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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 have suggested that one mechanism by which this can occur is through the action of neuroendocrine hormones. It appears that the immune and neuroendocrine systems communicate in a bidirectional regulatory circuit by virtue of common signal molecules and receptors. In this report we discuss findings of the nucleotide and amino acid sequence of lymphocyte-derived corticotropin (ACTH). Also presented are results that the hypothalamic releasing hormone for luteinizing hormone (LH) induces lymphocytes to synthesize a bioactive LH. Finally, we discuss our data that the ACTH receptor on the lymphocytes can inhibit mitogenesis and induces adherent leukocytes to produce TNF-alpha - Kaw...

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I. INTRODUCTION

It has been suggested that stressful situations can be a contributing factor in an individual's resistance to infections and tumors. We and others have shown that one mechanism by which this can occur is through the action of neuroendocrine hormones on the immune system (1,2). In addition, we have found that lymphocytes synthesize biologically active molecules identical to neuroendocrine hormones. Thus, both arms of a regulatory circuit between the immune and neuroendocrine systems exist and could provide bi-directional communication between the two systems. The basis of this form of interaction is the presence of common signal molecules and receptors in both systems. 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 activities.

The overall objective of the project has been 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. In the past we have shown that corticotropin releasing factor (CRF) and thyrotropin releasing hormone (TRH) induce lymphocytes to synthesize corticotropin (ACTH) and thyrotropin (TSH), respectively (3,4). For both cases this has recently been reproduced (5,6).

In this report we described the final proof that proopiomelanocortin (POMC) is produced in lymphocytes, characterization of ACTH receptors on splenocytes, immunoreactive luteinizing hormone (irLH) production, and the action of ACTH on TNF-α production.

II. METHODS

A. Amino acid and nucleotide sequencing of lymphocyte POMC. This project was a collaboration with Dr. J.E. Blalock and his group at the University of Alabama at Birmingham. For nucleotide sequencing, POMC mRNA isolated from C3H/FeJ mouse splenocytes was amplified by the polymerase chain reaction method (7). The cDNA was then cloned in M_{13}mp18 and mp19 bacteriophage on the E. coli host TG1 (8). Purified recombinant M13 ssDNA was then sequenced on an automated DNA sequencer.

The amino acid sequence was determined using purified irACTH from C3Heb/FeJ mouse splenocytes stimulated with bacterial lipopolysaccharide (LPS) (8). The majority of this irACTH is truncated approximately 3,000 rather than 4,500 daltons, and was purified by antibody affinity chromatography, gel filtration, and reverse phase HPLC (2). The sequencing was performed by the UTMB Cancer Center Core Facility on gas phase microsequencer and the derivitized amino acids quantitated by reverse phase by HPLC (9).

B. ACTH receptor studies

These studies consisted largely of identifying, through immunofluorescent staining, the cell populations that express ACTH receptors. A monospecific antiserum (10) prepared against purified mouse adrenal cell ACTH receptors and commercial monoclonal antibodies against cell surface markers were used. Enriched cell populations were prepared by standard procedures from Mishell and Shigii (12). T- and B-lymphocyte populations were

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prepared by absorption to nylon wool columns. Adherent cell populations were removed from B-cell populations or selected by adherence to plastic petri dishes. Cyclic AMP (cAMP) was measured in a radioligand competition assay previously described (12). In general, whole mouse spleen cell preparations or cultured cells were treated with varying doses of ACTH, CRF or forskolin. Supernatant fluids and cell pellets were assayed for cAMP.

C. <u>ACTH induction of TNF-α</u>

Adherent peripheral blood monocytes were prepared from normal donors (13). The cells were cultured for 24h in the presence of ACTH and/or INF-γ The supernatant fluids were collected and assayed for TNF cytolytic activity on actinomycin D treated L-cells (14). TNF-α activity was characterized by antibody neutralization of cytolytic activity.

D. <u>irLH production by lymphocytes</u>

Human peripheral blood mononuclear leukocytes were prepared by Ficoll-Hypaque density gradient separation. The cells were treated with luteinizing hormone releasing hormone (GnRH) at 1 μg/ml for 48h. Culture supernatant fluid was harvested and irLH was purified by antibody affinity column chromatography. Bound material was defined as irLH. Characterization by polyacrylamide gel electrophoresis, concanavalin A chromatography and reverse phase HPLC were by standard procedures (15).

LH bioactivity was measured by testosterone production from mouse Leydig cells. Mouse testicles were removed, dissociated, the Leydig cells cultured, then treated with irLH for 3h. Testosterone was quantitated in the Leydig cell culture supernatant fluids by RIA (16).

III. RESULTS

A. Nucleotide and amino acid sequence of lymphocyte-derived corticotropin: Endotoxin induction of a truncated peptide. In spite of marked similarities between the immune and neuroendocrine-derived substances, questions still remained as to the degree of relatedness. To test for precise molecular identity versus biochemical similarity for a corticotropin (ACTH)-like peptide from lymphocytes (17-23), we determined the amino acid sequence of this molecule as well as the nucleotide sequence of its cDNA in collaboration with Dr. Ed Blalock's group in Alabama. The nucleotide sequence encoding this peptide was identical to that of mouse pituitary ACTH (Figure 1). Elevated levels of lymphocyte irACTH were then induced with bacterial lipopolysaccharide (LPS) and the peptide(s) purified by antibody affinity chromatography and reverse phase high performance liquid chromatography (HPLC). The predominant irACTH species was approximately 3,000 daltons in size and its sequence was identical to pituitary $ACTH_{1-25}$ (Figure 1). These results conclusively demonstrate that lymphocytes produce authentic ACTH and transcribe its mRNA.

B. ACTH receptors on leukocytes: Cellular phenotype and function. We have made good progress in our ACTH receptor studies this past year. This stemmed from the initial findings of the project in which CRF was shown to induce lymphocytes to produce ACTH. As a structural approach to determine how ACTH (and indirectly CRF) may affect the immune system we have been characterizing the populations of leukocytes that express the receptor. Previously we had shown that the receptor was on some but not all B (50%) and T (20%) cells and macrophages (50%) (24). Since we also showed that thymocytes could upregulate the receptor, T-cell populations were examined in

mice more thoroughly this past year. Using a complement depletion procedure and immunofluorescence with an antiserum to the ACTH receptor, it appears that of nonstimulated T-cells of the helper subtype (L3T4+), only 20% are expressing the receptor (Table 1). The Lyt2 bearing cells could not be shown to express the receptor. Since thymocytes when stimulated with Con A upregulate ACTH receptor from nondetectable levels to over 90% expression, it is probable that both subtypes of T-cells express the receptor when stimulated. Upregulation of the receptor following Con A stimulation is common to most T-cells. Enriched splenic T-cells plus both mature and immature thymocytes (negative and positive for peanut agglutinin binding, respectively) up-regulate the ACTH receptor when stimulated by Con A.

Two human T-cell lines, S49A cells which stain positive for the ACTH receptor and the Molt 4 cells which are negative were examined for their sensitivity to ACTH induced cAMP production. ACTH would stimulate cAMP production in the S49A cells (and mouse spleen cells) but not the Molt 4 cells. Radioligand binding was also done, which correlated binding and cAMP production with antigenic presence of the ACTH receptor (12).

We expanded our cAMP studies this past year to look at cAMP levels in S49A cells in response to an antiserum that binds the ACTH receptor (anti-HTCA). This was to link the immunological and functional studies, by showing that they recognized and stimulated the same structure (25). As we hypothesized, the anti-HCTA increased the intracellular cAMP levels (76%) to within the range of that seen for ACTH treatment (97%). In addition, an even greater increase (155%) in intracellular cAMP was observed for cells preincubated with anti-HTCA and then treated with ACTH. This, less than additive increase, suggests that the ACTH filled the residual unoccupied ACTH

receptors and that there is an overlapping set of receptors activated by the anti-HTCA and ACTH.

Our major finding on ACTH receptors this year links the upregulation of ACTH receptors on T-cells with inhibition of T-cell mitogenesis. With Con A stimulated thymocytes, spleen cells, or enriched T-cells, ACTH inhibited the incorporation of ^{3}H-thymidine in parallel with the expression of ACTH receptors. ACTH inhibition was as great as 99% and dose responsive (Table 2). This provides direct support for reports in the literature that T-cell mitogenesis is inhibited by stress in the absence of glucocorticoids. This is certainly only one plausible mechanism, but it provides a new structural and mechanistic relationship to interpret behavioral effects on immune responses. To see if a known lymphokine was a major component in Con A induction of ACTH receptors, we treated mouse splenocytes with various crude and purified lymphokine preparations. Single purified preparations did not appear active by themselves. However, a crude preparation of interferon-γ (IFN-γ) would up-regulate the number of cells expressing ACTH receptors. Thus, it probably is a combination of factors regulating this receptor on lymphocytes.

C. <u>ACTH induction of tumor necrosis factor-α (TNF-α) by monocytes.</u>

Since 50% of "resting" macrophages express ACTH receptors we wondered if ACTH affected TNF production (Table 3). $ACTH_{1-39}$ (full length) induced TNF-α from the adherent fractions of human peripheral blood leukocytes (13). In addition, we also found that $ACTH_{1-39}$ will potentiate IFN-γ's induction of TNF-α from these cells. Since previous reports showed that ACTH inhibits INF-γ's activation of macrophages to a cytocidal state, our data raise questions concerning the relative role of TNF-α in macrophage mediated cytolysis. Also,

it adds yet another activity to the previous findings of ACTH modulation of macrophage function.

D. Luteogenic activity from human leukocytes.

In light of our previous findings that CRF and TRH induce ACTH and TSH, respectively; we wondered if gonadotropin releasing hormone, (GnRH) likewise would stimulate lymphocytes to produce leutinizing hormone (LH) (15). Using immunofluorescence and immunoaffinity chromatography, there appeared to be a basal level of immunoreactive (ir) LH which was increased approximately 3-fold by stimulation with GnRH. The physicochemical properties of irLH were examined by polyacrylamide gel electrophoresis, western blotting, reverse-phase HPLC, and Concanavalin A chromatography. In all cases the irLH appeared very similar, if not identical, to pituitary LH. The irLH incorporates radiolabeled carrier-free sulfate which indicates de novo synthesis and is a unique characteristic of LH compared to other gonadotropins. Additionally, we found that irLH induces testosterone production in vitro by cultured mouse Leydig cells and thus is biologically active (Table 4). This could be demonstrated at a minimal level using Con A affinity column enriched material alone, or in conjunction with forskolin, which has been reported to increase the Leydig cell's sensitivity to LH (26).

In a recent preliminary experiment we co-cultured human peripheral leukocytes with mouse Leydig cells. Our hypothesis was that if the leukocytes are producing irLH constitutively then this should induce testosterone production by the leydig cells. This is exactly what happened after 24 hours of culture. The testosterone production was dose-dependent, but unexpectedly it decreased with increased cell numbers. IL-1 will inhibit LH induced steroidogenesis, but this usually requires 48 hours (27). We are currently

investigating this response using antisera against LH or IL-1. In support of our findings is a report showing that LHRH will modulate development of the thymus (28). Thus, there is increasing evidence for a role of the peptide reproductive hormones in immune function.

IV. DISCUSSION

The results described above support our hypothesis that the immune and neuroendocrine systems can communicate through common signal molecules and receptors (1,2). In the case of ACTH, both the peptide and mRNA expressed in lymphocytes has the same sequence as the pituitary POMC gene products. This of course is not surprising, based on previous characterization of the peptide at structural and functional levels (2,17-23). Our finding that CRF stimulates leukocytes to produce the POMC products, ACTH and endorphins (3), has been replicated (5) and further demonstrates the common mechanisms. Interestingly, Kaveleers et al., (5) found that in their system CRF induced IL-2 which in turn induced endorphin production. Since, T-cells also have CRF receptors (29) there may be other mechanisms of action for CRF. Since the hypothalamic releasing hormones like CRF and TRH are now being found in the periphery and notably in the spleen (30), the action of these peptides on immune function is gaining a new importance.

The induction of irLH by GnRH extends this concept into the area of reproductive hormones. This is an area where immune and endocrine interactions have been found, but were generally attributed to androgens (31). Hall and Goldstein (32) have reported thymosin to stimulate release of pituitary gonadotropins. It now appears that the immune system itself is a source of LH (15). Additionally, GnRH has been found to reverse the thymic atrophy and partially restore immune responsiveness associated with aging

(28). It will be interesting to determine if irLH from leukocytes modulates this effect.

The ACTH receptor and immunomodulation reported above shows that the receptors are also common structures in the interaction between the two systems. The stimulation of ACTH receptor expression on thymocytes by mitogens suggests this receptor can be classified as an activation marker. The ability of ACTH to inhibit this mitogenic response seems opposite to that of IL-2 and may be a negative feedback mechanism in this regard. Experiments are underway to determine if ACTH inhibits IL-1 and/or IL-2 production in this system.

The ability of ACTH to induce TNF-α further demonstrates the multiple activities of ACTH on immune function. TNF-α induction has been thought to mediate activated macrophage killing of tumor cells. Koff and Dugan (33) found ACTH inhibited α-IFN activation of macrophage tumor cell killing. Therefore, **our data argues** that this killing may be through a mechanism other than TNF-**α**.

In summary, **the results described above show** in more **detail and** expand the idea that the immune and neuroendocrine systems can communicate through common signal molecules and receptors.

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TABLE 1

Distribution of ACTH receptor-positive cells among purified L3T4-positive T lymphocytes[a]

Antisera	% fluorescing cells[b]
anti-Ig	2 ± 2
anti-Thy 1.2	92 ± 0.2
anti-L3T4	89 ± 3
anti-Lyt2	4 ± 3
anti-ACTH receptor	16 ± 7
NRS control	1 ± 1

[a]L3T4-positive T cells were enriched from a 93% Thy 1.2-positive T cell population as described in Materials and Methods. Cells were analyzed for ACTH receptors and other lymphocyte surface markers by IF assay.

[b]Percentages shown reflect the mean ± standard deviation for 3 experiments.

Table 2. Effect of ACTH on Con A-stimulated thymocyte mitogenesis.

Treatment	$[^3H]$-thymidine incorporation (dpms)	Percent suppression[a]
Experiment 1:		
Media only	3768 ± 1162	
Con A only	54669 ± 3109	
Con A/ACTH (4 X 10^{-6}M)	3342 ± 1025	93.89 ± 1.87
Con A/ACTH (10^{-6}M)	4684 ± 1034	91.43 ± 1.89
Con A/ACTH (10^{-7}M)	5708 ± 520	89.56 ± 0.95
Con A/ACTH (10^{-9}M)	7018 ± 942	87.16 ± 1.72
ACTH only (4 X 10^{-6}M)	5017 ± 1097	
ACTH only (10^{-9}M)	4041 ± 715	
Experiment 2:		
Media only	3492 ± 1415	
Con A only	27411 ± 2686	
Con A/ACTH (4 X 10^{-6}M)	3320 ± 600	87.89 ± 2.19
Con A/ACTH (10^{-6}M)	5420 ± 2434	80.22 ± 8.88
Con A/ACTH (10^{-7}M)	9207 ± 4430	66.41 ± 16.16
Con A/ACTH (10^{-9}M)	6822 ± 2972	75.11 ± 10.84
ACTH only (4 X 10^{-6}M)	3702 ± 629	
ACTH only (10^{-9}M)	3401 ± 685	

[a]Percent suppression was calculated by determining the percentage of $[^3H]$-thymidine incorporation for Con A/ACTH treated cultures of that for Con A only treated cultures, subtracted from 100%.

5 X 10^6 thymocytes/ml were cultured in 1 ml volumes for 48 hours, in the presence or absence of Con A (1.25 μg/ml). ACTH was added every day of culture or equivalent volumes of media were added to non-ACTH treated wells. $[^3H]$-thymidine (5 μCi/well) was added 4-6 hours prior to cell harvest. Results shown are the mean ± standard deviation for triplicate cultures. These experiments are representative of 4 experiments which gave similar results.

Table 3. ACTH 1-39 induces a tumor necrosis factor-like activity.

Treatment	U/ml TNF Activity/10^4 cells
Media	<3
ACTH 10^{-6}M	100
10^{-7}M	30
10^{-8}M	<3
IFN-γ 100	70
30	30
10	<3

Legend to Table I

Adherent PBMs were prepared as described in Materials and Methods. ACTH 1-39 or IFN-γ were placed on the cells at the indicated concentrations in microtiter plates. Twenty-four hours later, supernatant fluids were harvested and tested for TNF activity on actinomycin D treated L-929 cells as described by Flick and Gifford (14). Briefly, 5 X 10^5 L-929 cells in microtiter plates were treated with 5 μg/ml actinomycin D (Cosmegen, Merck, Sharp and Dohme, West Point, PA) in EMEM 2%. Samples to be analyzed for TNF-α cytolytic activity were titrated on the cells. Twenty-four hours later, supernatant fluids were decanted and the cells stained with a solution of 20% methanol/1% crystal violet. Following removal of excess stain by exhaustive flushing with water, the plates were dried and then destained with Sorenson's buffer. The optical density of each well was then determined on an automated ELISA reader at a wavelength of 590 nm. One unit of TNF activity is defined as the reciprocal of the dilution at which the optical density of an experimental sample is 50% of that of an actinomycin D only treated control. Representative of three separate experiments.

Table 4 Effect CAE on testosterone production.

	Dilution	−F	+F
Medium		287 ± 45	597 ± 31
hLH (μIU)	22	398 ± 19	831 ± 188
	88	420 ± 20	921 ± 163
	442	834 ± 37	907 ± 252
	880	1223 ± 230	1048 ± 166
CAE [a]	10	153 ± 21	32 ± 1
	5.0	160 ± 12	136 ± 6
	3.3	163 ± 5	214 ± 11
	1.0	170 ± 28	276 ± 9
CAE + Ab	1.0	163 ± 20 [b]	256 ± 9 [c]

Leydig cells were cultured for 3 h with medium, hLH or Con A eluate (CAE) and treated with anti-hLH-β (Ab) or forskolin (F). At the end of the incubation, period cells were assayed for testosterone production by RIA. For hLH data are mean fg per cell ± SEM; for CAE, data are % respective control (medium or medium + F). Data are from duplicate cultures from two experiments. ND, not determined.

[a] 1.0 equals amount of material isolated from 2×10^7 GnRH stimulated PBL.

[b] $P < 0.06$ vs. CAE at 1.0 dilution.

[c] $P < 0.001$ vs. CAE + F at 1.0 dilution.

FIGURE 1. Nucleotide and amino acid sequence of lymphocyte-derived POMC.

Residue 1 10

Murine NH_2-Ser Tyr Ser Met Glu His Phe Arg Trp Gly Lys Pro Val Gly Lys Lys Arg Arg Pro
Pituitary 5'-TCC TAC TCC ATG GAG CAC TTC CGC TGG GGC AAG CCG GTG GGC AAG AAA CGG CGC CCG

Murine
Lymphocyte 5'-TCC TAC TCC ATG GAG CAC TTC CGC TGG GGC AAG CCG GTG GGC AAG AAA CGG CGC CCG

20 30 39

Val Lys Val Tyr Pro Asn Val Ala Glu Asn Glu Ser Ala Glu Ala Phe Pro Leu Glu Phe-COOH
GTG AAG GTG TAC CCC AAC GTT GCT GAG AAC GAG TCG GCG GAG GCC TTT CCC CTA GAG TTC-3'

GTG AAG GTG TAC CCC AAC GTT GCT GAG AAC GAG TCG GCG GAG GCC TTT CCC CTA GAG TTC-3'

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