

FLUORESCENCE MICROSCOPY FOR THE IN VITRO STUDY ON THE INTRACELLULAR DIGESTION
PROCESS IN PHAGOCYTIZED BACTERIA

AD 57-238
TF-67-61724

Translation No. 1927

SEPTEMBER 1966

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FLUORESCENCE MICROSCOPY FOR THE IN VITRO STUDY OF THE INTRACELLULAR DIGESTION
PROCESS IN PHAGOCYTIZED BACTERIA

[Following is the translation of an article by M. Ya. Korn, N. F. Gamaleya Institute of Epidemiology and Microbiology, AMN USSR, published in the Russian-language periodical Izvestiya AN SSSR, seriya biologicheskaya (Bulletin of the Academy of Sciences USSR, Biology Series), No 6, 1965, pages 913--918. It was submitted on 24 Nov 1964. Translation performed by Sp/7 Charles T. Ostertag Jr.]

The application of ordinary light and phase contrast microscopy in connection with cinemicrophotography for the in vitro study of phagocytosis made it possible to expose a whole series of morphological peculiarities of this process (Ivanitskaya, 1950; Policard et al., 1961; Hirsch, 1962). However, the cytochemical peculiarities of the process of digestion of phagocytized bacteria, which is of special interest, was studied mainly on fixed preparations (Braude et al., 1962). Fluorescence microscopy, which recently has been used successfully as a method for in vitro cytochemical analysis in various areas of biology, has still not found wide application for studying the mechanism of intracellular digestion during phagocytosis.

In the present report we will present several results of the fluorescent--microscopic study of the intracellular digestion of phagocytized bacteria.

Materials and Methods

In the capacity of phagocytizing cells we used amoebas (Ent. invadens), and also the leukocytes from the blood of horses and guinea pigs, and the exudate of white mice.

The cultures of Ent. invadens were obtained from S. A. Sarkisyan, who gave us help in mastering the method of cultivation, for which we express our sincere thanks.

As the objects of phagocytosis in the experiments on the amoebas we used primarily Bac. pseudoanthracis, and also staphylococcus, a toxigenic strain of Cl. perfringens, yeasts and horse erythrocytes.

In the tests with the leukocytes of blood and exudate the objects of phagocytosis were various species of bacteria (Bac. pseudoanthracis, the STI vaccine anthrax strain, various strains of enteric bacilli, and staphylococci).

The method of working with the amoebas was as follows: A 3--7 day culture of amoebas was mixed on a glass slide with a suspension of bacteria (or erythrocytes) in a solution of acridine orange, covered with a cover glass, and encased with paraffin. The end solution of acridine orange in the preparations comprised 1:50,000 -- 1:100,000.

The preparations were examined in a fluorescence microscope at a magnification of 400X (obj. 40X water immersion, oc. 10X). In several cases the preparations were used for fluorescence cinemicrophotography with the help of an electron-optical image brightness intensifier (Batslov et al., 1964; Korn, 1964).

It was established in preliminary tests that in 3--7 day cultures (in contrast to old cultures) at the stated concentrations of acridine orange the amoebas preserved their viability in preparations for a long time; when stored under conditions of room temperature, for a period of several days; during examination in a fluorescence microscope, up to 1.5 hours (period of observation). When amoebas from old cultures were examined in a fluorescence microscope they died in 20--30 seconds.

The tests with the blood leukocytes were performed in the following manner: Defibrinated, oxalated or heparinized blood from a horse or guinea pig was centrifuged for 5--10 minutes at 500--1000 rpm. The leukocytes found in the middle layer between the plasma and the erythrocytes were removed and transferred to a cover glass. The cover glass with the leukocytes was placed in a moist chamber for 15 minutes to one hour for the attachment of the leukocytes to the glass. Then to the drop containing the leukocytes we added the same drop by volume of a suspension of bacteria (2 billion microbial bodies/1 ml) in a solution of acridine orange with a calculation that its end concentration would comprise 1:50,000 -- 1:200,000 (it was preliminarily established that these concentrations do not inhibit phagocytosis).

After several minutes the cover glass was turned over and placed on a slide in such a manner that a distance of 0.2--1.0 mm remained between the cover and the slide. For this we first attached narrow strips of glass to the slide by using paraffin. The chamber formed was filled with a suspension of bacteria in a physiological solution of NaCl with 10% plasma or blood serum from the same species of animal from which the leukocytes being tested had come and encased with paraffin. The preparations were placed in an incubator or left on the microscope stage. Examination of the preparations in a luminescence microscope was carried out periodically for a period of 2--3 hours at magnifications of 400X and 700X (obj. 40X and 70X water immersion, oc. 10X). An analogous method of fixing preparations was used when studying phagocytosis of bacteria by leukocytes from white mouse exudate.

Results

The investigations showed that the fluorescence of phagocytized bacteria differed from the fluorescence of bacteria located extracellularly. Changes in the nature of fluorescence of the phagocytized bacteria did not depend on the species affiliation of the phagocytizing cells or on the species of the adsorbed bacteria being studied in our work.

With the dilutions of acridine orange used in our work the bacteria located outside of the cell hardly fluoresced. After their capture by the phagocytizing cells a gradual intensification takes place in the brightness of fluorescence, the bacteria begin to fluoresce a bright - green. It is interesting to note the significant increase in the brightness of that part of the bacteria which has still not been adsorbed by the phagocytizing cell (drawing). This was noticed especially well when studying large bacteria (Bac. pseudoanthracis). Gradually the color of fluorescence begins to change. At first it becomes yellow, and then fire red.

In the tests on blood leukocytes these changes in the brightness and color of fluorescence take place in approximately an hour. Further changes in the morphology of adsorbed bacteria depend both on their species affiliation and on the conditions of setting up the test. In the cells of Bac. pseudoanthracis dark circular non-fluorescing sectors appear and the cells preserve their form. Then the bacteria increase in volume and finally convert into a bright red granule. It should be noted that we were not able to observe the complete digestion of the phagocytized bacteria in all the tests.

When studying the phagocytosis of E. coli we often observed their conversion into protoplast-like formations, which corresponds to data available in the literature relative to other species of bacteria (Janssen et al., 1960). As a rule in one phagocytizing cell it was possible to detect several bacterial cells with a different color of fluorescence. Apparently this testifies to the difference in the time in which they were adsorbed. Under our conditions it was not possible to note any changes in the nature of fluorescence of the phagocytizing cells themselves.

In order to clear up if there was any connection between the changes in the nature of fluorescence and the intracellular death of the phagocytized microbes, we set up special experiments in which, along with living, the objects of phagocytosis were bacteria which had been boiled for 10--15 minutes. However, we did not detect any differences in the dynamics of the changes in fluorescence of phagocytized boiled bacteria in comparison with the living.

An attempt was also made to clear up if there was any connection between the above described changes in the intensity and color of fluorescence of the phagocytized bacteria and the action of lysozyme on them.

The results of the tests testified that the destruction of bacteria of various species (M. lysodeikticus, E. coli, Bac. subtilis, etc.) under the influence of lysozyme both under ordinary conditions and with the addition of complement (that is, under conditions close to those in which phagocytized bacteria are found) does not change the nature of their fluorescence in comparison with the unchanged bacteria in the control.

I. I. Mechnikov had already brought attention to the fact that phagocytized bacteria are stained more intensively with old solutions of vesuvin than those located outside the cells, connecting this phenomenon with the intracellular death of the bacteria.

In their review D. L. Rozental and A. S. Troshin (1963) point out that during the most diverse influences on cells, leading to their damage, an increase takes place in the sorption of dyes. This testifies to the structural rearrangements of the protein molecules. They also note that one of the reasons for the increase in colorability may be the dissociation of nucleoprotein complexes.

Many investigators studied metachromasy during the fluorescence of cells with acridine orange. Strugger, as is known, connected it with the death of the cell, considering that live bacteria fluoresced green, and dead - red. A further study showed that this provision could not be accepted unconditionally and that changes in the color of fluorescence are not always connected with the death of the cell (Meysel et al., 1961; Wolf, Aronson, 1961).

Works of recent years, connected with the study of the interaction of acridine dyes with nucleic acids, cleared up the reasons for the appearance of metachromatic staining during fluorochroming with acridine orange. These investigations showed that metachromasy is connected with the formation of dimers of fluorochrome and established certain regularities in its formation when cells were harmed (Wolf, Aronson, 1961) and during the denaturation of DNA (Borisova, Tumerman, 1964).

These data permit us to put forth several considerations concerning the reasons for the changes we observed in the color of fluorescence of phagocytized bacteria.

The most probable proposal that the changes in the nature of fluorescence in phagocytized bacteria is connected with their death within the adsorbing cell is not confirmed under our conditions, since the same changes were observed during the adsorption of bacteria killed by boiling.

It can also be proposed that the observed changes are connected either with a lowering of the pH within the phagocytizing cell or with the fact that

the concentration of fluorochrome within the cell is higher than in the surrounding medium.

However, these proposals are improbable based on the following considerations: 1) in both cases it is possible to expect a more rapid appearance of the changes than was observed in our tests; 2) we did not observe any changes in the nature of fluorescence of the phagocytizing cells themselves during the process of phagocytosis; 3) if the change in the color of the adsorbed bacteria is connected only with the higher concentration of dye within the phagocytes, then the fact that the cytoplasm of the phagocytizing cell fluoresces green and the bacteria being phagocytized - red remains unclear.

Therefore it can be proposed that the reason for the above described changes is the enzymatic breakdown (but not due to the action of lysozyme) of high molecular compounds and complexes, primarily nucleoproteins and protein molecules. This proposal agrees with the results obtained during a study of the ultraviolet fluorescence of phagocytized bacteria (Brumberg et al., 1964), and also with the data obtained during a study of the nature of micronecrotic foci, developing under the influence of ionizing radiation (Meysel, Manteyfel, 1963).

In this connection it is interesting to turn attention to the report that the color of the fluorescence of bacteria, fluorochromed with acridine orange, changed when treated with ultrasound, while the bacteria remained viable. The authors connect these changes with minute structural rearrangements in the DNA molecule (Tatake, Gopal-Aengar, 1963).

N. V. Gomzyakova and O. S. Yukov (1961), while studying phagocytosis of leukocytes by macrophages, detected within the macrophages leukocytes fluorescing a green as well as an orange and tomato red color. However, they come to the conclusion that the leukocytes, phagocytized by macrophages, do not change their inherent color of fluorescence, and consider that differences in the color of fluorescence are dependent on the fact that the macrophages absorb both living leukocytes and leukocytes which have been damaged. In the light of our observations it is possible to propose that differences in the color of fluorescence of phagocytized leukocytes may also depend on their intracellular digestion by macrophages.

Conclusions

1. Fluorescence microscopy ~~may be~~ used successfully for the in vitro study of the intracellular digestion of phagocytized bacteria.
2. During fluorochroming with acridine orange it is possible to observe changes in the brightness and color of fluorescence of phagocytized bacteria.

3. These changes were recorded (in a black-white image) with the help of a fluorescence cinemicrography technique ~~developed by us~~.

4. One of the possible reasons for changes in the brightness and color of fluorescence of phagocytized bacteria is the enzymatic destruction of high molecular biopolymers (proteins, nucleoproteins).

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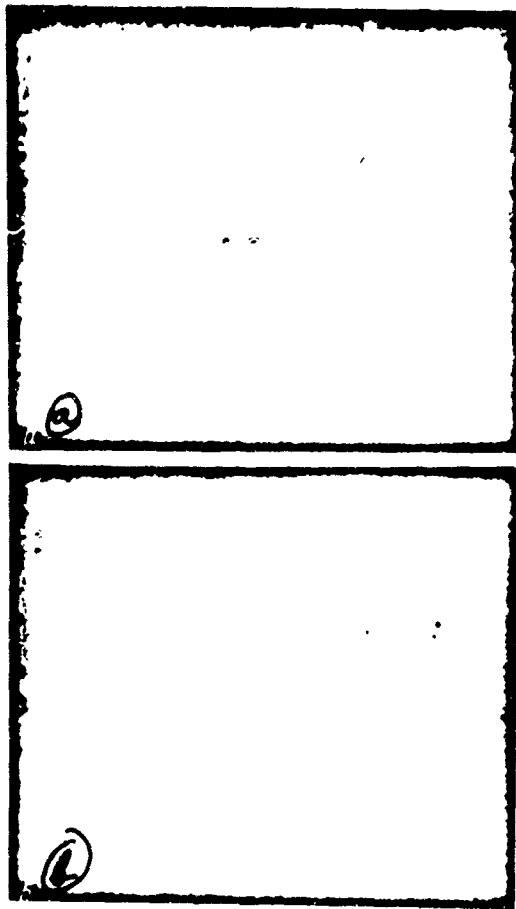
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Phagocytosis of Bac. pseudoanthracis by horse blood leukocytes. Phagocytized bacteria fluoresce brighter than bacteria in the surrounding medium.
a - an increase can be seen in the brightness of the sector of the bacterium being phagocytized which still has not been absorbed by the leukocyte.
b - the formation of pseudopodia is distinctly noticeable.
NL-2 microscope, obj. 70X (water immersion), gomal $\frac{1}{2}$ 5 λ . Filmed from an electron-optical brightness intensifier screen. Film AM-1 (45 GOST units), exposure 0.5 sec.