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STUDIES OF METABOLIC ALTERATIONS INDUCED BY STEROIDS IN MURINE LYMPHOCYTIC LEUKEMIA CELLS *IN VITRO*

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Abstract—Triamcinolone acetonide, an effective inhibitor of the reproduction of L5178Y murine neoplastic lymphoblasts both *in vivo* and in minute amounts in cell culture, depressed in these cells: (a) the uptake of several amino acids into protein, (b) the incorporation of adenine into nucleic acids, and (c) the conversion of glucose to carbon dioxide. A study of the sequence of the inhibition of several metabolic events in steroid-treated cells in culture demonstrated that the conversion of glucose-U-^{14}C into $^{14}CO_2$ was depressed before significant alterations in the uptake of glycine into protein could be detected. The inhibitory effects appeared to be related to that type of steroid structure generally associated with anti-inflammatory and gluconeogenic, as well as anti-leukemic, activities. At comparable concentrations, steroids which exhibited primarily androgenic activity had no inhibitory effects on the reactions studied in these cells.

INTRODUCTION

THE mechanism by which the steroid hormones influence cellular metabolism, although extensively investigated both *in vivo* and *in vitro*, remains largely unsolved. On the other hand, the effects of steroids upon cellular metabolism are well-documented. Thus, Cori and Cori[1] demonstrated in 1927 that fasted adrenalectomized rats could not maintain normal levels of blood sugar and liver glycogen. The deposition of liver glycogen in adrenalectomized mice and rats is increased greatly by the administration of cortisone.[2] Steroid-induced gluconeogenesis may be accomplished at the expense of the breakdown of tissue protein, and associated changes at the enzyme level have been demonstrated. Thus, Gavosto *et al.*, Rosen *et al.*, and other workers demonstrated that, following the administration of cortisone, the activities of glutamic-oxalacetic and glutamic-pyruvic transaminases in the liver are increased,[3, 4] and a similar increase in the activity of hepatic arginase occurs;[5] however, fasting also causes an increase in the levels of transaminase and arginase activities in the liver.[4, 6] It has been postulated that certain steroids may function as coenzymes in the transfer of hydrogen between the reduced and oxidized forms of diphosphopyridine and triphosphopyridine nucleotides.[7] Steroid specificity in the inhibition of glucose-6-phosphate dehydrogenase also has been reported;[8] in this case, however, pregnenolone, a precursor of several adrenocortico-steroids, dehydro-isoandrosterone and related steroids were active inhibitors. Evidence has also been presented that steroids influence

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electron transport,[9] the metabolism of fat[10, 11, 12] and nucleic acid, [13, 14] as well as the levels of citric acid in the blood and urine.[15]

Lymphocytes have been widely used in the investigation of the effect of steroids at the cell level.[16, 17] Of particular interest, however, have been studies of Jaffe and Fischer of this department (see Table 1)* which have shown that a variety of steroidal compounds that possess glucocorticoid activity not only profoundly inhibit the growth of murine L5178Y cells in culture, but also exert striking cytolytic effects upon ascitic populations of these cells *in vivo*. With the most potent compounds of this class, death of the great majority of ascitic tumor cells occurred following their exposure to minute concentrations of the steroids. Blecher *et al.*[18, 19] investigated the effects of various steroids upon normal (thymus) and malignant (Murphy–Sturm lymphosarcoma) lymphocytes and reported that several of these compounds effectively inhibit lymphocyte metabolism at the whole cell level, as well as in homogenates free of intact cells; however, androgenic and "mineral" steroids were the most active. The same authors have reported recently that, in their system, adenosine triphosphate may be the limiting factor responsible for the inhibition of glycolysis. An attempt to develop some of the biochemical aspects of the mechanism of inhibition of growth of the L5178Y cells by glucocorticoids has been made by measuring the uptake of amino acids, adenine and glucose by these lymphoblasts in cell culture, particularly as affected by triamcinolone acetonide (9α-fluoro-16α:17α-*iso*propylidene-dioxy-Δ^1-hydrocortisone). The observations made with this compound are described here.

METHODS AND MATERIALS

The substrates used, the position of the isotopic labels, and the specific radioactivity of each compound, respectively, were as follows: DL-tryptophan-3-^{14}C (3·8 μC/mg); L-glycine-2-^{14}C (16 μC/mg); DL-valine-1-^{14}C (0·27 μC/mg); adenine-8-^{14}C (14·8 μC/mg); glucose-U-^{14}C (23 μC/mg); glucose-1-^{14}C (3·2 μC/mg).

Either DBA/2 or AKR/2 $\times$ DBA/2 mice of either sex were used as sources of L5187Y cells, grown as ascites populations. Leukemic lymphocytes were harvested from ascites fluid on the seventh day after the intraperitoneal transplantation of about 2×10^6 cells. The animals were killed by neck dislocation; the cells were aspirated from the peritoneal cavity and transferred into a culture tube containing 5 ml of culture medium,[20] and centrifuged at 1000 **g** for 90 sec; the supernatant fluid was discarded and the cells were resuspended in 5 ml of culture medium. After adequate dilution of an aliquot of these resuspended cells, they were counted electronically (Coulter Counter) and transferred to 200–500 ml of culture medium, with or without horse serum (either 5 per cent or 10 per cent); in this manner a suspension of 1·5 to 9×10^5 cells per ml was obtained (a very small but variable proportion of erythrocytes and non-tumor cells were present); the suspensions of cells were then incubated at 37 °C. When radioactively labeled cells were to be used, exponentially dividing L5178Y cells were grown *in vitro* in the presence of a ^{14}C-labeled substrate; steroids were added to the cultures in order to produce concentrations such as those described under Results. In other experiments, the above-described pre-incubation period, in the presence or absence of a steroid, was followed by the addition of labeled

* The data presented in Table 1, shown with the kind permission of Dr. G. A. Fischer, have been selected from his findings with these and many other steroids tested on growing cultures of L5178Y cells. Detailed reports of these findings, as well as those of Dr. J. J. Jaffe obtained in mice, will be presented elsewhere.

substrate and incubation was continued at 37 °C for periods of time ranging from 10 min to 10·5 hr. At the end of the incubation period, the individual cell cultures were treated as follows: the cells were separated by centrifugation and washed twice with fresh medium free of horse serum, allowing an equilibration period of 10 min during each washing; the amount of radioactivity in the second washings always approached that of the background. In all experiments with ^{14}C-labeled glucose and in most other experiments, as indicated under Results, Warburg flasks were employed, with 0·2 ml of sodium hydroxide (2 N) in the center well and a solution of trichloroacetic acid (TCA) (20 per cent) in the side-arm, in order to permit estimations of the amount of $^{14}CO_2$ which was formed. The incubations were stopped after incubation for 1 hr at 37 °C and the evolved $^{14}CO_2$ was measured by adding a 0·1-ml aliquot of the sodium hydroxide solution of the center well to 10 ml of a mixture of toluene and absolute ethanol (2:1) (this contained, per liter, 2·7 g of 2:5-diphenyloxazole and 33 mg of 1:4-di-(2-(5-phenyloxazole))-benzene); measurements of the radioactivity were made in a Liquid Phosphor Counter. Separation of unreacted glucose from most of the compounds derived from glucose, in the TCA-soluble fractions and post-incubation media, was accomplished by adding to 3 ml of resin (Dowex-1 × 4 formate, 200–400) in 5-ml pipette-columns, an aliquot of the acid-soluble fraction or of the post-incubation medium; the column was then washed with 6 ml of water and eluted with 6 ml of formic acid (0·8 N). The free glucose was found in the water wash, while most of the metabolic derivatives of glucose occurred in the formic acid fraction.

For protein determination, the cells were precipitated four times with cold TCA (5 per cent) and twice with ethanol (95 per cent). The final protein precipitate was dissolved in sodium hydroxide (0·1 N). The hydrolysed nucleic acid fraction was obtained by precipitating the cells twice with cold TCA (5 per cent) and twice with ethanol (95 per cent); the precipitate was then resuspended in 2·5 ml of TCA (5 per cent), heated to 90 °C for 30 min and centrifuged; the protein precipitate was washed once with 2·5 ml of TCA (5 per cent) and centrifuged. The supernatant fraction of this TCA-wash was combined with the supernatant fluid separated from the hot TCA-precipitate, and the mixture was assayed for nucleic acids. Protein and nucleic acid fractions were assayed for radioactivity in the liquid scintillation counter (as described above). Protein was determined by a modified biuret method,[21] and the total nucleic acids estimated by measuring the amounts of deoxyribose and ribose present.[22, 23]

Stock solutions of steroids were prepared by dissolving the compounds in the culture medium with shaking at 37 °C for 48 hr; an average steroid concentration of about 100 μg per ml was obtained by this method. The final concentration of the steroid in the medium was measured in a Beckman DU-spectrophotometer.

RESULTS AND DISCUSSION

Triamcinolone acetonide, in concentrations ranging from 2 to 220 mμg per ml, inhibited cell growth (Fig. 1); the effect of this synthetic corticosteroid was the most marked of any of many compounds tested in culture (the results obtained in tests of a representative group of steroids are shown in Table 1). In contrast to the effect of triamcinolone acetonide, halotestin (9 α-fluoro-11β-hydroxy-17α methyl testosterone), in concentrations up to 200 mμg per ml (Table 2), did not influence either cell division or the uptake of glycine into cell proteins, whereas triamcinolone acetonide (100 mμg

per ml) markedly diminished both cell division and the incorporation of glycine into the proteins of the cells. In Table 2 are shown the results of experiments in which the actions of these steroids were compared; the results are expressed in terms of viable cell counts at the end of a 19-hr period. Rupture of the cell membranes and intense vacuolization of the cells were the distinguishing morphological signs which were regarded as the criteria of non-viability.

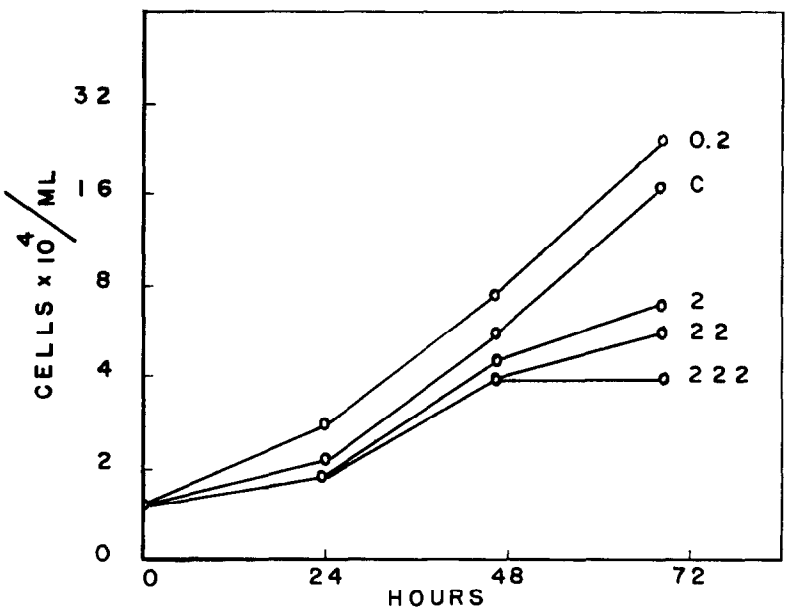

FIG. 1. L5178Y cells in culture medium containing 10 per cent horse serum. All cultures were carried out in duplicate (4·5-ml cultures). Triamcinolone acetonide was added at zero-time, in the concentrations recorded on the graph as mμg per ml. C on the graph represents a control culture to which no steroid had been added. The transfer of cells from mice to culture tubes, which was done at room temperature, induced a lag phase 3–6 hr during which growth did not occur.

TABLE 1. THE EFFECT OF STEROIDAL COMPOUNDS ON THE GROWTH OF L5178Y CELLS IN CULTURE

Agent	Concentration mμg/ml of culture medium*
Testosterone	500
Deoxycorticosterone	400
Hydrocortisone acetate	20
Triamcinolone	10
Triamcinolone acetonide	1–4

* In these experiments, the steroidal compounds were added to the culture medium[20] after exponential growth had been established. The concentrations given in the table refer to those required for 50 per cent inhibition of growth.

The assimilation of several radioactive substrates into cells was investigated and, as shown in Fig. 2, the conversion of glucose into CO_2 was inhibited at an earlier time and to a greater degree than was the incorporation of glycine into protein. After 3 hr in the presence of the steroid, glucose metabolism was inhibited by 30 per cent, while

TABLE 2. THE EFFECT OF TRIAMCINOLONE ACETONIDE AND OF HALOTESTIN ON THE UPTAKE AND INCORPORATION OF GLYCINE-2-^{14}C AND ADENINE-8-^{14}C INTO THE PROTEINS AND NUCLEIC ACIDS OF L 5178Y CELLS

Substrate added	Inhibitor		
	None	Triamcinolone acetonide	Halotestin
(500,000 cpm) Glycine-2-^{14}C	(cpm/mg*) 2800	(cpm/mg*) 1000	(cpm/mg*) 2950
Adenine-8-^{14}C	469,000	196,000	—
Duration of incubation (hr)	Cells × 10^5 per ml of medium		
0	3·5	3·5	3·5
19	6·8	3·1	6·2

* cpm/mg refers (a) with glycine-2-^{14}C, to cpm/mg of the *total protein* of the cells; (b) with adenine-8-^{14}C, to cpm/mg of *deoxyribose of the DNA* isolated from the cells.

L5178Y cells in 200-ml cultures (medium containing 10 per cent horse serum). The steroids, halotestin (2000 mμg per ml) and triamcinolone acetonide (500 mμg per ml), were added to the cultures at zero-time. The labeled substrates were added to the cultures 10 hr after the beginning of the incubation. The cultures were harvested after 19 hr of incubation and were assayed for radioactivity.

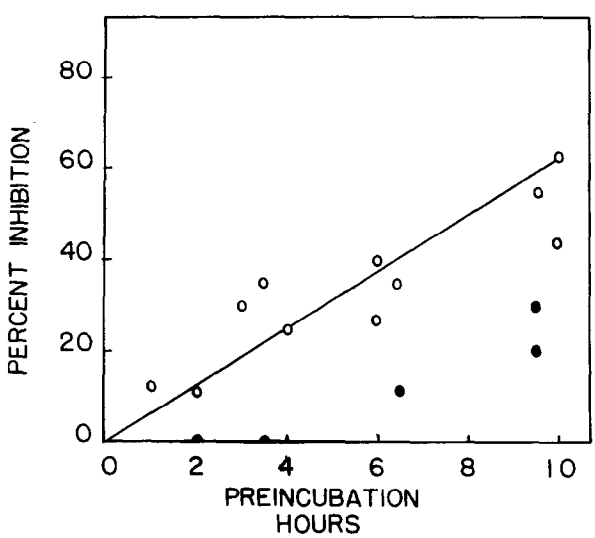

FIG. 2. L5178Y cells at 5 × 10^5 cells per ml in culture medium, without horse serum, were preincubated in the presence and absence of triamcinolone acetonide, 100 mμg per ml, harvested by centrifugation and resuspended in 3 ml of fresh medium containing no steroid. To the fresh medium, glucose-U-^{14}C (100,000 cpm) or glycine-2-^{14}C (100,000 cpm) was added and incubation at 37 °C was continued for 1 hr. The results represent percent inhibition of the uptake and metabolism of glucose into $^{14}CO_2$ "○" and the uptake and incorporation of glycine-2-^{14}C into proteins "●" in the steroid-treated cells.

no effect upon the uptake of glycine into proteins could be demonstrated. In three experiments in which the effects of glucocorticoids upon the metabolism of glucose was examined, the evolution of $^{14}CO_2$ from glucose-U-^{14}C was inhibited to a greater degree than was the evolution of $^{14}CO_2$ from glucose-1-^{14}C; the results are shown in Fig. 3. Using glucose-U-^{14}C as substrate, and assaying the acid-soluble fraction of these cells and the post-incubation medium, significant differences, indicating a

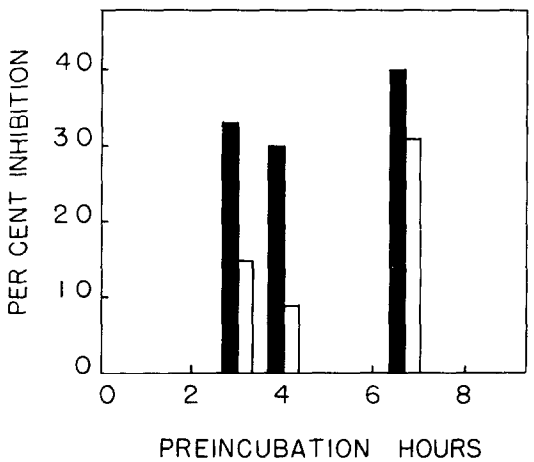

FIG. 3. L5178Y cells at 5 × 10^5 cells per ml of culture medium, containing 5 per cent horse serum, were pre-incubated in the presence and absence of triamcinolone acetonide, 20 mμg per ml, harvested by centrifugation and resuspended in 3 ml of fresh medium containing no steroid. To the fresh medium, glucose-U-^{14}C (150,000 cpm) or glucose-1-^{14}C (150,000 cpm) was added, and incubation at 37 °C was continued for 1 hr. On the graph, filled in bars and open bars represent percent inhibition of the uptake and metabolism into $^{14}CO_2$ of glucose-U-^{14}C and glucose-1-^{14}C, respectively.

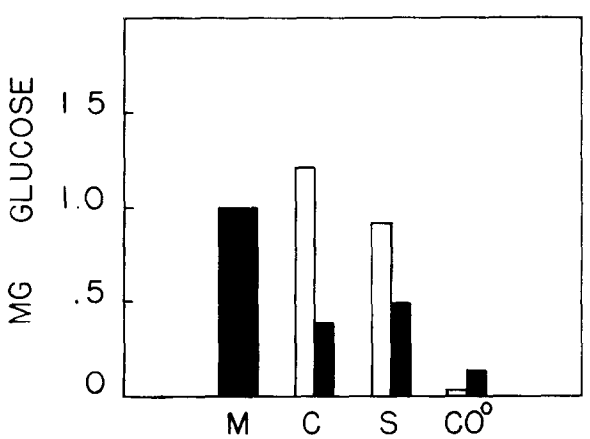

FIG. 4. L5178Y cells, pre-incubated for 4·5 hr in the presence or absence of triamcinolone acetonide, 100 mμg per ml, were harvested by centrifugation and resuspended in 1 ml of culture medium containing no steroid. The bar-graph depicts the relative amounts of glucose and lactic acid found in the acid-soluble fraction of the cells, relative to each mg of glucose found in the post-incubation medium (M-bar on the graph) after a 10-min incubation at 37 °C. Bars labeled "C", "S", and "CO°" depict free glucose (filled in bars) and lactic acid (open bars) found in the acid-soluble fraction of, respectively, control and steroid-treated cells incubated at 37 °C and control cells incubated at 0 °C.

marked inhibition of glycolysis in the steroid-treated cells, were found. Following pre-incubation of the cells for 4·5 hr, in the presence or absence of triamcinolone acetonide, the following ratios of lactic acid to free glucose were found in the acid-soluble fraction of the cells: controls, 3:1; steroid-treated, 1·85:1 (Fig. 4). When the cells were pre-incubated with the steroid for 24 hr, rather than 4·5 hr, the values were: controls, 3:1; steroid-treated, 1·1:1 (Fig. 5).

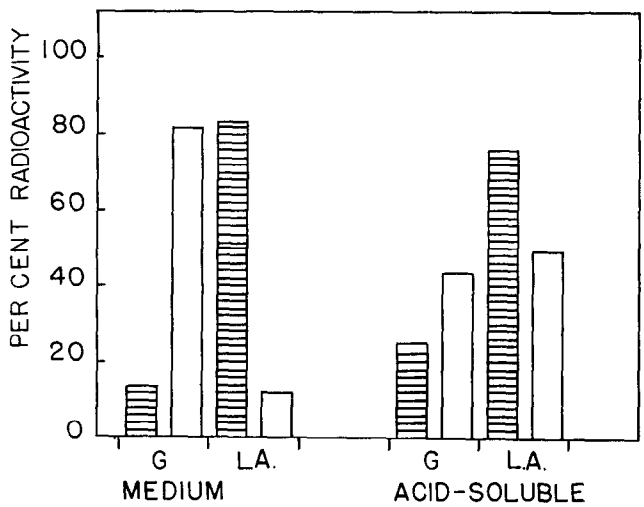

FIG. 5. L5178Y cells, pre-incubated in the presence or absence of triamcinolone acetonide, 100 mμg per ml, for 24 hr, were harvested by centrifugation and resuspended in 1·0 ml of culture medium containing no steroid. On the graph is shown the percent distribution of radioactivity in the post-incubation medium and in the acid-soluble fraction of control cells (cross-hatched bars) and steroid-treated cells (open bars), after an incubation period of 10 min at 37 °C. Before incubation the medium contained 1 mg of glucose (240,000 cpm) in a total volume of 1 ml. "G" on the graph represents free glucose and "LA" represents lactic acid.

Using exponentially dividing L5178Y cells which had been grown in culture in the presence of ^{14}C-labeled glycine, tryptophan or adenine, the turnover of protein and nucleic acids in lymphoblasts exposed to triamcinolone acetonide was investigated. After 4–8 doublings of the cell population in the presence of the radioactive substrate, the cells were harvested, washed and transferred to fresh medium free of labeled substrate and in the presence or absence of steroid. In these experiments with glycine, tryptophan and adenine, triamcinolone acetonide was added to the cultures at 0·1, 0·8 and 1·1 μg per ml, respectively, and the incubation was allowed to proceed for an additional 12–18 hr. Under these conditions, the results demonstrated no significant differences in the specific activity of the cell proteins and nucleic acid fractions of the steroid-treated cells, as compared to those of the controls (Table 3).

In summary, a new approach to the study of the effects of steroids upon whole cells *in vitro* has been described. The procedures offer the advantage of an *in vitro*-assay, using cells which grow in culture and in the mouse with comparable generation-times. The studies indicate that triamcinolone acetonide, in concentrations which

approach physiologic levels of corticosteroids, profoundly affects the metabolism and growth of the L 5187Y lymphoblasts in culture. The results suggest that this steroid exerts (a) a differential effect on metabolic pathways, as indicated by an early inhibition of glucose metabolism at a time when glycine uptake and incorporation into protein was not altered; (b) no significant effect on the transport of glucose into cells, even

TABLE 3. THE ABSENCE OF AN EFFECT OF TRIAMCINOLONE ACETONIDE ON THE TURNOVER OF PROTEIN AND NUCLEIC ACID*

Derivation of protein label	cpm/mg of protein			Derivation of nucleic acid label	cpm/mg of deoxyribose		
	Original	Control	Steroid		Original	Control	Steroid
Glycine-2-^{14}C	24,500	12,400	14,200	Glycine-2-^{14}C	474,000	252,000	287,000
Tryptophan-3-^{14}C	—	1,230	1,310	Adenine-8-^{14}C	672,000	493,000	495,000

* After growth of the L5178Y cells in culture in the presence of the respective radioactive substrate for 4–8 doublings, the cells were harvested, washed and transferred to fresh medium free of isotopically labeled compounds. At this time, the "*original*" level of radioactivity was determined. The cells were then re-incubated for 12–18 hr (a) in the absence of steroid ("*control*") and (b) in the presence of triamcinolone acetonide, 0·1 μg/ml for glycine-2-^{14}C, 0·8 μg/ml for tryptophan-3-^{14}C, and 1·1 μg/ml for adenine-8-^{14}C ("*steroid*").

though this is temperature-dependent and, therefore, energy-requiring; and (c) no influence on the catabolism of proteins and nucleic acids, as indicated by experiments with cells labeled with glycine, tryptophan or adenine.

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