations to Type B botulinus toxoid had any such adjuvant effect as had been shown by the Type A toxoid (Table VI). Indeed the presence of diphtheria toxoid, pneumococcus vaccine, and carbohydrate seemed to depress rather than augment the antigenicity of the Type B toxoid. No indication of cross immunization with the various antigens was obtained.

Discussion

These limited data indicate that mixing *C. botulinum* Type A toxoid with Type B toxoid may improve the immunizing properties of the latter for mice and possibly to a lesser degree for guinea-pigs, notwithstanding the fact that the two antigens when used singly induce no cross protection. This effect is quite aside from that produced by alum precipitation. The Type A toxoid

TABLE VI

NUMBER OF MICE SURVIVING MULTIPLE LETHAL DOSES OF BOTULINUS TYPE B TOXIN FOLLOWING IMMUNIZATION WITH TYPE B TOXOID ALONE OR IN MIXTURE WITH OTHER BACTERIAL ANTIGENS

Antigen mixture	State	Dose of Type B toxin No. 135		
		5 m.l.d.	50 m.l.d.	500 m.l.d.
Type B botulinus toxoid	Fluid	2/5	0/5	0/2
+ Type A botulinus toxoid	Fluid	5/5	3/4	2/4
+ tetanus toxoid	Fluid	3/4	0/3	—
+ diphtheria toxoid	Fluid	0/5	0/4	0/2
+ Pneumo. Type 2 vaccine	Suspension	0/5	0/5	0/5
+ Pneumo. Type 2 SSS	Solution	0/3	0/3	0/3
Type B botulinus toxoid	Alum	3/8	1/2	1/2
+ Type A botulinus toxoid	Alum	4/4	4/4	3/3
+ tetanus toxoid	Alum	1/4	0/2	0/2
+ diphtheria toxoid	Alum	0/5	0/1	—
+ Pneumo. Type 2 vaccine	Suspension	1/7	0/3	0/4
+ Pneumo. Type 2 SSS	Solution	0/5	0/2	0/2

did not exhibit this property when mixed with tetanus and diphtheria toxoid, nor was the immunizing action of Type B toxoids in mice increased by mixing with these unrelated antigens. Since the enhancing effect of Type A toxoid was limited to a botulinus toxoid of another type, the phenomenon may not have been entirely 'non-specific' as had appeared from the first review of the situation. Some basic similarity in the chemical constitution or the physiologic effects of the two types of botulinus toxoid may have contributed in some way to this unexpected immunologic response, a similarity that was not, however, sufficiently close to call forth a definite reciprocal immunity. The problem merits further detailed investigation.

In addition to their theoretical interest, these observations also appeared of some practical importance from the standpoint of antitoxin production. If divalent botulinus toxoids are highly effective in protecting against both Type A and Type B toxin, the number of injections and the period of immunization may be reduced to about half that needed were the two univalent toxoids to be administered consecutively.

References

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