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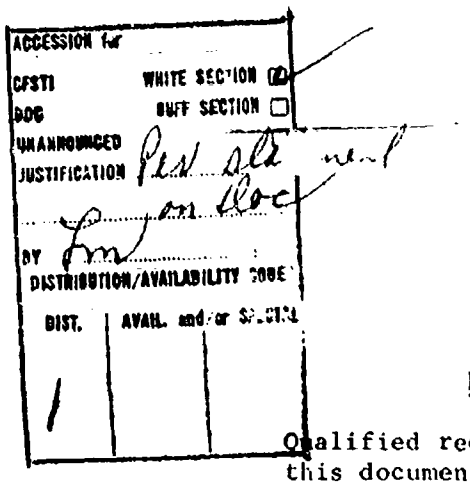

THE SIGNIFICANCE OF TYPE E BOTULINUM TOXIN ACTIVATION IN THE LABORATORY DIAGNOSIS OF BOTULISM

[Following is the translation of an article by T. I. Bulatova, Gamaleya Institute of Epidemiology and Microbiology, AMN, USSR, Moscow, published in the Russian-language periodical Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii (Journal of Microbiology, Epidemiology and Immunobiology), No. 8, 1964, pages 97-101. It was submitted on 14 Jan 1963. Translation performed by Sp/7 Charles T. Ostertag Jr.]

Of the six known causative agents of botulism (types A, B, C, D, E, F) only four have been detected in the Soviet Union -- types A, B, C and E. It has been established by investigations of recent years (Kravchenko et al., 1960, Shishulina, 1962) that not only types A and B botulism causative agents are widely distributed here, as this was considered earlier, but types C and E are encountered also -- types A, B, C and E have been found in the soil, A, B, and E in fish, and type E in water. According to the comprehensive investigations by the stated authors (6,804 tests) the causative agents of type E were isolated most often in various regions of the USSR, both in the soil and in fish. Type E was detected in 73.9% of the total number of positive tests. However, up until now in the communications of native investigators it is indicated that outbreaks of botulism in the Soviet Union are caused mainly by the type A and B causative agents. A unique case of type E botulism was described by Zavadovskaya in 1940.

In recent years, in connection with the issue (by the Gamaleya Institute of Epidemiology and Microbiology, AMN, USSR) of diagnostic type specific antibotulin sera of types A, B, C and E, outbreaks of type E botulism began to be detected more often (Donets, 1961; Sirota and Raynes, 1962; Litvinenko and Suvorov, 1962; Sergeyeva, 1962).

The causative agent of type E botulism is considered a weakly toxigenic microbe, since usually on artificial nutrient media it produces a weak toxin.

However, cases of botulism caused by it in humans testified to the high strength of this toxin. Since the discovery of this microbe (1934) more than 55 outbreaks of type E botulism have been described abroad. These

have encompassed 263 persons, out of which 88 died (Dolman et al., 1962), that is, the lethality was 34%.

Investigations conducted by us in 1954-1956 (Matveyev and Bulatova) and the recently published works of other authors make it possible to explain this apparent discrepancy (Sakaguchi et al., 1956-1959; Duff et al., 1956; Gordon et al., 1957).

By studying the toxin formation of Cl. botulinum type E in a mixed culture with Cl. sporogenes (18 strains) or Cl. putrificus (3 strains), we established that several strains of Cl. sporogenes stimulated the formation of type E botulinum toxin (strain No. 188-20), thereby confirming the data of Kushnir (1952). After being maintained for 6-8 days in an incubator at 28°, the filtrates of the mixed cultures and the pure culture of strain E No. 188-20 were titrated in mice, and simultaneously the neutralization reaction was set up with type specific serum type E. Only in one out of 18 tests with the Cl. sporogenes strain No. 3170 an increase of 100 times was noted in the strength of the botulinum toxin in comparison with its initial strength. The titer of the toxin of the pure strain usually equaled 1,000 Dlm in 1 ml, the titer of the toxin of the mixed culture reached 100,000 Dlm. The botulinum toxins obtained by incubation of a mixed culture were strictly type specific, and were completely neutralized by type specific antibotulinum serum type E.

During the joint incubation of botulism causative agents of types A, B and C with strain No. 3170, the strength of the toxin taken in the tests of the microbes was not increased.

In order to clear up the mechanism of the described phenomenon, we treated an 8-day filtrate and the cultural liquid of Cl. botulinum with the filtrate of a 2-3 day culture of strain No. 3170. The filtrates were combined in a ratio of 2:1, 1:1 and 1:2, and maintained in an incubator for 1, 2 and 24 hours at 37°. In a number of cases we observed a noticeable increase in strength of the botulinum toxin. However, it was less than during the combined incubation of Cl. botulinum type E and Cl. sporogenes No. 3170.

The facts obtained led us to the thought of the possible activation of type E botulinum toxin by a culture of Cl. sporogenes. The phenomenon of toxin activation by enzymes is described in Cl. perfringens type D (Turner and Rodwell, 1943). In the work by Ross et al. (1949) there is talk of the toxin activation of Cl. perfringens type E.

Assuming the presence of a prototoxin in Cl. botulinum type E, we attempted to activate both the sterile filtrates of this microbe, and the non-filtered cultural liquid, by treating them with enzymes. We combined 2 parts of a filtrate of a type E culture and 1 part of a 4% solution of

pancreatin in test tubes; in control test tubes physiological solution was added in place of pancreatin. The pH of the filtrates equaled 6.0. The mixtures were maintained at 37° for 5, 30 and 60 minutes and 2, 5 and 24 hours. Titration of the control mixture and the mixture activated with pancreatin showed that the strength of the toxin after activation with pancreatin had increased considerably. In the majority of tests the index of activation equaled 100 (incubation for 1--2 hours at 37°); if the strength of the initial toxin comprised 100--1,000 Dlm, then correspondingly the strength of the activated toxin reached 10,000 -- 100,000 Dlm in 1 ml.

The activity of the sterile filtrate treated with pancreatin was approximately the same as the activity of the whole culture treated with pancreatin. This testified that the liberation of toxin from the bacterial cells is not the main reason for the increase of toxicity after activation.

We also established an increase in toxicity in filtrates of a pure culture of type E following the action of pancreatin on them, at the same time that in filtrates, obtained as a result of the incubation of a mixed culture, the strength of type E botulinum toxin following the influence of pancreatin on them increased insignificantly (1.5 -- 2 times) or not at all. This speaks for the fact that the mechanism of action of Cl. sporogenes and pancreatin are the same, that is, this is a fermentation process, as the result of which a nonactive substance is converted into an active one.

Further tests on the activation of type E native botulinum toxins were conducted with other type E strains (table 1). It was established that in several strains, which formed weak toxins on nutrient media (2--10 Dlm), after their activation by pancreatin the strength of the toxin reached 5,000--10,000 Dlm/ml, that is, the index of activation equaled 500--1,000. At the same time, in other strains the index of activation did not exceed 10.

The results of similar tests on the activation of botulinum toxins of types A, B, and C were negative (the work was performed with 5-6 day filtrates of these toxins).

In tests on mice and guinea pigs we were able to show the significance of activation of type E botulinum toxin by enzymes by the toxic effect on animals. In the test we used the filtrate from a pure type E culture and a mixed culture. These were administered to the animals before and after activation. The mice received the toxin intravenously and orally, and the guinea pigs -- orally and subcutaneously (table 2).

The results of the tests showed that after activation of the filtrate of a pure strain by pancreatin, a significant increase was observed in the toxicity for mice following the intravenous administration and for guinea

pigs following the subcutaneous administration. At the same time, following the oral administration of the toxin to mice and guinea pigs no increase in toxicity was observed. In the mixed culture an increase in toxicity following the activation by pancreatin could not be detected neither following the parenteral administration to mice and guinea pigs nor following the oral administration.

The data obtained makes it possible to propose that a filtrate of a culture of type E, containing a little botulinum toxin and a large amount of prototoxin, was activated following oral administration in the digestive tract, therefore following the oral administration of an activated filtrate an increase in its toxicity could not be noticed. The results with toxin of a mixed strain make it possible to assume that this toxin is activated by the proteinase of Cl. sporogenes, therefore after activation by pancreatin, as it should have been expected, there was no increase in its toxicity not only following oral administration but also following subcutaneous and intravenous administration.

The high lethality during poisoning of humans with type E botulinum toxin becomes fully understandable, in spite of the fact that pure strains of type E possess a weak toxicity. Botulinum toxin and its prototoxin enter the human organism together with the food. The prototoxin is activated by enzymes in the digestive tract, therefore the destructive effect of the toxin on the organism is increased. Besides this, in the food product infected with the type E microbe there is the possibility of the activation of the toxin by proteinases of various origin (mainly attendant microflora) which also increases the destructive effect of the toxin on the organism.

In sanitary-epidemiological stations and other diagnostic laboratories, following the isolation of a causative agent which caused food poisoning with a clinical picture similar to botulism, often a toxin is not detected and a toxigenic strain is not found. In order to exclude the presence of type E Cl. botulinum in the materials being investigated, it is necessary that the nontoxic filtrates under study be subjected to activation.

In our investigations, conducted with extracts from various food products, in which we administered doses of type E botulinum toxin that were nonlethal for mice (0.1 - 0.01 - 0.001 Dlm/ml), the toxin could be exposed by a test on the mice only after activation with pancreatin (table 3).

We consider that the activation of extracts or filtrates should be conducted in all cases of laboratory investigation for botulism if they prove to be nontoxic during the initial investigations. Therefore, in the recently issued "Instructions on the sequence of investigation and registration of food poisonings with the method of bacteriological investigations

during food poisonings" (Medgiz, 1962), we have provided for the activation of extracts being investigated with pancreatin. This makes it possible to improve the laboratory diagnosis of botulism.

Sakaguchi (1959), during the investigation of the cultural liquid of several strains of Cl. botulinum type E on a 1-2 day growth prior to their activation with pancreatin, did not detect toxin in them in tests on mice, whereas after activation 1 ml contained 20 Dlm of the toxin.

With the help of pancreatin activation Shishulina (1962) was able to increase the percentage of detection of the causative agent of type E botulism during investigations of soil samples.

Conclusions

1. On artificial nutrient media, Cl. botulinum produces a toxin and a nontoxic prototoxin. The latter may be transformed into a toxin due to the action of various enzymes (pancreatin, proteinase of Cl. sporogenes).

2. When botulinum prototoxin type E reaches the digestive tract its activation by enzymes takes place. As a result of this the toxic effect on the organism is strengthened.

3. During the investigation of various materials (food products, discharges from a patient, section material, original seedings) for the presence of botulinum toxins, it is necessary to subject the nontoxic filtrates to activation by pancreatin, and after this set up biological tests and the neutralization reaction with antibotulinum serum type E.

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Table 1

Activation of type E botulinum toxin by pancreatin

Strain	Titer of toxin (in Dlm/ml)		Index of activation
	Before activation	After activation	
188-20	1 000	100 000	100
153	100	1 000	10
Ped-1	10	100	10
Ped-2	10	5 000	500
Ped-3	10	10 000	1 000
Ped-4	2	1 000	500
P-34	100	1 000	10
HV-8	50	5 000	100
20-18	100	1 000	10
115-35	50	1 000	20
186-2	20	1 000	50
Sergeyeva	10 000	100 000	10

Table 2

Influence of activation by pancreatin on the toxicity of type E Cl. botulinum for guinea pigs and mice during various methods of administration

Culture from which toxin obtained	Toxin utilized in test	Value of lethal dose (in ml) for various animals			
		Mice		Guinea pigs	
		Intravenous	Orally	Subcutaneous	Orally
Strain E No 188-20 Mixed	Before activation	0.001	0.2	0.05	0.5
	After activation	0.00001	0.2	0.0005	0.5
	Before activation	0.00002	0.2	0.0005	0.5
	After activation	0.00001	0.2	0.0005	0.5

Table 3

Toxicity of extracts, containing botulinum toxin type E, before and after activation with pancreatin

Strain from which toxin obtained	Before activation		After activation		Activation
	Concentration of toxin in extract (Dlm)	Result of biological test	Concentration of toxin in same extract (in Dlm)	Result of biological test	
188-20 or 115-35	1 0.1 0.01	++ 00 00	>10<100 >1<10 <1	++ ++ 00	10 times
Ped-2 or Ped-3	1 0.1 0.01 0.001	++ 00 00 00	>100<1000 >10<100 >1<10 <1	++ ++ ++ 00	100 times