THE DETECTION OF CLOSTRIDIUM BOTULINUM BY MEANS OF LUMINESCENT ANTIBODIES

COMMUNICATION I. THE PRODUCTION OF SPECIFIC LUMINESCENCE IN CLOSTRIDIUM BOTULINUM BY TREATMENT WITH A LUMINESCENT IMMUNE SERUM

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One of the most complicated and, at the same time, important divisions of microbiology is the rapid identification of microorganisms, both in pure culture and, in particular, when mixed with other microorganisms in different substrates. In order to solve this problem, it is possible to use the method, developed and recommended by Coons and his coworkers [5, 6], of direct detection of bacterial and viral antigens by means of luminescent immune sera. Many reports have recently appeared in the literature [7, 8, 10] of the successful use of this method for the rapid identification of a whole series of bacteria, viruses and yeasts. Much work has been done on the direct detection of various pathogenic microorganisms in objects from the external environment, such as: the causative organism of pseudoglanders [11], the typhoid bacillus [1], the streptococcus [9] and the agents of dysentery and anthrax [2, 4] and paratyphod fever [3].

In the present research, our aim was to obtain a luminescent botulinus antiserum for the identification of the microorganism Clostridium botulinum.

METHOD

In this work we used botulinus rabbit antisera, prepared by the hyperimmunization of animals with an anaculture of Cl. botulinum (strain 98 type A). In addition, normal rabbit sera were used as controls. Films were prepared in physiological saline from a suspension, in one group of experiments, of 2-day cultures of anaerobes grown on blood agar and, in the other group, from 2-day cultures grown in liquid media (casein medium, saccharose broth). The suspensions contained 5 x 10^8 bacterial cells in 1 ml. The globulin fractions were obtained from the immune and normal rabbit sera by triple precipitation with half-saturated ammonium sulfate solution and subsequent dialysis against physiological saline to remove the SO_4^{2-} ions. The protein content of the immune sera and their globulin fractions was determined by a refractometric method; an electrophoretic analysis was carried out. The immune serum contained 8.16% of protein. Its titer in the agglutination reaction was 1:300. The globulin fraction contained not less than 1% of protein, and electrophoretic examination showed absence of albumins in it. The globulin fraction was twice as active serologically as the original serum.

Conjugates of the globulins of the normal and immune sera with luminescent dyes were obtained. The dye used was liquid fluorescein isocyanate *.

Combination of the globulin with the fluorescein isocyanate was carried out by the method of Coons and Kaplan [7], in a water - dioxane - acetone medium in the cold, with constant agitation for 18 hr. In this mixture, the protein content did not exceed 2% and the dioxane content 15%; the isocyanate solution was added in a dose of 5 mg of dye to 100 mg of protein. At the end of the reaction, in order to remove the uncombined dye, the conjugate was dialyzed against physiological saline for 2-3 days; it was then precipitated 4-6 times with a half-saturated solution of ammonium sulfate, followed by dialysis to remove SO_4^{2-} ions. The precipitation was continued until the dye was completely removed from the supernatant fluid.

In order to remove nonspecific luminescence, the luminescent serum was treated with powdered liver from white mice †.

The films were stained as follows: A drop of the bacterial suspension was placed on a fat-free glass slide and dried at room temperature, after which it was fixed for 30 min in ethyl alcohol and dried in air. A few drops of undiluted luminescent immune serum were poured on to the films, placed in a humid chamber, so that the entire film was covered with serum. After 30 min the films were washed with several samples of physiological saline at pH 7.4, rinsed with distilled water and dried in air. As controls, we used films stained with luminescent normal rabbit serum. The completed films were examined beneath the MUF-ZM microscope, equipped with an OI-23

* The dye was kindly supplied by our colleague G. I. Mikhailov of the Institute of Chemical Reagents.

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luminescent illuminator. As a source of light we used a SVD-120A mercury lamp. Light filters SS-4, SS-8 and ZhS-17 or 2N were used, with an apochromatic objective of 90 x and an eyepiece of 7 x.

RESULTS

When the films were stained with luminescent botulinus antiserum, a specific luminescence of the Cl. botulinum cells was clearly observed, whereas the other species of microorganisms were not luminescent. The Cl. botulinum cells showed a bright green luminescence against the dark background of the film, and the morphology of the cells be distinguished. The fluorescent cells had the appearance of bacilli with rounded ends; in some films racket-shaped forms could be seen. The periphery of the cells shone more brightly than the central area.

In certain films shapeless conglomerates, showing a bright yellow or green luminescence, could sometimes be seen, but these could be differentiated without difficulty from the luminescent microorganisms because of the features of the luminescence as described above.

In order to establish the specificity of the luminescence after treatment with the luminescent botulinus antiserum, 13 strains of Cl. botulinum‡ type A, five strains of type B, three strains of type C, one strain each of types D and E, and also 38 strains of other species of microorganisms.

The experimental results, summarized in Table 1, demonstrate the specificity of the action of the luminescent botulinus antiserum. After staining with the luminescent antiserum, the Cl. botulinum cells showed a specific green luminescence, whereas if coliform, anaerobic (except Cl. botulinum), staphylococcal and anthracoid cells were treated with the same serum, no such luminescence was observed.

It must be mentioned that isolated cells of other species of bacteria possessed a hardly perceptible, pale green luminescence, but these cells resembled ill-defined formations, without characteristic morphological features. Thus the staining of Cl. botulinum cells, fixed to glass slides, with a luminescent antiserum enabled microorganisms of this species to be identified from other species: anaerobes, coliforms, staphylococci and anthracoids.

We found no essential difference in the intensity of the luminescence of all the strains which we studied of Cl. botulinum types A, B, C, D and E. In films prepared from cultures grown in liquid media, the microorganisms were more intensely luminescent than in the films from cultures grown on solid media. It must be borne in mind that tested strains differed in their ability to produce toxin. They included such highly toxic strains as strain 98 type A and strain 255 type B, as well as strains of low toxicity such as strain 13 type A and strain 346 type B, yet there was no difference in the intensity of luminescence.

In order to determine the sensitivity of the method, films were prepared from suspensions of Cl. botulinum cells of different concentrations in physiological saline. The investigation was carried out with three strains of type A and one strain of type B. Part of the film was examined under the luminescent microscope, and another part was stained by Gram's method and examined under the ordinary microscope with the same magnification.

The results given in Table 2 show that solitary Cl. botulinum cells, brightly luminescent, could be found in the films prepared from suspensions of microorganisms with concentrations of 1,000,000 and 500,000 bacterial cells in 1 ml, by luminescent microscopy.

As a result of this work, we thus succeeded in preparing a luminescent botulinus antiserum. When cultures of Cl. botulinum were stained with this serum, an intensive, characteristic luminescence of the Cl. botulinum cells was obtained. This specific luminescence may be used for the identification of this species of microorganism in microscopic preparations. When the films were treated with a luminescent normal rabbit serum, no staining of the microorganisms took place. **

TABLE 1. Intensity of Specific Luminescence of Bacteria, Stained with Luminescent Botulinus Antiserum

Species and type of microorganism	Number of strains	Intensity of luminescence
Cl. botulinum		
type A	13	++++
» B	5	++++
» C	3	++++
» D	1	++++
» E	1	++++
Cl. tetani	2	—
Cl. welchii	2	—
Cl. oedematiens	2	—
Cl. histolyticum	2	—
Cl. septicum	2	—
Cl. sporogenes	2	—
Cl. putrificum	1	—
B. subtilis	4	—
B. mesentericus	3	—
B. anthracoides	2	—
Staphylococcus	2	—
E. coli	14	—

Legend: ++++ clear luminescence of bacterial cells; - absence of luminescence.

‡ Most of the strains were obtained from the L. A. Tarasevich State Control Institute for Vaccines and Sera; 14 strains of Escherichia coli were isolated in the microbiological laboratory of the Institute of Nutrition.

** The author expresses his gratitude for the advice given by E. A. Kabanova, of the Department of Epidemiology of the N. F. Gamaleya Institute of Immunology, Epidemiology and Microbiology.

TABLE 2. Sensitivity of the Method of Staining with Luminescent Antibodies

No. of microorganisms in 1 ml of suspension	Mean no. of microorganisms in a field of vision of the ordinary microscope	Mean no. of microorganisms in a field of vision of the luminescent microscope
1	Whole field of vision	Whole field of vision
100 000 000	20	20
10 000 000	5	5
1 000 000	1 in 10 fields of vision	1 in 5 fields of vision
500 000	1 in 25 fields of vision	1 in 25 fields of vision
100 000	—	—

SUMMARY

The author obtained a conjugate of botulinus rabbit antiserum with fluorescein isocyanate, possessing immunological specificity. In staining the films of pure cultures of Cl. botulinum by the luminescent antiserum, an intensive green luminescence of these microorganisms was obtained. When preparations of other anaerobic cultures, E. coli, staphylococci or anthracoids were stained with the same conjugate, the specific luminescence was absent. In this way it is possible to reveal solitary Cl. botulinum cells in the films prepared from suspensions with a concentration of up to 500,000 bacterine cells per 1 ml. The method of staining by luminescent antibodies makes it possible to identify Cl. botulinum in microscopic preparations.

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