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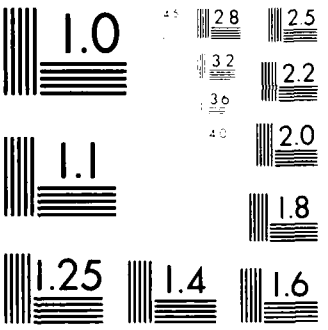
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It has been known for many years that stressful situations can be a contributing factor in the development of a variety of bacterial, viral and neoplastic diseases. Specifically, decreased immuno-competence seems to account for the increased susceptibility of stressed hosts for these disease states. We and others have shown that one mechanism by which this can occur is through the action of neuroendocrine hormones on the immune system. Conversely, we and others have shown lymphocytes synthesize biologically active molecules very similar, if not identical, to neuroendocrine hormones. Thus, it appears that the immune and neuroendocrine systems communicate in a bidirectional regulatory circuit by virtue of common signal molecules and receptors. This report summarizes data showing that hypothalamic hormones which were classically thought only to act on the pituitary also stimulate the immune system. Thus, these hormones released by the brain can serve as a direct link between the central nervous and lymphoid systems. This may explain in part how stressful situations can result in decreased resistance to infections and tumors.

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## Background

It has been known for many years that stressful situations can be a contributing factor in the development of a variety of bacterial, viral and neoplastic diseases (1,2). Specifically, decreased immuno-competence seems to account for the increased susceptibility of stressed hosts for these disease states. We and others have shown that one mechanism by which this can occur is through the action of neuroendocrine hormones on the immune system (3, see 4 for review). Conversely, we and others have shown lymphocytes synthesize biologically active molecules very similar, if not identical, to neuroendocrine hormones (4-6). Thus, it appears that the immune and neuroendocrine systems communicate in a bidirectional regulatory circuit by virtue of common signal molecules and receptors. The significance of this relationship is just beginning to be determined, but initially it appears to be a mechanism whereby behavior and stress can enhance susceptibility to disease or affect healing. Thus, if true, the implications would be major, especially for the military. Once understood, it may be possible to block this stress effect, thereby preventing disease and the subsequent inefficiency or disruption of training and other missions.

## Specific Aims

The overall objective of the project is to characterize the molecules and mechanisms by which the immune and neuroendocrine systems interact. In particular, this project is aimed at determining if the hypothalamus can modulate immune responses directly by hypothalamic hormones or indirectly through activation of other neuroendocrine tissues. More specifically the aims of the original project include:

1. Characterization of lymphocyte immunoreactive corticotropin (ir-ACTH) induced by corticotropin releasing factor (CRF).

2. To determine if CRF induces lymphocytes to make immunoreactive (ir) endorphins.

3. To determine if other hypothalamic releasing factors (RF) stimulate ir hormone production by lymphocytes.

4. Characterization of RF immunomodulatory activity.

## Results

The results of the initial two years research have been summarized below in general as they relate to the specific aims.

### 1. Corticotropin Releasing Factor (CRF) induction of ir-ACTH and endorphins

In vitro, CRF was observed to cause the de novo synthesis and release of leukocyte derived ACTH and β-endorphin. While it occurred at about 10 fold higher concentrations, arginine vasopressin (AVP) alone was observed to have

intrinsic CRF activity. At concentrations that are frequently used on cultured pituitary cells, CRF and AVP together acted in an additive fashion to induce these proopiomelanocortin (POMC) derived peptides and such induction was blocked by dexamethasone. Thus, leukocytes seem quite similar to anterior pituitary cells with respect to control of the POMC gene by positive hypothalamic signals (CRF and AVP) and feedback inhibition by a synthetic glucocorticoid hormone (7). Interestingly, while control of the leukocyte POMC gene may be similar to that of anterior pituitary cells, the processing of its products appears somewhat different. For instance, while Newcastle disease virus (NDV) and CRF cause the production of POMC peptides with the molecular weight of ACTH (1-39) and β-endorphin, lipopolysaccharide LPS elicits the production of corticotropin (ACTH) and endorphins which correspond to the molecular weight of ACTH (1-24 to 26) and α or γ endorphin (see section 2). Although the relative contribution to alternate processing of the stimuli as opposed to the possible different leukocyte types which are responding to the stimuli are presently unknown, these findings nontheless point to alternate proteolytic cleavages of POMC as have been previously observed in the anterior and intermediate lobe of the pituitary as well as the hypothalamus. Of course, these results also suggest that cells of the immune system differ from virtually all other extrapituitary tissues where the major proteolytic cleavages are similar to those in the intermediate lobe of the pituitary gland. For example, though we detect β-endorphin, we have yet to observe the production of an α-melanocyte stimulating hormone (MSH)-like peptide. Further, LPS induction of an ir-ACTH with a molecular weight of approximately 2.9k suggests a quite novel processing pathway. Such differential pathways which are both unique and in some instances composites of those seen in the anterior and intermediate lobe of the pituitary gland.

As a final characterization of the CRF induced ACTH and endorphins, we decided to purify and amino acid sequence the molecule. This was made feasible over the last year due to the University's acquisiton of an ultra sensitive gas phase sequenator and a technical improvement in our ability to produce larger quantities of ir-ACTH. A radioimmunoassay (RIA) for ACTH has been assessed as a means to follow small quantities of ir-ACTH during purification and to provide further information on antigenic similarities. This is a commercial RIA (Immunonuclear) which uses an antiserum against ACTH 1-24 and correlates well with biological activity. The RIA is specific, it will react only with ACTH 1-24 and ACTH 1-39, not alpha MSH (ACTH 1-13) or β-endorphin. Also, the RIA will detect ACTH at concentrations as low as 1 pg/ml. Based on the parallel slopes, the ir-ACTH is antigenically identical with the ACTH standard. Using this RIA, various inducers were compared for maximum ACTH production by lymphocytes. CRF and bacterial LPS, both described in our initial report induced more ir-ACTH than the prototype inducer, NDV (3). Since the LPS induced ACTH appears to be shorter (8), and therefore probably easier to sequence, we chose this system for the initial intrinsicly radiolabeled ($^3$H)ACTH awaiting availability of the sequenator. The amino acid analysis suggested both ACTH 1-24 and ACTH 1-39 were present. However, amino acids in which there would be a one to one ratio between the species correlated highly (Table 1). We now have 60 nmoles of radiolabeled irACTH for sequencing once the two ACTH species are separated.

Table 1. Amino Acid Composition of Lymphocyte-derived ACTH

| Amino Acid | Expected | Observed |
|---|---|---|
| Ala | - | - |
| Arg | 3 | 2.3 |
| Asx | 0.1 | 2 |
| Cys | 0 | 0 |
| Glx | 1.5 | 4-5 |
| Gly | - | - |
| His | 1 | 1 |
| Ile | 0 | 0 |
| Leu | 0-1 | 2 |
| Lys | 4 | 4 |
| Met | 1 | 1 |
| Phe | 1-3 | 2.4 |
| Pro | 3-4 | 3-4 |
| Ser | 2-3 | 2 |
| Thr | 0 | 0 |
| Trp | - | - |
| Tyr | 2 | 1.2 |
| Val | 3 | 3 |

Recent reports from other laboratories have confirmed our finding of POMC prodution by leukocytes at the level of mRNA. Westly et al., (9) using a system identical to ours, detected POMC related mRNA in mouse spleen cells induced with NDV. Also, Lolait et al., (10) has detected POMC mRNA and post translational processing of endorphins in spleen macrophages.

2. Bacterial endotoxin induction of leukocyte derived ir-ACTH and endorphins.

Previous reports have shown that there is an endogenous opioid component associated with pathophysiologic responses to endotoxin. It has been shown that these responses are alleviated by naloxone, a specific opiate antagonist. Another study indicated that leukocytes may mediate some of those responses since leukocyte depletion alleviated the LPS effects. In view of the above reports, as well as the finding that leukocytes produce ir endorphins and ACTH when stimulated with NDV or CRF, we postulated that leukocytes may serve as an extrapituitary source of endorphins produced in response to bacterial endotoxin. In order to test this hypothesis, human peripheral blood leukocytes, as well as mouse spleen cells, were cultured in vitro with LPS for 48 hours. The LPS (i.e., endotoxin) was shown to induce de novo synthesis of ir-ACTH and ir-endorphins (8). The leukocyte derived ir-ACTH had a molecular weight of approximately 2900 daltons and demonstrated similar bioactivity to pituitary derived ACTH. The lymphocyte derived ir-endorphin comigrated with $\alpha$ and $\gamma$ endorphin at approximately 1800 daltons and was shown to bind to brain opiate receptors. These findings imply that leukocyte derived endorphins may be involved in the pathophysiologic response to endotoxin.

3. Are any of the other characterized hypothalamic RF's able to stimulate ir hormone production in lymphocytes.

Human peripheral lymphocytes (HPL) or mouse spleen cells were treated with thyrotropin releasing hormone (TRH), growth hormone releasing hormone (GHRH) and luteinizing hormone releasing hormone (LHRH) and then tested for possible thyrotropin (TSH), growth hormone (GH), and lutropin production by an indirect immunofluorescent (IF) assay which employed monospecific antisera against the respective hormone. Table 2 shows that each RF (TRH, GHRH and LHRH) caused a positive IF reaction for the expected pituitary hormone (TSH, GH and LH, respectively). Thus, the induction of leukocyte derived hormones by hypothalamic RFs seems to be a general feature of the circuitry between the immune and neuroendocrine systems.

Since these hypothalamic releasing factors have been shown to induce a positive immunofluorescence reaction (Table 2) for the respective pituitary hormones, we began to structurally characterize the products. irLH is the product we have initially examined. It appears to be synthesized de novo since (3H) labeled amino acids are incorporated into material that binds to an anti-LH B-chain antibody affinity column. There may be some endogenous irLH produced since a small amount of radiolabeled material from the mock preparation also bound to the column. Gel filtration sizing of the radiolabeled irLH shows it to migrate in the same fraction as our LH B-chain marker. Thus, LHRH like CRF appears to induce lymphocytes to synthesize the same peptide hormone these factors classically induce in the pituitary gland. Preparation of larger quantities of irLH with a higher efficiency of radiolabeling will enable us to determine if the irLH is a two chain molecule like pituitary LH.

As shown in Table 2, GHRH treated lymphocytes stained positive by immunofluorescence with antisera to human growth hormone. Preliminary experiments indicate that (3H) amino acids can be incorporated into material that binds to an antibody affinity column. Also in a very preliminary experiment, transfer of GHRH treated mouse splenocytes to Snell dwarf mice caused a small but significant gain in weight (0.1g over 16 days) when compared to mock treated controls. This suggests that the GHRH induces lymphocytes to produce a biologically active irGH.

In conjunction with these studies described above, is a search to determine the spectrum of hormones that lymphocytes can synthesize and the variety of inducing stimuli. One such stimuli that induces a novel hormone-like molecule is a mixed leukocyte culture. Lymphocytes from individuals with different ABO blood types cultured for 5 days were found to synthesize a molecule identical with the placental hormone, human chorionic gonadotropin (hCG). irhCG has the same molecular weight as a radioiodinated hCG marker. Evidence that couples with similar HLAs can often overcome conception problems following transfusion of the female with leukocyte, major histocompatibility antigens (11) is an intriguing suggestion that lymphocyte derived hCG could play a role in the reproduction cycle or generation of diversity.

4. Immunomodulatory activity of hypothalamic RFs.

Originally, we found that CRF could act directly on the immune system by inducing lymphocytes to synthesize irACTH and endorphins (7). In preliminary experiments during the first year, CRF was found also to enhance

Table 2. Induction of ir hormones in lymphocytes by hypothalamic releasing hormones.

| Releasing Factor | ir Hormone Induced | Day of Maximum Production | % Positive Cells (+SD) |
|---|---|---|---|
| CRF | ACTH | 2 | 60-90[a]* |
| | Endorphin | 2 | 60-90 |
| TRH | TSH | 1 | 20 + 12 |
| LH-RH | LH | 1 | 35 + 5 |
| | FSH | 1 | 20 + 5 |
| GH-RH[b] | GH | 1 | 20 + 2 |

Human peripheral blood lymphocytes were prepared by buoyant density centifugation on Ficoll-Hypaque gradients and incubated with various releasing hormones (0.1 ug/ml) as indicated. The fixed cells were stained by an indirect immunofluorescent technique with antisera specific for the indicated neuroendocrine hormones. Background levels of nonspecific staining for the above results were 5% or less.

[a]CRF alone stimulated up to 60% of the lymphocytes to produce ACTH and endorphin and could be enhanced (up to 90%) when AVP (100 ng/ml) was included.

[b]Murine spleen cells were used for this experiment.

the in vitro production of IgM antibody to sheep red blood cells (SRBC). Not only is antibody production enhanced to a T-lymphocyte dependent antigen, but also to Brucella abortus-tri-nitrophenol (BA-TNP) which is a relatively T-cell independent antigen. This suggests that the CRF effect may be primarily on the B-lymphocyte. The dose required for enhancement was sub nanomolar and as we previously found with ACTH, the immunomodulatory effect is blocked by the reducing agent 2-mercaptoethanol (2-ME) (12). In the case of ACTH, the receptor is labile in the presence of reducing agents (13). With CRF it appears that enhancement of plaque forming cells (PFC) is only visible under suboptimal conditions and therefore can not be detected with 2-ME driving the response to maximum antibody production. CRF must be present initially in the culture for maximum enhancement of the response and if added after day 3 it has no effect. This compares with ACTH (12) suppression and TSH enhancement of the PFC response to SRBC both of which need to be present initially for maximum modulation. Although not shown, CRF did not shift the kinetics of antibody production. Also not shown is that AVP did not further enhance the effect of CRF as it does with ACTH production by pituitary cells and lymphocytes.

Currently, we are trying to determine the mechanism of CRF's enhancement. Since CRF induces lymphocytes to produce ACTH, comparable doses of ACTH were added to the PFC cultures. The result, a biphasic curve in which at very low doses ACTH enhances and as we found previously high doses are suppressive (12). Therefore, CRF may act indirectly by inducing low concentrations of irACTH which in turn enhance the PFC response.

Although not shown, our first experiments adding LHRH to the BA-TNP PFC assay showed 300 to 500% enhancement in the number of plaques at levels below 20 ng/ml. This experiment needs to be repeated and the LHRH checked for endotoxin contamination but preliminarily it appears that another hypothalamic releasing hormone has immunomodulatory activity.

5. CRF activity of monokines.

Hepatocyte-stimulating factor and interleukin-1 are proteins produced by monocytes in response to inflammatory challenge. Neither of these monokines had direct effects on steroid production by cultured adrenocortical cells. Both monokines stimulated pituitary cells (AtT-20) to release ACTH; interleukin-1 was equipotent with a combination of CRF and AVP and hepatocyte-stimulating factor (HSF) was at least three times as effective (15). The synthetic glucocorticoid, dexamethasone, inhibited production of HSF by cultured monocytes. These results indicate an axis between monocytes and pituitary and adrenocortical cells which may play a role in regulating host defense.

6. Enhancement of the in vitro antibody response by thyrotropin.

The pituitary hormone TSH has been shown to enhance in a dose dependent manner the in vitro antibody response (14). Highly purified preparations of bovine and human TSH enhanced up to 375% the number of cells producing antibody to SRBC. TSH had to be present prior to 24-48h of the the initiation of culture for enhancement of the antibody response. Since SRBC are a T-lymphocyte dependent antigen we next determined the possible immunoregulatory function of thyrotropin on lymphocytes immunized with a T-independent antigen Brucella abortus - TNP, (BA-TNP) and the cellular components involved in such function. TSH enhanced the in vitro antibody response to BA-TNP as determined by direct PFC assays (16). Cell depletion studies showed that the TSH effect, while

independent of macrophages, required the presence of T cells. Thus pituitary, and possibly leukocyte, TSH appears to function as a lymphokine which may act via T cells to augment antibody production.

7. Characterization of an ACTH receptor on leukocytes.

Previously, while studying the effect of ACTH on the in vitro antibody response we reported that iodinated ACTH bound specifically with high affinity to mouse spleen cell membranes (12). Thus suggesting, that ACTH suppressed the antibody response through interaction with an ACTH receptor on the spleen cells. IF CRF enhances the PFC response through induction of ir-ACTH, it would help in determining the mechanism to know which lymphoid cells bear functional ACTH receptors. Using a specific antiserum to the pituitary ACTH receptor (17), mouse spleen cells have been fractionated by standard methods into subpopulations and stained by immunofluorescence. The receptor appears to be present on both T (23%) and B (47%) lymphocytes and macrophages but not the entire population of any of these types of cells. Furthermore, mouse thymocytes express essentially no ACTH receptors unless stimulated such as with the T-cell mitogen, concanavalin A and then over 90% of the cells express the receptor after 3 days of culture. Thus, modulation of the ACTH receptor may be an important aspect of immune regulation and may be a necessary mechanism for CRF modulation of immune responses, especially if mediated indirectly by ir-ACTH.

In a related series of experiments, human peripheral blood lymphocytes were examined by a receptor binding experiment for ACTH receptors using 125-I radiolabeled ACTH. The binding appeared to be very similar to the binding of ACTH to adrenal cells (18). There appear to be two binding sites of high and lower affinity, Kds of 0.02 nM and 1.8 nM respectively. When lymphocytes from an individual with a clinically apparent defect in adrenal ACTH receptors (ACTH insensitivity syndrome) were analyzed by ACTH binding studies the binding appeared to be very low affinity of a nonspecific nature. Thus, there appears to be a functional correlation between peripheral ACTH receptors and central adrenal ACTH receptors.

8. Generation of a soluble IFN-gamma inducer by oxidation of galactose residues on macrophages.

Although not an original specific aim, one of the investigators was able to lend his expertise of this area to a related study on mechanisms of lymphokine induction.

Depletion of macrophages from human peripheral blood mononuclear cells (PBMC) caused a marked decrease in galactose oxidase and sodium periodate, but not a calcium ionophore, stimulated Interferon-$\gamma$ (IFN-$\gamma$) production (19). Reconstitution of such depleted cultures with galactose oxidase treated macrophages, but not lymphocytes, restored IFN-$\gamma$ levels to those of control nonfractionated PBMC. Thus, galactose oxidase seemed to act on macrophages which in turn stimulated lymphocyte production of IFN-$\gamma$. Unlike human cells which have terminal galactose residues on glycoproteins, murine cell glycoproteins terminate their oligosaccharide component in the order N-acetyl-neuraminic acid followed by D-galactose, N-acetyl-glucosamine, and glycoprotein. Galactose oxidase or sodium periodate only activated murine macrophages to stimulate lymphocyte IFN-$\gamma$ production after exposing D-galactose residues by the removal of the terminal N-acetyl-neuraminic acid residues with neuraminidase. Removal of such exposed terminal glactose residues with B-galactosidase

inhibited the effect of galactose oxidase on murine macrophages. Taken together, these results strongly suggest that oxidation of terminal galactose residues on macrophages is the initial site of action of galactose oxidase and sodium periodate. Studies with Boyden chambers have shown that galactose oxidase-treated macrophages released a soluble factor which stimulates lymphocyte production of IFN-$\gamma$ . Based on these findings, it appears that the oxidation of terminal galactose residues on the surface of macrophages leads to the induction and transmission of a soluble signal for lymphocyte production of IFN-$\gamma$ .

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