MODIFICATIONS TO THE CHROMATIN OF LYMPHOCYTES BY MITOGENS AND THE MECHANISMS OF THEIR ACTION

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Investigation of the fluorescence of acridine orange, bound with the chromatin of circulating blood lymphocytes of rats, thymocytes, and their nuclei, by a cytospectrofluorometric method showed that the lymphocytes have the lowest initial level of fluorescence. The addition of phytohemaglutinin (PHA) activates the DNP-complexes of intact lymphocytes, as shown by an increase in the intensity of fluorescence. The initial intensities of luminescence of the DNP-complexes of the thymocytes and of their nuclei, incubated in medium not containing serum, were found to be both equal and maximal. Addition of PHA led to no further activation. Melting of PHA-stimulated lymphocytes, and also of the thymocytes of their nuclei, was accompanied by displacement of the curve toward lower temperatures and by a change in its profile.

An important problem in molecular biology is that of the state of the secondary structure of DNA as a component of the nucleoprotein complex and the degree of blocking of the nuclear template by protein in the various stages of the mitotic cell cycle. Naturally the most complete solution to this problem is possible only by direct determination of the corresponding parameters of the DNA and DNP in vivo. One of the most successful methods used to investigate these problems is that of microfluorometry, described adequately by Rigler [4, 5].

The object of the present investigation was to study the following problems: to what extent does the initial physiological state of the cell influence the degree of possible activation of its chromatin by a mitogen; does activation of the chromatin take place indirectly through the cytoplasm or can this process be induced directly in cell nuclei; what is the degree of participation of components of the medium in which the cells are cultivated and, in particular, of the blood serum in the activation of cell chromatin.

EXPERIMENTAL METHOD

The cells used as test objects belonged to a polymorphic series – circulating blood lymphocytes and thymocytes of rats, for they are sufficiently similar morphologically and also, to some extent, functionally. However, whereas thymocytes characteristically have intensive DNA synthesis and a high mitotic index, the circulating blood lymphocytes exhibit a low intensity of DNA synthesis, and mitoses are almost completely absent in them. Some information on the possible differences between the cells in their initial state has been given elsewhere [2, 3, 6].

Male rats weighing 130–150 g were used in the experiments. Preparations were made as follows.

Circulating Blood Lymphocytes of Rats. Heparinized rat blood (100 units heparin [ml]) was diluted with Eagle's medium (four drops to 10 ml medium) and poured into Petri dishes with cover slips (18 × 18 mm) lying on the bottom. The cover slips with adherent cells were placed in a Teflon holder 5 min later and dipped into cold (4°) fixing solution (a 1:1 mixture of ethanol and acetone). In parallel tests,

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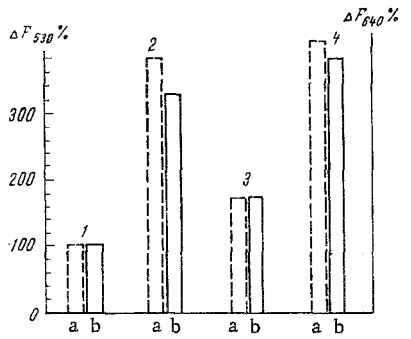

Fig. 1. Relative values of intensities of fluorescence after incubation for 5 min: a) 640 nm; b) 530 nm. 1) Intact lymphocytes; 2) thymocytes without serum; 3) thymocytes with 10% serum; 4) nuclei of thymocytes in 0.25 m sucrose. Ordinate, here and in Fig. 2: values of ΔF_{530} and ΔF_{640}.

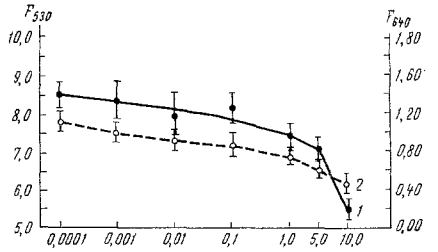

Fig. 2. Intensity of luminescence of DNP from thymocytes in different concentrations of autologous serum: 1) ΔF_{530}; 2) ΔF_{640}. Abscissa, concentration of serum (in %).

phythohemaglutinin (PHA) ("P"-PHA, Wellcome, England) was added to the incubation medium before the beginning of incubation in a concentration of 40 μg/ml; in this case the lymphocytes were incubated for 60 min at 37°C, after which they were placed in the fixing solution.

Rat Thymocytes. The thymus was taken from rats immediately after decapitation and a suspension of the thymocytes made in Eagle's medium at 37°C (10^4 cells/ml medium); the cells were fixed as described above to the surface of cover slips, and then dipped in cold fixing solution. In some experiments the cells were stimulated by PHA (40 μg/ml) for 60 min at 37°C. During incubation of the thymocytes with autologous serum, concentrations of between 0.0001 and 10% serum were used. The serum was obtained by centrifuging blood from recently killed animals for 15 min at 2000 rpm.

Nuclei of Rat Thymocytes. The nuclei were isolated by a slightly modified method of Allfrey and Mirsky [1]. The resulting nuclear residue was diluted in 0.25 M sucrose (10^4 nuclei per ml) and poured into Petri dishes with cover slips. After incubation the slips with the adherent nuclei of the thymocytes were placed in cold fixing solution.

Staining, fixing, and the immediate preparation of the specimens were carried out as described by Rigler [4, 5]. Fluorescence was measured on a single-beam microfluorometer built on an Ortholux (Leitz) luminescence microscope. An immersion objective (95.7, A-1.1) was used. Fluorescence was excited by light with γ = 360-405 nm (glass filter) from a xenon lamp. The fluorescence spectrum of the specimens was analyzed with the aid of interference filters with maxima of transmission in the 530 and 640 nm regions.

The lymphocytes (stimulated with PHA and intact), thymocytes, and thymocyte nuclei were melted by a slightly modified Rigler's technique. The time the specimens were kept in cold standard salt solution and the time of their taking through alcohols (from 20 to 2 min) were reduced, so that the degree of renaturation of the DNA was minimized and higher values of the coefficient α (F_{530}/F_{640}), a measure of the thermal denaturation of the DNA in the composition of the cell chromatin, could be obtained.

EXPERIMENTAL RESULTS

Incubation of the lymphocytes, thymocytes, and their isolated nuclei for 5 min showed that intact lymphocytes have the lowest intensity of luminescence. The intensity of luminescence of DNP from thymocytes incubated in medium containing 10% autologous serum was on the average 50% higher than that of intact lymphocytes; thymocytes incubated in medium not containing serum had an intensity of luminescence 200% higher than that of the lymphocytes. An even greater difference was found between the lymphocytes and the isolated thymocyte nuclei (Fig. 1).

The "dye-binding" properties of DNP thus increase in the order: intact lymphocytes, thymocytes incubated with 10% autologous serum, thymocytes incubation without serum, and thymocyte nuclei. A connection is known to exist between the degree of activation of the cell DNP and its "dye-binding" properties. In the series of cells and nuclei examined, an increase in the "dye-binding" properties of the cell DNP was observed in the course of its activation. Thymocytes incubated in medium not containing serum and the

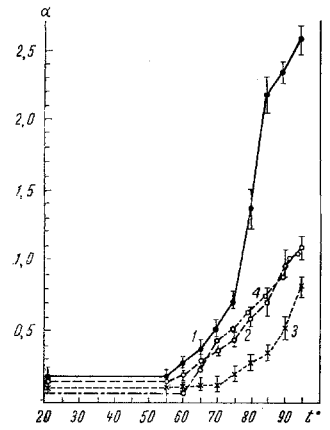

Fig. 3. Melting of DNP of intact and PHA-stimulated lymphocytes, thymocytes, and thymocyte nuclei: 1) thymocytes without serum; 2) thymocyte nuclei; 3) intact lymphocytes; 4) lymphocytes stimulated by PHA. Abscissa, temperature (in °C); ordinate, coefficient α (F_{640}/F_{530}).

nuclei isolated from them had similar values of the intensity of luminescence (Fig. 1). This indicates the invalidity of the hypothesis that changes in the permeability of the cell membrane to acridine orange takes place during activation of cells.

The marked difference between the intensity of luminescence of DNP-complexes of thymocytes in medium containing 10% autologous serum and in medium without serum made it necessary to investigate the relationship between the degree of activation of the DNP-complexes of thymocytes and the serum concentration. With an increase in the serum concentration, the intensity of luminescence of DNP-complexes of the thymocytes fell (Fig. 2), i.e., activation of the thymocyte chromatin depends on the serum concentration.

To study the ability of the DNP of the cells to undergo additional activation, cells were stimulated with a standard mitogen (PHA). As a result of incubation for 60 min in the presence of PHA, the intensity of green luminescence was increased by 2.6 times (compared with incubation for 5 min), and the intensity of red luminescence was increased by 2.4 times. In addition, the "dye-binding" properties of the DNP were increased, although to a lesser degree, in lymphocytes incubated for 60 min without PHA. This activation of unknown etiology will be called spontaneous.

DNP of thymocytes incubated in medium not containing serum was virtually not activated by PHA, and the spontaneous activation under these circumstances likewise was very slight. In the case of DNP from thymocytes incubated in medium with 10% autologous serum, considerable activation by PHA, and also spontaneous activation, were observed. In isolated thymocyte nuclei, neither activation by PHA nor spontaneous activation was observed.

It is thus evident that activation of the DNP-complex of the lymphocyte nuclei can be induced by the addition of a mitogen (PHA) to the incubation medium, and also by reducing the concentration of autologous serum in the medium. The degree of this additional activation is determined by the initial level of activation of the cell DNP.

To analyze the effects obtained and described above, it was decided to use the method of melting the cell DNP. It might be expected that activated cells would give a high amplitude of melting; on the other hand the possibility could not be ruled out that investigation of the melting of DNP in the composition of these objects could give information on possible differences in the secondary structure of their DNA. The study of thermal denaturation curves of the DNP from intact and PHA-stimulated lymphocytes showed that the DNP from PHA-stimulated lymphocytes began to melt at lower temperatures than DNP from intact lymphocytes. In addition, by modifying Rigler's method it was possible to detect the presence of a plateau in the melting curves of PHA-stimulated lymphocytes, indicating two stages in the process of melting of the DNP. Melting of DNP from thymocytes incubated in medium without serum and of isolated thymocyte nuclei began within the temperature range 55-60°C. This temperature was 10-15° lower than the temperature at which DNP from intact lymphocytes began to melt (Fig. 3). The coefficient α reached the following values at 95°C: for intact lymphocytes 0.9-1.1; for thymocytes, 2.4-2.8.

The "activated" DNP is thus more thermolabile than the DNP from cells with a lower degree of activation. Displacement of the melting curve of the DNP of activated cells toward the region of lower temperatures suggest specific interaction with a particular protein (possibly the nonhistone fraction) belonging to part of the genome of the activated cell, leading to partial destabilization of the DNP-complex.

LITERATURE CITED

1. V. G. Allfrey and A. E. Mirsky, Proc. Nat. Acad. Sci (Washington), 48, 1590 (1962).
2. B. H. Gledhill et al., Exp. Cell Res., 41, 652 (1964).
3. D. Killander and R. Rigler, Exp. Cell Res., 54, 163 (1969).
4. R. Rigler, Acta Physiol. Scand., 67, Suppl., 1267 (1966).

5. R. Rigler et al., Exp. Cell Res., 55, 215 (1969).
6. N. P. Ringertz and H. Bollund, Exp. Cell Res., 55, 205 (1969).