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EFFECTS OF GLUCOCORTICOIDS ON NUCLEIC ACID SYNTHESIS IN MOUSE LYMPHOMA CELLS GROWING *IN VITRO* AND ON NUCLEI ISOLATED FROM THESE CELLS*

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Abstract—Mouse lymphoma 388 cells, growing *in vitro*, are inhibited in their growth rate by glucocorticoids. Studies with whole cells incorporating thymidine into DNA, uridine into RNA, and leucine into protein suggested an early (12 hr) depression of thymidine incorporation, with less effect on uridine or leucine. DNA and RNA polymerase activities in these cells were studied more directly by examining a broken cell system. Washed nuclei isolated from ML-388 cells incorporate nucleoside and deoxynucleoside triphosphates into RNA and DNA. Using a washed nuclear system, we have shown that glucocorticoids have no direct effect on either RNA or DNA polymerase activity. Nuclei taken from cells exposed to glucocorticoids for 12 or 24 hr show no consistent differences when compared to control preparations with respect to their ability to synthesize DNA. RNA synthesis is depressed about 30 per cent in nuclei taken from steroid-treated cells.

PREVIOUS investigations have demonstrated that low doses of glucocorticoids inhibit the rate of growth of mouse lymphoma cells, ML-388, growing *in vitro*.[1] Structure–activity relationships showed that the growth inhibition effect parallels the anti-inflammatory potency of various steroids. Similar results have been observed in studies *in vitro* with both thymocytes[2] and fibroblasts.[3] Further studies relating to the mechanism of steroid action in these cells were focused on steroid-mediated inhibition of protein synthesis.[4] With respect to lymphoid cells in general, some recent investigations have supported the contention that glucocorticoids effect a decrease in the rate of incorporation of labeled precursors into DNA,[2, 5–7] while other investigations have placed greater emphasis on the steroid-mediated inhibition of uptake of labeled precursors into RNA[2, 5, 8–10] in this type of cell.

In this communication, we extend the investigations on mouse lymphoma cells growing *in vitro* to the effect of glucocorticoids on the incorporation of nucleic acid precursors into both DNA and RNA by the intact cells and by nuclei isolated from these cells.

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MATERIALS AND METHODS

Cell culture. Mouse lymphoma cells (ML-388) were originally obtained from Robert A. Roosa, The Wistar Institute, Philadelphia, Pa. They are maintained on Eagle's minimal medium (MEM), obtained from General Biochemicals Corp., supplemented with serine (10^{-3} M), pyruvate (10^{-4} M), and 6% calf serum, also obtained from General Biochemicals Corp. Stock cultures were maintained at 37° in Roux bottles containing 70 ml medium, and were subdivided every 3 or 4 days. The cell line is known to be free of mycoplasma, both by routine arginine deiminase assay[11] and by testing for colonial growth on agar plates. (We are indebted to Dr. Leonard Hayflick, Stanford University, for the agar plate testing of these cultures for mycoplasma.)

Chemicals. Actinomycin D was a gift from Merck, Sharp & Dohme Research Laboratory, Rahway, N. J.; fluocinolone acetonide (6α,9α-difluoro-11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione-16,17-acetonide) was a gift from Syntex Laboratories, Palo Alto, Calif. Radioactive uridine and thymidine were purchased from New England Nuclear Corp., Boston, Mass. Radioactive DL-leucine, deoxyadenosine triphosphate and adenosine triphosphate were purchased from Schwarz Bioresearch, Inc., Orangeburg, N.Y., as was unlabeled dCTP, dGTP and TTP. GTP, UTP and CTP were purchased from Calbiochem, Los Angeles, Calif. ATP and dATP were obtained from Sigma Chemical Co., St. Louis, Mo.

Dose–response assays and cell-counting technique. Cells from stock cultures were suspended in fresh growth medium and replicate samples distributed in 15 ml medium in 6-oz bottles as described previously.[1] The steroid (or vehicle) was added on the following day, to cultures containing 1–2 × 10^5 cells per bottle. Steroid was added in 0·15 ml of a dilute ethanol solution such that the final concentration of ethanol never exceeded 0·005%, a concentration that did not produce any effect on cell growth. Experiments were terminated on the fifth day after inoculation by carefully pouring off the spent medium and allowing the bottles to drain for 10–15 min. The cells were suspended in 10 ml saline solution (0·9% NaCl) and 1 ml was diluted with 9 ml saline for counting in the Coulter cell counter. Three replicate bottles were used to determine each value.

Whole cell incorporation studies. Cells were distributed in 6-oz prescription bottles. Steroid (or vehicle) was added on day 2, during the early log phase of growth, when the cell count was 2–4 × 10^5 cells per bottle. At various times thereafter, radioactive precursors were added in 0·1 ml of aqueous solution. Separate bottles received (in triplicate): uridine-2-^{14}C, 16·7 mμmoles; thymidine-methyl-^{3}H, 0·45 mμmole; or DL-leucine-1-^{14}C, 155 mμmoles. One hr later, the medium was carefully poured off, and the adherent cells were gently rinsed with Earle's salt solution at pH 7·2 and allowed to drain. The cells were collected in 10 ml saline solution (0·9% NaCl) and 1 ml of the cell suspension was used for counting in the Coulter counter, while 8 ml was used for the determination of acid-insoluble radioactivity as described previously.[12]

Isolation and purification of nuclei. Cells were grown in Roux bottles for 3 days. While the cells were still in the log phase of growth, steroid or vehicle was added. After 12 or 24 hr, the cells were collected by decanting the medium, gently washing the adherent cells two times with warm Earle's salt solution, and scraping the cells into cold Earle's salt solution. The cell suspension was centrifuged for 10 min at 600 *g* in the cold. All of the following procedures were carried out at 0–4°. The cell pellet was suspended in four times the cell volume of a hypotonic solution of 0·01 M Tris buffer,

pH 7·5, and 0·1 mM EDTA. After 8 min in ice, the suspension was homogenized with 20 strokes of a tight-fitting pestle in a Dounce-type glass homogenizer. After homogenization, hypertonic buffer (1·43 M NaCl, 0·11 M KCl, 0·033 M $MgCl_2$, 0·033 M $CaCl_2$ and 0·11 M Tris, pH 7·5) was added ($\frac{1}{10}$ volume) to bring the broken cell suspension to isotonicity, and the mixture was homogenized once again with 5 strokes of the Dounce homogenizer. In experiments studying incorporation of nucleoside triphosphates in broken whole cell preparations, the hypotonic homogenized cells were brought to isotonicity with $\frac{1}{4}$ volume of a concentrated sucrose solution (0·5 M Tris, pH 7·5; 1·25 M sucrose; 5 mM $CaCl_2$).

The cell suspension was centrifuged at 600 *g* for 10 min and the supernatant decanted. The nuclear pellet was washed twice by suspending in approximately 10 ml of an isotonic sucrose solution (0·25 M sucrose; 0·05 M Tris, pH 7·5; 1 mM $MgCl_2$) and centrifuging at 600 *g*. After the final wash, the nuclei were suspended in the same isotonic sucrose solution adjusted to the appropriate pH for the assay to be performed. The nuclei were diluted such that the concentration of nuclear protein nitrogen of the final washed nuclear suspension was between 100 and 150 μg/ml. Aliquots (0·4 ml) of this suspension were used for enzyme assays and portions were assayed for protein nitrogen. Optical microscopic examination of the nuclear suspension showed apparently intact nuclei with well delineated nucleoli, very little cytoplasmic debris, and no whole cells.

Nuclear assay system. To measure RNA polymerase activity of the isolated nuclei, each incubation tube contained the following substances in a final volume of 1 ml: 0·05 μmole ATP-U-^{3}H (81 mc/m-mole) in 0·05 ml; 1 μmole each of unlabeled GTP, CTP and UTP in 0·05 ml; 0·50 ml of incubation buffer (200 μmoles/ml Tris, pH 8·0; 10 μmoles/ml $MgCl_2$; 40 μmoles/ml NaCl; 140 μmoles/ml KCl; 20 μmoles/ml cysteine). The reaction was started by adding 0·40 ml of nuclear suspension. Further additions were made in a volume of 0·05 ml. The assay mixture was routinely incubated for 10 min in a shaking water bath at 37°, and the reaction was terminated by the addition of 3 ml of an ice-cold solution of 5% TCA containing 0·05 M $Na_4P_2O_7$.

The cold acid-insoluble precipitate was centrifuged at 12,000 *g*, washed once by resuspension in cold TCA containing pyrophosphate, and recentrifuged. The pellet was resuspended in cold TCA and collected by filtration with mild suction onto 2·4 cm Whatman glass filter paper discs. The discs were washed with 3 ml of the cold 5% TCA solution containing pyrophosphate, and once with 3 ml of cold water. The glass paper discs were then dried and placed in a liquid scintillation counting vial. Ten ml of scintillator solution (4 g 2,5-diphenyloxazole and 0·1 g 2,2-*p*-phenylenebis-(5-phenyloxazole) per liter of toluene) was added and the radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer.

The radioactivity obtained for each sample was corrected for adsorbed radioactivity by subtracting the radioactivity present in identical samples, which were precipitated with TCA immediately after addition of the nuclear suspension (zero time). These values, as well as radioactivity obtained on incubation for 10 min at 0°, were less than 10 per cent of the values obtained in the complete system incubated for 10 min at 37°.

To measure DNA polymerase activity, each incubation tube contained the following substances in a final volume of 1 ml: 0·01 μmole dATP-U-^{3}H (1 c/m-mole) in 0·05 ml; 0·5 μmole each of dGTP, dCTP and TTP in 0·05 ml; 0·50 ml of incubation buffer,

same composition as in RNA system but at pH 7·5; and 0·4 ml of nuclear suspension. The usual incubation time was 120 min, but in some experiments, using Mn^{2+} instead of Mg^{2+}, 60 min of incubation was used.

Collection of the acid-insoluble material, correction for zero time incorporation (also less than 10 per cent of control incorporation), and determination of radioactivity were performed as already described for the RNA polymerase assay.

Chemical assays. Protein determinations were carried out using the method of Oyama and Eagle.[13]

RESULTS

Effect of glucocorticoids on growth of ML-388 cells. These cells were originally shown to be sensitive to glucocorticoids by Gabourel and Aronow in 1962,[1] and their characteristic response has not substantially changed since that time. Depending somewhat on the size of the initial cell inoculum (the fewer the cells, the greater the sensitivity to glucocorticoids), a cortisol concentration of 10^{-7} M suffices to inhibit cellular growth by 50 per cent over a 4- or 5-day growth period, while concentrations of 10^{-9} to 10^{-10} M fluocinolone acetonide can inhibit the cells to the same degree.

Influence of fluocinolone acetonide upon the rate of incorporation of DNA, RNA and protein precursors. The rate of synthesis of RNA, DNA and protein was examined in these cells by studying the incorporation of appropriate precursors over a 1-hr incubation period (all precursors are incorporated linearly over this time) at various times after the addition of 5×10^{-7} M fluocinolone acetonide to cultures in the early log phase of growth. This concentration of fluocinolone acetonide reliably reduced the cell count of treated cultures by 50 per cent in comparison to control cultures within 48 hr.

The extent of incorporation of radioactive leucine, uridine and thymidine over a 1-hr period at 6, 12 and 24 hr after addition of fluocinolone acetonide is shown in Fig. 1. These precursors are known to be incorporated into protein, RNA and DNA, respectively, and we confirmed this by using the same procedures described by Pratt *et al.*[14] in a study utilizing rat thymocytes.

There was no marked steroid effect at 6 hr, but at 12 hr thymidine incorporation was inhibited (Fig. 1). By 24 hr, the incorporation of all three precursors was depressed. The cell count data showed little difference between treated and control cultures until 48 hr, although in other experiments a clear depression of cell count may be seen as early as 24 hr. A number of experiments of the same design were carried out. Calculations of incorporation per 10^5 cells in the treated and control groups were obtained at various times after steroid addition and the results expressed as per cent of control incorporation (Table 1). There is a substantial and significant depression of thymidine incorporation into DNA 12 hr after steroid addition, a small depression of leucine incorporation, and little or no effect on uridine incorporation into RNA. By 24 hr, the per cell incorporation of all three precursors was substantially less than that of control cells in log phase of growth, although in a quantitative sense the depression of thymidine incorporation seemed to be the parameter most affected.

Since data of the type shown in Fig. 1 and Table 1 can be markedly affected by changes in pool sizes and specific activities of the phosphorylated nucleoside derivatives within the cell, experiments were performed using thymidine and uridine at high and at low specific activity. Such experiments are capable of indirectly detecting

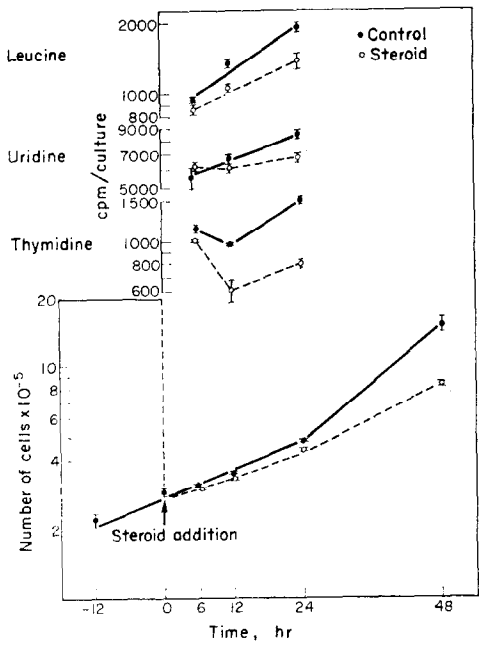

FIG. 1. Effect of fluocinolone acetonide, 5×10^{-7} M, on the incorporation of radioactive thymidine, uridine and leucine by mouse lymphoma cells. Fluocinolone acetonide was added during the logarithmic phase of growth to replicate cultures of ML-388 cells. At various intervals thereafter, 1-hr incubations were carried out with one of the following additions: 0·45 mμmole thymidine-methyl-^{3}H (6·7 c/m-mole), 16·7 mμmoles uridine-2-^{14}C (30 mc/m-mole), or 155 mμmoles DL-leucine-1-^{14}C (31 mc/m-mole). At the end of each incubation the cells were counted, and the cold acid-insoluble material was collected and assayed for radioactivity as described in the text. Each of the values presented in the chart represents the mean and standard errors of 3 replicate cultures.

TABLE 1. EFFECTS OF FLUOCINOLONE ACETONIDE ON CELL GROWTH AND PRECURSOR INCORPORATION IN MOUSE LYMPHOMA CELLS*

Time after steroid addition (hr)	Cell count	Thymidine incorporation	Uridine incorporation	Leucine incorporation
3 (2)†	105 ± 1	108 ± 11	90 ± 6	96 ± 8
6 (5)	96 ± 3	107 ± 7	100 ± 3	98 ± 7
12 (8)	94 ± 4	69 ± 3‡	96 ± 5	85 ± 4‡
24 (7)	84 ± 6§	68 ± 5§	84 ± 5§	82 ± 5‡
48 (9)	58 ± 6‡			

* Fluocinolone acetonide, 5×10^{-7} M, was added to replicate cultures during logarithmic phase of growth. At various intervals thereafter, 1-hr incubations were carried out in triplicate cultures with radioactive labeled thymidine, uridine or leucine as described in the legend of Fig. 1. Calculations of counts per minute of precursor incorporated per 10^{5} cells in the treated and control groups were obtained, and the results (at each time point) are expressed as per cent of control incorporation ± the standard error of the per cent.

† The number in parentheses represents the number of experiments at each time point.

‡ Significantly different from control at $P < 0.01$.

§ Significantly different from control at $P < 0.05$.

TABLE 2. EFFECT OF FLUOCINOLONE ACETONIDE ON INCORPORATION OF NUCLEOSIDES INTO MACROMOLECULAR MATERIAL UNDER CONDITIONS OF HIGH AND LOW PRECURSOR SPECIFIC RADIOACTIVITY*

Exp.	Specific radioactivity of precursor	Thymidine incorporation (cpm/10^5 cells) Control	+Steroid	Ratio of steroid to control	Uridine incorporation (cpm/10^5 cells) Control	+Steroid	Ratio of steroid to control	Cell count (cells/culture × 10^{-5}) Control	+Steroid	Ratio of steroid to control
A	High	1080 ± 74	289 ± 15	0·27†	1747 ± 16	1185 ± 118	0·68†	5·9 ± 0·35	3·4 ± 0·29	0·57†
	Low	323 ± 42	154 ± 10	0·48†	442 ± 37	159 ± 36	0·36†			
B	High	1052 ± 219	590 ± 194	0·56†	2256 ± 605	1452 ± 27	0·64‡	10·4 ± 0·54	6·0 ± 0·20	0·58†
	Low	258 ± 55	160 ± 24	0·62†	247 ± 9	187 ± 8	0·76†			
C	High	1049 ± 195	428 ± 71	0·41†	1953 ± 295	1531 ± 151	0·78‡	10·6 ± 1·4	5·6 ± 0·39	0·53†
	Low	329 ± 82	139 ± 13	0·42†	270 ± 36	162 ± 27	0·60†			

* Lymphoma cells in the logarithmic phase of growth were incubated for 24 hr with fluocinolone acetonide (5×10^{-7} M). After 24 hr, the medium (containing steroid or vehicle) was changed and the replicate 15-ml cultures were incubated for an additional hour with one of the following: 0·45 mμmole thymidine-methyl-^{3}H (6700 mc/m-mole; high specific activity); 22·5 mμmoles thymidine-methyl-^{3}H (134 mc/m-mole; low specific activity); 9 mμmoles uridine-2-^{14}C (55 mc/m-mole; high specific activity); or 450 mμmoles uridine-2-^{14}C (1 mc/m-mole; low specific activity). The acid-insoluble material was collected at the end of the 1-hr incubation and radioactivity determined as described in Methods. Note that the amount of radioactivity per culture is kept constant in high and low specific activity incubations. The figures in the table represent the means and standard errors of three replicate cultures.

† Significantly different from control at $P < 0.05$.

‡ Not significantly different from control.

changes in precursor pool sizes.[14–16] The results (Table 2) showed that dilution of the specific activity of the precursor thymidine or uridine by a factor of 50 reduced the incorporation of labeled material by about 75 per cent.

There is considerable variability in the degree of steroid-induced inhibition seen under conditions of high and low specific activity (Table 2), but in most cases there was a significant inhibition of incorporation seen under both conditions. The average inhibition of thymidine-^{3}H incorporation in the three experiments was 59 $\pm$ 8 per cent under conditions of high specific activity and 49 $\pm$ 6 per cent under conditions of low specific activity. These two values could not be shown to be significantly different by statistical analysis. With respect to uridine-^{14}C, the average inhibition in the three experiments was 30 $\pm$ 4 per cent under conditions of high specific activity and 43 $\pm$ 12 per cent under conditions of low specific activity. Once again, the degree of inhibition observed under these two conditions could not be shown to be significantly different. It should be noted that there was a somewhat greater inhibition of both cell growth and thymidine and uridine uptake in these experiments than in those presented in Table 1.

The nuclear DNA and RNA synthesizing system. A more direct assay of cellular RNA and DNA polymerase activity was sought by measuring the rate of incorporation of radioactive nucleoside triphosphates into acid-insoluble material by intact nuclei isolated from ML-388 cells. Nuclei were obtained by swelling cells in a hypotonic medium, homogenizing, and washing the nuclei in several sucrose centrifugations as described in Materials and Methods. The final suspension of washed nuclei was found to be active in incorporating ribonucleoside triphosphates and deoxyribonucleoside triphosphates into cold acid-insoluble material. Some of the characteristics of the two systems are presented in Fig. 2. RNA synthesis is linear for 10 min, is inhibited by actinomycin D, has a pH optimum at about 8·0, and has a divalent cation requirement which is satisfied by either Mn^{2+} (at 2–3 mM) or Mg^{2+} (at about 5 mM). Mn^{2+} is slightly better than Mg^{2+}, and Ca^{2+} will not satisfy the divalent ion requirement. The reaction is linear with respect to the amount of nuclear protein up to about 60 μg protein nitrogen.

In experiments not presented, it was found that the product of incorporation of ATP-^{3}H was solubilized by digestion with RNase but was resistant to DNase. It was hydrolyzed by alkali or hot acid treatment. Radioactive adenosine (in place of ATP-^{3}H) was not incorporated and nonradioactive adenosine did not depress the incorporation of ATP-^{3}H. Adenosine monophosphate and adenosine diphosphate were incorporated, presumably by being converted in part to the triphosphate. Incorporation was greatest with ATP-^{3}H. The addition of purified DNA does not stimulate the incorporation of ATP-^{3}H.

dATP incorporation in these washed nuclei remains linear for at least 2 hr, is also sensitive to inhibition by actinomycin D, and has a pH optimum at about 7·5. The divalent cation requirements are similar to those required for ATP incorporation, save that Mn^{2+} at 1–2 mM is substantially better than Mg^{2+} at its optimal concentration of 3–5 mM. The reaction remains linear with respect to nuclear protein up to 80 μg protein nitrogen.

In experiments not shown, it was found that the product of the DNA reaction was sensitive to digestion by DNase and resistant to RNase. The product was resistant to alkaline digestion but was hydrolyzed by hot acid. Radioactive deoxyadenosine (in

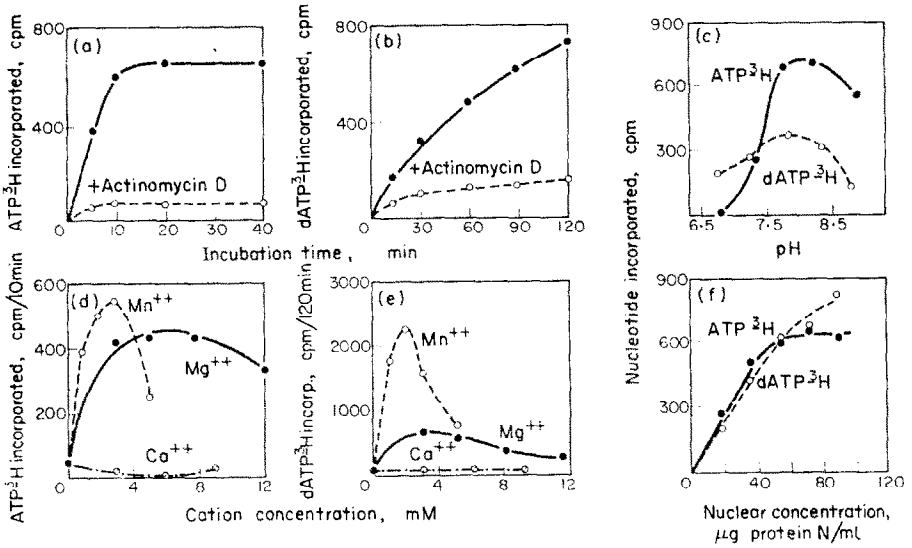

FIG. 2. Characteristics of DNA and RNA synthesis in suspensions of washed nuclei from mouse lymphoma cells. Nuclei were isolated as described in Materials and Methods. (a) Replicate 1-ml nuclear suspensions containing 39 μg nuclear protein nitrogen were incubated for varying times with ATP-^{3}H; 20 μl ethanol (-●-●-) or 20 μl ethanol containing 20 μg actinomycin D (-○- -○-) was added to each incubation. (b) Nuclear suspensions, each containing 51 μg nuclear protein nitrogen, were incubated with dATP-^{3}H for varying times in the presence (-○-○-) or absence (-●-●-) of actinomycin D, 20 μg/ml. (c) The pH of replicate aliquots of the final nuclear suspension (containing 65 μg protein nitrogen), as well as the incubation buffer for each assay, was adjusted to the appropriate pH value and incubations were carried out with ATP-^{3}H (-●-●-) for 10 min or with dATP-^{3}H (-○- -○-) for 120 min; the final pH values indicated on the abscissa were measured in each incubation with a glass electrode midway during the incubation. (d) Nuclei were suspended after the final wash in 0·25 M sucrose, 0·05 M Tris without $MgCl_2$; 0·05 ml of aqueous divalent salt solution was added to 1·0 ml of the usual incubation mixture without $MgCl_2$. The final incubation vessels (containing 63 μg nuclear protein nitrogen) were incubated with ATP-^{3}H for 10 min. $MgCl_2$, -●-●-; $MnCl_2$, -○- -○-; $CaCl_2$, -⊘-⊘-. (e) Incubations containing 63 μg nuclear protein nitrogen were prepared as described for (d) and dATP-^{3}H was incorporated for 120 min. (f) Varying amounts of nuclei were incubated with ATP-^{3}H for 10 min (-●-●-) or with dATP-^{3}H for 120 min (-○- -○-).

place of dATP-^{3}H) was not incorporated and nonradioactive deoxyadenosine did not depress the incorporation of dATP-^{3}H.

The ribonucleotide requirement for RNA synthesis (Table 3) revealed that omission of one or more of the nucleoside triphosphates reduced the incorporation of ATP-^{3}H substantially. The relatively good incorporation seen in the absence of CTP may be due to the presence of cytidine nucleotides contaminating one of the other nucleoside triphosphates, probably UTP. Unlabeled deoxynucleoside triphosphates are not able to replace the ribonucleoside triphosphates in this system.

Similar experiments for DNA synthesis (Table 4) showed that omission of any one deoxynucleoside triphosphate reduced incorporation of dATP-^{3}H by only approximately 50 per cent in either an Mg^{2+}-stimulated system or an Mn^{2+}-stimulated system. Omission of a second triphosphate reduced the incorporation to about 30–40 per cent of the complete system, while omission of all three complementary deoxy-

TABLE 3. THE REQUIREMENT FOR RIBONUCLEOSIDE TRIPHOSPHATES IN RNA SYNTHESIS BY ML-388 NUCLEI*

Components	Incorporation of ATP-^{3}H	
	(cpm)	(% of incorporation in complete system)
Complete system	696	100
Minus CTP	357	51
Minus GTP	174	25
Minus UTP	77	11
Minus CTP, GTP	167	24
Minus CTP, UTP	70	10
Minus GTP, UTP	97	14
Minus CTP, GTP, UTP	74	11

* Nuclear suspensions containing 64 μg protein nitrogen were incubated for 10 min as described in Materials and Methods, except as noted in the table. In the case of the omission of one or two unlabeled nucleoside triphosphates, the amounts of the remaining unlabeled nucleoside triphosphates were adjusted such that the total amount of unlabeled nucleoside triphosphate was the same as in the complete system.

TABLE 4. THE REQUIREMENT FOR DEOXYRIBONUCLEOSIDE TRIPHOSPHATES IN DNA SYNTHESIS BY ML-388 NUCLEI*

Components	Incorporation of dATP-^{3}H in the presence of Mn^{2+}†		Incorporation of dATP-^{3}H in the presence of Mg^{2+}‡	
	(cpm)	(% of incorporation in complete system)	(cpm)	(% of incorporation in complete system)
Complete system	2260	100	732	100
Minus dCTP	1136	50	363	50
Minus dGTP	1030	46	451	62
Minus TTP	832	37	394	54
Minus dCTP, dGTP	631	28	286	39
Minus dCTP, TTP	659	29	356	49
Minus dGTP, TTP	536	27	301	41
Minus dCTP, dGTP, TTP	143	6	57	8

* The incubation conditions are as described under Materials and Methods except as noted in the table. In the case of the omission of one or two unlabeled nucleoside triphosphates, the amounts of the remaining unlabeled nucleoside triphosphates were adjusted such that the total amount of unlabeled nucleoside triphosphate was the same as in the complete system.
† The nuclei (64 μg protein nitrogen) were incubated for 60 min.
‡ The nuclei (65 μg protein nitrogen) were incubated for 120 min.

nucleoside triphosphates reduced incorporation to less than 10 per cent of that seen in the complete system. Nucleoside triphosphates (not shown) could not replace the deoxynucleoside triphosphates in this system.

Effect of fluocinolone acetonide treatment of the intact cell on the ability of nuclear preparations to incorporate ATP-^{3}H and dATP-^{3}H. Nuclei were isolated from cells that had been treated with fluocinolone acetonide (5×10^{-7} M) for 24 hr and were then compared to control nuclei in their ability to incorporate ATP-^{3}H and dATP-^{3}H into cold acid-insoluble material. A typical experiment is shown in Table 5, in which both an Mg^{2+}-stimulated and an Mn^{2+}-stimulated nuclear system were studied. In

TABLE 5. DNA AND RNA SYNTHESIS IN NUCLEI PREPARED FROM LYMPHOMA CELLS*

Labeled precursor	Nuclear incorporation in the presence of Mg^{2+}			Nuclear incorporation in the presence of Mn^{2+}			Broken cell suspension incorporation in the presence of Mn^{2+}		
	Control	+Steroid	Ratio steroid to control	Control	+Steroid	Ratio steroid to control	Control	+Steroid	Ratio steroid to control
dATP-^{3}H	13·63	14·61	1·08	57·12	55·25	0·97	41·65	39·75	0·95
ATP-^{3}H	10·24	8·05	0·79	9·38	6·88	0·73	7·59	5·96	0·79

* The lymphoma cells had been grown for 24 hr in the presence of 5×10^{-7} M fluocinolone acetonide. Control and steroid-treated cells were collected and nuclei were prepared as described under Materials and Methods. The final nuclear preparations were suspended in the sucrose buffer at pH 7·5. Incubations were carried out with $MgCl_2$ for 120 min or with $MnCl_2$ for 60 min. Broken cell incubations were carried out for 60 min in the case of dATP-^{3}H and for 10 min with ATP-^{3}H. The same final suspension of nuclei was used for both DNA and RNA synthesis in each group. The results are expressed as cpm of precursor incorporated per μg of nuclear or broken cell protein nitrogen.

addition, incorporation of the nucleoside triphosphates in the cruder broken cell homogenate was examined. In this experiment, nuclei obtained from cells exposed to fluocinolone acetonide for 24 hr showed DNA polymerase activity not detectably different from that of control cells, while RNA polymerase activity was depressed approximately 20 per cent.

A total of seven experiments were carried out with nuclei prepared from lymphoma cells after 24 hr of exposure to fluocinolone acetonide (5×10^{-7} M). Effects on DNA synthesis were small and not constant, with a mean dATP-^{3}H incorporation of 15·3 cpm/μg nuclear protein nitrogen in nuclei isolated from vehicle-treated cells and 14·6 cpm/μg nuclear protein nitrogen in nuclei prepared from steroid-treated cells. RNA synthesis, however, was consistently depressed, with a mean incorporation of ATP-^{3}H of 8·7 cpm/μg nuclear protein nitrogen in nuclei from control cells and 6·6 cpm/μg nuclear protein nitrogen in nuclei from treated cells.

Fluocinolone acetonide, at either 5×10^{-7} M or 5×10^{-5} M, had no appreciable effect on either RNA or DNA synthesis in a Mg^{2+}- or Mn^{2+}-stimulated system when added directly to a washed nuclear incubation system (Table 6).

TABLE 6. EFFECT OF DIRECT ADDITION OF FLUOCINOLONE ACETONIDE TO NUCLEAR SUSPENSIONS PREPARED FROM LYMPHOMA CELLS*

Labeled precursor	Concn of fluocinolone acetonide (M)	Incorporation in presence of Mg^{2+}†	Ratio of treated to control	Incorporation in presence of Mn^{2+}‡	Ratio of treated to control
dATP-^{3}H	0	14·7		26·7	
	5×10^{-7}	13·6	0·93	26·1	0·98
	5×10^{-5}	12·4	0·84	23·9	0·90
ATP-^{3}H	0	11·7		10·1	
	5×10^{-7}	10·8	0·92	10·3	1·02
	5×10^{-5}	10·6	0·90	10·8	1·07

* Nuclear suspensions were prepared and incubated as described under Materials and Methods, except that a single final nuclear suspension in sucrose solution, pH 7·5, was made and replicate suspensions were delivered to each incubation vessel. The steroid was added in 50 μl of 10% ethanol and the control incubations received the appropriate amount of vehicle. The results are expressed as cpm per μg of nuclear protein nitrogen.

† Incubations were carried out in 5 mM $MgCl_2$ for 120 min in the case of dATP-^{3}H and for 10 min for ATP-^{3}H.

‡ dATP-^{3}H incubations were carried out for 60 min in 2 mM $MgCl_2$ and incubations with ATP-^{3}H were carried out for 10 min in 3 mM $MgCl_2$.

DISCUSSION

Gabourel and Comstock[4] studied precursor incorporation in ML-388 cells and found approximately equal depressions in incorporation of protein, RNA and DNA precursors occurring as early as 6 hr after steroid addition. Our results are somewhat different and, in the studies described here, these cells demonstrate substantially the same reaction to glucocorticoids in terms of the inhibition of DNA, RNA and protein precursor uptake as has been demonstrated in mouse fibroblasts growing *in vitro*[12] and in lymphosarcoma P1798.[6] The ML-388 cell system is, however, much more variable, both in terms of the rate of cell growth and the degree of steroid response, than are L-929 fibroblasts. The inhibitions of precursor uptake in the whole cell which are observed here could be interpreted to indicate that there was a steroid-mediated inhibition in the rate of DNA, RNA and protein synthesis in these cells. The same

results, however, would have been observed if the effect of the steroid were simply to increase the pool size of nonradioactive precursors or, in the case of the nucleosides, their phosphorylated derivatives. Several laboratories have reported that there are increases in RNase activity in lymphosarcoma cells[17, 18] and of both RNase[19, 20] and DNase[19, 21] activity in thymocytes after treatment with glucocorticoids. Such an increase in degradative enzyme activity might be expected to result in an expansion of the pool sizes of nonradioactive nucleotides. When the precursor pool size was expanded 50-fold in experiments with ML-388 cells (Table 2), there was no substantial change in the steroid effect. We may conclude that large changes in the nucleotide pools are probably not occurring and the observed inhibition of precursor uptake by prior treatment with fluocinolone acetonide cannot be accounted for by alterations in these pool sizes. When the same type of experiment was carried out on thymus cell suspensions[14] or thymus gland slices,[15] the steroid-mediated inhibition of deoxynucleoside incorporation at high precursor specific activity was found to be obliterated when the precursor was diluted with large amounts of the nonradioactive compound.

In order to study in more detail the steroid-mediated inhibition of labeled nucleoside uptake into DNA and RNA, it was felt desirable to have a system in which the direct incorporation of nucleoside triphosphates into DNA and RNA could be studied. A nuclear system circumvents some of the difficulties of interpretation attendant to labeled nucleoside uptake by the whole cell. Difficulties such as transfer of the precursor from the medium to the cell, phosphorylation to the triphosphate, and dilution of the precursor pools by products of cellular metabolism may be avoided.

Using the isolated washed nuclear system from ML-388 cells, we have shown, first, that potent glucocorticoids have no detectable direct effect on the rate of synthesis of either RNA or DNA (Table 6). Second, nuclei taken from cells exposed to glucocorticoids for 12–24 hr do not show any pronounced change with respect to their ability to polymerize DNA precursors (Table 5). Finally, these nuclei do show a depressed rate of synthesis of RNA (Table 5), a finding in agreement with the work of Fox and Gabourel[8] and Nakagawa and White[10] in studies with thymocytes.

The lack of any depression of dATP incorporation in nuclei isolated from steroid-treated cells implies that the biochemical locus of inhibition of incorporation of thymidine observed in whole cells is not simply an inhibition of DNA polymerase activity or content, or a change in the template activity of the DNA in the chromatin complex. There may be alterations in the rate of transport of precursor into the cell or depression in the endogenous synthesis of four complementary deoxyribonucleoside triphosphates required for DNA synthesis. Another possibility is that some other enzymatic function important in DNA replication, but not measured in our nuclear incorporation assay, may be affected. The activity of endonucleases, ligase and possibly other components of the replication process in the nuclear preparation are not at present known. Depressions in these functions may account for the observed inhibition of thymidine incorporation in the whole cell with little or no discernible effect on dATP-^{3}H incorporation in the isolated nuclear system.

There is, of course, the possibility that steroid treatment results in the killing of a certain percentage of the cells, or that there is a change in the cell population so that fewer cells are in the synthesis phase of the division cycle. We have not carried out vital staining, mitotic indices, or radioautographic studies which would be required to answer these questions. In the isolated nuclear system, however, there is no apparent

steroid effect on DNA synthesis (Table 5) whether activity is expressed on the basis of incorporation per microgram of protein nitrogen or per microgram of DNA. It follows, then, that such nuclei as could be isolated from steroid-treated cells seem indistinguishable from nuclei obtained from nonsynchronized cells in log phase of growth. There is no indication of a differential recovery of nuclei from control or treated cells. It is noteworthy that cortisol treatment has been demonstrated to alter the cell cycle of squamous epithelial cells of the mouse forestomach.[22] Assuming either that only a portion of the cell population is affected or that there are effects in the direction of partial cell synchrony, the difference between the inhibition of thymidine incorporation in whole cells versus the lack of inhibition of nuclear incorporation of dATP remains unexplained.

The long time lag between introduction of the steroid and observed alterations of nucleic acid synthesis argues against the postulation that such alterations represent primary effects of the steroid. In thymocytes, early effects on the uptake both of nucleic acid precursors[23] and glucose[24] into the cell have been demonstrated recently.

Although it seems increasingly unlikely that the effects of glucocorticoids on nucleic acid synthesis are primarily responsible for the growth-inhibitory effects of steroids, nevertheless, these alterations in nucleic acid metabolism are important in our understanding of the entire sequence of molecular events involved in this steroid response.

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