EFFECT OF ADENOSINE AND ADENINE NUCLEOTIDES ON BLAST TRANSFORMATION OF RAT THYMOCYTES INDUCED BY CONCANAVALIN A

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A severe inherited disease, manifested as a lesion of the immune system and associated with absence or a considerable decrease in intracelluar adenosine deaminase (ADA) activity has recently been found in children. Since one direct result of this enzyme deficiency may be the accumulation of adenosine in the patient's tissues and body fluids, its role in immune function has attracted close attention of research workers [7]. Adenosine has been shown to have a toxic action on lymphocytes and, in particular, to inhibit their proliferation induced by mitogens [6, 8, 10, 11]. The mechanism of this action of adenosine on immune cells is not clear, although several suggestions have been put forward to explain it [3, 12]. According to one of them, the action of adenosine on lymphocytes is effected from outside, through specific plasma membrane receptors connected with the adenylate cyclase complex. According to another suggestion, it acts on intracellular metabolism while accumulating in the lymphocytes.

To understand the mechanism of the role of adenosine in immune cell function, it is important to compare its action on blast transformation of lymphocytes activated by concanavalin A with the action of other adenosine derivatives and, in particular, the adenine nucleotides.

EXPERIMENTAL METHOD

Thymocytes were isolated from thymus glands of Wistar rats (age 1.5-2 months) and cultured at 37°C in a concentration of 10^7 cells/ml in medium RPMI-1640, containing HEPES (20 mM), $NaHCO_3$ (1.5 mg/ml), penicillin (50 units/ml), streptomycin (50 μg/ml), and human group IV serum heated to 56°C (5%). Thymocyte activation was induced by the addition of concanavalin A (from Calbiochem, USA) to the cell culture in a final concentration of 5 μg/ml. Adenine nucleotides and adenosine (from Reanal, Hungary), ADA isolated by the method in [9], and the other test substances were added to the cell suspension simultaneously with concanavalin A. ADA was added to the culture medium in a dose of 20 μg/ml, sufficient to produce deamination of 1 millimole adenosine in 3 min. The blast transformation reaction of the thymocytes was judged after culture for 72 h by measuring incorporation of ^{3}H-thymidine, which was added to the cells 24 h before determination of its incorporation into DNA. Filters with thymocytes precipitated on them with 5% TCA were immersed in vessels containing scintillation fluid ZhS-1 and their radioactivity was measured in an SL-30 counter (from Intertechnique, France).

To study the extracellular conversion of adenosine, AMP, ADP, and ATP the thymocytes were incubated for different times in culture medium in the presence of one of the above-mentioned substances, after which they were sedimented by centrifugation for 3 min at 3000 rpm. Adenosine and the individual adenine nucleotides were isolated from the supernatant after precipitation of the proteins with perchloric acid and neutralization of the solution with KOH, by chromatography on Dowex 1 in the H^+ form [5]. To determine the content of adenosine and adenine nucleotides at the zero point each of them was added to the supernatant obtained by centrifugation of the original thymocyte suspension, after treatment with perchloric acid.

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TABLE 1. Effect of Various Substances on ^{3}H-Thymidine Uptake by Thymocytes Activated by Concanavalin A (M ± m)

Substance	^{3}H-thymidine uptake, % of control	Substance	^{3}H-thymidine uptake, % of control
Control	100,0	ADA (1 mM)	89,0±3,3 n=6
Adenosine (1 mM)	45,0±4,1 n=5	ATP (5 nM)	104,9±9,2 n=3
AMP (1 mM)	51,7±3,2 n=6	ATP (5 μM) + cAMP (10 mM)	98,1±8,0 n=3
AMP (1 mM) + ADA (1 mM)	82,3±5,8 n=4	ATP (5 μM) + cAMP (10 mM)	115,1±6,1 n=3
ADP (1 mM)	47,6±5,1 n=5	PP_i (1 mM)	39,0±4,5 n=4
ADP (1 mM) + ADA (1 mM)	75,0±2,3 n=3	NH_4Cl (1 mM)	97,6±3,6 n=4
ATP (1 mM)	83,6±1,9 n=5	Curantyl (10 μM)	0,5±0,046 n=3
ATP (1 mM) + ADA (1 mM)	72,0±2,8 n=5	Papaverine (200 μM)	5,4±1,2 n=3

Legend. PP_i Pyrophosphate, n) number of experiments.

TABLE 2. Conversion of Extracellular Adenine Nucleotides and Adenosine by Thymocytes in Culture

Incubation time, min	Extracellular substance (1 mM)	Concentration in culture fluid, μM			
		adenosine	AMP	ADP	ATP
0	ATP	1,7	12,7	37,8	837,2
	ADP	2,3	39,5	775,6	8,5
	AMP	4,7	784,1	4,7	0,6
	Adenosine	840,8	7,4	2,2	0,0
10	ATP	1,7	23,3	61,0	715,2
	ADP	3,7	51,0	735,4	13,8
	AMP	5,6	810,2	4,2	0,6
	Adenosine	862,0	3,0	0,4	0,0
20	ATP	2,6	36,7	151,4	627,1
	ADP	3,2	57,0	702,1	8,5
	AMP	5,2	722,0	1,8	0,0
	Adenosine	767,2	2,8	0,4	0,0
40	ATP	1,3	60,1	182,4	465,5
	ADP	3,3	59,0	696,1	19,8
	AMP	6,2	656,8	3,4	0,2
	Adenosine	583,2	1,4	0,0	0,0
80	ATP	—	—	—	—
	ADP	5,8	76,2	690,5	13,3
	AMP	5,6	676,3	3,0	0,0
	Adenosine	620,8	4,7	0,4	0,0

The content of adenine nucleotides and adenosine in the fractions was determined by a fluorescence method, based on interaction between the adenine base and glyoxal hydrate trimer [13], in the following modification. Into 1-ml glass ampuls were introduced 50 µl of 0.1 N HCl, 100 µl of 1 M glyoxal hydrate timer solution (from Merck, West Germany), and 300 µl of glacial acetic acid. The ampuls were then sealed and incubated in boiling water bath for 3 h, after which they were cooled to room temperature, opened, and the fluorescence of their contents was measured on a spectrofluorometer (from Hitachi, Japan) at excitation and emission wavelengths of 328 and 382 nm, respectively. The intensity of fluorescence was determined against standard solutions of adenosine and adenine nucleotides. The corresponding solutions without adenine and its derivatives served as the controls.

EXPERIMENTAL RESULTS

Adenosine was found to induce marked depression of blast transformation of thymocytes stimulated by concanavalin A (Table 1). This action of adenosine was evidently unconnected with the accumulation of ammonium ions, formed from it as a result of the adenosine deaminase reaction, in the culture medium, for ammonium ions do not affect ^{3}H-thymidine uptake by mitogen-stimulated cells.

AMP and ADP had the same inhibitory action on thymocyte proliferation as adenosine, but it was considerably abolished by the addition of ADA to the culture medium (Table 1). Hence it follows that the action of these nucleotides on thymocytes is effected through their extracellular conversion into adenosine. This conversion of adenine nucleotides can take place because of the presence of the corresponding ectoenzymes in the plasma membrane of the lymphocytes [1].

The inhibitory action of ATP on incorporation of ^{3}H-thymidine into concanavalin A-stimulated thymocytes was weaker than that of the above-mentioned nucleotides and adenosine. It would be logical to suggest that ATP exerts its own suppressor action on thymocyte proliferation in the same way as ADP and AMP, after hydrolysis to adenosine. However, exogenous ADA did not abolish the action of ATP on thymocyte proliferation (Table 1). In this connection it can be postulated, for example, that the effect of ATP on blast transformation of thymocytes is due to inorganic pyrophosphate, which may be formed from it through the action of ectopyrophosphatase [1], and which has a stronger inhibitory effect on ^{3}H-thymidine uptake by concanavalin A-stimulated cells than the action of adenosine (Table 1), thereby making the effect of this nucleoside. At the same time, it may be that adenosine is formed from ATP more slowly than from AMP and ADP, and for this reason it does not exhibit its action on thymocytes activated by the mitogen. In this case it may be more effectively removed by ADA, whose activity begins to appear and subsequently increases in the medium during lymphocyte culture *in vitro* [10]. The special effect of ATP compared with that of adenosine and the other adenine nucleotides on blast transformation of lymphocytes activated by different mitogens has also been observed by other workers [6] and requires further study.

Adenine nucleotides added to thymocytes in culture are dephosphorylated, but no adenosine accumulated in the culture medium during the period of investigation. If the concentration of adenosine added to the cells was about 10^{-3}M, the amount which accumulated in culture fluid from adenine nucleotides was small — about 10^{-6}M (Table 2). Since these different concentrations of adenosine were bound to have opposite actions on the adenylate cyclase activity of the cells [2], it is difficult to imagine that inhibition of lymphocyte proliferation by this nucleotide was due to its extracellular influence through adenylate cyclase. It is most likely that adenosine realizes its regulatory action on activated lymphocytes only after its penetration into the cell. This hypothesis is supported by the fact that curantyl and papaverine, which block cellular adenoise transport [3, 4], complete inhibited blast transformation of thymocytes (Table 1).

The discovery of cAMP-dependent ectoprotein-kinases suggests that this cyclic nucleotide may affect cell function from outside, through phosphorylation of surface proteins of the plasma membrane [1]. However, no significant effect of cAMP and ATP when added to the culture medium in sufficient concentrations for the protein kinase reaction to take place on incorporation of ^{3}H-thymidine into stimulated thymocytes could be found (Table 1).

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DEMONSTRATION OF A DIFFERENTIAL ANTIGEN OF ACTIVATED T AND B LYMPHOCYTES (ACA-1) ON TUMOR CELLS

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The view has recently been formed in immunology that differentiation of cells is accompanied by changes in the antigenic properties of their cell surfaces [2, 8]. Antigenic markers of intact T and B lymphocytes and of their subpopulations have now been discovered [1, 6]. After stimulation of lymphocytes by monspecific mitogens or antigens, several workers have found antigenic markers of the stimulated lymphocytes [3, 7, 11, 13, 14]. The writers showed previously that a special ACA-1 antigen (activated cell antigen), which is not found on intact T and B lymphocytes, is present on the surface of activated T and B lymphocytes [2-4, 5].

The aim of this investigation was to demonstrate this antigen on intensively proliferating tumor cells of different histogenetic origin.

EXPERIMENTAL METHOD

Mice of both sexes and of the C57BL/6 $(H\text{-}2)^b$, $C57BL/10_{Sn}$ $(H\text{-}2^b)$, C3H $(H\text{-}2^k)$, A/Sn $(H\text{-}2^a)$, BALB/c $(H\text{-}2^d)$, CBA $(H\text{-}2^k)$, (CBA × C57BL/6) F_1 $(H\text{-}2^{k/b})$ lines, weighing 18-20 g, were obtained from the Stolbovaya Nursery, Academy of Medical Sciences of the USSR.

Ascites forms of tumors Sa-1 $(H\text{-}2^a)$, MCh-11 $(H\text{-}2^b)$, and EL-4 $(H\text{-}2^b)$ were generously provided by B. D. Brondz, and an ascites form of tumor AG-22 by Yu. A. Rovenskii (All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR). Serial passages of the tumors were carried out every 8-13 days by intraperitoneal injections of tumor cells into intact recipients ($5 \cdot 10^6$-$10 \cdot 10^6$ cells per mouse). C3H mice were used for passage of hepatoma AG-22, A/Sn mice for sarcoma Sa-1 and $C57BL/10_{Sn}$ or C57BL/6 mice for T lymphoma EL-4 and tumor MCh-11.

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