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HUMAN LYMPHOCYTE SUBPOPULATIONS: EFFECTS OF GLUCOCORTICOIDS *IN VITRO*

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SUMMARY

Glucocorticoid receptor levels and steroid induced inhibition of nucleic acid precursors incorporation have been determined in circulating lymphocytes of healthy donors on subpopulations isolated by immunoabsorbant column and density gradient centrifugation. The number of steroid binding sites and the affinities of the receptors for the tracer are not significantly different in B and T + Null cells fractions. [^{3}H]-uridine incorporation tends to be in general more sensitive to steroid action in B cells than in T + Null cells, whereas the situation is reversed for [^{3}H]-thymidine.

INTRODUCTION

Recent reviews have emphasized the variable glucocorticoid sensitivity of lymphoid cells [1–4]. There are considerable species differences in the magnitude of the response to glucocorticoids: rats, mice and rabbits appear particularly steroid sensitive species whereas guinea-pig, monkey and man are relatively resistant [1]. However, even in a given species or in cultured cell lines, lymphoid cells are not uniformly susceptible to steroid action. In the $S_{49}1A$ lymphoma cell line and in the P_{1798} cultured lymphosarcoma, the onset of corticoresistant clones has been described [5–7]. In the mouse, cell mediated immunity which is a function of thymus derived cells, seems more sensitive to the inhibitory effects of steroid than humoral immunity, a function of B cells [4]. Moreover, even in one cell lineage, changes of steroid sensitivity occur during development which appear to follow an alternative pattern of resistance and sensitivity. The molecular bases of these steroid sensitivity variations are essentially unknown. Several authors postulate that steroid resistance could be associated with a decrease in the number of cellular glucocorticoid receptors [5, 6, 8, 9]. This concept is questionable since it has been recently demonstrated that steroid resistant cells could possess as many receptors as sensitive ones [7, 10–15]. In order to further investigate the effects of steroids *in vitro* on circulating human lymphocytes, we have measured the binding of [^{3}H]-dexamethasone and the steroid induced inhibition of [^{3}H]-uridine and [^{3}H]-thymidine incorporations in lymphocyte subpopulations separated by anti-f(ab')$_2$ column and BSA gradient.

MATERIALS AND METHODS

1. *Isolation of lymphocytes*

Enriched leukocyte suspension from normal donors (9 male subjects, 25–35 years old) were obtained by leukopheresis using a cell separator (Haemonetics model 30) Leukocyte suspensions were diluted in Hank's balanced salt solution and incubated for 30 min at 37°C with 2 mg/ml of carbonyl iron powder (Kochlight Laboratories) with continuous stirring. The suspension was then layered on Ficoll-metrizoat and centrifuged at 400 *g* for 30 min at 18°C to remove polymorphonuclear leukocytes and phagocytes.

2. *Separation of lymphocytes on immunoabsorbant column*

T and B lymphocytes were separated using an immunoabsorbant column according to the method of Schlossman and Hudson [16] as modified by Thierry *et al* [17]. 2×10^8 lymphocytes were layered on the top of the column. Elution of T lymphocytes was complete after the passage of 50 ml of separation medium (RPMI 1640 buffered with 1% Hepes, supplemented with 15% heat inactivated fetal calf serum and 2% of 0.02% EDTA solution) at a flow rate of 1 ml/min. Elution of B lymphocytes was obtained with 30 ml of the same medium containing 10 mg/ml of human gamma globulin (Cohn fraction II, Pentex).

3. *Separation of lymphocyte subpopulations on a bovine serum albumin gradient*

T lymphocytes were further fractionated on a discontinuous gradient [18] prepared by superposition of the following BSA solutions adjusted to a density of 1.095, 1.072, 1.064 and 1.058 g/cm^3 at 25°C. Lymphocyte suspension was layered at the top of the gradient and centrifuged for 30 min. at 5000 *g* (4°C). Lymphocyte subpopulations were collected at the interfaces, washed and resuspended in medium 199 (Gibco).

4. *Viability and membrane markers*

Viability of the cells was estimated by the trypan blue exclusion procedure. The control of the efficiency

of the separation was estimated by the E rosette test and the surface immunoglobulins as previously described [19].

5. Binding experiments

[^{3}H]-Dexamethasone ([^{3}H]-DM, 23 Ci/mmol, Amersham centre, U.K.) was used as tracer because, unlike cortisol, it has low affinity for the plasma cortisol binding globulin. The methodology used for [^{3}H]-DM binding assays has been described in detail elsewhere [10, 11]. In each experiment, the cells were incubated either with [^{3}H]-DM alone or in the presence of 300-fold excess of unlabelled dexamethasone in order to determine the specific binding of the tracer.

6. Steroid induced inhibition of nucleic acid precursor incorporation

To determine *in vitro* the steroid sensitivity of lymphocytes we measured the incorporation of tritiated nucleosides in the absence (control) or presence of non radioactive dexamethasone. These experiments were performed either in short term assays (4 h) or over 24 h periods. The procedure used for short term experiment has been previously described [10, 11, 20]. In 24 h experiments the lymphocyte suspensions were preincubated for 22 h at 37°C in a 5% CO_2 in air atmosphere, in the absence or presence of 10^{-6} M non-radioactive dexamethasone, and then received 1 μCi of either [^{3}H]-uridine (26 Ci/mmol) or [^{3}H]-thymidine (30 Ci/mmol). After 2 h additional incubation the reaction was terminated by addition of ice-cold 5% trichloroacetic acid. The precipitates were filtered on Whatman GF/A filters and washed three times with 10 ml of ice-cold 5% trichloroacetic acid. The radioactivity collected on the filters was counted by liquid scintillation spectrometry.

RESULTS

1. Membrane markers and viability of the different fractions

The efficiency of the separation and the immunological markers of the subpopulations obtained are shown in Table 1. The percentage of E rosette forming cells (E-RFC) and of cells with surface immunoglobulins (Sm–Ig) in the unseparated population (after monocyte depletion) were 66.6 ± 2.7% and 13 ± 1.9% respectively (n = 9). The effluent population recovered from the anti-f(ab')$_2$ column contained 83.5 ± 1.7% E-RFC and was called T + Null cell population. The population eluted from the column by the immunoglubulin was strongly enriched in B cells (70.4 ± 5.8% with Sm–Ig). The T + Null population was further fractionated on a BSA gradient: a large proportion (62.4 ± 15.3%) of the cells layered on the gradient was recovered in fraction d = 1.072; this fraction contained nearly 90% (88.9 ± 1.4%) E-RFC. In Table 1 are also presented the viability of the different cell subpopulations after 22-h incubation in the presence or absence of 10^{-6} M dexamethasone. As can be seen, the steroid had no effect on lymphocyte viability.

2. Binding of [^{3}H]-dexamethasone

Figure 1 shows the specific binding of [^{3}H]-dexamethasone as a function of the tracer concentration in the unseparated lymphocyte population and in various lymphocyte subpopulations. Scatchard analysis [21] of these binding curves gave straight lines consistent with the existence of a single class of binding sites. The number of binding sites and the affinities of the receptors for the tracer are not significantly different from a lymphocyte subpopulation to another. The number of binding sites is in the range of 3600 to 6000 sites per cell, and affinities are: $K_{D\,37°C} = 5\text{–}7 \times 10^{-8}$ M.

Table 1. Recovery, membrane markers and viability at 24 h of the various lymphocyte subpopulations studied (mean value ± SD, n = 9)

Cell populations	Recovery (%)	Viability (%) Control	Viability (%) Dex. 10^{-6} M	E-RFC (%)	Sm–Ig (%)
Unseparated populations	—	>95	>95	66.6 ± 2.7	13 ± 1.9
Anti-f(ab')$_2$ column					
Effluent population (T + Null cells)	68 ± 7.3	92.3 ± 6.3	88.4 ± 9.6	83.5 ± 1.7	1.8 ± 0.4
Eluted population (B cells)	13 ± 3.4	84.3 ± 7.1	75 ± 9.0	6.7 ± 9.0	70.4 ± 5.8
BSA gradient					
Fraction d = 1.058	0.3 ± 0.2	—	—	—	—
Fraction d = 1.064	17.6 ± 14.4	92 ± 10.1	83.4 ± 9.6	74.7 ± 3.7	2.5 ± 0.6
Fraction d = 1.072	62.4 ± 15.3	95.9 ± 4	90.9 ± 7.8	88.9 ± 1.4	1.7 ± 1.3
Fraction d = 1.095	7.1 ± 6.3	89 ± 9.5	84.2 ± 14.3	80.8 ± 2.1	2.4 ± 0.5

The viability of the cells, estimated by trypan blue exclusion, was determined after 22-h incubation at 37°C in the presence or absence of 10^{-6} M dexamethasone. The percentage of recovery after separation procedures refer to the initial number of cells applied either to the columns or to the gradients.

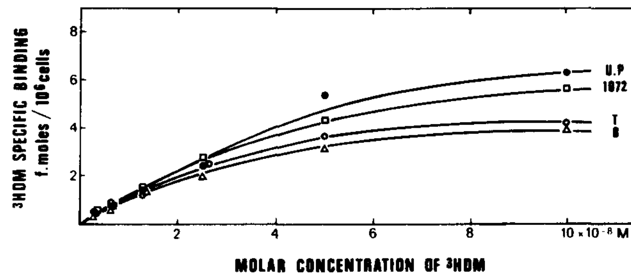

Fig. 1. The specific binding of [^{3}H]-DM as a function of tracer concentration in unseparated lymphocyte population (UP), in population eluted from immunoabsorbant column (T + Null and B cells) and in fraction d = 1.072 obtained by gradient centrifugation. The results are expressed as f.moles per 10^6 cells ($n = 5$).

3. *Inhibition of nucleic acid precursor incorporation by dexamethasone*

Short-term experiments: it has been shown previously in corticosensitive species (i.e. mouse or rat) that incubation of lymphocytes with glucocorticoids induces after 4 h a significant (60–70%) decrease of [^{3}H]-uridine incorporation into RNA [10, 11, 22]. In contrast, a 4-h incubation of circulating lymphocytes from normal volunteers in the presence of 10^{-6} M dexamethasone produced only a moderate inhibition of [^{3}H]-uridine incorporation ($26.1 \pm 6\%$, $n = 5$), Fig. 2. This value is very close to that obtained previously in another group of normal volunteers [20]. In T + Null and B cell populations the extent of the inhibition was not significantly different whereas the slope of the dose response curves appeared different: in T + Null cells the steroid induced inhibition of uridine incorporation reached a plateau level at a concentration of 10^{-6} M; whereas in B cells and in fraction d = 1.064 the process appeared unsaturable. As we have not tested the effects of higher dexamethasone concentrations, the meaning of this difference remains uncertain, but it could perhaps reflect different mechanism of steroid action.

As also shown in Fig. 2, the levels of spontaneous uridine incorporation (i.e. in the absence of steroid) were not significantly distinct from one fraction to another.

Twenty-four-hour experiments. The variability among the subjects examined precluded the demonstration of statistically significant differences between the subpopulation of lymphocytes, either in terms of level of incorporation or of steroid induced inhibitions (Figs 3 and 4). However, some general trends are apparent: for example, the level of [^{3}H]-uridine incorporation in B cells is consistently higher than in T + Null cells, in agreement with the results of Huang *et al.*[23], and in parallel the percentage of inhibition of [^{3}H]-uridine incorporation, after incubation with dexamethasone, is higher in B cells than in T + Null cells. Second, in T + Null cells subfractions, the effect of steroid on [^{3}H]-uridine incorporation follows the order: fraction 1.064 > fraction 1.072 > fraction 1.095. Finally in T + Null cells subfractions, the effect of steroid on [^{3}H]-thymidine in-

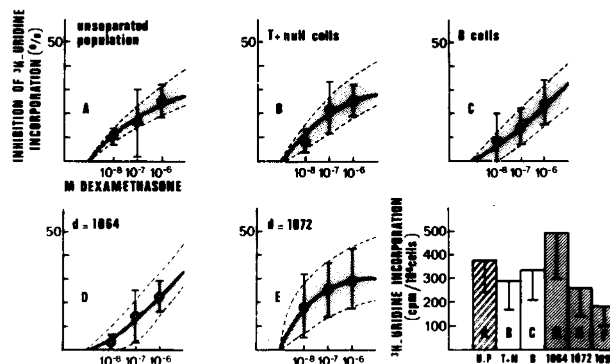

Fig. 2. Steroid induced inhibition of [^{3}H]-uridine incorporation in 4 h experiments. The cells are preincubated for three hours at 37°C in the absence or presence of various concentrations of dexamethasone and then received 1 μCi of [^{3}H]-uridine for a 60-min pulse. The levels of trichloroacetic acid precipitable precursor incorporation in the absence of steroid (control) are shown in the right part of the figure, expressed as c.p.m./10^6 cells. The percentage of steroid induced inhibition of precursor incorporation are the mean values ($\pm$SD) of five different experiments: A, unseparated population; B, T + Null cells; C, B cells; D, T + Null fraction d = 1.064; E, T + Null fraction d = 1.072.

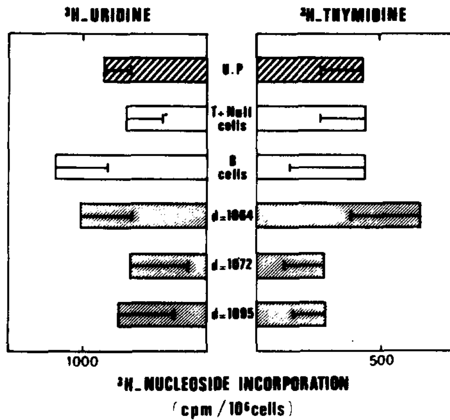

Fig. 3. 24-h experiments: incorporation of [^{3}H]-uridine (left) and [^{3}H]thymidine (right) in cells preincubated for 22 h at 37°C in the absence of steroid (control), after a 120-min pulse with 1 μCi of tritiated precursor. The results are expressed as c.p.m./10^6 viable cells, n = 5.

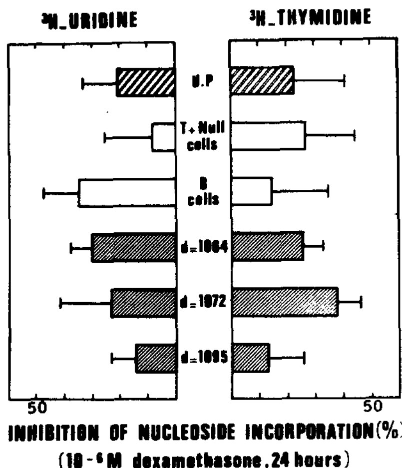

Fig. 4. 24-h experiments: inhibition of [^{3}H]uridine (left) and [^{3}H]-thymidine (right) incorporation in the presence of 10^{-6} M unlabelled dexamethasone (mean value ± SD, n = 5).

corporation are generally more pronounced than in B cells (particularly for fractions 1.064 and 1.072). The fact that each fraction contains cells which incorporate [^{3}H]-thymidine suggests that there is no specific localization of proliferating cells.

DISCUSSION

The number of steroid binding sites in different subpopulations of human circulating lymphocytes are not significantly different. This result and the values of steroid receptors per cell are in good agreement with the recent report of Lippman and Barr[14]. The quantitation of the number of receptors, however, provides only minimal and perhaps misleading information on the cell sensitivity to glucocorticoids.

Glucocorticoids, as previously demonstrated [14, 20], affect *in vitro* nucleoside incorporation in both T and B cells. Due to individual variations, there are no statistically significant differences in steroid induced inhibitions between subpopulations. However, [^{3}H]-uridine incorporation appears to be in general more sensitive to steroid action in B cells than in T + Null cells, whereas the situation is reversed for [^{3}H]-thymidine incorporation. In any case, no significant cell lysis, at 24 h, could be demonstrated in dexamethasone treated versus control samples. This is in good agreement with the fact that man is a relatively corticoresistant species [1–4] and also with the results of Schreck who stated that human lymphocytes are not lysed even after 7 days incubation *in vitro* in the presence of 10 μg/ml of cortisol succinate [24]. It is therefore possible that glucocorticoids affect first metabolic functions of lymphocytes leading to a rapid inhibition of RNA and protein synthesis [lymphokines, immunoglobulins 25, 28] and that cell lysis is only a late effect of the drug. These steroid effects could vary according to the degree of immunological maturation of the cells or the stage of proliferation, for example blocking the progression of the cells in G_1 phase [26, 27]. The two T + Null cells subpopulations (d = 1.072 and d = 1.095) have been shown previously to exhibit identical membrane markers (E and EAC rosettes) but also to respond differently to mitogens and to show different electrophorectic mobility [19]. The steroid sensitivity of these two subpopulations also appears markedly different, particularly for [^{3}H]-thymidine incorporation which is poorly inhibited by dexamethasone in fraction d = 1.095. Thus, even with assay systems that provide only rough parameters, there appear to be observable differences between these two T + Null fractions, which are repeated in the *in vitro* effects of glucocorticoids.

Evaluation of steroid sensitivity appears complicated since the drug susceptibility depends simultaneously on different parameters, such as the immunological origin of the cell, the degree of maturation or differentiation and the stage of proliferation. The application and extension of studies such as reported here to cell subpopulation enriched on the basis of specific immunological function, could provide information both on the characterization of subpopulation and on prediction of *in vivo* steroid effect.

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