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DETECTION OF INTRACELLULAR BOTULINUM E TOXIN BY FLUORESCENT ANTIBODY TECHNIQUE*¹

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INTRODUCTION

Methods for detection of botulinum toxin in cultures or food samples are based on injection of cultural supernatants or extracts into sensitive experimental animals, generally mice. The mice are observed for survival and symptoms of botulism during a 96 hour period and the specificity of the reaction is checked by protecting mice with botulinum antitoxin. This biological test is specific and sensitive but it is time consuming and also rather expensive if a large number of samples have to be examined.

Fluorescent antibody technique has been applied in the detection of diphtheria toxin⁴⁾ and staphylococcus enterotoxins^{1,2)}. The present paper describes the development of a similar technique for the detection of botulinum type E toxin. This technique will be of value in rapid screening of the effect of a number of factors on toxin production, and is also a useful tool to distinguish non-toxic or hypotoxigenic type E strains from toxic strains.

MATERIALS AND METHODS

Origin of *C. botulinum* cultures

C. botulinum type E, toxic strains: Minnesota, from Dr. G. YORK, University of California, Davis, USA. B 103-6, isolated from mud sample in Greenland, Dr. H. O. PEDERSEN. 1663/61 and 3324/61, from Wellcome Research Laboratory, Beckenham, England. E-50, from Dr. C. E. DOLMAN.

C. botulinum type E, atoxic strains: 13 BPL, 7 BPL2, 20 FPL, and 14 APL3, isolated from mud samples in the north sea, Torry Research Station, Aberdeen, USA. GB 3 and S-9, from Dr. D. A. KAUTTER, Food and Drug Administration Laboratory, Washington, D. C., USA. These strains are closely related to the toxic strains and stained with Well-

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come fluorescent antisera prepared against boiled, toxic E cells³).

C. botulinum type A and type B, toxic strains: 62A and 32B, from Dr. G. YORK, University of California, Davis, USA.

Partially purified antitoxin (horse origin)

Lyophilized type E antitoxic serum containing 10 antitoxic units per ml was obtained from Communicable Disease Center, Atlanta, Georgia, USA and used through the experiments. Ten ml of the serum was mixed with 10 ml of saturated ammonium sulfate solution and kept half an hour at room temperature to precipitate gamma globulins. The precipitate was collected by centrifugation and redissolved in distilled water. The ammonium sulfate precipitation was repeated two times and the final precipitate was dissolved in distilled water and dialyzed overnight at 2°C against 0.02 molar phosphate buffer pH 7.2 containing 0.9% sodium chloride (saline phosphate buffer).

The dialyzed preparation was absorbed with an equal volume of wet packed, boiled cells from a 15 hour culture of *C. botulinum* type E Minnesota. The absorption took place for one hour at 37°C followed by 18 hours at 2°C. The absorbed serum was recovered by centrifugation and the absorption was repeated- this time using not-boiled cells from atoxic cultures 13 BPL and 20 FPL of *C. botulinum* type E.

Preliminary experiments indicated that the described absorption is necessary to avoid staining of atoxic *C. botulinum* E strains which are frequently found together with toxic *C. botulinum* type E.

The purity of the prepared antitoxin was checked by gel diffusion technique against concentrated, toxic culture filtrates of *C. botulinum* type E. Only one precipitation line was formed which indicates that the botulinum antitoxin was the only relevant antibody which was present in significant amounts.

Fluorescent rabbit anti-equine gamma globulin

A preparation labeled with fluorescein isothiocyanate was obtained from Nutritional Biochemical Corp. Five ml of the preparation was placed on a DEAE-cellulose column which had been equilibrated with 0.05 molar phosphate buffer pH 7.2. After the serum had

TABLE 1 *Fluorescent staining of C. botulinum type E Minnesota, 24 hour culture, with fluorescent rabbit anti-equine gamma globulin (see the text)*

PREPARATION	INTENSITY OF STAINING
20X diluted original gamma globulin solution	+
Gamma globulin fractions from DEAE cellulose column	
Fraction 1	—
Fraction 2	±
Fraction 3	+
Fraction 4	+

been absorbed in the column a small volume of buffer was percolated through. Elution was then carried out with the same kind of buffer containing stepwise increasing concentrations of sodium chloride: 0.1 molar (fraction 1), 0.25 molar (fraction 2), 0.5 molar (fraction 3), and 1.0 molar (fraction 4). The ability of the four fractions to stain toxic *C. botulinum* E cells was tested by direct staining technique (without antitoxin). As seen in table 1, fractions 2 to 4 give unspecific reaction. Fractions 2 to 4 were discarded and fraction 1 was retained to be used for staining as described in the following.

Preparation of smears

Smears were made on cover-slips from proteose peptone cultures. The preparations were air dried and fixed in 95 % ethanol, 100 % acetone, 100 % methanol or 10 % formaldehyde at room temperature or at -20°C . All fixation methods except methanol at -20°C gave negative staining results with toxic strains of *C. botulinum* type E. The best staining was obtained with preparations which had not been fixed but only air dried. However, frequently a large number of cells are lost during washing when fixation is omitted even if acetone cleaned cover-slips are used. Preparations of the atoxic *C. botulinum* type E cultures 13 BPL and 20 FPL were made and stained in the same way. These preparations were used as controls of the specificity of the toxin staining reaction.

Staining

The staining was carried in the following way. A few drops of the partially purified antitoxin were placed on the cover-slip preparations which were then incubated in a moist atmosphere for one hour at 37°C . The preparations were drained and air dried after rinsing twice with saline phosphate buffer and once with distilled water (step 1).

The dried preparations were covered with a few drops of the fluorescent rabbit anti-equine gamma globulin solution and left in a moist atmosphere for half an hour at 37°C . Washing and drying was then carried out as before (step 2).

The stained preparations were mounted in 10 % glycerol phosphate buffer and microscopic examination was carried out with a Zeiss fluorescent microscope using high power dry objective.

RESULTS

The results of staining and microscopy of *C. botulinum* cultures are shown in table 2, and figure.

Various tests carried out to check the specificity of the staining reaction gave the following results.

- 1 There was no staining of cells from toxic *C. botulinum* type E cultures when type A or type B antitoxin was substituted for type E antitoxin in step 1 of the staining reaction.
- 2 Staining was also negative when toxic *C. botulinum* type E preparations were treated with unlabeled rabbit anti-equine gamma globulin between step 1 and step 2 of the staining reaction.
- 3 Atoxic *C. botulinum* type E preparations were not stained after they had been suspended in the supernatant fluid from toxic *C. botulinum* type E cultures.
- 4 Cultures of atoxic *C. botulinum* type E, cultures of *Staphylococcus aureus*, and cultures

TABLE 2 *Staining of various strains of C. botulinum by indirect fluorescent antibody technique (type E antitoxin)*

CELLS USED	RESULT
<i>C. botulinum</i> E atoxic strains	
13 BPL, 7 BPL 2, 20 FPL, 14 APL 3 S-9, GB 3	No staining
<i>C. botulinum</i> E toxic strains	
B 103-6, Minnesota, 1663/61, 3324/61, E-50	Staining
<i>C. botulinum</i> E toxic strains (same as above)	
heated 10 min 100°C	No staining
<i>C. botulinum</i> 62A	No staining
<i>C. botulinum</i> 32B	No staining

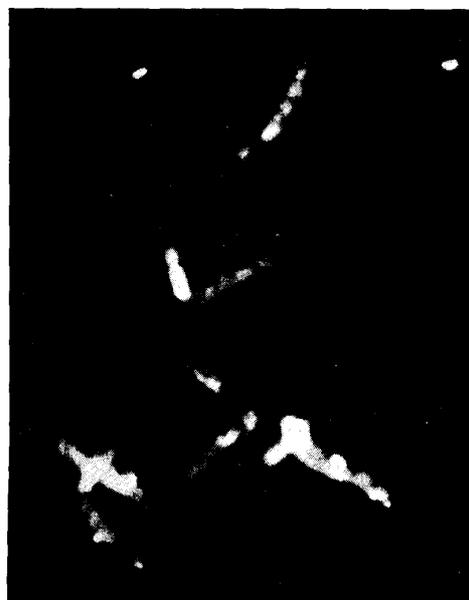
Note: The cells used for staining were grown 12~24 hours at 30°C in 2% proteose peptone containing 1.5% yeast extract, 1% glucose and 0.1% sodium thioglycollate.

FIGURE *Twenty-four hour cultures of C. botulinum type E strain Minnesota (A) and strain 3324/61 (B) stained for toxin by indirect fluorescent antibody technique*

(A)



(B)



of the putrifactive clostridium PA 3679, all gave negative staining results.

5 Cells from toxic cultures of *C. botulinum* types A and B gave negative staining results.

The best staining of *C. botulinum* type E was observed with 24 hour cultures grown in 2% proteose peptone containing 1.5% yeast extract, 1% glucose, and 0.1~0.2% sodium

thioglycollate. Positive results were also obtained with very young, 6 hour, cultures grown from an inoculum of vegetative cells or spores. Forty-eight hour cultures still contained intact fluorescent cells but also much debris originating from the lysing cells. This was even more pronounced in 96 hour cultures although still some intact fluorescent cells could be detected. Cells from blood agar colonies generally gave a weak staining reaction possibly because this medium is suboptimal for toxin production. Staining reaction was also carried out using cells grown in various other media; meat piece medium, 5% trypticase medium, brain-heart infusion medium. All the cultures gave positive results but the proteose peptone medium was found best.

Cells from 12 to 14 hour proteose peptone cultures, stained very brightly, after they had been washed three times with saline containing 0.1% sodium thioglycollate. Washed cells actually made better preparations since they stick better to the cover-slip and make fixation unnecessary. Another way to overcome the problem of fixation is to stain the cells on Millipore filters as described by GENIGEORGIS & SADLER (1966).

No attempts were made to measure the sensitivity of the fluorescent antibody technique as compared to the traditional mouse test. But it was observed that 6 hour proteose peptone cultures which were positive by the fluorescent antibody technique contained less than 100 MLD when tested by intraperitoneal injection in mice after trypsin activation.

Attempts were made to apply direct staining technique using partially purified type E antitoxin which had been labeled with fluorescein isothiocyanate and isolated by chromatography on DEAE-cellulose. However, preliminary experiments indicated that the direct staining technique is not as sensitive as the indirect and it also tended to give unspecific reactions.

Preliminary staining experiments were carried out with *C. botulinum* type A, using the principles as described for *C. botulinum* type E. In this case the antitoxin was absorbed only with boiled *C. botulinum* type A cells since atoxic *C. botulinum* type A cultures were not available. The results were promising and the staining reaction seemed to be specific since no staining was obtained with toxic cultures of *C. botulinum* type B and type E.

SUMMARY

Intracellular botulinum type E toxin was detected by indirect fluorescent antibody technique. The specificity of the staining reaction was examined and discussed.

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