MICROFLUORIMETRIC STUDY OF THERMAL DENATURATION OF THE NUCLEAR DNP-COMPLEX OF LYMPHOCYTES STAINED WITH ACRIDINE ORANGE

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The addition of phytohemagglutinin (PHA) to a culture of human lymphocytes increases by 21% the intensity of the green ($\lambda = 530$ nm) nuclear fluorescence of cells fixed and stained to equilibrium in a 1.12×10^{-6} M solution of acridine orange. The melting profiles of the nuclear DNP-complex of the lymphocytes are represented by curves with two maxima at 65 and 85°C. On heating to temperatures above 90°C the intensity of fluorescence diminishes. The intensity of fluorescence of cells cultivated in medium with PHA is increased in the region of the maxima.

Changes in the melting profile of the DNP-complex of individual cell nuclei caused by physiological activation of the cells have been described [3, 5, 6]. The value of $\alpha = F_{640}/F_{530}$ (where F_{640} and F_{530} are the intensities of fluorescence at wave lengths of 640 and 530 nm, respectively), corresponding to the degree of DNP denaturation, has been recorded experimentally. Subsequent investigation [1] showed, however, that heating to temperatures preceding the melting temperature of DNA (55-65°C) leads to considerable changes in adsorption of the dye by the DNP complex of the cell nuclei.

To make a detailed study of this phenomenon the temperature-dependent changes in the degree of adsorption of the dye on the DNA of cells in various physiological states were investigated.

EXPERIMENTAL METHOD

During the work it was the aim to exclude procedures which could lead to changes in the native properties of the DNP-complex from the technique as suggested previously [5]. For this purpose, the medium in which denaturation was carried out did not contain formaldehyde and the process of denaturation was inhibited by transferring the specimens rapidly to fixing solution at a temperature of 0°C [1]. The procedure of acetylation, directly preceding the staining of the specimens, likewise was excluded because it could lead to uncontrollable changes in the connections between the PO_4-groups of the DNA and the protein.

In view of observations made with respect to nonequilibrium staining of cells, in the present investigation the specimens were stained to equilibrium in a solution of acridine orange of low concentration (1.12×10^{-6} M). Under these conditions the molecules of the dye are linked together by a strong (hydrophobic) type of interaction with the double-helical regions of the DNA virtually without forming aggregates fluorescing in the red region of the spectrum (the ratio between the intensities of fluorescence of the cells stained to equilibrium at 640 nm and the intensity of fluorescence at 530 nm was 0.016).

Cultures of white blood cells were prepared from the blood of healthy donors (15 ml), taken under sterile conditions from the cubital vein into a tube containing 200 i.u. heparin made up in 1 ml Ringer's solution. The tubes were incubated at an angle of 45° for 60 min; plasma containing white blood cells was drawn off and diluted with Eagle's medium to a concentration of 5×10^5 cells/ml and poured into plastic Petri dishes with dry coverslips (18 × 18 mm) on the bottom. The height of the layer of liquid culture me-

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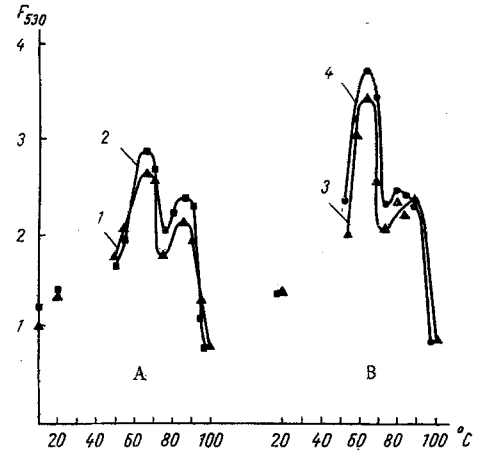

Fig. 1. Changes in intensity of fluorescence of cell nuclear DNP measured at $\lambda = 530$ nm (in conventional units). A) Cells incubated for 60 min in medium without PHA (1) and in medium with PHA (2); B) Cells incubated for 240 min in medium without PHA (3) and in medium with PHA (4). Abscissa, incubation temperature (in deg.); ordinate, intensity of fluorescence of cells grown for 1 h in medium without PHA (triangles) and in medium with PHA (squares) and stained immediately after fixation (cells not incubated in SSC).

dium was 5 mm. Phytohemagglutinin (PHA; Difco-P, USA) was added to some of the cultures to a final concentration of 40 μg/ml. The experiments were carried out at 37°C. The cells were grown for 60 and 240 min (long enough for activation of the cell DNP by PHA [7]). After cultivation for 60 and 240 min the coverslips with the attached cells were removed, rinsed twice by immersion in physiological saline (37°C), picked up in a Teflon holder, and transferred to the fixing solution (acetone–ethanol, 1:1), in which they were fixed for 1 h. Some of the specimens used to obtain the melting profiles were then transferred to a salt and sodium citrate solution (SSC) [1] and incubated in it for 20 min at various temperatures, after which the holder with the coverslips was quickly transferred to the fixing solution at 0°C, in a vessel of sufficient capacity (600 ml) and height (≥150 mm). The cells were fixed a second time for 1 h. After fixation all the specimens (i.e., those kept all the time in the fixing solution and those replaced in it after incubation at various temperatures) were taken through a series of alcohols: absolute ethanol, 96°, 60°, 30°, to water, transferred to buffer (pH 4.1), and stained in a buffered solution of acridine orange for 30 min. After staining the coverslips were placed in a drop of acridine orange solution on slides, and the edges of the coverslips were mounted with Entellan. Fluorimetry was carried out on the specimens 20 min after preparation. The fluorescence of the specimens was excited with an incandescent lamp (maximal intensity of exciting light at $\lambda = 420$ nm). The intensity of fluorescence was recorded with the FÉU-15V instrument. Blood samples from 13 donors were grown in culture for 60 min and samples from five donors for 240 min.

EXPERIMENTAL RESULTS

Measurement of the intensity of fluorescence of cells grown for 1 h and stained after the first fixation (cells not incubated in SSC) showed that addition of PHA to the culture increased the intensity of fluorescence by 21% ($\Delta = 0.21 \pm 0.08$, $n = 11$, $P < 0.05$, difference method; here and in all subsequent cases Δ signifies the difference between the intensity of fluorescence of cells grown in medium with and without PHA, n the number of differences, and P the criterion of significance). The results are shown as two points on the ordinate in Fig. 1. The same figure gives curves showing the intensity of fluorescence of the cells measured at $\lambda = 530$ nm as a function of the incubation temperature. It will be clear from Fig. 1 that incubation of the cells at 20°C led to some increase in the intensity of fluorescence compared with that of cells not incubated in SSC and stained immediately after fixation. The melting profiles are curves with two maxima at 65 and 85°C (the first and second maxima, respectively). On heating to temperatures above 90°C the intensity of fluorescence fell sharply. The melting curve of cells grown in medium containing PHA coincided to some extent with the melting curve of cells incubated in medium without PHA, but in the regions of 65 and 85°C the intensity of fluorescence of the cells stimulated by PHA was relatively higher (at 65°C $\Delta = 0.33 \pm 0.1$; $n = 13$, $P < 0.02$). Incubation of the cells for 240 min did not significantly alter the melting profiles. These also were curves with maxima of intensity of fluorescence at 65 and 85°C. A marked decrease in the intensity of fluorescence was observed on heating to temperatures above 90°C. With an increase in the duration of incubation (Fig. 1, curves 1 and 3) an increase in the intensity of fluorescence was observed in the region of the first maximum (at 60°C $\Delta = 0.97 \pm 0.21$; $n = 5$, $P < 0.01$).

These experiments showed that stimulation of the cells by PHA for 1 h increases adsorption of the dye by 21%. This figure is less than that given previously [2], but it is considered that the equilibrium method of staining allows the true increase in adsorption of dye to be recorded.

The melting profiles are curves with maxima of intensity of fluorescence at 65 and 85°C. The complex character of the relationship between the intensity of fluorescence and the temperature cannot yet be explained. The presence of two maxima can be connected with differences in the temperature stability of

the individual components of the DNP. Presumably the increase in intensity of fluorescence in the region of the maxima is connected with dissociation of the protein. The increase in intensity of fluorescence can perhaps be partly attributed to the fact that removal of the protein component leads to conformational changes in the structure of the DNA, with a consequent increase in the orderliness of the secondary structure and a corresponding increase in adsorption of the dye. The decrease in intensity of fluorescence in the temperature region 65-75°C can be explained by denaturation of the DNA fraction with reduced resistance to heat. The sharp decrease in the intensity of fluorescence observed on heating above 90°C is evidently due to denaturation of the DNA. Within this range of temperatures, on account of loss of the helical structure of the DNA, the strong type of binding (intercalation) is reduced and this causes a decrease in the intensity of green fluorescence [4]. An increase in the duration of cultivation to 240 min increased the intensity of fluorescence in the region of the first maximum. The addition of PHA to the medium also led to an increase in the intensity of fluorescence in the region of the maxima after cultivation for 60 min. Presumably both factors produce structural changes in the chromatin, changes in the composition of the protein part, and a relative increase in the content of easily dissociated components.

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