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Immunology

Immunosuppressive effects of corticotropin and melanotropin and their possible significance in human immunodeficiency virus infection

(neuroimmunomodulation/neuropeptides/AIDS/neutral endopeptidase 24.11)

ERIC M. SMITH*†, THOMAS K. HUGHES, JR.†, FARHAD HASHEMI†, AND GEORGE B. STEFANO‡

Departments of *Psychiatry and †Microbiology, University of Texas Medical Branch, Galveston, TX 77550; and ‡Multidisciplinary Center for the Study of Aging, State University of New York, Old Westbury, NY 11568

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ABSTRACT **The activation of human granulocytes and invertebrate immunocytes was found to be suppressed by corticotropin (ACTH) and melanotropin (MSH). In spontaneously active granulocytes both neuropeptides caused significant conformational changes indicative of inactivity plus a reduction in their locomotion. Significant inactivation of human granulocytes by ACTH required 2 hr, that by MSH only 20 min. The addition to the incubation medium of phosphoramidon, a specific inhibitor of neutral endopeptidase 24.11, blocked inactivation of granulocytes by ACTH. Radioimmunoassay for MSH of supernatant fluids from granulocytes incubated with ACTH demonstrated a time-dependent increase in MSH. These data strongly indicate that the effect of ACTH is largely due to its conversion to MSH by granulocyte-associated neutral endopeptidase. Parallel experiments with immunocytes from the mollusc *Mytilus edulis* gave similar results, indicating the universality of this phenomenon. Our finding that the human immunodeficiency virus, among several viruses, induces ACTH and MSH production in H9 T-lymphoma cells suggests an important role of these neuropeptides in the immunosuppression characteristic of such infections.**

Broadly based comparative studies demonstrate that neuropeptides play important roles in immunoregulatory processes (1, 2). Not only do they convey neural directives to the immune system but also they function as autoregulatory factors within the immune system (1–3). Many neuropeptides tested thus far have shown stimulatory effects on granulocyte (4) and invertebrate immunocyte (5) activity as determined by conformational and locomotory responses. Two neuropeptides, corticotropin (ACTH) and melanotropin (MSH), however, demonstrate inhibitory effects (6).

MSH is a proteolytic cleavage product of proopiomelanocortin (POMC), the polyprotein precursor for ACTH, and corresponds to ACTH-(1–13). Although this precursor–product relationship of ACTH and MSH is well documented in the brain and neuroendocrine system, it has only been suggested in regard to the immune system (4). Some previous reports are consistent with the concept that some of the effects of ACTH on the immune system are due to its conversion to MSH. For example, the inhibitory effect of MSH on polymorphonuclear cell migration and superoxide dismutatase induction is much more potent than that of ACTH (4). ACTH can directly alter immune responses, including the *in vitro* production of antibody (7, 8) and induction of interferon γ (IFN-γ) (9), inhibition of macrophage activation (10), induction of tumor necrosis factor (11), and modulation of invertebrate hemocyte phagocytotic activity (12).

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Conversion of ACTH to MSH is suggested by the fact that pharmacological doses and relatively long periods of time are required to obtain the effects reported in the present study. Furthermore, proteolytic enzymatic activities, such as neutral endopeptidase 24.11 (NEP; EC 3.4.24.11), also known as the lymphoid surface antigen CD10 (13), are present in high quantities in granulocytic cells of human and invertebrate origins (14). Also, proteolytic processing of ACTH has been reported in leukocytes stimulated by bacterial lipopolysaccharide (15). Thus, there is a precedent to suggest that ACTH could be processed to MSH within the lymphoid system.

The present study examines the mechanism of the inhibitory effects of ACTH and MSH on granulocyte conformation and locomotory responses. We present data that both substances contribute to the inhibitory effects, but MSH is the more potent and faster acting of the two. It also provides evidence that ACTH is processed to MSH in this system by a granulocyte-associated cell surface enzyme, NEP. The participation of these hormones in the immunosuppression seen in certain viral diseases is suggested by our finding that the human immunodeficiency virus (HIV) induces highly significant levels of ACTH and MSH in a cultured T-cell line.

MATERIALS AND METHODS

Cells. Human blood was obtained from volunteer donors at the Dana–Farber Cancer Institute for cellular image analysis or the University of Texas Medical Branch Blood Bank for granulocyte preparation. Granulocyte pellets were prepared by Ficoll–Hypaque density gradient centrifugation and dextran sedimentation (16) and utilized within 6 hr of collection as noted elsewhere in detail (2, 5).

H9 cells and HIV-1 (strain SK-1) were gifts from Miles Cloyd (University of Texas Medical Branch, Galveston) and cultured by standard methods as previously described (17). H9 cells (3.5×10^5) were inoculated with HIV at an estimated multiplicity of infection of 1. The cultures were incubated at 37°C for the indicated times. Supernatant fluids were saved for radioimmunoassay and the cells were saved for indirect immunofluorescent staining as previously described for ACTH and p24 antigens (18). For comparative studies with immunocytes of the mollusc *Mytilus edulis* (mussel) subtidal animals were collected from the shore of Wading River, Long Island Sound, New York.

Cellular Inactivation Assay. The examination of the effect of ACTH and MSH on cellular preparations was carried out as previously described (5, 14). Briefly, blood or hemolymph was incubated on albumin-coated slides with the agents and

Abbreviations: ACTH, corticotropin (adrenocorticotropic hormone); MSH, melanotropin (melanocyte-stimulating hormone); POMC, proopiomelanocortin; IFN-γ, interferon γ; NEP, neutral endopeptidase 24.11; HIV, human immunodeficiency virus.

the angiotensin-converting enzyme inhibitor captopril (19) or the NEP inhibitor phosphoramidon (13) at 37°C for human and at room temperature for the invertebrate cells for the specified incubation times.

Microscopy. The cell preparations were examined by use of phase-contrast and Nomarski optics, coupled with a Zeiss Axiophot microscope. Measurements were taken by utilizing the Zeiss Videoplan/Vidas and American Innovision image analysis systems as previously described (20). Before the various cells were recorded, specific images were converted to binary images after "frame grabbing." Simultaneously, specific cells were photographed with a time-lapse video synchronization system (JVC). Changes in cellular conformation based on measurements of cellular area and perimeter were mathematically expressed by use of the form-factor-pe (FF) calculation of the Zeiss Vidas analysis system, whereby the formula $(4 \times \pi \times \text{area})/\text{perimeter}^2$ provides mean numerical values. The lower this number, the larger is the cellular perimeter and the more ameboid the cellular morphology. The numbers of activated cells were obtained by counting cells automatically that exhibited areas greater than 145 μm^2, as contrasted with inactive cells whose areas were less than 115 μm^2. Mean values were derived from 15 individual readings taken from different cells. The individual points on the graphs represent the means of six to eight mean values. The final mean values did not vary by more than 5%.

Radioimmunoassays. ACTH and MSH were both quantitated by use of commercial radioimmunoassay (RIA) kits (Incstar, Stillwater, MN) (21). Sensitivity ranges for these assays were 15–500 pg/ml for ACTH and 20–600 pg/ml for MSH. Cross-reactivity was less than 0.1% according to the kits' specifications and as measured with peptide standards. Assay procedures were followed according to the instructions. Briefly, supernatant fluids from the granulocyte cultures were incubated with the primary antiserum to the peptides, nonspecific material was washed out, ^{125}I-labeled peptides were added, and the bound radioactivity was quantitated. Nonspecific binding was subtracted, and the percent of bound radioactivity compared with the zero standard was extrapolated from an empirically derived standard curve.

Reagents. Phosphoramidon (13), MSH (α form), and porcine ACTH-(1–39) were purchased from Sigma and captopril (19) was a gift from Margaret A. Shipp (Dana-Farber Cancer Institute, Boston). Antisera to ACTH-(1–24) was purchased from ICN and the monoclonal antibody preparation to p24 (M26) was a gift from Miles Cloyd.

RESULTS

Previous studies with human polymorphonuclear cells (4) and invertebrate immunocytes (6) have demonstrated that MSH can modulate immune responses. In the present study, we determined whether ACTH, like MSH, inhibits the activation of human granulocytes (Fig. 1) and *Mytilus* immunocytes (Fig. 2). Activation is expressed by a flattening of the cell and an increase in cell perimeter, resulting in a decrease of the form factor value to approximately 0.4 (22).

In control preparations, inactive granulocytes and *Mytilus* immunocytes are rounded and measure approximately 60–121 μm^2. In a viewing field of approximately 300 cells, 6–8% of the cells commonly become spontaneously active in 1 hr (6), and this increases to approximately 14% after 5 hr of culture (Figs. 1*A* and 2*A*). By virtue of the video analysis system, individual, activated cells were monitored, and the results are expressed as a percent of the activated cell population (with 100% representing the total number of activated cells at the start of the incubation period).

The addition of ACTH or MSH (0.1 μM) to the incubation medium caused conformational changes in the majority of the granulocytes that were spontaneously active (Fig. 1 *A* and *B*)

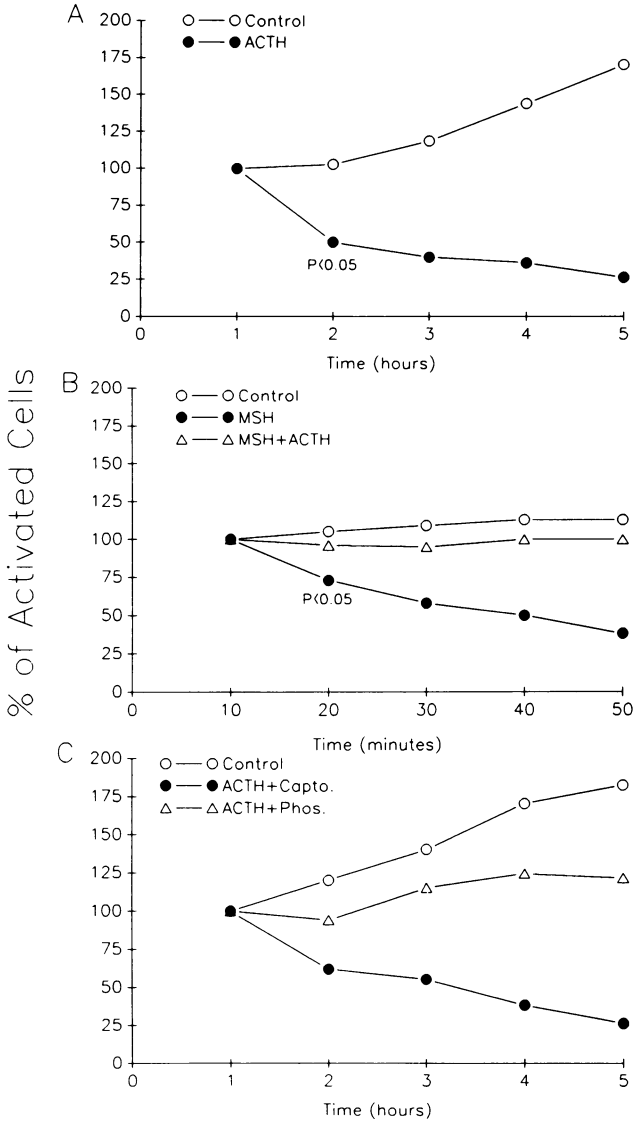

FIG. 1. Inactivation of human granulocytes by ACTH and MSH. Enriched granulocytes were incubated with 0.1 μM ACTH (*A*), 0.1 μM MSH with or without ACTH (*B*), or with ACTH with 100 μM captopril or 100 μM phosphoramidon (*C*). Activation was measured by video analysis and expressed as percent of activated cells in the control at the start of the incubation. $P < 0.05$ is in comparison with the control and is true for later points on the same curve.

by the end of the observation periods. The conformational changes that these cells underwent while becoming inactive included withdrawal of pseudopodia and rounding. A comparison of the effects of ACTH and MSH at peak response times (Fig. 1 *A* and *B*) revealed form factors of 0.80–0.91 for every cell in the culture, indicating that all cells are round. ACTH significantly inhibited the activation of human granulocytes within 2 hr (Fig. 1*A*), whereas MSH appears to exert its effect within 20 min (Fig. 1*B*). It should be noted that as the observation period is extended there is an increase in the number of spontaneously active cells. An interesting comparative observation is that *Mytilus* immunocytes responded more slowly to ACTH and MSH suppressive action than human granulocytes (Fig. 2 *A* and *B*).

The slower onset of inactivation with ACTH as compared to MSH suggested that the peptide was converted to MSH. In both human granulocyte and *Mytilus* immunocyte membranes, NEP has been shown to be present (14). Therefore, tests were performed with the NEP inhibitor phosphorami-

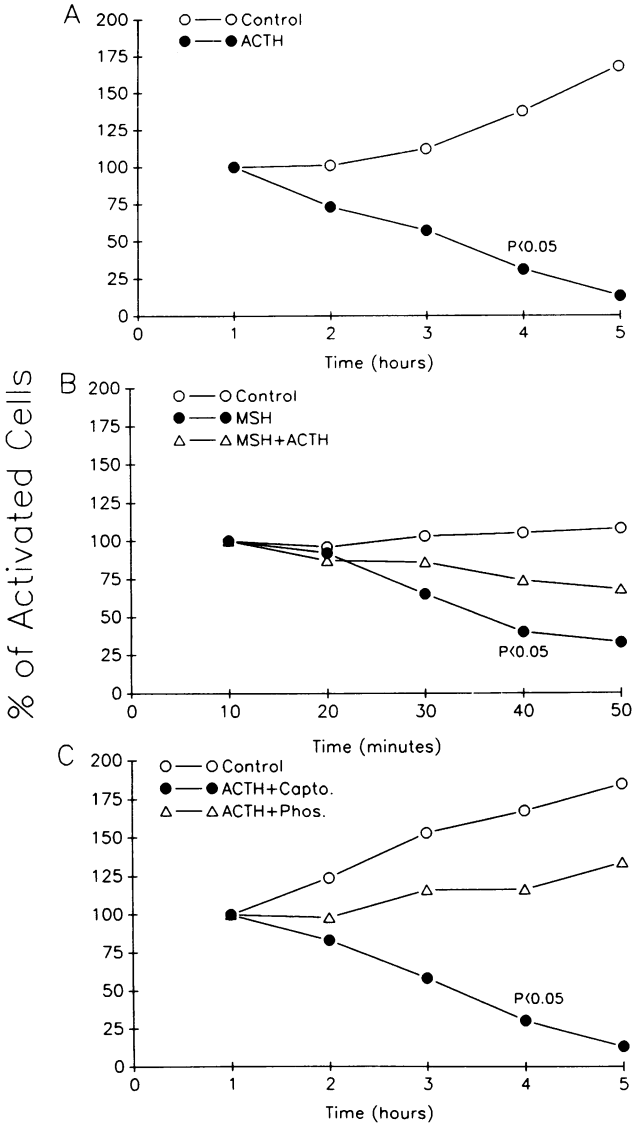

FIG. 2. Inactivation of *Mytilus edulis* immunocytes by ACTH (*A*), MSH with or without ACTH (*B*), or ACTH with captopril or phosphoramidon (*C*). Same concentrations as in Fig. 1.

don at its previously determined effective dose (6). Phosphoramidon prevented the ACTH inactivation of the spontaneously active state for both cell types (Figs. 1*C* and 2*C*). Captopril, an inhibitor of another endopeptidase, angiotensin-converting enzyme, was without effect. This result strongly suggests that MSH is an important signal molecule in causing the previously described cellular immunosuppression. Moreover, this finding is further supported by the experiment noting that ACTH can antagonize cellular immunosuppression caused by MSH (Figs. 1*B* and 2*B*).

Inhibition of proteolytic cleavage and inactivation of immunocytes by ACTH further supported the hypothesis of ACTH conversion to MSH. To address this, relative ACTH and MSH levels were measured in human granulocytes treated with ACTH. With some variability in the absolute quantity, we consistently found a time-dependent decrease in ACTH and an increase in immunoreactive MSH over the phosphoramidon-treated cultures (Fig. 3). No cross-reactivity could be detected for the peptides in either radioimmunoassay to explain this rise in MSH. Phosphoramidon treatment reduced the ACTH drop and delayed the rise in MSH. It is interesting to note the similarity in kinetics for the

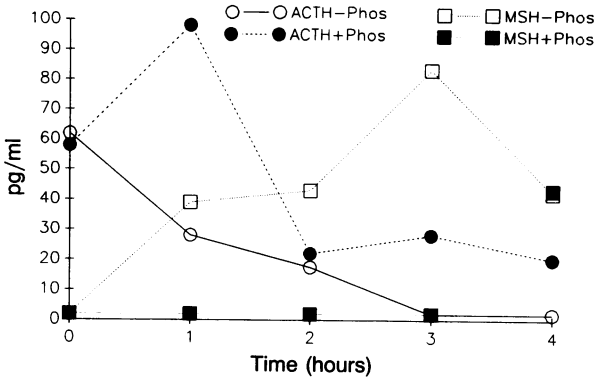

FIG. 3. Conversion of ACTH to immunoreactive MSH by human granulocytes. Enriched granulocytes (1×10^7 per ml) were incubated for the indicated times with ACTH at 0.5–1 ng/ml in the presence or absence of phosphoramidon (100 μM). Culture supernatant fluids were radioimmunoassayed for residual ACTH and newly generated MSH. ACTH or MSH + and − Phos refers to the neuropeptide measured in the supernatant fluid from cultures either treated with phosphoramidon or untreated. The data are representative of five experiments.

degradation of ACTH and the significant rise in MSH to that of the cellular inactivation (Figs. 1 and 2).

Since MSH is degraded by NEP (23), we hypothesized that MSH in our system would be degraded also, and that phosphoramidon treatment should result in greater inhibition of granulocyte activation by MSH. Fig. 4*A* shows greater inhibition of granulocyte activation in cultures treated with MSH and phosphoramidon versus MSH alone. A similar effect on *Mytilus* immunocytes is seen in Fig. 4*B*.

It has been shown that viruses will stimulate leukocytes to synthesize POMC (1, 18, 24, 25). In addition, we have preliminary evidence suggesting that HIV induces lymphocytes to produce ACTH (26). The infection and suppression of the immune system, plus the fact that functioning of the

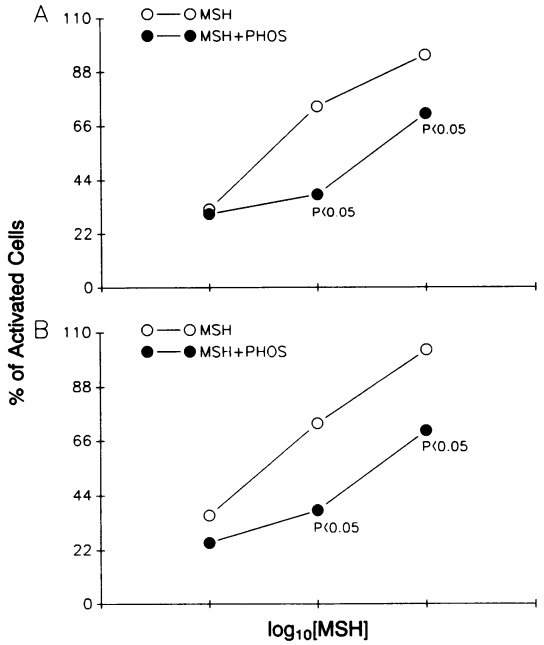

FIG. 4. Enhancement of cellular inactivation by MSH in the presence of phosphoramidon. Human granulocytes (*A*) and *Mytilus* immunocytes (*B*) were treated with the indicated doses of MSH plus or minus 100 μM phosphoramidon, and activation was measured by video analysis.

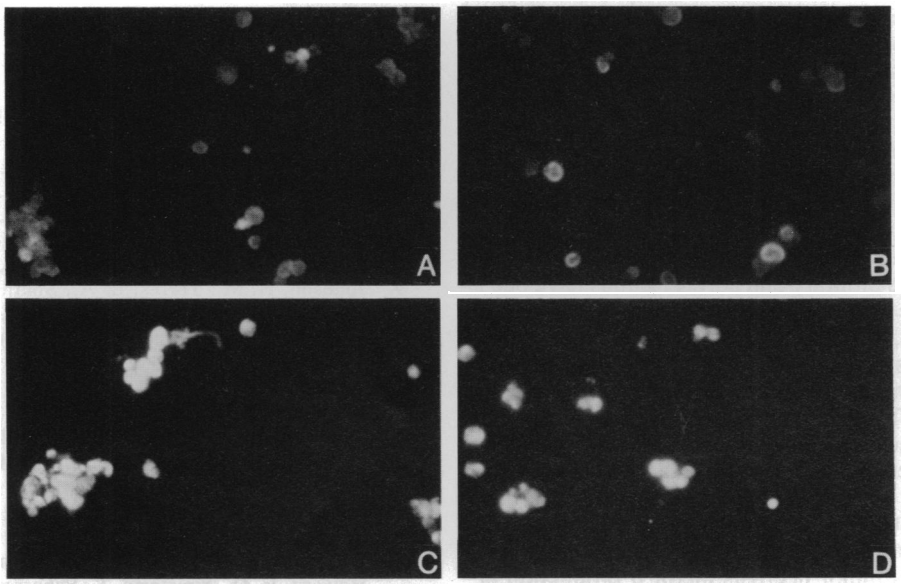

FIG. 5. Expression of ACTH and HIV nucleocapsid (p24) antigens in HIV-infected H9 cells. Infected cells were stained by immunofluorescence with normal rabbit serum at 45 days after infection (*A*), with anti-ACTH at 25 days after infection (*B*), with anti-ACTH at 45 days after infection (*C*), or with anti-p24 at 45 days after infection (*D*).

pituitary and adrenal glands can be altered in AIDS, led us to examine this virus for induction of ACTH and MSH.

Fig. 5 shows that HIV infection of H9 T-lymphoma cells induced the production of intracellular ACTH. Noninfected H9 cells or normal rabbit serum-stained controls exhibited only background staining for ACTH or the HIV p24 antigen. To determine if ACTH-like peptides are secreted and processed to MSH, radioimmunoassays were performed on supernatant fluids of cultures. Table 1 shows both ACTH and MSH to be present. Significant amounts of MSH were found, and on a molar basis this suggests that there are approximately 2 to 3 times more MSH than ACTH molecules.

DISCUSSION

Previous reports have shown that the presence of ACTH and MSH, individually, can modulate immune responses. The data presented here provide compelling evidence that the cellular immunosuppression attributed to ACTH may actually be a composite of ACTH and MSH actions. Specifically, we have shown that MSH acts rapidly, within minutes of application to the cells. Conversely, ACTH requires hours, which is sufficient time for its processing into MSH. ACTH also blocks MSH activity, but only when added concomitantly, presumably by competing with MSH for binding to its receptor. Since there appears to be some tolerance of the MSH receptor for ACTH (27), the data are consistent with the view of competition.

Alternatively, ACTH receptors could be present on the cells in addition to MSH binding sites. However, they would have to be slower acting and have lower affinity than the MSH binding sites and somehow inhibit intracellular action of MSH. Thus, processing of ACTH to MSH is supported by kinetics and similarity of action, detection of MSH subsequent to addition of ACTH, plus inhibition of the proteolytic mechanism thought to generate MSH.

Table 1. ACTH and MSH production by HIV-infected H9 cells

Time, days	Hormone conc., pg/ml	
	ACTH	MSH
0	ND	ND
15	32.0 ± 0.1	40.0 ± 4.2
22	47.5 ± 12.0	ND
35	29.0 ± 5.6	42.0 ± 2.8
58	53.5 ± 9.2	38.5 ± 2.1

Supernatant fluids from HIV-infected cultures were collected at the indicated times after infection and radioimmunoassayed for ACTH or MSH. Results are mean ± SD. ND, none detected.

Since ACTH does bind with high affinity to lymphocytes (28, 29) and activates their intracellular pathways (30, 31), it undoubtedly has its own direct immunomodulatory effects. The effects of ACTH described here probably are a consolidation of ACTH and MSH activities, dependent upon the cell types and numbers, kinetics, and the presence of multiple other regulatory factors such as lymphokines and corticosteroid hormones.

The results suggest that at least part if not all of the proteolytic processing of ACTH to MSH is phosphoramidon sensitive. This strongly implicates NEP (13, 14) as one of the proteases involved. This would be a mode of operation for NEP not previously described and one different from the classical trypsin-like cleavage generally thought to process POMC (32). These results do not eliminate the possibility of other proteolytic enzymes being involved. A carboxypeptidase or an endopeptidase plus several newly cloned convertases have been shown to generate an ACTH-(1–16) fragment and one or all of these enzymes may be phosphoramidon sensitive (32). Harbour *et al.* (15) reported an acid-dependent proteolytic activity associated with B lymphocytes that is induced by bacterial endotoxin. This enzyme truncates ACTH-(1–39) to a species approximately 24 residues in length, a result which indicates that it will be important to determine the relationships of these enzymes in immunomodulation. NEP cleavage occurs at the amino side of hydrophobic residues (13). A candidate site on ACTH for cleavage by NEP could be residues 12-13 (Pro-Val), generating ACTH-(1–12). Such a fragment should retain MSH-like immunoreactivity and bioactivity. In addition, blockage of MSH degradation by phosphoramidon (Fig. 3) is consistent with NEP activity (23). Degradation of MSH by NEP could represent another immunoregulatory mechanism.

Shipp *et al.* (14) found a regulation of enkephalin signals in lymphoid cells by coexpression of cell surface opioid receptors and the CD10/NEP enzyme. Considered in this context,

our results showing induction of ACTH by HIV in a T-cell line illustrate the broad implications of this ACTH → MSH conversion mechanism. Its universality is suggested, since additional viruses have been shown to induce ACTH formation in lymphocytes. Our HIV results do contrast with those of Oates *et al.* (33), but their failure to find induction of POMC mRNA after infection may be due to differences in the length of infection time.

We are not certain what roles may be played by ACTH and MSH in AIDS or other viral infections. However, alterations in the functions of the pituitary and adrenal glands have been reported and may be a common feature of virus infections in general (24, 25, 33, 34). ACTH from lymphocytes may contribute to these systemic effects (24) as undoubtedly do other cytokines such as interleukin 1 (21, 35). Presumably, the induction of ACTH and MSH formation is adaptive for either the virus or the host. MSH or other related fragments might be active in enhancing or inhibiting HIV replication directly. The ability to inactivate granulocytes means that neuropeptides debilitate host defense mechanisms, particularly those that might protect against opportunistic infection. Also, ACTH induces tumor necrosis factor α production *in vitro* (11), which if it occurred *in vivo* might contribute to the wasting seen in AIDS.

The overall means by which HIV compromises the host's immune system is not known. There are many incongruous features of HIV infection, such as that the number of infected cells is too low to account for the magnitude of immunodeficiency (36, 37). The overall effect is probably due to multiple factors ranging from manipulation of normal immune functions to cytotoxic and interfering activities of viral proteins (see ref. 38 for review).

When considered with our findings, it becomes apparent that the immunosuppression can result from many causes. MSH has been shown to have multiple immunomodulatory effects such as antipyresis, inhibition of polymorphonuclear cell mobilization, and inhibition of cytokine production (4, 39). ACTH inhibits production of IFN-γ (7) and activation of macrophages by IFN-γ (10). ACTH is induced by a number of stimuli, either at the pituitary gland or lymphoid sites, ranging from "stress" and circadian rhythms to pathogenic stimuli (viruses, bacterial lipopolysaccharide, tumor cells) (1). Since suppressed immune responses are associated with many of these conditions, ACTH's processing into MSH, which has a stronger and faster effect, may be a fundamental mechanism for debilitating host defenses. Additionally, since the conversion of ACTH to MSH may require NEP found on the surface of only certain lymphoid cells, the immunosuppressive phenomenon may initially reside at the local level and then be followed by a broader suppression of other cells in the vicinity of the MSH.

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