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THE QUANTITATIVE DETERMINATION OF THE TOXIC FACTORS PRODUCED BY *CLOSTRIDIUM BOTULINUM* (VAN ERMENGEM, 1896) TYPES C AND D

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ABSTRACT

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The Lf values of the C_1, C_2 and D factors produced by *Clostridium botulinum* types C and D were compared with the results of *in vivo* quantitative tests. It was found that the flocculation test is a reliable method of determining these antigens quantitatively.

INTRODUCTION

In a previous study involving *Clostridium botulinum* types $C\alpha$, $C\beta$ and D, Jansen (1971) showed that type $C\alpha$ produces factors C_1, C_2 and D; type $C\beta$, factor C_2; and type D, factors C_1 and D when cultured by the method of Sterne & Wentzel (1950) (S.W. method). Following on this work it was essential to develop a reliable, relatively simple method of assay in order to determine quantitatively the amount of the different factors produced and their antigenic characteristics in relation to the production of a vaccine.

Some of the currently available tests have decided disadvantages. The LD_{50} value depends on the amount of toxin present. When, however, the organisms are cultured for several days, as is current practice in *Cl. botulinum* vaccine production, much of the toxin is converted to toxoid and thus represents antigen which cannot be determined by this method. Other tests relying on the use of experimental animals, such as the determination of the L_+ value and the total antitoxin combining power (TCP) of the antigen are sufficiently reliable, but more indirect, time-consuming and expensive than *in vitro* tests. The object of this study was to determine whether the flocculation test could replace *in vivo* methods.

MATERIALS AND METHODS

The toxins used were produced by the S.W. method.

The ideal would have been to perform the tests in this series of experiments with monospecific antitoxins against factors C_1, C_2 and D (Jansen, 1971). However, due to the exacting requirements for their preparation, they were considered too valuable for extensive use. It was also deemed important to establish whether the routine quantitative tests could be done with less specialized standard laboratory antitoxins. Consequently, monospecific antitoxins were used only in the *in vivo* tests to eliminate the toxic factors ancillary to the main one under investigation. The objective was, therefore, to prepare three antitoxic sera containing antibodies mainly against factors C_1, C_2 and D respectively.

For the preparation of a C_1 antitoxin, *Cl. botulinum* type $C\alpha$ was grown in Robertson's meat broth at 37°C for five days. The toxic fraction in the clarified culture filtrate was precipitated with ammonium sulphate at a concentration of 21 g per 100 ml after first removing the precipitate obtained with 19 g per 100 ml. The precipitate was washed three times with a solution containing 21 g ammonium sulphate per 100 ml isotonic saline, redissolved in a minimal volume of saline and dialyzed against distilled water at 4°C for 8 days. The resulting toxic solution was toxoided by the addition of formalin to a final concentration of 0,3 per cent and precipitated with 2 per cent alum at pH 6,0 (APT). The APT was then used for immunizing a horse according to Table 1.

TABLE 1 Schedule of immunizing a horse with *Cl. botulinum* type $C\alpha$ APT

Day	ml APT subcut.
1	2
43	5
112	5
125	10
133	20
136	40
141	80

The horse was bled one week after the last injection and its serum refined by the method of Pope (1939).

For the preparation of a C_2 antitoxin, *Cl. botulinum* type $C\beta$ was cultured by the S.W. method for ten days. By serial fractionation of the clarified culture filtrate with ammonium sulphate two precipitates were obtained. The first precipitate obtained with ammonium sulphate at a concentration of 27 g per 100 ml was only slightly toxic after being washed, redissolved and dialyzed free of sulphate, while the second precipitate (27 g to 39 g ammonium sulphate per 100 ml) was much more toxic. The second precipitate was toxoided, alum-precipitated and used for the immunization of a horse.

The antitoxin against factor D was produced by growing *Cl. botulinum* type D in Robertson's meat broth at 37°C for five days. The toxic culture filtrate was clarified and serially fractionated with ammonium sulphate. The fractions were washed, redissolved, dialyzed free of sulphate and then tested for toxicity. The fraction precipitated by ammonium sulphate in the range of 20 g to 25 g per 100 ml was the most toxic. This was used for the immunization of a horse.

The antitoxin against factor D was produced by growing *Cl. botulinum* type D in Robertson's meat broth at 37°C for five days. The toxic culture filtrate was clarified and serially fractionated with ammonium sulphate. The fractions were washed, redissolved, dialyzed free of sulphate and then tested for toxicity. The fraction precipitated by ammonium sulphate in the range of 20 g to 25 g per 100 ml was the most toxic. This was used for the immunization of a horse.

The L_+ value of the toxic factors was determined by mixing the clarified culture filtrate (diluted 1:10 in physiological saline for C_1 and D and undiluted for C_2) with decreasing quantities of antitoxin. The toxic factors not concerned in the test were neutralized by

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adding excess monospecific antitoxin, e.g. when determining the C_1 factor, C_2 and D were neutralized. The toxin-antitoxin mixtures were left at room temperature for one hour and then injected intravenously in volumes of 0,2 ml into white mice weighing an average of 25 g. The results were recorded for three days.

The Ramon flocculation test, as modified by Glenny & Okell (1924) for the titration of diphtheria toxin and antitoxin, was used. The culture filtrates were diluted 1:10 and quantities of 1,0 ml measured into tubes 8 × 1 cm. The refined antitoxin was added to the tubes in increasing quantities and the mixtures shaken mechanically in a waterbath at 38 °C.

The L_f and L_+ values of the toxic culture filtrates were expressed in terms of the volume of serum equivalent to 1,0 ml of toxic solution. This was done because Jansen (1971) demonstrated that the International Standard Antitoxin was of no value in measuring the individual factors.

For the quantitative determination of toxoided factors their total combining power (TCP) was determined by the method used by Mason (1935) for the β antigen of *Cl. welchii* type B. For each toxoid the corresponding toxin was rendered specific by neutralizing the irrelevant elements with monospecific antitoxins. The same method of calculating the number of toxoid units in a solution was applied.

Results

The results of the comparison between the L_f and L_+ values as determined for each factor on a different series of toxic culture filtrates are recorded in Table 2.

By applying the t-test (Snedecor & Cochran, 1969) to the sets of values for each factor in Table 2, it is obvious that there is no difference between the two assay methods at the 1 per cent level of significance. Furthermore the correlation coefficient for the two sets of values under factor C_1 is 0,95, for C_2 it is 0,8 and for D 0,99.

Because the toxoided culture filtrate flocculated readily when mixed with antitoxin under the conditions described above, the values obtained by the flocculation test were compared with the results of the TCP test for each factor on a separate series of formolized culture filtrates. The results are given in Table 3.

From Table 3 it is clear that for each factor the results of the two tests arrange the toxoids in the same order of potency.

Discussion

According to the results recorded in Table 2 the values obtained by the *in vivo* and *in vitro* methods for the same toxic culture filtrates coincide remarkably well. The reason why so many of the filtrates have the same potency can be explained by the fact that they were drawn from different flasks of the same batch of cultures.

A statistical examination shows convincingly that the *in vitro* test results in the same antigen measurement as the *in vivo* for all three the factors.

When the toxoided antigen is determined quantitatively by the flocculation test and its total combining power is measured by an *in vivo* method, the results also coincide rather well (Table 3). This leads to the conclusion that the flocculation test is acceptable for measuring the quantities of C_1, C_2 and D antigens of *Cl. botulinum* types C and D.

Summary

The L_+ and L_f values of toxic culture filtrates of *Cl. botulinum* types C and D were determined with respect to factors C_1, C_2 and D. The L_f values of toxoided culture filtrates were also compared with their total antitoxin combining power. For each of the three factors it was found that the flocculation test could be relied upon to measure the antigen quantitatively.

Table 2 The L_f and L_+ values for factor C_1, C_2 and D determined on separate series of culture filtrates

Factor	C_1		C_2		D	
Value	L_f ml serum	L_+ ml serum	L_f ml serum	L_+ ml serum	L_f ml serum	L_+ ml serum
	0,08	0,08	0,008	0,008	0,03	0,04
	0,04	0,04	0,008	0,008	0,02	0,02
	0,06	0,06	0,008	0,008	0,02	0,02
	0,08	0,08	0,008	0,01	0,02	0,02
	0,06	0,06	0,01	0,01	0,03	0,03
	0,08	0,08	0,01	0,01	0,03	0,04
	0,06	0,06	0,01	0,01	0,03	0,04
	0,04	0,04	0,01	0,02	0,02	0,02
	0,10	0,10	0,02	0,01	0,01	0,02
	0,04	0,04	0,03	0,03	0,008	0,01
	0,04	0,04	0,01	0,01	0,008	0,01
	0,04	0,06	0,02	0,03	0,008	0,01
	0,04	0,04	0,02	0,02		
	0,08	0,10	0,02	0,02		
	0,04	0,04				
	0,04	0,04				
	0,04	0,04				
	0,04	0,04				
	0,02	0,04				
	0,02	0,02				
	0,04	0,04				
	0,06	0,06				
	0,02	0,02				
	0,04	0,06				
	0,06	0,06				
	0,08	0,08				
	0,06	0,06				
	0,03	0,04				

TABLE 3 The results obtained with the flocculation test (L_f) and combining power test (TCP) done on toxoided culture filtrates

Factor	C_1		C_2		D	
Value	L_f ml serum	TCP Units/ml	L_f ml serum	TCP Units/ml	L_f ml serum	TCP Units/ml
	0,01	6,4	0,002	4,0	0,004	60
	0,012	12,8	0,002	4,0	0,008	120
	0,02	12,8	0,004	8,0	0,015	240
	0,03	25,6	0,004	8,0	0,01	160
	0,03	25,6	0,006	16,0	0,015	240
	0,03	25,6	0,006	16,0	0,02	240
	0,04	25,6	0,008	16,0	0,02	240
	0,05	51,2	0,008	16,0	0,02	240
	0,06	51,2	0,008	32,0	0,03	480
	0,06	51,2	0,008	16,0	0,03	640
	0,06	51,2	0,01	32,0	0,03	640
	0,08	102,4	0,01	32,0		
			0,01	32,0		
			0,02	64,0		
			0,02	64,0		
			0,02	64,0		
			0,02	64,0		
			0,02	64,0		
			0,03	64,0		

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