EFFECT OF LYMPHOKINES ON DNA STRUCTURE IN HUMAN LYMPHOCYTES CULTURED IN VITRO: CONNECTION WITH THE cAMP AND OLIGO-A SYSTEM

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Cultivation of human peripheral blood lymphocytes and activation of lymphocytes by mitogens are accompanied by changes in the secondary structure of the DNA of these cells [1]. The formation of single-stranded breaks in DNA has also been found during differentiation of monkey myoblasts [2], of murine erythroleucosis cells [3], of precursor cells of the granulocytic-macrophagal series [4], and of promyelocytic leukemic cell line HL-60 [5]. Leukocyte proliferation induced by antigens and mitogens is known to be preceeded by sequetion of several lymphokines and an increase in the number of their receptors. The aim of the present investigation was accordingly to study the effect of interferons (IFN) and interleukins (IL), and also of compounds which can mediate their action, such as cAMP and 2',5'-oligoadenylate (oligo-A), and the effect of lymphokines on activity of 2',5'-oligo-A-synthetase (OAS) in lymphocytes and on lymphocyte proliferation.

EXPERIMENTAL METHOD

Recombinant IFNα_2 (from the "Ferment" Research-Production Combine, Vilnius) and γ (provided by Corresponding Member of the Academy of Sciences of the USSR E. D. Sverdlov), recombinant IL-2 BRMP (Ministry of the Medical and Biological Industry, USSR), and a purified preparation of human IL-1 (provided by V. A. Simbirtsev) were used. The 2',5'-oligoadenylate trimer (ApApAp) was synthesized by S. N. Mikhailov (Institute of Molecular Biology, Academy of Sciences of the USSR). Dibutyryl-cAMP, ATP ("Sigma") and poly(I)·poly(C) ("Pharmacia") were commercial products. ^{3}H-ATP ("Isotop" or "Amersham") was used after preliminary purification on DEAE-cellulose (DE-52, from "Whatman").

Mononuclear human leukocytes were isolated from fresh heparinized blood by the method in [6], by centrifugation through a Ficoll–Paque density gradient ("Pharmacia") and washed with Hanks' medium. The resulting specimens contained 80-90% of lymphocytes and 10-20% of monocytes. The cells were suspended in complete medium RPMI1640 with 10 mM HEPES and 10% PBS, and incubated in plastic flasks in a CO_2-incubator, with a CO_2 concentration of 5%.

The DNA structure of intact cells and at different times after addition of the test substances was studied by the direct fluorescent method with ethidium bromide as described in [7], and in the modification in [8]. The rate of alkaline denaturation of DNA was determined as the percentage of DNA remaining in the form of double-stranded DNA (dDNA) after incubation of the cell lysate for 1 h at 15°C, and after establishment of the denaturing value (pH 12.8) in the course of 30 min at 0°C, calculated by the equation:

$$D = \frac{P - B}{T - B},$$

where: B denotes background fluorescence of the samples determined after treatment of the lysate with ultrasound and alkali (pH 12.8) for total denaturation of the DNA, T denotes total fluorescence, determined by dDNA and fluorescent impurities without treatment with alkali, and P denotes fluorescence determined by the DNA fractions remaining behind in the form of dDNA after alkaline denaturation, and by fluorescent impurities. This value is

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TABLE 1. Effect of Lymphokines, cAMP, Oligo-A, Actinomycin D, and Cyclohexaimide on Structure of DNA of Lymphocytes (fraction of DNA preserved in the form of DS DNA after incubation of lysates of intact cells at 15°C, pH 12.8, taken as 100%; absolute value of this parameter for control cells at each time of investigation given in parentheses)

Conditions of lymphocyte culture	Rate of alkaline denaturation of DNA, in percent of control		
	3	18	72
Intact	100,0±5,0	100,0±5,6	100,0±7,0
	(79,4±4,0)	(63,0±4,5)	(49,3±5,6)
1000 U/ml IFNα_2	87,2±7,6	124,1±1,5*	126,5±9,8*
500 U/ml IFNγ	88,4±2,9	126,5±6,6*	96,3±0,6
5,0 U/mlIL-1	99,4±4,0	121,1±6,7*	117,9±1,5*
10,0 U/ml IL-1	90,6±1,0	125,1±9,4*	
50,0 U/ml IL-2	86,7±4,3	104,9±3,7	
100 U/ml IL-2	91,4±0,5	107,4±7,9	104,2±2,3
1000 U/ml IFNα_2 + 5 μg/ml conA		105,9±3,4	149,1±2,3*
Actinomycin D			
0,01 μg/ml	73,4±0,6*		
0,05 μg/ml	52,2±2,9*		
0,1 μg/ml	54,5±0,5*	61,6±2,9*	
1,0 μg/ml	58,5±1,0*	22,8±11,6*	
Cycloheximide			
1 μg/ml	83,1±4,7*	75,8±1,7*	
10 μg/ml	83,8±3,5*	63,5±0,7*	
100 μg/ml	93,1±5,0*	21,0±0,1*	
1 μg/ml	117,1±5,8		
10^{-8} μg/ml	119,4±5,6		
10^{-7} μg/ml	118,6±7,8		
1 mM dibutyryl-cAMP	100,0±7,0		
	(46,0±4,3)		
1 1 mM dibutyryl-cAMP	145,2±8,9*		
10^{-8} oligo-A	140,7±0,9*		
10^{-7} oligo-A	155,8±14,8*		

Legend. *p < 0.005) differences from control significant.

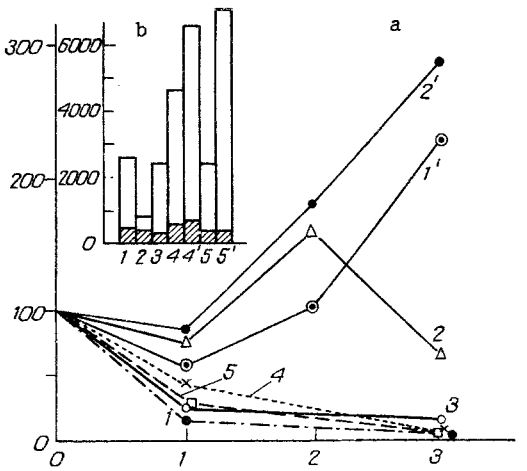

Fig. 1. Effect of lymphokines of OAS activity and on DNA biosynthesis in intact and conA-stimulated human lymphocytes: a) abscissa, incubation time of lymphocytes with lymphokines, indicated by numbers; ordinate, OAS activity (in percent). OAS activity in freshly isolated lymphocytes taken as 100%. b) ordinate, incorporation of ^{3}H-thymidine into DNA of control (shaded columns) and conA-stimulated lymphocytes (unshaded columns) after incubation for 72 h with lymphokines indicated by numbers. 1) Control lymphocytes, 1') conA 5 μg/ml, 2) IFNα_2 1000 U/ml; 2') IFNα_2 1000 U/ml + conA 5 μg/ml; 3) IFNα 500 U/ml; 4) IL-1 5 U/ml; 4') IL-1 10 U/ml; 5) IL-2 50 U/ml; 5') IL-2 100 U/ml.

determined by the number of single-stranded breaks and of alkali-labile sites, which are converted in the course of alkaline lysis into breaks. Fluorometry was carried out on a "Jasco FP_{550}" fluorometer, with excitation wavelength λ = 520 nm and emission wavelength λ = 590 nm.

The intensity of DNA biosynthesis was determined as incorporation of ^{3}H-thymidine (1 uCi/well, 40 mCi/mmole) during incubation for 2 h with cells in a 96-well plate ("Costar") in complete medium 1640 with 10% PBS. Cells for radiometry were harvested on filters and the acid-soluble products were washed off with 5% TCA, using an automatic cell "Harvester" ("Flow Laboratories").

Activity of OAS was determined by the method in [9] after partial purification on poly(I)·poly(C)-agarose. The lymphocytes were lysed for 30 min at 0!C in buffer containing 10 mM HEPES, pH 7.6, 10 mM KCl, 2 mM $(CH_3COO)_2Mg$, 7 mM 2-mercaptoethanol, and 0.5% NP-40, in the ratio of 10^7 cells to 1 ml of buffer. The extract was collected during centrifugation of the lysate for 10 min at 10,000 g in the cold. Next, 100 µl of extract was applied to 100 µl of poly(I)·poly(C)-agarose (AGPOLY), equilibrated in buffer A (10 mM HEPES pH 7.6, 50 mM KCl, 2 mM Mg $(CH_3COO)_2$, 7 mM 2-mercaptoethanol and 20% glycerol), in plastic test tubes, and incubated for 15 min at room temperature, and then for 15 min at 30°C, with occasional mixing. Proteins not bound with the poly(I)·poly(C) were removed by washing 3 times with buffer A. OAS bound with poly(I)·poly(C)-agarose (100 µl) was resuspended in a reaction mixture (final volume 300 l) of the following composition: 20 mM HEPES pH 7.6, 50 mM KCl, 25 mM Mg $(CH_3COO)_2$, 7 mM 2-mercaptoethanol, 5 mM ATP, 10 mM creating phosphate, 0.16 µg/ml of creating kinase, 0.1 mg/ml of poly(I)·poly(C), and 1 µl of ^{3}H-ATP (1 mCi/ml), and incubated for 18 h at 30°C. The reaction was stopped by heating for 5 min at 90°C. The ^{3}H-oligo-A was collected by eluting with 1 ml of 150 mM KCl in buffer A, and after dilution with 2 ml of buffer A without KCl, it was separated from ^{3}H-ATP and its breakdown products on coluns of 0.6 × 1, DE-52, equilibrated in buffer B (10 mM HEPES pH 7.6, 50 mM KCl, 2 mM Mg $(CH_3COO)_2$) during washing of the columns with 20 ml of buffer B and elution of the ^{3}H-oligo-A with 3 ml of 300 mM KCl in buffer B. Radioactivity was determined after addition of ZhS-107 scintillation fluid by radiometry on a liquid-scintillation counter. OAS activity was expressed in nanomoles ^{3}H-AMT incorporated into oligo-A per 10^6 lymphocytes. The results were subjected to statistical analysis by Student's test.

EXPERIMENTAL RESULTS

The study of OAS activity in lysates of human lymphocytes incubated for 3 days in vitro in the presence of conA or various lymphokines (see Fig. 1) revealed a decrease in activity of this enzyme to 14-25% after only 24 h, and to 1-6% of its initial level after 3 days in the control cells (1A). On incubation of the lymphocytes in the presence of IFN_{α_2} (2A) OAS activity after 24 h was reduced only to 80% of the original level, and exceeded it by 50% on the second day, then fell to 50% by the 3rd day, i.e., addition of IFN_{α_2} to the incubation medium significantly increased the OAS activity of the lymphocytes. On incubation of lymphocytes with IFN_{α}, IL-1, and IL-2 for 24 h, higher OAS activity also was observed (3A, 4A, and 5A respectively), but OAS activity by the 3rd day was virtually indistinnguishable from the control values. On incubation of lymphocytes with conA (1'A) OAS activity after 24 h was only 55% lower than the initial values, it rose to the original level by the 2nd day, and significantly (by 2.2 times) exceeded it by the 3rd day. Incubation of lymphocytes with simultaneous addition of IFN_{α_2} and conA led to an even higher level of OAS activity — to 90, 180, and 285% of the initial value ont he 1st, 2nd, and 3rd days respectively (2'A). Under these circumstances lymphokines had virtually no effect on the level of incorporation of ^{3}H-thymidine into DNA of the control lymphocytes, IFN_{α}, IL-1, and IL-2 had a commitogenic action, whereas IFN_{α_2} blocked proliferation of conA-stimulated lymphocytes (Fig. 1b), confirming the familiar properties of these lymphokines. A decrease in OAS activity during culture of intact lymphocytes may be connected with release of this enzyme from the cells into the culture medium, as has already been noted in the literature [10], although the causes of this phenomenon are not clear. The increase in OAS activity in conA-stimulated lymphocytes is evidently determined by its induction by interferons, IL-1, and IL-2, secreted in the process of activation of lymphocytes, since each of these lymphokines, on incubaton for 24 h with intact lymphocytes, causes the strongest and most prolonged activation (or induction) of the enzyme.

The results of the study of the effect of these lymphokines on the structure of DNA of the lymphocytes are given in Table 1.

It follows from these results that IFN_{α_2}, after 18-82 h, IFN_{γ}, after 18 h, and IL-1, after 18 h and also, to a lesser degree after 72 h, reduce the rate of alkaline denaturation of lymphocytic DNA. The ability of secondary messengers, involved in the realization of the biological action of IFN, namely cAMP and oligo-A, to simulate its stabilizing action on the structure of DNA of intact and conA-stimulated lymphocytes, also was demonstrated. We know that the biological action of IFN may be connected with inhibition of transcription and translation. We therefore compared the action of IFN we discovered on the structure of lymphocytic DNA with the effect of inhibitors of RNA and protein biosynthesis, namely antinomycin D and cycloheximide (Table 2). We found that both inhibitors, unlike the action of lymphokines, cAMP, and oligo-A, induced an increase in the number of alkali-labile sites in lymphocytic DNA. These findings suggest the existence of short-living proteins and of their mRNAs, which maintain the structure of DNA in intact human peripheral blood G_0 lymphocytes. The effect of IFN and IL-1 on the structure of DNA, however, is realized not through the inhibition of transcription and translation, but by a more complex system of regulation.

Recently new nonphosphorylated oligoadenylates, whose accumulation correlates with the level of cell proliferation [11], have been found in stimulated lymphocytes and identified [11]. Their function may possible be linked with stabilization of the structure of DNA of proliferating cells.

Thus the preparations of lymphokines which we investigated had a commitogenic (IL-1, IL-2, IFN_{γ}) or an antiproliferative (IFN_{α_2}) action and caused an increase in OAS activity after incubation for 18 h with intact lymphocytes, whereas IFN_{α_2} and conA caused a longer increase of its activity, to 72 h. The same lymphokines stabilized the structure of lymphocytic DNa; this effect, moreover, was simulated by secondary messengers specific for the IFN system, namely cAMP and oligo-A.

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