MORPHOLOGICAL AND CYTOCHEMICAL NATURE OF RED ACRIDINE ORANGE GRANULES

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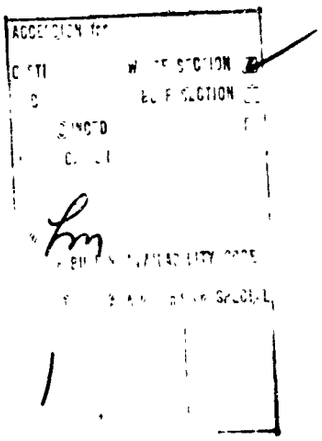

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MORPHOLOGICAL AND CYTOCHEMICAL NATURE OF RED ACRIDINE ORANGE GRANULES

[Following is the translation of an article by A. V. Zelenin, Institute of Molecular Biology, USSR Academy of Sciences, Moscow, published in the Russian-language periodical Izvestiya AN SSSR, seriya biologicheskaya (Bulletin of the USSR Academy of Sciences, Biology Series), No 6, 1965, pages 925--928. It was submitted on 24 Nov 1964. Translation performed by Sp/7 Charles T. Ostertag Jr.]

The increasing use of fluorescence microscopic investigations necessitates the thorough study of the cytochemical foundations and the cytochemical authenticity of this method.

Among the unresolved problems of fluorescence cytochemistry an important place belongs to the problem concerning the nature of the cytoplasmatic granules, detected regularly in cells treated intravitally with acridine orange fluorochrome (2,8-bis-dimethylaminoncridine). These granules are characterized by a bright red fluorescence, usually have a round, but sometimes rod-shaped form, and have a tendency to be localized close to the nucleus in the area of the Golgi apparatus. The red cytoplasmatic granules (RCG) are detected only in cells investigated intravitally and are not preserved during fixation.

After it was found that acridine orange (AO) was capable of producing red fluorescing complexes when combined with RNA (Meysel, Korchagin, 1952; Bertalanffy, Bickis, 1956), the opinion was put forth, and it received a quite wide dissemination, that RCG is the product of the combination of acridine orange with cytoplasmatic cellular RNA (Zelenin, 1961; Zelenin, Khrushchov, 1964; Robbins et al., 1964). However, papers appeared in the literature in which the authors doubted the feasibility of using RCG to make a judgment concerning cellular RNA (Eichner, 1959; Wolf, Aronson, 1961).

The investigation of whether or not the total cellular RNA (or any of the types of RNA) is the RCG substrate was the primary part of this work.

We analyzed the process of the formation of granules in the cytoplasm of neutrophilic leucocytes and demonstrated (Zelenin, Krushchov, 1964) that in any case the formation of granules in the neutrophils cannot be explained by the formation of complexes of AO with cytoplasmatic RNA since in these cells it is practically lacking.

A detailed study was undertaken of the fluorescence microscope picture of tissue culture cells, treated intravitally with acridine orange

(Zelenin, Lyapunova, 1961). This investigation showed that a large number of RCG is formed in a living cell under those conditions of fluorochroming during which the structures, known to be rich in RNA (nucleoli in the interphase, chromosomes in a dividing cell), fluoresce green.

Interesting results could be obtained in experiments on the vital fluorochroming of cells in tissue cultures, subjected to the influence of a hypotonic medium. It turned out that under certain experimental conditions it was possible to obtain a situation where following treatment with acridine orange the granules and all the remaining cytoplasm in the cell fluoresced red at the same time. This cytoplasm, based on morphology and the color of fluorescence, was quite analogous to the cytoplasm of fixed cells fluorochromed with acridine orange. Without a doubt the fluorescence of these cells is connected with the complex formation of acridine orange with ribosome RNA.

The interrelationship of the red granules with cytoplasmatic RNA was also investigated in experiments with the removal of RNA from the living cell and with the suppression of its synthesis.

An investigation was made of the capability of RNAase to penetrate the protoplasm of living cells (in particular, amoebas) by means of pinocytosis. It turned out that insertion of cells of the parasitic amoeba E. invadens into a medium containing RNAase in a concentration of 5 mg/ml leads to the complete disappearance or, at any rate, a sharp decrease in the amount of cytoplasmatic RNA. At the same time there is no change in the ability for granule formation by amoebas treated with RNAase.

In the next series of experiments (Zalmanzon et al., 1965) it was shown that treatment of tissue culture cells with large doses of a specific inhibitor of RNA synthesis, the antibiotic actinomycin D or its analog the antibiotic aurantine, even after 24 hours following the onset of the action, did not lead to an inhibition in the formation of RCG under the influence of acridine orange.

It is also necessary to mention the data of Mayor (1964), who demonstrated that the intravital action by acridine orange on cells, infected with the RNA containing virus of poliomyelitis, leads to the emergence of foci with a bright green (but not red) fluorescence in the cytoplasm of these cells.

The combination of the facts cited makes it possible to accept the fact that RCG are not a complex of acridine orange with ribosome RNA. In connection with this it is appropriate to recall the opinion of Wolf and Aronson (1961) that the ribonucleoprotein of the living cell does not form fluorescing complexes with the acridine orange dye. Such complexes are formed only with the complete (during fixation) or partial (during the reversible action -- paranecrosis) denaturation of RNA.

Data, obtained in experiments with actinomycin D and aurantine, made it possible to also exclude matrix RNA as a substrate for the granules. As special experiments showed (Zalmanzon et al., 1965), after 24 hours following the onset of action of these antibiotics matrix RNA is absent in the cell (in respect to actinomycin D see also Davidson et al., 1963). Nevertheless the ability for granule formation remains normal in cells treated with these antibiotics.

Somewhat more complex is the situation with the third fraction of RNA -- transfer RNA. There are certain facts which substantiate the proposal that transfer RNA also does not take part in the formation of RCG. The first of these facts is the presence of granules in amoebas treated with RNAase. According to certain data (Brachet, Six, 1959; Robin, 1962) ribonuclease, having penetrated into the living cell, attacks and subjects to separation primarily transfer RNA.

As the second fact it is necessary to keep in mind the very small amount of transfer RNA in the cell in respect to the total dry substance of the cell. At the same time the RCG occupy a significant, and in some cases even a large, part of the cytoplasm.

All that has been said gives a basis to assert that red cytoplasmatic granules, formed in cells which have been intravitally fluorochromed with acridine orange (and certain other deriviatives of acridine), are not a complex of fluorochrome with RNA. It is not possible to make any conclusions concerning the exchange of RNA * based on these granules, their quantity, brightness of fluorescence and location.

Establishment of the fact that RCG are not a complex of AO with cytoplasmatic RNA and are not agglutinated under the influence of AO with ribosomes, as this is asserted by Venetta and Snure, (1961) impelled the additional attempts for a clarification of the composition of RCG.

Certain investigators have expressed the opinion that the RCG are mitochondria fluorochromed with AO (Borchet, Helmke, 1950; Austin, Bishop, 1959). We studied the interrelationships of RCG with mitochondria with the help of the combined methods of microscopic investigation: A parallel study of the cells using fluorescence and phase contrast microscopes, and a simultaneous treatment of the cells with acridine orange and Janus green.

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*It is necessary to stress that the discussion deals only with an investigation of a living cell. The feasibility of the fluorescence microscopic detection of nucleic acids (including RNA) in fixed preparations with the help of the fluorochrome acridine orange at the appropriate pH is firmly established and widely used in histochemistry.

The investigations showed that the RCG and mitochondria (at least in the cells of a tissue culture) are morphologically independent structures (see also Wittekind, 1964).

In a special series of tests we made attempts to use the method of differential centrifuging for determination of the nature of the granules. We used liver as the object of investigation. In it, as preliminary tests showed, typical RCG are formed as the result of the intravenous administration of AO to the animal. It turned out that the granules did not decompose during homogenization of the liver tissue in a solution of saccharose. During differential centrifuging it was possible to obtain a subcellular fraction, enriched to the maximum with RCG and sedimenting at 1600 g (Zelenin, Lyapunova, 1964).

An electron microscopic investigation was carried out on the granule fraction (Zelenin et al., 1965). It was established that the granules are rounded bodies, surrounded by a thin membrane whose appearance calls to mind a myelin membrane. It was possible to expose the ultrastructures of the granules in preparations prepared with the help of the negative contrast method. In some cases the granules are homogeneous formations, in others they contain numerous tubules, strands and folds, creating an unusual network.

Without a doubt the visualization of the structure of the granules makes it possible to relate them to lysosomes or structures similar to lysosomes (Novikoff, 1961). An analogous conclusion concerning the lysosome nature of the RCG was arrived at simultaneously by Robbins et al. (1964), who observed that the RCG contain acidic phosphatase and apparently are so-called multivesicular bodies. It is very probable that the fluorescence microscopic investigation of cells fluorochromed with acridine orange will turn out to be a valuable method for the intravital detection of lysosome and lysosome-like structures in them.

At the same time the establishment of the lysosome nature of RCG left unresolved the problem concerning the chemical nature of substances on which acridine orange, when it is adsorbed on them, gives a red fluorescence to the granules. This is connected with the formation of dimers of the stated fluorochrome.

The facts presented in the first part of the article make it possible to refute the proposal that RNA is the chemical substrate of red fluorescence. The situation with acidic mucopolysaccharides is more complex. As is known, if acridine orange binds with them it also produces a red (dimeric) fluorescence. The problem requires further investigation. From previous considerations in this connection the only fact that can be noted is that RCG are revealed only at a pH relatively close to neutral (no lower than 5), while acidic mucopolysaccharides (in any case, sulfating) produce a red fluorescence at very low pH values (Armstrong, 1956; Wolf, Aronson, 1961).

It is also necessary to remember the circumstance that in many cells acidic mucopolysaccharides are lacking completely or are contained in very insignificant numbers, while numerous RCG are observed after treatment with acridine orange in the overwhelming majority of animal cells. Therefore it seems very probable that the red fluorescence develops in the granules not as a result of the combining of AO with acid mucopolysaccharides, but due to the adsorption of this fluorochrome on hydrolase enzymes, which are found in great number in lysosomes.

Conclusions

1. A complex investigation was carried out on the nature of red fluorescing cytoplasmatic granules, developing in living cells treated with acridine orange.

2. It was shown that these granules are lysosomes or lysosome-like structures of cells that have accumulated acridine orange. It has been established that no conclusions can be made on the amount and exchange of RNA based on a study of the red cytoplasmatic granules.

3. It is proposed that the red fluorescence of the granules is connected with the adsorption of acridine orange by proteins (enzymes) contained in lysosomes or lysosome-like structures.

4. The data obtained may be the foundation for the fluorescence microscopic detection of lysosomes in living cells.

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