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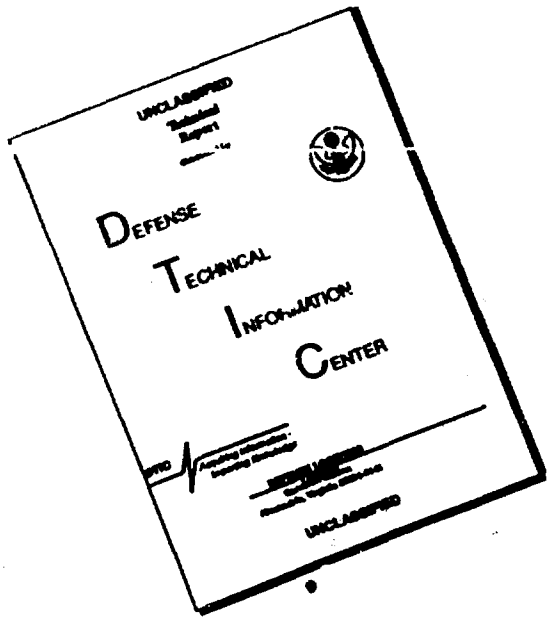

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METHOD OF PROCESSING BACTERIOLOGICAL PREPARATIONS WITH FLUORESCENT ANTIBODIES

[Following is a translation of an article by I. O. Dashkevich, S. I. D'yakov, V. M. Ekrtmn and I.V. Osipova in the Russian-language journal Zh. Mikrobiologii, Epidemiologii i Immunobiologii (J. of Microbiology, Epidemiology, and Immunobiology), Vol XXXIII, No 7, Moscow, 1962, pp 101-107.]

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The luminescent microscopy examination of microorganisms by the method of fluorescent antibodies has recently been increasingly employed in microbiological practice, especially for a more rapid identification of the causative agents of a number of infectious diseases. (Whitaker and coauthors, 1958; Goldwasser and Kisling, 1958; Kabanova and coauthors, 1960, et al.). However, together with positive experience of the practical use of this method, reports have recently been appearing concerning the insufficient specificity of the luminescent-serological method of examination. For instance, in the diagnosis of dysentery (La Brec and coauthors, 1959), salmonellosis (Thomason, Cherry and Edwards, 1959) and other infections, phenomena have been described of cross-staining of non-pathogenic microorganisms, considerable autofluorescence of some objects, etc. Presumably, the cause of some defects of the immuno-fluorescent method may be the unexplained methodical problems connected with the details of preparation and processing of the preparations with labeled antibodies. Therefore, a more thorough study of the methods of processing of bacteriological preparations with fluorescent antibodies should be considered as an important stage in the perfection of this very promising investigation method.

The task of the present work was a study of the role of methods of preparation and fixation of bacteriological smears, the duration and thermal regimen of their staining with fluorescent antibodies, as well as the regimen of washing the preparations.

Tests were conducted with dysentery (Flexner type 'c') and typhoid, as well as tularemic, plague, and anthrax vaccine strains (a total of 20 strains). As controls we use the strains of B. coli, dysentery bacilli of other varieties and serological types, salmonellae, and microorganisms of other species (a total of 30 strains).

For the staining of preparations, we employed the following set of fluorescent conjugats: typhoid gamma-globulin (series No 317, 324, 404), adsorbed Flexner serum (series No 26), monoreceptor serum, Flexner type 'c' (series No 318 and 45), tularemic gamma-globulin (series No 3), antirabbit chicken (series No 399 and 405) and sheep (series No 13) gamma-globulin, antihorse rabbit gamma-globulin (series No 7 and 8), and normal rabbit gamma-globulin (series No 14). In our work with the indirect method of fluorescent antibodies, during the first stage we employed rabbit's dysentery and salmonella diagnostic sera of the Leningrad Institute of Vaccines and Sera and of the Institute of Epidemiology and Microbiology imeni Pasteur.

The study of preparations was carried out with Soviet apparatus for luminescent microscopy ML-1 and OI-17 (light-filters: UFS-3, FS-1, SS-4 + SS-8) with the use of additional light-filters: SZS-7, SZS-14, and BS-8; as protective filters we employed light-filters T-2N, ZhS-3, and ZhS-18.

The smears for processing with fluorescent antibodies were prepared by dropping and drying one or several drops (depending on the size of the smear and concentration of microorganisms in the suspension) of the microbial suspension on carefully degreased object slides.

In the preparation of a smear from a culture with a solid nutritive medium, it is necessary to prepare the microbial suspensions preliminarily with physiological solution, distilled water, or sterile pipe water with a concentration of not more than 10-100 million microbial cells in one ml. For the dilution of bacterial suspensions to maximum concentration, detected microscopically, it is preferable to use distilled water, and not a physiological solution. The preparation of a smear from a culture with a liquid nutrient

medium it is advisable to take it directly from the broth. The composition of the nutrient medium (the presence of carbohydrates and certain other ingredients) usually exerts no substantial effect on the quality of the specific cellular fluorescence and the coloring of the background. The exceptions are certain complex protein media containing milk, yolk, and similar products.

Fixation of the preparations was carried out by chemical and physical methods. For chemical fixation we tested the following fluids: methyl alcohol (1, 3, 5, 10, and 20 minutes), Nikiforov's mixture (3, 5, 10, and 15 minutes), ethyl alcohol (3, 5, 10, and 20 minutes), choroform (5, 10, and 20 minutes), formalin vapor (1, 3, and 5 minutes), etc. The chemical fixation was carried out at various temperatures: minus 20-15°; +24°; +16-22°; and 37°. The physical method of fixation consisted of heating of the smear on a gas flame for 5, 10, 30 seconds, one minute, and more.

It has been established that the use of various methods and lengths of fixation, as well as various temperature regimen had no essential effect on the specificity of microbial antigens and their property of uniting with the fluorescent antibodies. The employed methods of fixation of preparations had no substantial effect on the intensity of the specific fluorescence of homologous microbial cells and produced no emergence of non-specific fluorescence in the heterologous microorganisms.

The investigation results enable us to recommend for practical purposes the fixation of preparations by heating as the simplest and quickest method. However, taking into account the characteristics of processing of preparations with causative agents of particularly dangerous infections, as well as the peculiarities of antigenic structure of some microbial species, the method of fixation by heating cannot be regarded as universally applicable. In working with causative agents of particularly dangerous infections, it is preferable to employ chemical methods of fixation. For this purpose, the majority of fixing fluids is acceptable, with the exception of chemical fluids containing compounds of heavy metals which quench fluorescence.

The fixed preparations prior to their staining with fluorescent antibodies do not require supplementary processing in the majority of cases. However, in some instances, in order to reduce or completely eliminate the autofluorescence of the object and the background fluorescence, the

following methods of supplementary processing are recommended: washing with buffer solutions with pH = 7.0 to 7.4, solutions of iodine, fuchsin, methylene blue, and other substances which quench the proper fluorescence of the object; procesing with benzine, xylol, and other organic solvents, in order to remove fatty substances which, as a rule, possess bright fluorescence; treatment with normal non-fluorescent serum for the saturation of non-specific adsorption bonds with protein, etc.

In staining by the direct method, a drop of fluorescent immune serum is placed on the fixed smear which is then placed in a moist chamber at various temperatures: 4, 16, 26, 37, 40, and 50°. At the indicated temperature, the smears were kept for 1, 5, 15, 30 minutes, one or two hours, or more. During processing, one must avoid the dessication of the drops of fluorescents conjugates, since this often leads to the appearance of non-specific fluorescence of the cells and background. After washing and drying, the smears were examined under a luminescent microscope.

Our investigations have established that at low temperatures (2° to 4°) the specific staining of microbial cells proceeded noticeably slower. For instance, upon staining for 5, 15, and 30 minutes with a given fluorescent antibody at this temperature, the homotypical microbial cells, as a rule, scarcely fluoresced (+). Upon staining for an hour, or longer, an appreciable specific luminescence of microbial cells usually appears (++). Under these circumstances, if the principal mass of cells in the preparation showed mild fluorescence, some isolated cells fluoresced more intensely (+++). Staining of the preparations for two hours or longer led to the appearance of a more uniform fluorescence of the homologous microbial cells; however, their luminescence still remained insufficiently bright (++ and +++).

Staining of the smears at 14-16° for 20-30 minutes led to the appearance of a marked specific fluorescence of the cells (++ and +++); however, the uneven coloration of some microbial cells remained. At 18-22°, the preparations stained much faster and fluoresced brighter; to obtain luminescence of medium intensity (+++), 5-10 minutes sufficed; upon staining for 15-20 minutes, a bright, specific luminescence was observed (+++ and ++++).

At 37°, a bright luminescence (+++) of homologous microbial cells was noted within three to five minutes. With the increase of processing time of the preparations to 10-15

minutes, the intensity of fluorescence of the cells increased (to ++++). Further prolongation of staining did not appreciably increase the intensity of the specific luminescence of microbial cells. Staining of the preparation for an hour, or longer, was often accompanied by the appearance of non-specific coloration of the heterotypical microorganisms and background.

A higher temperature (40-50°) led to a reduced contrast and a decrease of the specific fluorescence of cells. Hence, 37° should be considered as the optimum temperature at which the fastest and specific coloration of the microorganisms takes place with the direct method of fluorescent antibodies, and 5-10 minutes (but not more than 30) minutes should be considered as an adequate period for processing the smear with the fluorescent substance.

The indirect method of processing of preparations was carried out in two stages. During the first stage, a drop of non-fluorescent immune serum was placed on the fixed smear. Following the contact, the preparation was washed and dried. During the second stage, a drop of fluorescent antirabbit or antihorse serum was placed on the preparation. The smear was then washed again and, after drying, was examined under a luminescent microscope.

As in the staining with the direct method, we investigated the importance of the temperature regimen and duration of processing of the preparations with immune non-fluorescent sera during the first stage, and with labeled antisera globulins during the second stage. It can be seen in the cited table of data that, with the indirect method of processing the preparations, the intensity of specific fluorescence of microbial cells was determined by the effect of the same factors as with the direct method. At low temperatures (two to four degrees), the process of coloration of microbial cells was considerably retarded. Upon processing the preparation during the first and second stage for 20-30 minutes, a mild fluorescence (+ and ++) of isolated cells was noted. Processing of the preparations for two hours during each stage led to the increased fluorescence of isolated cells (++ and +++).

At 16-18°, and especially at 24°, the duration of staining of the smears was considerably reduced. Ten minutes during the first stage and 10-20 minutes during the second stage were usually sufficient for the obtaining of specific cellular fluorescence of adequate luminosity (+++ and ++++).

Subsequent increase of the duration of the contact of sera with the microbial cells (one to two hours for the first stage, and 2 to 24 hours for the second) was accompanied by the appearance of non-specific luminescence of the heterologous microorganisms. At 37°, the fastest coloration of microbial cells took place. For instance, a short (5-10-minute) contact with immune globulins was required at each stage for the obtaining of a specific bacterial fluorescence of a sufficiently high luminosity (+++). Taking into account the various degrees of activity of immune sera, the processing of preparations at each stage should be carried out for 10-15 minutes, but not more than 30 minutes, since a longer processing of the smears leads to the appearance of non-specific fluorescence of the heterologous cells and background.

Thus, the data on the investigation of the effect of various temperatures and the duration of interaction of labeled immune sera with microbial cells in the smear attest to the importance of the evaluation of these factors in the regimen of processing the bacteriological preparations by the direct as well as the indirect method. These factors condition to a considerable extent the intensity and rate of inducing a specific luminescence in homotypical microorganisms.

The following washing liquids were tested for cleansing the preparations and their applied specific sera or fluorescent conjugates: buffer solutions of various molar concentration and of various pH values (from 5.0 to 8.0), physiological solution (pH = 7.0 to 7.4), distilled and running water. The duration of washing the preparations varied from one, to two minutes, to several hours. The washing method consisted of immersing the smears in the washing fluid, or their processing in running water.

As a result of observations, we established the necessity of a thorough washing off of the excess of non-reacted immune serum gamma-globulins in both staining methods. The composition of washing fluid was of no particular importance. The most suitable washing fluids proved to be the solution of a phosphate buffer 0.15 M (pH = 7.2 to 7.6) and the physiological solution (pH = 7.2). Washing of the preparations with these fluids should be carried out at room temperature for no less than 10 minutes. Variations in the pH of the phosphate buffer within a 5.0 to 8.0 range had no particular effect on the specific fluorescence of the microbial cells. Prolonged washing of the preparation with a phosphate buffer or a physiological solution (20-30 minutes, and longer) somewhat increased the contrast character of the image, at the expense of the reduction of background luminescence.

Intensity of Fluorescence of Microbial Cells, Depending on the Temperature Regimen and Duration of Staining by the Indirect Method of Fluorescent Antibodies

(1) Вид бактерий в препарате	(3) Время окрашивания люминесцирующей сывороткой					
	(4) 5 минут	(5) 15 минут	(6) 30 минут	(7) 1 час	(8) 3 часа	
(9) Температура холодильника от +2 до +4°						
Гомологичный (2)	—	—	+.—	++.+	++.+	10 минут (3)
Гетерологичный (3)	—	—	—	—	—	
Гомологичный (2)	—	—	+.—	+	++.+	20 минут (5)
Гетерологичный (3)	—	—	—	—	—	
Гомологичный (2)	—	—	+.—	++.+	+.++	1 час (6)
Гетерологичный (3)	—	—	—	—	—	
Гомологичный (7)	—	—	+.	+.++	+++.	2 часа (8)
Гетерологичный (8)	—	—	—	—	++.+	
Гомологичный (7)	—	+.—	+.	+.++	+++.	24 часа (9)
Гетерологичный (8)	—	+.—	+.—	+.—	++.+	
(10) Температура комнаты от +20 до +24°						
Гомологичный (2)	+++	++++	++++	++++	++++	10 минут (3)
Гетерологичный (3)	—	—	—	—	+.—	
Гомологичный (2)	++++	++++	++++	++++	++++	20 минут (5)
Гетерологичный (3)	—	—	—	—	+	
Гомологичный (2)	++++	++++	++++	++++	++++	1 час (6)
Гетерологичный (3)	—	—	—.+	+	+	
Гомологичный (2)	++++	++++	++++	++++	++++	3 часа (8)
Гетерологичный (3)	—	—	+	+	+	
Гомологичный (2)	++++	++++	++++	++++	++++	24 часа (9)
Гетерологичный (3)	—	+	+	++	++	
(10) Температура термостата +37°						
Гомологичный (2)	++++	++++	++++	++++	++++	10 минут (3)
Гетерологичный (3)	—	—	+.—	+	+	
Гомологичный (2)	++++	++++	++++	++++	++++	20 минут (5)
Гетерологичный (3)	—	—	+	+	+	
Гомологичный (2)	++++	++++	++++	++++	++++	1 час (6)
Гетерологичный (3)	+	+	+.++	++	++	
Гомологичный (2)	++++	++++	++++	++++	++++	3 часа (8)
Гетерологичный (3)	+	+	+.++	++	+++.	

Annotation: +, ++, +++, ++++ denotes the intensity of fluorescence of the microbial cells.

1 -- Type of bacteria in the preparation; 2 -- Duration of

staining with a non-fluorescent serum; 3 -- 5 minutes... 15 min... 30 min...; 4 -- One hour... two hours...; 5 -- Duration of staining with a fluorescent serum (second stage); 6 -- Temperature of the refrigerator from +2° to +4°; 7 -- Homologous; 8 -- Heterologous; 9 -- Room temperature from +20° to +24°; 10 -- Temperature of the thermostat +37°.

Distilled water proved least suitable for washing of smears, since it did not ensure the complete removal of serum proteins from the surface of the object slide. Good results have been obtained with rinsing of the preparations in running water. Five to ten minutes usually sufficed for this purpose. To accelerate the procedure, the smear can be rinsed in running water for one to two minutes.

The results of our investigations attest to the necessity of a thorough washing of preparations, so as to remove the non-reacted serum globulins in both methods of staining. In practical laboratories, the washing of preparations should be carried out in running water for ten minutes.

Conclusions

1. The method of fixation of bacteriological smears for luminescent microscopy has no essential effect on the capacity of microbial cells of being stained with specific fluorescent antibodies. The fixation can be achieved by heating, or with chemical fluids which do not contain heavy metal compounds.

2. The temperature regimen and duration of processing of the preparations with non-fluorescent and fluorescent immune sera, via direct and indirect methods, are important factors which condition the intensity and rate of specific staining of the microbial cells. The optimum conditions for smear processing, when the fastest interaction of the antigen with the labeled antibodies takes place, is 37° temperature and exposure for five to ten minutes, but not higher than 30 minutes. Staining at a lower temperature may be of practical value for the reduction of cross staining.

3. Washing of bacteriological smears following their processing with immune globulins can be carried out by immersing them in a container with washing fluid (physiological solution, phosphate buffer), or in running water for a period of ten minutes. To accelerate processing, the smears can be rinsed under a stream of pipe water for one to two minutes.

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[English Summary]

CONTRIBUTIONS TO THE METHOD OF PROCESSING BACTERIOLOGICAL PREPARATIONS WITH FLUORESCENT ANTIBODIES

The work deals with preparation, fixation and staining of bacteriological preparations with fluorescent antibodies (direct and indirect methods). The authors' investigations have demonstrated that the temperature regimen and the duration of smear staining by means of fluorescent antibodies are important factors, conditioning the intensity and specificity of microbial cells fluorescence. The composition of the washing liquids and the methods of fixation of the preparations had no essential effect on the capacity of bacterial cells to be stained with specific fluorescent antibodies.

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