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organisms in the field of vision only 6 were fluorescent, and in another preparation (strain No. 2207), of 5 cells in the field of vision only 2 fluoresced brightly. It may be suggested that the absence of fluorescence in individual cells is connected with a

TABLE 3. THE RELATION BETWEEN INTENSITY OF FLUORESCENCE AND AGGLUTINATION REACTION TITRE (FLUORESCENT DYSENTERY ANTISERA)

| Organisms | Intensity of fluorescence | No. of strains | Agglutination titre | | |
|---|---|---|---|---|---|
| | | | 1 : 960 1 : 3840 | 1 : 30 1 : 120 | 0 |
| Dysentery | + + + + | 6 | 6 | 0 | 0 |
| | + + | 2 | 0 | 2 | 0 |
| | − | 3 | 0 | 2 | 1 |
| | | 7 | | | |
| *Esch. coli* | − | 3 | 0 | 0 | 3 |

variation of their capacity for agglutination, though this problem requires further study.

As a result of the work we have carried out, therefore, we have succeeded in preparing a fluorescent dysentery antiserum which may be used to produce specific fluorescence in dysentery bacilli and to identify them in microscopical preparations.

*Translated by* A. P. FLETCHER

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# FLUORESCEIN-LABELLED ANTIBODY FOR THE DETECTION OF THE ANTHRAX BACILLUS—I*

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THE Ascoli precipitation reaction is widely used for the laboratory diagnosis of anthrax in the investigation of raw leather, animal carcasses and other materials. However, although the precipitation reaction is considered to be a sensitive method, it requires the presence of several millions of bacteria in the material under investigation. On several occasions it was insufficient to carry out only one Ascoli reaction and further bacteriological study was necessary. The process of isolating the anthrax bacillus for bacteriological study and the determination of the differential characteristics for this anthracoid and other saprophytes is not only lengthy, but the complex cultural,

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morphological and biochemical properties and the pathogenicity for animals must also be studied. As to the desirability and necessity of studying the serological properties of anthrax there is a difference of opinion among various authors.

Yet, experiments by Ascoli, Valentini and others have shown that the precipitation reactions used for the study of extracts of anthrax and pseudoanthrax bacilli are of a group nature.

Furthermore, Tomesik, Ivanovich and Bruckner state that the somatic antigens of anthrax and pseudoanthrax are chemically identical and, in their opinion, this explains the absence of a high specificity in the precipitation reaction.

However, Pokshishevski's detailed study of this problem (41 anthrax and 23 pseudoanthrax strains) showed that there is a quantitative difference in the precipitation reaction which permits the differentiation between *B. anthracis* and pseudoanthrax and their antigenic complexes. Thus culture extracts from anthrax bacilli gave a positive precipitation reaction with a 1 : 50 dilution of antigen while those of pseudoanthrax gave it only in dilution 1 : 5.

Sukharetskii points to the possibility of differentiating between the polysaccharides of anthrax and pseudoanthrax by diluting the anthrax antiserum and determining the titre of the precipitation reaction. The author used a precipitating serum diluted with normal horse or rabbit serum, both of which were previously tested for the absence of unspecific reactions. This permitted the determination of the true precipitation reaction titre by the dilution of the serum and not of the antigen, and by this titre the antigens could be differentiated. Thus, if the anthrax serum precipitated the antigen in dilutions of 1 : 20 to 1 : 50, it only gave a positive reaction with the full concentration or the 1 : 2 dilution of the pseudoanthrax antigen.

The problem of increasing the specificity of precipitating serum and the method of preparing extracts of anthrax bacilli for the precipitation reaction have not been sufficiently reflected in the literature. Nevertheless, the few publications on the subject convinced us of the possibility of using under defined conditions serological reactions based on the precipitation reaction for the differential diagnosis of anthrax from pseudoanthrax and other saprophytes. The basic problem for this appeared to be the necessity for increasing the specificity of precipitating sera.

The object of this work was a study of the possible use of fluorescent sera for detecting anthrax bacilli by fluorescence microscopy of smears.

An earlier publication has dealt with the essentials of the use of the method of fluorescent antibody and the ways of preparing it. The essentials of the method consist of the determination of an unknown antigen with a known specific antiserum conjugated with a fluorescent dye; antigen treated with fluorescent antibody acquires the ability to fluoresce when observed in blue or ultra-violet light.

There are numerous indications in the literature of the use of the fluorescence of labelled antibodies with the object of detecting viral and rickettsial antigens in tissue: rickettsias, mumps virus (Coons and Snyder, 1950), poliomyelitis virus (Buckley, 1956), influenza virus (Liu Chen, 1955) and occasional works on the detection of bacteria —enterococci (Hobson, 1955) and melioidosis (Moody, Goldman and Thomason, 1956).

The labelling of the globulin fraction of precipitating anthrax antiserum in our work was carried out with Soviet fluorescein isocyanate (the fluorescein isocyanate was produced in the Institute for Chemical Reagents by a group of scientific workers under the leadership of G. I. Mikhailov). The globulin fraction, freed from inert proteins, was used to increase the specificity of the precipitating antisera.

The globulin fractions (the fluorescent sera were prepared by M. M. Pishchurina and A. I. Glubokina of the Biochemistry Department, Gamaleia Institute of Epidemiology and Microbiology) were prepared by sedimentation with ammonium sulphate

according to Coons' method and globulin fractions containing 30-60 mg of protein per millilitre of serum were used for labelling with fluorescein isocyanate. The purity of the globulin fractions was checked by paper electrophoresis.

The conjugation of the globulin fractions of the precipitating sera with fluorescein isocyanate was carried out according to the method of Coons and Kaplan as follows: the isocyanate solution was added to a mixture of dioxane and an aqueous solution of protein in proportion of 5 mg of dye to 100 mg of protein. The reaction was allowed to proceed for 18 hr in the cold. After the completion of the reaction the excess of dye was removed by dialysis lasting 3-4 days, followed by a 3–4 times repeated precipitation with ammonium sulphate and finally by a further dialysis for 3 days to separate the ammonium sulphate from the conjugate. The sera so treated were sufficiently free from unconjugated excess dye and this was proved by the absence of fluorescence during the observation of the dialysation water in ultra-violet light.

However, to exclude unspecific fluorescence (the mechanism of this fluorescence has not been studied to any extent) the fluorescent sera were treated with a powder made from the liver of white mice, as recommended by Coons and Kaplan (1955). The powder was prepared as follows: the liver of the animals was washed in 0·15 M saline solution and thoroughly ground in a porcelain mortar after which an equal volume of saline and 4 volumes of acetone were added. After allowing to stand, to sediment the suspended liver particles to the bottom of the cylinder, the supernatant was decanted and the sediment was again washed with 0·15 M saline several times until the supernatant contained a minimal amount of haemoglobin. An equal volume of 0·15 M saline and 4 volumes of acetone were added to the washed sediment and the mixture was shaken; after standing, the supernatant fluid was poured off, 4 volumes of acetone were added, the upper layer and the residue was filtered off through a Buchner funnel. The mass thus obtained was dried for 12 hr at 37° C and afterwards ground to a powder.

For the treatment of the fluorescent serum 80–100 mg of the powder were used for each millilitre of serum. After thorough shaking the suspension was allowed to stand at room temperature for 1 hr, the suspension was then centrifuged or filtered through moist filter paper in a U-shaped funnel. Two such treatments produced a serum which had lost its unspecific fluorescence.

The quality of the original anthrax serum and its globulin fractions was tested in a precipitation reaction with standard anthrax and pseudoanthrax antigens (for this part of the work we used the findings of Prof. S. G. Kolesov of the Scientific Research Institute of Veterinary Control, for which we wish to thank him). Apart from the normal precipitation reaction we also used a horizontal precipitation reaction in agar.

The last-named method was carried out as follows: 0·7% saline agar was mixed with an equal volume of antigen and 1 ml of this solution was poured into a tube. After this had set an intermediate layer consisting of 0·5 ml of 0·35% saline agar was poured on top. When this had set we poured 0·5 ml of either the experimental serum or of the fraction on top of it. The reaction was conducted at 20–37° C and recorded after 24–48 hr.

Sera which gave a precipitation reaction with the standard antigen by the usual method in 30 sec were used for conjugation with fluorescein isocyanate, and also those which gave it in agar in 24 hr; in our experiments the reactions were carried out with dilutions of the standard antigen up to 1 : 8 (Table 1).

The precipitation reaction in agar gave encouraging results in the study of the precipitating properties of fluorescent sera, whereas the normal precipitation reaction did not permit the observation of the ring of precipitate at the interface between antigen and fluorescent serum.

TABLE 1. CHARACTERISTICS OF THE PRECIPITATING PROPERTIES OF ANTHRAX ANTISERA

| Dilution of standard anthrax antigen | Undiluted | 1 : 2 | 1 : 4 | 1 : 8 | Pseudo-anthrax | Saline |
|---|---|---|---|---|---|---|
| Serum No. 9 | ++++ | ++++ | ++++ | ++++ | − | − |
| | ++++ | ++++ | ++++ | ++++ | − | − |
| Globulin fraction of | ++++ | ++++ | ++++ | ++++ | − | − |
| serum No. 9 | ++++ | ++++ | ++++ | ++++ | − | − |
| Serum No. 183 | ++++ | ++++ | ++++ | ++++ | − | − |
| | ++++ | ++++ | ++++ | ++++ | − | − |
| Globulin fraction of | ++++ | ++++ | ++++ | ++++ | − | − |
| serum No. 183 | ++++ | ++++ | ++++ | ++++ | + | − |
| Globulin fraction of serum No. 8 | − | − | − | − | − | − |

Numerator [*sic*]—standard precipitation reaction; denominator [*sic*]—precipitation reaction in agar.

For the study of the possibility of producing differential fluorescence of bacteria preparations were made by various methods using several methods of fixation, and also by treating viable bacterial cells. Various methods of removing background fluorescence from the preparation were also studied.

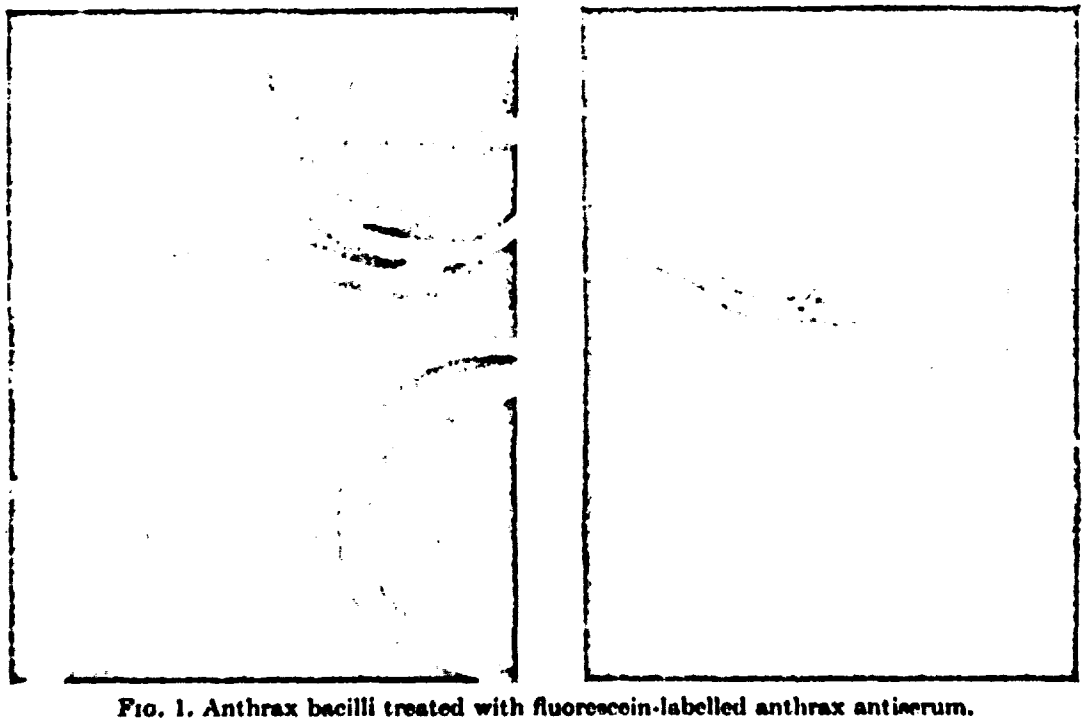

FIG. 1. Anthrax bacilli treated with fluorescein-labelled anthrax antiserum.

This part of the work was carried out using vaccination strains (I and II Tsenkov vaccine, strain STI-Ginsburg vaccine) and also with anthracoid strains, pseudoanthrax and other Gram-positive saprophytes. All strains were obtained from the All-Union Institute of Veterinary Medicine and the Veterinary Control Institute.

The best results were obtained with the following method of preparation and treatment: the fixed smear made from the investigated material was treated with fluorescent serum for ½–1 hr, then thoroughly washed in several volumes of buffer solution and

dried. This facilitated the removal of fluorescent background from the preparation, which depended on the amount of free fluorescent serum in the preparation which was not bound to the bacteria.

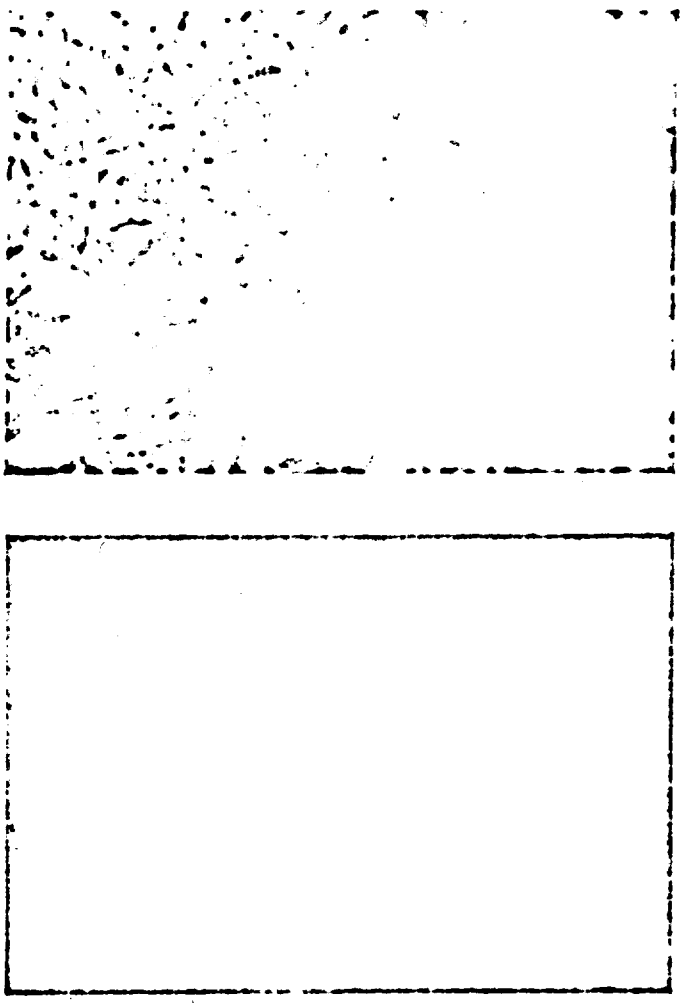

FIG. 2. Pseudoanthrax bacilli (above—phase contrast; below—bacteria treated with fluorescein-labelled anthrax serum).

All preparations treated with fluorescent serum were studied at magnifications of 100, 450 and 630 in ultra-violet and blue light, using light source 01–17 and lamp SVD-120A or SVDSH-150-3, and also by phase-contrast microscopy.

For the preservation of preparations the smears were enclosed in a buffered solution of glycerol under a cover glass (9 parts of glycerol to 1 part of phosphate buffer pH 7).

Apart from the vaccination strains we also studied anthrax strains kindly sent to us by Ia. Meshcheriakov, Microbiological Laboratory, All-Union Experimental Veterinary Institute.

When making preparations directly from agar medium the differentiation of individual bacteria was made difficult when great masses of bacteria were present in the smear.

The preparation of smears from bacterial suspensions containing 20–30 cells per field of view permitted the observation of the fluorescence of individual bacteria. The observation of preparations at magnifications of 450–630 in blue as well as ultra-violet

light showed that the anthrax bacilli give a bright yellowish-green fluorescence of individual cells against the generally dark background of the preparation. The vaccination strains (Tsenkov strain II and STI) also gave fluorescence after treatment with fluorescent anthrax antiserum, although this was less intense. These showed brighter and more intense fluorescence at the periphery than in the centre of the cells (Figs. 1 and 2). The pseudoanthrax strains studied and others from the group of Gram-positive bacteria did not show fluorescence (Table 2).

TABLE 2. INTENSITY OF FLUORESCENCE OF ANTHRAX BACILLI TREATED WITH FLUORESCEIN LABELLED ANTISERUM

| Serum / Bacteria | Anthrax antiserum | | Normal serum |
|---|---|---|---|
| | Not absorbed | Preliminary absorption | |
| Anthrax 4 | +++ | ++ | ± |
| Pseudoanthrax 6 | ± | ± | ± |
| Gram-positive 4 | − | − | − |
| Vaccination strains 2 | ++ | ++ | ± |
| Enteric 3 | − | − | − |

Control preparations made by treating other bacterial species, for instance *Esch. coli*, with fluorescent anthrax serum, or by treating anthrax bacilli with normal rabbit serum, showed either weak or no fluorescence. Consequently one must assume that the use of such a test would be of insufficient value for the study of the specificity of the fluorescence of bacteria.

Precipitating anthrax sera labelled with fluorescein isocyanate were prepared and the results of this study show that it is possible to diagnose and identify anthrax from pseudoanthrax and other Gram-positive bacteria by its bright fluorescence.

*Translated by* K. A. ALLEN

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