Antibodies against Cortisol Block Suppressive Effects of Corticosteroids on Lymphocytes *In Vitro* (43373)

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Abstract. **Lymphocyte transformation assays were used to test the ability of antibodies against cortisol to reduce bioactivity of corticosteroids *in vitro*. Mononuclear cells were separated from whole bovine blood and cultured in the presence of PHA alone, PHA + steroid, PHA + steroid + anticortisol, or PHA + steroid + anti-bovine serum albumin. Tritiated thymidine uptake was determined for all groups during the last 24 hr of a 72-hr culture period by scintillation counting. Polyclonal anticortisol against cortisol-bovine serum albumin conjugated in the 21 position was more effective in blocking cortisol activity than monoclonal anticortisol built against conjugates in the 3 position. The steroids that suppressed PHA-induced lymphocyte proliferation in a concentration-dependent manner were: cortisol, corticosterone, dexamethasone, prednisolone, 11-deoxycortisol, and 11-deoxycorticosterone. Aldosterone, cortisone, cholesterol, estradiol, and progesterone did not exhibit concentration-dependent effects and, thus, were not considered suppressive. These concentration-independent steroids were also the least suppressive (with the exception of aldosterone). Anticortisol was able to reduce bioactivity of suppressive corticosteroids that had an 11-hydroxy group, suggesting the antibody was primarily made against this site. Anti-BSA was not effective in blocking corticosteroid activity, but it did enhance proliferation of lymphocytes if added in combination with weakly suppressive steroids. Anticortisol also had an enhancing effect when added with some weakly suppressive steroids. We conclude that antibodies against cortisol are capable of reducing bioactivity of steroids that strongly suppress lymphocyte proliferation. Additionally, the 11-hydroxy group may be an important antigenic determinant of steroid molecules.** [P.S.E.B.M. 1992, Vol 199]

Corticosteroids have been found to suppress lymphocyte proliferation induced by mitogens, most likely by inhibiting mitogen-induced DNA synthesis (1–4). These effects may occur indirectly, via inhibition of both interleukin 2 production by lymphocytes and their ability to respond to interleukin 1 (1, 5).

Corticosteroids may also act directly on lymphocytes. Intracellular glucocorticoid receptors have been found in human lymphocytes (6), and were found to vary in number according to the stage of the cell cycle (7). Direct actions of corticosteroids on lymphocytes include: changes in intracellular ratios of cyclic nucleotides, alteration of plasma membrane structure, and disruption of ion channels. These actions likely prevent synthesis of new proteins, such as lymphokines, or surface antigens, which are necessary for a subsequent immune response (8–11).

The suppressive effects of corticosteroids are likely responsible for stress-associated diseases such as the bovine respiratory disease complex (shipping fever), in which normally nonpathogenic bacteria are allowed to cause pneumonia. Shipping fever is found to occur most frequently during stressful situations such as dehorning, branding, shipping, and handling of feedlot cattle. These activities have been shown to increase plasma concentrations of cortisol (12–17) and decrease lymphocyte blastogenesis (18). Our hypothesis was that suppressive effects of corticosteroids could be overcome by reducing bioavailability of these steroids.

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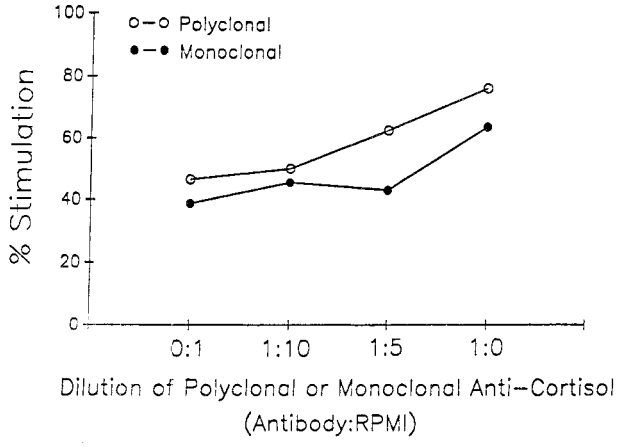


Figure 1. Ability of polyclonal and monoclonal anticortisol to overcome cortisol-induced suppression of lymphocyte proliferation *in vitro*. Increasing the concentrations of both polyclonal and monoclonal anticortisol from 0/1 (no anticortisol) to 1/0 (undiluted) bound increasing amounts of cortisol, thus allowing increased proliferation of lymphocytes.

The objectives of the present study were to determine whether the suppressive properties of cortisol could be blocked *in vitro* by antibodies against cortisol, and to determine the type of antibody necessary to achieve these effects. In addition, we wished to examine the suppressive properties of various corticosteroids and the effect of anticortisol on the suppression, thereby gaining insight into the binding site of the antibody by comparing steroid structures with antibody effectiveness.

Materials and Methods

Blood was collected from feedlot heifers by aseptic jugular venipuncture in heparinized tubes (Becton Dickinson and Co., Rutherford, NJ). Whole blood was diluted 1/3 with calcium-magnesium-free Hanks' balanced salt solution within 20 min of collection. White blood cells were separated by adding Histopaque-1077 (Sigma, St. Louis, MO) to diluted whole blood and centrifuging for 30 min at 2000*g* and 22°C. Mononuclear leukocytes collected from the diluted blood-Histopaque interface were washed three times with calcium-magnesium-free Hanks' solution containing 10% Alsevers solution, then collected by centrifugation for 10 min at 1000*g* and 4°C. The washed cells were resuspended in 3 ml of complete medium (complete RPMI). Complete RPMI contained RPMI 1640 (Sigma), 10 m*M* Hepes buffer, 2 m*M* L-glutamine, 100 units of penicillin, 100 μg/ml of streptomycin, and 8% fetal calf serum. Lymphocytes were counted using a Neubauer hemocytometer (American Optical, Buffalo, NY) and stored for 1 to 24 hr at 4°C in complete RPMI before culturing.

Steroids. Dexamethasone (1,4-pregnadien-9α-flouro-16α-methyl-11β,17α,21-triol-3,20-dione; Azium; Schering Veterinary, Kenilworth, NJ) and prednisolone (1,4-pregnadien-11β,17α,21-triol-3,20-dione; Solu-Delta-Cortef; Upjohn, Kalamazoo, MI) were diluted in complete RPMI so that culture medium contained 0.1, 0.01, or 0.001 ng of dexamethasone/ml and 1.0, 0.1, or 0.01 ng of prednisolone/ml. Other steroids were obtained from Steraloids (Wilton, NH), dissolved in absolute ethanol, then diluted in complete RPMI (without fetal calf serum). Ethanol content did not exceed 1.0% in solutions and did not affect lymphocyte proliferation at these concentrations (data not shown). Concentrations of steroids in culture medium were chosen to represent physiologically circulating levels of steroids and/or relative potency and were as follows: cortisol (4-pregnen-11β,17α,21-triol-3,20-dione), 1.0, 0.1, and 0.01 ng/ml; cortisone (4-pregnen-17α,21-diol-3,11,20-trione), 8.0, 0.8, and 0.08 ng/ml; corticosterone (4-pregnen-11β,21-diol-3,20-dione), 1.0, 0.1, and 0.01 ng/ml; 11-deoxycortisol (4-pregnen-17α,21-diol-3,20-dione), 10.0, 1.0, and 0.1 ng/ml; 11-deoxycorticosterone (4-pregnen-21-ol-3,20-dione), 10.0, 1.0, and 0.1 ng/ml; aldosterone (4-pregnen-11β,21-diol-3,18,20-trione), 50.0, 5.0, and 0.5 ng/ml; progesterone (4-pregnen-3,20-dione), 1.0, 0.1, and 0.01 ng/ml; estradiol (1,3,5(10)-estratrien-3,17β-diol), 1.0, 0.1, and 0.01 ng/ml; and cholesterol (5-cholesten-3β-ol), 100.0, 10.0, and 1.0 ng/ml.

Antibodies. Polyclonal anticortisol and anti-bovine serum albumin (BSA) antibodies were collected from rams immunized with cortisol-BSA conjugate (Sigma) or against BSA, respectively. All rams were initially injected subcutaneously with 1.0 mg of antigen dissolved in 1.0 ml of 0.9% saline and emulsified in 1.0 ml of complete Freund's adjuvant. Booster immunizations of antigen emulsified in incomplete Freund's adjuvant were given three to four times. Serum was harvested, passed through a 0.45-μm filter, aliquoted in 3-ml vials, and stored frozen at −20°C. Titers (dilution of anticortisol at which 50% [^{3}H]cortisol bound) of polyclonal anticortisol were determined on all immunized rams by radioimmunoassay (19). Titers from one ram were consistently around 1/400 and antiserum from this ram was used throughout the remainder of the study. Serum from this ram was calculated to contain 4.4×10^{-12} moles of anticortisol/20 μl.

Monoclonal anticortisol-3 was obtained from BioPacific (Emeryville, CA), diluted to 0.5 mg/ml of RPMI, and stored at −20°C. Undiluted monoclonal anticortisol solution was calculated to contain 2.3×10^{-9} moles/20 μl.

Lymphocyte Transformation Assay. Lymphocyte proliferation was quantified by incorporation of [^{3}H] thymidine into lymphocyte DNA by the methods described by Tjoelker *et al.* (20). Briefly, bovine lymphocytes (2×10^5) were added to each well in a volume of 125 μl of complete RPMI. Phytohemagglutinin (PHA) diluted in RPMI was added to cells at 0.05 μg/well. In

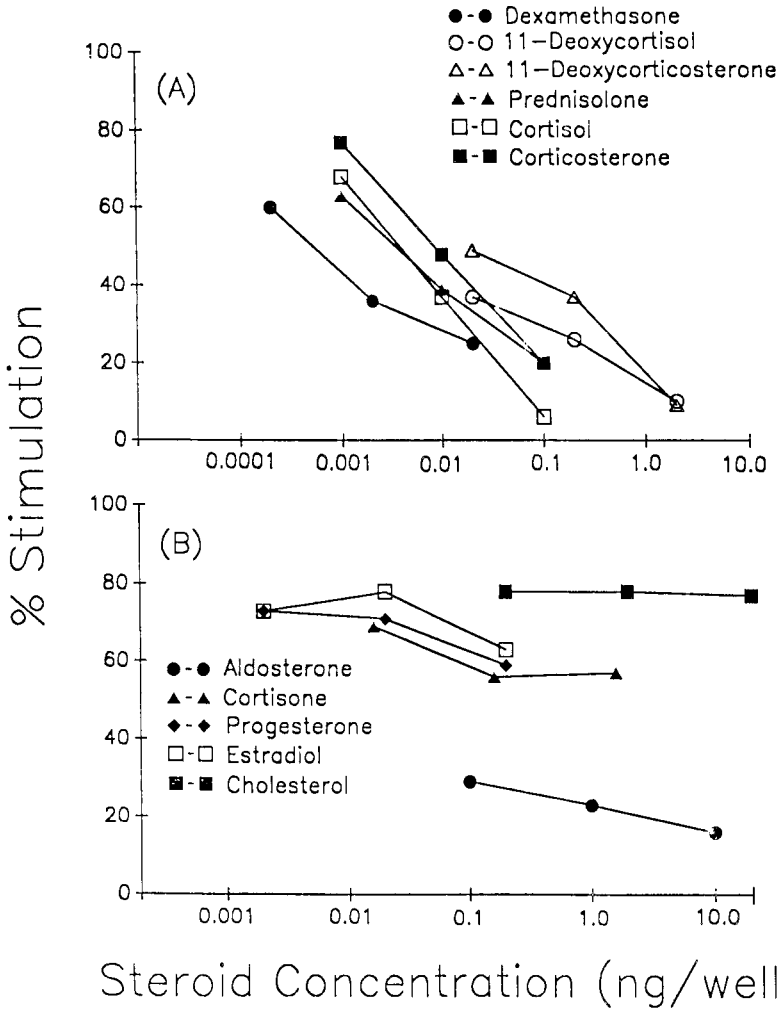


Figure 2. (A) Steroids in which increasing concentrations caused a decrease in lymphocyte proliferation (concentration dependent) and were different from mitogen alone ($P < 0.05$). (B) Steroids in which increasing concentrations did not inhibit mitogen-induced proliferation of lymphocytes at the concentrations used in this study.

order to determine the dilution of anticortisol necessary to overcome the suppressive effects of steroids, both polyclonal and monoclonal antibodies were added to wells at dilutions of 1/0 (undiluted), 1/5, and 1/10 in a volume of 20 μl/well. Cells, PHA, anticortisol, and steroid were cultured in a final volume of 195 μl of complete media at 38°C and 5% CO_2 for 48 hr. Tritiated thymidine (1.0 μCi/well), (20 μl of 6.7 Ci/mmol of thymidine; NEN, Wilmington, DE) was added and cells were further cultured for 24 hr. Cells were collected on glass microfiber filters (Whatman, Clifton, NJ) using a cell harvester. Filters were air dried for 4 hr, placed in vials with 4 ml of scintillation fluid, and counted for 1 min in a scintillation counter (Beckman Instruments, Fullerton, CA).

Statistical Analysis. In order to evaluate the response of lymphocytes to various treatments, a stimulation index (SI) was calculated as follows: SI = cpm (well with PHA)/mean cpm of wells containing no PHA. The ability of steroids to suppress lymphocyte proliferation was determined by calculating percentage of suppression as follows: % suppression = [1-(SI treatment/SI control treatment)]*100. Percentage of suppression was calculated using (i) steroid + PHA as treatment with PHA alone as control treatment; (ii) steroid + PHA + anticortisol as treatment with PHA + anticortisol as control treatment; and (iii) steroid + PHA + anti-BSA as treatment with PHA + anti-BSA as control treatment. For clarity of presentation, percentage of stimulation was calculated (1 − % suppression), and reflects graphically the ability of PHA to stimulate lymphocyte proliferation in the presence of various treatments (y axis of Figs. 1, 2, and 3).

Differences in the amount of lymphocyte proliferation in response to various treatments were determined using the general linear models procedure of the statistical analysis system (21). In cases in which treatment differences were found ($P < 0.05$), individual treatment effects were determined using least significant difference (22). Concentration dependency was deter-

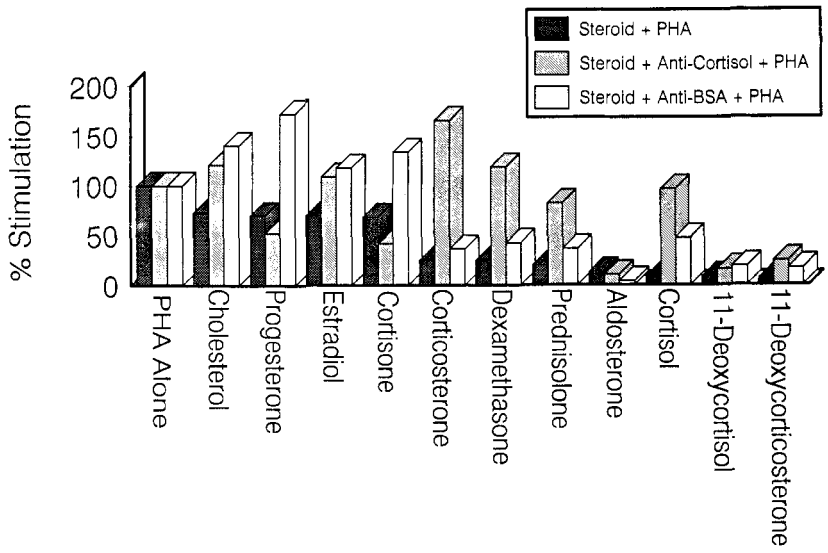


Figure 3. Response of lymphocytes to PHA + steroid, PHA + steroid + anticortisol, and PHA + steroid + anti-BSA. Steroids were most suppressive when percentage of stimulation was closest to 0% compared with PHA alone (100%). When steroids were blocked by anticortisol, stimulation of lymphocytes by PHA returned to nearly 100%. Anti-BSA did not block steroid activity, but did enhance proliferation of lymphocytes, especially with weakly suppressive steroids.

mined by regression analysis of response curves for individual steroids (22).

Results

Antibodies against cortisol were capable of overcoming cortisol-induced suppression of lymphocyte proliferation in eight separate experiments. Increasing concentrations of both polyclonal and monoclonal anticortisol caused a decrease in the ability of 1.0 ng/ml of cortisol to suppress lymphocyte proliferation, with maximal blocking of cortisol activity occurring when anticortisol was undiluted (Fig. 1). Undiluted polyclonal anticortisol (4.4×10^{-12} mole/20 μl) had the greatest cortisol-blocking effect and was used in all subsequent experiments.

Suppressive effects of steroids that were classified as concentration-dependent are illustrated in Figure 2A. Suppression by steroids that were not concentration-dependent is shown in Figure 2B. Steroids that were both concentration-dependent and different ($P < 0.05$) from PHA alone were: cortisol, corticosterone, dexamethasone, prednisolone, 11-deoxycortisol, and 11-deoxycorticosterone. Other steroids (aldosterone, cortisone, cholesterol, estradiol, and progesterone) were not considered suppressive at the concentrations used in this study because of the lack of concentration-dependent response. The highest concentration of each steroid was used for subsequent experiments.

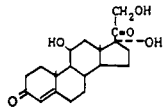
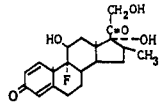
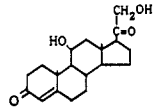
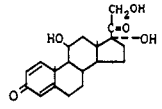
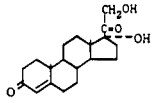
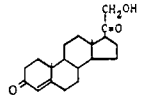
The suppressive ability of individual steroids and effect of anticortisol or anti-BSA on this suppression is illustrated in Figure 3. The amount of lymphocyte proliferation in wells with steroid plus PHA was less ($P < 0.05$) than the amount for wells with PHA alone for all steroids. Concentration-dependent steroids, with the exception of aldosterone, were more suppressive than steroids that did not exhibit concentration dependency. Antibodies against cortisol negated the suppressive effects of corticosterone, dexamethasone, cortisol, and prednisolone ($P < 0.05$). Suppressive steroids that were not significantly affected by anticortisol were 11-deoxycorticosterone and 11-deoxycortisol. Steroids that were most suppressive in culture with PHA alone were also most suppressive when anti-BSA was added ($P < 0.05$). Steroids that were only slightly suppressive with PHA alone enhanced lymphocyte proliferation in the presence of anti-BSA ($P < 0.05$).

Discussion

In the present study, polyclonal antibodies against cortisol were more effective in blocking the effects of cortisol than monoclonal anticortisol. Polyclonal antibodies were made by immunization with BSA bound to the 21 position of cortisol, and monoclonals against cortisol with BSA bound in the 3 position. Furthermore, polyclonal antibodies built against 3-position conjugates of cortisol-BSA failed to affect suppressive properties of corticosteroids (data not shown). From these results, it appears that the end of the steroid molecule away from the 21 position may be responsible for biologic activity. However, the use of polyclonal anticortisol does not provide conclusive evidence as to the active site of steroid molecules.

Cortisol was found to decrease lymphocyte proliferation in the presence of PHA as a mitogen. Other investigators have found PHA-stimulated lymphocytes to be unresponsive to cortisol (23). These investigators, however, used a higher concentration of PHA and slightly different assay procedures. Additionally, lym-

Table I. Structural Relationship Between Steroids and Ability of Polyclonal Anticortisol to Overcome Steroid-Induced Suppression of Lymphocyte Proliferation

Suppressive steroid	Structure	Blocked by anticortisol?
Cortisol		Yes
Dexamethasone		Yes
Corticosterone		Yes
Prednisolone		Yes
11-Deoxycortisol		No
11-Deoxycorticosterone		No

phocytes isolated by our protocol may be from a different subset, as the response to mitogens differs with different lymphocyte populations (24). Other investigators have found lymphocytes from cows (25) and sheep (26) to have decreased proliferation in the presence of PHA and cortisol.

Only steroids that significantly suppressed lymphocyte proliferation in the presence of PHA and suppressed in a concentration-dependent manner were classified as suppressive. Different concentrations of most steroids were used and, therefore, comparison of suppressive abilities of steroids based on equal concentrations was not made in the present study. Relative potencies of different steroids have been documented (27).

The inability of estrogen and progesterone to block lymphocyte proliferation was somewhat surprising in light of previous findings in which these steroids prevented uptake of [^{3}H]thymidine into human lymphocytes (28). These investigators, however, used high concentrations of steroids (up to 20 μg/ml) and concentrations used in the present study (1.0–0.01 ng/well) were closer to those commonly assayed in bovine blood. Progesterone may act physiologically as an immunosuppressive agent during the luteal phase of sheep and, probably, pregnancy, times when local concentrations of progesterone may reach 450 ng/mg of uterine tissue (29).

While undiluted anticortisol serum was effective in blocking the suppressive effects of some steroids, it was not effective in blocking others. As shown in Table I, polyclonal antibodies made in the present study were only effective if the steroid molecule contained an 11-hydroxy group. This finding leads us to believe that our antibody primarily binds to the 11-hydroxy and somehow prevents biologic activity, most likely by preventing uptake of the steroid molecule through the cell membrane. Steroid-binding proteins in serum have been found to prevent uptake of steroids by cells (30), although the site at which they bind to the steroid molecule is unknown.

Lymphocyte proliferation was increased (above PHA-steroid-treated cells) when weakly suppressive steroids were added in combination with anticortisol or anti-BSA. Possibly, an unknown serum factor is capable of interacting with certain steroids to stimulate lymphocyte proliferation above that of PHA alone. Alternatively, these steroids may be metabolized by lymphocytes into molecules that can be utilized for growth by the cells.

From the results of this study, we conclude that antibodies against cortisol are capable of blocking suppressive effects of cortisol *in vitro*. Additionally, polyclonal antibodies produced in the present study appear to be made against the 11-hydroxy position of steroids, implying that this is an important antigenic site of steroid molecules.

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