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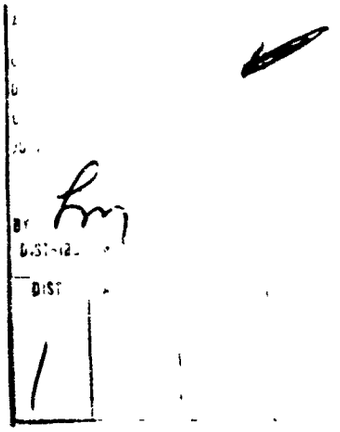

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METHOD OF FLUORESCENT ANTIBODIES IN THE STUDY OF THE ANTIGENIC STRUCTURE OF BACTERIA

[Following is the translation of an article by Ye. N. Levina, N. F. Gamaleya Institute of Epidemiology and Microbiology, USSR Academy of Medical Sciences, Moscow, published in the Russian-language periodical Izvestiya Akademii Nauk SSSR, Seriya biologicheskaya (Bulletin of the Academy of Sciences USSR, Biology Series) No 6, 1965, pages 909--912. It was submitted on 24 Nov 1964. Translation performed by Sp/7 Charles T. Ostertag Jr.]

Numerous investigators have dealt with the problems of studying the antigenic structure of microorganisms. The results of these investigations were based on the establishment of the basic schemes of the antigenic composition of bacteria, leading to the O, Vi, H and K-antigens (Kaufman, 1959). In these tests immunological and biochemical methods were used mainly. These were namely the agglutination reaction with corpuscular antigens and the reactions of precipitation, neutralization and complement fixation with various soluble antigens, obtained by means of destruction of microbial cells, mainly by chemical influences (trypsin, trichloracetic acid, etc.). On the basis of the results of these investigations, methods were developed for the preparation of various prophylactic preparations (vaccines), and also serological methods for the identification of pathogenic microorganisms. However, the majority of these investigations did not resolve many problems connected with explaining the mechanism of certain immunological reactions, for example, the reaction of somatic agglutination, problems of the localization of antigens in the microbial cell and their connection with the individual organelles of the cell, problems on the identity of antigens isolated from the microbial cells with antigens found in the whole microbial cells, etc. Along with other methods of investigation (reaction of precipitation in agar, immunoelectrophoresis, disintegration methods, etc.) for studying problems of the antigenic structure of microorganisms, great importance has been acquired in recent years by the method of fluorescent antibodies (Repentigny, Frappier, 1956; Thomason et al., 1957; Poetschke et al., 1957; Mikhaylov, Stanislavskiy, 1963).

This method makes it possible to study the localization and topography of individual antigen complexes directly in the whole microbial cell, without disrupting its morphological organization and functional structure, and to establish the bond of various cellular organelles with antigenicity, toxicity, virulence and other properties which are characteristic for a given species of microorganisms. This opens up new possibilities in the study of the antigenic structure of the microbial cell of pathogenic microorganisms. The combination of the method of fluorescent antibodies with the various immunological, biochemical and cytochemical methods is a promising course in the study of the antigenic structure of pathogenic microorganisms.

In this work we studied the feasibility of using the method of fluorescent antibodies for determining the localization of antigens in a microbial cell. For determining the chemical nature of individual antigens we used cytochemical methods, namely treating preparations of microbial cells with enzymes (Pepsin, trypsin, ribonuclease, hyaluronidase, lysozyme). As the object of study we selected the anthrax causative agent, since it was the least studied in respect to antigenic structure and it had a number of peculiarities of a morphological (capsular, capsule-less and spore forms) and immunological nature (identity of serological reactions in anthrax microbes with certain strains of sporiferous aerobes).

Methods

The study was carried out on vaccine strains of the II vaccine Tsenkovskogo No 71/12 and the STI strain, and also on certain virulent strains of Bacillus anthracis.

The cultures for obtaining capsular forms were incubated at 37° on a GKI medium (State Control Institute imeni Tarasevicha) containing 60% Hanks solution and 40% bovine serum (Arkhipova, 1962). For obtaining capsule-less we used cultures on meat-peptone agar at 37°, and for obtaining spore forms -- on a medium of casein digest.

Cell membranes were obtained by a 10 minute disintegration of the cultures, in the phase of stationary growth, on a disintegrator of the Yevatsugova-- Zhvanetskoy design with the subsequent centrifuging and washing off of the fraction of membranes with distilled water at 5000 and 8000 rpm. The degree of destruction of the cells was controlled with the help of phase contrast, light and electron microscopy. Protoplasts were obtained from microbial cells in the logarithmic phase of growth with a 1--2 hour action of penicillin in a dose of 100--200 units/ml.

From the various forms of the anthrax microbe - capsular, capsule-less, spore - and organelles we prepared smears on slides, fixed them and treated them with fluorescent sera for the characterization of antigens of the microbes being studied. Fluorescent sera were obtained by precipitating commercially prepared anthrax sera (these sera were called membrane luminescent sera, MLS), from locally prepared capsular anthrax sera (capsular luminescent sera, CLS; Levina, Arkhipova, 1964), and from locally prepared spore anthrax sera (spore luminescent sera, SLS), by means of combining the globulin fractions of the latter with fluorescein isothiocyanate according to the accepted method (Chadwick et al., 1958; Riggs et al., 1958; Blagoveschenskiy, Kulberg, 1962; Ogiyevetskaya-Pishchurina et al., 1963).

For determining the chemical nature of the antigens the preparations were treated with crystalline enzymes with subsequent staining with the corresponding fluorescent sera (Levina, Kats, 1964).

Observations were carried out in a ML-2 fluorescence microscope with 90X objective and 5X eyepiece and diaphragm of 1.1; 1.6; 2.5. Microphotographs were made on RF-3 film with exposures of 10,20 and 30 seconds.

Results

During the treatment of capsular cells of a vaccine strain of Bac. anthracis with capsular luminescent sera (CLS) luminescence of the entire layer of the capsule is observed (figure 1). Capsular luminescent sera, obtained by immunization with a specially isolated surface capsular antigen (SCLS), caused the luminescence of only the surface layer of the capsule (figure 2).*

In the capsular cells treated with MLS the membrane of the cells, a thin fringe along the periphery of the capsule and the transverse bands passing through the body of the capsule were illuminated dimly (figure 3). A brighter luminescence of these elements developed under the influence of trypsin and hyaluronidase on the capsular cell (figure 4).

Following treatment of capsule-less cells of Bac. anthracis with membrane luminescent sera, fluorescence was localized on the cell membrane. This explains why these fluorescent sera are named membrane (figure 5). Capsule-less cells treated with capsular luminescent serum and spore luminescent serum did not produce fluorescence (figure 6).

Following the treatment of spore forms of Bac. anthracis with spore sera a bright fluorescence of the spore membrane was observed (figure 6). When the spore forms were treated with MLS a dimmer fluorescence of the spore membranes was given off (figure 7).

When preparations of membranes of capsule-less cells were treated with MLS the fluorescence bore a diffuse nature with a clearer fluorescence at the edge of the membrane (figure 8). Following treatment with MSL the protoplasts of capsule-less forms of the No 71/12 vaccine strain also fluoresced. In the periphery of the protoplast individual sectors were fluorescent, which apparently indicates remnants of a cellular membrane in these forms (figure 9).

In this manner the study of the antigenic structure of Bac. anthracis with the help of the fluorescent antibody method made it possible to separate various antigenic complexes, having a specific localization in the microbial cell and possessing a definite antigenic specificity in respect to the various forms of Bac. anthracis (capsular, capsule-less and spore).

Thus the capsular forms of the anthrax microbe contain capsule antigens characterized by specificity from other forms of Bac. anthracis and consist of surface capsular antigens exposed by CLS and SCLS and inherent capsular antigens, located deep in the capsule and revealed by CLS and MLS.

*These sera, in contrast to CLS, which are obtained by immunization with the whole microbial capsular cell, were named the surface capsular luminescent sera (SCLS).

Membrane antigens of capsule-less cells, just as of capsular cells, are exposed by MLS, while the latter are more clearly revealed following destruction of the capsular antigens by certain enzymes, for example, trypsin (figure 4).

Membrane antigens of spore forms possess a significantly greater specificity than those of capsule-less forms. This is supported by the following: Membrane luminescent sera cause the fluorescence not only of microbial capsule-less cells of Bac. anthracis, but also of certain strains of spore aerobes, while spore luminescent sera cause the fluorescence primarily of spore forms of Bac. anthracis (table).

For determining the chemical nature of individual antigens of the microbial cell we made use of the ability of certain enzymes to selectively destroy specific chemical complexes of the microbial cell which possess antigenic properties.

The effect of the enzymes can be judged indirectly based on the change in the nature and the degree of fluorescence of the microbial cell following treatment with the appropriate fluorescent sera.

Thus, following the action of pepsin on capsular cells of Bac. anthracis the fluorescence of the entire capsular layer was removed during treatment with CLS (figure 10), and there was no change in the degree of fluorescence of the cellular membrane of both the capsular and capsule-less forms. The action of trypsin reduced the degree of fluorescence of the capsule during treatment with CLS (figure 11) and exposed the fluorescence of the cellular membrane and individual luminous septa and punctate formations in the body of the capsule during treatment with MLS (figure 4). In capsule-less cells the action of trypsin led to a lessening of the degree of fluorescence of the cell membrane (figure 12). Following the action of lysozyme on capsular cells (treated with CLS) the nature of capsule fluorescence changed; the luminous mass of the capsule was broken up and the inner portion of the capsule was not fluorescent (figure 13). In capsule-less cells (following treatment with MLS) there was a sharp disruption in the degree of fluorescence of the cell membrane (figure 14) and individual luminous formations were revealed in the body of the cell. When preparations of capsular cells were treated with CLS, then following the action of hyaluronidase the surface layer of the capsule had the form of a brightly luminous band, while the main portion of the capsule was destroyed (figure 15). During the action of hyaluronidase and ribonuclease and following the extraction of fats in capsule-less cells (treated with MLS) a reduction in the degree of fluorescence of the cell membranes takes place and a porous fluorescence of the protoplasm of the cell is revealed. This is similar to the fluorescence following the action of lysozyme (figure 14).

Thus the study of the localization of antigens in the microbial cell, conducted by the fluorescent antibody method, was supplemented with materials from the study of the chemical nature of individual antigens.

It was shown that the capsular antigens have a complex composition and consist of surface capsular antigens which are localized in its outer layer and are selectively removed by pepsin and partially by trypsin. This speaks for their protein nature. Apart from these antigens, in the main thickness of the capsule, antigens are localized which have a complex polysaccharide-protein composition and are sensitive to a wide spectrum of enzymes -- trypsin, hyaluronidase, lysozyme.

The antigens of the cellular membrane represent a wide complex of substances which are sensitive to lysozyme, fat solvents, hyaluronidase, ribonuclease, and partially trypsin.

The antigens of the cytoplasm in capsule-less cells are exposed by MLS only following the disruption of the completeness of the cell wall under the influence of enzymes (lysozyme and others).

Conclusions

1. The use of the fluorescent antibody method made it possible to expose the individual antigens of the microbial cell and to show their localization in specific organelles -- the capsule, membrane, and apparently the cytoplasm

2. The combined application of immunological methods and methods of cytochemistry made it possible to establish that the antigen complexes of various organelles are characterized by a specificity of antigen composition connected with their chemical differences.

3. It was shown that microorganisms, possessing a capability to exist in various forms - capsular, capsule-less and spore, are characterized by an antigenic specificity, distinct for each of these forms.

4. This example of the use of various complex methods for studying the microbial cell(fluorescent antibody method, methods of cytochemistry, luminescent and light microscopy, methods of disintegration of the microbial cell) is proof of the expediency and future possibilities of using these methods in studying the antigenic structure of microorganisms.

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Fig. 1. Capsular cells of vaccine strain 71/12, treated with CLS.

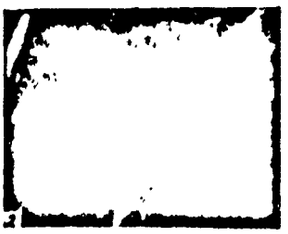

Fig. 2. Capsular cells of vaccine strain 71/12, treated with SCLS.

Fig. 3. Capsular cells of vaccine strain 71/12, treated with MLS.

Fig. 4. Capsular cells of vaccine strain 71/12, treated with trypsin and MLS.

Fig. 5. Capsule-less cells of vaccine strain, treated with MLS.

Figure 6. Spore and capsule-less cells of the STI vaccine strain, treated with SLS.
a - capsule-less cells

Figure 7. Spore and capsule-less cells of the STI vaccine strain, treated with MLS.
b - spores

Figure 8. Membranes and capsule-less cells of the STI vaccine strain, treated with MLS.

Figure 9. Protoplasts of the 71/12 vaccine strain, treated with MLS.

Fig. 10. Capsular cells of 71/12 vaccine strain, treated with pepsin & CLS.
Fig. 11. Capsular cells of 71/12 vaccine strain, treated with trypsin & CLS.
Fig. 12. Capsule-less cells of 71/12 vaccine strain, treated with trypsin and MLS.
Fig. 13. Capsular cells of 71/12 vaccine strain, treated with lysozyme & CLS.
Fig. 14. Capsule-less cells of 71/12 vaccine strain, treated with lysozyme and MLS.
Fig. 15. Capsular cells of 71/12 vaccine strain, treated with hyaluronidase and CLS.

Fluorescence of Bac. anthracis and Bac. cereus following treatment with fluorescent antirax sera

Form		Fluorescent sera						Number of strains
		Membrane			Spore			
		Fluorescence						
		Bright	Dim	None	Bright	Dim	None	
Bac. cerus	Capsule-less	20	12	76	0	0	108	108
	Spore	2	4	102	3	20	85	108
Bac. anthracis	Capsule-less	43	0	0	0	5	38	43
	Spore	7	53	0	58	1	1	60