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ANALYSIS OF THE SECONDARY STRUCTURE OF DNA DURING ACTIVATION OF LYMPHOCYTES BY MITOGENS

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The development of the immune response is accompanied by differentiation and proliferation of immunocompetent cells under the influence of the antigen. To study the molecular mechanisms of lymphocyte activation, stimulation of peripheral blood mononuclear leukocytes by mitogens is used as a model of the immune response in vitro [9]. The state of the structure of DNA of the lymphocytes during activation has not been studied, although it is known that the secondary structure of DNA of resting and proliferating cells differs [1, 2], and their differentiation also is accompanied by changes in the structure of DNA [5].

The aim of this investigation was to study the secondary structure of DNA of human peripheral blood lymphocytes during activation by mitogens on the rate of alkaline denaturation of DNA in cell lysates. The process of activation was monitored by determining the increased intensity of DNA and RNA biosynthesis, by the use of ^{3}H-thymidine and ^{3}H-uridine.

EXPERIMENTAL METHOD

Human mononuclear leukocytes were isolated from fresh heparinized blood by the method in [4], using centrifugation through a Ficoll—Paque gradient ("Pharmacia"), and after washing with Hanks' medium, they were used as lymphocytes, for those cells accounted for 80-95% of the preparations obtained. To obtain highly purified lymphocytes the fraction of mononuclear leukocytes was centrifuged through fetal bovine serum (FBS) with 5 mM EDTA to remove platelets, after which they were subjected to adhesion on plastic to remove monocytes [7], and then to filtration through a plastic column with nylon wadding to remove any contaminating monocytes and B lymphocytes [6]. Lymphocytes isolated in this way did not proliferate on the addition of concanavalin A (conA), confirming the high degree of purification of the monocytes. The intensity of DNA and RNA synthesis was determined by measuring incorporation of ^{3}H-thymidine (1 μCi/well, 40 mCi/mmole) and ^{3}H-uridine (10 μCi/well, 40 mCi/mmole), during incubation for 2 h with cells in a 96-well panel (Costar) in complete medium RPMI-1640 with 10 mM HEPES and 10% FBS, and in special cases, in Iscov medium without FBS in a CO_2 incubator. ConA was added to the lymphocytes in a concentration of 4 μg/ml. For radiometry the cells were collected on filters and washed to remove acid-soluble products with 5% TCA by means of an automatic cell harvester (Flow Laboratories). The structure of DNA of the intact cells and at various times after addition of conA was studied by the direct fluorometric method using ethidium bromide as described in [3], in the modification [8]. The rate of alkaline denaturation of DNA was estimated as a percentage of DNA remaining in the double-stranded form (dsDNA) after incubation of the cell lysate at 15°C for 1 h after establishment of the denaturing pH of 12.8 for 30 min at 0°C, calculated by the equation $D = [(P - B)/(T - B)] \cdot 100$, where B denotes the background fluorescence of the samples determined after treatment of the lysates with ultrasound and alkali (pH 12.8) for complete denaturation of DNA, T denotes total fluorescence, determined by dsDNA and fluorescent contaminants without treatment with alkali, and P denotes fluorescence determined by DNA fractions remaining in the dsDNA form after alkaline

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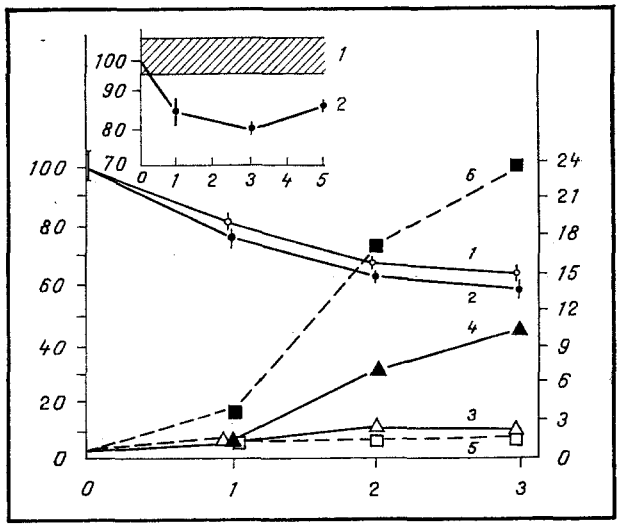

Fig. 1. Changes in secondary structure of DNA (1, 2) and intensity of biosynthesis of DNA (△, 3, 4) and RNA (□, 5, 6) in intact (1, 3, 5) and conA-activated (2, 4, 6) human peripheral blood lymphocytes. Abscissa, time from beginning of incubation, in days; in inset — in hours; ordinate, on left — value of D in % of initial level; right — index of stimulation of ^{3}H-thymidine or ^{3}H-uridine incorporation.

denaturation. This value is determined by the number of single-stranded breaks and of alkali-labile sites, which are converted into breaks during alkaline lysis. Fluorometry was carried out on a "Jasco FP 550" fluorometer at excitation wavelength 520 nm and emission 590 nm. Statistical analysis was carried out by Student's method and correlation analysis on a Hewlett Packard HP 85 microcomputer.

EXPERIMENTAL RESULTS

In preparations of mononuclear leukocytes analyzed immediately after isolation the fraction of dsDNA under standard conditions of lysis was 79.7 ± 4.0%. This value was taken as 100% for analysis of the changes caused by the various procedures.

Transferring the peripheral blood lymphocytes into culture was accompanied by profound changes in the structure of the cellular DNA (Fig. 1, curve 1). The factors provoking this phenomenon could be the mitogenic action of the FBS used for culture or the DR-antigens of the monocytes and B lymphocytes on interaction with the T-cell receptor of the lymphocytes, for during work with mononuclear leukocytes a very weak degree of spontaneous stimulation of DNA biosynthesis often occurs: by 1.5-2 times compared with the initial level (Fig. 1, curve 3). However, neither replacement of the fetal serum by autologous serum nor culture of the cells on serum-free complete Iscov medium, nor purification of the lymphocytes from monocytes and B cells by adhesion on plastic and on nylon wadding (Table 1) had any effect on the character of curve 1. The high degree of purification of the lymphocytes under these circumstances was verified by the absence of stimulation of DNA biosynthesis 72 h after treatment with conA. The stimulus for the change in the structure of DNA of the intact lymphocytes on the transfer of these cells into culture may perhaps have been interaction of the cell membrane with the surface of the culture vessel. Determination of the value of D in the cells of lines cultured for a long time showed that for the K-562 line it is 32% (logarithmic phase — 63% — stationary phase), for the Jurkat line of T-cell lymphoma 40-50%, which constitutes 40-79% of the value of D for freshly isolated intact lymphocytes. This means that 24-48 h after transfer of the lymphocytes into culture the structure of their DNA closely resembled that of DNA of lines transplanted in vitro, and this phenomenon is evidently linked with early stages of the G_0–G_1 transition.

Activation of peripheral blood lymphocytes by conA caused profound changes in the structure of the DNA of these cells in the early period after addition of the mitogen: the decrease in D was 15-25% after 1-3 h compared with intact cells (Fig. 1, inset, curve 2). This phenomenon also was reproduced on addition of the mitogen to lymphocytes 18-24 h after isolation, when spontaneous changes in DNA structure were already taking place in the cells (Fig. 1, curve 1). After 18-72 h

TABLE 1. Secondary Structure of DNA and Intensity of DNA and RNA Synthesis by Mononuclear Leukocytes and by Lymphocytes Purified on Nylon Wadding, after Stimulation with conA (value of D of mononuclears and lymphocytes immediately after isolation taken as 100%, index of stimulation as a conventional unit)

Parameter	Mononuclear leukocytes		Lymphocytes	
Rate of alkaline denaturation				
immed. aft. select.	100±4,6		100±5,9	
1 days later	64,0±5,6		58,4±9,0	
Index of stimulation relative to incorpotion of ^{3}H-thymidine (1) and ^{3}H-uridine (2)	1	2	1	2
immed. aft. select.	1,0±0,2	1,0±0,2	1,0±0,1	1,0±0,1
1 days later	1,6±0,1	1,6±0,1	0,6±0,1	1.0±0,1
3 days later	1,7±0,2	1,6±0,1	0,9±0,1	1,1±0,1
3 days later + conA	6,6±0,3	8,6±0,2	1,1±0,2	5,0±0,1

similar changes were observed in the structure of DNA of both activated and intact lymphocytes. Differences in the value of D amounted to only 5-15% (Fig. 1, curves 1 and 2), whereas in relation to stimulation of RNA biosynthesis (Fig. 1, curves 5 and 6) after 18 h, and RNA and DNA synthesis (Fig. 1, curves 3 and 4) after 48-72 h, the conA-activated lymphocytes exceeded the intact cell levels by 4 times and 26 and 10 times respectively. Similar results were obtained by the use of PHA and staphylococcal enterotoxin A as mitogens. Incidentally, the degree of labilization of the structure of DNA was proportional both to the intensity of DNA biosynthesis, estimated as incorporation of ^{3}H-thymidine, and to the intensity of transcription, estimated as incorporation of ^{3}H-thymidine; however, on regression analysis no significant correlation was found between the intensity of incorporation of ^{3}H-thymidine or ^{3}H-uridine (in cpm) and the rate of alkaline denaturation of DNA (as a percentage of the value of D), and the equations of the regression lines and the coefficient of correlation were $y_1 = 5924 - 56x$ and $y_2 = 146951 - 1362x$, and $r_1 = 11.4$ and $r_2 = 11.4$ respectively. In fact, the most significant changes in the structure of DNA under the influence of the mitogen took place in the first few hours after exposure, when the intensity of incorporation of ^{3}H-thymidine and ^{3}H-uridine was minimal (Fig. 1). The rapid change in the structure of DNA during activation of the lymphocytes was evidently determined by transmembrane transmission of the activating signal into the cell nucleus, and is one of the early stages of activation of the genome.

This investigation thus shows that one of the early events of lymphocytes activation by mitogens is labilization of the secondary structure of DNA, recorded as an increase in the rate of alkaline denaturation of DNA in cell lysates. It has also been shown that culture of intact human peripheral blood lymphocytes in vitro is accompanied by a change in the secondary structure of their DNA and, with respect to the number of alkali-labile connections it closely resembles the structure of DNA of transplantable human cell lines. It is suggested that this phenomenon is linked with early stages of the G_0–G_1 transition.

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