Glucocorticoid Effects on Lipopolysaccharide-stimulated Murine B-Cell Leukemia Line (BCL₁) Cells¹

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ABSTRACT

The effects of glucocorticoids were studied in lipopolysaccharide (LPS)-stimulated splenic murine B-cell leukemia line one (BCL₁) cells. At 24 hr, LPS caused a 3-fold increase in [³H]-thymidine incorporation compared to similarly cultured unstimulated cells. Triamcinolone acetonide (TA) and dexamethasone at a concentration of 10⁻⁶ M reduced [³H]-thymidine incorporation 80 and 53%, respectively, while estradiol at concentrations of 10⁻¹⁰ to 10⁻⁶ M had no effect. A 500-fold excess of corticosterone or progesterone blocked the response of 10⁻⁸ M TA by 42 and 38%, respectively, indicating that the glucocorticoid response could be inhibited by antiguclocorticoids. The maximum rate of thymidine incorporation in LPS-stimulated cells occurred at 24 hr, a time at which 10⁻⁵ M TA present in parallel cultures from the initiation of LPS stimulation showed a 79% reduction in [³H]thymidine incorporation. If TA was added at any time after the initiation of LPS stimulation, the degree of decrease in nucleotide incorporation was not as marked. Therefore, maximum TA effect in LPS-stimulated BCL₁ cells occurred when TA was added to cultures at the onset of mitogen stimulation. We also measured glucocorticoid-specific receptor in whole cells both before LPS stimulation and in BCL₁ cells incubated 24 hr in the presence of LPS. The equilibrium dissociation constant, the number of sites/cell, and the hormone specificity of the glucocorticoid receptor were similar prior to and at the peak of mitogen stimulation.

INTRODUCTION

The availability of BCL₁ presents a unique opportunity to study the effects of glucocorticoids in proliferating B-cells. The tumor is monoclonal (25) as evidenced by: (a) the surface immunoglobulin is IgM, all bearing λ light chains; (b) the immunoglobulin receptors appear to have identical V regions as evidenced by highly cross-reactive idiotypic determinants; and (c) the secreted immunoglobulin is also IgM with λ light chains and bears the surface idiotypes. Whereas most lymphoid tumors are actively proliferating but appear to be locked into a fixed differentiative stage, this is not the case for the BCL₁ cell line. BCL₁ cells can be stimulated in vitro by the B-cell mitogen, LPS to both proliferate and differentiate into immunoglobulin-secreting cells. Although the LPS response is not as pronounced as that seen in normal murine spleen cells (7), the spleen cell population passed by BCL₁ cell inoculation into host animals has an advantage in being comparatively homogeneous after about 6 weeks. Splenic BCL₁ cells are available in large numbers which also facilitates correlating glucocorticoid effects with receptor characteristics in stimulated B-cells.

Examination of the literature reveals very few reports which critically study the effects of corticosteroids directly on B-lymphocytes during different stages of differentiation. Our previous studies have shown that LPS-stimulated murine B-cells were glucocorticoid sensitive and that, during proliferation, this sensitivity was mediated through the glucocorticoid receptor (16). Because the proliferating B-cell becomes progressively less sensitive to glucocorticoid treatment with time (17), it was of interest to study BCL₁ cells prior to and at the peak of LPS-induced mitogenesis. The large number of available cells would also make it possible to study glucocorticoid receptors in the BCL₁ cells. Thus, these tumor cells offer an ideal tool to study biochemical events in homogeneous population of B-cells during differentiation.

MATERIALS AND METHODS

Chemicals. [1,2,4-³H]TA (specific activity, 26 Ci/mmol) was obtained from Amersham/Searle Corp. (Chicago, Ill.). [methyl-³H]dThd (specific activity, 2 Ci/mmol) was purchased from New England Nuclear (Boston, Mass.). LPS (Escherichia coli serotype 055:B5) and unlabeled steroids were obtained from Sigma Chemical Co. (St. Louis, Mo.). RPMI 1640 with 25 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.2), and HBSS, pH 7.2, were purchased from Grand Island Biological Co. (Grand Island, N. Y.). Fetal calf serum (Lot 200014) was obtained from K. C. Biological (Lenexa, Kans.).

BCL₁ Cells. BCL₁ cells were passed by injection of 1 x 10⁶ cells i.p. into male BALB/c mice. After about 6 weeks, spleens were removed aseptically and placed in HBSS at 4°, and all further steps were performed under sterile conditions. Spleens were gently teased to isolate lymphocytes, the debris was removed by sedimentation, and the cells were washed 3 times at 4° in HBSS by centrifugation at 400 x g for 10 min. On the average, each spleen contained at least 1 x 10⁹ cells. Initial cell viability using trypan blue dye exclusion was 90%.

dThd Incorporation. Optimal conditions for dThd incorporation were determined. Cells were used at a concentration of 2 x 10⁶ cells/ml in RPMI 1640 supplemented with 5% fetal bovine serum, 2 x 10⁻³ M glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 2-mercaptoethanol at a final concentration of 5 x 10⁻⁴ M. LPS (E. coli serotype 055:B5) at a concentration of 50 μg/ml gave a 3- to 4-fold increase in [³H]dThd incorporation from 24 to 48 hr. Cells (0.180 ml) were incubated in Costar 96-well tissue culture clusters (Belloco Glass, Inc., Vineyard, N. J.) with various glucocorticoids in RPMI 1640:ethanol added to wells at a 10-fold concentration in a volume of 0.02 ml under sterile conditions to give the final concentrations indicated. The ethanol concentration per well was less than 0.01%. Incubations were for 48 hr in 5% CO₂ in air at 37°. [³H]dThd (1 μCi/well) added to each well at 42 hr, and the cells were collected and washed 6 hr later on glass fiber filters using a MASH II harvester (Microbiological Associates, Bethesda, Md.). Filters

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³The abbreviations used are: BCL₁, B-cell leukemia line one; LPS, lipopolysaccharide; TA, triamcinolone acetonide; dThd, thymidine; RPMI 1640, Roswell Park Memorial Institute Tissue Culture Medium 1640; HBSS, Hank's balanced salt solution.

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were dried, and the radioactivity was counted in 4 ml of 0.4% Omnifluor:xylene. The counting efficiency was 49%.

To determine the effect of glucocorticoids on BCL, cells at various time points after LPS stimulation, \(10^{-5}\) M TA was added to cultures at 6-hr intervals beginning at Time 0. \([\text{H}]\text{dThd}\) was added in every case 6 hr prior to harvesting the cell cultures. Quadruplicate determinations were made, and the mean of 2 experiments was calculated. For each data point, the variability was less than 8%. Using this protocol, (a) the rate of \([\text{H}]\text{dThd}\) incorporation over a 48-hr period was determined, (b) the effect of \(10^{-5}\) TA on \([\text{H}]\text{dThd}\) incorporation at these various time periods was measured, and (c) the effect of a constant exposure time of the cells to TA could be monitored.

Whole-Cell Glucocorticoid-binding Assay. Glucocorticoid receptor-binding studies were performed as described previously (19). The washed BCL, cells were used at a concentration of \(1.33 \times 10^7\) cells/ml in HBSS. Cells (0.6 ml) were added to tubes containing various concentrations of \([\text{H}]\text{TA}\) without (to determine total binding) or with 100-fold excess unlabeled TA (to determine nonspecific binding) or other steroid hormones for competition studies, to give the final steroid concentration indicated. Cells were incubated at 22° for 2 hr in a shaking water bath. They were gently vortexed at 30-min intervals. After 2 hr, the cells were collected mechanically onto glass fiber filters and washed with HBSS. The filters were dried overnight at room temperature, placed in 4 ml of scintillation fluid described above, and the radioactivity was determined. Exact hormone concentrations were determined by counting 0.05-ml aliquots of each \([\text{H}]\text{TA}\) concentration in 0.04% Omnifluor:25% Triton X-114:xylene. Tritium efficiency was 41%. Specific binding at each concentration was determined (total binding minus nonspecific binding), and Scatchard analysis was performed (20).

In order to compare glucocorticoid receptor characteristics in BCL, cells prior to and at the peak of LPS-induced mitogenesis, some of the lymphocytes were incubated at a concentration of \(1 \times 10^7\) cells/ml with LPS (50 \(\mu\)g/ml) in culture medium for 24 hr in sterile Costar flasks at 37°. Flasks were placed in 5% CO\(_2\) in air and rocked for the incubation period. At 24 hr, the BCL, cells were washed 3 times at 4° in HBSS by centrifugation at 400 \(\times\) g for 10 min. The cell concentration was adjusted to \(1.33 \times 10^7\) cells/ml in HBSS. The whole-cell binding assay and competition experiments were performed as described above.

RESULTS

dThd Incorporation in LPS-stimulated BCL, Cells. BCL, cells at a concentration of \(2 \times 10^6\) cells/ml showed a 3-fold increase in \([\text{H}]\text{dThd}\) incorporation at 24 hr when stimulated with LPS at a concentration of 50 \(\mu\)g/ml (data not shown). When hormones were added with the LPS (Chart 1), the incorporation of \([\text{H}]\text{dThd}\) was decreased at 24 hr by dexamethasone and TA in accordance with their reported affinities for the glucocorticoid receptor (10). At a concentration of \(10^{-8}\) M, \([\text{H}]\text{dThd}\) incorporation was reduced 80 and 53% in the presence of TA and dexamethasone, respectively. Over a concentration range of \(10^{-10}\) to \(10^{-6}\) M, estradiol had no effect on \([\text{H}]\text{dThd}\) incorporation. Thus, BCL, cells were specifically sensitive to glucocorticoids as measured by dThd incorporation.

Cortisolone and progesterone, steroids with antiglucocorticoid action (13), were used in conjunction with TA to determine whether steroid effects on \([\text{H}]\text{dThd}\) incorporation were glucocorticoid specific and mediated by the glucocorticoid receptor. At a concentration of \(10^{-8}\) M, \([\text{H}]\text{dThd}\) incorporation was reduced 80% in the presence of cortisolone and progesterone, respectively. Over a concentration range of \(10^{-10}\) to \(10^{-6}\) M, estradiol had no effect on \([\text{H}]\text{dThd}\) incorporation. Thus, BCL, cells were specifically sensitive to glucocorticoids as measured by dThd incorporation.

Cortisolone and progesterone, steroids with antiglucocorticoid action (13), were used in conjunction with TA to determine whether steroid effects on \([\text{H}]\text{dThd}\) incorporation were glucocorticoid receptor mediated. At a concentration of \(10^{-8}\) M, TA caused an 80% reduction in \([\text{H}]\text{dThd}\) incorporation, which was partially blocked by 5 \(\times\) \(10^{-8}\) M cortisolone or progesterone (Chart 2). At this concentration, cortisolone or progesterone alone had no effect on nucleotide incorporation. A 500-fold excess of cortisolone or progesterone was used in these competition experiments due to the lower affinity of these antiglucocorticoids for the glucocorticoid receptor (3, 24) and reports that greater than 300-fold excess progesterone was required to block hydrocortisone inhibition of \([\text{H}]\text{dThd}\) incorporation in chick embryo fibroblast cells (6). Also, antiglucocorticoid concentrations greater than \(5 \times 10^{-8}\) M were toxic for LPS-stimulated lymphocytes. These results, in conjunction with data showing no estradiol effect on \([\text{H}]\text{dThd}\) incorporation during LPS stimulation, suggest that the TA effect on proliferating cells is glucocorticoid specific and mediated by the glucocorticoid receptor.
We were interested to see if TA caused a uniform effect on dThd incorporation at all times following LPS stimulation. To eliminate the problems associated with differences in the duration of steroid present in the incubation medium, measurements of the rate of dThd incorporation were made at 6-hr intervals. TA was added at either the initiation of LPS stimulation or at 6-hr intervals thereafter in a concentration of $10^{-5}$ M. As seen in Chart 3, addition of TA concomitant with LPS stimulation resulted in a reduction of [3H]dThd incorporation at each time interval throughout the incubation period when compared to cultures without TA. The peak rate of dThd incorporation was measured at 24 hr, a time at which TA treatment initiated at Time 0 resulted in a 79% reduction of dThd incorporation from that seen in non-hormone-treated, LPS-stimulated cells. In non-LPS-stimulated cultures, the level of dThd incorporation decreased after 24 hr due to general loss of cell viability of the BCL1 cells in culture (data not shown). However, the stimulated:control ratio after 24 hr remained constant, so that the effect of LPS was still apparent at these later times. With the addition of TA at later times following LPS stimulation, there was a reduction in the rate of dThd incorporation observed at each subsequent interval, but the magnitude of this reduction was progressively less than that seen when steroid was added at Time 0. In order to examine the effect of constant time exposure of the cells to glucocorticoid, the data were replotted as shown in Chart 4. The percentage of inhibition of [3H]dThd incorporation in steroid-treated cells was 80% at 24 hr for cells to which TA was added at Time 0. When TA was added at 6 or 12 hr after LPS, the magnitude of the decrease was less, i.e., [3H]dThd incorporation was inhibited 50 to 35%, respectively, when hormone was present for 24 hr.

Glucocorticoid Receptor-binding Experiments. The state of the glucocorticoid receptor in LPS-stimulated cells at 24 hr was of particular interest. At this time, non-steroid-treated cells were undergoing a maximum rate of [3H]dThd incorporation. We were interested to see whether changes had occurred in the receptor at 24 hr after LPS stimulation when compared to receptor in nonstimulated cells. As seen in Chart 5A, the specific binding of [3H]TA to the glucocorticoid receptor was similar at 0 and 24 hr. Saturation at both times occurred at about 10 nm TA. A Scatchard plot (20) of these data is shown in Chart 5B. At both 0 and 24 hr, Scatchard analysis indicates a class of high-affinity binding sites with equilibrium dissociation constants of 2.7 and 3.4 nm, respectively. This result is comparable to reported dissociation constants of approximately 5 nm in normal mouse spleen cytosol (9) and 18 nm for mouse spleen cells (4). The estimated number of sites per cell calculated at 0 and 24 hr, 6500 and 7900, respectively, is also similar to reports for mouse spleen cells in which about 6000 receptors/cell were found (4, 12). The affinity of the receptor for TA in these BCL1 cells and the number of receptors are in accordance with receptors seen in normal murine spleen cells. More importantly, with LPS stimulation, the affinity and number of receptors/cell are not appreciably altered. However, there may be a class of binding sites with lower affinity in the non-LPS-stimulated cells. Future studies using the cytosol fraction will allow a better analysis of the binding characteristics. As seen in Chart 6, competition of other steroids for the glucocorticoid receptor was also unaltered. Addition of unlabeled TA, corticosterone, or dexamethasone resulted in a 77 to 84% reduction of the total [3H]TA binding. Interestingly, progesterone, which also acts as an antiglucocorticoid under some conditions (3), competed effectively for the receptor.
Glucocorticoids and BCL₁, Cells

The B-cell leukemia, BCL₁, provides a useful cell system in which to study glucocorticoid effects on proliferating B-cells. Like normal B-cells (16, 17), these monoclonal cells are stimulated by LPS, resulting in an increase in dThd incorporation (7). In addition, LPS-activated BCL₁ cells are glucocorticoid sensitive; dexamethasone and TA at concentrations greater than $10^{-9}$ M reduce dThd incorporation. Although the minimum concentration of TA which inhibits [³H]dThd incorporation is about 10-fold greater than seen in normal LPS-stimulated murine B-lymphocytes (16), dThd incorporation is nevertheless inhibited by synthetic steroids.

The results from this study suggest that glucocorticoid sensitivity decreased with time during LPS-induced proliferative events. Addition of $10^{-5}$ M TA with the initiation of LPS stimulation resulted in a 79% decrease in [³H]dThd incorporation at 24 hr. The magnitude of this decrease was markedly less when the hormone was added to the stimulated cells at later times after LPS addition. Addition of TA 6 hr after LPS stimulation resulted in a maximal inhibition of only 57% within the subsequent 24-hr incubation. Similar results have also been obtained by us with normal B-cells in mixed spleen cell cultures (17). Future studies will examine the antibody production by LPS-stimulated BCL₁ cells when TA is added at progressively later times following LPS activation. Together with the dThd incorporation studies, we will then have a better understanding of hormone sensitivity during differentiation.

The decreased dThd incorporation observed when TA was added at subsequent times after LPS stimulation is not due simply to a reduction in the duration of hormone exposure, as seen when the time of hormone exposure is normalized (Chart 4). Earlier reports of glucocorticoid effects on peripheral blood lymphocytes or thymocytes (14, 15) had suggested that lectin stimulation could decrease the sensitivity of these cells to steroids. However, these studies did not control for exposure time to the steroid, so their results may have been due simply to decreased contact time with hormone. More recently, Smith et al. (21) suggested that concanavalin A-stimulated peripheral lymphocytes were sensitive to glucocorticoids regardless of their state of activation. In their studies, exposure time to steroid was kept constant. Our report is the first study of a homogeneous population of B-cells triggered by a B-cell mitogen in which glucocorticoid effects on dThd incorporation were studied prior to and at various times after the initiation of mitogenesis.

There was little change in the number of glucocorticoid receptor sites per cell in LPS-stimulated BCL₁ cells during the maximum rate of dThd incorporation. Studies with concanavalin A-stimulated human peripheral blood lymphocytes (2) or synchronized HeLa cells (1) have correlated increases in receptor number during blast transformation with high rates of dThd incorporation. The reported increase in receptor number in mitogen-stimulated cells could be the result of an increase in the number of cells preparing for mitosis. However, we did not find an increase in glucocorticoid receptor number in BCL₁ cells at the peak of dThd incorporation. It has been reported that at least 35% of BCL₁ cells respond to LPS (9), and the rate of dThd incorporation in our studies is sufficient to indicate that a mitogenic response has occurred. With reported increases of 2- to 3-fold in receptor number during peak dThd incorporation (1, 2), the number of BCL₁ cells that might show increased receptor number should have been sufficient to increase the number of observed sites per cell in the population as a whole. Likewise, BCL₁ cells at the peak of dThd incorporation showed a decreased sensitivity to glucocorticoids, a response that would be unexpected if receptor numbers had increased significantly.

The presence of glucocorticoids at the initiation of LPS stimulation could presumably be preventing dThd incorporation in preparation for cell division or selectively killing those cells which are responding to LPS. Changes in the glucocorticoid responses at later times could be the result of the cells differentiating past a critical glucocorticoid-sensitive phase, but by 24 hr these cells retain the full complement of steroid receptor. Work with human T-cells shows that glucocorticoid sensitivity cannot be assessed solely on the basis of receptor number and affinity (5). Human T-lymphocytes isolated from peripheral blood have been separated into 2 populations on the basis of their Fc receptor, $T₀$ and $T_m$. These 2 groups of cells have similar glucocorticoid receptor-binding properties and receptor capacities, but $T₀$ cells are less sensitive than are $T_m$ cells to dexamethasone as measured by dose response to steroid during peak concanavalin A response. Thus, receptor number is not the only criterion for predicting glucocorticoid response.

Changes in glucocorticoid responsiveness could be due to alterations at other steps in the classical steroid hormone model. Binding to the receptor and the number of sites available in the cytoplasm have been shown to be important in some studies of glucocorticoid-sensitive and -resistant acute lymphocytic leukemia blast cells (10), although the presence of receptor does not assure sensitivity (11). The physicochemical characteristics of the receptor as well as subsequent nuclear events could determine the result of steroid-receptor interaction. In the mouse lymphoma P1798, glucocorticoid-sensitive and -resistant cell line variants have been selected which show different immunochromatographic properties as well as physicochemical properties (22, 23). Glucocorticoid receptor complexes from corticosensitive cells had a Stokes’ radius of 58 to 62 A and were retained by immunocaffinity column, while corticosteroid-resistant receptor complexes with a Stokes’ radius of 27 to 28 A were not retained. Thus, resistance in these cells may be a function of their physical properties.
The mechanism of changes in glucocorticoid responsiveness during mitogenic stimulation is not clear at this time. Despite an increased rate of dThd incorporation at 24 hr, the receptor number did not increase in these cells. Further work with this cell line is clearly indicated. The availability of a homogeneous population of mitogen-stimulatable cells will be helpful in working out a cohesive picture of the events occurring during cell proliferation.

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