Previous research in this laboratory demonstrated that protein deficient (PD) mice have elevated serum corticosterone, decreased macrophage function, and increased susceptibility to infection. The goal of this project was to determine how corticosterone affects selected aspects of macrophage activity. Peritoneal exudate cells from mice were stimulated with IFN-γ and LPS, then treated with corticosterone. At the concentration found in PD mice, corticosterone greatly inhibited macrophage MHCII expression, IL-12 secretion and TNF-α secretion, but only slightly inhibited NO production. Corticosterone interferes with IL-12 upregulation at the transcriptional level, as determined using semi-quantitative RT-PCR. IL-12 secreted by macrophages cultured with corticosterone stimulated T-lymphocyte secretion of IFN-γ in proportion to its concentration as determined by ELISA, whereas T-lymphocytes treated with corticosterone did not respond to IL-12. These findings indicate that elevated corticosterone may be a primary cause of increased susceptibility to infection in PD mice.
EFFECTS OF CORTICOSTERONE ON SELECTED ASPECTS OF MACROPHAGE AND T-CELL ACTIVITY

A Thesis

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INTRODUCTION

Protein malnourishment is a severe condition affecting one in every four children worldwide according to the World Health Organization (27). A child maintained on a protein deficient diet not only has developmental problems, but also an increased susceptibility to infectious diseases. Diarrheal diseases are among the most common sources of childhood death due to infectious disease, including those caused by Rotavirus, *Vibrio cholerae*, *Escherichia coli*, *Shigella dysenteriae*, and *Salmonella typhimurium* (27).

Our laboratory uses a mouse model to assess the effects of protein malnourishment, and focuses primarily on the immune system. Previous work in the laboratory has characterized mice maintained on a protein deficient (PD) diet in comparison to their littermates maintained on a protein sufficient (PS) diet. PD mice have a decreased body weight, thymic atrophy, splenic atrophy, and a decreased number of lymphocytes in the blood compared to PS mice (5, 6, 10, 25, 26). These characteristics of PD mice are the result of a glucocorticosteroid hormone, corticosterone, being produced at a level much higher than that of their PS littermates (5).

Corticosterone is secreted by the adrenal glands in response to stress such as that caused by protein malnourishment (2). When an individual has a protein deficient diet, corticosterone aids in recycling amino acids to help the body maintain protein synthesis as well as stimulating glycogen production (7). Corticosterone forms a reversible complex with one of two carrier proteins, albumin or transportin, for transport in the blood (2). When corticosterone is released from its carrier protein, it passively diffuses through the plasma membrane of a cell into the cytosol where it binds the glucocorticoid receptor (GR) complex. The GR-corticosterone complex is then translocated to the nucleus where the GR can form homodimers. GR homodimers can either bind specific DNA sequences known as glucocorticoid responsive elements (GREs) or bind transcription factors directly (2). In either case, they ultimately activate or suppress expression of certain genes (28). Corticosterone has been implicated in suppression of cytokine genes and other genes important for mounting an effective immune response, rendering it immunosuppressive (3, 23, 28).
Utilizing our mouse model, the susceptibility of PD mice to infection with *S. typhimurium*, a facultative intracellular Gram-negative bacterium, was compared to that of PS mice. Mice maintained on a PD diet for 21 days had a 25- to 40-fold decrease in the median lethal dose (MLD), indicating significantly decreased resistance of PD mice to *S. typhimurium* infection (25, 26). *S. typhimurium* can infect and live within host phagocytic cells, thus requiring a cell-mediated immune response to conquer the infection. Therefore, the increased susceptibility of PD mice to infection by *S. typhimurium* indicates a breakdown in cell-mediated immunity.

Cell-mediated immunity is critical in the response to facultative intracellular pathogens since antibody-mediated immunity is of little to no assistance in clearing pathogens sheltered inside host cells. Cell-mediated immunity involves multiple cell types, each of which has a critical role in the overall response leading to destruction of intracellular pathogens. A breakdown in the ability of any cell involved in cell-mediated immunity to respond when faced with infection can result in collapse of an effective response.

The immune response to facultative intracellular pathogens begins with antigen presenting cells (APC). The APC, which in the case of *S. typhimurium* is often a macrophage, will phagocytose the bacterium, enclosing it in a membrane-bound vesicle termed a phagosome. The phagosome is then fused with lysosomes, acidic vesicles containing digestive enzymes, to form a phagolysosome. Inside the phagolysosome, bacterial antigens are digested into small fragments, which can be presented bound to major histocompatibility complex class II (MHCII) (29). In the context of MHCII, bacterial antigen fragments can be recognized by T-cells, thus helping induce their differentiation and proliferation. MHCII is constitutively expressed at low levels on the surface of the cell, but can be upregulated by a T-cell secreted cytokine, interferon-gamma (IFN-γ) (24).

As macrophages process and present antigen, they also secrete chemical messengers, known as cytokines. Cytokines are signaling molecules responsible for cell-to-cell communication allowing the immune response to function properly. In the case of intracellular pathogens, the initial cytokines secreted are important in two main ways: (1) they polarize the T-helper cell population towards a cell-mediated immune response; and
they increase the phagocytic activity of macrophages. In particular, the macrophage secretes interleukin-12 (IL-12), which induces T-cells to secrete IFN-γ, and tumor necrosis factor alpha (TNF-α), which enhances phagocytosis.

IL-12, a 70kDa heterodimeric cytokine composed of a 35-kDa subunit (p35) and a 40-kDa subunit (p40), is secreted at low levels by macrophages in response to LPS stimulation (32). IL-12 and MHCII-presented antigen induce naive T-helper (Th) cells to differentiate into Th1 cells, which are responsible for IFN-γ secretion. In return, IFN-γ enhances secretion of IL-12 by macrophages stimulated with LPS (32). The mRNA for the smaller p35 subunit of IL-12 has been detected in most cell types, whereas the p40 mRNA and the p40 subunit itself are only produced by cells that secrete the biologically active IL-12(p70) heterodimer (17). The p35 subunit is not directly secreted from a cell unless covalently linked to the p40 subunit. The p40 subunit, on the other hand, is capable of forming a homodimer that can be secreted and is capable of binding the IL-12 receptor, which can antagonize the activity of the IL-12(p70) heterodimer both in vivo and in vitro (9, 32).

TNF-α is another macrophage-secreted cytokine that is induced by LPS. It is responsible for increasing the phagocytic activity of macrophages as well as inducing their production of nitric oxide (NO), secretion of other cytokines, and tumoricidal activity. TNF-α can also cause pathology if secreted in excess, such as in the case of rheumatoid arthritis or toxic shock (21). Due to the potential for damaging effects, it is critical to have tight control over TNF-α expression to prevent excess cytokine secretion.

Macrophages stimulated by LPS and/or IFN-γ are the primary cells that produce NO (19). NO is a water-soluble gas that has important roles in multiple systems of the body including the nervous, vascular and immune systems. NO has anti-microbial and anti-tumor activities because its toxicity creates an environment that is not suitable for survival and replication of various cells (19, 31).

T-cells are critical in mounting an effective cell-mediated immune response. Given that PD mice have increased susceptibility to infection and a decreased number of T lymphocytes, adoptive transfer experiments were performed to determine if reconstitution of the cells was sufficient to restore immune function. Using spleen cells to replenish the lymphocytes was not sufficient to provide PD mice with normal levels of
resistance to *S. typhimurium* (8). This prompted investigation of the functional status of the various cells involved in cell-mediated immunity in the presence of corticosterone.

To begin determining where the breakdown(s) occurred in the cell-mediated immune response to *S. typhimurium* in PD mice, MHCII expression was evaluated. To assess MHCII expression on the surface of macrophages cultured with corticosterone, a novel cell culture ELISA system was devised. Macrophages cultured with IFN-γ had an increased level of MHCII expression, which was inhibited by corticosterone (25, 26).

In addition to investigating the expression of MHCII on macrophages in the presence of corticosterone, production of selected cytokines by spleen cells from PD mice was characterized. To determine if a T-helper cell bias was occurring during T-cell differentiation, levels of interferon-gamma (IFN-γ) and interleukin-4 (IL-4) were assessed using spleen cells removed from PS and PD mice 10 days post infection. IFN-γ is secreted by a subset of T-cells known as T-helper type 1 (Th1) cells, whereas IL-4 is secreted mostly by T-helper type 2 (Th2) cells. T-helper cells are undifferentiated until they are exposed to certain cytokines. IL-12 secreted by macrophages fosters differentiation of T-helper type 0 (Th0) cells into Th1 cells whereas IL-4 fosters differentiation of Th2 cells. Th1 cells are important in cell-mediated immunity whereas Th2 cells are important in antibody-mediated immunity. The amount of IFN-γ produced by cells from PD mice was not reduced, but the amount of IL-4 was decreased, indicating a shift during T-cell differentiation to a Th1 cell-mediated response (8). These experiments used cells which were removed from PD mice and cultured without corticosterone; therefore, they may not be truly representative of what is occurring in vivo.

It was the goal of this project to evaluate the effect corticosterone has on macrophage and T-cell activity, to further understand the role of corticosterone in the increased susceptibility of PD mice to *S. typhimurium*. Alteration of macrophage activity in the presence of corticosterone was assessed by quantification of NO production, TNF-α secretion and IL-12 secretion in addition to upregulation of MHCII expression. Because IL-12 secretion was profoundly affected by corticosterone, the mechanism by which this occurs was also examined. Finally, the ability of T-cells to secrete IFN-γ when cultured with corticosterone was evaluated in vitro.
MATERIALS AND METHODS

Mice, Diets and Cells

Cells from adult female CD2F1 mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were used in all experiments. The mice were maintained on Purina Mills Laboratory Rodent Diet 5001 (23.4% protein; Richmond, IN); they had unlimited access to food and deionized water.

Peritoneal exudate cells (PECs) were acquired in a manner similar to that described by Matsuura et al. (19). Mice were injected intraperitoneally with 2ml of sterile Brewer’s thioglycollate broth (Becton Dickinson, Franklin Lakes, NJ) 4 days prior to cell collection. Mice were killed by carbon dioxide inhalation, then PECs were removed by washing the abdominal cavity with tissue culture medium (TCM) which was comprised of RPMI-1640 (Cellgro, Herndon, VA) containing 5% bovine growth serum (HyClone, Logan, UT), 10mM HEPES (Sigma Aldrich, St. Louis, MO), 100U/ml penicillin and 100µg/ml streptomycin (Sigma Aldrich). The cells were washed twice by centrifugation at 300xg for 10min, followed by aspiration of the TCM and suspension in 10ml TCM. The number of viable cells per ml was determined by trypan blue exclusion. To determine the percentage of macrophages in PECs, 200µl of the cell suspension was deposited onto a microscope slide using a cytocentrifuge (Wescor Cytopro Model 7620, Logan, UT) at 300rpm for 3min with medium acceleration. The cells were then Wright stained and counted by light microscopy. More that 90% of PECs were macrophages.

Cell Culture for Secretion Assays

PECs were adjusted to 2x10^6 cells/ml and 300 µl was added to each well of a 24-well Costar plate (USA Scientific, Ocala, FL). To stimulate cells to produce NO, TNF-α and IL-12, LPS and IFN-γ were added. IFN-γ (Peprotech, Rocky Hill, NJ), with a specific activity ≥1x10^7 Units/mg, was added at a final concentration of 100ng/ml. S. typhimurium LPS (Sigma Aldrich) was added at a final concentration of 10ng/ml. To determine its effect on macrophage activity, corticosterone was added to selected cultures to reach a final volume of 600 µl per well. Corticosterone (Sigma Aldrich) was dissolved in 95% ethanol and filter-sterilized. The final concentrations of corticosterone used were 10, 100, 600 and 1000ng/ml. To control for the effect of ethanol on cells, all corticosterone samples, including the 0ng/ml corticosterone control, contained 0.006%
final ethanol concentration. The cells were incubated in a humidified incubator at 37°C in a 95% air, 5% CO₂ atmosphere. Supernatant fluids were collected at appropriate times and stored at -80°C until assayed for NO, TNF-α and IL-12 (p70) as described below.

**MHC Class II (Ia<sup>d</sup>) and Mac-1 Expression**

PECs from adult mice were used to assess MHC class II (Ia<sup>d</sup>) expression. Cells were added to a Falcon Microtest 96-well flat bottom tissue culture treated plate (Fisher Scientific) at 100µl of 2x10<sup>6</sup> cells/ml per well. The cells were incubated for 4h at 37°C in a 95% air, 5% CO₂ atmosphere to allow attachment of adherent cells. Non-adherent cells were removed by pouring the liquid from the plate and blotting the remainder onto clean Kimwipes. The cells were then stimulated with 100µl of IFN-γ in TCM to a final concentration of 100ng/ml and treated with corticosterone (0, 10, 100, 600 or 1000ng/ml). After the cells were incubated for 48 h at 37°C in a 95% air, 5% CO₂ atmosphere, the TCM was removed and the cells were fixed with 1% paraformaldehyde in 0.13M NaCl (pH 7.4), for 20min at 25°C. The wells were washed three times with PBS, then blocked with 300µl PBS containing 5% skim milk (Sanalac Nonfat Dry Milk; Irvine, CA), 10% bovine serum (Sigma Aldrich), and 5% normal rabbit serum (Sigma Aldrich) at 37°C for 30min. Biotinylated anti-Ia<sup>d</sup> monoclonal Ab (mAb) (2µg/ml, Pharmingen, San Diego, CA) or biotinylated anti-Mac-1 mAb (0.25µg/ml, Pharmingen) was added to appropriate wells (100µl/well). The plate was incubated for 45min at 37°C prior to being washed three times with PBS. Streptavidin-HRP (R&D Systems) was diluted according to the manufacturer’s directions and 100µl was added per well. The plate was incubated 20min at room temperature in the dark, the wells were washed three times with PBS, then blotted dry. TMB substrate (Sigma Aldrich) was added (100µl/well) and the plate was incubated at room temperature in the dark for 20min. Stop solution (2N H<sub>2</sub>SO₄) was added (50µl per well) and the Absorbance was determined as described earlier.

**Griess Assay for Nitric Oxide (NO) Production**

NO was quantified as follows. Equal volumes (100µl) of Griess reagent (Sigma Aldrich) and supernatant fluid were mixed in wells of a NUNC flat-bottom 96-well plate (Fisher Scientific, Pittsburgh, PA). Assay plates were incubated 10min at room
temperature and the absorbance of each well was read at a wavelength of 570nm on a Bio-Rad Benchmark Microplate Reader (Hercules, CA). A 2-fold dilution series of a solution of sodium nitrate (Sigma Aldrich), diluted in TCM, was used to generate a 7-point standard curve, with a maximum concentration of 200µM.

**TNF-α Enzyme Linked Immunosorbant Assay (ELISA)**

A murine TNF-α ELISA development kit was purchased from R&D Systems (Minneapolis, MN). The protocol provided in the manual was followed. In brief, a NUNC flat-bottom 96-well plate (Fisher Scientific) was coated overnight at room temperature with 100µl per well of anti-TNF-α capture antibody diluted to a concentration of 0.8µg/ml with phosphate buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 8.1mM Na₂HPO₄, 1.5mM KH₂PO₄, pH 7.2). The wells were washed three times with wash buffer (0.05% Tween 20 in PBS), and then blocked with reagent diluent (1% BSA in PBS, pH 7.2, 0.2µm filtered) for a minimum of 1h. The wells were washed with wash buffer three more times, then thoroughly drained before 100µl of sample or standard was added to each well. The plate was incubated for 2h at room temperature, wells were washed with wash buffer, anti-TNF-α detection antibody (100µl per well at 150ng/ml) was added, and the plate was incubated at room temperature for 2h. The wells were then washed with wash buffer and incubated with 100µl of streptavidin conjugated to horseradish-peroxidase (streptavidin-HRP) in the dark for 20min. After washing the wells as before, 100µl of tetramethylbenzidine (TMB) liquid substrate (Sigma Aldrich) was added to each well and the plate was incubated in the dark at room temperature for 20min. Stop solution (2N H₂SO₄) was added to each well (50µl/well) before the plate was read at 450nm and corrected to 540nm using a Bio-Rad microplate reader. To correct for the Absorbance of the plastic, the reader was set to determine the Absorbance at wavelengths of both 450nm and 540nm; the Absorbance of the plastic at 540nm was then subtracted from the Absorbance at 450nm. A 7-point standard curve was generated using 2-fold dilutions in reagent diluent (1% BSA in PBS, pH 7.2) with a maximum concentration of 2000pg/ml TNF-α.

**TNF-α Bioassay**

L929 cells (ATCC; Rockville, MD) were harvested from a confluent monolayer cell culture as follows. The monolayer was washed with 5ml PBS and then incubated at
room temperature for 10min with 1ml trypsin-EDTA solution (Sigma Aldrich). The cells were then suspended in TCM and the suspension was centrifuged at 300xg for 10min to sediment the cells. The cells were washed once with TCM, then suspended in TCM, adjusted to 4 x 10^5 cells/ml, and 100µl aliquots were placed in each well of a Falcon flat bottom 96-well tissue culture treated plate (Fisher Scientific). The plate was incubated at 37°C in a 95% air, 5% CO$_2$ atmosphere for 1h to allow the cells to attach to the plastic. The TCM was then removed and 100µl of sample or standard was added to the wells. Finally, 100µl of 2µg/ml actinomycin-D (Amresco, Solon, OH) was added to each well. After incubation at 37°C in a 95% air, 5% CO$_2$ atmosphere for 18-20 hours, the supernatant fluid was removed and the wells were washed with approximately 300µl PBS per well. The cells were then stained with 50µl 0.2% crystal violet in methanol:water (1:4) at room temperature for 10min. The wells were washed five times with PBS and blotted dry on Kimwipes after each wash. Crystal violet retention by intact cells was assessed by addition of 100µl of 70% ethanol to each well and the plate was gently shaken. The Absorbance of the solubilized crystal violet was then read at 540nm on a Bio-Rad microplate reader. To control for crystal violet retention, the medium on control wells, containing actinomycin-D-treated cells, was replaced with 200µl per well of Triton-X 100 (0.5% Triton-X 100 in PBS) for 30min. The contents of the wells were mixed by pipetting the solution up and down gently, stained and solubilized with ethanol as before. The Absorbance of this control was then subtracted from the Absorbance of all other wells prior to analysis of data. An 8-point standard curve was generated using 2-fold dilutions in TCM with a maximum concentration of 133pg/ml recombinant mouse TNF-α (Peprotech) with a specific activity ≥ 1x10^7 Units/mg.

**IL-12 (p70) ELISA**

A mouse IL-12(p70) ELISA development kit was purchased from R&D Systems. The protocol provided in the manual was followed. In brief, a NUNC 96-well flat-bottom plate (Fisher Scientific) was coated overnight at room temperature with 100µl of anti-IL-12(p70) capture antibody at a concentration of 4µg/ml. The wells were washed three times with wash buffer and blocked with blocking buffer (1% BSA, 5% sucrose in PBS with 0.05% NaN$_3$) for a minimum of 1h. After the wells were washed three times with wash buffer, 100µl of sample or standard was added to them. After 2h incubation,
the wells were washed and 100µl of 400ng/ml anti-IL-12(p70) detection antibody was added per well. After the plate was incubated at room temperature for 2h, the wells were washed with wash buffer and then incubated with 100µl of streptavidin-HRP in the dark for 20min. After washing the wells three times with wash buffer, 100µl of TMB liquid substrate (Sigma Aldrich) was added to each well and the plate was incubated at room temperature in the dark for 20min. Stop solution (2N H₂SO₄) was added to each well (50µl per well) before the Absorbance was determined as described earlier. A 7-point standard curve was generated using 2-fold dilutions in reagent diluent (1% BSA in PBS, pH 7.2) with a maximum concentration of 1500pg/ml IL-12.

**IL-12 Bioassay**

The IL-12 bioassay protocol was modified from Bost et al. (9). The spleen from an adult female CD2F1 mouse was placed in 10ml of TCM. The cells were disaggregated from the organ by gentle agitation of spleen fragments between frosted slides to create a single cell suspension. The suspension was pelleted at 300xg for 10min. The supernatant fluid was removed and the cells were suspended in 5ml of TRIS-NH₄Cl (0.017M tris[hydroxymethyl]aminomethane; 0.14M NH₄Cl; pH 7.2) and incubated at 37°C for 2min to lyse red blood cells. Calf serum (Sigma Aldrich) was then layered under the suspension before it was pelleted by centrifugation for 10min at 250xg. The supernatant fluid was removed and the cells were washed with TCM. The cells were counted by trypan blue exclusion and adjusted to 10⁷cells/ml.

A Falcon Microtest II 96-well flat-bottom tissue culture treated plate was coated with 100µl of 2µg/ml anti-IL-12p70 antibody (R&D Systems) overnight at room temperature. The wells were then washed three times with PBS before being incubated for 6h with either 100µl of sample or standard. The wells were washed three times with TCM, then 10⁶ spleen cells were added (100µl per well) and the plate was incubated at 37°C in a 95% air, 5% CO₂ atmosphere for 48h. The supernatant fluid was then removed and assayed for IFN-γ by ELISA as described below. In experiments that measured the effect of corticosterone on the ability of spleen cells to produce IFN-γ, 100µl of corticosterone dilutions in TCM were added per well to reach a final concentration of 0, 10, 100, 600 or 1000ng/ml. To control for the effect of ethanol on
cells, all corticosterone samples, including the 0ng/ml corticosterone controls, contained 0.006% ethanol.

**IFN-γ ELISA**

A Mouse IFN-γ DuoSet ELISA Development kit was purchased from R&D systems. The protocol outlined in the manual was followed. A NUNC 96-well flat-bottom plate (Fisher Scientific) was coated overnight at room temperature with 100µl of 4µg/ml per well of anti-IFN-γ capture antibody. The wells were washed three times with wash buffer before incubation at room temperature with blocking buffer (1% BSA in PBS with 0.05% NaN₃) for about an hour. The wells were washed three times with wash buffer and then 100µl of either sample or standard was added per well. The plate was incubated for 2h at room temperature and then washed. Anti-IFN-γ detection antibody was added, 100µl per well of a concentration of 400ng/ml, and the plate was incubated at room temperature for 2h. The plate was washed three times and blotted dry before 100µl streptavidin-HRP was added per well. After incubating for 20min at room temperature in the dark, 100µl of TMB substrate (Sigma Aldrich) was added to each well. Following 20min incubation in the dark at room temperature, stop solution (2N H₂SO₄) was added (50µl/well) and the Absorbance was read as described earlier. A 7-point standard curve was generated using 2-fold dilutions in reagent diluent (0.1% BSA, 0.05% Tween 20 in Tris-buffered saline (20mM Tris base, 150mM NaCl, pH7.2)) with a maximum concentration of 2000pg/ml IFN-γ.

**Reverse Transcription Polymerase Chain Reaction (RT-PCR)**

RNA was purified from cells using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer’s directions. Cells were suspended in 1ml Tri-Reagent, and then transferred into a 1.5-ml Eppendorf tube (USA Scientific). Chloroform was added (3ml per ml of Tri-Reagent) and the tubes were shaken vigorously by hand for 15sec. The tubes were incubated for 10min at room temperature, and then centrifuged at 8,000g for 10min. The aqueous phase (400µl) was transferred to a new tube with a pipetter and mixed vigorously with 0.5ml absolute isopropanol (Amresco) to precipitate the RNA. The tubes were incubated at room temperature for 15min before being centrifuged at 8,000g for 8min. The pellet, containing all forms of RNA, was
washed with 75% ethanol (Amresco) before being suspended in 20µl of formazol (Molecular Research Center, Inc.).

Reverse transcription of RNA to cDNA was accomplished using the Invitrogen reverse transcriptase II kit (Carlsbad, CA). Each reaction contained: 1µl of the RNA preparation in formazol; 1µl oligo(dT) 12-18bp at a concentration of 500µg/ml; 4µl 2.5mM dNTP mix (Fisher Scientific); and 6µl RNase/DNase free water (Sigma Aldrich). The tubes were incubated for 5min at 65°C, then chilled for 2min on ice. To each tube, 4µl of 5X standard buffer, 2µl of 0.1M DTT, and 1µl of ribonuclease inhibitor (RNase-out, Fisher Scientific) was added. The tubes were then placed in an MJ Research PTC-200 Peltier Thermal Cycler and the reverse transcription program was begun. After template denaturation at 42°C for 2min, 1µl of reverse transcriptase was added per tube. The program was restarted, 42°C for 50min, 70°C for 15min, and then held at 4°C until removed from the machine.

Specific amplification of an approximately 500bp region of IL-12p40 message, in addition to a 140bp region of internal control β-actin message, was accomplished using the Eppendorf Taq PCR kit (Fisher Scientific). The primers (Integrated DNA Technologies, Coralville, IA) for the reactions were as follows: IL-12p40 Forward 5’-GGC CAG TAC ACC TGC CAC AAA GG-3’; IL-12p40 Reverse 5’-GCT GAC CTC CAC CTG TGA G-3’; β-actin Forward 5’-CGA GCA GGA GAT GGC CAC TG-3’; and β-actin Reverse 5’-GGC TGG AAA AGA GCC TCA GGG-3’. Each 100µl of reaction contained 1µl of 20µM IL-12p40 Forward primer, 1µl of 20µM IL-12p40 Reverse primer, 1µl of 20µM β-actin Forward primer, 1µl of 20µM β-actin Reverse primer, 10µl of 10X Buffer, 74.5µl H2O, 8µl 2.5mM dNTP mixture, 0.5µl Taq (5U/µl), 1µl of 25µCi/µl [α-32P]dATP (Perkin-Elmer, Wellesley, MA), and 2µl of template. The PCR reaction was performed in a Perkin-Elmer GeneAmp 2400 PCR System (Wellesley, MA). Amplification was performed with the following program: 94°C for 2min, followed by the beginning of the cycle at 94°C for 1min; 58°C for 2min; and 75°C for 1.5min, cycled 30 times, followed by a 5min incubation at 72°C and held at 4°C until removed from the thermal cycler.
The PCR products were separated at 50V for 45-60min, until the 100bp loading dye marker was about 1-2cm from the end of the gel, in an 8% polyacrylamide gel (4ml Acyl/Bis 29:1, 2ml 10x TAE buffer, 13.6ml H$_2$O; 50µl TEMED, 200µl 10%APS; all reagents from Amresco). A Molecular Dynamics phosphoimaging screen (Amersham Bioscience, Piscataway, NJ) was then exposed to the gel at room temperature overnight. The screen was scanned using a Storm Scan phosphoimaging system (Amersham Bioscience). Band intensities were quantified and standardized against the loading control, β-actin, using ImageQuant software (Molecular Dynamics, Amersham Bioscience).

**Analysis of Data and Statistics**

Experiments were repeated at least 2 times. Percent inhibition was calculated by subtracting the experimental group value from the appropriate control value and then dividing the difference by the control value. The result was then expressed as a percent by multiplying the value by 100.

Michael R. Hughes, Statistical Consulting Service, Department of Mathematics and Statistics, Miami University, performed the statistical analyses. Statistical significance of differences among experimental values was determined using ANOVA adjusted for Dunnett multiple comparisons versus a control. Values of $p \leq 0.05$ were considered significant.
RESULTS

A breakdown in cell-mediated immunity appears to be responsible for increased susceptibility of PD mice to *S. typhimurium*. Elevated levels of serum corticosterone in these mice may be causing this effect. Therefore, the goal of this research was to understand how corticosterone affects the macrophages and T-cells involved in cell-mediated immunity.

Effect of Corticosterone on Macrophage Expression of MHCII

To assess MHCII expression, a novel ELISA system developed previously in this laboratory (25) was used to determine the optimal level of stimulation and then to determine the effect corticosterone has on MHCII expression. MHCII is constitutively expressed on the surface of PEC macrophages at a low level, but can be upregulated. Mac-1, a macrophage specific marker, is expressed on the surface of cells at a constant level, which allowed more effective determination of changes in MHCII expression. Upregulation of MHCII expression was stimulated with IFN-γ. Maximal MHCII expression was detected after 48h of stimulation with 100ng/ml IFN-γ (Table 1). Stimulation of PECs with IFN-γ did not induce an increase in Mac-1 expression, but did induce a 71% increase in the amount of MHCII on the cell surface (Fig. 1A). A range of corticosterone concentrations that bracketed the average (600ng/ml) observed in PD mice was used to determine its effect on MHCII upregulation (6). When PECs were cultured with 10ng/ml corticosterone there was no inhibition of the IFN-γ-stimulated increase in MHCII present on the cell surface. A slight decrease (36%) in MHCII expression occurred in the presence of 100ng/ml corticosterone. Upregulation of MHCII expression was inhibited 62% when cells were cultured with 600ng/ml corticosterone and it was inhibited 65% in the presence of 1000ng/ml corticosterone (Fig. 1A). In contrast, corticosterone, at any concentration, did not affect the expression level of Mac-1 (Fig. 1B).

Effect of Corticosterone on Macrophage Production of NO and Secretion of TNF-α, and IL-12

Macrophage activity was further assessed by quantifying the production of NO and secretion of TNF-α and IL-12. To optimize secretion, PECs were cultured for 48h
Table 1. Optimization of MHCII expression.

<table>
<thead>
<tr>
<th>IFN-γ (ng/ml)a</th>
<th>MHCII Expression (Absorbance) b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.91 (+/- 0.12) c</td>
</tr>
<tr>
<td>10</td>
<td>1.00 (+/- 0.06)</td>
</tr>
<tr>
<td>100</td>
<td>1.23 (+/- 0.15)</td>
</tr>
</tbody>
</table>

a PECs were stimulated with the indicated concentrations of IFN-γ for 48h.
b MHCII (Ia\(^d\)) expression was assessed by ELISA.
c Data are expressed as mean +/- standard deviation (n=4).
Figure 1. Corticosterone inhibition of MHCII upregulation. PECs were stimulated with 100ng/ml IFN-γ and treated with corticosterone, as indicated, for 48h. A. The relative amount of MHCII (Ia<sup>d</sup>) expression on the cell surface was determined by ELISA using anti-Ia<sup>d</sup> antibody. B. To serve as a control, the relative amount of Mac-1 present on the cell surface was determined by ELISA using anti-Mac-1 antibody. Data are expressed as mean +/- standard deviation (n=4). * indicates significance at $p \leq 0.05$ compared to the 0ng/ml corticosterone control.
with 0, 1, 10, or 100ng/ml of LPS from *S. typhimurium* together with 100ng/ml of IFN-γ, the concentration used in the MHCII experiments. The supernatant fluid from these cultures was then assayed to determine the amounts of NO, TNF-α and IL-12. As indicated by the data in Table 2, optimal stimulation was a combination of 10ng/ml LPS and 100ng/ml IFN-γ. Once the optimal stimulation had been determined, the effect of corticosterone on the production of NO and secretion of TNF-α and IL-12 by PECs was assessed.

PECs stimulated with 10ng/ml LPS and 100ng/ml IFN-γ produced high levels of NO. This production was not inhibited by 10, 100 or 600ng/ml corticosterone. In the presence of 1000ng/ml corticosterone, however, a slight (12%) inhibition of NO production was observed (Fig. 2).

The effect of corticosterone on TNF-α secretion was assessed by ELISA. PECs were stimulated with 10ng/ml LPS, 100ng/ml IFN-γ and selected concentrations of corticosterone for 48h, at which time the amount of TNF-α in the supernatant fluid was quantified. High levels of TNF-α were secreted by stimulated PECs. This secretion was not inhibited by 10ng/ml corticosterone. Upregulation of TNF-α secretion was, however, inhibited in a dose dependent manner by corticosterone at concentrations of 100ng/ml and above. Corticosterone inhibited PEC secretion of TNF-α by 32% at 100ng/ml, by 42% at 600ng/ml, and by 48% at 1000ng/ml (Fig. 3A). The biological activity of TNF-α secreted by corticosterone treated PEC cultures was assessed using a bioassay. Supernatant fluids from PECs cultured with corticosterone induced cell death in a manner proportional to the amount of TNF-α they contained, as quantified by ELISA (Fig. 3B).

The IL-12 secretion induced by 10ng/ml LPS and 100ng/ml IFN-γ stimulation was also inhibited by corticosterone in a dose-dependent manner. Corticosterone, at 10ng/ml, caused a 38% inhibition of IL-12 upregulation, whereas at 100ng/ml it inhibited upregulation by 68%. IL-12 upregulation was inhibited 78% by 600ng/ml corticosterone and an 85% inhibition occurred at 1000ng/ml corticosterone (Fig. 4A). A bioassay was used to determine the biological activity of IL-12 secreted by PECs treated with corticosterone. In the absence of corticosterone, PECs secreted high levels of IL-12,
Table 2. Optimization of NO Production and of Secretion of IL-12 and TNF-α.

<table>
<thead>
<tr>
<th>LPS (ng/ml)(^a)</th>
<th>NO (um)(^b)</th>
<th>IL-12 (pg/ml) (^c)</th>
<th>TNF-alpha (Units) (^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72</td>
<td>451</td>
<td>334</td>
</tr>
<tr>
<td>10</td>
<td>75</td>
<td>2924</td>
<td>1000</td>
</tr>
<tr>
<td>100</td>
<td>72</td>
<td>1244</td>
<td>1001</td>
</tr>
</tbody>
</table>

\(^a\) PECs were stimulated with LPS, as indicated, in addition to 100ng/ml IFN-γ for 48h.

\(^b\) The culture supernatant fluid was assessed for NO by Griess assay.

\(^c\) The culture supernatant fluid was assessed for IL-12 by ELISA.

\(^d\) The culture supernatant fluid was assessed for TNF-α by bioassay.
Figure 2. Corticosterone inhibition of NO Production. PECs were stimulated with 10ng/ml LPS and 100ng/ml IFN-γ and treated with concentrations of corticosterone, as indicated, for 48h. The culture supernatant fluid was then assayed by Griess assay to quantify NO produced. Data are expressed as mean +/- standard deviation (n=3). * indicates significance at $p \leq 0.05$ compared to the 0ng/ml corticosterone control.
NO Production

Corticosterone (ng/ml)

NO (µm)

0 10 100 600 1000

*
Figure 3. **Corticosterone inhibition of TNF-α secretion.** TNF-α was quantified in the culture supernatant fluid of PECs stimulated with 10ng/ml LPS, 100ng/ml IFN-γ, and treated with corticosterone, as indicated, for 48h. **A.** The amount of TNF-α secreted was quantified by ELISA. Data are expressed as mean +/- standard deviation (n=3). ** indicates significance of \( p \leq 0.005 \) and *** indicates significance of \( p \leq 0.001 \) compared to the 0ng/ml corticosterone control. Data shown are representative of 3 experiments. **B.** The bioactivity of TNF-α secreted in the presence of corticosterone was quantified by bioassay. Data shown are representative of three replicates from an individual experiment.
A

TNF-α Secretion

B

TNF-α Bioactivity
Figure 4. Corticosterone inhibition of IL-12 secretion. A. PECs were stimulated with 10ng/ml LPS and 100ng/ml IFN-γ, and treated with corticosterone, as indicated, for 48h. The amount of IL-12 in the culture supernatant fluid was quantified by ELISA. B. The culture supernatant fluid containing IL-12 was used to stimulate IFN-γ secretion by splenic T-cells for 48h. IFN-γ secretion in these T-cell culture supernatant fluids was quantified by ELISA. Data are expressed as mean +/- standard deviation (n=3). * indicates significance at $p \leq 0.05$, ** indicates significance at $p \leq 0.005$ and *** indicates significance at $p \leq 0.0001$ compared to the 0ng/ml corticosterone control.
A

IL-12 Secretion

Corticosterone (ng/ml)

**

***

IL-12 (pg/ml)

B

IL-12 Bioactivity

Corticosterone (ng/ml)

* 

**
which stimulated high levels of IFN-γ secretion by splenic T-cells. PECs cultured with 600ng/ml corticosterone secreted a low level of IL-12 and therefore stimulated T-cells to secrete a low level of IFN-γ. The amount of IFN-γ produced by T-cells was found to be proportional to the amount of IL-12 present in the sample (Fig. 4B).

Due to the critical role of IL-12 in mounting an effective cell-mediated immune response, it was important to see how quickly after exposure of PECs to corticosterone inhibition of IL-12 secretion occurred. To answer this question, a time course experiment was performed in which IL-12 secretion was assessed at 12, 24 and 48h post corticosterone treatment. Inhibition of IL-12 secretion by corticosterone occurred as early as 12h and was maintained for 48h. An equivalent degree of dose-dependent inhibition of IL-12 secretion was detected at all time points (Fig. 5).

**Effect of Corticosterone on IL-12 mRNA Expression**

To determine the effect corticosterone has on IL-12 transcription, semi-quantitative RT-PCR was used to determine the mRNA level for p40, the inducible subunit. Because the results of the previous experiment indicated that inhibition of IL-12 secretion occurred as early as 12h after exposure to corticosterone, this time point was used for these experiments.

Stimulation of PEC macrophages with 10ng/ml LPS and 100ng/ml IFN-γ induced a high level of IL-12p40 mRNA expression. Treatment with corticosterone decreased the level of p40 mRNA in a dose dependent manner. Corticosterone at 10ng/ml inhibited p40 mRNA expression by 50%, and at 100ng/ml corticosterone inhibited by 72%. Increasing the concentration of corticosterone to 600ng/ml, the level detected in PD mice, resulted in 79% inhibition of p40 mRNA expression, and at 1000ng/ml, corticosterone inhibited p40 mRNA expression by 84% (Fig. 6).

**Effect of Corticosterone on IL-12-Induced T-cell Synthesis of IFN-γ**

The effect of corticosterone in the ability of T-cells to respond to mouse rIL-12 was assessed. In the absence of rIL-12, spleen cells did not secrete detectable levels of IFN-γ, whether corticosterone was present or not. In the presence of rIL-12 at 250, 500 or 1000pg/ml, however, secretion of IFN-γ by T-cells was proportional to the amount of rIL-12 present (Fig. 7). Addition of corticosterone as 600ng/ml inhibited IFN-γ secretion in all the spleen cell cultures, independent of the IL-12 concentration (Fig. 7). Next, the
Figure 5. Time course of corticosterone inhibition of IL-12 secretion. PECs were stimulated with 10ng/ml LPS and 100ng/ml IFN-γ and treated with corticosterone, as indicated. After incubation for 12, 24 or 48h, the culture supernatant fluid was removed and assayed by ELISA to quantify IL-12 secretion. Data are expressed as mean +/- standard deviation (n=3). *** indicates significance at $p \leq 0.0001$ when compared to the 0ng/ml corticosterone control for the appropriate time point.
Time Course of IL-12 Secretion

IL-12 (pg/ml) vs. Corticosterone (ng/ml)

- 0 hours
- 12 hours
- 24 hours
- 48 hours

Significant differences indicated by ***.
Figure 6. Corticosterone inhibition of IL-12p40 mRNA expression. PECs were stimulated with 10ng/ml LPS and 100ng/ml IFN-γ and treated with corticosterone, as indicated, for 12h before the RNA was harvested for semi-quantitative RT-PCR. mRNA was separated on an 8% polyacrylamide gel. A phosphoimaging screen was then exposed to the gel at room temperature overnight. The screen was scanned using a Storm Scan phosphoimaging system, the results of which are displayed in panel A. Lanes correspond to corticosterone treatment groups shown in the bar graph below. B. Graphic representation of IL-12p40 mRNA expression normalized to β-actin expression. The intensities of the bands in the gel image were quantified using ImageQuant software.
A

mRNA gel image

<table>
<thead>
<tr>
<th>IL-12p40</th>
<th>500bp</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>β-actin</th>
<th>130bp</th>
</tr>
</thead>
</table>

B

Normalized mRNA IL-12p40 levels

<table>
<thead>
<tr>
<th>Corticosterone (ng/ml)</th>
<th>Ratio (IL-12p40/β-actin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.25</td>
</tr>
<tr>
<td>10</td>
<td>0.20</td>
</tr>
<tr>
<td>100</td>
<td>0.15</td>
</tr>
<tr>
<td>600</td>
<td>0.10</td>
</tr>
<tr>
<td>1000</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Figure 7. IFN-γ secretion dependence on IL-12 concentration and inhibition by corticosterone. Spleen cells were stimulated with IL-12, as indicated, and treated with 600ng/ml corticosterone for 48h. The culture supernatant fluid was assayed by ELISA to quantify IFN-γ secretion. Data are expressed as mean +/- standard deviation (n=3). ** indicates significance at $p \leq 0.005$ and *** indicates significance at $p \leq 0.0001$ when compared to the 0ng/ml corticosterone control.
IFN-γ Secretion

- 0 ng/ml Corticosterone
- 600 ng/ml Corticosterone

![Bar chart showing IFN-γ secretion levels with different IL-12 concentrations. The x-axis represents IL-12 (pg/ml) and the y-axis represents IFN-γ (pg/ml). There are significant differences indicated by *** and ** symbols.](image-url)
effect of varying the corticosterone concentration was determined. Because 1000pg/ml of rIL-12 elicited the highest IFN-γ response from T-cells, this concentration was used in these experiments. In the absence of corticosterone, 1000pg/ml rIL-12 resulted in secretion of about 1600pg/ml of IFN-γ. At 10ng/ml corticosterone, production of IFN-γ by T-cells was inhibited 74%. Further, concentrations of corticosterone at or above 100ng/ml completely inhibited IFN-γ secretion (Fig. 8).
Figure 8. Dose dependence of corticosterone inhibition of IFN-γ secretion. Spleen cells were stimulated with 1000pg/ml IL-12 and treated with corticosterone, as indicated, for 48h. IFN-γ in the culture supernatant fluid was quantified by ELISA. Data are expressed as mean +/- standard deviation (n=3). *** indicates significance at $p \leq 0.0001$ compared to the 0ng/ml corticosterone control.
IFN-γ Secretion

![Graph showing IFN-γ secretion levels in relation to corticosterone concentrations. The x-axis represents corticosterone levels in ng/ml (0, 10, 100, 600, 1000), and the y-axis represents IFN-γ levels in pg/ml (0, 10, 100, 600, 1000). There are bars indicating secretion levels with error bars. The graph includes asterisks to indicate statistical significance.](attachment:Graph.png)
DISCUSSION

A protein deficient (PD) diet causes increased secretion of corticosterone, a glucocorticoid (GC) hormone induced by stress, which is responsible for increased amino acid turnover (7). Previous research in this laboratory has characterized PD mice and found that this elevated serum corticosterone leads to thymic atrophy, splenic atrophy and leucopenia (5, 6, 10, 25, 26). Another characteristic of PD mice is increased susceptibility to infection with *S. typhimurium*, which may be corticosterone-related (25, 26). The mechanism by which corticosterone decreases the resistance of PD mice to *S. typhimurium* is multifaceted and not well understood, even though GCs have been shown to mediate macrophage dysfunction in malnutrition (12), and to inhibit cytokine secretion (1). Consequently, understanding the effects corticosterone has on macrophages and T-cells and their ability to initiate a cell-mediated immune response was the goal of this project.

An in vitro system was developed in which PECs from mice maintained on a PS diet were used to assess the effects of corticosterone on macrophages. To begin determining the effects of corticosterone on macrophage activity, MHCII expression was evaluated. When IFN-γ stimulated PEC macrophages were treated with corticosterone at 600ng/ml, the average concentration found in PD mice, they exhibited diminished MHCII upregulation. Macrophage activity was also assessed by determining the levels of production of NO and secretion of TNF-α, and IL-12 in PEC cultures stimulated with LPS and IFN-γ. Stimulated PECs treated with 600ng/ml corticosterone exhibited only slight inhibition of NO production, but had significantly decreased levels of TNF-α and IL-12 secretion. The dose dependent inhibition of IL-12 secretion occurred within the first 12h the cells were cultured with corticosterone and continued through 48h. Further characterization revealed that corticosterone treatment decreased the level of IL-12p40 mRNA in stimulated PECs. IL-12 induced dose-dependent IFN-γ secretion by splenic T-cells, even when the IL-12 was produced in the presence of corticosterone. In contrast, splenic T-cells cultured with 600ng/ml corticosterone did not secrete IFN-γ in response to rIL-12. Taken together, these results indicate that corticosterone causes inhibition of macrophage and T-cell activities that could interfere with development of an effective cell-mediated immune response.
Antigen presentation is critical in mounting an immune response against pathogens. MHCII is constitutively expressed at a low level to allow initial antigen presentation to T-cells which, together with IL-12, induces IFN-γ secretion (24, 20). The IFN-γ produced then induces upregulation of MHCII expression, enhancing the process. In the assay system used here, stimulation with IFN-γ induced upregulation of MHCII expression by 70% above the constitutive level. Corticosterone, at concentrations of 600ng/ml or higher, inhibited the upregulation. It is important to note that constitutive levels of MHCII are not affected, which indicates that corticosterone does not completely knock out MHCII gene expression, but only inhibits upregulation of gene activity by IFN-γ. Other studies have shown that corticosterone prevents upregulation of MHCII expression (12, 28), but the role corticosterone plays is not yet clear. Three cis-acting elements (W, X and Y) located upstream of the MHCII genes must be activated to upregulate these genes. GC-mediated inhibition of MHCII expression involves blocking the binding of the X-box DNA binding protein (29). Upregulation of MHCII genes also requires a series of DNA binding proteins (RFX, X2BP/CREB and NF-Y) that must interact with a transcriptional coactivator, CIITA (20). CIITA, the master regulator of MHCII, can be upregulated by IFN-γ stimulation, and its levels correlate with MHCII expression (24, 20). The CIITA promoter IV region contains three elements, including a gamma activation sequence (GAS) element that binds STAT-1, an E-box that binds USF-1, and an interferon regulatory factor-1 (IRF-1) binding site. Additionally, STAT-1 binds to the IRF-1 promoter, increasing the level of IRF-1 available to bind to the CIITA promoter (20). GCs have been shown to inhibit MHCII expression by blocking STAT-1 production. However, this inhibition requires at least 24h pre-exposure to GC, to allow the intracellular levels of STAT-1 to decline, prior to IFN-γ stimulation (13). Inhibition of MHCII expression by this mechanism would not have been seen in the in vitro experiments in the present study, because IFN-γ and corticosterone were added simultaneously. However, this mechanism could be acting in PD mice, which are exposed to 600ng/ml corticosterone prior to infection (25).

Stimulating PEC macrophages with both IFN-γ and LPS induced a high level of NO production, which was only slightly inhibited at the highest level of corticosterone tested, 1000ng/ml. Consistent with these findings, others have shown that GC-mediated
inhibition of NO production did not occur in cells stimulated with both LPS and IFN, although it did occur in cells stimulated with LPS alone (16). NO production induced by LPS and IFN-\(\gamma\) results from transactivation due to promoter binding of LPS-induced transcription factors STAT-1 and IRF-1 (15, 19). GC-mediated inhibition of inducible nitric oxide synthase (iNOS) gene expression has been shown to occur via blocking NF-\(\kappa\)B transactivation and mRNA destabilization (15, 16). The slight inhibition demonstrated in the present study may have resulted from the fact that IFN-\(\gamma\) was able to largely overcome these inhibitory effects of corticosterone. However, this would not be the case in PD mice, because their production of IFN-\(\gamma\) will most likely be inhibited by corticosterone, as shown by other results from this study. NO production by macrophages is important in creating an environment unfavorable to bacterial viability and replication (19, 31). Consequently, if NO production is inhibited in PD mice, where corticosterone levels are elevated, bacteria would be able to grow and divide more readily, potentially resulting in a deadly infection.

Inhibition of TNF-\(\alpha\) secretion by corticosterone also occurred in a dose-dependent manner at concentrations of 100ng/ml and higher, demonstrating its sensitivity to corticosterone. The TNF-\(\alpha\) secreted by corticosterone treated cells was found to be functional using a bioassay system. The promoter for the TNF-\(\alpha\) gene requires multiple transcription factors in addition to NF-\(\kappa\)B, including CRE/AP-1, NF-IL6, Ets factors and NF-AT (14, 21). There is an enhancer region located downstream which also requires NF-\(\kappa\)B (14). AP-1, NF-\(\kappa\)B and NF-AT activities have previously been shown to be affected by corticosterone; therefore, interference with these transcription factors is likely to play a key role in inhibition of TNF-\(\alpha\) by corticosterone in this system (3, 14, 23, 30). TNF-\(\alpha\) is first produced as a membrane bound protein that is released after cleavage by a protease. GCs have been implicated in altering the activity of many proteases and therefore may also prevent active secretion of TNF-\(\alpha\) (14). TNF-\(\alpha\) is important in inducing production of NO and increasing the phagocytic activity of macrophages (21). Inhibition of TNF-\(\alpha\) secretion would allow for bacterial replication due to decreased NO production, whereas decreased phagocytic activity of macrophages
would limit the amount of antigen presentation and the cell-mediated response from progressing.

IL-12 secretion was inhibited by corticosterone in a dose-dependent manner. This inhibition occurred within the first 12h PEC macrophages were cultured with corticosterone and was maintained through 48h. The bioactivity of IL-12 secreted in the presence of corticosterone was not affected; it was able to induce IFN-γ secretion by splenic T-cells in proportion to the amount of IL-12 present according to ELISA. This proportional secretion of IFN-γ indicates that the IL-12 secreted by macrophages treated with corticosterone is functionally capable of stimulating T-cells to secrete IFN-γ in a normal fashion.

Given the critical role of IL-12 in initiating cell-mediated immunity and fostering Th1 differentiation, the mechanism by which corticosterone inhibits the secretion of IL-12 was investigated further. To get increased secretion of biologically active IL-12, the amount of the p40 subunit has to be upregulated so it can dimerize with the constitutively expressed p35 subunit. If there are more p40 subunits than p35 subunits, p40 can homodimerize, forming a molecule able to bind the IL-12 receptor and ultimately preventing its binding of the p70 heterodimer (9). To determine the effect of corticosterone on IL-12 transcription, the p40 mRNA level was quantified by semi-quantitative RT-PCR. IL-12p40 mRNA levels decreased in a manner dependent on the concentration corticosterone, and the degree of the decrease correlated with the level of IL-12 secreted from the cell. These findings indicate that inhibition of IL-12 by corticosterone was the result of corticosterone somehow preventing transcription of the p40 gene or affecting mRNA stability.

As described earlier, IL-12 is a heterodimeric cytokine composed of two subunits. Although the p35 promoter contains multiple binding sites for the LPS stimulated transcription factor, NF-κB (32), p35 is not secreted from the cell unless covalently bound to the p40 subunit (18). The p40 promoter contains transcription factor binding sites for NF-κB, Ets-2, AP-1 and members of the IRF-1 and C/EBP families (4, 17). Using gel shift assays in a human macrophage system, the binding activities of both NFκB and AP1 have been shown to decrease in the presence of GC, resulting in inhibition of IL-12 secretion (17). Yet another laboratory investigated the effect of GCs
on the IFN-γ-induced signaling pathway in human peripheral blood mononuclear cells, and determined that GCs inhibit activation of STAT-1 (13). Given these findings, I propose that inhibition of IL-12 secretion by corticosterone in the present in vitro PEC system is most likely due to a combination of inhibition of STAT-1 dependent signaling and/or decreased activity of NFκB and AP1.

Previous research in the laboratory measured the level of IFN-γ secretion in an ex vivo culture system and concluded that it was not affected when cells from PD mice were stimulated in culture (8). Given the nature of these experiments and their results, a concern arose because these cells were removed from the in vivo conditions of the PD mouse. Therefore, it became important to determine the effect that culturing the cells in the presence of corticosterone would have. Consequently, in the present research, an in vitro bioassay system allowed for the effects of corticosterone on macrophages and T-cells to be assessed separately. The ability of T-cells to respond to IL-12 when they were cultured in the presence of corticosterone was drastically decreased. Research from other laboratories has also demonstrated inhibition of IFN-γ secretion in the presence of GCs (11, 22). The mechanism of IFN-γ inhibition by GCs was shown not to depend on direct binding of GR to GREs; therefore, it is likely to be due to blocking of the activity of transcription factors such as NF-κB (Reichardt, 2001). Another laboratory looked at the signaling of the IL-12 receptor and determined that inhibition of STAT4 phosphorylation, independent of the phosphorylation of the Janus kinases, contributes to GC induced inhibition of IFN-γ secretion (11). Inhibition of IFN-γ gene transcription prevents stimulation of macrophage activity, including upregulation of MHCII expression and IL-12 secretion, and indicates a corticosterone-induced shift away from a Th1 response.

In PD mice, elevated serum corticosterone causes many different negative effects on the immune system, resulting in decreased resistance to infection. Due to the high level of corticosterone in PD mice, their macrophages present exogenous antigen fragments only on constitutively expressed MHCII molecules, and secrete limited amounts of IL-12 and TNF-α. Faced with these decreases in MHCII-Ag presentation and IL-12 secretion in the presence of corticosterone, the T-cells are not able to secrete adequate amounts of IFN-γ. The small amount of IFN-γ that results limits the progression of cell-mediated immunity because macrophages are not activated. This is
compounded by the fact that corticosterone also blocks T-cell secretion of IFN-γ. As a result, corticosterone induced inhibition of both macrophage activity and T-cell secretion of IFN-γ prevents development of cell-mediated immunity and ultimately increases a protein malnourished individual’s susceptibility to infection.
REFERENCES


