# The synthesis and release of adrenocorticotropic hormone (ACTH) and related proopiomelanocortin (POMC) peptides by peripheral mononuclear leukocytes (PML)



by

Brett Lee Fundak

A Thesis Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
MASTER OF SCIENCE

Department: Veterinary Physiology and Pharmacology
Major: Physiology



Iowa State University
Ames, Iowa

1990

TABLE OF CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| $\alpha$ | alpha |
| ABC | avidin-biotinylated-peroxidase complex |
| ACD | acid citrate dextrose solution |
| ACTH | adrenocorticotropic hormone |
| $\beta$ | beta |
| BSA | bovine serum albumin |
| CLIP | corticotropin-like peptide |
| ConA | concanavalin A |
| cpm | counts per minute |
| CRF | corticotropin releasing factor |
| DAB | 3,3'-diaminobenzidene |
| DMEM | Dulbecco's Modified Eagles Medium |
| EDTA | ethylenediaminetetraacetic acid |
| EtOH | ethyl alcohol |
| FeLV | feline leukemia virus |
| $\gamma$ | gamma |
| ICC | immunocytochemistry |
| IgG | immunoglobulin gamma |
| IL-1 | interleukin 1 |
| IL-2 | interleukin 2 |
| ir | immunoreactive |
| KIU | kallikrein inhibitory units |
| kDa | kilodalton |
| LCM | leukocyte culture media |
| LPH | lipotropin |
| ml | milliliter |
| MSH | melanocyte stimulating hormone |
| PAGE | polyacrylamide gel electrophoresis |
| PMA | phorbol-12-myristate-13-acetate |
| PML | peripheral mononuclear leukocytes |
| POMC | proopiomelanocortin |
| PPO | 2,5-diphenyloxazole |
| RIA | radioimmunoassay |
| SDS | sodium dodecyl sulfate |
| SEM | standard error of the mean |

# ABSTRACT

The presence of POMC peptides in PML was investigated using immunocytochemistry. Virtually all PML obtained from cow, pig, dog, and rat donors stained positively for immunoreactive ACTH (ir-ACTH). Bovine PML also stained positively for ir-met-enkephalin and porcine PML for ir-$\beta$-endorphin.

The release of ir-ACTH by bovine PML was investigated by radioimmunoassay (RIA). The assay characteristics were determined. The RIA sensitivity was observed to be 11.1 ± 1.8 pg/ml ACTH ($\bar{x}$ ± SEM, n=11), yet the release of ir-ACTH from bovine PML was not detected under basal or stimulated conditions.

After characterizing the presence of POMC peptides in PML, a potential role was hypothesized. The ability of POMC peptides to modulate the functions and activities of immune cells has been observed. A summary of POMC peptide effects on immune cells has been included in the General Discussion.

# GENERAL INTRODUCTION

The immune system is composed of many different cell types. For example, the lymphocytes that function in acquired immunity are not constrained to a specific tissue; however, they are found to be concentrated in secondary lymphoid organs: the lymph nodes and the spleen (Mota, 1986). The lymphocytes, like all immune cells, are carefully regulated to control the nature and magnitude of their response to foreign material. Two types of regulatory signalling are observed to occur in the control of a immune response (Janeway et al., 1985).

The first type of regulatory signalling involves direct cell-to-cell contact. For interaction to take place, membrane receptors of one cell recognize and bind the ligands on another cell. No interaction will take place if the receptor and ligand are not complementary in structure: 'lock and key' recognition. This type of signalling occurs at many stages of an immune response (Unanue and Allen, 1987).

The second type of regulatory signalling is mediated by soluble peptides. After stimulation, some immune cells release soluble peptides, cytokines, into the extracellular fluid. These cytokines then modulate the activities of any cell possessing the necessary receptors. For example, interleukin 1 (IL-1) is released by activated macrophages and functions with antigen presentation to initiate helper T-lymphocyte differentiation and maturation (Widman, 1989).

Macrophages are not unique in this respect, lymphocytes have also been observed to release cytokines. The cytokines released by immune cells are a heterogenous group of peptides. For example, considerable evidence

suggests that the proopiomelanocortin (POMC) peptides may function as potential cytokines. These peptide hormones are more traditionally associated with the anterior pituitary (the most important POMC peptides are ACTH, β-endorphin, and met-enkephalin); however, the synthesis of POMC peptides by macrophages and lymphocytes has been reported (Smith and Blalock, 1981; Lolait et al., 1984; Bost et al., 1987; Buzzetti et al., 1989).

In addition to local production by immune cells, POMC peptides have been implicated in regulating the nature and magnitude of a immune response. For example, adrenocorticotropic hormone (ACTH) has been reported to suppress the production of antibodies by B lymphocytes (Johnson et al., 1982), and to block the activation of peritoneal macrophages by σ-interferon (Koff and Dunegan, 1985). β-endorphin has been observed to enhance the proliferative response of rat spleenic lymphocytes to T-lymphocyte mitogens (Gilman et al., 1982) and, β-endorphin and met-enkephalin have been observed to enhance the cytotoxic activities of natural killer cells (Mathews et al., 1983; Faith et al., 1984; Froelich and Bankhurst, 1984; Kay et al., 1984; and Mandler et al., 1986). Clearly, POMC peptides can modulate the activities of an immune response within the body. To strengthen the view that POMC peptides act as cytokines, it is, of course, necessary to establish that POMC peptides are stored and released by PML. Assessment of the immunoactivity of POMC peptides is not sufficient evidence to assign POMC peptides to the PML-derived-cytokine category. Hence, further information on the synthesis and release of POMC peptides by PML was required to determine the peptides role in controlling

a developing immune response.

Accordingly, PML of humans and mice have been reported to store immunoreactive adrenocorticotropic hormone (ir-ACTH) and related POMC peptides (Lolait et al., 1985; Harbour-McMenamin et al., 1985; Smith et al., 1985; Bost et al., 1987; Meyer et al., 1987). However, these data were derived from only two different mammalian species. To gain further support for the hypothesis that POMC peptides released from mammalian PML serve a general function in controlling an immune response, it was necessary to show the synthesis and release of POMC peptides from PML of other mammalian species. The purpose of the current study was to further characterize the synthesis and release of POMC peptides from a broad spectrum of mammalian species. To accomplish this goal, the current study was divided into two separate and complementary sections.

In the first series of experiments, the storage of ir-ACTH and related POMC peptides by PML of cows, pigs, dogs, and rats was investigated using immunocytochemistry (ICC). ICC is a technique that utilizes the specificity of the antibody/antigen interaction to identify the presence or absence of an antigen in a tissue section or cell preparation. This antigen can be structural, cytoplasmic, or nuclear in nature.

After it was successfully demonstrated that PML stored ir-POMC peptides (e.g., ACTH, $\beta$-endorphin, and met-enkephalin), a second series of experiments was performed to detect the release of ir-ACTH from PML. To study the release of ir-ACTH, bovine PML were cultured at several different concentrations and then subjected to a variety of treatments. The media obtained from the cultures of bovine PML was quantitated for ir-ACTH

content by radioimmunoassay (RIA).

The secretagogues that stimulate the release of ir-ACTH from bovine PML were not known, therefore, the bovine PML were treated with two pharmacological agents: (1) A23187, a calcium ionophore that increases cytoplasmic calcium ion concentrations in treated cell types, and (2) phorbol-12-myristate-13-acetate (PMA), a phorbol ester that activates protein Kinase C in treated cell types. These are both major signalling pathways associated with cell secretion. Bovine PML were also treated with Concanavalin A (ConA) and corticotropin releasing factor (CRF), both of which have been reported to stimulate increases in ir-ACTH content of human and mouse PML (Besedovsky et al., 1981; Harbour-McMenamin et al., 1985; Smith et al., 1986; Kavelaars et al., 1989).

The results of these two complementary studies served to characterize the ability of mammalian PML to synthesize, store, and release POMC peptides. The current studies were designed to establish if POMC peptides might function as potential cytokines. After characterizing the presence of POMC peptides in mammalian PML, it was possible to speculate on the function of POMC peptides in immunoregulation.

# SECTION I. THE SYNTHESIS OF ADRENOCORTICOTROPIC HORMONE (ACTH) AND RELATED PROOPIOMELANOCORTIN (POMC) PEPTIDES BY PERIPHERAL MONONUCLEAR LEUKOCYTES (PML)

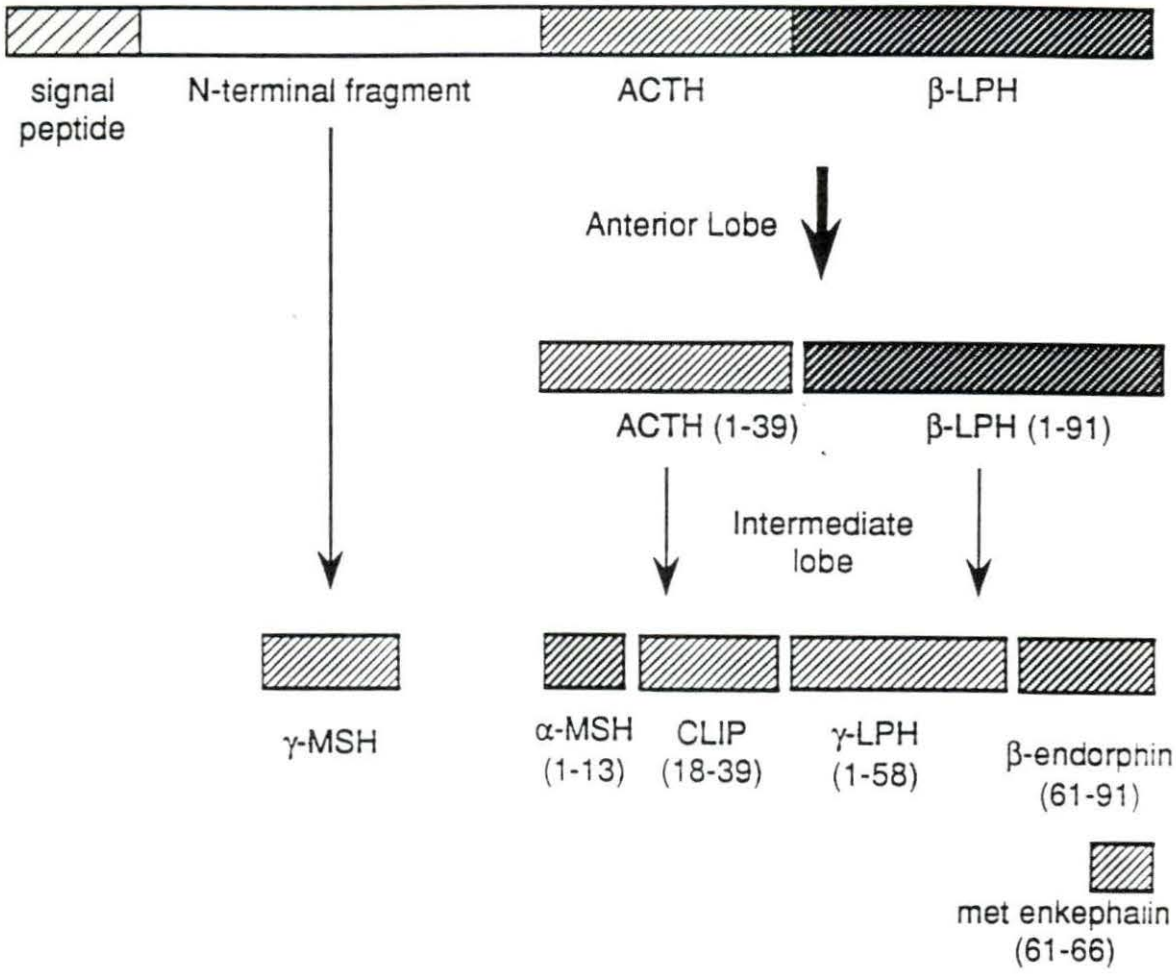
## Introduction and Literature Review

ACTH is a polypeptide, thirty-nine amino acids in length, produced primarily in the anterior pituitary gland. It is related to a large family of peptides through the precursor, POMC protein. As seen in Figure 1.1, the POMC protein is processed in the anterior lobe of the pituitary to produce ACTH, $\alpha$-melanocyte stimulating hormone ($\alpha$-MSH), and $\beta$-lipotropin ($\beta$-LPH). Further processing of these POMC products occurs in the intermediate lobe of the pituitary to yield $\gamma$-MSH, corticotropin-like peptide (CLIP), $\gamma$-LPH, and $\beta$-endorphin.

ACTH has been noted to exert a wide range of effects on a variety of immune cell types. The **in vitro** production of antibodies by mouse spleen B lymphocytes is suppressed by the administration of ACTH to the cultures (Johnson et al., 1982). ACTH has also been ascribed a role in the release of cytokines by mouse spleenocytes: administration of ACTH to spleenocyte cultures suppresses the production of $\gamma$-interferon- a cytokine involved in anti-viral responses (Johnson et al., 1984). Finally, ACTH has been observed to block the induction of mouse peritoneal macrophages to the tumoricidal state by $\gamma$-interferon (Koff and Dunegan, 1985).

In contrast to the suppressor functions associated with ACTH, $\beta$-endorphin has been observed to enhance the proliferative response of rat spleenic lymphocytes to the T lymphocyte mitogens Concanavalin A and

Figure 1.1: Processing of the proopiomelanocortin (POMC) molecule in the anterior and intermediate lobes of the pituitary gland. The precursor POMC protein contains the sequence for $\gamma$-melanocyte stimulating hormone ($\gamma$-MSH). In the anterior lobe POMC is processed to produce adrenocorticotropic hormone (ACTH, amino acids 1-39), and $\beta$-lipotropin ($\beta$-LPH, 1-91). These two peptides are further processed in the intermediate lobe. ACTH is cleaved to yield $\alpha$-MSH (1-13) and corticotropin-like peptide (CLIP, 18-39) while $\beta$-LPH is cleaved to yield $\gamma$-LPH (1-58) and $\beta$-endorphin (61-91). The first six amino acids of $\beta$-endorphin contain the sequence for met-enkephalin (61-66). (Modified from S. Reichlin: In: **Williams Textbook of Endocrinolgy**, 7th ed., JD Williams and DW Foster, eds., Saunders, Philadelphia, 1985, p. 502)


signal peptide
N-terminal fragment
ACTH
β-LPH
Anterior Lobe
ACTH (1-39)
β-LPH (1-91)
Intermediate lobe
γ-MSH
α-MSH (1-13)
CLIP (18-39)
γ-LPH (1-58)
β-endorphin (61-91)
met enkephalin (61-66)

phytohemagglutinin (Gilman et al., 1982). β-endorphin and met-enkephelin have been shown to enhance the cytotoxic activities of natural killer cells (Mathews et al., 1983; Faith et al., 1984; Froelich and Bankhurst, 1984; Kay et al., 1984; and Mandler et al.,1986).

It is possible that the effects of ACTH and β-endorphin are complementary in action and act to enhance one capacity of the immune response while suppressing another. In order to better understand the role of the POMC related peptides in the developing immune response, it is necessary to determine if the peptides are produced locally at the site of stimulation or at some other site.

If the POMC peptides are produced elsewhere, then the amount of peptide needed to affect an immune response is greatly increased. The peptide must enter the circulatory system and circulate through the body before interacting with the cells involved in acquired immunity. In addition, an efficient feedback mechanism would be required to achieve the level of control exhibited by the immune system during antigenic stimulation.

To circumvent this situation, it is possible that ACTH, β-endorphin, and other POMC peptides are produced locally by the immune cells themselves. Several lines of evidence support the hypothesis that PML synthesize and release POMC related peptides.

The expression of the POMC gene has been demonstrated in PML of several mammalian species. Human PML express the gene for POMC as demonstrated by the specific binding of a labelled DNA probe to a Northern blot of the mRNA (Buzzetti et al., 1989). Bovine PML activated with bovine herpesvirus-1 **in vitro** have been observed to express the gene for POMC. The mRNA of the

activated bovine PML was observed to contain a sequence which was bound by a specific labelled human DNA probe for the POMC message (Westly and Splitter, 1987). Activated mouse T-helper cells have also been observed to produce mRNA for preproenkephalin (Zurawski et al., 1986). Given that immune cells expressed the gene for POMC related peptides, it was predicted that POMC proteins would also be synthesized by the immune cells.

Several different approaches were used to study the synthesis of POMC peptides by PML. The first method involved the staining of tissue sections or cell preparations by immunocytochemistry (ICC). Cells that have been positively stained by ICC procedures are termed immunoreactive (ir). Immunoreactive $\beta$-endorphin has been identified in mouse spleen macrophages (Lolait et al., 1984) by indirect immunofluorescence ICC.

If the tissue section or cell preparation failed to stain positively for the peptide of interest, it was possible to increase the amount of stored peptide by pre-exposing the cells to mitogenic stimuli. Human PML have been shown to be capable of producing $\beta$-endorphin and ACTH following a wide variety of treatments. In particular, human PML produce ir-$\beta$-endorphin and ir-ACTH after: (1) **in vitro** infection with Newcastle's disease virus (Smith et al., 1982); (2) **in vitro** stimulation with the endotoxin lipopolysaccharide (Harbour-McMenamin et al., 1985); (3) **in vitro** stimulation with corticotropin releasing factor (Smith et al., 1986); and (4) **in vivo** administration of a typhoid vaccine (Meyer et al., 1987).

A second alternative for detecting peptides produced in small quantities was utilized in the current study. Instead of increasing protein stores with mitogenic stimuli, the signal amplification of the technique was

improved. Several variations of ICC methodology have been devised that increase the degree of signal amplification; however, before discussing improvements to the ICC, it is first necessary to understand the principles of the technique.

ICC is a technique that enables one to detect the presence or absence of a specific antigen in a tissue or cell preparation. The detection of the antigen is based on the primary antibody raised against the molecule of interest. A primary antibody attaches to any molecule in the cell which contains the antigenic determinants recognized by the primary antibody. Several different methodologies may be used to locate cells which bind the primary antibody. These methods can be divided into two basic groups: direct and indirect labelling of the primary antibody.

The simplest is the direct method. In this case the primary antibody is labelled directly. The label conjugated to the primary antibody can be radioactive (e.g., $I^{125}$ labelled immunoglobulin), enzymatic (e.g., peroxidase conjugated to the immunoglobulin), or fluorescent (e.g., isothiocyanate conjugated to the immunoglobulin).

The direct method is the least sensitive in detecting cellular peptides. A single peptide antigen is labelled by only the primary antibody. If the peptide is small, then, at most, several primary antibodies can bind to it. A three to four fold amplification of the signal is probably the best that can be achieved by direct ICC.

The purpose of indirect methodology is to increase the amplification of the signal. In the indirect method, a second molecule that attaches specifically to the primary antibody is labelled. Once again there are

several methods of labelling this molecule. In general, the second molecule is an antibody that has been raised against the primary antibody. For example, if the primary antibody is isolated from a rabbit inoculated with the antigen of interest, then the secondary antibody will be an anti-rabbit-immunoglobulin antibody.

In the indirect ICC methodology the sensitivity is greatly increased. Consider a simple case where three primary antibodies can bind the peptide; furthermore, assume that four labelled secondary antibodies can bind a single primary antibody. In this situation, the signal has been amplified twelve fold; a significant improvement in resolution over the direct method.

Even greater amplification can be obtained by the indirect avidin-biotinylated-peroxidase complex (ABC) method, the most sensitive of ICC methodologies. In brief, a tissue section or cell preparation is incubated first with a primary antibody raised against the molecule of interest. Next, a biotinylated secondary antibody that recognizes the primary antibody is incubated with the tissue section or cell preparation. The antigen-primary-secondary-antibody complex is then localized by adding the ABC reagent to the tissue section or cell preparation.

The ABC reagent is formed by incubating a suspension of avidin, a protein containing four high affinity binding sites for biotin with biotinylated horseradish peroxidase. During the incubation, many large complexes of avidin and biotinylated peroxidase are formed. Upon adding the ABC reagent to the tissue section or cell preparation, the unoccupied avidin domains remaining on individual ABC complexes are bound to the

biotinylated secondary antibodies.

The antigen labelled by the primary-secondary-antibody-ABC is resolved by incubating the cell preparation in a solution containing an electron donor like 3,3'-diaminobenzidene (DAB). The reducing agent is locally oxidized by the peroxidase enzyme in a ABC to form an amorphous precipitate at the site of antigen binding. The precipitate is visible on the tissue sections or cell preparations when examined under a light microscope.

In the ABC method there are multiple points where the signal is amplified. Besides the initial amplification of the signal by twelve due the binding of the primary and secondary antibodies, assume that the biotinylated secondary antibodies are bound by two of ABC complexes. Furthermore, although the ABC complex has not been characterized, assume that four peroxidase enzymes are bound in a single complex. Thus, the signal is amplified at least 96 times and probably more by the ABC method. Clearly the degree of signal amplification achieved by the ABC methodology is greater than that achieved by simple indirect methods.

It should be recognized, however, that all ICC methodology has some limitations. While it is possible to determine if the cell/tissue in question stores a molecule that can interact with the primary antibody, it is not possible to determine with exact nature of the reacting antigen or when the antigen first appears in the cell. Moreover, ICC provides no proof of actual hormone release, although it is commonly assumed that positively staining cells also release product.

The second method for studying the cytoplasmic constituents of cells is gel electrophoresis. The gel matrix separates a mixture of proteins on the

basis of size. As the protein mixture moves through the gel matrix, the migration of the larger proteins through the gel is impeded more than that smaller proteins. Thus, proteins of similar gel mobility concentrate into discrete bands as the protein mixture moves through the gel. These protein bands can be further characterized by a technique known as Western blotting.

In Western blotting, the proteins in the gel are transferred (blotted) onto a nitrocellulose or nylon membrane. The membrane is then screened for the protein of interest with an antibody raised against the protein. To determine where the antibody has bound on the blot, the antibody can be labelled either directly or indirectly (see above). The positions of the labelled proteins are determined by radioautography or chromogens.

The Western blot provides more information about a cellular protein than ICC. Not only does Western blotting enable a researcher to assess the ability of a cell preparation to synthesis a protein, it also provides an estimate of protein size and of the quantities produced in the cell.

In summary, the above investigations indicate that cells of the immune system are capable of the local production of ACTH and $\beta$-endorphin. The following series of experiments were performed to further characterize the synthesis of POMC proteins by PML. The primary question addressed was this: do PML of species other than humans and mice store POMC peptides?

## Materials and Methods

**Animals:** Blood of the donor animal was collected into a syringe containing 5ml of an Acid Citrate Dextrose (ACD) solution for each 45ml of

whole blood collected. ACD was made by dissolving 132g sodium citrate, 58g citric acid, and 150g dextrose into three liters of reagent grade water. For each experiment, a fresh supply of blood was utilized. The methods of collection were as follows:

Rat: Each experiment required approximately 30ml of fresh rat blood. Each Harlem Sprague-Dawley rat provided about 5 to 6ml of whole blood. A rat was placed into a large jar containing a wad of cotton soaked with diethyl ether. As soon as the rat lost consciousness, it was removed from the jar and placed under a fume hood. Anesthesia was maintained with a funnel that had its neck stuffed with cotton that was then drenched with diethyl ether.

With a syringe containing the ACD solution ready, the skin of the abdomen was peeled back to expose the underlying abdominal muscles. The abdominal cavity was opened with a mid-line incision using a pair of scissors. Next, the intestines were moved to the one side to expose the kidneys -sometimes it was necessary to remove the adipose tissue to expose the kidneys. Rostral to the kidney, and running parallel to the spinal column was the large dorsal aorta. The needle of the syringe (18-20 gauge) was inserted into the artery and the blood slowly withdrawn. The procedure was repeated on additional rats until at least 30ml of fresh rat blood was obtained.

Canine: Each experiment required approximately 200ml of fresh canine blood. Each greyhound provided 50ml of blood and four donors were used in

a single experiment.

While one person restrained the dog, its head was tilted back and slightly to one side to expose the jugular vein. Fourty-five ml of whole blood was collected by venepuncture into a 50ml syringe containing 5ml of ACD. The syringe was then inverted several times before the procedure was repeated on another greyhound.

Bovine: The blood used in the bovine experiments was provided by Dr. Roth's group in the Department of Veterinary Microbiology and Preventative Medicine. The whole blood was collected by venepuncture into a 250ml sterile screw top bottle containing 25ml ACD on the morning of the experiment.

Porcine: The blood used in the porcine experiments was kindly provided by two sources: the ISU Meat Laboratory and Dr. Roth's research group. The whole blood was collected by venepuncture on the morning of the experiment. As before, the sample contained 5ml of ACD per 45ml of whole blood.

**Leukocyte preparation:** The isolation procedure for the mononuclear peripheral leukocytes was the same for each species. Whole blood containing 5ml ACD/45ml whole blood was divided into an even number of 50ml plastic centrifuge tubes and spun on the Centra-8 centrifuge (International Equipment Company) at 850g for 22 minutes. After centrifugation, the samples were placed in a laminar flow hood. The buffy coat, the top of the

plasma/cell interface, was drawn off along with 5ml of plasma and placed into a 15ml plastic centrifugation tube. To each 15ml centrifugation tube was added 5ml of a density gradient (Sepracell-MN, Sepratech Corporation). After adding the Sepracell-MN, each tube was capped, inverted several times and spun on the Centra-8 centrifuge at 1900g for 22 minutes.

Following centrifugation with the Sepracell-MN density gradient, a distinct white band of mononuclear leukocytes was apparent just below the meniscus in each centrifuge tube. The white band was collected from each tube with a sterile Pasteur pipet and combined into a single 15ml centrifuge tube which was then filled to capacity with Hank's Balanced Salt Solution (Gibco). The sample was triturated with a sterile Pasteur pipet, and spun on a Clinical centrifuge (International Equipment Company) at setting 6 for five minutes. Next, the supernatant was decanted from the tube and 5ml of Hank's medium was added back before the pellet was triturated with a sterile Pasteur pipet and filtered through a sterile 25$\mu$m mesh into a fresh 15ml centrifugation tube. The filtrate was then diluted to 10ml with fresh Hank's medium, the tube inverted several times, and an aliquot was taken for a cell count on a hemocytometer.

The sample was then spun on the Clinical centrifuge at setting 6 for 5 minutes; the supernatant was discarded and the pellet was diluted with Hank's Balanced Salt Solution to at a concentration of $2 \times 10^6$ cells per ml of solution. The cells thus obtained were a mixture of mononuclear leukocytes. Approximately 5% of the preparation were monocytes and the rest was a mixture of T lymphocytes and B lymphocytes (information provided by the Sepratech Corporation).

**Murine pituitary preparation:** The anterior lobe of the pituitary was isolated from a freshly killed Harlem Sprague-Dawley rat and placed into a sterile siliconized petri dish (6cm diameter) containing 2ml of Spinner's-0.1% bovine serum albumin (BSA) which was made by adding 1.1ml penicillin-streptomycin (Gibco) and 2.6ml 4% BSA to 100ml of Spinner's Minimal Essential Medium (Gibco). The petri dish was placed in a laminar flow hood where the anterior lobe was transferred to a larger sterile, siliconized petri dish (10cm diameter), rinsed several times with fresh Spinner's-0.1% BSA, and cut into approximately $1mm^3$ pieces with sterile scalpel blades.

The pieces were next transferred to a siliconized spinner's flask containing 10ml of a 1mg/ml trypsin solution in Spinner's-0.1% BSA that was filtered through a Millex-GV filter. The flask was gassed through a sidearm for 20 seconds with 5% $CO_2$/ 95% air, placed in a 37°C water bath and left to incubate with stirring for 50 minutes. After stirring, the suspension was triturated under a laminar flow hood using a sterile pipet, gassed again with 5% $CO_2$/ 95% air, and placed in a 37°C water bath to incubate for an additional 30 minutes. This procedure was repeated at 20 minute intervals thereafter until the pituitary pieces were no longer visible.

After dispersing the pituitary cells, they were transferred to a 15ml centrifugation tube, made up to 15ml with Spinner's and spun on a Clinical centrifuge (IEC) at setting 5 for 10 minutes. The supernatant fluid was decanted, 5ml Spinner's was added to pellet which was then triturated with a Pasteur pipet. An aliquot was removed for a cell count on a hemocytometer, then the suspension was made to 15ml with Spinner's and spun

on a Clinical centrifuge at setting 5 for 10 minutes.

The pellet was resuspended in enough DMEM-0.1% BSA (100ml Dulbecco's Modified Eagles Medium, Gibco; 1.1ml penicillin streptomycin (10,000 units/ml), Gibco; 2.6ml 4% BSA) to achieve a concentration of 2 to 4 x $10^6$ cells per ml of medium and transferred to a sterile plastic petri dish. The cells were placed in a humidified incubator (Queue Cell Culture Incubator, Queue Systems, Inc.) at 37°C, 5% $CO_2$/ 95% air, for 3 hours before the DMEM solution was decanted and replaced with 2ml of DMEM-10% horse serum (100ml DMEM, Gibco; 1.1ml penicillin streptomycin, Gibco; 11.3ml horse serum, Gibco). The pituitary cells were then incubated overnight in a humidified Queue incubator at 37°C, 5% $CO_2$/ 95% air.

Prior to staining, the pituitary cells were retrypsinized from the plastic petri dish as follows. The DMEM-10% horse serum was poured off and replaced by 2ml of a trypsin solution (2.5mg trypsin, 10ml Spinner's-0.1% BSA). The bottom of the dish was tapped lightly after 5 minutes. The solution was transferred with rinsing into a 15ml centrifuge tube after 10 minutes. The centrifuge tube was made to 15ml with Spinner's and spun on a Clinical centrifuge at setting 5 for 10 minutes. The supernatant fluid was decanted, the pellet resuspended in 5ml of Spinner's and an aliquot was removed for a cell count on a hemocytometer. The suspension was then made to 15ml with Spinner's and spun for an additional 10 minutes. The supernatant was again decanted and the cells were suspended in sufficient DMEM-0.1% BSA to achieve a concentration of 2 x $10^6$ cells per ml of medium.

**Leukocyte immunocytochemistry:** One hundred $\mu$l of peripheral mononuclear

leukocytes (PML) at a concentration of 2 x $10^6$ cells per ml of Hank's medium were placed inside a hydrophobic ring that had been inscribed at one end of a poly-l-lysine coated microscope slide using a PAP Pen (Daido Sangyo Co., LTD.). The slides were then placed inside ICC trays and transferred to a Queue incubator at 37°C, 5% $CO_2$/ 95% air, for 30 minutes to allow attachment of the PML to the slides. After thirty minutes, the slides were gently washed three times with Tris buffer that was made up as follows. 6.06g Trizma base and 8.77g NaCl were dissolved in one liter of doubled distilled water; the resulting solution was adjusted to a pH of 7.6 with 1.0M HCl.

After rinsing with the Tris buffer, the slides were submerged in ice cold ethanol at 4°C for 10 minutes to fix the cells on the slides. The slides were then washed three times with Tris and dried outside the PAP ring with light suction. A solution of hydrogen peroxide (1ml 30% hydrogen peroxide, 10ml double distilled water) was then placed inside the PAP rings for five minutes. Hydrogen peroxide treatment of PML destroyed endogenous peroxidase activity.

The slides were then rinsed with Tris and suction dried outside the PAP ring. Next, diluted normal goat serum (180$\mu$l normal goat serum, Vectastain ABC Kit, Vector; 9.82ml Tris) was placed inside the PAP rings containing the PML for thirty minutes. Normal goat serum was added to prevent non-specific binding of the antibodies to the PML.

After thirty minutes the normal goat serum was removed with light suction, and replaced with the primary antibody (anti-human ACTH immunoglobulin, rabbit raised, Dako Corporation). The slides were

incubated with the primary antibody for ninety minutes in humidified ICC trays. The primary antibody was prepared in dilutions of 1:100 to 1:2000 (v:v) by diluting the stock antibody solution (as obtained from Dako) with Tris. This antibody was obtained from whole serum.

At the end of ninety minutes, the slides were washed three times with Tris and then dried with suction before the biotinylated secondary antibody (biotinylated anti-rabbit goat immuno-globulin, ABC Kit) was added to the interior of the PAP rings. The secondary antibody was allowed to incubate on the slides for thirty minutes before the slides were washed off by three Tris rinsings and then suction dried.

The inside of the PAP rings were next flooded with a preformed solution of the avidin-biotinylated-peroxidase complex (ABC Kit, Vector) and allowed to incubate for forty-five minutes. The avidin-biotinylated-peroxidase complex (ABC) was formed incubating 90$\mu$l of 'A' and 90$\mu$l of 'B' (reagents supplied in the Vectastain ABC KIT, Vector) together in 9.88ml of Tris buffer for at least twenty minutes. 'A' contained a suspension of avidin, a protein with four high affinity binding sites for biotin. 'B' contained a suspension of a multi-biotinylated horseradish peroxidase enzyme. After incubating together for twenty minutes, a number of large complexes, the ABC reagent, were formed that contained multiple copies of the peroxidase enzyme.

The slides were then washed three times with Tris, placed into slide holders, and developed for 5 minutes in the peroxidase developing solution. This solution was formed by combining 150ml of a filtered Tris Solution containing 1mg/ml 3,3'-diaminobenzidene (DAB) and 150ml of a 5% nickel

sulfate solution containing 200$\mu$l 30% hydrogen peroxide. DAB was oxidized by the bound ABC complex to form an amorphous precipitate at the site of the reaction. This nickel enhanced precipitate was visible under a light microscope.

Finally, the slides were rinsed three times in distilled water, rinsed for 5 minutes in tap water, and dehydrated in the following ethanol series for one minute each: 50% EtOH, 70% EtOH, 80% EtOH, 95% EtOH, 100% EtOH, 50% EtOH/ 50% xylene, 100% xylene. After dehydrating the slides, coverslips were mounted on the slides with Quick-Mount (Daido Sanygo Co., LTD.).

In each experiment, a set of duplicate slides was run for each treatment. A series of controls were run to reduce the possibility of non-specific staining. These controls included the following: (1) omission of the primary antibody, (2) omission of the secondary antibody, (3) omission of the primary and secondary antibodies, (4) addition of normal rabbit serum instead of primary antibody at same dilution as primary antibody, (5) a serial dilution of the primary antibody, and (6) preabsorption of the primary antibody with 50ng/ml ACTH for 90 minutes at room temperature. In addition, red blood cells were subjected to the same staining procedure to serve as a negative control.

Each experiment was replicated three times or more.

**Pituitary immunocytochemistry:** The anterior pituitary gland was isolated and subjected to staining to serve as a positive control. It has been reported that 5-10% of the cells in the anterior pituitary produce

ACTH (Childs et al., 1989) and thus, a similar percentage of the dispersed pituitary cells were expected to stain positively for ir-ACTH in this laboratory.

The procedure followed for staining the pituitary cells was the same as the immunocytochemistry of the leukocytes, as described in detail above, except for fixation of the cells. 100$\mu$l of the retrypsinized pituitary cells at a concentration of 2 x $10^6$ cells per ml of DMEM-0.1% BSA were placed inside the PAP ring inscribed at one end of a poly-l-lysine coated microscope slide. The slides were placed in humidified ICC trays and transferred to a Queue incubator at 37°C, 5% $CO_2$/ 95% air for 45 minutes.

After incubating for 45 minutes, the slides were rinsed three times with Tris and then suction dried outside the PAP rings. The slides were then covered with a 9:1 B-5 fixative (68g/l mercuric chloride, 13.61 g/l sodium acetate) to formaldehyde solution for 10 minutes. After ten minutes, the slides were bathed in 95% ethanol for one minute and then in double-distilled water for one minute. The slides were then subjected to the following series of 1 minute baths: Lugol's Iodine (10.15 g/l iodine, 19.92 g/l sodium iodide); doubled-distilled water; sodium thiosulfate, 19.85 g/l; and Tris.

Following the last bath in Tris, the slides were suction dried outside the PAP rings and covered with a solution of saponin (0.5mg/ml) for thirty minutes. Next, the slides were rinsed three times with Tris, suction dried outside the PAP ring and then covered with a hydrogen peroxide solution. The procedure after this point was the same as described above for leukocyte ICC.

**Western blot:** The western blot was performed on the lysate of bovine PML to confirm the synthesis of ir-ACTH. After the final spin in the leukocyte isolation procedure (see above) and before bovine PML are resuspended, 2ml of lysis buffer (1.25ml NP40; 250$\mu$l of 10,000 KIU/ml transylol; 1ml 0.5M Hepes- pH 7.25; dilute to 25ml with double distilled water) was added to the pellet in the centrifugation tube. The mixture was next vortexed for several minutes and then spun on a Clinical centrifuge at setting 6 for 10 minutes. The supernatant fluid obtained from the lysate was decanted from the centrifuge tube and then respun on the Clinical centrifuge for an additional 10 minutes at setting 6. This supernatant fluid was either loaded on a polyacrylamide gel or frozen in 50$\mu$l aliquots at -20°C for later use.

In addition to the supernatant lysates, $I^{125}$-ACTH, ACTH, and cytochrome c were loaded into separate wells of the polyacrylamide gel. $I^{125}$-ACTH was loaded to serve as a visible marker of ACTH migration in the gel; it did not require labelling by the antibody to be detected by radioautography. ACTH was loaded on the gel to serve as a positive control for the resolution of the blot. If the unlabelled ACTH was not visible in the radioautograph of the blot, then the antigen labelling procedure had failed. Cytochrome c was loaded on the gel to serve as a molecular weight marker; it had a known mass of 10.5 kDa.

Pouring the Gel: The 15% polyacrylamide gel was poured just before the protein sample was loaded. After constructing the mould for the gel and sealing the edges with 1% agarose, 15ml of degassed 15% acrylamide solution

was mixed with 7.5$\mu$l of N,N,N',N'-tetramethylethylenediamine (TEMED) and 10$\mu$l of 10% ammonium persulfate and quickly transferred to the mould with a Pasteur pipet. The gel was covered with an overlay solution and allowed to polymerize for thirty minutes. The overlay solution was prepared by mixing 20ml of Tris buffer (181.5g/l Trizma adjusted to a pH of 8.9), 1ml of 1% SDS, and 79ml of distilled water.

Running the Gel: After the gel had polymerized, the overlay solution was poured off and the stacker added. The stacker was made from a 5ml of degassed 5% acrylamide solution mixed with 2.5$\mu$l of TEMED and 50$\mu$l of 10% ammonium persulfate and then transferred to the top of the gel with a Pasteur pipet. After the stacker solution was added to the top of the 15% polyacrylamide gel, a comb was inserted to form the lanes for running the protein.

The comb was removed from the gel after thirty minutes, and the resulting wells were rinsed with distilled water and dried with a needle attached to a vacuum. The gel was then inverted, the bottom spacer of the mould removed, and the space was filled with 1% agarose-SDS. Once the spacer had set, the gel was sealed onto the polyacrylamide gel electrophoresis (PAGE) apparatus with 1% agarose-SDS.

The PAGE apparatus reservoirs were filled with running buffer (172.8g glycine; 36g Trizma base; 6g SDS; diluted to six liters with distilled water) after each lane of the gel had been loaded with the appropriate protein sample or standard. Each well was loaded with either 30$\mu$l of solution containing a 1:1 mixture of sample to 2x SDS loading buffer (25ml

5.7% Tris solution- pH 6.9, 20ml glycerol, 8ml 0.1% bromphenol blue, 43ml distilled water, 4g SDS, and 200$\mu$l $\beta$-mercaptoethanol/5ml of buffer) or 1x SDS loading buffer (5ml 2x SDS loading buffer diluted to 10ml with distilled water). Some samples were heat treated (100°C, 2 minutes) before being loaded in the wells.

A power supply was then attached to the PAGE apparatus and the gel was allowed to run until the bromphenol blue indicator entered the bottom spacer. The power was then turned off and the gel was blotted.

Blotting the Gel: The resolved gel was soaked for thirty minutes in degassed transfer buffer (16.3g Trizma base; 1.2l methanol; 54g glycine; diluted to 6 liters with double distilled water). The soaked gel was then placed in a sandwich made with the transfer cassette (Transfor apparatus, Hoefer Scientific Instruments). The sandwich consisted of: (1) a Dacron sponge on the first half of the cassette, (2) two sheets of Whatman 3mm filter paper, (3) a nitrocellulose membrane cut to the size of the gel and presoaked in the transfer buffer, (4) the resolved gel soaked in transfer buffer, (5) two additional pieces of 3mm Whatman filter paper, and (6) the second half of the cassette. The cassette was then placed in the Transfor apparatus, four liters of the degassed transfer buffer were added, and a power supply was connected with the anode on the nitrocellulose side of the cassette.

The voltage was adjusted to 100V at the beginning of the transfer, and the current was kept below 0.9 mA. The electrotransfer was continued for three hours before the cassette was disassembled and the blot resolved.

Resolving the Blot and Gel: The blot was resolved using radiolabelled $I^{125}$ Protein A as follows. The nitrocellulose membrane was incubated with a 1:200 solution of normal rabbit serum for thirty minutes at room temperature with mild shaking. Next, the rabbit serum was poured off and membrane was incubated with 1:100 solution of anti-human ACTH immunoglobulin (Dako Corporation) for two hours at room temperature with mild shaking. The membrane was then rinsed three times with Tris for ten minutes each time. After rinsing, the membrane was soaked in a solution containing $5 \times 10^5$ cpm of $I^{125}$ Protein A in 2% nonfat powdered milk (dissolved in doubled distilled water) for 2.5 hours with gentle shaking. Following the Protein A incubation, the membrane was rinsed three times with Tris for 10 minutes each time.

To facilitate localization of the standards, the membrane was stained in Ponceau C reagent and the position of the unlabelled standards were marked with radioactive lead. The membrane was then wrapped in plastic wrap, and exposed at -70°C to a pre-flashed Kodak X-ray film for 10 days.

After blotting the polyacrylamide gel, it was stained in Coomassie Blue, impregnated with 2,5-diphenyloxazole (PPO) by soaking it in a 20% PPO dimethyl sulfoxide solution, placed on a piece of filter paper cut to the size of the gel, dried on a gel dehydrator, and then wrapped in plastic wrap. Finally, the gel was exposed to a pre-flashed Kodak X-ray film at -70°C for 10 days.

# Results

**Immunocytochemistry validation:** ICC was performed on the peripheral mononuclear leukocytes (PML) derived from the following mammalian species: rat, bovine, porcine, and canine. Each mammalian species was probed for the synthesis of adrenocorticotropic hormone (ACTH). In addition, the synthesis of other proopiomelanocortin (POMC) peptides by PML was examined in bovine and porcine species. The bovine PML were tested for the synthesis of met-enkephalin and the porcine PML were tested for $\beta$-endorphin synthesis.

A series of control slides were run in parallel to serve as an indicator of non-specific binding and antibody specificity. These controls, as mentioned in Materials and Methods, were of several distinct types. The first was the omission of either the primary and/or the secondary antibody. As expected, deletion of either the primary or secondary antibody resulted in abolition of the staining. The replacement of the primary antibody by normal rabbit serum (NRS) of the same dilution resulted in a series of slides that could serve as indicators of non-specific binding. In the photomicrographs that follow, pictures of the control slides to which the NRS was added accompany the pictures of the slides to which the primary antibody was added.

The specificity of the anti-ACTH antibody was demonstrated by preabsorbing the antibody with 50ng/ml ACTH for one and a half hours at room temperature. The slides to which the preabsorbed antibody was added exhibited a level of staining similar to the NRS slides. A photomicrograph

of a preabsorbed anti-ACTH antibody can be seen in Figure 2.3b for the bovine PML.

Two additional controls were run to further characterize the specificity of staining observed in the experiments. A negative control was run in which red blood cells of the bovine were subjected to the ICC protocol. Red blood cells are not known to produce ACTH or other POMC peptides; thus, as expected these cells were not stained by ICC. As an additional check on the specificity of staining, a positive control was run. For this control, the dispersed cells of the anterior pituitary gland of a Harlem Sprague-Dawley rat were plated on the slide. Five to ten percent of the cells in the pituitary gland are reported to be corticotropes (Childs et al., 1989). Hence, it was expected that 5 to 10% of the dispersed cells would be stained after being subjected to the ICC. As seen in Figure 2.5a, 4% - 6% (duplicate slides) of the pituitary cells were stained by the ICC and thus, positive for ir-ACTH synthesis.

As a final check on the specificity of the ICC protocol, the primary antibody was serially diluted. It was observed that as the concentration of the primary antibody was decreased, the intensity of staining also decreased. This pattern of staining was observed for each of the species probed for ACTH production. A clear example of this pattern was observed in a series of photomicrographs of the canine PML (Figures 2.8-2.11).

A summary of the results for each animal can be found under the appropriate animal heading. The quantitative assessments of the staining patterns observed for PML of each species are accompanied by representative photomicrographs.

**Immunocytochemical detection of ACTH and met-enkephalin in bovine PML:** The bovine PML stained positively for ir-ACTH (Figure 2.1a) and ir-met-enkephalin (Figure 2.2a). At a primary antibody dilution of 1:400 for the ACTH antibody and the met-enkephalin antibody, 100% of the PML were observed to stain positively for ir-ACTH and ir-met-enkephalin. The amount of non-specific binding was negligible as evident in the respective control NRS photomicrographs (Figures 2.1b, 2.2b).

Pre-absorption of the primary antibody with the ACTH antigen at a concentration of 50pg/ml effectively blocked staining of the bovine PML (Figure 2.3b). The level of staining observed for the preabsorbed antibody was comparable to the level of non-specific staining observed on the control NRS slide (Figure 2.3c).

**Immunocytochemical detection of ACTH in rat PML and pituitary cells:** The rat PML stained positively for ir-ACTH (Figure 2.4a). On each slide to which the ACTH antibody was added at a 1:400 dilution, 100% of the PML present were stained positively by the ICC. The slides to which 1:400 NRS was added were observed to show minimal staining (Figure 2.4b).

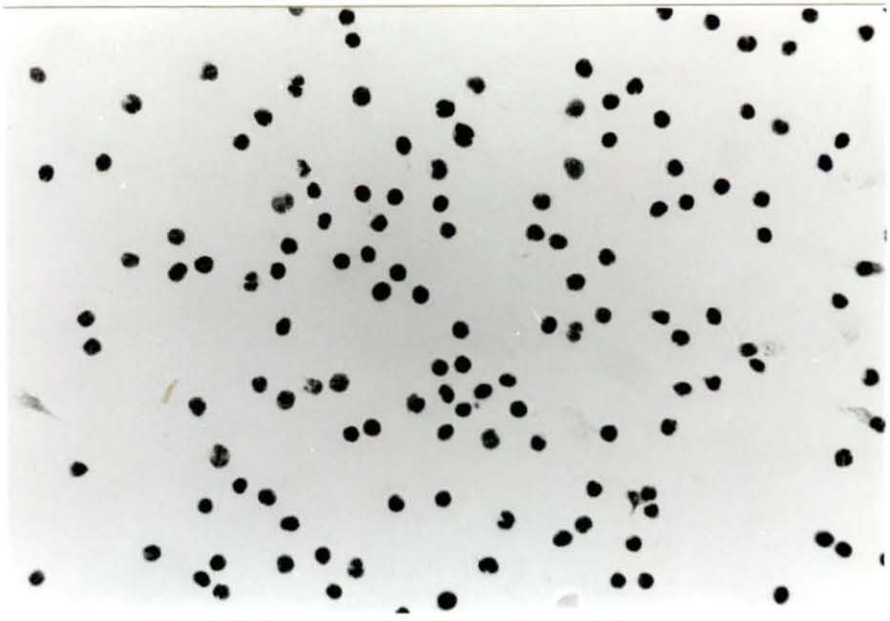
The dispersed pituitary cells were exposed to the primary antibody at a dilution of 1:1600. As seen in Figure 2.5a, 4% - 6% (duplicate slides) of the pituitary cells stained positively for ir-ACTH. Once again the addition of 1:1600 NRS in the place of the primary antibody was observed to abolish staining of the cells (Figure 2.5b).

**Immunocytochemical detection of ACTH and ß-endorphin in porcine PML:** The porcine PML stained positively for ir-ACTH (Figure 2.6a) and ir-$\beta$-endorphin (Figure 2.7a). At a primary antibody dilution of 1:400 for the ACTH antibody and 1:200 for the $\beta$-endorphin antibody, it was clearly apparent that 100% of the porcine PML present were stained by the ICC. At the same respective concentration of NRS, the amount of staining observed was negligible (Figures 2.6b, 2.7b).

**Immunocytochemical detection of ACTH in canine PML:** The canine PML stained positively for ir-ACTH (Figures 2.8a, 2.9a, 2.10a, and 2.11a). At a 1:400 dilution of the primary ACTH antibody, the canine PML were observed to be deeply stained (Figure 2.8a). As the primary antibody was further diluted the intensity of staining was observed to decline. At a 1:500 dilution of the ACTH antibody, the staining of the PML was still quite pronounced in 100% of the cells present on the slide (Figure 2.9a). When the ACTH antibody was further diluted to 1:800, the PML were observed to display some degree of differential staining (Figure 2.10a); however, 100% of the cells were still showing some degree of staining. Finally at a 1:1000 dilution of the primary antibody, the proportion of cells staining positively for ir-ACTH dropped below 100% (Figure 2.11a). At each corresponding dilution of NRS, the degree of staining observed was negligible (Figures 2.8b, 2.9b, 2.10b, and 2.11b).

Figure 2.1a: In the top photomicrograph bovine PML have been incubated with the ACTH antibody at a 1:400 dilution. Notice that 100% of the cells shown have stained positively for ir-ACTH systhesis

Figure 2.1b: In the bottom photomicrograph bovine PML have been incubated with non-immune normal rabbit serum at a 1:400 dilution. The degree of staining exhibited by these cells was negligible when compared to the cells exposed to the anti-ACTH antibody



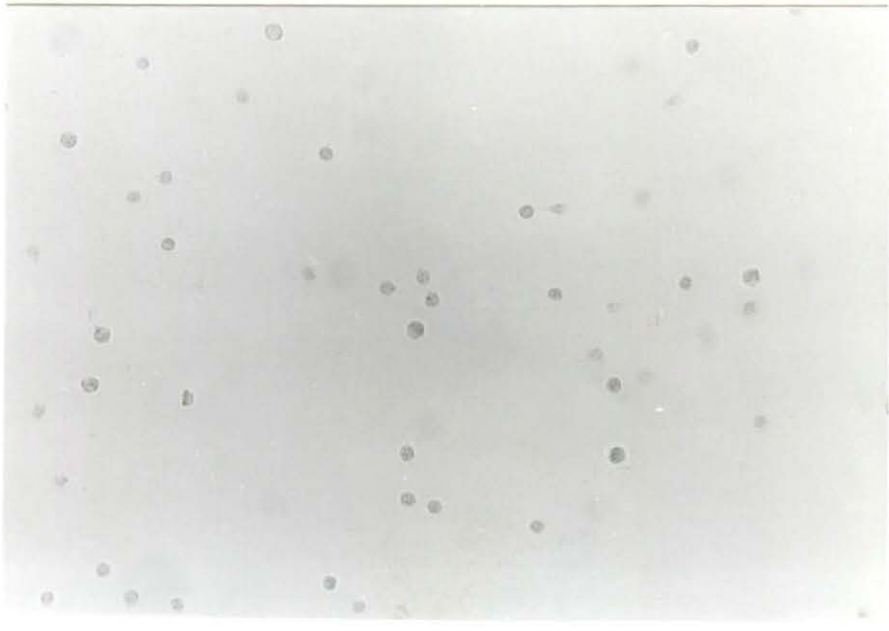
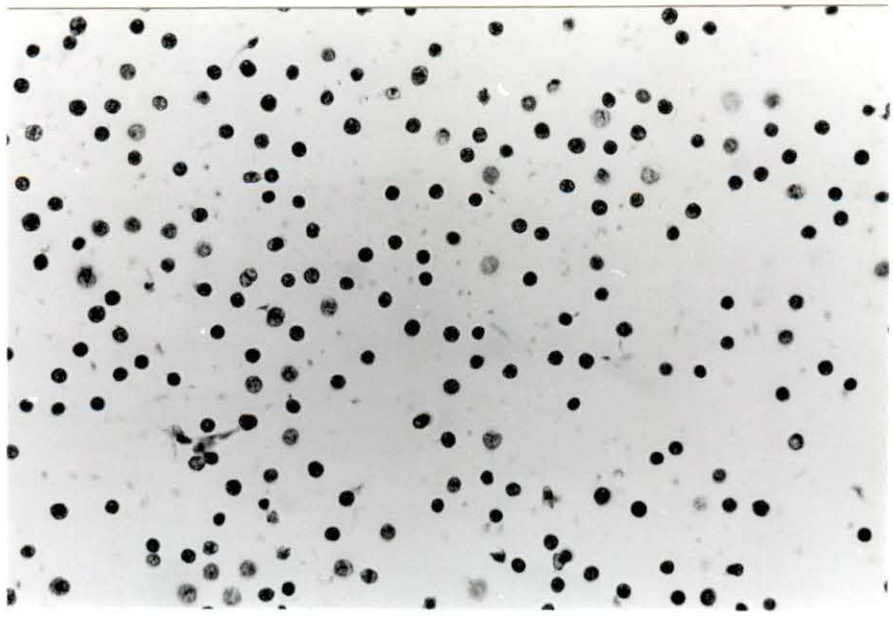


Figure 2.2a: In the top photomicrograph bovine PML have been incubated with the anti-met-enkephalin antibody at a 1:400 dilution. Notice that 100% of the cells shown have stained positively for ir-met-enkephalin synthesis

Figure 2.2b: In the bottom photomicrograph bovine PML have been incubated with non-immune normal rabbit serum at a 1:400 dilution. The degree of staining exhibited by these cells was negligible when compared to the cells exposed to the anti-met-enkelphalin antibody



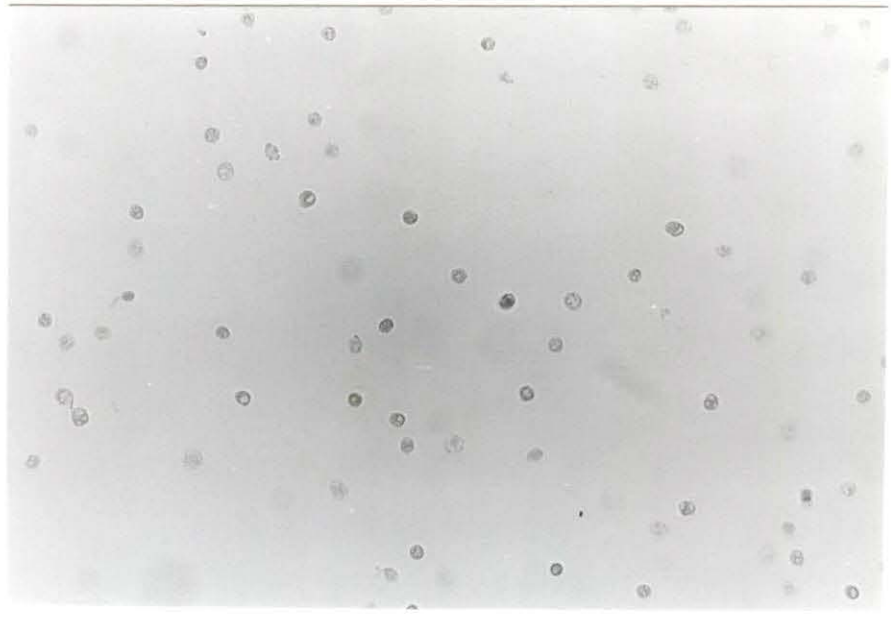
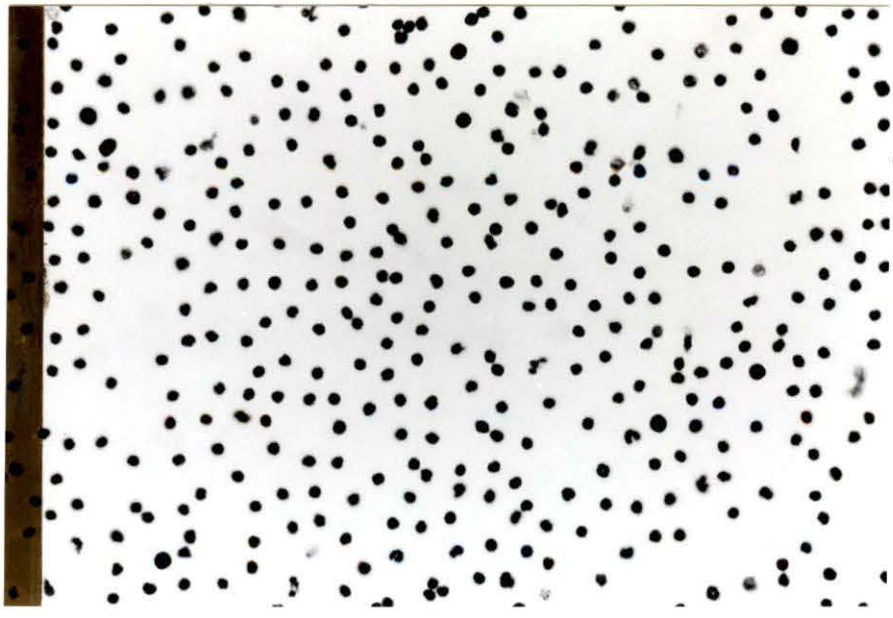


Figure 2.3a: In the top photomicrograph bovine PML have been incubated with the anti-ACTH antibody at a 1:400 dilution. Notice that 100% of the porcine PML shown have stained positively for ir-ACTH synthesis

Figure 2.3b: In the bottom photomicrograph bovine PML have been incubated with the anti-ACTH antibody at a 1:400 dilution. The anti-ACTH antibody was preabsorbed with ACTH by incubating the diluted antibody in a 50pg/ml ACTH solution for ninety minutes at room temperature. The degree of staining exhibited by these cells was negligible when compared to bovine PML incubated with the untreated anti-ACTH antibody at the same dilution



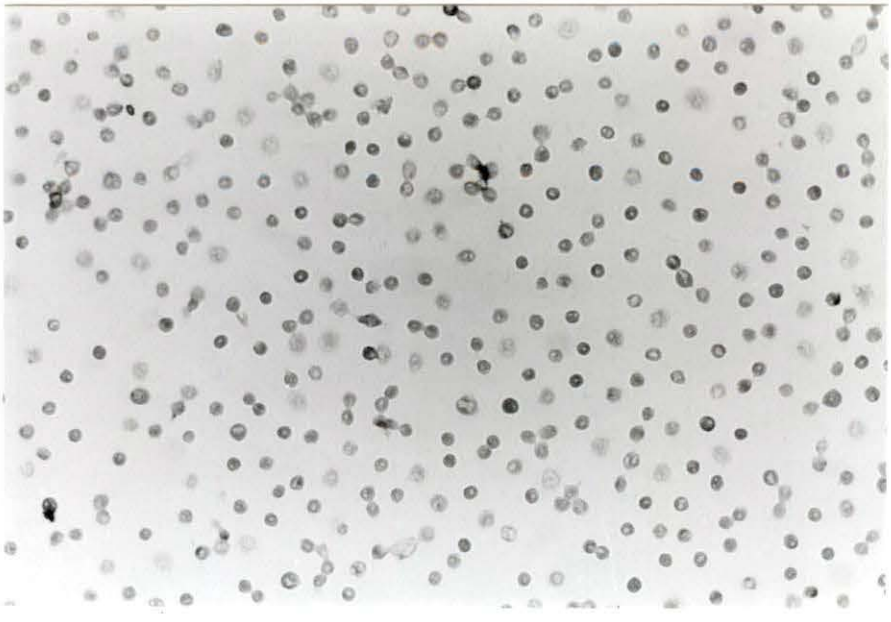


Figure 2.3c: In the photomicrograph on the right, bovine PML have been incubated with non-immune normal rabbit serum at a 1:400 dilution. The degree of staining exhibited by these cells was negligible when compared to bovine PML incubated with the untreated anti-ACTH antibody at the same dilution

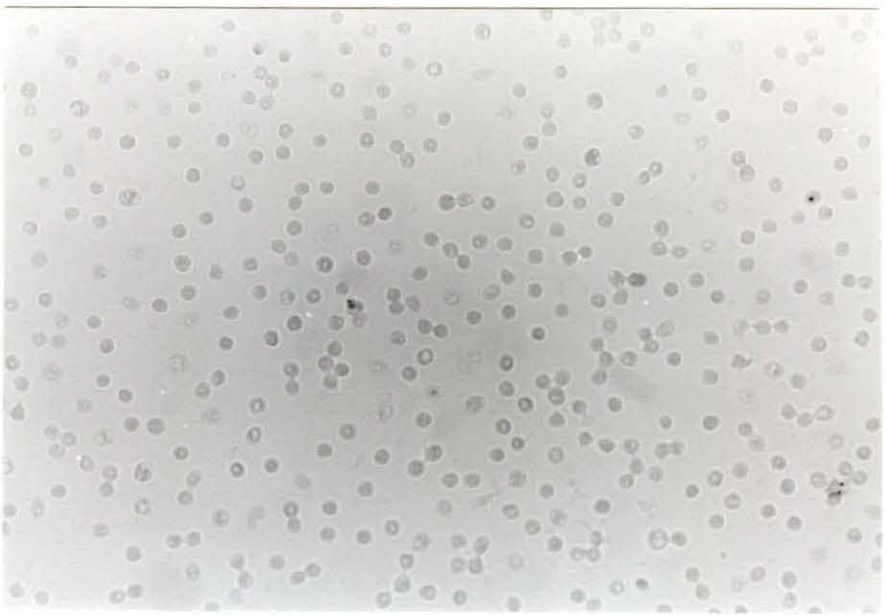
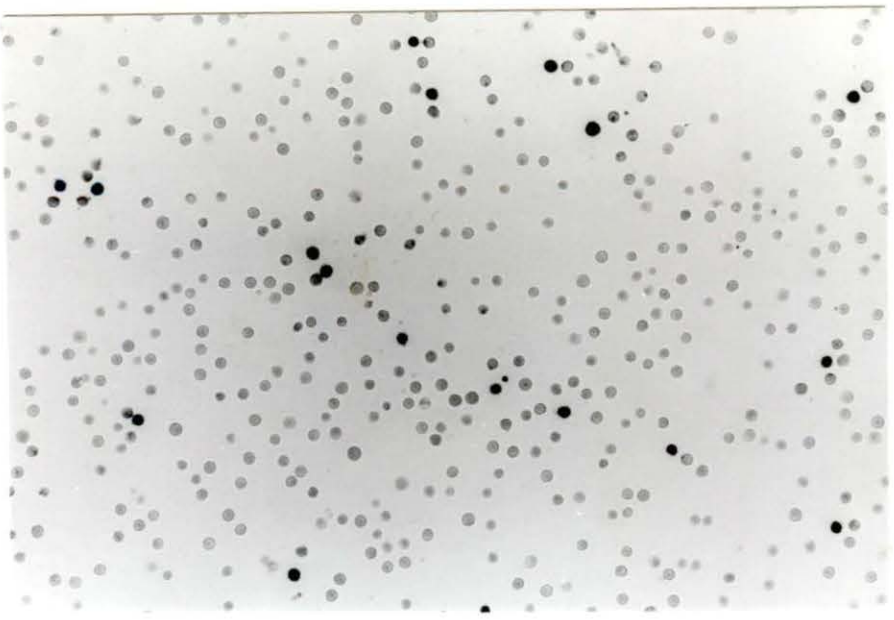


Figure 2.4a: In the top photomicrograph murine PML have been incubated with the anti-ACTH antibody at a 1:400 dilution. Notice that 100% of the cells shown have stained positively for ir-ACTH synthesis

Figure 2.4b: In the bottom photomicrograph murine PML have been incubated with non-immune normal rabbit serum at a 1:400 dilution. The degree of staining exhibited by these cells was negligible when compared to the cells exposed to the anti-ACTH antibody

Figure 2.5a: In the top photomicrograph murine pituitary cells have been incubated with the anti-ACTH antibody at a 1:1600 dilution. Notice that 4% - 6% (duplicate slides) of the dispersed pituitary cells shown have stained positively for ir-ACTH synthesis

Figure 2.5b: In the bottom photomicrograph murine pituitary cells have been incubated with non-immune normal rabbit serum at a 1:1600 dilution. The degree of staining exhibited by these cells was negligible when compared to the stained corticotropes exposed to the anti-ACTH antibody



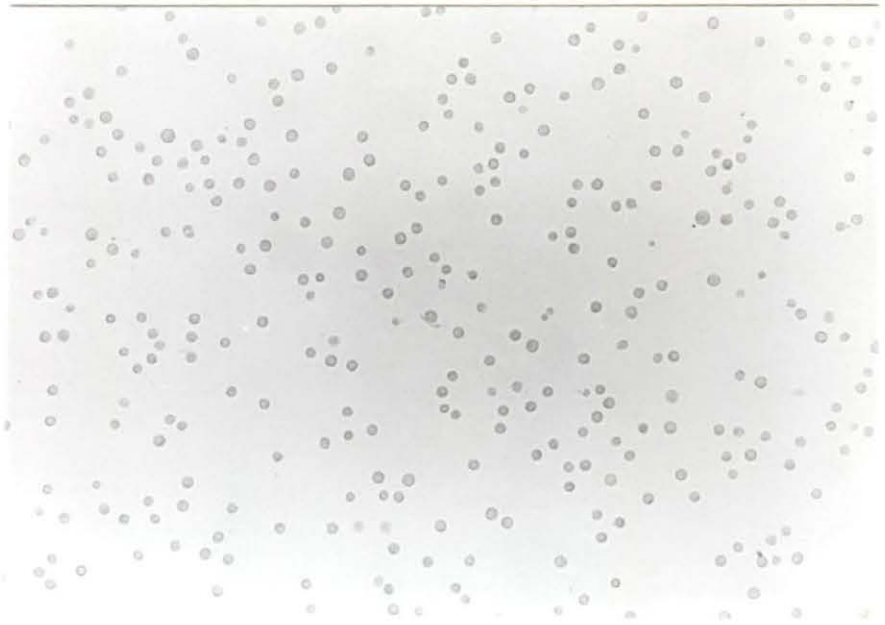


Figure 2.6a: In the top photomicrograph porcine PML have been incubated with the anti-ACTH antibody at a 1:400 dilution. Notice that 100% of the porcine PML shown have stained positively for ir-ACTH synthesis

Figure 2.6b: In the bottom photomicrograph porcine PML have been incubated with non-immune normal rabbit serum at a 1:400 dilution. The degree of staining exhibited by these cells was negligible when compared to porcine PML exposed to the anti-ACTH antibody at the same dilution

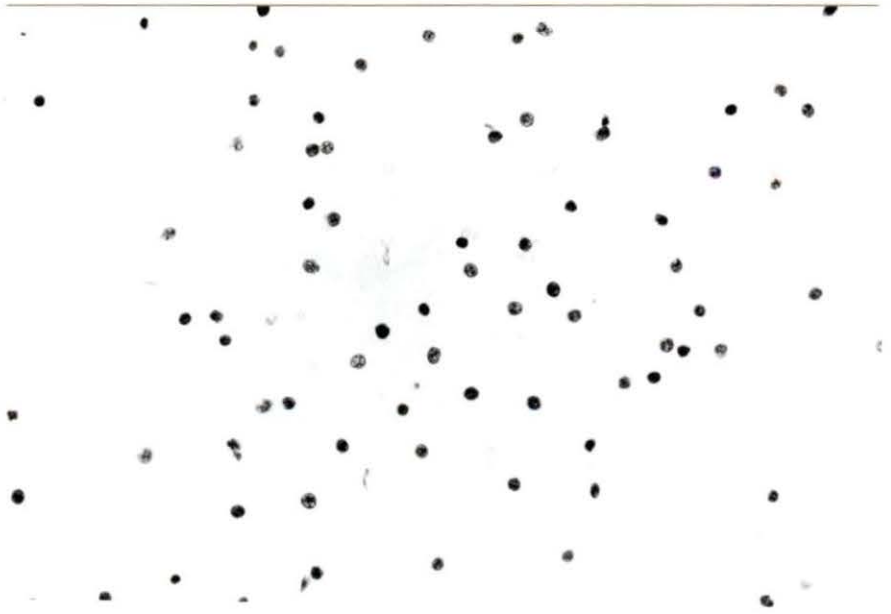
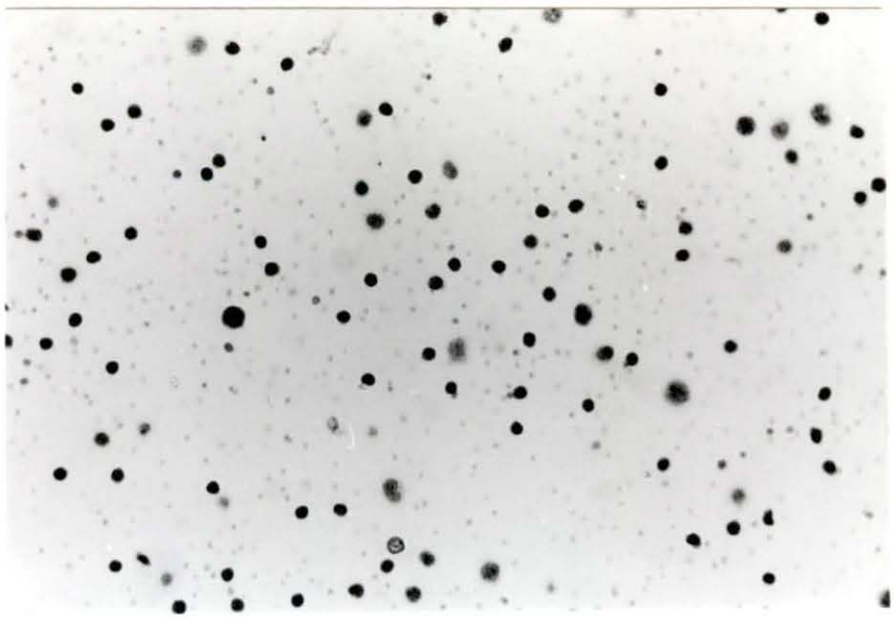


Figure 2.7a: In the top photomicrograph porcine PML have been incubated with the anti-$\beta$-endorphin antibody at a 1:200 dilution. Notice that 100% of the porcine PML shown have stained positively for ir-$\beta$-endorphin synthesis

Figure 2.7b: In the bottom photomicrograph porcine PML have been incubated with non-immune normal rabbit serum at a 1:200 dilution. The degree of staining exhibited by these cells was negligible when compared to porcine PML exposed to the anti-$\beta$-endorphin antibody at the same dilution



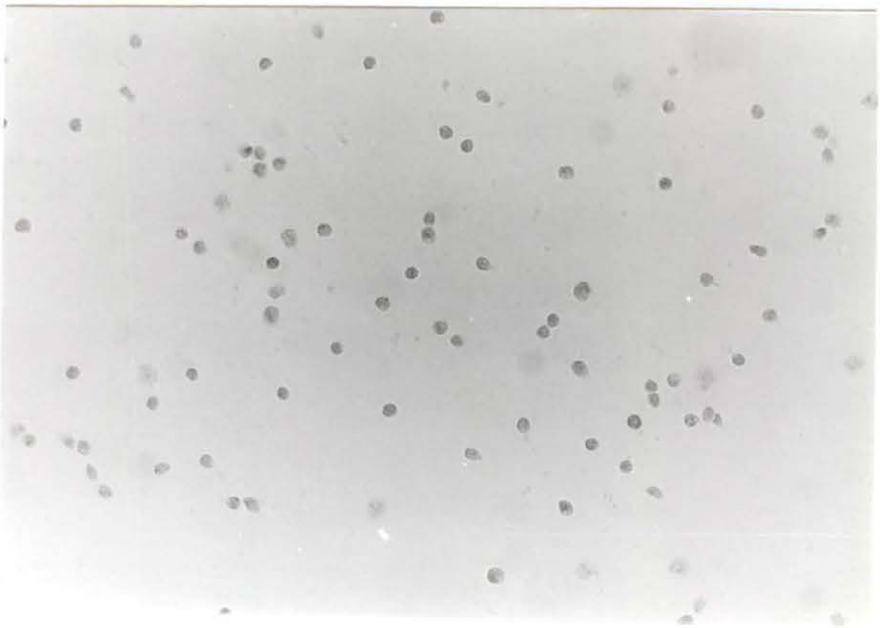
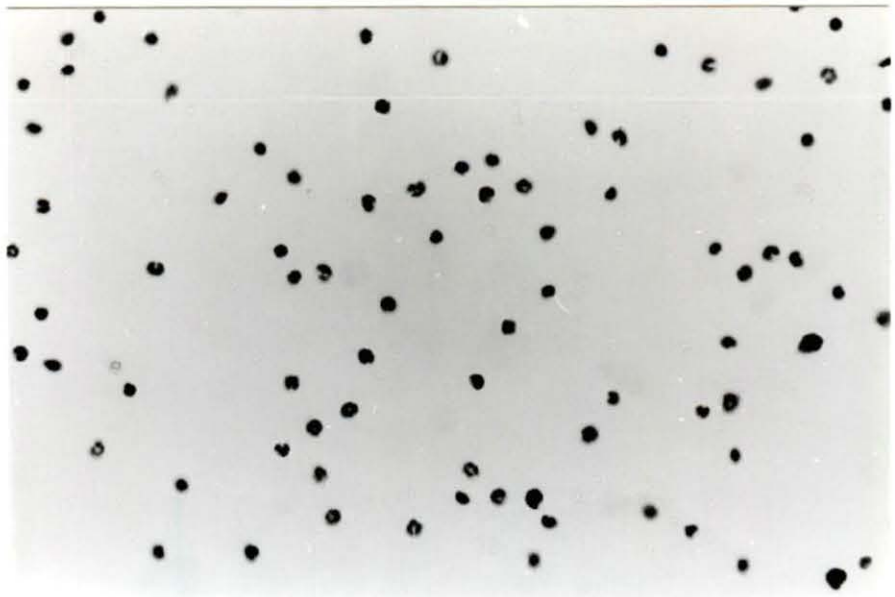


Figure 2.8a: In the top photomicrograph canine PML have been incubated with the anti-ACTH antibody at a 1:400 dilution. Notice that 100% of the canine PML shown have stained positively for ir-ACTH synthesis

Figure 2.8b: In the bottom photomicrograph canine PML have been incubated with non-immune normal rabbit serum at a 1:400 dilution. The degree of staining exhibited by these cells was negligible when compared to canine PML exposed to the anti-ACTH antibody at the same dilution



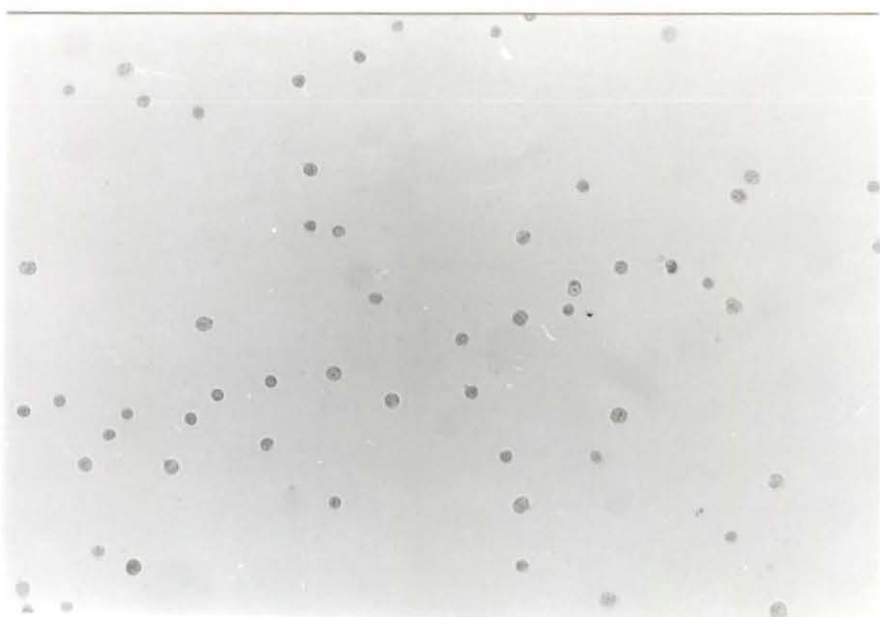
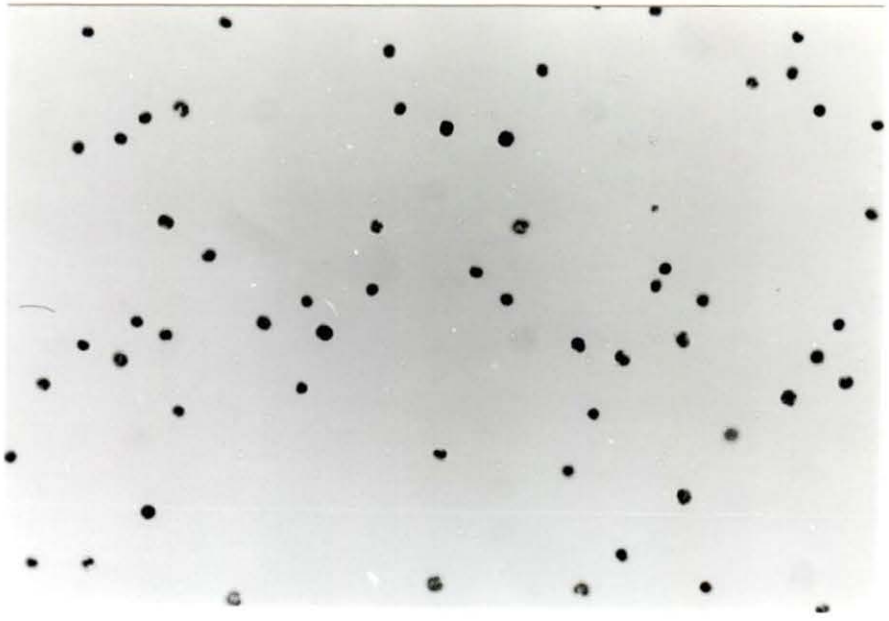


Figure 2.9a: In the top photomicrograph canine PML have been incubated with the anti-ACTH antibody at a 1:500 dilution. Notice that 100% of the PML shown have stained positively for ir-ACTH synthesis. The intensity of staining was observed to be decreased below the level observed at the 1:400 dilution of the anti-ACTH antibody

Figure 2.9b: In the bottom photomicrograph canine PML have been incubated with non-immune normal rabbit serum at a 1:500 dilution. The degree of staining exhibited by these cells was negligible when compared to the PML exposed to the anti-ACTH antibody at the same concentration



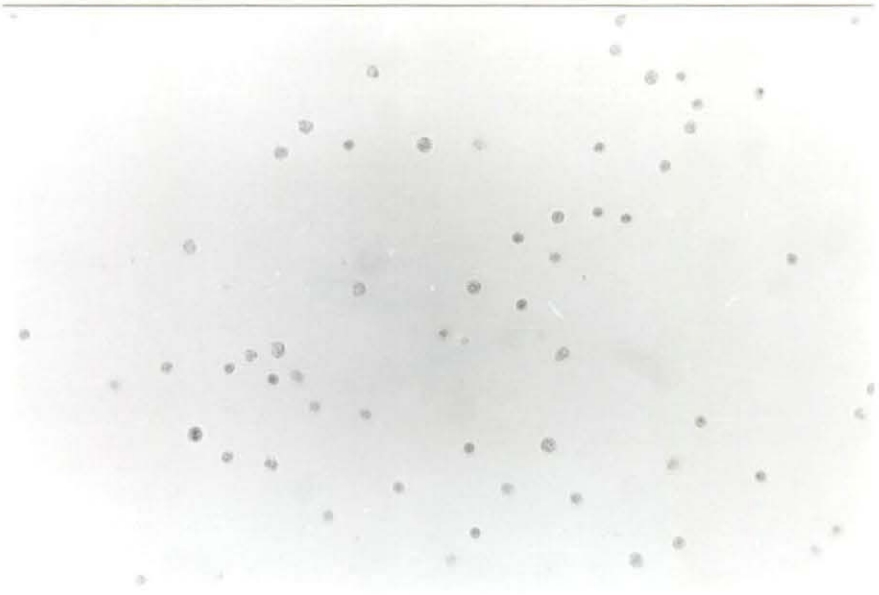


Figure 2.10a: In the top photomicrograph canine PML have been incubated with the anti-ACTH antibody at a 1:800 dilution. The PML were observed to display some degree of differential staining at this antibody dilution; however, 100% of the canine PML shown stained positively for ir-ACTH synthesis

Figure 2.10b: In the bottom photomicrograph canine PML have been incubated with non-immune normal rabbit serum at a 1:800 dilution. The degree of staining exhibited by these cells was negligible when compared to the PML exposed to the anti-ACTH antibody at the same concentration

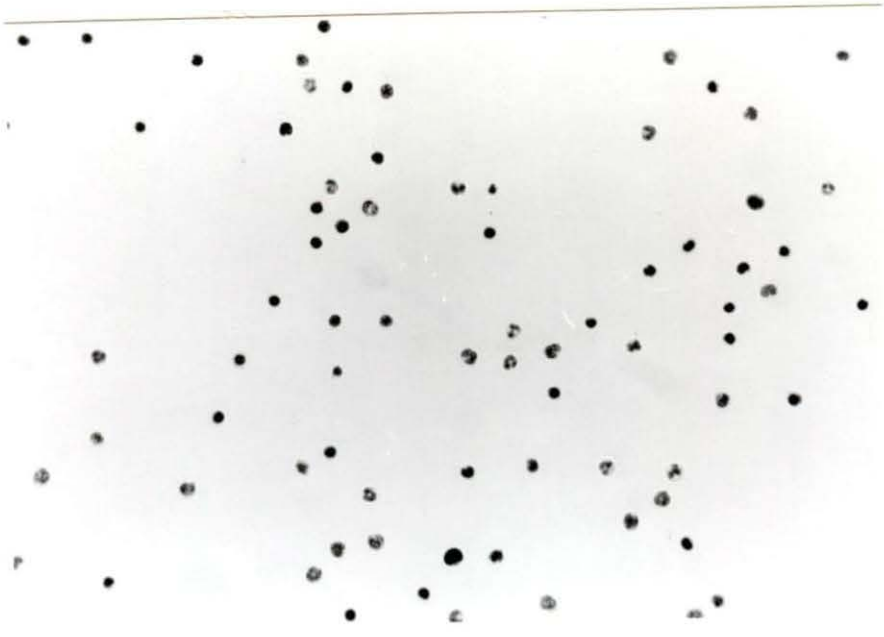
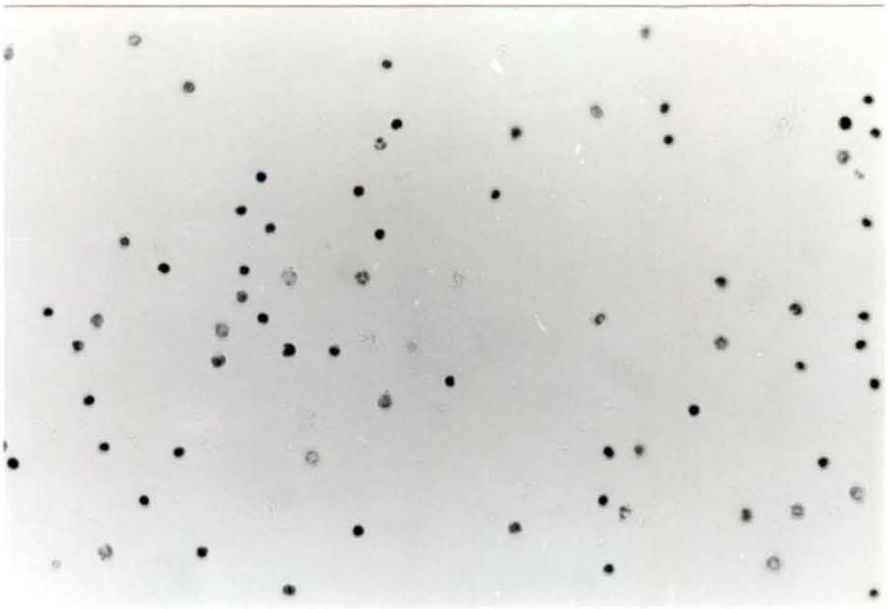


Figure 2.11a: In the top photomicrograph canine PML have been incubated with the anti-ACTH antibody at a 1:1000 dilution. The PML were observed to display a large degree of differential staining at this antibody dilution. The percentage of PML stain positively for ir-ACTH synthesis has dropped below 100%. It was apparent that percentages of cells observed to stain positively for ir-ACTH production could be modified by higher dilutions of the primary antibody

Figure 2.11b: In the bottom photomicrograph canine PML have been incubated with non-immune normal rabbit serum at a 1:1000 dilution. The degree of staining exhibited by these cells was negligible when compared to canine PML exposed to the anti-ACTH antibody at the same concentration



**Western blot of the lysate from bovine peripheral mononuclear leukocytes:** The lysate of the bovine PML was loaded into two lanes of a 15% polyacrylamide gel. In another lane, cytochrome c was loaded to serve as the molecular weight marker; it has a known mass of 10.5 kDa. To characterize the movement of ACTH in the gel, pure samples of labeled $I^{125}$-ACTH and unlabelled ACTH were also loaded on the gel.

After running the lysate on the gel, a distinct pattern of bands could ube observed in the lanes into which the lysate was loaded. To determine if ACTH was present in the lysate, the gel was blotted and the blot was probed for ACTH using an ACTH antibody. After blotting the gel, both it and the nitrocellulose membrane were placed on pre-flashed Kodak X-ray film for ten days at -70°C.

The radioautographs revealed that $I^{125}$-ACTH migrated to a position below that of cytochrome c; thus, as expected, the 5kDa ACTH migrated further in the gel than 10.5kDa cytochrome c. Furthermore, $I^{125}$-ACTH formed a broad diffuse band in the gel. It was not possible to determine if ACTH was present in the lysate. No band corresponding to the position of ACTH could be seen in a lane into which the lysate was loaded.

Neither the presence nor the absence of ACTH in the bovine PML lysate could be confirmed by the Western Blot.

## Discussion

The results of the present study suggest that peripheral mononuclear leukocytes (PML) from a broad spectrum of mammalian species synthesize pro-opiomelanocortin (POMC) peptides. All PML examined by the avidin

biotinylated peroxidase complex (ABC) immunocytochemical method stained positively for presence of ir-ACTH. In addition to ir-ACTH, several other POMC peptides can be detected in PML. In particular, ir-$\beta$-endorphin was identified in porcine PML and ir-met-enkephalin was identified in bovine PML. These results partially agree with other reports on the synthesis of POMC peptides by the PML.

Using an indirect immunofluorescence technique, 25% of the PML derived from humans with a ACTH deficiency were reported to stain positively for ir-ACTH. Six hours after administrating a typhoid vaccine, the percentage of the human PML stained positively for ir-ACTH increased to 50% (Meyer et al., 1987).

A strain of mouse B-lymphocytes were observed to be 66% positive for ir-ACTH (Bost et al., 1987). Once again an indirect immunofluorescence procedure was utilized. In contrast, in the current study, 100% of the PML isolated from the Harlem Sprague-Dawley rats were positively stained for ir-ACTH when the ABC method was used. Clearly, the percentage of mouse PML that stained positively for ir-ACTH (66%) was closer to the observed percentage of rat PML that stained positively for ir-ACTH (100%) than to the percentage of human PML that stained positively for ir-ACTH (25%).

Several different explanations account for these observed discrepancies. In the studies of Meyer et al. (1987) and Bost et al. (1987), a different type of indirect immunocytochemistry was utilized to probe for the presence of ir-ACTH; the secondary antibody was conjugated to fluorescein isothiocyanate. However, the ABC technique was used in the current study and it has been reported to be more sensitive than other indirect

immunocytochemical techniques (Hsu et al., 1981a, b). Thus, a reduction in technique sensitivity provides one explanation for the differences observed between the previous and the current studies.

Species variation in ir-ACTH production may provide an alterative explanation for the observed differences in the number of PML staining positive for ir-ACTH. It is possible that the role ir-ACTH plays in a immune response may differ in the various mammalian species; however, it is clear that PML of mammalian species are capable of producing ir-ACTH.

The Western blot was unable to confirm the immunocytochemical results obtained for the bovine PML; however, the procedure was not fine tuned for the separation of ACTH from the supernatant fluids of bovine PML lysates. In order to facilitate the detection of ir-ACTH by Western blot at least two improvements will be required in the future.

First, it will be necessary to selectively concentrate ir-ACTH from the lysates of PML before gel electrophoresis. One method to concentrate a protein of interest is immunoprecipitation. Not only will the protein become more concentrated during the precipitation, it will also be partially purified by the specificity of the precipitating antibody.

Second, the lysate should be separated on a more suitable gel matrix. The 15% polyacrylamide gel is best suited for the separation of large molecular weight proteins; small proteins tend to form diffuse bands. The spreading of the labelled ACTH into a diffuse band makes detection of the protein by Western blot difficult. If the lysate contains only a small amount of ir-ACTH, then spreading the protein over a large area (diffuse band) decreases the amount of protein, antibody, and $I^{125}$-Protein A bound

per unit area. Thus, the X-ray film may not receive adequate exposure to register the presence of a band and the protein may go undetected.

The Western Blot offers considerable promise for the study of ir-ACTH synthesis by the PML. Once the system is fine tuned for the separation and detection of small proteins, it will be possible to simultaneously test the supernatant fluids of the lysates of PML cultures for multiple POMC peptides. The lysate supernatant solutions will yield information on the contents of the cell. Cells can be subjected to different treatments, and the supernatants of the lysates can be assessed for the ability of a given treatment to induce the synthesis of POMC products.

Even though the presence of ir-ACTH in the supernatant lysates obtained from bovine PML was not detected by the Western blot, another study provides indirect evidence of POMC peptide synthesis by bovine PML. Bovine PML infected with bovine herpesvirus-1 have been demonstrated to express the POMC gene (Westly and Splitter, 1987). It was unlikely that transcription of the gene for POMC would occur in bovine PML without the eventual translation of the mRNA into peptide synthesis.

# SECTION II. THE RELEASE OF ADRENOCORTICOTROPIC HORMONE (ACTH) BY BOVINE PERIPHERAL MONONUCLEAR LEUKOCYTES (PML)

## Introduction and Literature Review

The first part of this study was concerned with the sucessful detection of stored immunoreactive-ACTH (ir-ACTH) and related POMC peptides by PML isolated from a broad spectrum of mammalian species. However, ICC provided no information on the release of POMC peptides from PML. In this section, the release of ir-ACTH by bovine PML was examined by radioimmunoassay (RIA).

The release of ACTH and POMC related peptides by PML has been reported. Human PML cultured with corticotropin releasing factor (CRF) have been observed to release ir-ACTH and ir-$\beta$-endorphin into culture media (Smith et al., 1986). The ir-ACTH molecule was observed to have a relative molecular weight of 4.5kDa (the molecular weight of pituitary derived ACTH) and to exert a trophic action similar to that of ACTH on cultured mouse adrenal cells.

Human B-lymphocytes have also been observed to release $\beta$-endorphin in the absence of CRF. The release of $\beta$-endorphin was dependent on the presence of monocytes and was blocked by anti-interleukin 1 antibodies. Apparently the release of $\beta$-endorphin by human B-lymphocytes was mediated by interleukin 1 (IL-1).

Besides human PML, only mouse B-lymphocytes have also been observed to release ir-ACTH (Bost et al., 1987). Culture media obtained from the cultures of mouse B-lymphocytes was purified on an affinity column. The

media eluted from the anti-ACTH (1-13) affinity column was observed to contain a peptide with a molecular weight and steroidogenic activity similar to pituitary derived ACTH.

The release of POMC peptides by PML in other mammalian species has not been investigated. If the synthesis and secretion of POMC peptides by PML of mammalian species is a recurrent theme, then it strengthens the view that these peptides serve an immunoregulatory role.

This part of the study concerns the release of ir-ACTH by bovine PML. An attempt was made to identify potential secretagogues of ir-ACTH and to measure levels of ir-ACTH released from bovine PML under basal and stimulated conditions. The culture media of treated and untreated bovine PML was assessed for ir-ACTH content using a radioimmunoassay.

## Materials and Methods

**Animals**: Bovine blood was collected by venepuncture into a 250ml sterile screw top bottle containing 25ml of an Acid Citrate Dextrose (ACD) solution. The ACD solution was made by dissolving 132g sodium citrate, 58g citric acid, and 150g dextrose into thrjee liters of reagent grade water. The PML cultured for each experiment were isolated from blood drawn that same morning.

**Leukocyte preparation**: Whole blood collected by venepuncture into a 250ml sterile screw top bottle containing 10ml of ACD was divided evenly between four 50ml centrifuge tubes and then spun on a Centra-8 centrifuge (International Equipment Company) at 850g for 22 minutes. After

centrifugation, the samples were placed in a laminar flow hood. The buffy coat, the top of the plasma/cell interface, was drawn off along with 5ml of plasma from each 50ml tube and placed into a 15ml centrifuge tube. To each 15ml centrifuge tube was added 5ml of a density gradient: Sepracell-MN (Sepratech Corporation). After adding the Sepracell-MN, each tube was capped, inverted several times and spun on the Centra-8 at 1900g for 22 minutes.

Following centrifugation with Sepracell-MN, a distinct white band was apparent just below the meniscus in each centrifuge tube. The white band containing PML was collected from each tube with a sterile Pasteur pipet and combined into a single 15ml centrifuge tube which was then filled to capacity with Hank's Balanced Salt Solution (Sigma). The sample was triturated with a sterile Pasteur pipet, and spun on a Clinical centrifuge (International Equipment Company) at setting 6 for five minutes. Next, the supernatant fluid was decanted from the tube and 5ml of Hank's medium was added again before the pellet was triturated with a sterile Pasteur pipet and filtered through a sterile 25$\mu$m mesh into a clean 15ml centrifuge tube. The filtrate was then diluted to 10ml, the tube was inverted several times, and an aliquot was taken for a cell count on a hemocytometer.

The sample was then spun on the Clinical centrifuge at setting 6 for five minutes; the supernatant fluid was discarded and the pellet was diluted with leukocyte culture medium (LCM) to a concentration of $10^6$ cell/ml. LCM was made by adding 15ml fetal calf serum (Gibco), 1ml penicillin-streptomycin (Gibco), and 1ml fungizone (Gibco) to 100ml of Media 199 (Gibco). Next, 1ml of the leukocyte suspension was then

aliquoted to each well in a 24 well incubation tray (Corning Cell Wells, Corning Glass Works). The tray was then placed into a humidified Queue incubator set at 37°C, 5% $CO_2$/ 95% air, and the cells were allowed to incubate for 24 hours before treatments were applied.

**Murine pituitary preparation:** The anterior lobe of the pituitary was isolated from a freshly killed Harlem Sprague-Dawley rat and placed into a sterile siliconized petri dish (6cm diameter) containing a solution of Spinner's-0.1% bovine serum albumin (BSA) which was made by adding 1.1ml penicillin-streptomycin (Gibco) and 2.6ml 4% BSA to 100ml of Spinner's Minimal Essential Medium (Gibco). The petri dish was placed in a laminar flow hood where the anterior lobe was transferred to a larger sterile petri dish (10cm diameter), rinsed several times with fresh Spinner's-0.1% BSA, and cut into approximately $1mm^3$ pieces with sterile scalpel blades.

The pieces were next transferred to a siliconized spinner's flask containing 10ml of a 1mg/ml trypsin solution in Spinner's-0.1% BSA that was filtered through a Millex-GV filter. The flask was gassed through a sidearm for twenty seconds with 5% $CO_2$/ 95% air, placed in a 37°C water bath, and left to incubate with stirring for 50 minutes. After stirring, the solution was triturated under the laminar flow hood using a sterile Pasteur pipet, regassed through the sidearm, and left to incubate for another 30 minutes. This procedure was repeated at 20 minutes intervals until the pieces of the anterior pituitary were no longer visible.

After dispersing the pituitary cells, they were transferred to a 15ml centrifuge tube, made up to 15ml with Spinner's and spun on a Clinical

centrifuge (IEC) at setting 5 for 10 minutes. The supernatant fluid was decanted, 5ml Spinner's was added to the pellet which was triturated with a Pasteur pipet. An aliquot was removed for a cell count in a hemocytometer, then the suspension of pituitary cells was diluted to 15ml with Spinner's and spun on a Clinical centrifuge at setting 5 for 10 minutes.

The pellet was resuspended in leukocyte culture media (LCM) to achieve the concentration of pituitary cells desired for the experiment. The pituitary cells were dispensed in 1ml aliquots to each of the wells in a 24 well culture tray (Corning Cell Wells, Corning Glass Works). The tray was then placed inside a humidified Queue incubator set at 37°C, 5% $CO_2$/ 95% air, and left to incubate for 24 hours before the treatments were applied.

**Cell culture treatments:** Treatments were applied in an identical manner in each experiment and on each type of cell culture. After incubating in the Queue incubator for 24 hours, the 24 well culture trays containing the cultured cells were removed from the incubator and placed in the laminar flow hood. Next, 250$\mu$l of culture media was removed from each well with a sterile pipet and replaced with 250$\mu$l of fresh media containing the appropriate treatment. The cells were allowed to incubate with the treatments in a humidified Queue incubator at 37°C for 6 hours.

After six hours, aliquots of culture media were then taken from each well, placed into vials, and frozen at -20°C until assayed for ACTH by radioimmunoassay (RIA).

The following is a listing of the treatments applied to the leukocytes: (1) leukocyte culture media (LCM; control), (2) 100ng/ml ACTH (porcine),

(3) 6.5 μg/ml Concanavalin A (ConA), (4) 50nM corticotropin releasing factor (CRF), (5) 6.5 μg/ml ConA and 50nM CRF, (6) 10nM and 100nM phorbol-12-myristate-13-acetate (PMA), (7) 1 μM and 5 μM calcium ionophore, A23187, and (8) 1:80 and 1:10,000 dimethyl sulfoxide to LCM (dimethyl sulfoxide was the vehicle of PMA and A23187).

The pituitary cells were treated with either LCM (control) or 50nM CRF.

**Radioimmunoassay (RIA):** The procedure followed was provided by the IgG Corporation (P.O. Box 120052, Nashville, Tennessee, 37212-0052) with some slight modifications. On the first day of the procedure, the samples to be assayed were melted on ice along with the ACTH standard (IgG-pACTH, IgG Corporation) and the ACTH antibody (IgG-ACTH-1, IgG Corporation). While the samples were melting, the RIA buffer was freshly mixed from an ice cold phosphate ethylenediaminetetraacetic acid (EDTA) buffer (8.94g/l sodium phosphate dibasic, 4.74g/l disodium EDTA dihydrous, and 0.2g/l sodium azide) by adding 1μl Triton X-100 and 250 KIU aprotinin (Sigma) per ml of buffer.

ACTH has been observed to be a very labile protein. Hence, the RIA was performed at 4°C to limit the extent of protein degradation. Several additional steps were taken to minimize the degradation of ir-ACTH in the system. Triton X-100 was added to limit the extent of non-specific binding of the ir-ACTH to the polypropylene tubes. Aprotinin, a protease inhibitor, was added to inhibit ir-ACTH degradation by proteases in the system.

After the samples were melted, the standards were prepared by taking 100

$\mu$l of standard ACTH concentrate (1ng/$\mu$l) and adding to 9.9ml of RIA buffer in a tube labelled 'A'. After vortexing, 100$\mu$l of solution A was added to 4.9ml of LCM in a tube labelled 'B', thereby obtaining a standard solution of 200pg/ml ACTH. The remaining standards (100pg/ml, 50pg/ml, 25pg/ml, 12.5pg/ml, 6.25pg/ml, and 3.125pg/ml) were made by double dilutions.

The entire procedure was carried out at 4°C with all reagents chilled on ice or in a refrigerator. On the first day, 100$\mu$l of antibody (1:100 dilution of stock antibody to RIA buffer) and 100$\mu$l of either standard or sample were added to numbered 12 x 75mm polypropylene tubes. The tubes were then vortexed and placed in a rack on ice. The racks were covered with plastic wrap, moved to a refrigerator at 4°C, and left to incubate for 24 hours.

In a single assay, each standard was replicated three times; each media sample was replicated twice. In addition, a series of standards (100pg/ml, 25pg/ml, and 5pg/ml) were mixed and frozen for subsequent experiments. These standards were used to measure intra-assay and inter-assay coefficients of variation.

On the second day, after the antibody and sample had incubated at 4°C for 24 hours, the racks were repacked in ice and moved back to the lab. Next, 100$\mu$l of a 20,000 cpm/ml solution of $I^{125}$ ACTH (Amersham) in RIA buffer was added to each tube. After vortexing, the tube was returned to the rack, covered with plastic wrap and moved into a refrigerator at 4°C for an additional 24 hours of incubation.

On the third day, the racks containing the samples were repacked in ice and moved into the lab where 100$\mu$l of 1:40 normal rabbit serum (Calbiochem)

in RIA buffer and 100μl of anti-rabbit-immunoglobulin antibody (1unit/100μl, Calbiochem) were added to the tubes. The tubes were then vortexed, covered with plastic, and moved into a refrigerator at 4°C for 4 hours of incubation. After 4 hours, 1.5ml of ice cold 6% polyethylene glycol in 0.9% NaCl was added to each tube. The tubes were then transferred to centrifuge tube holders and spun on a Beckman centrifuge (Model J-6B) at 4°C, 1900g for one hour. After centrifugation, the tubes were placed in an ice bath and individually decanted by slowly inverting the tube while removing the supernatant with a syringe attached to a vacuum source. Finally, the tubes were loaded on a gamma counter (Compugamma Gamma Counter, LKD Instruments, Inc.), counted, and then discarded.

The standard curve was computed on a Hewlett-Packard 41C calculator using the Clinical Lab and Nuclear Medicine Pac. In brief, the program performs the calculations for a logit/log plot of the data. After input of the standard data, the program computes a slope, a correlation coefficient, an intercept, and the least-squares regression line for the standards. Next, the cpm obtained for the media samples was input to determine if ir-ACTH was secreted into the media by the cell culture. The data was reported in terms of picograms of ACTH detected per ml of media.

**ACTH degradation:** To study the degradation of ACTH in culture conditions, a sterile solution of 100pg/ml ACTH in leukocyte culture media (LCM) was prepared in a laminar flow hood and then dispensed in 1ml aliquots to each well in a 24 well culture tray (Corning Cell Wells, Corning Glass Works). The tray was next transferred to a humidified Queue

incubator set at 37°C, 5% $CO_2$/ 95% air, then left to incubate. Two-hundred and fifty $\mu$l aliquots of the culture media were taken from a different set of four wells at 4, 6, 12, 24, 48, and 78 hours. Each aliquot was placed in a vial, capped, and transferred to a freezer at -20°C. The samples were later assayed for ir-ACTH by RIA.

## Results

**Assay validation:** The radioimmunoassay (RIA) utilized in the following series of experiments was modified from a procedure supplied by the IgG Corporation. The assay characteristics listed below were based on the data obtained from 11 separate assays. These observed characteristics agreed well with those supplied by the manufacturer of the RIA.

The trace binding was observed to average 39.7% ± 2.2% ($\bar{x}$ ± SEM, n=11). This binding refers to the percentage of labelled ACTH that was bound by the ACTH antibody under assay conditions and was obtained from the cpm bound in polypropylene tubes to which only media (LCM), labelled ACTH, and the ACTH antibody were added. To achieve maximum sensitivity for the detection of ACTH, IgG Corporation recommended that 30-35% of the total cpm added per tube should be bound.

The sensitivity was 11.1pg/ml ± 1.8pg/ml ($\bar{x}$ ± SEM, n=11). This amount of ACTH was required to reduce $B_0$ by 10%. The 50% binding range was 42.6pg/ml ± 5.19pg/ml ($\bar{x}$ ± SEM, n=11). The upper limit of the assay, when the sample count was reduced to 20% of $B_0$, was 100.0pg/ml ± 10.6pg/ml ($\bar{x}$ ± SEM, n=11).

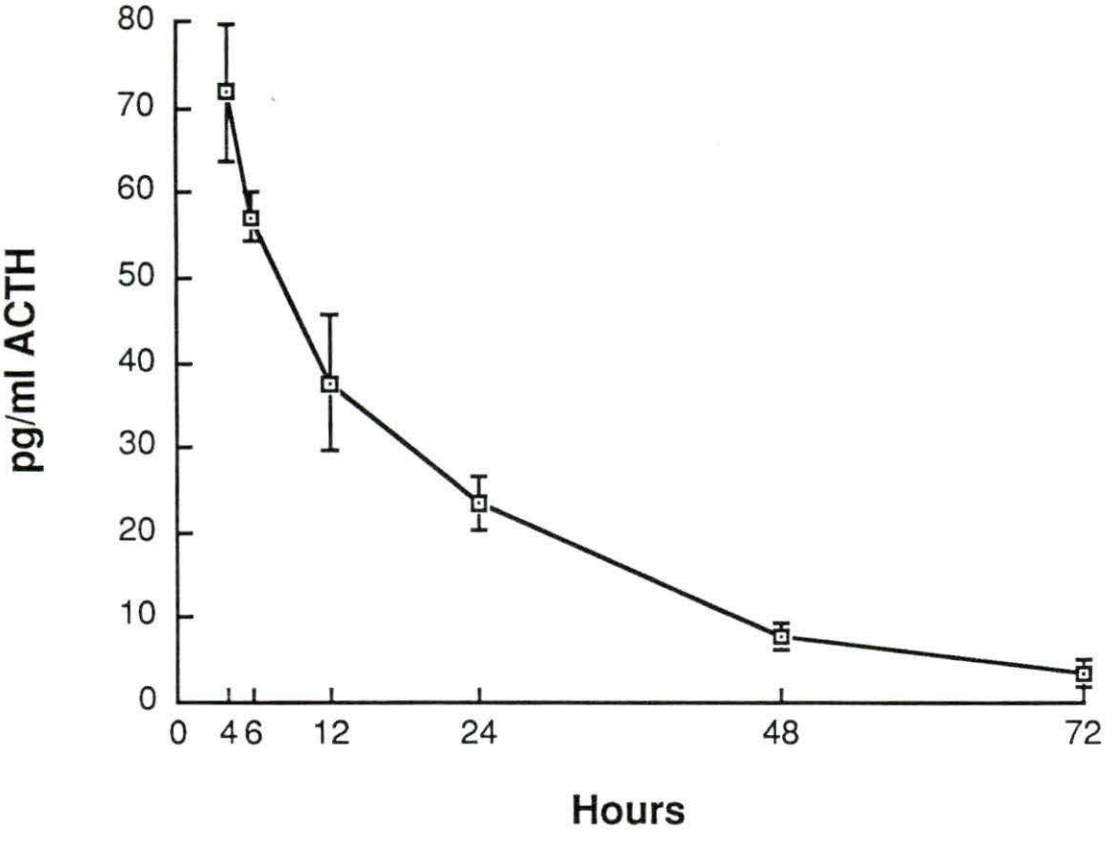
The degree of variation observed between the 11 experiments was within acceptable limits. The inter-assay coefficient of variation was determined for three separate stock solutions of ACTH (100pg/ml, 25pg/ml, and 5pg/ml). The coefficients of variation were 8.9%, 18.3%, and 19.5%, respectively.

The intra-assay coefficient of variation was measured in a single assay on a standard solution of 25pg/ml and was determined to be 8.8% (n=10).

The cross reactivity of the antibody used in these experiments was tested, the following information was provided by the IgG Corporation: ACTH(1-39), 100%; ACTH(1-24), 170%; ACTH(1-18 $NH_2$), 170%; ACTH(1-16 $NH_2$), 0.4%; ACTH(5-24), 128%; ACTH(7-24), 151%; ACTH(9-24), 11%; ACTH(11-24), 0.1%; ACTH(13-24), <0.1%; ACTH(15-24), <0.1%; ACTH(17-24), <0.1%; ACTH(18-39), <0.2%; ACTH(5-18), 101%; $\alpha$MSH, <0.2%; human-$\beta$MSH, <0.4%; human-$\beta$-endorphin, <0.5%; human-$\beta$LPH, <1.3%; human-LPH, <0.8%.

**ACTH degradation:** ACTH was observed to be rapidly degraded under culture conditions. Upon subjecting a solution of 100pg/ml ACTH in LCM to cell-free culture conditions, ACTH was observed to have a half life of approximately 8 hours. The temporal pattern of degradation was determined by taking samples of the spiked media at discrete times. The media samples were later quantitated for ACTH content using RIA. The results on the degradation of ACTH have been displayed in Figure 3.1. After 48 hours of incubation at 37°C, the amount of ACTH that could be detected in the spiked media had approached the sensitivity of the RIA (11.1 pg/ml, see above).

Figure 3.1: The degradation of ACTH under culture conditions. ACTH had a half life of approximately 8 hours when incubated in cell free leukocyte culture media (LCM) placed in a humidified Queue incubator at 37°C, 5% $CO_2$/95% air. The valves were reported as the mean ± the standard deviation (n=4)

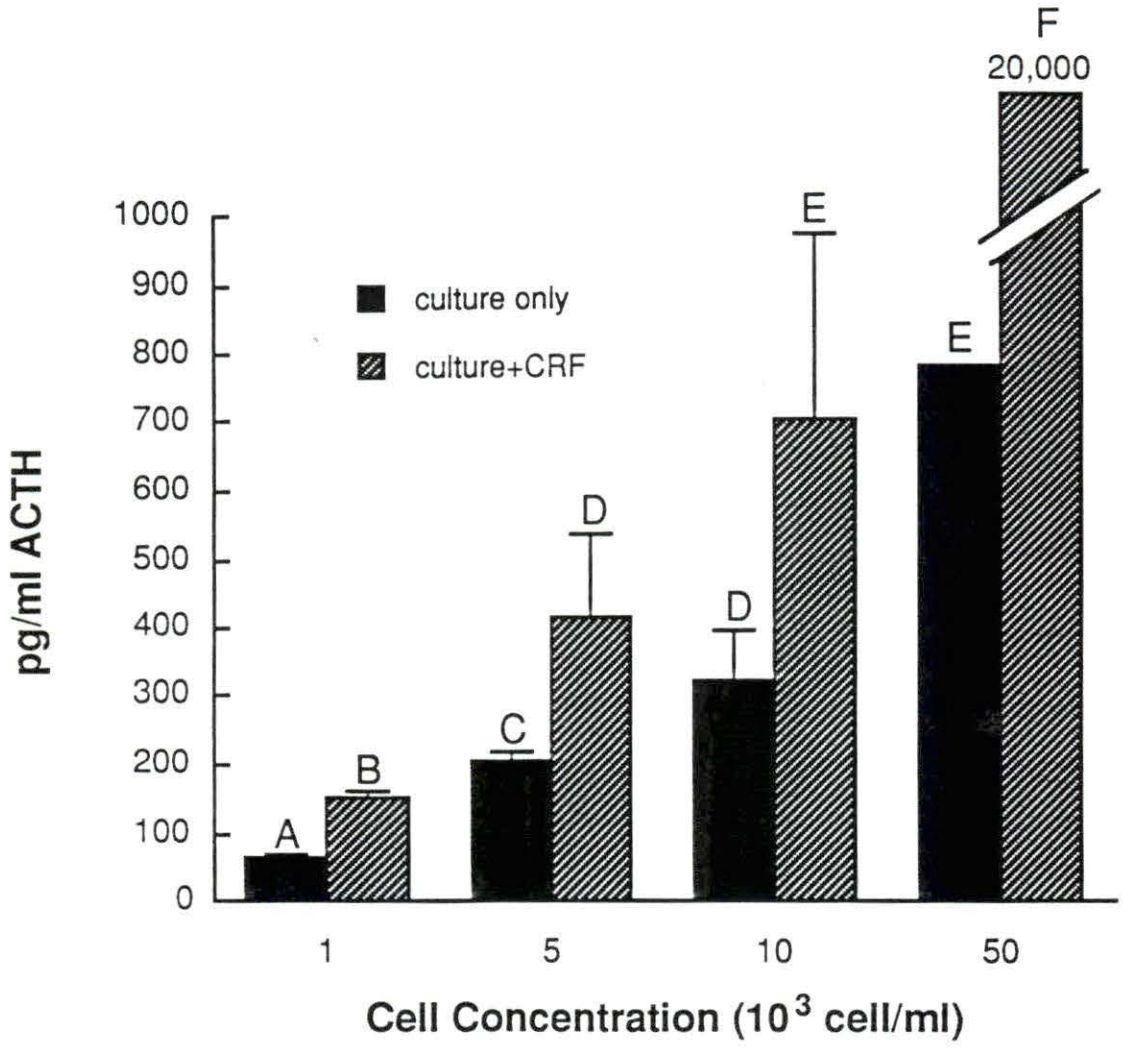

80
70
60
50
40
30
20
10
0
pg/ml ACTH
0
4
6
12
24
48
72
Hours

**ACTH release by rat pituitary cells:** Rat pituitary cells cultured under the same conditions as the bovine PML were observed to release ir-ACTH into the culture media. As the concentration of pituitary cells per ml LCM was increased, a concomitant increase in ir-ACTH release was observed (Figure 3.2). The addition of a solution of 50nM corticotropin releasing factor (CRF) to the LCM was observed to increase the amount of ir-ACTH released. CRF stimulated pituitary cells released significantly more ($P > t=0.05$ or less) ir-ACTH than non-stimulated pituitary cells cultured under the same conditions and at the same cellular concentrations (Figure 3.2).

**ACTH release by bovine PML:** To detect the basal release of ir-ACTH by bovine PML, they were cultured at a concentration range of $10^4$ to $10^6$ cells per ml of LCM. Media samples from the cultured bovine PML were taken after 24, 48, and 72 hours of incubation; however, no basal release of ir-ACTH was detected. Higher cell concentrations were not quantitated for ir-ACTH release because the PML were observed to agglutinate. The agglutination of bovine PML prevented the uniform distribution of these cells within the LCM and hence to the culture wells.

To minimize the degradation of the released ir-ACTH in treated bovine PML cultures, media samples were taken 6 hours after the treatments were applied to the cultures (see Materials and Methods: Cell culture treatments). Treatment of the bovine PML with two putative secretagogues, CRF and ConA, was not observed to stimulate the release of ir-ACTH from the cells. Treatment of bovine PML with pharmacological agents (PMA and

Figure 3.2: The release of ir-ACTH by dispersed pituitary cells under culture conditions. After 24 hours of incubation in a humidified Queue incubator at 37°C, 5% $CO_2$/ 95% air, media samples were quantitated for ir-ACTH content by RIA. As the cell concentration was increased, the amount of ir-ACTH released into the media increased. Treatment of the pituitary cells with corticotropic releasing hormone (CRF) resulted in a significant rise in the levels of ir-ACTH released into the media. All bars marked with a different letter are significantly different at the 5% level ($P > t = 0.05$ or less). Values were reported as the mean ± the standard deviation (n=4)


F
20,000
E
E
D
D
C
B
A
culture only
culture+CRF
pg/ml ACTH
1000
900
800
700
600
500
400
300
200
100
0
1
5
10
50
Cell Concentration (10 3 cell/ml)

A23187) was also not observed to stimulate the release of measurable amounts of ir-ACTH into the culture media.

## Discussion

The RIA procedure was validated in the course of this study. The assay characteristics have been summarized above (see Results section). In brief, the RIA was capable of detecting ir-ACTH at the 11.1pg/ml level. The RIA was capable of detecting the release of ir-ACTH under the employed culture conditions as demonstrated by the release of ir-ACTH from rat pituitary cells. Furthermore, it was possible to stimulate release of ir-ACTH from the pituitary cells by applying a known corticotropin secretagogue, CRF.

The inability to detect the release of ir-ACTH by bovine PML in the present study is at odds with the observations of its release from human (Smith et al., 1986) and mouse PML (Bost et al., 1987). This discrepancy has several explanations. (1) The amount of ir-ACTH released from bovine PML was insufficient for detection by the RIA protocol used in this study. (2) The released ir-ACTH was degraded before it could be detected. (3) The bovine PML were not stimulated to release ir-ACTH into the culture media or (4) the cows that served as donors of the PML were stressed or otherwise perturbed by outside influences. Each of these explanations will be discussed below.

(1) The amount of ir-ACTH secreted by human PML is minute. To detect the presence of ir-ACTH by radioimemunoassay, $2 \times 10^7$ human PML were lysed in 2ml of 0.01M HCl before the lysate was concentrated and quantitated. A

median value of 29pg of ir-ACTH was observed (Buzzetti et al., 1989). If bovine PML synthesize a similar amount of ir-ACTH, then even the complete release of all the ir-ACTH produced by all $10^6$ bovine PML in a single culture well was below the sensitivity of the RIA used in the current study.

To detect the release of ir-ACTH, the media of cultured mouse B-lymphocytes was passed through an immunoaffinity column conjugated with anti-ACTH antibody. Several of the eluted fractions were observed to a contain a steroidogenic material (Bost et al., 1987). The eluted fraction which induced the largest increase in corticosterone production by an adrenal cell lines corresponded with the 5kDa fraction. Recall that ACTH has a molecular mass of about 5kDa. The eluted fractions were further characterized for the presence of ir-ACTH by RIA. It was found that 43ml media eluted from the affinity column contained an average of 875pg of an ir-ACTH molecule (43ml of media contained $4.5 \times 10^7$ cells).

In order to detect the presence of ir-ACTH in the supernatant fluids of the mouse B lymphocytes, it was necessary to selectively concentrate the molecules of interest on an affinity column. The supernatant fluids of the bovine PML were not subjected to any type of purification before being assayed by the RIA. If the bovine PML secreted amounts of ir-ACTH comparable to that observed for human PML and mouse B lymphocytes, the levels secreted were at least ten fold below the sensitivity of the RIA.

(2) ACTH was degraded rapidly in culture conditions. It was observed to have a half life of 8 hours under cell free culture conditions. The media samples quantitated by RIA in the current experiments were taken 6 hours

after treatments were applied. Clearly, if ir-ACTH was released by bovine PML after treatment, it was subject to prompt degradation.

(3) The release of ir-ACTH by bovine PML may not have been triggered. The regulatory signals that control the release of ir-ACTH and other POMC peptides from bovine PML have not been identified. It was hoped that the pharmacological agents (PMA and A23187) would elicit a release of cytoplasmic granules contained in the bovine PML. Several mitogenic agents were also utilized that have been shown to induce the synthesis and/or release of POMC peptides from the PML of humans and mice (Besedovsky el al, 1981; Smith et al., 1986). However, none of the treatments were observed to induce the release of detectable amounts of ir-ACTH from bovine PML.

(4) The donors from which PML were isolated may have been suffering from sub-clinical infection. Many of the animals were housed communally; thus, the possibility existed for the horizontal transmission of disease between the individual donor animals.

This pre-exposure to pathogens may affect the ability of PML to synthesize and release cytokines. To test this hypothesis, culture PML isolated from the blood of a pathogen free animal to the RIA protocal and assay for ir-ACTH.

Summary: the ability of bovine PML to release ir-ACTH could not be confirmed. Proof of release awaits the employment of increasing sensitive assays and/or methods of selectively concentrating the proteins of interest. One possible candidate is the Western blot which should prove useful in the characterization of PML ability to synthesize and release the various POMC peptides.

# GENERAL DISCUSSION

The present study shows that the synthesis of ir-ACTH by PML occurs in a broad spectrum of mammalian species: cow, pig, dog, and rat; however, the release of ir-ACTH by PML has only been characterized in humans and mice (Harbour-McMenamin et al., 1985; Bost et al., 1987; Meyer et al., 1987). Several other peptides of proopiomelanocortin (POMC) origin have also been identified in PML. In particular, ir-$\beta$-endorphin has been observed in pigs (current study), in humans (Harbour-McMenamin et al., 1985; Smith et al. 1986; and Kavelaars et al., 1989) and in mice (Lolait et al., 1984). Immunoreactive met-enkephalin has been identified in cattle (current study). Expression of the gene for POMC has also been reported in bovine PML infected with bovine herpesvirus-1 (Westly and Splitter, 1987). It is apparent that PML isolated from mammalian species are capable of expressing and releasing POMC related peptides.

The release of ir-ACTH by bovine PML was not detected in the current study. However, as mentioned before (see Discussion in SECTION II), the release of ir-ACTH by human PML and mouse B-lymphocytes was determined to be on the order of 10-60 pg per $10^7$ cells. Bovine PML were cultured at $10^6$ cells/ml in the current study. If bovine PML were to release ir-ACTH at a level similar to mice or humans, then only 1-6pg would be released by $10^6$ cells. These concentrations of ir-ACTH were below the limits of the RIA to detect (sensitivity was 11.1pg/ml ACTH).

The observation of POMC synthesis and release by PML does not address the function these peptides play in controlling the development of an immune response. If POMC peptides are capable of directly modulating the

functions of the PML during antigenic stimulation, then PML must possess specific receptors for POMC peptides. Several studies have demonstrated the existence of POMC receptors on the surface of PML.

The presence of morphine and met-enkephalin receptors on the surface of human T-lymphocytes was reported when these two peptides were observed to suppress and enhance, respectively, the formation of sheep red blood cell rosettes (Wybran et al., 1979). Incubation of the T-lymphocytes with morphine at a concentration of $10^{-10}$ M to $10^{-7}$ M for one hour was observed to suppress the number of rosettes formed by the T-lymphocytes. Incubation of the T-lymphocytes with met-enkelphin ($10^{-7}$ M to $10^{-4}$ M) increased the number of rosettes formed.

$\beta$-endorphin receptors have also been reported on human PML. $I^{125}$ -D-Ala-endorphin was observed to bind specifically to cultured human PML (Hazum et al., 1979). The binding of the labelled endorphin was partially inhibited by human $\beta$-endorphin at a concentration of $10^{-9}$ M and completely inhibited at $10^{-5}$ M. Additional opiate receptors have been observed on murine (mouse) spleenocytes (Carr et al., 1989).

Finally, the presence of ACTH receptors on human PML has been reported (Smith et al., 1987). These receptors were shown to be similar in number and affinity to adrenal cell ACTH receptors.

Given that PML possess receptors for POMC peptides, then it is reasonable to assume that POMC peptides are capable of directly affecting activities of PML. A number of POMC peptides have been observed to affect the activation, proliferation, and activities of PML and other immunocompetent cells. A summary of the actions of POMC peptides follows.

ACTH and α-endorphin can inhibit the release of antibodies by mouse B-lymphocytes (Johnson et al., 1982) when applied at 0.5 μM concentration. ACTH has also been implicated in repressing the release of γ-interferon from mouse spleen cells. When the mouse spleen cells were exposed to concentrations of ACTH in the range of 1 to 3μM, the spleen cells released less ir-γ-interferon than those cells cultured in media alone (Johnson et al., 1984). γ-interferon concentration was assessed by neutralization with a specific antiserum. Later, it was determined that ACTH blocked the capacity of γ-interferon to activate mouse peritoneal macrophages to cytotoxic state (Koff and Dunegan, 1985). The concentration required to block the activation by γ-interferon was 0.5-1μM ACTH. Whether ACTH directly or indirectly blocked the release or the action of γ-interferon remains uncertain; however, ACTH apparently can suppress the activation of macrophages by γ-interferon.

β-endorphin and met-enkephalin, on the otherhand, have been observed to enhance the release of γ-interferon by ConA stimulated human PML (Brown and Van Epps, 1986). The release of γ-interferon as measured by indirect enzyme-linked immunosorbent assay was enhanced when β-endorphin was present in concentrations ranging from $10^{-14}$ M to $10^{-10}$ M or when met-enkephalin was present in concentrations ranging from $10^{-13}$ M to $10^{-11}$ M. At higher concentrations, the release of γ-interferon was not augmented by either peptide.

β-endorphin has also been observed to enhance or suppress the proliferation of lymphocytes depending on the timing of the administration. Rat spleen cells cultured in the presence of ConA and β-

endorphin incorporated more labelled thymidine than those cultured with ConA alone. The effect was apparent at a concentration of 1ng/ml β-endorphin (Gilman et al., 1982).

On the other hand, human lymphocyte proliferation was observed to be repressed by the addition of β-endorphin to the cultures (McCain et al., 1982). The human lymphocytes were cultured with phytohemagglutinin, a T-lymphocyte mitogen, in the presence and absence of β-endorphin. At nanomolar concentration, β-endorphin was observed to repress the uptake of labelled nucleic acids; however, the suppression was evident only if β-endorphin was applied at the same time as the mitogenic stimulus.

Finally, both β-endorphin ($10^{-14}$ M) and met-enkephalin ($10^{-9}$ M) are found to enhance the cytotoxicity of human natural killer cells. The enhanced cytotoxicity was measured by the release of $Cr^{51}$ from target cells (Mathews et al., 1983; Faith et al., 1984; Froelich and Bankhurst, 1984; Kay et al., 1984; and Mandler et al., 1986).

Thus, it is apparent that PML are capable of synthesizing, releasing, and interacting with POMC related peptides; however, are these actions of the POMC peptides of physiological importance or are the observed activities of POMC peptides merely an artifact of the experiment conditions? To address this question, one must consider the environment were a immune response is initiated.

Acquired immunity is mediated and initiated by lymphocytes in the secondary lymphoid organs- the spleen and the lymph nodes. The lymph node contains numerous cells types supported by meshwork of sinusoidal fibers in a tough collagen capsule. Lymphocytes are concentrated in the cortical and

paracortical regions of a node, while macrophages are found in the medullary regions. The cells are present in high concentrations; thus, intimate contact occurs between immune cells. Two basic types of interactions occur at a local level: cell to cell contact, and the release of cytokines. The anatomy of a node ensures that the cytokines, even though released in minute quantities, are effective in playing their respective roles in the control of a immune response. The autocrine and paracrine routes of the released cytokines are favored within a lymph node.

The POMC peptides released by PML may exert actions beyond the local environment. The level of action being dependent on the strength of the stimulating signal. With mild stimulation, POMC peptides act at the local level within the lymph node. Under extreme stimulation, POMC peptides may reach sufficient concentration to affect systemic function.

Several systemic effects of cytokines have been noted. In sudden acute infections, the cytokines reach a sufficient concentration to exert systemic action. Hypophysectomized mice infected with Newcastle's disease virus have been observed to display a time-dependent increase in corticosterone plasma levels. The increase was attributed to the release of a corticotropic peptide by viral stimulated lymphocytes (Smith et al., 1982). IL-1 has been implicated in the release of ACTH from the anterior pituitary (Fukata et al., 1989) and the induction of fever (Widman, F.K., 1989).

Considering the local release POMC peptides by PML, it is probable that the ir-POMC peptides identified in the PML function as cytokines. ACTH, $\beta$-endorphin, and other POMC peptides have been shown to affect the activities

of PML. The presence of ir-ACTH, ir-$\beta$-endorphin, and ir-met-enkephalin in PML of several mammalian species has been demonstrated. In the current study the presence of ir-ACTH in PML of cows, pigs, dogs, and rats was found. Finally, the presence of specific receptors for POMC peptides has been detected. Given this information, it is reasonable to assume that ir-POMC peptides produced by PML are partially responsible for controlling the course of a immune response.

At the present, the signals that fine tune an immune response against the stimulating antigenic material are incompletely defined. There would be obvious advantages to understanding the pathways that influence the various cell types involved in the immune response.

As an example, consider an infectious disease of felines that suppresses the immune system: feline leukemia virus (FeLV). FeLV suppresses the ability of helper T-lymphocytes to release stimulating cytokines and of B-lymphocytes to release of IgG antibodies (Rojko and Olsen, 1984; Ogilvie et al., 1988). Felines with a persistent FeLV infection may develop secondary infections, which are often fatal due to immunosuppression. If the regulatory signals released by helper T-lymphocytes could be supplied exogenously, then the effects of FeLV could be bypassed and immune function could be restored. Some felines demonstrate improved clinical signs and clearance of viremia when heterologous interferons are included with antibiotics in the FeLV therapy (Weiss, 1988). Additional cytokines have yet to be tested for their efficacy in terminating the FeLV infection. However, before any logical approach can be taken to the administration of cytokines for immunomodulatory effects, it will be necessary to identify

the structure, action, and source of the cytokine. One set of proteins that may function as cytokines are the POMC peptides released by PML.

In summary, it is possible that the ir-POMC peptides identified by the current study serve an important regulatory role in the immune response. The PML of cows, pigs, rats, and dogs stained positively for ir-ACTH. Furthermore, human and mouse PML have been observed to synthesize, release, and response to POMC peptides. Given this information, it is reasonable to hypothesize that POMC peptides synthesized and released by mammalian PML are cytokines that function in controlling immune responses.

## REFERENCES

Alvarez-Mon, M., J. H. Kehrl, and A. S. Fauci. 1985. A potential role for adrenocorticotropin in regulating human B lymphocyte functions. J. Immunol. 135:3823-3826.

Besedovsky, H. O., A. Del Ray, and E. Sorkin. 1981. Lymphokine-containing supernatants from Con-A stimulated cells increase corticosterone blood levels. J. Immunol. 126:385-387.

Bohme, M. W. J., K. Becker, W.A. Scherbaum, G. Jirikowski, E. Spath-Schwalbe, H. L. Fehm, and E. F. Pfeiffer. 1987. No expression of ir-ACTH on stimulated peripheral blood leukocytes. Horm. Metab. Res. 19:670-671.

Bost, K. L., E. M. Smith, L. B. Wear, and J. E. Blalock. 1987. Presence of ACTH and its receptor on a B lymphocytic cell line: a possible autocrine function for a neuroendocrine hormone. J. Biol. Regul. Homeost. Agents 1:23-27.

Brown, S. L. and D. E. Van Epps. 1985. Suppression of T lymphocyte chemotactic factor production by the opioid peptides $\beta$-endorphin and met-enkephalin. J. Immunol. 134:3384-3389.

Brown, S. L. and D. E. Van Epps. 1986. Opioid peptides modulate production of interferon-$\gamma$ by human mononuclear cells. Cellular Immunol. 103:19-36.

Buzzetti, R., L. McLoughlin, D. Scavo, and L. H. Rees. 1988. A critical assessment of the interactions between the immune system and the hypothalamo-pituitary-adrenal axis. J. Endocrinol. 120:183-187.

Buzzetti, R., L. McLoughlin, P. M. Lavender, A. J. L. Clark, and L. H. Rees. 1989. Expression of Pro-opiomelanocortin gene and quantification of adrenocorticotropic hormone-like immunoreactivity in human normal peripheral mononuclear cells and lymphoid and myeloid malignancies. J. Clin. Invest. 83:733-737.

Carr, D. J. J., B. R. DeCosta, C. H. Kim, A. E. Jacobson, V. Guarcello, K. C. Rice, and J. E. Blalock. 1989. Opioid receptors on the cells of the immune system: evidence for $\delta$- and k- classes. J. Endocrinol. 122:161-168.

Childs, G. V., J. Lloyd, G. Unabia, and D. Rougeau. 1989. Growth and secretory responses of enriched populations of corticotropes. Endocrinology 125:2540-2549.

Dave, J. R., L. E. Eiden, D. Lozovsky, J. A. Waschek, and R. L. Eskay. 1987. Calcium-independent and calcium-dependent mechanisms regulate corticotropin releasing factor stimulated proopiomelanocortin peptide secretion and messenger ribonucleic acid production. Endocrinology 120:305-310.

Faith, R. E., H. J. Liang, A. J. Murgo, and N. P. Plotnikoff. 1984. Neuroimmunomodulation with enkephalins: enhancement of human natural killer (NK) cell activity **in vitro**. Clin. Immunol. Immunopathol. 31:412-418.

Froelich, C. J., and A. D. Bankhurst. 1984. The effect of $\beta$-endorphin on natural cytotoxicity and antibody dependent cellular cytotoxicity. Life Sci. 35:261-265.

Fukata, J., T. Usui, Y. Naitoh, and H. Imura. 1989. Effects of recombinant human interleukin-1a, -1b, 2 and 6 on ACTH synthesis and release in the mouse pituitary cell line AtT-20. J. Endocrinol. 122:33-39.

Ganong, W. F. 1987. Review of Medical Physiology. 13th ed. Appleton and Lange, San Mateo, California.

Gilman, S. C., J. M. Schwartz, R. J. Milner, F. E. Bloom, and J. D. Feldman. 1982. e$\beta$-endorphin enhances lymphocyte proliferative responses. Proc. Natl. Acad. Sci. USA 79:4226-4230.

Harbour-McMenamin, D., E. M. Smith, and J. E. Blalock. 1985. Bacterial lipopolysaccharide induction of leukocyte-derived corticotropin and endorphins. Infect. and Immun. 48:813-817.

Hazum, E., K.-J. Chang, and P. Cuatrecasas. 1979. Specific nonopiate receptors for $\beta$-endorphin. Science 205:1033-1035.

Heijnen, C. J., C. Beavers, A. Kavelaars, and R. E. Ballieux. 1986. Effect of $\alpha$-endorphin on the antigen induced primary antibody response of human blood B cells **in vitro**. J. Immunol. 136:231-216.

Hsu, S.-M., L. Raine, and H. Fanger. 1981a. A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. Am. J. Clin. Pathol. 75:734-738.

Hsu, S.-M., L. Raine, and H. Fanger. 1981b. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J. Histochem. Cytochem. 29:577-580.

Janeway, C. A., Jr., K. Bottomly, J. Horowitz, J. Kaye, B. Jones, and J. Tite. Modes of cell:cell communication in the immune system. 1985. J. Immunol. 135:739s-742s.

Johnson, H. M., E. M. Smith, B. A. Torres, and J. E. Blalock. 1982. Regulation of **in vitro** antibody response by neuroendocrine hormones. Proc. Natl. Acad. Sci. USA 79:4171-4174.

Johnson, H. M., B. A. Torres, E. M. Smith, L. D. Dion, and J. E. Blalock. 1984. Regulation of lymphokine ($\gamma$-interferon) production by corticotropin. J. Immunol. 132:246-250.

Kavelaars, A., R. E. Ballieux, and C. J. Heijnen. 1989. The role of IL-1 in the corticotropin-releasing factor and arginine-vasopressin induced secretion of immunoreactive $\beta$-endorphin by human peripheral blood mononuclear cells. J. Immunol. 142:2338-2342.

Kay, N., J. Allen, and J. E. Morley. 1984. Endorphins stimulate human peripheral blood lymphocyte natural killer activity. Life Sci. 35:53-59.

Koff, W. C., and M. A. Dunegan. 1985. Modulation of macrophage mediated tumoricidal activity by neuropeptides and neurohormones. J. Immunol. 135:350-354.

Lolait, S. J., A. T. W. Lim, B. H. Toh, and J. W. Funder. 1984. Immunoreactive $\beta$-endorphin in a subpopulation of mouse spleen macrophages. J. Clin. Invest. 73:277-280.

Lolait, S. J., J. A. Clements, A. J. Markwick, C. Cheng, M. McNally, A. I. Smith, and J. W. Funder. 1986. Pro-opiomelanocortin messenger ribonucleic acid and posttranslational processing of beta endorphin in spleen macrophages. J. Clin. Invest. 77:1776-1779.

Mandler, R. N., W. E. Biddison, R. Mandler, and S. A. Serrate. 1986. $\beta$-endorphin augments the cytolytic activity and interferon production of natural killer cells. J. Immunol. 136:934-939.

Mathews, P. M., C. J. Froelich, W. L. Sibbit, Jr., and A. R. Bankhurst. 1983. Enhancement of natural cytotoxicity by $\beta$-endorphin. J. Immunol. 130:1658-1662.

McCain, H. W., I. B. Lamster, J. M. Bozzone, and J. T. Grbic. 1982. $\beta$-endorphin modulates human immune activity via non-opiate receptor mechanisms. Life Sci. 31:1619-1624.

Meyer, W. J., III, E. M. Smith, G. E. Richards, A. Cavallo, A. C. Morrill, and J. E. Blalock. 1987. **In vivo** immunoreactive adrenocorticotropin (ACTH) production by human mononuclear leukocytes from normal and ACTH-deficient individuals. J. Clin. Endocrinol. Metab. 64:98-105.

Mota, I. 1986. Tissue and cells of the immune system. Pp. 1-34. In: Bier, O. G., W. D. da Silva, D. Gotze, and I. Mota, eds. **Fundamentals of Immunology**. 2nd. ed. Springer-Verlag, New York, New York.

Ogilvie, G. K., M. B. Tompkins, and W. A. F. Tompkins. 1988. Clinical and immunologic aspects of FeLV-induced immunosuppression. Vet. Microbiol. 17:287-296.

Payan, D. G., and E. J. Goetzl. 1985. Modulation of lymphocyte function by sensory neuropeptides. J. Immunol. 135:783s-786s.

Rojko, J. L. and R. G. Olsen. 1984. The immunobiology of the feline leukemia virus. Vet. Immunol. Immunopathol. 6:107-165.

Smith, E. M., and J. E. Blalock. 1981. Human lymphocyte production of corticotropin and endorphin-like substances: association with leukocyte interferon. Proc. Natl. Acad. Sci. USA 78:7530-7534.

Smith, E. M., W. J. Meyer, and J. E. Blalock. 1982. Virus-induced corticosterone in hypophysectomized mice: A possible lymphoid adrenal axis. Science 218:1311-1312.

Smith, E. M., D. Harbour-McMenamin, and J. E. Blalock. 1985. Lymphocyte production of endorphins and endorphin mediated immunoregulatory activity. J. Immunol. 135:779s-782s.

Smith, E. M., A. C. Morrill, W. J. Meyer, III, and J. E. Blalock. 1986. Corticotropin releasing factor induction of leukocyte derived ACTH and endorphins. Nature 321:881-882.

Smith, E. M., P. Brosnan, W. J. Meyer, III, and J. E. Blalock. 1987. An ACTH receptor on human mononuclear leukocytes. New England J. Medicine 317:1266-1269.

Unanue, E. R., and P. M. Allen. 1987. The immunoregulatory role of the macrophage. Hosp. Prac. 22:87-104.

Van Epps, D. E., and L. Saland. 1984. $\beta$-endorphin and met-enkephalin stimulate human peripheral blood mononuclear cell chemotaxis. J. Immunol. 132:3046-3053.

Weiss, R. C. 1988. Immunotherapy for feline leukemia, using staphylococcal protein A or heterologous interferons: immunopharmacologic actions and potential use. J.A.V.M.A. 192:681-684.

Westly, H. J. and G. A. Splitter. 1987. Expression of IL-1, IL-2,IL-2R and POMC genes in the blood of mononuclear cells following activation with bovine herpesvirus-1. Fed. Proc. 46:780. Abstract No. 2706.

Westly, H. J., A. J. Kleiss, K. W. Kelley, P. K. Y. Wong, and P.-H. Yuen. 1986. Newcastle disease virus-infected spleenocytes express the proopiomelanocortin gene. J. Exp. Med. 163:1589-1594.

Whitcomb, R. W., W. M. Linehan, L. M. Wahl, and R. A. Knazek. 1988. Monocytes stimulate cortisol production by cultured human adrenocortical cells. J. Clin. Endocrinol. Metab. 66:33-38.

Widman, F. K. 1989. Clinical Immunology. F. A. Davis Co., Philadelphia.

Wybran, J., T. Appelboom, J.-P. Famaey, and A. Govaerts. 1979. Suggestive evidence for receptors for morphine and methionine-enkephalin on normal human blood T lymphocytes. J. Immunol. 123:1068-1070.

Zurawski, G., M. Benedik, B. J. Kamb, J. S. Abrams, S. M. Zurawski, and F. D. Lee. 1986. Activation of mouse T-helper cells induces abundant preproenkelphin mRNA synthesis. Science 232:772-775.

## ACKNOWLEDGEMENTS

This work was supported by a Grant from the National Institute of Health (NIH Grant HD-22786).

I could not have completed the graduate work without the help and support of several individuals. In particular, I extend my gratitude to Dr. Michael Taylor for keeping me on the right path and teaching me the finer points of critical research.

To Cheryl Clark for teaching me the ICC technique and for her technical assistance in performing the procedure.

To Fangi Yu for teaching me the essentials of gel electrophoresis and the Western blot.

Finally, I wish to acknowledge Dr. Richard Engen, Jennifer Drisko, Dr. James Roth, Tom Skadow, and the ISU Meat Lab for supplying me with the fresh blood samples from which the PML were isolated.