Glucocorticoid Receptors in Peripheral Blood Lymphocytes from Bovine Leukemia Virus-infected Cows with Persistent Lymphocytosis

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ABSTRACT

Bovine leukemia virus-infected cows with persistent lymphocytosis have an expanded population of B-lymphocytes in the peripheral blood that is sensitive to glucocorticoids in vitro and in vivo. We examined peripheral blood lymphocytes from cows with persistent lymphocytosis for the presence of specific glucocorticoid receptors. Steroid binding in intact cells was determined by a whole cell competitive binding assay using \[^{1}H\]dexamethasone. The binding of the glucocorticoid to receptor was characterized in terms of affinity, specificity, and kinetics of the reaction. We found that peripheral blood lymphocytes from three cows with persistent lymphocytosis had 5000 to 6600 specific glucocorticoid-binding sites/cell. Compared with that reported for human lymphoid cells, glucocorticoid receptors in the bovine lymphocytes were found to have a greater affinity for the steroid with an association rate that was three times faster and a dissociation rate that was less than one-half of the former. We examined the biological half-life of hydrocortisone in the normal cow and found it to be 69.3 min, which is shorter than that reported for other domestic species and humans. The kinetics and affinity of the steroid binding may explain why in vivo glucocorticoid sensitivity was demonstrated in these animals despite the fact that elevated levels of plasma corticoids were not maintained. These results suggest that glucocorticoid sensitivity may be influenced by the nature of the binding reaction between steroid and receptor.

INTRODUCTION

Glucocorticoids affect a variety of lymphocyte functions (5). It is generally accepted that these effects are influenced by both the number of GR's present in the cell and the nature of the steroid-receptor interaction (1). Several investigators have shown a correlation between the presence of GR's in lymphoid cells and the response to steroids in vitro and in vivo (2, 8, 11, 12, 16, 22). How the binding reaction itself relates to the drug effect, however, has not been well documented.

BLV infection in cattle is associated with both a benign lymphoproliferative condition called persistent lymphocytosis and lymphosarcoma, a lymphoid cancer. It is now well established that in cows with PL, the increase in PBL is largely due to an expanded B-cell population (20, 26, 33) which is BLV negative (9), undergoes a high rate of blastogenesis in tissue culture (19), and probably represents an immunological response to viral antigens or tumor cells (3). We have recently shown that this reactive lymphocyte population is sensitive to glucocorticoids both in vitro and in vivo (3). Moreover, systemic treatment with prednisolone acetate causes a selective depletion of these cells from the circulation even when significant elevations in total plasma corticoids are not maintained.

In this study, we have examined PBL from cows with PL for the presence of GR's. We have characterized the binding of glucocorticoid to receptor in terms of affinity, specificity, and kinetics of the reaction. A possible relationship between the nature of the binding reaction of the steroid hormone to receptors in PBL from cows with PL and the glucocorticoid sensitivity of these cells is postulated.

MATERIALS AND METHODS

Three BLV-infected cows with PL were selected from Jersey Herd BF of the Leukemia Research Unit at New Bolton Center, the large animal clinic and research facility of the University of Pennsylvania School of Veterinary Medicine. Animals selected from this herd were clinically normal adult cows 7 to 11 years of age with total lymphocyte counts ranging from 19,000 to 40,000 lymphocytes/cu mm, of which 84 to 92% carried surface immunoglobulins detectable by membrane immunofluorescence. All 3 cows had antibodies to internal virus antigen, and their PBL induced syncytia in bovine embryo spleen monolayer cell cultures (7).

Cell suspensions were prepared from fresh heparinized peripheral blood collected aseptically by jugular venipuncture. PBL were separated from the peripheral blood by discontinuous density gradient centrifugation over Ficoll-Hypaque, washed 3 times in EBSS at 4°C, and suspended at a cell concentration varying between 5 and 10 x 10⁶ cells/ml in Medium 199. Lymphocyte suspensions prepared in this way contained greater than 95% mononuclear cells.

Specific glucocorticoid binding was measured using a whole cell competitive binding assay described by Lippman and Barr (13). \[^{1}H\]Dexamethasone (6,7-\[^{3}H\]dexamethasone; specific activity, 37.6 Ci/mmol; New England Nuclear, Boston, Mass.) in ethanol was diluted in Medium 199 to yield a concentration 10 times the final concentration desired. Final ethanol concentration was less than or equal to 0.1%. Dexamethasone was selected over hydrocortisone because it will not bind transcortin, corticosteroid-binding globulin (15), which may be included in small amounts of serum proteins that remain adherent to cells throughout the purification procedure. Aliquots of 0.1 ml
of the 10-fold concentration of \([^{3}H]dexamethasone\) were placed in six 12- x 75-mm glass tubes/concentration of radioactive steroid tested. Into 3 of these tubes was placed 0.1 ml Medium 199. A solution of unlabeled dexamethasone (0.1 ml) at a concentration calculated to provide a 100-fold excess of cold steroid was placed into the remaining 3 tubes. An aliquot of 0.8 ml of the cell suspension was then delivered into each tube, resulting in the final desired concentration of cells and \([^{3}H]dexamethasone\). This resulted in 6 tubes containing the same concentrations of cells and radiolabeled dexamethasone with 3 of these tubes containing a 100-fold excess of unlabeled competing glucocorticoid which were used to determine non-specific binding. These tubes were gently mixed and incubated at 21 °C in a shaking incubator for 2 hr. Every 15 min, the cells were gently resuspended by brief mixing. Kinetic studies described below indicate that this interval exceeds that required for maximal binding. After incubation, 2 ml of cold EBSS were rapidly added to each tube, and the cell pellets were collected by centrifugation at 600 x g for 5 min at 4°C, resuspended, and washed twice more in ice-cold EBSS. Viability as determined by trypan blue exclusion was greater than 90% under these assay conditions. After the final wash, the cell pellets were resuspended in 0.5 ml of EBSS, and the suspension was transferred to liquid scintillation vials along with 2 aliquots (0.5 ml) used to rinse each tube. The samples were counted in 12 ml Scintisol (Isolab, Inc., Akron, Ohio) in a Packard Tri-Carb liquid scintillation counter using an automatic standard. The counting efficiency for each sample was calculated using a quench curve and ranged from 21 to 27%. Cytoplasmic binding sites/cell were calculated, and the equilibrium dissociation constants were obtained from Scatchard analysis (28).

The plasma half-life of hydrocortisone in the cow was studied by placing an i.v. catheter in the jugular vein of a 7-year-old 538-kg Guernsey cow in good health. Twenty-four hr following catheterization, 260 mg of hydrocortisone 21-sodium succinate (Sigma Chemical Co., St. Louis, Mo.) dissolved in a 20% ethanol solution were administered in the uncatheterized jugular vein. Heparinized blood samples were collected at intervals of 15 min for the first hr, 30 min for the next 2 hr, and hourly for a total of 24 hr. The samples were centrifuged at 600 x g for 10 min within 15 min of collection. The plasma was removed and stored at —20°C until assayed for total corticoids using a competitive protein-binding assay described in detail by Murphy (18).

RESULTS

The affinity of glucocorticoid binding was determined by measuring the uptake of \([^{3}H]dexamethasone\) at varying concentrations ranging from 1.9 x 10^{-10} to 1.3 x 10^{-8} M. The binding curves obtained using PBL from 3 cows with PL are shown in Chart 1A. Both the number of sites/cell (5000 to 6600) and the shape of the curves are similar with approximately 90% saturation at a concentration of 3.3 x 10^{-9} M dexamethasone. Chart 1B shows the data replotted for the 3 curves by the Scatchard technique (28). Parallel straight lines were obtained indicating similar dissociation constants (0.87 to 1.1 x 10^{-8} M). The linear relationship obtained is consistent with radiolabeled dexamethasone binding to a single class of receptor sites of uniform affinity.

Evidence for specificity of binding is presented in Charts 2...
and 3 which show the ability of unlabeled steroids present in molar excess to compete with $10^{-8}$ M $[^3H]$dexamethasone. The amount of $[^3H]$dexamethasone bound in PBL from BF 238 and BF 250 in the presence of a labeled:unlabeled dexamethasone ratio of 1:1 is approximately 50% of that bound with only the labeled steroid present. The biologically active glucocorticoid hydrocortisone will also compete with labeled dexamethasone, whereas androstenedione, a biologically inactive steroid, and the sex steroids, estrogen and progesterone, compete relatively little (Chart 3).

The rates of association and dissociation between dexamethasone and specific glucocorticoid-binding sites were examined. Chart 4 shows the time course of specific binding of dexamethasone to GR at 21°. At a concentration of steroid sufficient to saturate GR, the reaction has approached equilibrium after 20 min. If second-order kinetics applies in this reaction, a plot of time versus $\log_{10}$ (unbound dexamethasone concentration/unbound specific GR concentration) should give a straight line. Results are seen in the inset and are consistent with second-order kinetics in the binding reaction (27).

The dissociation reaction of the dexamethasone:GR complex was also examined. After the GR was allowed to become saturated with radiolabeled dexamethasone, a 100-fold excess of cold dexamethasone was added as a chase. Specifically bound counts were examined at various time intervals (Chart 5). If the dissociation reaction follows first-order kinetics, then a plot of $\log_{10}$ (bound steroid concentration) versus time should be linear (27). This was found to be true as shown in the inset.

From the association and dissociation rate constants, an overall equilibrium constant ($K_o$) was calculated to be $1.0 \times 10^{-10}$ M as compared to the $8.7 \times 10^{-10}$ to $1.1 \times 10^{-9}$ M determined from the equilibrium data presented in Chart 1.

We studied the total plasma corticoid levels in a normal cow following an i.v. injection of hydrocortisone 21-sodium succinate as described in "Materials and Methods." Chart 6 shows the corticoid levels immediately preceding and for the first 3 hr following the bolus injection. Plasma levels of the corticoid returned to preinjection levels within 45 min. The data were analyzed using a 2-compartmental model (NONLIN Program, Upjohn Co.), and the elimination rate ($t_{1/2} \beta$), or biological half-life, was calculated to be 69.3 min$^{-1}$ with a regression coefficient of -0.9.
but found that the sensitivity of these cells to metabolic inhibition by glucocorticoids did not change.

It is possible that cells may contain apparently adequate or even increased numbers of cytoplasmic receptor activity and yet fail to respond to glucocorticoids due to a defect in one of the several sites distal to that of the initial binding of steroid to receptor. This phenomenon has been observed in rodent and human leukemic cells (14) as well as in glucocorticoid-resistant mutant cell lines (29, 34). Lippman and Barr (13) have also reported that although glucocorticoids do appear to exert differential effects on subpopulations of normal human PBL (T-versus B-lymphocytes), purified T- and non-T-lymphocytes contain equivalent amounts of GR which, by binding affinity and specificity measurements, are indistinguishable from each other (13), suggesting that these differential effects are not mediated by differences in the glucocorticoid receptors.

Glucocorticoid sensitivity varies from species to species as well as among different subpopulations of lymphocytes (4, 5). Although it is generally accepted that the mechanism of physiological action of glucocorticoids is similar between species, it is clear that the effects these drugs exert on the immune responses differ among corticosteroid-resistant species (e.g., guinea pig and human) and those which have been found in vivo to be much more corticosensitive (e.g., mouse and rat) (4). Differences in glucocorticoid pharmacokinetics may account for some of this variation in in vivo effects. How events at the receptor level relate to these species differences has yet to be established.

Recently, Kraft et al. (10) compared the binding affinity of glucocorticoid receptors in guinea pig splenic cells with those of the mouse and found that the former had a 20-fold lower affinity than did its corticosensitive counterpart. The lower binding affinity in the guinea pig correlated with the relatively high concentration of glucocorticoid necessary for half-maximal inhibition of thymidine incorporation in vitro and was associated with the relatively high circulating levels of free cortisol in these species.

Whether the cow is a corticosensitive or corticoresistant species is not yet clear. However, observations by these authors as well as by others (17, 21, 24) suggest that cow lymphocytes are relatively resistant to glucocorticoids in vivo. This may be in some measure due to the fact that it is difficult to maintain high plasma corticoid levels in the cow, the normal
level of which is only about 5 to 10 ng/ml or 10% of that in humans (30). Evidence of this was presented in a previous report (3) where we were unable to maintain elevated plasma corticoid levels by administering 1 g prednisolone acetate. One reason for this may be that ruminants have less than 10% of the cortisol-binding globulin levels seen in human plasma (12). Compatible with these observations is our finding here that the biological half-life of hydrocortisone in the cow is relatively short (69.3 min; Chart 6) compared with that of other species (25, 29).

A possible explanation to the question of why such profound glucocorticoid effects on PBL in cows with PL were observed without maintaining elevated plasma corticoid levels is provided by our data showing the affinity and kinetics of the glucocorticoid binding to the receptor in these cells. In the bovine, 90% saturation of the GR sites took place at a concentration of about 3.3 × 10⁻⁹ M [³H]dexamethasone. This is in contrast with a steroid-sensitive human lymphoid cell population in which only 70% of the receptor sites were saturated using the same technique at a concentration of 8 × 10⁻⁹ M of the radiolabeled steroid as reported by Yarbro et al. (35). Furthermore, the association rate of the binding reaction was 3 times faster in the bovine cells, requiring only 20 min to saturate the receptor sites as compared with 60 min in the human cells.

Finally, the rate of dissociation of the glucocorticoid from the receptor was considerably slower in the bovine where 60% of the specifically bound steroid was still bound after 7 hr of incubation in the presence of a 100-fold excess of unlabeled dexamethasone as compared with that observed in experiments by Yarbro et al. where specifically bound [³H]dexamethasone was no longer observed after incubation with the unlabeled steroid for the same period of time. The greater affinity of the glucocorticoid binding to the specific receptor along with the increased association rate and decreased dissociation rate of the binding reaction in the bovine lymphocytes may explain why these glucocorticoid effects were observed in the face of only minor or transient increases in plasma corticoid levels.

REFERENCES
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