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Ob ukorennoi diagnostike sibirskoi lazvy s
primeneniem luminisatsionnykh antitel

[On the application of luminescent antibodies for
accelerated diagnosis of anthrax]

Veterinariia 36(7):69-75. July 1959. Moskva.
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(In Russian)

Using the present methods, approximately two or three days are needed for the identification of isolated microorganisms; in lucky cases, it can be done in several hours. However, for the final confirmation of the presence of the anthracis pathogen in pathologic material seven to ten days are often required.

The isolation and identification of the Bac. anthracis in objects of the outer habitat (soil, water, forage and others) is a very complicated and time-consuming task, consequently it is natural that many researchers seek to find a new accelerated and simple diagnostic method for the identification of the Bac. anthracis and other pathogenic microorganisms.

During the last years, our national researchers (MEISEL', LEVINA, KABANOVA, PISHCHIURINA and others) as well as scientists of foreign countries (Koontz, Caplan, Moody, Goldman, Thompson and others) were intensively working on the elaboration of a method of luminescent microscopy and utilizing tagged antibodies

for purposes of an early diagnosis. In 1942, this method was introduced by Albert Koontz for the first time, whereas in our country M. N. MEISEL¹ and collaborators, E. N. LEVINA, A. E. KABANOVA, and M. M. PISHCHURINA are the pioneers in this field. [Begin. p. 70].

We started the study of luminescent microscopy in 1955; our purpose was to find a new method for an early diagnosis. We conducted a series of experiments on the staining of pathogenic bacteria, using for these purposes fluorochromes (special dyes of strong fluorescing capacity). The microorganisms of different species, treated with the same dyes, acquired in our experiments brilliant monotypic luminescence. As a result of our work, we came to the conclusion that it is not possible to identify and to differentiate microorganisms by using ordinary fluorescent dyes. We utilized for these purposes, and for the detection of bacteria in objects of the outer habitat, tagged antibodies.

The first tests on the application of tagged antibodies for the detection of anthrax bacteria were conducted by E. N. LEVINA¹. According to her data, the anthrax pathogen emits a brilliant green-yellow light, whereas the luminescence of vaccinal strains is somewhat dimmer; the cells of anthracoids and of gram-positive bacteria did not luminesce at all. The author came to the conclusion that "as the result of the conducted

¹Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii, 1958, no.1.

work, tagged isocyanate-fluorescein precipitating anthracic sera were obtained, and the possibility was proved that anthrax bacilli, because of the difference of their brilliant light, can be differentiated from anthracoids and from other gramm-positive bacteria".

E. N. LEVINA used only cultures in her work. Pathological material and objects of the outer habitat infected with Bac. anthracis, had not been subjected to the tests. We decided to apply this method for the detection of the anthracic pathogen in the pathological material of cadavers of animals that had died of anthrax, and also for the search of the pathogen in objects of the outer habitat (hay and oats). The mechanism of immunobiological reactions is the basis of this method.

The majority of researchers consider that specific antibodies are usually concentrated in the globulin fractions of immune serum. Sedimentation, caused by ammonium sulfate $[(\text{NH}_4)_2\text{SO}_4]$, separates the globulin fractions from the fractions of albumin [protein], and combines them with the luminescent chlorochrome dyes. The obtained complex of antibody-fluorochrome was used for the detection of the corresponding microbes in the smear. In this case the antibody + antigen reaction is obtained; it can be detected in luminescent microscopy because of the fluorochrome, which became chemically combined with the antibody. The antibodies are specifically adsorbed on the surface of the

related microbe. Thus, a stable combination is produced, which cannot be destroyed by washing.

We utilized as fluorochrome the isocyanate fluorescein prepared by G. I. MIKHAILOV (Vsesoiuznyi nauchno-issledovatel'skii Institut khimicheskikh reaktivov) [All-Union Scientific-Research Institute of Chemical Reactives]. Isocyanate-fluorescein is of brilliant green fluorescence of which the wavelength, in regard to its sensibility, almost corresponds with that of the retina of the eye.

We used for the tagging, globulin fractions of active precipitating anti-anthrax sera of different series, prepared by the Orlov Biopiant, whereas for the combination of globulins with the chlorochromes we applied on the whole the Koontz-Caplan method.

The method for the preparation of the luminescent anti-sera consists in the following: a most immune serum must be selected, and if it contains some admixtures, it must be filtered. To the serum, one half of the ~~of the~~ [liter] volume distilled water must be added; this must be done gradually (drop by drop) in the cold, (temperature: 3 to 4°) while stirring constantly. The mixture of serum and water must be placed into the refrigerator for 10 to 15 minutes. Thereafter, the globulin fraction of the serum should be sedimented by means of several precipitations and semi-saturation with ammonium sulfate $[(\text{NH}_4)_2\text{SO}_4]$. To the diluted

serum an equal volume of the saturated solution of ammonium sulfate must be added drop by drop in the cold. The serum mixture with the solution of ammonium sulfate must be kept in the cold for 1.5 hours. Thereafter, it must be centrifuged at 3,000 revolutions p. m. for 20 minutes. The supernatant liquid must be decanted after centrifugation, and the sediment diluted with cool distilled water up to the amount of the former volume of the serum.

The re-sedimentation and centrifugation must be done three to four times, whereupon the sedimented liquid becomes transparent. The globulin deposit must be diluted with cold water, and the volume of the dilution must reach the initial amount; thereafter, it must be transferred to a cellophane bag; in order to liberate it of ammonium sulfate, dialysis must be conducted. Against the physiological solution of sodium chloride pH = 7.2 to 7.4 dialysis must be carried out in the cold, using an electric mixer.

Dialysis is completed when no traits of ammonium sulfate are present. The presence of the latter must be tested by a 10 per cent solution of barium chloride.

After the analysis, the volume of the serum must be measured and the content of albumin [protein] established. For these purposes the ^{Kjeldahl} K'el'dal [Queldale?] micromethod must be applied.

After the content of albumin in the serum is established,

it must be inter-combined with the isocyanate-fluorescein (conjunction). For these purposes the following reactive mixture must be prepared: globulin + physiological solution + buffer solution + diäxan [diäxon] + acetone + isocyanate-fluorescein. The duration of the conjunction of isocyanate-fluorescein with albumin [Begin. p. 71] [protein] takes approximately 18 hours in the cold, by mixing constantly.

The isocyanate-fluorescein, which did not unite with the albumin [protein], must be removed by means of dialy is against the buffered solution of sodium chloride at pH = 7.1 to 7.2. Dialysis in the cold will take approximately three hours.

Therefore, using ammonium sulfate, the globulins must be re-sedimented, and centrifugation, as formerly described, must follow. In order to remove ammonium sulfate, dialysis must be carried out for three to four more days.

In order to remove the residue of the non-combined isocyanate-fluorescein and the intermediate products of its preparation, to the tagged serum white-mice-liver-powder must be added (1 g of the powder per 10 ml of serum). For adsorption, after thorough mixing, the mixture must be kept at room temperature for one hour; thereafter it must be centrifuged for 20 minutes at 3,000 revolutions p. m. The serum must be decanted from the sediment, filtered through a Seitz filter, and distributed into ampules under sterile conditions

Our own experiments. The precipitating anthracis tagged serum, prepared according to the method mentioned above, was tested in three experimental series:

1. For the identification of the cultures of the following bacteria: Bac. anthracis of the strains of Tsenkovskii's and STI vaccine, from cultures of Bac. anthracoides, Bac. pseudoanthracis, Bac. subtilis, Bac. mesentericus, Bac. megatherium, Bac. mycoides, Bac. perfringens, Vibrion septique and others;
2. for the detection of the pathogen of anthrax in organs and tissues of animals that had died of this disease;
3. for the detection of Bac. anthracis in hay and oats.

Working method. For these experiments were used: 24 agar and bouillon cultures of seven strains of Bac. anthracis (No. 63, 64, 65, 66, 67, 68, and 69) and of older ones; of two strains of Bac. anthracoides (No. 86 and 103); Bac. pseudoanthracis, vaccinal strains of the first and second Tsenkovskii's vaccines and of the STI vaccine; two strains (type C) of Bac. perfringens; two strains of Vibrion septique; Bac. subtilis, Bac. mesentericus; Bac. anthracoides and Bac. pseudoanthracis (the latter we obtained from O. A. POLIAKOVA), the anaerobe strains, obtained from GNKI, and strains of other soil microbes which were received from the Scientific Control Institute imeni Tarasevich.

By means of a platinum loop we planted the microbes into one drop of the physiological solution that was preliminarily

placed onto the subject slide. Drops of the bouillon cultures were also placed drop by drop onto the slides. The smears were thin and even; they were dried and fixed according to the following three methods: one part of the smears was treated with methyl alcohol for five minutes; the other one, with ethyl alcohol, for 15 to 20 minutes; whereas the third one was fixed on the flame. For fixation the alcohol was poured directly onto the smear.

After the fixation, one drop of the fluorescent serum was placed onto the preparation; it was evenly distributed on the surface of the smear. The smears were treated with the tagged serum the following way: a) 30 minutes at room temperature; b) 30 minutes in the incubator at a temperature of 31 to 38°; c) five to ten minutes, slightly heated over the flame. Thereafter, the preparation was thoroughly washed using the physiological solution during 15 to 20 minutes. Preparations, stained by this method, were carefully observed under a luminescent microscope of phase-contrast construction. For the luminescent microscopy, an opaque illuminator was used (illuminator OI-17 with the lamp SVD-120A). In case immersion object glass was used, the buffered solution of glycerin (9 parts of glycerin and one part of buffered physiological solution pH=7.0) and not "Kedrol" [Cedrol?] was applied.

In the second experimental series, 22 white mice were infected with strains of Tsenkovskii's vaccines. The cadavers of

these mice were dissected, and their internal organs examined by the following methods: bacteriologically (the klatch-preparations were stained according to Gram's); by luminescent microscopy (the imprints of the organs on the slides were treated with tagged serum, as it was mentioned above, and by seeding on nutrient media [meat-pepton agar (MPA) and meat-pepton bouillon (MPB)]).

In the third series, the pathogen of anthrax and the strains of Tsenkovskii's vaccines were added to the samples of hay and oats by admixing the cultures to the latter, and also by sedimenting aerosols of the cultures on samples of forage in the chamber.

Many of these samples were also subjected to the effect of ethylene oxide (the experiments with ethylene oxide were conducted by U. V. PANTELEEV). The remaining samples were not treated.

The infected forage samples were washed with small portions of physiological solution. From the wash-cuts were made preparations which were dried, fixed by methyl alcohol, and treated with the tagged serum as mentioned above.

In the first series over 1,160 preparations were treated by luminescent serum (See table on page 72).

Other preparations treated with normal tagged horse-blood-serum served as control in all cases.

Methyl alcohol turned out to be the best fixing means in

our experiments. Most clear results were obtained in luminescent microscopy after treating the preparations under incubator conditions with tagged serum for 30 minutes; this was followed by compulsory washing with the physiological buffer solution for 15 to 20 minutes. Anthrax microbes emit brilliant green-yellow luminescence. [Begin. p. 72] A green-yellow circle, in form of a capsule, surrounds the microbial cell; the microbial cell itself is dark, brownish-gray, and lies within the circle. The spores containing microbial cells luminesce too; the spores, however, are visible by phase-contrast microscopy only. Spores separately located from the groups, gave away a very dim light, without a circle, whereas the spores of the anthracic strain 68 did not luminesce at all.

The microbes of vaccinal strains emit a less brilliant yellow-green light, however, some of them emit the same clear light as the microbes of anthrax.

Anthracid bacilli yield dim luminescence; in the majority of the cases, only shadows of the microbic cells were visible; the spores do not luminesce. The character of the luminescence of microbial cells of the anthracoid strain 86 resembled that of the pathogen of anthrax. However, after a thorough study of the luminescence of the microbes of this strain, it was evident that there was no even circle surrounding the anthracoid cell as it was seen around the microbes of anthrax. The circle around the

anthracoid cells is irregular and uneven, and not clearly expressed either; luminescence of the microbial cell is dense, and separated cells can be hardly distinguished in the chains by microscopy.

The microbes of pseudoanthrax, when treated with tagged precipitating anthracic serum, yield also a well-expressed luminescence resembling that of anthracoids. The microbial cells emit a dim light, and the circle surrounding them is not clear and hardly visible. Some of the microbial cells of *Bac. pseudoanthracis* of strain 104 emit a brilliant green-yellow light without a well-visible circle around the cell. The sporogenic microbial cells luminesce dimly, and appear in the luminescent microscope in the form of shadows.

The hay bacillus (*Bac. subtilis*) does not yield luminescence after the treatment with anthracic precipitating tagged serum. Only a weak luminescence of the spores and of some single little rods could be observed.

The potato bacillus (*Bac. mesentericus*), its spores and some of the adjacent rods emit also a dim light.

Hyphomycetes (mold fungi), mycelium and spores yield a brilliant green-yellow light after the treatment with tagged and even with normal sera; however, due to their peculiar morphology, they can be easily differentiated from microbes of anthrax.

The cabbage bacillus (*Bac. magatherium*), the rhizomorphous bacilli (*Bac. mycoides*, the cocci, staphylococci), paratyphoid

bacteria, (Bac. suispestifer, Bac. Gärtneri, Bac. Breslau), the intestinal rod, paracoli, Bac. perfringens and Vibrion septique do not luminesce in microscopy after the treatment with the anthracic precipitating tagged serum; they can be detected in phase-contrast luminescence only. In some of the 15 to 30 day-old cultures of spores of Bac. perfringens a very weak light can be observed.

Thus, our experiments on using the tagged anthracic serum for identification were not very successful, since we were not able to differentiate microbes of anthrax from other soil bacilli, especially not from those of anthracoids and pseudoanthrax. Some of these strains emit a non-specific light [Begin. p. 73] which cannot be easily differentiated, by unexperienced workers, from the light of the anthracic pathogen.

In order to omit group luminescence, we decided to carry out adsorption of the serum by microbial cultures luminescing with the anthracic tagged serum of variant degree.

After its first dialysis, the serum (globulin fraction) was adsorbed by a 24-hour agar culture of Bac. anthracoides, Bac. pseudoanthracis, Bac. mesentericus, and Bac. subtilis. The cultures of these microbes were washed off by the obtained serum, thereafter they were placed into the incubator (thermostat) for 2 hours. The mixture was shaken every 10 to 15 minutes. Thereafter both, the mixture of the serum and the cultures were kept

at room temperature for 12 hours, whereas in the cold (4 to 8°) they were kept for 24 hours; the preparation was shaken systematically. Thereafter the serum was centrifuged, filtered through the Gutz filter, its content of albumin [protein] was established, and the reactive mixture was prepared.

The obtained tagged, adsorbed, precipitating anthracic serum reduced group luminescence. The treated preparations of anthracoids and pseudoanthrax cultures did not luminesce; very seldom some of the little rods emitted a dim light in the form of shadows. *Bac. mesentericus* and *Bac. subtilis* did not yield luminescence, but their spores emitted a dim light that could hardly be noted.

The anaerobes (*Bac. perfringens*, *Vibrio septique*), the intestinal rod, paratyphoid bacteria, cocci and others did not luminesce at all. The mold fungi emitted light as before.

During the treatment with the adsorbed serum of anthracic microbes, a clear green-yellow luminescent circle (capsule) surrounded the microbic cells. The strains of Tsenkovskii's vaccines and those of the ST1 vaccine emitted the typical luminescent light, however, it was not as clearly expressed as that of the anthracic pathogen.

In order to detect the pathogen of anthrax in the tissues and organs of 18 dead mice, we obtained smears-replicas of the blood, the lungs, spleen, liver, and muscles and treated them

with a tagged serum, as described above. Fifty four smears of blood were investigated, 72 smears of the spleen, 36 smears of the lungs, 54 smears of the liver, 36 smears of kidneys, and 72 smears of the muscles. While examining these smears-replicas, a strong luminescence of the background hindered the work. Because of the strong luminescence of the background of the spleen, liver, and blood preparations, we were not able to detect the pathogen in luminescent microscopy, however, in smears stained according to Gram's method the pathogen could be easily observed. The background of the muscle preparation luminesced less intensively, and this permitted us to detect easily the anthracic pathogen. After treating the organs with the tagged anthracic serum and tissues of mice that had died of paratyphoid, luminescence was less intensive as in the previous cases. Consequently, the intensive luminescence of the organs and tissues of animals that have died of anthrax, obtained by treating them with the specific tagged sera, may serve to a certain degree as a selective and diagnostic sign.

In the third experimental series, we examined the wash of oats and hay of different breeds prior to infecting them with the anthracic pathogen and vaccinal strains, and after the infection.

After treating the wash preparations of oats and hay with the tagged serum, sometimes luminescing green-yellow crystals,

fungi of different shapes (triangles, squares and others), albumin particles of oat grains, pieces of stalks and the like were observed in the field of vision.

Cells of Bac. anthracis, treated with the tagged anthrax serum could be easily detected in samples of oats and hay that were mixed with a small amount (tens of thousands) of the microbes, however, in the smears stained according to Gram's, the pathogen of anthrax could not be detected.

Conclusion

1. The adsorbed tagged precipitating anthracic serum, obtained by us, is a specific preparation for the detection of the pathogen of anthrax in objects of the outer habitat (oats and hay), and also for the identification of cultures.

2. Due to the brilliant green-yellow luminescence of the circles (capsules) surrounding the microbial bodies, it is easy to detect the Bac. anthracis in oats and hay within 1 to 1.5 hours.

3. Due to the intensive luminescence of the preparations treated with tagged serum and obtained from organs and tissues of animals that died of anthrax, the method of luminescing antibodies to be used for the diagnosis of anthrax in materials obtained from the cadavers of animals, must be improved.

9/21/59