Ovine Corticotropin-Releasing Factor Increases Endocrine and Immunologic Activity of Avian Leukocytes *In Vitro* (43204)

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Abstract. **Treatment of splenic leukocytes from Cornell K strain male chickens (homozygous at the B^{15} locus of the major histocompatibility complex) with ovine corticotropin-releasing factor (oCRF), before their co-incubation with naive chicken adrenal cells, resulted in an increase in corticosterone production. Supernatants from the oCRF-treated splenic leukocytes caused a time-dependent increase in corticosterone production when incubated with chicken adrenal cells. Adding oCRF directly to chicken adrenal cells did not increase corticosterone production. Pretreatment of peripheral leukocytes with oCRF increased their activity in a concanavalin A mitogen assay. Thus, chicken leukocytes stimulated with corticotropin releasing factor appear to increase the production of an "adrenocorticotropin-like" substance (adrenocorticotropin-like because it increases corticosterone production by adrenal cells), and increased their cell-mediated immune activity.** [P.S.E.B.M. 1991, Vol 196]

Evidence indicates that, in addition to the pituitary, an adrenocorticotropin (ACTH)-like peptide is produced by some, if not all, leukocytes. Cultured human mononuclear leukocytes have been shown to produce an ACTH-like peptide when induced by virus (1). The presence of this ACTH-like peptide was determined by immunofluorescent staining of the lymphocytes with antiserum specific to ACTH-(1–13) amide (1). This ACTH-like peptide (immunoreactive ACTH or ir-ACTH) is similar to pituitary ACTH because (i) it has the same molecular weight (1); (ii) both pituitary and lymphoid cells synthesize pro-opiomelanocortin, the precursor protein for ACTH (2); (iii) both leukocyte ACTH and synthetic ACTH stimulate steroid production in cultured Y-1 adrenal tumor cells (3, 4); and (iv) production of both is suppressed by dexamethasone *in vivo* (5). Leukocyte ACTH has been identified in mouse splenic monocytes (6) and is synthesized by B cells when stimulated by bacterial lipopolysaccharide (LPS) (3). In addition, evidence exists that human peripheral mononuclear leukocytes possess ACTH receptors similar in affinity and number to adrenal-cell ACTH receptors (7).

A rise in plasma corticosterone followed infection with Newcastle disease virus in hypophysectomized mice, and mononuclear splenocytes showed positive immunofluorescence to antiserum against ACTH-(1–13) amide (5). Both responses were suppressed by dexamethasone (5).

Lymphocytes from White Rock female chickens also produce an ACTH-like substance (8). Lymphocytes from chickens injected with *Salmonella pullorum* antigen, co-incubated with adrenal cells, cause a significant increase in corticosteroid production over non-challenged controls (8).

Human peripheral leukocytes, incubated in various concentrations of synthetic ovine CRF, produce ir-ACTH and β-endorphin (4), as determined by a specific antibody immunofluorescence response to ACTH-(1–13).

The present study was done to determine if CRF stimulates ACTH-like activity in chicken leukocytes as measured by bioassay on isolated avian adrenal cells, and whether the CRF altered the mitogenic activity of avian leukocytes.

Materials and Methods

Experimental Animals. Cornell K-strain Single Comb White Leghorn male chickens, which are homo-

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Received June 25, 1990. [P.S.E.B.M. 1991, Vol 196]
Accepted November 20, 1990.

0037-9727/91/1964-0390$3.00/0

zygous at the B^{15} locus of the major histocompatibility complex (9), were used in all experiments. Six birds were used as spleen donors and eight birds were used as adrenal donors in each of the first two experiments (leukocyte-adrenal cell incubation and leukocyte supernatant cell incubation).

Isolation of Splenic Leukocytes. Birds were sacrificed by cervical dislocation. Spleens were dissected, trimmed of fat and connective tissue, minced, and expressed through a coarse mesh screen into RPMI 1640 media. The suspension was transferred to 50-ml centrifuge tubes, vortexed, and allowed to stand for 6 min to allow the heavier cells and pieces of tissue to settle. The suspended leukocytes were decanted and concentrated by centrifugation for 7 min at 220*g*. Cells were resuspended in 4 ml of fresh RPMI 1640 medium, layered onto 3 ml of Ficoll-paque (1.077 g/cm^3; Pharmacia Fine Chemicals, Piscataway, NJ), and centrifuged for 30 min at 220*g*. The white cell layer (upper cell layer) was removed and washed in RPMI 1640 medium. Cells were kept on ice during and between all steps. Live-dead counts were made using a hemocytometer and trypan blue exclusion.

Splenic Leukocyte Cell Culture. Ten million leukocytes, suspended in 100 μl of RPMI 1640 medium, were added to 800 μl of RPMI 1640 medium containing 2% chicken serum stripped of endogenous steroids with alkaline-washed charcoal (Norit A, 50 mg·ml^{-1}; Fisher Scientific, Atlanta, GA) (10) and of ACTH with silica (QUSO G-32; PQ Corp., Valley Forge, PA) (11) in 24-well plates (3.5 ml/well). To these cultures, ovine corticotropin-releasing factor (oCRF) (Dr. A. V. Schally, Veterans Administration Hospital, New Orleans, LA) was added in appropriate concentrations as described below. These cultures were then incubated in 24-well plates at 37°C in an atmosphere of 5% CO_2 for 48 hr. Harvested cells were transferred to 5-ml plastic tubes and centrifuged for 15 min at 220*g*. The supernatant was saved and cells were resuspended in 3 ml of fresh RPMI 1640 medium. Supernatants or cells were used according to experimental protocol. Total cell counts and viability were determined as before.

Isolation of Adrenal Cells. Nonantigen-stimulated male chickens were sacrificed by cervical dislocation and their adrenal glands were excised and pooled into incubation medium (5.0 mg·ml^{-1} bovine serum albumin dissolved in ice-cold Krebs-Ringer-Hepes buffer with glucose). Adrenals were trimmed of connective tissue and fat, minced, and placed into a 50-ml conical centrifuge tube containing 2 ml of digestion medium [2.0 mg·ml^{-1} bovine serum albumin, 2.0 mg·ml^{-1} collagenase, and 0.1 mg·ml^{-1} lima bean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) dissolved in Krebs-Ringer-Hepes buffer with glucose]. The tube was capped, placed on its side in a Dubnoff shaker, and incubated with shaking for 20 min at 38°C. The incubate was expressed through a series of five pipette tips with decreasing tip diameter. Each tip was used 20 times. The supernatant was then poured into a 15-ml centrifuge tube kept on ice. This process was repeated twice to produce a fine mixture of cells, then the combined supernatants were filtered through a double layer of cotton gauze and centrifuged at 1085*g* for 10 min at 4°C. The supernatant was decanted, and cells were washed in 10 ml of incubation medium and recentrifuged. The supernatant was again decanted and the cells were resuspended in 4 ml of incubation medium. Cell viability and number were determined using a hemocytometer and trypan blue exclusion.

Adrenal Cell-Leukocyte Incubation. One million adrenal cells, 10^7 leukocytes (primed with either 0, 5, 50, or 500 ng/ml oCRF), 700 μl of RPMI 1640 medium, 800 μl of bovine serum albumin incubation medium (5.0 mg·ml^{-1} bovine serum albumin dissolved in Krebs-Ringer-Hepes buffer with glucose), and 300 μl of stripped chicken serum were placed in 50-ml round-bottomed tubes and incubated in a Dubnoff shaker for 2 hr in a 95% O_2:5% CO_2 atmosphere at 38°C. Cultures were then transferred to 12- × 75-mm test tubes and centrifuged for 15 min at 650*g*. An aliquot of each supernatant was saved to measure corticosterone.

Adrenal Cell-Leukocyte Supernatant Incubation. Supernatants instead of cells from the 48-hr, CRF-primed splenic leukocyte cell culture were used in this experiment. The experiment was performed identically to the adrenal cell-leukocyte incubation, except that 1 ml of the splenic leukocyte supernatants was substituted for the 10^7 leukocytes in 700 μl of RPMI 1640 medium and cells were incubated for 4, 6, and 12 hr in addition to 2 hr. Concentrations of 0, 50, 500, and 1000 ng/ml oCRF were used. Because the supernatants contained oCRF, 1-ml controls of the same oCRF concentrations used above were substituted for the supernatants to determine whether oCRF had a direct effect on adrenal cells.

Corticosterone Radioimmunoassay. Total corticosterone was measured by radioimmunoassay (Cambridge Medical Diagnostics, Billerica, MA) and counted on a gamma counter (1272 Clinigamma; LKB-Wallach, Gaithersburg, MD).

Mitogen Assay. Three milliliters of blood were collected from six 8-week-old control birds via the ulnar vein. Cells were isolated as before and cell number and viability were determined as before. Ten million leukocytes were suspended in wells containing 100 μl of RPMI 1640 supplemented with 2% fetal calf serum and 50 μl of mitogen [5 or 10 μg/ml concanavalin A (Con A; Sigma)]. For each concentration of mitogen used, four concentrations of CRF (0, 4, 20, and 40 ng/ml) were used. Cells were then incubated under 5% CO_2 at 37°C. Following a 36-hr incubation, 1 μCi of [^{3}H]thymidine in 50 μl of RPMI 1640 medium supple-

mented with 10% fetal calf serum was added to each well. Cells were again incubated under 5% CO_2 at 37°C for 12 hr to allow for [^{3}H]thymidine uptake. Cells were then harvested onto glass fiber filters using a cell harvester (Dynatec). These filters were then put into scintillation vials, and uptake of [^{3}H]thymidine was measured as cpm using a scintillation counter (1209 Rackbeta; LKB-Wallach). Identical studies were also performed using bacterial LPS, but these studies were inconclusive.

Data Analysis. Regression analysis was used to evaluate the data for the leukocyte-adrenal cell culture and the leukocyte sueprnatant-adrenal cell culture using the Statistical Analysis System (SAS Institute Inc., Gary, NC) General Linear Models Program after treatment levels were first transformed into logarithms. Because the logarithm of zero results in an error, the appropriate regression equation is

$$\text{corticosterone} = B_0 + B_1 \log (\text{level of CRF} + B_2)$$

where B_0 is the intercept, B_1 is the slope of the line, and B_2 is the value to be added to each level of CRF (Statistical Consulting Service, Statistics Department, The Pennsylvania State University).

Data for the proliferative response of leukocytes were analyzed using analysis of variance (SAS). Data were normalized by subtracting the counts of 0 μg/ml Con A from their respective 5 and 10 μg/ml Con A values. Then, by assigning 0 μg/ml CRF a value of 100%, counts•resulting from incubation with different CRF concentrations were compared as deviations from the 0 μg/ml CRF. (In other words, each value of CRF was divided by its corresponding 0 μg/ml CRF value and the data were presented as percent deviation from 0 μg/ml CRF). A two-way analysis of variance with interaction was used to determine the differences between treatment groups (oCRF and mitogen). Where appropriate, means were compared by Duncan's multiple range test.

Results

CRF Effects on Leukocyte Stimulation of Corticosterone Production by Adrenal Cells. A significant ($P < 0.002$) positive linear relationship was observed between oCRF concentration and corticosterone production (Fig. 1). No corticosterone was detected in the incubation medium or RPMI 1640 medium; the stripped serum contained 0.3 ng/ml of corticosterone.

CRF Effects on Leukocyte Supernatant Stimulation of Corticosterone Production by Adrenal Cells. Shown in Figure 2 is corticosterone production by adrenal cells incubated with oCRF-primed leukocyte supernatants. Two-hour incubation of supernatants with adrenal cells resulted in no significant changes in ability to stimulate adrenal cells over a range of 0–1000 ng/ml oCRF (Fig. 2A). However, after 4 hr of incubation with adrenal cells, a positive linear relationship was observed between CRF concentration and corticosterone production ($P < 0.03$) (Fig. 2B). The positive linear relationship was also seen after 6 hr of incubation with adrenal cells ($P < 0.0004$) (Fig. 2C). By 12 hr, the linear relationship was no longer apparent (Fig. 2D). Identical concentrations of oCRF added directly to adrenal cells and incubated along with samples described above showed no linear relationship at any time during the experiment. Two-hour cultures of adrenal cells alone (no leukocytes or oCRF) produced 6.7 ng of corticosterone/ml of incubate without any additional stimulus. Addition of increasing concentrations of oCRF did not increase corticosterone production above this amount when incubated with adrenal cells alone. These results were consistent throughout the 4-, 6-, and 12-hr cultures. As before, no corticosterone was detected in the incubation medium and RPMI 1640 medium, whereas the stripped serum contained 0.3 ng/ml corticosterone.

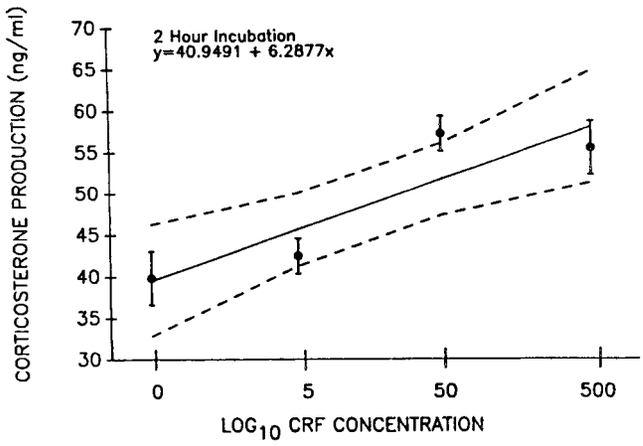

Figure 1. The production of corticosterone by 1 × 10^6 naive adrenal cells incubated for 2 hr with 1 × 10^7 leukocytes primed for 48 hr with increasing levels of oCRF. The regression line is represented by the solid line; the 95% confidence intervals are represented by the dashed lines. Each point represents the mean of six individuals ± SE. A positive linear relationship exists between oCRF concentration and corticosterone ($P < 0.002$).

CRF and Proliferating Response of Leukocytes to Mitogen Stimulation. The 48-hr Con A mitogen assay showed that a concentration of 40 ng/ml oCRF caused a significant ($P < 0.05$) increase in [^{3}H]thymidine uptake over samples containing only 0, 4, and 20 ng/ml oCRF (Fig. 3). Leukocytes stimulated with 4 ng/ml oCRF had 99.3% of the activity of non-oCRF stimulated leukocytes, whereas leukocytes stimulated with 20 ng/ml had 101.6% of the activity of nonstimulated leukocytes. The activity of leukocytes stimulated with 40 ng/ml had 114.5% of the activity of nonstimulated leukocytes.

Discussion

This study involved the activity of avian leukocytes after stimulation with oCRF. The results of the leuko-

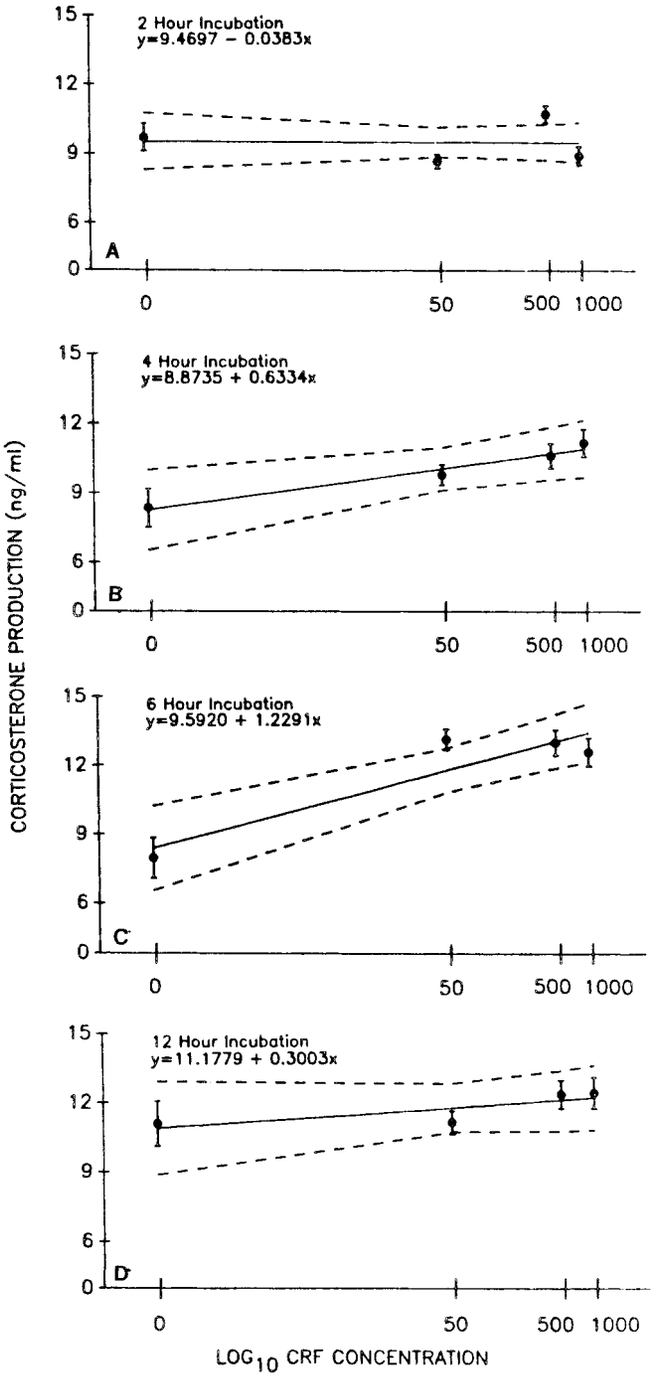

Figure 2. The production of corticosterone by 1×10^6 naive adrenal cells incubated for 2 (A), 4 (B), 6 (C), and 12 hr (D) with the supernatants from 1×10^7 leukocytes primed for 48 hr with increasing levels of oCRF. The regression line is represented by the solid line; the 95% confidence intervals are represented by the dashed lines. Each point represents the mean of six individuals ± SE. A positive linear relationship between oCRF concentration and corticosterone production was found at 4 ($P < 0.03$) and 6 hr ($P < 0.0004$). The relationships at 2 and 12 hr were not significant ($P > 0.05$).

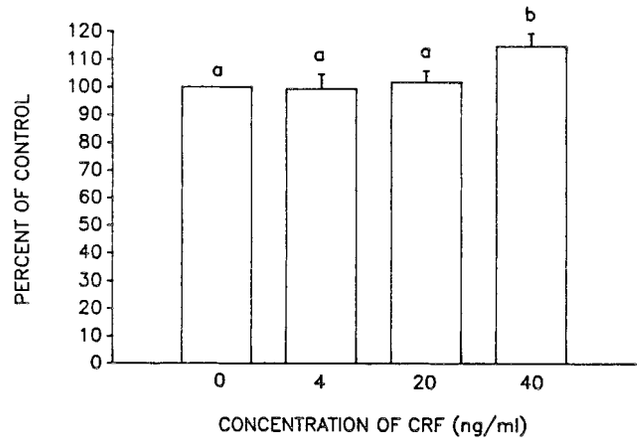

Figure 3. The effect of 48-hr Con A mitogen stimulation on 1×10^7 48-hr oCRF-primed leukocytes. A concentration of 40 ng/ml oCRF caused a significant ($P < 0.05$) increase in [^{3}H]thymidine uptake over samples containing 0, 4, and 20 ng/ml oCRF. Each bar represents the mean of six individuals ± SE.

cyte-adrenal cell incubation indicated that there was a positive linear relationship between concentration of oCRF and corticosterone produced. Presumably, the adrenal cells are stimulated by an ACTH-like substance produced by avian leukocytes. An ACTH-like substance has been shown to be produced by human leukocytes (1), mouse splenocytes (2), and chicken splenocytes (8). The quantity of oCRF necessary to stimulate leukocytes is similar to the amount of oCRF necessary to stimulate pituitary cells *in vitro*. Concentrations of oCRF from 10 ng/ml to 100 ng/ml have been found to increase pro-opiomelanocortin-related peptides from 5.4×10^4 human anterior pituitary cells (12).

The data from the leukocyte supernatant-adrenal cell culture show that the ACTH-like substance acts in a time-dependent manner. After 2 hr of incubation, adrenal cells incubated in supernatants of oCRF-stimulated leukocytes showed no increase in ability to stimulate corticosterone production over non-oCRF-stimulated leukocyte supernatants. However, following 4- and 6-hr incubations, there was a significant relationship between oCRF stimulation and corticosterone production. By 12 hr, however, the corticosterone output of the adrenal cells appears to have reached a maximum regardless of oCRF stimulation. Corticosterone output by adrenal cells incubated in non-oCRF-stimulated leukocyte supernatants had risen to the level of output by adrenal cells incubated in oCRF-stimulated leukocyte supernatants. These findings are supported by the work of Davis and Siopes (13), who found a significant increase in corticosterone production 6 hr after injection of ACTH in turkey poults. The effect of this leukocyte-produced ACTH-like substance on adrenal cells is comparable with the effect of ACTH. ACTH (10^{-7} ng/ml) substituted for leukocyte supernatants and cultured for 2, 4, 6, and 12 hr with adrenal cells produced 10.5, 11.1, 15.5, and 12.9 ng/ml corticosterone, respectively (unpublished results). Because oCRF was present in the supernatants, the possibility that it

might contribute an additional signaling function cannot be entirely ruled out. However, adrenal cells without leukocyte supernatants showed no increase in corticosterone production with increasing concentration of oCRF. Additionally, oCRF-primed leukocytes incubated with adrenal cells exhibited a dose response after the oCRF was removed. There is also evidence of leukocyte production of thyrotropin (14), human chorionic gonadotropin (15), and growth hormone (16).

Not only do cells of the immune system produce endocrine peptide hormones, but they also possess peptide hormone receptors (7, 17, 18). The results of the mitogen stimulation experiment show that a concentration of 40 ng/ml oCRF was capable of increasing the activity of Con A-stimulated leukocytes significantly over non-oCRF-stimulated leukocytes. Because Con A is a T cell mitogen (19), these stimulated leukocytes are most likely T cells. This evidence of T cell stimulation suggests that avian T cells may possess a receptor for CRF. Evidence exists of other peptide hormone receptors on lymphocytes. Lymphocytes have been shown to possess receptors for growth hormone (17), prolactin (18), growth hormone-releasing hormone, and thyrotropin-releasing hormone (20). Both mouse (21) and human (7) leukocytes possess ACTH receptors. Because lymphocytes and pituitary cells possess receptors that recognize releasing hormones, it is possible that these releasing hormones (or fractions of these releasing hormones) elicit a different response from leukocytes than from pituitary cells, depending on the initial stimulus.

Alternatively, CRF may stimulate another subpopulation of leukocytes to secrete a substance that stimulates T cells. Macrophages can stimulate T cells through the production of lymphocyte-activating factor (22), now known as interleukin (IL) 1. Con A stimulates the production of mouse IL-1 (22). CRF may enhance this production. T helper cells secrete IL-2, which activates and maintains proliferation of mitogen-stimulated T cells (23). Thus, CRF may enhance the mitogenic response by binding to macrophages and causing an increase in T cell activity through IL-1 or by binding to T cells and increasing their activity directly or indirectly through the secretion of IL-2. Whereas it is possible that T cells are not the prime producers of ir-ACTH, all peripheral blood mononuclear leukocytes, monocytes, macrophages, and T and B lymphocytes will probably synthesize ir-ACTH if properly stimulated (24).

These results support previous reports that leukocytes have the ability of producing a substance with ACTH-like activity capable of causing the production of corticosterone by adrenal cells; however, to our knowledge, this is the first report of CRF effects in birds. The data on the leukocyte supernatant adrenal cell culture indicate that peak production of this substance occurs at about 6 hr for chicken splenocytes. Pretreatment of leukocytes with 40 ng/ml CRF significantly increased their response to Con A mitogen stimulation. These experiments provide further evidence that leukocytes can both recognize and produce peptide hormones, such as ACTH.

The authors wish to thank Dr. A. V. Schally of the Endocrine and Polypeptide Laboratory, Veterans Administration Hospital, New Orleans, LA, for furnishing the oCRF.

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