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SENSITIVITY OF LYMPHOCYTES TO GLUCOCORTICOIDS

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SUMMARY

Subpopulations of T- and B- lymphocytes may be distinguished by their sensitivity to Dexamethasone (9L-fluoro-16L-methyl-11β, 17, 21-trihydroxypregna-1,4-diene-3,20-dione). The RNA-synthesizing system of lymphoid cells at different stages of differentation displays distinct sensitivity to Dexamethasone. The rate of RNA synthesis in thymocytes of normal organisms is eventually lowered under the action of the hormone. The glucocorticoid effect on RNA biosynthesis in T_1-lymphocytes, however, has been found to be of a biphasic character (stimulation followed by inhibition), whereas the RNA synthesizing system of B- and T_2- lymphocytes proved to be nonresponsive to the hormone under similar conditions. It is concluded that a complete loss of sensitivity of the RNA-synthesizing system to the hormone or a drastic diminution of the capacity to respond to it observed in peripheral blood lymphocytes taken from cancer patients may be due to a shift in the relative proportion of various types of lymphocyte populations in favour of the nonreactive lymphocytes.

INTRODUCTION

It is currently held that immunodepression favours growth and progression of tumors induced both by viruses and chemicals. Steroid hormones are known to play a regulatory role in cell proliferation and differentiation and they are at the same time widely used as drugs. Therefore the study of the mechanisms of the hormone control of the lymphocyte metabolism at various stages of their differentiation is warranted. Immunogenesis and the induction of the immunological response is a complex process involving various cell populations. It includes several stages: migration of stem precursor cells from the bone marrow, their differentiation to T- and B-lymphocytes, cooperation of T- and B-lymphocytes resulting in the formation of plasma cells—producers of antibodies. Humoral immune responses include the interaction between B-cells and T-cell "helpers". Cell-mediated immunity requires at least two subpopulations of T-lymphocytes: T-cell "helpers" (T_1-lymphocytes) and T-Cell "killers" (T_2-lymphocytes) which differ from each other in their localization and sensitivity to corticosteroids [1–4].

We studied the sensitivity to Dexamethasone of the RNA-synthesizing system of mouse thymocytes, T_1- and T_2-lymphocytes, B-lymphocytes as well as that of peripheral blood lymphocytes in donors and in patients with various malignant tumors. In addition an attempt has been made to elucidate the mechanism of action of glucocorticoids on lymphoid cells.

MATERIALS AND METHODS

Animals

CBA-mice and hybrids (CBA × C57B1/6)F_1 were used. Animals were fed normal diet.

B-cells

The spleen of so called "B-mice" was used as a source of the population enriched in B-cells. To prepare "B-mice". $2 \cdot 10^7$ bone marrow cells from mice previously treated with anti-C57B1/6 lymphocyte serum, were administered into the lethally irradiated syngenic recipients. The spleen of "B-mice" presumably does not contain T-cells, though the mice they were obtained from were not subjected to thymectomy. "B-mice" were killed on day 7 after the transplantation of medullar cells [3].

T-cell subpopulations

To obtain T-cell populations the method suggested by Tïgelaar [5] was used. Spleen cells of adult normal mice were administred to the lethally irradiated syngenic animals 24 h later. The spleen of irradiated mice contained mainly T_1-cell population and the lymph nodes—T_2-cell population.

Peripheral blood lymphocytes of healthy persons were separated from the total blood leukocyte pool by sedimentation on a nylon column for 30 minutes in an atmosphere containing 95% air and 5% CO_2 at 37°C. The suspensions studied contained 95–98% of small lymphocytes.

Nuclei were obtained from the lymphoid cells by homogenization (Potter–Elvejhem homogenizer) in a medium containing 0.32 M sucrose, 0.02 m $MgCl_2$ followed by centrifugation at 1000 rev./min for 10 min. Purity of the nuclei was controlled by phase contrast microscopy. DNA was determined by the Burton method [6].

Activity of DNA-dependent RNA-polymerase (E.C.2.7.7.6.) was studied in a cell-free system containing the nuclei with all additions needed including all the four nucleoside triphosphates one of which ([H^3]-UTP) was labelled [7]. DNA-dependent RNA-polymerase of the nuclear extracts was subjected to a partial purification on a 5–20% sucrose gradient [8].

RNA was separated by the method of Georgiev [9]. Samples of nuclear RNA were fractionated on a linear sucrose gradient. RNA containing poly-A sequences were separated using poly-U sepharose columns by the method of Vassart [10]. [^{3}H]-Uridine served as an RNA-precursor. The radioactivity of the labelled RNA preparations was measured in a Nuclear Chicago Mark-2 scintillation counter, effectivity 30%.

Analysis of Dexamethasone receptors of peripheral blood lymphocytes obtained from healthy persons was performed by the method of Gehring [11]. Specific binding by cytosol was assessed by the difference in [^{3}H]-Dexamethasone incorporation in the absence and in the presence of unlabeled steroid. [^{3}H]-Dexamethasone was used in the concentration of 10^{-9}–10^{-8} M. The radioactivity was measured in a Nuclear Chicago Mark-2 scintillation counter, effectivity 30%.

Analysis of the rosette-formation was carried out by the Biozzi method as modified by Pukhalski [12, 13].

RESULTS

It is generally known that steroid hormones inhibit RNA synthesis in animal thymus gland. The most pronounced effect in our studies was achieved at a dose 0.125 mg/25g Dexamethasone per mouse.

DNA-dependent RNA-polymerase activity of the thymocyte nuclei was found to be markedly reduced within 30 min of intraperitoneal administration of Dexamethasone to an animal, and was fully depressed 3 h after exposure to the hormone. In the following studies 0.125 mg Dexamethasone per 25g mouse was injected and the response was examined after 30 min and 3 h—the optimal time to detect the Dexamethasone effect on the cellular RNA-synthesizing system.

In T_1-cells, however, a decrease in the DNA-dependent RNA-polymerase activity of the nuclei and nuclear extracts as well as a reduction in the [^{3}H]-RNA specific radioactivity were observed only after 3 h of exposure to the hormone. Moreover, in contrast to thymocytes, a marked increase in the above parameters was observed.

Under the same conditions the glucocorticoid did not affect RNA-synthesis in mouse B- and T_2-lymphocytes. Table 1 presents the results of an analysis of the incorporation of the pulse label into lymphocyte RNA. Figures 1 and 2 illustrate the sensitivity of Mg^{++}-dependent and Mn^{++}-dependent RNA polymerase. α-amanitin sensitive RNA-polymerase in nuclei and nuclear extracts of thymocytes and T_1-lymphocytes. Data on DNA-dependent RNA-polymerase activity of B- and T_2-lymphocytes are not shown since no changes in enzyme activities after the exposure of the hormone were observed (Table 1).

Thus the RNA-synthesizing system of the mouse lymphocyte populations studied responds differently

Table 1. Effect of Dexamethasone on the incorporation of [^{3}H]-uridine in lymphocyte and thymocyte RNA

Cell		Specific radioactivity (d.p.m/mg RNA × 10^{-3})		
		Nucleus	Cytoplasm	Total
Thymocytes	control	95 ± 7	198 ± 8	293 ± 15
	30 min D	85 ± 4	123 ± 9	208 ± 13
	3 h D	72 ± 11	98 ± 3	170 ± 14
T_1-	control	81 ± 3	217 ± 6	298 ± 9
	30 min D	76 ± 8	544 ± 28	620 ± 36
	3 h D	52 ± 4	183 ± 15	235 ± 19
T_2-	control	81 ± 5	217 ± 26	298 ± 31
	30 min D	112 ± 14	193 ± 7	305 ± 21
	3 h D	82 ± 9	191 ± 15	273 ± 24
B-	control	120 ± 14	90 ± 8	210 ± 22
	30 min D	145 ± 6	89 ± 7	234 ± 13
	3 h D	140 ± 8	90 ± 18	230 ± 26

8 Animals were used for each experimental point; Dexamethasone (D) was injected to mice intraperitoneally. The animals were killed 30 min and 3 h later. [^{3}H]-uridine (15 μCi/25g weight) was administered intraperitoneally 30 min prior to killing. "Control"—the animals which were injected with 0.14 M NaCl.

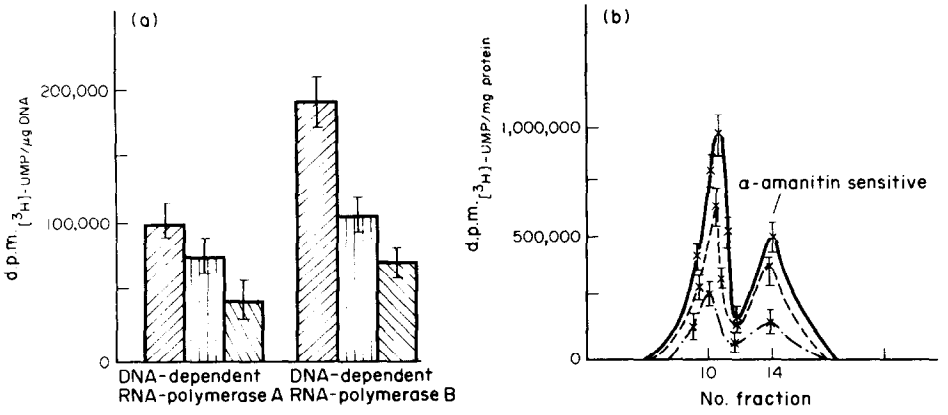

Fig. 1. Activity of DNA-dependent RNA-polymerase of nuclei and nuclear extracts of thymocytes. (a) Activity of DNA-dependent RNA-polymerase of nuclei; (b) Activity of DNA-dependent RNA-polymerase in nucleus extracts. Symbols: —— the animals which were injected with 0.14 M NaCl; --- the animals which were injected with Dexamethasone for 30 min; —·— the animals which were injected with Dexamethasone for 3 h. Dexamethasone at 0.125 mg/25 g of mouse weight was injected intraperitonally. The values represent the average of 4 experiments.

to steroid. The RNA synthesizing system of thymocytes is inhibited, T_2- and B-lymphocytes appear not to be responsive and T_1-lymphocytes are characterized by a biphasic response.

Sensitivity to Dexamethasone of RNA-synthesizing system of peripheral blood lymphocyte in man

In order to find out whether the same features will manifest themselves in humans we studied the action of Dexamethasone on the RNA-synthesizing system of peripheral blood lymphocytes from both healthy persons and patients with various malignant tumors.

T-lymphocytes of normal human peripheral blood responding to PHA by blast-transformation amount to 65–80% of the total lymphocyte population [1].

To obtain a more homogeneous lymphocyte population a specific purification procedure was used (Table 2). The technique described increases significantly the relative content of presumed T-lymphocytes, judging from the results of the specific test-rosette-formation by lymphocytes with heterologous erythrocytes. Thus, lymphocytes prepared by this method appeared to be more homogeneous in terms of cell composition and preserved their immune reactivity since they were able to form rosettes.

Lymphocytes of healthy persons not stimulated with PHA

These lymphocytes, obtained by the above method, were incubated *in vitro* with Dexamethasone for 6 and 20 h in the 199 medium or Henks solution containing 20% autologous serum.

It is known that steroid hormones inhibit metabolism of PHA-transformed cells, in particular the activity of both Mg^{++} and Mn^{++} activated DNA-dependent RNA-polymerase is suppressed by the hor-

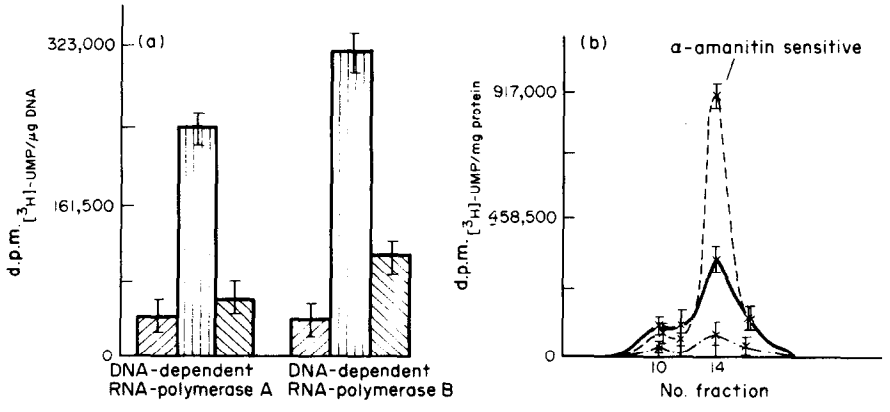

Fig. 2. Activity of DNA-dependent RNA-polymerase of nuclei and nuclear extract of T_1-lymphocytes. (a) Activity of DNA-dependent RNA-polymerase of nuclei; (b) Activity of DNA-dependent RNA-polymerase in nuclear extracts. Symbols: —— animals which were injected with 0.14 M NaCl; --- the animals which were injected with Dexamethasone for 30 min; —·— the animals which were injected with Dexamethasone for 3 h. Dexamethasone at 0.125 mg/25 g of mouse weight was injected intraperitoneally. The values represent the average of 4 experiments.

Table 2. Composition of cell elements in leukocyte pool of the human donors' blood

Cell	Previous to passing through the column (%)	After that (%)
Granulocytes	39	1–3
Monocytes	3	—
Lymphocytes	58	99–97
Erythrocytes	many	—
Thrombocytes	many	—
Rosette-formation	15.4 ± 4.1	77.7 ± 0.4

mone. However, as to the resting cells, conflicting opinions exist. Some authors maintain that these cells are completely insensitive to glucocorticoids even when used in a wide range of concentrations, from 1 to 100 μg/ml [14]. We searched for the optimal conditions to detect the response of RNA-synthesizing systems to the hormone in resting and PHA-stimulated lymphocytes from the human peripheric blood by means of the measuring the activity of this enzyme (activated by Mg^{++} and Mn^{++} ions) in both kinds of cells. We used commercial hormone preparations in concentrations of 1 to 80 μg/ml. A maximal inhibition of both Mg^{++} and Mn^{++} activated DNA-dependent RNA-polymerase by Dexamethasone was observed at the concentration of the latter of 60 μg/ml. A similar measurement of the activity of DNA-dependent RNA-polymerase at various concentrations of Dexamethasone was carried out with resting cells as well. This hormone stimulated the enzyme activity in all the concentrations used (Figs. 3,4).

In the following studies these lymphocytes were incubated *in vitro* with 60 μg/ml Dexamethasone and the response was examined after 6 and 20 h—the optimal time to detect the Dexamethasone effect on the cellular RNA-synthesizing system. The cell number was brought up to $6 \cdot 10^6$ per ml, 5 and 19 h later [^{3}H]-uridin (100 μCi/ml) was added to the culture, and after 45 min of subsequent incubation the nuclear and cytoplasmic RNA was analysed. As seen from Table 3, 6 h after exposure to Dexamethasone a stimulation of RNA synthesis was observed, whereas after a 20 h exposure RNA synthesis was inhibited. These changes are more evident when the rate of cytoplasmatic RNA synthesis is determined using the pulse-label technique. No significant changes in the specific radioactivity of nuclear RNA were observed under similar conditions.

Additional data concerning the character of cytoplasmic RNA synthesis, which was induced 6 h after the hormone injection, were obtained when this RNA was analyzed by sucrose density gradient. Figure 5 shows that the bulk of the RNA is characterized by a sedimentation coefficient of 10–15 *S*. Furthermore, the analysis of this RNA on the poly-U sepharose column shows that the specific activity of RNA

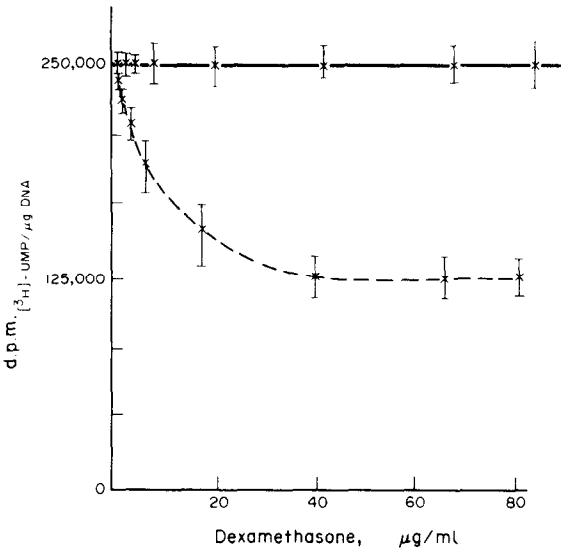

Fig. 3. The activity of DNA-dependent RNA-polymerase B PHA-transformation lymphocytes of peripheral blood of man at various concentrations of Dexamethasone. Symbols: —— lymphocytes treated with PHA and 0.14 M NaCl for 6 h *in vitro*; --- lymphocytes treated with PHA and Dexamethasone for 6 h *in vitro*. The values represent an average of 6 experiments.

enriched in poly-A sequences is highly increased (Table 6).

The above data indicate that the induced RNA belongs to the class of mRNA. A biphasic character of the effect of Dexamethasone on RNA synthesis under conditions described was confirmed by the results represented in Fig. 6a. After 6 h of incubation with Dexamethasone Mn^{++}-dependent RNA-polymerase was activated, whereas after 24 h the RNA-polymerase reaction was found to be inhibited. Simi-

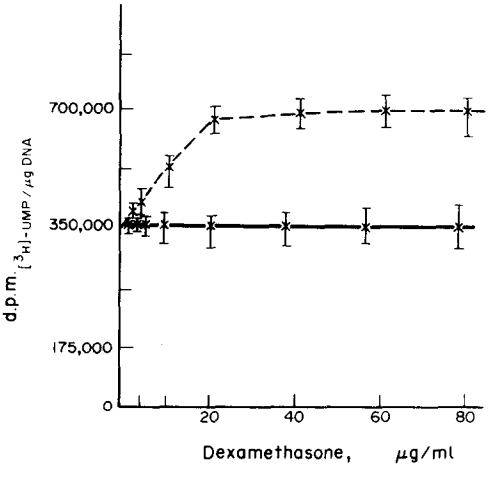

Fig. 4. The activity of DNA-dependent RNA-polymerase B resting lymphocytes of peripheral blood of man at various concentrations of Dexamethasone. Symbols: —— lymphocytes treated with 0.14 M NaCl for 6 h *in vitro*; --- lymphocytes treated with Dexamethasone for 6 h *in vitro*. The values represent an average of 6 experiments.

Table 3. Effect of Dexamethasone on RNA synthesis in lymphocytes of the peripheral donor blood

PHA-stimulation	Dexam. treatment	Specific radioactivity d.p.m./mg RNA × 10^{-3}		
		Nucleus	Cytoplasm	Total
–	none	740 ± 47	23 ± 13	763 ± 60
	6 h	602 ± 22	2305 ± 68	2907 ± 90
	20 h	300 ± 35	10 ± 2	310 ± 37
+	none	7100 ± 83	1300 ± 27	8400 ± 111
	6 h	993 ± 64	710 ± 84	1703 ± 149
	20 h	800 ± 95	500 ± 48	1300 ± 143

Mean values of 6 experiments are given. 300 ml of periphery blood was used.

lar results were obtained with the lymphocyte RNA-polymerase partially purified by sucrose density-gradient centrifugation. Analysis of the distribution of the radioactivity shows that the Mn^{++}-dependent, α-amanitin sensitive fraction of RNA-polymerase is distinctly activated within this time interval of steroid–cell interaction. On the contrary incubation of the steroid with lymphocytes for 24 h evokes an inhibition of the polymerase B responsible for the synthesis of DNA-like RNA (Fig. 6b).

Studies of the effect of Dexamethasone on rosette-formation by T-lymphocytes in the nonpurified leukocytic population from 32 healthy subjects showed that the addition of 60 μg/ml Dexamethasone increased the rosette number as compared to the control (in 10 subjects—2–6 times, in 18–30—70%, in 4—insignificantly). This effect did not depend on the blood group. Differences between experimental and control samples determined by Student's *t*-test were statistically significant ($P < 0.05$).

It is obvious that changes in the rate of *in vitro* RNA synthesis induced by the glucocorticoid in human blood lymphocytes are of the same biphasic character that was revealed in T_1-cells *in vivo*: phase 1—the synthesis of messenger RNA is stimulated by the activation of Mn^{++}-dependent RNA-polymerase; phase 2—the synthesis is suppressed.

Human donor lymphocytes stimulated with PHA

Treatment of peripheral lymphocytes with PHA causes a strong stimulation of RNA synthesis at the very early stages of blast-transformation. Dexamethasone injected in the culture simultaneously with PHA prevents stimulation of RNA-synthesis.

Table 4 illustrates the results of the analysis of cytoplasmic RNA on the poly-U sepharose column. In the hormone-treated lymphocytes a reduced synthesis of the RNA enriched in poly-A sequences is observed. Analysis of nuclear RNA by sucrose density gradient also manifests an inhibitory effect of the steroid, namely a decrease in [^{3}H]-uridine incorporation into total nuclear RNA (Fig. 7).

The effect of Dexamethasone on the activity of DNA-dependent RNA-polymerase in nuclei and nuclear extracts of PHA-stimulated lymphocytes was studied (Fig. 8). The results of these experiments apparently contradict those concerning the products of the reaction. Indeed, some increase in DNA-depen-

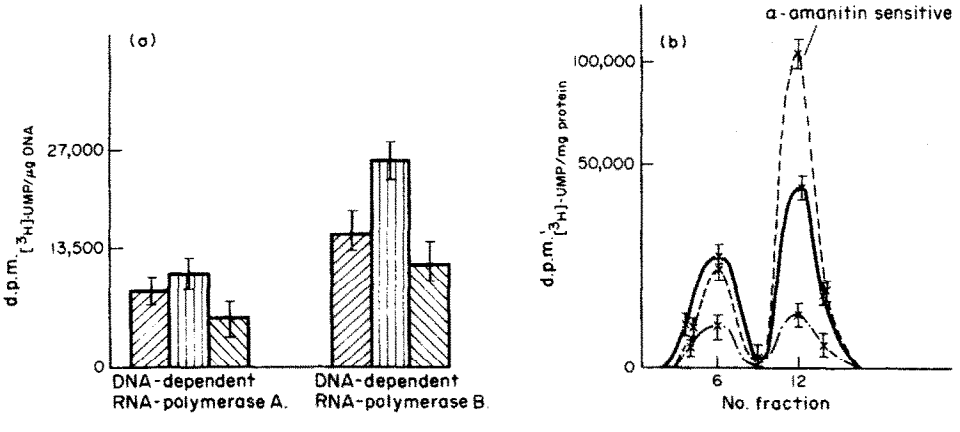

Fig. 5. Activity of DNA-dependent RNA-polymerase of nuclei and nuclear extracts of lymphocytes of peripheral donors blood. (a) Activity of DNA-dependent RNA-polymerase of nuclei; (b) Activity of DNA-dependent RNA-polymerase in nucleus extracts. Symbols: —— lymphocytes treated with 0.14 M NaCl; – – – lymphocytes treated with Dexamethasone for 6 h; —·— lymphocytes treated with Dexamethasone for 20 h. Dexamethasone at 60 μg/ml was added to the lymphocytes culture *in vitro*. Values represent an average of 5 experiments.

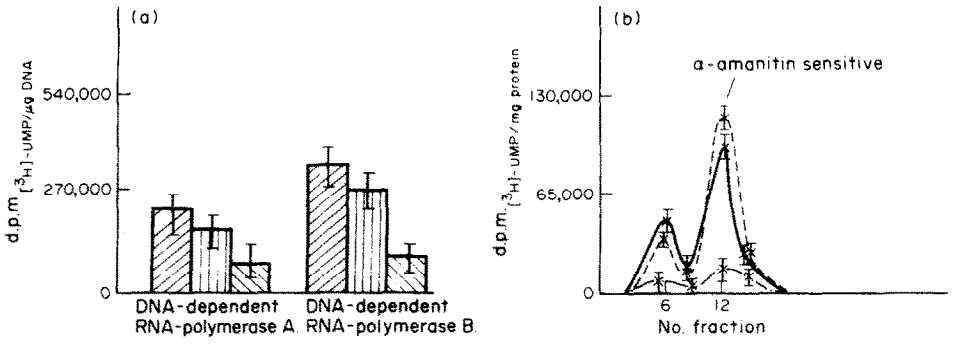

Fig. 6. Activity of DNA-dependent RNA-polymerase of nuclei and nuclear extracts of PHA-treated lymphocytes of the peripheral donor blood. (a) Activity of DNA-dependent RNA-polymerase of nuclei. (b) Activity of DNA-dependent RNA-polymerase of nuclear extracts. Symbols: —— lymphocytes treated with 0.14 M NaCl; --- lymphocytes treated with Dexamethasone for 6 h; —·— lymphocytes treated with Dexamethasone for 20 h. Dexamethasone at 60 μg/ml was added to the lymphocytes culture *in vitro*. Values represent an average of 5 experiments.

dent RNA-polymerase activity was observed immediately after injection of the hormone. It is conceivable that some factor suppressing the enzyme activity could be lost during the procedure of nuclei isolation and purification of the RNA polymerase.

Thus the experimental data presented here indicate that even at the early stages of blast-transformation the inhibitory effect of the steroid on the RNA-synthesizing system stimulated by PHA could be observed. It is likely that this effect is easily detectable in terms of a suppression of the synthesis of mRNA enhanced by the mitogen in the absence of the hormone.

Table 4. Effect of Dexamethasone on poly(A) containing RNA (adsorbed on the poly(U) sepharose column) from the donor lymphocytes stimulated with PHA.

PHA-stimulation	Dexam. treatment	Specific radioactivity (d.p.m./mg RNA × 10^{-3})	
		Nucleus	Cytoplasm
—	none	21 ± 5	130 ± 3
	6 h	79 ± 5	1625 ± 8
+	none	64 ± 7	23 ± 4
	6 h	29 ± 3	14 ± 7

300 ml of donor's blood was used.

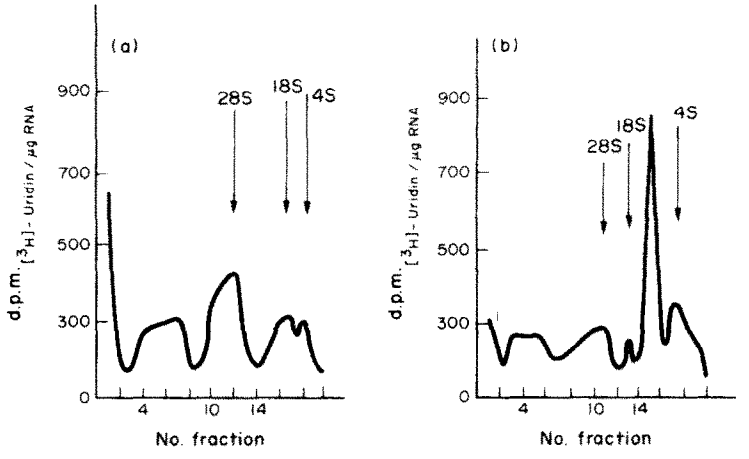

Fig. 7. Sedimentation profiles of nuclear RNA of human blood lymphocytes. Centrifugation of saccharose density gradient 5–20% at 37,000 rev/min for 280 min. Dexamethasone (60 μg/ml) was added at the zero time of incubation. [^{3}H]-Uridin (100 μCi/ml) was administered for 45 min. (a) Lymphocytes treated with 0.14 M NaCl; (b) lymphocytes treated with Dexamethasone for 6 h.

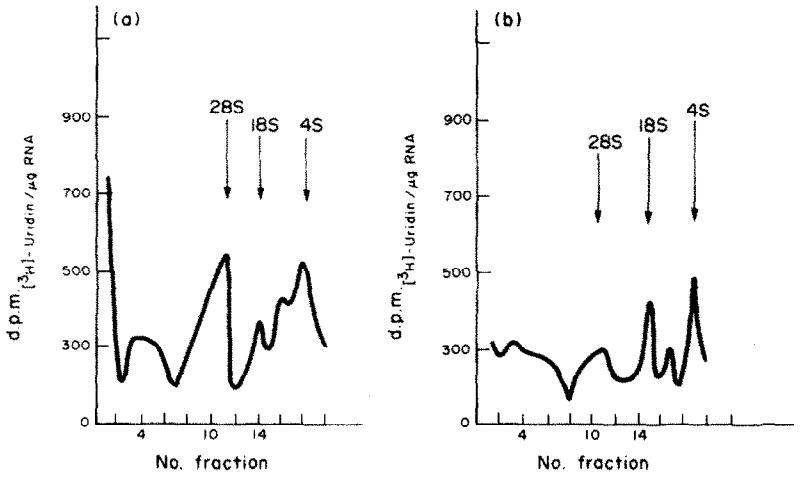

Fig. 8. Sedimentation profiles of nuclear RNA human blood lymphocytes stimulated by PHA. Centrifugation of saccharose density gradient 5–20% at 37000 rev./min for 280 min. Dexamethasone (60 μg/ml) was added at the zero time of incubation. [^{3}H]-uridin (100 μCi/ml) was administered for 45 min. (a) Lymphocytes treated with 0.14 M NaCl; and PHA; (b) Lymphocytes treated with Dexamethasone and PHA.

Table 5. Binding of Dexamethasone by lymphocytes during PHA-induced blast-transformation

	Association ($K_{ass} \times 10^9\ M^{-1}$)	The number of binding sites ($n \times 10^{-13}$ M/mg)
Control	2.66	0.9
Experiment	4.75	2.85

Table 6. Activity of DNA-dependent RNA-polymerase in nuclei of donor lymphocytes and those of patients with melanoma, sarcoma and lung cancer

		Activity of DNA-dependent RNA-polymerase (d.p.m./μg DNA)	
Lymphocytes	*n*	Mg^{++}-dependent	Mn^{++}-dependent
Donors	32	4340 ± 1120	11270 ± 3020
Lung cancer	18	23480 ± 5242	36640 ± 5498
Melanoma	26	25970 ± 1890	20480 ± 8938
Sarcoma	20	29000 ± 5920	80100 ± 6000

n—The number of cancer patients.

Lymphocytes of healthy subjects stimulated with PHA 24 hours before their treatment with the hormone

Hormone treatment of lymphocytes 24 h after PHA was introduced into the system caused no suppression of RNA synthesis (results not shown). Study of the receptor of such lymphocytes showed that in these cells, the number of hormone binding sites increases whereas the association constant remains unchanged (Table 5). It is possible that these lymphocytes correspond to T_2-lymphocytes in terms of their maturity.

Lymphocytes of patients with different malignant neoplasms

No data in the literature concerning the activity of DNA-dependent RNA-polymerase in lymphocytes of patients with melanoma, lung cancer or sarcoma have been available. Lymphocytes of cancer patients are known not to differ from normal ones by their morphology but they do display certain distinct functional features. Amongst these are a reduced susceptibility to the mitogenic action of PHA and a diminished ability to form rosettes with heterologous erythrocytes [15, 16]. RNA-synthesis was studied as an index of the degree of sensitivity of DNA-dependent RNA-polymerase to glucocorticoids (Tables 6). A significant difference was revealed between Mg^{++}- and Mn^{++}-dependent RNA polymerase in lymphocyte nuclei derived from healthy persons and cancer patients. In the latter the RNA-polymerase activities were found to be higher than those in the former by a factor 6 and 2 for melanoma, 5 and 4 for lung cancer and 7–8 times for sarcoma, respectively.

In lymphocytes of patients with sarcoma, melanoma and lung cancer the sensitivity of DNA-dependent RNA-polymerase to PHA and Dexamethasone was shown to be reduced in comparison to that of healthy-person lymphocytes (data not shown).

DISCUSSION

The response of lymphocyte populations to glucocorticoids, irrespective of the final effect, is apparently implemented through the inhibition or biphasic changes in the synthesis of specific RNA [17, 18]. It is widely held that glucocorticoids can affect tissue metabolism only in a "no" or "yes" manner, e.g. inhibit nucleic acid and protein synthesis in lymphocytes and stimulate the above processes in such organs as the liver. However, this point seems uncertain. The data presented above indicate that the first steps in both instances may have a common feature, namely, the activation of RNA-synthesis, whereas the directions of following steps are opposite to each other in the above target-tissues. The second phase of the glucocorticoid effect is more pronounced in lymphoid tissue than in the liver, particularly at the early stages of maturity.

In the RNA-synthesizing system of insensitive lymphocyte populations no significant changes induced by the hormone could be detected. It seems that 20 h of treatment is the critical period that permits one to distinguish sensitive cells from insensitive ones. Fluctuations in the content of glucocorticoid receptors hardly determines the sensitivity of lymphocytes to the hormone, and later steps of the hormone action may be crucial, e.g. the initial level of the DNA-dependent RNA-polymerase activity. Apparently, when the latter is very high the cell loses its sensitivity to the hormone.

The reduced sensitivity of lymphocytes from cancer patients to PHA and Dexamethasone observed by us correlates with an elevated activity of DNA-dependent RNA polymerase in these cells as compared with those of donors. Blinov demonstrated that indeed lymphocytes of patients with chronical lymphoid leukaemia, which are not responsive to PHA, are characterized by a high DNA-dependent RNA-polymerase activity [19]. It is likely that T-cells responsive to glucocorticoids are affected by tumor growth first resulting in an impairment of the cell-mediated immunity.

Thus the experimental data presented here suggest that determination of alterations in the sensitivity of the RNA-synthesizing system of lymphoid tissue to glucocorticoids permits the evaluation of heterogeneity of T-cell population. This analysis, together with the reaction of blast-transformation, may be used for the assessment of the immunological status of the host.

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