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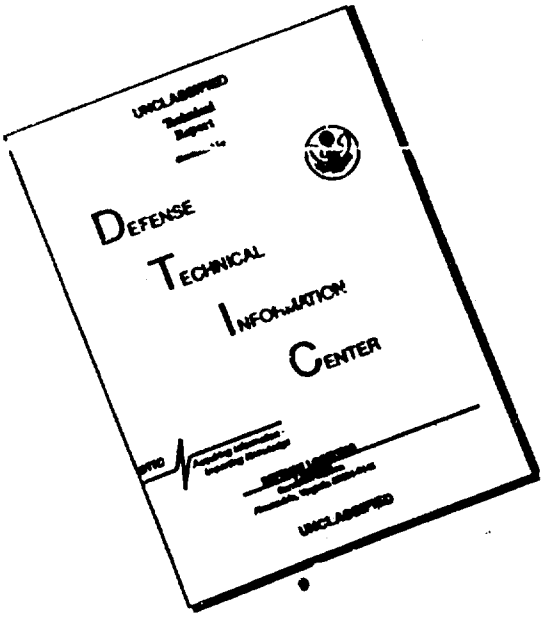

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Detection of Cl.Botulinum with the Aid of Luminescent Antibodies

Report I

Specific Luminescence of Cl.Botulinum Obtained During Their Processing with Luminescent Immune Serum

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One of the most difficult and, at the same time, important subdivisions in microbiology is the question of a quick identification of microbes in a pure culture and, particularly, in a mixture with other microbes in various substrates. In order to solve this problem, one can use a method prepared and offered by COONS et al. (5,6), by which bacterial and viral antigens are detected with the aid of luminescent immune sera. Recently, a great number of reports (7,8,10) appeared in the literature in connection with a successful application of this method to obtain a quick identification of numerous bacteria, viruses and yeasts. A substantial number of experiments was devoted to direct detection of various pathogenic microbes in the objects of outer habitat,

namely: a causal agent of pseudoglanders (11), typhous microbe (1), streptococcous (9), dysentery and anthrax (2,4), as well as a causal agent of paratyphoid infections (3).

Our current objective was to obtain a luminescent antibotulinal serum for identification of Cl. botulinum bacteria.

Experimental Method

We used in experiments antibotulinal rabbit sera obtained by way of hyperimmunization of animals with the anaculture of Cl. botulinum (strain 98, type A). We also used normal rabbit sera for control purposes. Smears for one group of experiments were prepared in a physiological solution from a suspension of 2-day anaerobic cultures grown on blood agar and, for the other group, - from 2-day cultures grown in liquid media (casein medium, sugar broth). The suspensions contained 500,000,000 microbic cells per 1 ml. The globulin fractions were obtained from immune and normal rabbit sera by way of a triple reprecipitation with a semisaturated solution of ammonium sulfate and by a subsequent dialysis against the physiological solution until the $SO_4^{=}$-ions have been removed. Then, we determined by refractometric method the protein content in obtained immune sera and in their globulin fractions; we also conducted the electrophoretic analysis. The immune serum contained 8.16% of protein. Its titer was 1:300, following the agglutination reaction. The globulin fraction contained no less than 1% of protein, while the electrophoretic analysis showed the absence of proteins. The serological globulin fraction was twice as active as the initial serum.

We obtained with a luminescent stain the conjugates of globulins of normal and immune sera. We used liquid fluorescein isocyanate*) for a stain.

The combining of globulin with isocyanate was accomplished according to the method of COONS and KAPLAN (7) in aqueous aceto-dioxane medium, in the cold and during a constant mixing for 18 hours. The amount of protein in this mixture did not exceed 2% and that of dioxane, 15%; the isocyanate solution was added from estimated 5 mg of stain per 100 mg of protein. In order to remove noncombined stain after the reaction, the conjugate was dialyzed against the physiological solution for 2 to 3 days, then it was reprecipitated 4 to 6 times with a semisaturated solution of ammonium sulfate and a subsequent dialysis was made in order to remove the SO_4''-ions. The reprecipitation was carried out until the stain was completely removed from the precipitating liquid.

In order to remove a nonspecific luminescence, the luminescent serum was processed with pulverized liver of white mice**).

We stained the preparations in the following manner: we placed a drop of bacterial suspension on a degreased (microscope) slide and allowed it to dry at room temperature; subsequently, we fixed it (within 30 minutes) in ethyl alcohol and again allowed it to air-dry. Having the preparations in a humidor chamber, we put

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*) - We obtained the stain through the courtesy of G.I. MIKHAILOV, a member of the Chemical Reagents Institute.

**) - We familiarized ourselves with the preparation method of tracir sera at the Department of Biochemistry, N.F.GAMALEI'S Institute of Immunology, Epidemiology and Microbiology.

several drops of undiluted luminescent immune serum in such a way that the dab of suspension was covered with the serum. Later, the preparations were washed for 30 minutes in few batches of physiological solution at pH 7.4, then rinsed with distilled water and air-dried. The preparations stained with luminescent normal rabbit serum were used for control purposes. Finished preparations were inspected under the MUF-ZM microscope equipped with a luminescent condenser OI-23. The source of light was a mercury vapor lamp SVD-120A. We also used light filters SS-4, SS-8 and ZHS-17, or ZS, as well as the apochromatic objective 90X and the eyepiece 7X.

Results of Experiments

With the staining of smears with a luminescent antibotulinal serum, we observed a bright specific luminescence of Cl. botulinum cells, but other types of microbes showed no luminescence. Cl. botulinum microbes shined with a bright green color on a dark background of the preparation and, at the same time, one could recognize the morphology of cells. The shining cells appeared like bacilli with rounded ends; in some preparations one could see the shapes similar to rockets. The periphery of bacilli shined brighter than their central part.

In some preparations one could notice, at times, shapeless conglomerations that shined brightly with a yellow or green color; they could be readily distinguished among luminescent microbes owing to the afore-described distinctive luminescence features.

In order to determine the specificity of luminescence at the

Table 1

Intensity of Specific Luminescence of Bacteria Stained with Anti-botuline Luminescent Serum

Species and type of microbe	Number of Strains	Intesity of luminescence
Cl. botulinum, type A	13	++++
type B	5	++++
type C	3	++++
type D	1	++++
type E	1	++++
Cl. tetani	2	-
Cl. perfringens	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	-
B. coli	14	-

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

time of processing with luminescent antibotulinal serum, we tested 13 strains of Cl. botulinum*) type A, five strains type B, three strains type C, one of each type D and E, as well as 38 strains of other types of microbes.

*) - We obtained most strains from L.A.TARASEVICH'S State Control Institute for Vaccines and Sera; 14 strains of B. coli were separated at the Microbiological Laboratory of the Institute of Nutrition.

The experiments presented in the form of a summary Table No.1 point out the specificity of the luminescence effect of antibotulinal serum. After staining Cl. botulinum microbes with a luminescent antiserum, they shined with a specific green color, and suchlike luminescence was not observed when we processed with the same serum the following bacteria: coliform bacterium, anaerobes (except Cl. botulinum), staphylococci and anthracoids.

It should be noted that individual cells of other types of bacteria showed hardly noticeable pale-green color; the cells resembled indistinctly outlined formations without characteristic morphological features. Thus, the smears of Cl. botulinum fixed on a slide and stained with a luminescent antiserum permitted us to identify botulinum bacteria among other types, such as: anaerobes, coliform bacterium, staphylococci and anthracoids.

We did not observe any essential difference in the intensity of luminescence among examined by us all strains of Cl. botulinum, i.e. of the A, B, C, D and E types. Microbes shined more intensely in preparations made of cultures grown in liquid media, than on smears of cultures grown on solid media. One should also consider that the examined strains were dissimilar as to their toxicity. Among them were highly toxic (e.g. strain 98, type A and strain 255, type B) and moderately toxic strains (e.g. strain 13, type A and strain 346, type B), however there was no difference in the intensity of luminescence.

In order to determine the sensitivity of the method, we pre-

Table 2

Sensitivity of Staining Method with the Aid of Luminescent Antibodies

Quantity of microbes in 1 ml of suspension	Average number of microbes in the field of vision of usual microscope	Average number of microbes in the field of vision of luminescent microscope
1 billion	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 20 fields of vision
100,000	—	—

pared in a physiological solution smears of suspensions of Cl. botulinum microbes in various concentrations. The investigation included three strains of type A and one strain of type B. One part of the preparations was studied under a luminescent microscope and the other part, stained according to Gram's method, was examined under the usual microscope with the the same magnification.

The findings presented in Table 2 indicate that, during luminescent microscopy, uniquely bright and shining cells of Cl. botulinum were detected in the preparations made of microbic suspensions with 1,000,000 and 500,000 microbic cells concentrations per 1 ml.

Thus, as a result of successfully accomplished work, we were able to prepare a luminescent antibotulinal serum. Following the staining of Cl. botulinum cultures with this serum, we obtained

a characteristic intense luminescence of Cl. botulinum microbes. This specific luminescence can be used in connection with microscopic preparations to identify the type of microbes in question. However, no staining of microbes was effected during processing of preparations with a luminescent normal serum of a rabbit*).

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Summary (copied)

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