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GLUCOCORTICOIDS AND LYMPHOCYTES

II. Cell Cycle-Dependent Changes in Glucocorticoid Receptor Content[1]

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To study variations in glucocorticoid receptor levels during the cell cycle, we have separated mitogen-stimulated human peripheral lymphocytes and rat lymph node cells by unit gravity sedimentation and measured glucocorticoid binding in the resultant fractions. By morphologic criteria and thymidine incorporation, the fractions were separated into populations of G_0 and G_1 phase and S and post-S phase cells. A 2- to 3-fold increase in glucocorticoid receptor sites per cell, for cells in the S and post-S phase over those in G_0 and G_1, was observed with both nonstimulated rat lymph node cell suspensions and concanavalin A-stimulated human peripheral lymphocytes. These observations together with those from other studies indicate that formation of new glucocorticoid receptors near the S phase may be a general phenomenon in proliferating cells. We propose that this increase in glucocorticoid receptors during the cell cycle may explain the increase in glucocorticoid receptors in mitogen-stimulated lymphocytes.

Lymphocytes from mammalian species have been studied extensively as target tissues for glucocorticoid hormones. Recently we and others have noted that lymphocytes activated *in vivo* and *in vitro* by a variety of mitogenic and antigenic stimuli show an increase in both the intracellular concentration and number of glucocorticoid receptors per cell (1, 2). Because the increase in glucocorticoid receptor sites could be blocked with inhibitors of RNA and protein synthesis, it has been suggested that the increase represents induction of the receptor protein by mitogen (2).

An alternative explanation is one we have suggested (1) on the basis of the work of Cidlowski and Michaels (3) who found that synchronized HeLa cells exhibited a 2- to 3-fold increase in glucocorticoid receptor site content during late G_1. If a similar variation in receptors with stages of the cell cycle occurs in lymphocytes, then upon either mitogen- or antigen-induced activation, an increase in glucocorticoid receptor content would be expected as a large number of cells move into the later phases of the cell cycle.

To test this hypothesis we have separated mitogen-stimulated human peripheral lymphocytes and rat lymph node cell suspensions by unit gravity sedimentation and studied glucocorticoid binding in the resulting synchronized cell suspension. Unit gravity sedimentation has frequently been used to synchronize proliferating cells, and has several advantages over other means of obtaining synchronous cell populations for studying cell cycle-dependent fluctuations in the levels of biologically important molecules (4–7). First, it avoids the use of blocking agents such as colchicine, which might have effects in addition to blocking progression through the cycle. Second, since the cells are separated after reaching a point in the cycle, sedimentation avoids the need for reculture after synchronization (which inevitably leads to a loss of synchrony). Third, and possibly more important, sedimentation permits simultaneous measurement of a given parameter on cells in various phases of the cycle, thus allowing more meaningful comparisons of results from the different phases. These advantages made this an ideal technique for testing our hypothesis.

MATERIALS AND METHODS

Animals, adrenalectomy, and preparation of cell suspensions. Adult male Sprague Dawley rats were adrenalectomized and lymph node cell suspensions were prepared as described (8). Viability was determined by exclusion of 0.4% trypan blue (Gibco, Grand Island, N. Y.).

Isolation and mitogen stimulation of human lymphocytes. Peripheral blood (50 to 100 ml) was drawn from normal donors and defibrinated by shaking gently for 10 to 15 min with 3-mm glass beads to remove monocytes and platelets. Lymphocytes were separated by discontinuous density gradient centrifugation over Ficoll-hypaque (9). Cells at the interface were pooled and washed twice in 50 ml RPMI[3] 1640 (Grand Island Biological Supply, Grand Island, N. Y.) with 10% fetal calf serum, (FCS), 50 units/ml penicillin, 50 μg/ml gentamicin, and 300 μg/ml glutamine at 1×10^6 cells/ml. Con A (Miles-Yeda Rehoveth, Israel) was added to a final concentration of 20 μg/ml and cultures were incubated in plastic tissue culture flasks at 37°C in 5% CO_2 in humidified air for 60 to 80 hr before use.

Unit gravity sedmentation. A lucite sedimentation chamber was built according to the design recommended by Miller and Phillips (7) to accomodate the necessary number of cells (2.5×10^9) and allow sufficient sedimentation distance to obtain the required separation. The diameter was 15 cm (O.D.), height was 5.5 cm, and was volume 780 ml. A 108° angle with the walls

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[3] Abbreviations used in this paper: Dexamethasone, 9α fluoro-16α-methyl-11β,17,21 trihydroxypregn-1,4-diene-3,20-dione; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

of the chamber was machined at the top and bottom to minimize overlap between layers of cells upon emptying the chamber. To fill the sedimentation chamber and form the gradient, 20 ml of Dulbecco's phosphate-buffered saline (Grand Island Biological Co.), pH 7.35, were added through the bottom of the chamber followed by 25 ml of cell suspension at 1×10^7 cells/ml. The gradient was then formed beneath the cell suspension (taking care not to disturb the layer of cells) by pumping fluid at 7.2 ml/min from three serially connected lucite chambers containing (in order, starting from the final mixing chamber) 50 ml of 5% FCS, 300 ml of 15% FCS, and 300 ml of 30% FCS in PBS with 0.1% (w/v) ethylene diaminetetraacetic acid. This produced a FCS gradient with a sharp initial rise in concentration necessary to allow sedimentation at 10^7 cells/ml without mixing of cell layers by "streaming" as described previously (6, 7). Cells were allowed to sediment for a total of 200 to 260 min for Con A-stimulated cells and 340 to 400 min for rat lymphocytes before displacement through the top of the chamber with a solution of 7% (w/v) sucrose and 0.29% (w/v) NaCl at a rate of 7.2 ml/min into 50 14.4-ml fractions. A longer sedimentation period was necessary for rat lymphocytes since these cells are smaller than Con A-stimulated cells and thus have a slower sedimentation rate. All sedimentations and fractionations were carried out at 3°C. All solutions used for sedimentation of cells were passed through a 0.45-μm filter. Cell size determination on cells in the fractions were done by using a Coulter Counter Model B with a sizing attachment (Coulter, Hialeah, Fla.).

(^{3}H)thymidine incorporation. To measure rates of (^{3}H)thymidine incorporation, cells were first sedimented and fractionated as described above. Cells from each fraction were washed once in 15 ml RPMI 1640 containing 25 mM HEPES buffer at 3°C, and resuspended in 1 ml of the same medium. Aliquots (100 μl) of this suspension were added to microtiter plates (Costar, Cambridge, Mass.) wells containing 100 μl of (^{3}H)thymidine (1.9 Ci/mole, Schwarz-Mann, Orangeburg, N. J.), 10 μCi/ml in medium. The microtiter plates containing the cell suspension were incubated 2 hr at 37°C in humidified room air with 5% CO_2. At the end of the incubation cells were harvested and the incorporation of (^{3}H)thymidine was determined as described (8). Counts per minute incorporated were adjusted for the number of cells in the initial 100-μl aliquot.

Glucocorticoid receptors. Glucocorticoid receptors were assayed by using an adaptation of methods previously described in detail (10–12). To study nuclear dexamethasone binding in each of the 50 synchronous fractions, cells were first incubated with 5 to 10 nM (^{3}H)dexamethasone (New England Nuclear, Boston, Mass., 30.7 Ci/mmole) for 30 min at 37°C. They were then washed twice in 50 ml of ice cold phosphate-buffered saline (pH 7.4) at 3°C, centrifuged at 400 × G for 5 min, and resuspended in phosphate-buffered saline at 0°C containing 3% FCS and 0.1% ethylene-diaminetetraacetic acid. Cells were fractionated by unit gravity sedimentation as described above. The cells in each fraction were washed twice at 3°C by centrifugation at 400 × G for 5 min through 15 ml RPMI 1640 with 25 mM HEPES buffer, and resuspended in 1 ml of the same medium. Of this cell suspension, 20 μl were used for cell counting, 100 μl was incubated with (^{3}H)thymidine as described above, and the remaining 880 μl was centrifuged to a pellet. The supernatant was carefully aspirated, and the cells lysed by rapidly adding 1.2 ml of 1.5 mM $MgCl_2$ at 3°C with vigorous mixing. After incubating 15 min at 3°C to complete lysis, the suspension was again mixed vigorously and centrifuged at 12,000 × G for 4 min. The tips of the tubes with the resulting nuclear pellets were cut off and counted in 5 ml of Bray's solution at an efficiency of 40%. Data were expressed as cpm bound/10^6 cells. This method does not allow correction for nonsaturable binding but does permit measurement of dexamethasone binding in each of the partially synchronous fractions.

To allow correction for nonsaturable binding, suspensions of lymph nodes or mitogen-stimulated cells were first sedimented and fractionated. Fractions were pooled from the slowly sedimenting cells (which were characterized by small size, absence of (^{3}H)thymidine incorporation, absence of mitoses, and few morphologic blast cells), and from the rapidly sedimenting cells (characterized by larger size, high rates of thymidine incorporation, numerous mitoses, and frequent blast cells). The pooled fractions were washed twice in medium at room temperature by centrifuging 5 min at 500 × G, and suspended in 100 μl of RPMI 1640 with 25 mM HEPES buffer. Aliquots were removed for cell counts, thymidine incorporation, Wright-Giemsa stains, protein determination, and measurement of glucocorticoid receptors. Glucocorticoid receptors were assayed by incubating cells with 10 nM (^{3}H)dexamethasone with and without 1 μM dexamethasone for 30 min at 37°C. Cells were then lysed by diluting the cell suspension 60-fold in 1.5 mM $MgCl_2$ at 3°C and incubating for 15 min. After centrifuging at 12,000 × G for 4 min at 3°C, the supernatant was carefully aspirated and the tip of the tube containing the nuclear pellet was cut off, placed in 5 ml of Bray's solution, and counted at an efficiency of 40%. Counts bound in the presence of 1 μM unlabeled dexamethasone were subtracted from counts bound in the absence of competing unlabeled steroid to give saturable nuclear dexamethasone binding, which, as shown in the previous paper (8), displays the characteristics of glucocorticoid receptors.

RESULTS

Characteristics of fractionated cell populations. Figure 1 shows the distribution of cells in the fractions collected after sedimentation of human Con A-stimulated lymphocytes. Red blood cells and nonviable cells were seen only in Fractions 1 to 12. Results from these fractions are ignored in the following discussion since we have found in separate experiments that they lack glucocorticoid binding activity. Slowly sedimenting mature lymphocytes were present in fractions 10 to 20 and more rapidly sedimenting immature blastoid cells were seen in fractions 20 to 50 (Fig. 1, bottom). Mitoses were present in fractions 45 to 50 and made up 3 to 6% of the total cells in Con A-stimulated lymphocytes. Monocytes sedimented more slowly than blasts and constituted from 2 to 5% of the cells in fractions 30 to 40%. The cells in fractions 13 to 50 were greater than 95% viable by trypan blue exclusion.

Figure 2 shows the distribution of cells in the fractions collected after sedimentation of rat lymph node cell suspensions. Red blood cells and nonviable cells were seen in fractions 10 to 24. Morphologic blast cells were seen in fractions 36 to 48. Mature lymphocytes were present in all fractions, however, they constituted less than 50% of the cells in fractions 44 to 48. Plasma cells and monocytes sedimented more slowly than blasts and made up less than 6 and 4%, respectively, of the cells in fractions 30 to 40. The cells in fractions 24 to 50 were greater than 95% viable by trypan blue exclusion.

Evidence that sedimented cells are separated into synchronous populations. Unit gravity sedimentation has been useful for synchronizing a variety of proliferating cells (6–8). Evidence that there is synchronization in our fractions comes from several sources: 1) Mitoses were seen only in fractions 45 to 50. In these fractions the mitotic index was 20 to 100 times as high as in the

initial suspension, indicating a 20- to 100-fold enrichment of M-phase cells in these fractions. 2) (^{3}H)thymidine incorporation was highest in fractions 40 to 50 from both cell types (Figs. 1 and 2). Earlier fractions, corresponding to morphologically mature lymphocytes, showed less than 1/100 to 1/1000 of the rate of (^{3}H)thymidine incorporation seen in fractions 40 to 50, indicating a 100- to 1000-fold higher concentration of S-phase cells in these fractions. 3) The morphology of the cells in each fraction also indicate synchronization. Mature lymphocytes appeared in distinct peaks in both cell preparations (Figs. 1 and 2). Contamination of the peak with other viable cell types was 5 to 10% for Con A-stimulated cells (Fig. 1) and less than 2% for rat lymph node cell suspensions (Fig. 2). Morphologic blasts were seen in late fractions, and reached 85 to 97% of the total for Con A-stimulated cells and 40 to 60% for rat lymph node suspension. This indicates a 20- to 60-fold enrichment of blasts in these fractions. There was a continuous increase in cell size from the G_0 and G_1 cells to the post S phase cells (data not shown). For the purposes of presenting our results we have designated those fractions containing morphologically mature, small cells not incorporating (^{3}H)thymidine as "G_0 and G_1" and those fractions containing large, blastoid cells with frequent mitosis and incorporating large amounts of (^{3}H)thymidine as "S and post-S-phases".

Glucocorticoid receptor studies. To study glucocorticoid receptors as a function of the cell cycle we have taken two approaches. First, in order to demonstrate a relationship between the beginning of the S phase and an increase in dexamethasone binding, rates of (^{3}H)thymidine incorporation and binding of (^{3}H)dexamethasone were studied in synchronized cells after unit gravity sedimentation. Typical experiments are illustrated in Figure 3*A* for human Con A-stimulated lymphocytes, and Figure 3*B* for rat lymph node cell suspensions. In each case an increase in (^{3}H)dexamethasone binding was seen in the later fractions, corresponding to the S and post-S-phases. In the rat lymph node cell suspensions the level of glucocorticoid binding per cell in fractions incorporating (^{3}H)thymidine formed a plateau at levels 2 to 3 times the value seen in fractions not incorporating (^{3}H)thymidine. Possibly because synchronization of the Con A-stimulated cell suspensions was less precise, distinct plateaus of (^{3}H)dexamethasone binding were not seen;

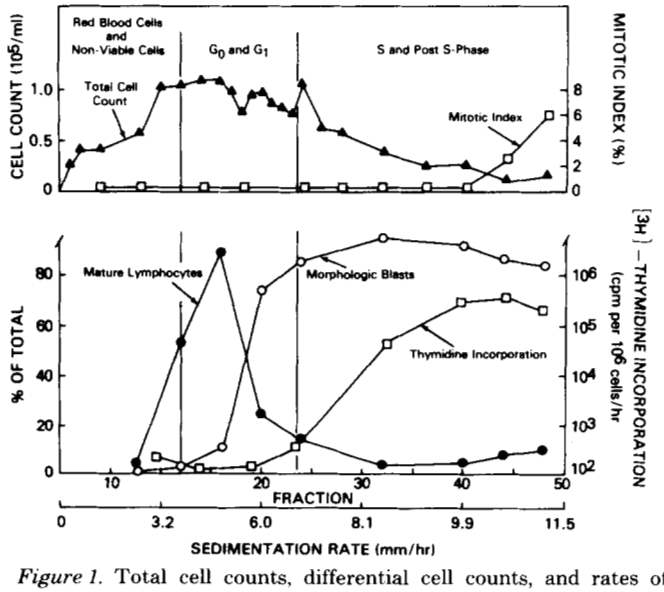

Figure 1. Total cell counts, differential cell counts, and rates of thymidine incorporation after unit gravity fractionation of Con A-stimulated human peripheral lymphocytes.

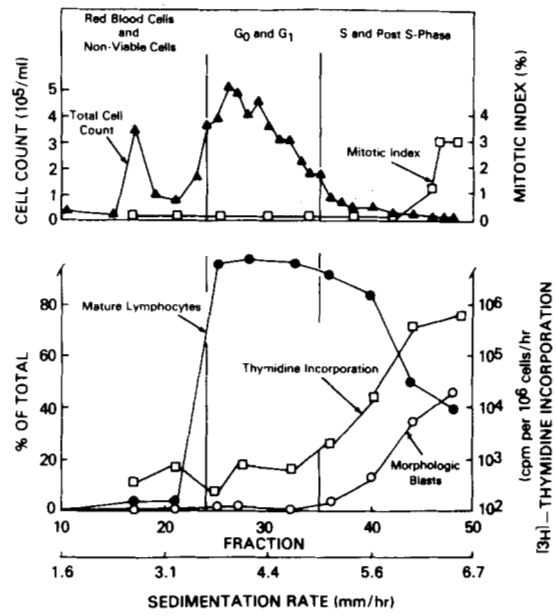

Figure 2. Total cell counts, differential cell counts, and rates of thymidine incorporation after unit gravity fractionation of rat lymph node suspensions.

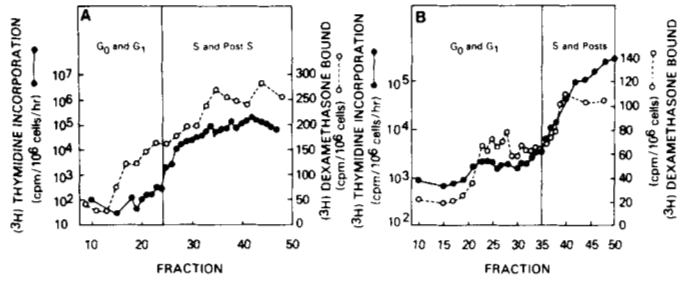

Figure 3. (^{3}H)thymidine incorporation (●) and (^{3}H)dexamethasone binding (○) in: (*A*) unit gravity fractionated Con A-stimulated human peripheral lymphocytes and (*B*) rat lymph node cells.

nevertheless, the level of glucocorticoid binding per cell was always 2- to 3-fold higher in the S and post-S-phase cells compared with cells in G_0 and G_1.

To allow correction for nonsaturable binding of dexamethasone, cells were fractionated, and those fractions before and after the sharp rise in (^{3}H)thymidine incorporation were pooled and washed to remove any cortisol present in the FCS. These pooled fractions were then assayed for specific dexamethasone binding as described in *Methods*. In the case of Con A-stimulated cells, cells from the initial suspension and nonstimulated cells from the same donor were also assayed. In each case, 1.5- to 4-fold higher levels of saturable dexamethasone binding per viable white cell were observed in the pooled fractions incorporating (^{3}H)thymidine (S and post-S-phase) compared with those incorporating only a small amount of (^{3}H)thymidine (G_0 and G_1) (Table I). Nonstimulated controls had slightly lower levels of saturable (^{3}H)dexamethasone binding than the pooled fractions of G_0 and G_1 cells. This is probably due to slight contamination of these pooled fractions by S-phase cells, as suggested by the higher levels of (^{3}H)thymidine incorporation compared to nonstimulated controls. Similar results were obtained with rat lymph node cell suspensions. S and post-S-phase cells specifically bound approximately 2 times as much (^{3}H)dexamethasone per cell as did the G_0 and G_1 cells (Table I). However, no increase was observed in the number of recep-

TABLE I

Saturable binding of dexamethasone to unit gravity sedimented lymphocytes

	Human Peripheral Lymphocytes				Rat Lymph Node Cells	
	Unstimulated[a]	Con A-stimulated 72 hr				
		Unfractionated	G_0 and G_1	S and Post-S	G_0 and G_1	S and Post-S
Specific dexamethasone binding[b]	40 ± 11	75 ± 21	59 ± 9	158 ± 17	49 ± 4	102 ± 20
Per μg protein[c]	260 ± 40	220 ± 5	250 ± 30	280 ± 80	N.D.[d]	N.D.
(^{3}H)Tdr incorporation[e]	15 ± 6	1410 ± 510	470 ± 105	7210 ± 1230	21 ± 6	2200 ± 810
Percent blasts	1	42	5	81	1	51
Percent lymphocytes	96	57	94	17	99	43
Percent monocytes	2	1	1	2	0	3
Percent PMN[f]	1	0	0	0	0	0
Percent plasma cells	0	0	0	0	0	3

Values represent the means and ranges of two experiments.

[a] Cultured for 72 hr.

[b] Expressed as cpm saturably bound per 10^6 cells.

[c] Expressed as millions of saturable binding sites per μg protein.

[d] Not determined.

[e] Expressed as cpm/10^5 cells.

[f] Polymorphonuclear leukocyte.

tors per milligram of cellular protein in Con A-stimulated lymphocytes (Table I).

DISCUSSION

Our results show that with synchronized cell populations obtained by unit gravity sedimentation of Con A-stimulated human peripheral lymphocytes and cell suspensions of rat lymph nodes, increased levels of glucocorticoid receptor sites per cell can be demonstrated in fractions corresponding to the S and post-S-phases. In contrast, there is little or no change when the results are expressed as receptors per milligram cell protein.

These findings are consistent both with those of Cidlowski and Michaels (3), who found that HeLa cells synchronized by double thymidine block showed a 2- to 3-fold increase in glucocorticoid receptor sites per cell during late G_1, and with our hypothesis (1) that the increase in glucocorticoid receptor sites that follows mitogen stimulation (1) or *in vivo* antigen stimulation (8) reflects an increase in the fraction of cells in phases of the cell cycle preceding cell division, which we have shown contain 2 to 3 times the number of receptors as do G_0 and G_1 cells. In particular, the following points established by our studies support this hypothesis: a) within the stimulated population, only cells in the late phases of the cycle contain large numbers of receptors (Table I), thus exposure to mitogen or antigen is not enough to increase the receptor content; b) cell populations with the highest level of receptor sites also have the greatest proportion of cells in late phases of the cell cycle (Fig. 2, Table I); c) changes in receptor number can be seen in lymphocytes passing through the cell cycle in the absence of mitogen; and d) the time course and degree of increase in glucocorticoid receptors roughly parallels the increase in protein synthesis and content in mitogen-stimulated cells (Reference 1 and Table I).

The alternative explanation proposed by Neifeld *et al.* (2) for the increase in receptor site number, namely, that mitogens induce glucocorticoid receptors, is based largely on the observation that the increase can be blocked by cyclohexamide and actinomycin D. These inhibitors, however, also block macromolecular synthesis associated with preparation for cell division and block cell division itself. This evidence is equally compatible with our hypothesis, which we find more plausible for the reasons mentioned above. Primarily, that simple exposure to mitogen is not sufficient to increase the number of receptors and the increase in glucocorticoid receptors is paralleled by an equal increase in other cellular proteins, i.e., that it is not specific. We propose that an increase in glucocorticoid receptor sites per cell accompanies *in vivo* and *in vitro* stimulation of human and rat lymphocytes, and that these changes are related to synthesis of new receptors before cell division rather than to specific induction of the receptor protein. Our observations, together with those of Cidiowski and Michaels (3), suggest that synthesis of glucocorticoid receptors during or near the S phase may be a general phenomenon among proliferating cells.

These findings may be of some practical importance in interpreting the results of steroid receptor assays in leukemia and lymphoma. We and others (13, 14) have found that cells from human leukemias and lymphomas vary widely in the number of glucocorticoid receptors per malignant cell. It is also known that the malignant cell population from patients with leukemia and lymphoma have differing but characteristic proportions of cells in the various phases of the cell cycle. The observation that lymphoid cells show cell cycle-dependent variations in the number of glucocorticoid receptors per cell therefore suggests that in leukemic cell populations there may be a correlation between levels of glucocorticoid receptors and the proportion of cells in S and post-S-phases.

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