

SHORT ANALYTICAL REVIEW

Methionine Enkephalin: A New Cytokine—Human Studies

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The effects of methionine enkephalin (met-enkephalin) on human immune function are reviewed. This pentapeptide functions to upregulate, or enhance, immune function in the majority of donor samples at low doses and suppresses at high doses. The influence of this molecule is shared by the central nervous, neuroendocrine, and immune systems. Cells from each of these systems possess receptors for met-enkephalin and have the ability to process met-enkephalin from its prohormone, proenkephalin A. Studies have shown that this molecule is capable of enhancing immune function in patients with cancer or AIDS. It is proposed that this molecule be classified as a cytokine. © 1997

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INTRODUCTION

Recent studies have shown that enkephalins and endorphins are produced by cells of the immune system during periods of antigen and/or mitogen activation of the lymphocytes—the major specific reactive cells of the immune system (1). Various peptide fragments derived from the prohormones, proopiomelanocortin, and proenkephalin A have been studied and described (2), and substantial literature documents the immunomodulatory influences of these fragments, particularly β -endorphin, methionine enkephalin, and leucine enkephalin (3). Each of these peptides has been reported to induce immunostimulation at low doses and immunosuppression at high doses in the majority of donor samples (4). Similar biphasic responses have been reported for a variety of cytokines such as interleukin 2 and several interferons (5). This review is designed to focus on the immunological influences of methionine enkephalin (met-enkephalin) on human cells and to

highlight clinical analysis of influence of met-enkephalin in volunteers and patients suffering from cancer or AIDS.

Major differences have been appreciated between the enkephalin and endorphin fragments because they preferentially bind to μ (β -endorphin), δ (methionine enkephalin), epsilon (β -endorphin), or κ receptors (dynorphin) (2). Mu and kappa ligands act primarily to downregulate the immune system while delta and epsilon ligands act to upregulate the immune system (6, 7). Considerable data suggest that subreceptors also exist. This is the case with respect to the delta receptors in the immune system but not in the central nervous system (8–10). Therefore, this review will focus on the influence of methionine enkephalin on the δ receptors in human cells, both *in vitro* and *in vivo*, and thus to avoid known problems attributable to species and strain differences, which have been documented by Fischer (11).

Methionine enkephalin binds with high affinity for the δ receptors but low affinity to the μ receptor, while β -endorphin has high affinity for the μ and epsilon receptors and low affinity for the δ receptor. These differences in receptor affinities may account for differences in immunological influences reported for methionine enkephalin and β -endorphin (3, 4, 11–13). Indeed, Mazumder *et al.* (14) have recently reported δ opioid receptor-selective peptides to have marked immunostimulant activities in both normal persons and patients suffering from leprosy and tuberculosis while μ receptor-selective peptides appear to act immunodepressive in the same subjects.

ORIGIN OF ENKEPHALINS

The enkephalins were originally described as the endogenous ligands for morphine receptors in the brain (15). Plotnikoff *et al.* showed that the enkephalins exhibit antidepressant, antianxiety, and anticonvulsant

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activities (16). Enkephalins are normally produced in adrenal glands as well as in the hypothalamus (17). In a normal healthy state, enkephalins are released in a 6:1 ratio of methionine enkephalin to leucine enkephalin, into the adrenal venous plasma (18). In 1979, Wybran *et al.* (19) reported that normal human T lymphocytes possess receptors for methionine enkephalin, and subsequently the presence of opiate receptors on human phagocytic leukocytes was reported (20). Recent studies have shown that human peripheral blood lymphocytes contain proenkephalin A messenger RNA and release endorphins and proenkephalin A-derived peptides including methionine enkephalin (21, 22). Similar findings have been reported in rats and mice (23–25). Studies with human peripheral blood lymphocytes suggest that phytohemagglutinin mitogen stimulation of lymphocytes provokes release of methionine enkephalin peptides (1, 2, 22, 26–30).

ENKEPHALIN RECEPTORS ON HUMAN IMMUNE CELLS

Wybran *et al.* (19), in a historic discovery, reported that human T cells possess binding sites for methionine enkephalin and morphine and that these molecules can be displaceable by the opiate antagonist naloxone. This initial discovery was followed by a series of studies by Wybran *et al.* showing that both opioid (naloxone-reversible) and nonopioid (naloxone-nonreversible) binding sites for neuropeptides are present on lymphocytes (31–34). Miller *et al.* confirmed the early reports that methionine enkephalin increased sheep red blood cell rosetting of lymphocytes in normal subjects and also in some patients with lymphoma (35–37). Direct binding of naloxone to human lymphocytes and platelets has been reported by Mehrishi and Mills (38). Additionally, leucine enkephalin has been shown to bind to cultured T lymphocytes of a human lymphoid T cell line (Jurkat) (39). Wybran and Schandene (40) demonstrated that methionine enkephalin increased expression of (CD 2) OKT10, Leu 11 (CD 16), and TAC surface antigens (CD 25) on leukocytes. This increase was also shown to be reversed by treatment with naloxone. Finally, human granulocytes and monocytes have been shown to express high-affinity binding to dihydromorphine and naloxone but not to 3H-D-Ala-D-Leu-enkephalin (20, 41).

Of significance are reports of Hucklebridge *et al.* (42) that methionine enkephalin stimulates *in vitro* proliferation, in the absence of other mitogens, of human peripheral blood lymphocytes after an incubation period of 4 to 5 days. This stimulation appears to occur via δ opioid receptors. This stimulation of lymphocytes by met-enkephalin is antagonized by high but not by low concentrations of naloxone, indicating that the μ

receptors from which agonists are displaced by very low doses of naloxone are not involved (8, 42).

Methionine enkephalin, β -endorphin, and ACTH have been found in homogenates of bone marrow cells of leukemic children (43). Proenkephalin A activity has been found in human chronic lymphoblastic leukemia cells (44). The enkephalins have been shown to have high affinity for the δ receptor (2). In light of this it is surprising that methionine and leucine enkephalin have been reported to have major differences in their effects on immune cell activity. Miller *et al.* (37) reported that methionine enkephalin increases rosette formation of T cells in lymphoma patients over a wide dilution range (10^{-2} to 10^{-14} M) whereas leucine enkephalin enhanced only at one dilution (10^{-14} M). Similarly Roscetti *et al.* (45) reported that methionine enkephalin had a biphasic influence on blastogenesis induced by Candida antigen, causing stimulation in eight donors (101.1% over controls) and suppression in five donors (20.7% below controls) whereas the effect of leucine enkephalin was "ambiguous." Furthermore, Mendelsohn *et al.* (46) found no specific binding for [3 H]Ala²-D-Leu⁵-enkephalin (a δ ligand) on human mononuclear cells. These reports suggest that δ subreceptors (heterogeneity in the receptors) for the enkephalins are probably present on immune cells. We propose that the δ -1 receptor possess high affinity for methionine enkephalin while the δ -2 receptor has high affinity for leucine enkephalin (47).

ENKEPHALIN EFFECTS ON RELEASE OF CYTOKINES

Methionine enkephalin causes increases in interleukin 2 production when human peripheral blood mononuclear cells are incubated with PHA and methionine enkephalin at concentrations between 10^{-9} and 10^{-6} M (40). Similarly, interferon gamma production in concanavalin A-stimulated human mononuclear cells was increased by both β -endorphin and methionine enkephalin, at concentrations between 10^{-14} and 10^{-10} M (48). Naloxone did not prevent this effect, indicating that this influence represents a nonopioid effect (48).

ENKEPHALIN INFLUENCES MITOGEN-INDUCED PROLIFERATION OF LYMPHOID CELLS

Most *in vitro* studies have shown a biphasic effect of the opioids on phytomitogen stimulation of immune cells. Low doses of opioids tend to enhance proliferative responses to phytomitogens while high doses tend to act as suppressors of these same responses. In our own analyses we observed differences in phytomitogen responses to opioids that appeared to be a function of mitogen concentration, low vs moderate vs high mitogen levels. Perhaps high mitogen levels result in a ceil-

ing effect that exerts a negative feedback influence, resulting in inhibition of T cell proliferation (bimodal effects—bell shaped curve)? It is often difficult to compare findings between laboratories because many reported studies used only a single concentration of phyto mitogen and these concentrations often vary among laboratories. Threshold changes may be more physiological and therefore the use of suboptimal mitogen concentrations may be the most informative way of looking at this relationship. Another problem with comparing studies from different laboratories is that the reported culture time of immune cells in the presence of mitogen has ranged from 72 hr to 7 days. Recognizing all of these difficulties, we still think it worthwhile to review the reported influence of opioids on mitogen stimulation of immune cells while recognizing that inconsistencies may exist.

In vitro studies. Wybran (34) originally reported that only leucine enkephalin at a concentration of 10^{-5} M increased PHA-induced lymphoid cell blastogenesis if PHA had been used at a suboptimal concentration. Roscetti *et al.* (45) studied proliferation of human peripheral blood mononuclear cells induced by *Candida* antigen in 7-day cultures. Methionine enkephalin stimulated proliferation in eight donors (101.7% over controls) while proliferation was inhibited in five donors (20.7% below controls). Sizemore *et al.* (49) studied the effects of leucine enkephalin and its metabolic fragments on the regulatory activity of immune cells induced by concanavalin A. Leucine enkephalin and two of its metabolic fragments were found to increase the numbers of T helper and T suppressor cells in 48-hr cultures in a bimodal response. Hucklebridge *et al.* (42) reported that methionine enkephalin enhances stimulation of *in vitro* proliferation of human peripheral blood lymphocytes when cultured in the presence of suboptimal concentrations of Con A for a period of 4 to 5 days. This enhancement appears to occur as consequences of stimulation of the cells via δ opioid receptors. Similar results were obtained by Kharkevich and Kadagidze (50) with PHA and methionine enkephalin.

In contrast to the above-described preceding reports, Prete *et al.* (51) cultured lymphocytes for 1 to 72 hr in the presence of optimum levels of PHA plus interleukin 2. They found that neither β -endorphin, dynorphin, nor methionine enkephalin had any effect on the PHA-induced blastogenesis of these cells. Zbrog *et al.* reported that methionine enkephalin exerted no influence on PHA-induced blastogenesis of immune cells from normal subjects but depressed the response in cells from uremic patients (52). Antibody synthesis to tetanus toxoid was inhibited by methionine enkephalin at low concentrations and increased by high levels of methionine enkephalin (53) (bimodal response).

In vivo studies. Intravenous treatment of normal volunteers and cancer and AIDS patients with methionine enkephalin (20 to 100 μ g/kg) increased blastogenesis of lymphocytes exposed to suboptimal and optimal concentrations of PHA, Con A, and pokeweed mitogen in 70 to 80% of the subjects tested (54, 55). The *in vivo* physiological concentrations of methionine enkephalin achieved which influenced the responses of immune cells normally ranged from 10^{-10} to 10^{-12} M. Biphasic responses of methionine enkephalin to phyto mitogens *in vitro* have been observed over wide concentration ranges. Methionine enkephalin is found in human blood in a picogram (10^{-12} M) range (56). Therefore, differences seen between *in vitro* and *in vivo* may be a function of the concentrations of methionine enkephalin employed, as well as influences of optimal vs suboptimal mitogen concentrations, and duration of incubation of the cells with the enkephalins and/or phyto mitogens.

ENKEPHALIN EFFECTS ON NATURAL KILLER (NK) CELLS

Faith *et al.* reported that methionine enkephalin and leucine enkephalin (10^{-6} to 10^{12} M) increased NK cell activity in peripheral blood leukocytes from normal volunteers and cancer patients (57, 58) at effector to target cell ratios of 100:1, 33:1, and 11:1 (57, 58). Maximal increases were particularly observed in those individuals who had low baseline levels of NK cell activity. Mathews *et al.* (59) found that methionine enkephalin (10^{-6} to 10^{-12} M) increased NK cell activity in healthy subjects when the NK activity was evaluated at an effector to target cell ratio of 50:1. Wybran reported similar findings at an effector to target cell ratio of 80:1, employing methionine enkephalin concentrations ranging from 10^{-5} to 10^{-8} M (34). Oleson and Johnson observed that methionine enkephalin increased NK cell activity of individuals with low baseline activities while NK cell activity was decreased in cells from individuals with high baseline activities (60). Puente *et al.* (61) reported that cells from 7 of 15 volunteers had at least a 20% increase in NK cell activity following treatment with methionine enkephalin (10^{-6} to 10^{-12} M). Oleson *et al.* (62) observed increases in NK cell activity in cells from AIDS patients when the latter were treated *in vitro* with leucine enkephalin, at concentrations ranging from 10^{-8} to 10^{-10} M.

In contrast to these findings, Prete *et al.* (51) reported that methionine enkephalin diminished NK cell activity at target to effector cell ratios of 200:1 when the cells were incubated for 6 to 72 hr in the presence of IL2. Kastin *et al.* (63) could not produce significant effects of met-enkephalin on NK cell activity when cells from healthy subjects ranging in age from 23 to 79 were treated with methionine enkephalin. The great

majority of *in vitro* studies supports the view that methionine enkephalin does indeed stimulate an increase in NK cell activity. The two negative reports mentioned may result from methodological differences or differences in the subjects analyzed; for example, target to effector ratios as well as incubation times.

ENKEPHALIN EFFECTS ON GRANULOCYTES AND MONOCYTES

Foris *et al.* (64) reported that methionine enkephalin in low concentrations (10^{-7} to 10^{-9} M) stimulates the antibody-dependent cellular cytotoxicity (ADCC) activity of polymorphonuclear (PMN) leukocytes following a 60-min preincubation. Higher concentrations of methionine enkephalin (10^{-6} to 10^{-5} M) suppressed ADCC activity via naloxone-sensitive opioid receptors. Nagy *et al.* found that methionine enkephalin increased reactive oxygen species produced by peripheral mononuclear leukocytes from patients with type-2 diabetes mellitus following a 60-min incubation of the cells with the enkephalin (65). In a subsequent study methionine enkephalin was shown to increase the intracellular killing capability of human PMNs (as did f-Met-Leu-Phe-FMLP following a 30-min incubation) (66). The authors proposed that the mechanism of this enhancement includes lipoxygenation and increased leukotriene B₄ synthesis. Marotti *et al.* (67) observed a biphasic effect of methionine enkephalin on the production of superoxide by human PMNs. When these cells were incubated with methionine enkephalin for periods of 10 to 30 min the production of superoxide was enhanced in cells from individuals with low baselines while it was suppressed in cells from individuals with high baselines. However, Simpkins *et al.* (68) reported that leucine enkephalin and methionine enkephalin inhibit superoxide release by human phagocytic cells following 15 to 30 min incubation of the cells with the neuropeptide. Slaoui-Hasnaoui *et al.* (69) studied the effects of leucine enkephalin, β -endorphin, and dynorphin on the respiratory burst of human PMNs. They found that none of the tested opioid peptides affected resting oxidative metabolism in the PMNs. However, the opioid peptides suppressed a phorbol myristate acetate (PMA)-stimulated respiratory burst in a bell-shaped dose response of the PMNs. Conversely, if the opioid peptides were first exposed to activated oxygen species, they enhanced the PMA-stimulated respiratory burst in human PMNs in an inverted U-shaped dose response. Peck found that superoxide production was not increased in macrophages following 3 days of incubation with methionine enkephalin (70). Superoxide formation in human phagocytes induced by a maximally stimulatory concentration of PMA was unaffected by methionine enkephalin but was enhanced by

N-formyl-L-methionyl-L-phenylalanine (71). Fulop *et al.* (72) reported biphasic effects of methionine enkephalin on extracellular cytotoxicity and cAMP levels in human PMNs.

These studies taken together indicate that methionine enkephalin induces biphasic responses over a broad range of dose concentrations and that these effects may be influenced by incubation times and the age of the subject from which the cells are taken. The majority of reports indicates that methionine enkephalin increases PMN activities as exhibited by increased cytotoxicity and superoxide formation. Van Epps and Saland (73) originally reported that methionine enkephalin stimulated human mononuclear cell chemotaxis and that this stimulation was blocked by naloxone. It was shown by Fischer and Falke (74) that methionine enkephalin induces cell elongation of PMNs while the proteolytically stable enkephalin analogs D-met-Pro-enkephalinamide (DMPE) and D-al^a-D-Leu-enkephalin (DADLE) are inactive in this respect. This finding indicates that this reaction is mediated by delta-type subreceptors, since DMPE and DADLE bind to delta receptors in the brain but not in the immune system.

Methionine enkephalin stimulates the adherence of human neutrophils to serum protein-coated slides (75). This indicates that the enkephalins may influence inflammatory responses perhaps by altering the interaction between circulating cells and endothelin via an influence on adhesion molecules. Boogaerts *et al.* (76) showed that methionine enkephalin increases prostacyclin production and thus may dampen immune-triggered, granulocyte-induced endothelial damage. Perez-Castrillon *et al.* (77) speculate that the functional defect in chemotactic response of monocytes obtained from intravenous drug abusers may be due to a receptor effect (downregulation) or could be attributed to a nonspecific action on the monocytes. They showed a decrease in chemotactic function in monocytes from control subjects when these cells were treated with the selective μ and δ receptor agonists DAGO and DPDPE, suggesting that the response is receptor mediated and that the opioids play an important role in the depression of monocyte chemotaxis that may be observed in intravenous drug abusers. Methionine enkephalin induction of adherence, conformational changes, and locomotor activity in human granulocytic cells have been confirmed by the studies of Stefano *et al.* (78). It is interesting to note that enkephalin analogs may have different affinities for δ receptors, again suggesting the evidence of specific receptors with different influences on the function of immune cells.

IN VIVO CLINICAL STUDIES

Normal volunteers. The extensive *in vitro* analyses performed with cells obtained from human donors have

been replicated in large measure by several *in vivo* clinical studies carried out in normal volunteers. In a series of studies methionine enkephalin was administered to 14 normal volunteers by intravenous infusion as a single dose delivered over a 30-min period in dose ranges of 1 to 250 $\mu\text{g}/\text{kg}$ (54, 79–85). This treatment with methionine enkephalin was found to increase the number of cells in T cell subsets (CD 4, CD 8, and CD 2 cells), enhance proliferation induced by the mitogen PHA, ConA, and pokeweed mitogen, and increase numbers of T cell rosettes, total circulating lymphocytes, B lymphocytes, and NK cells. Subjects with low baseline NK cell activity exhibited increased NK cell activity following methionine enkephalin treatment while those with high baseline activities exhibited a slight decrease in NK cell function. No major signs of toxicity were observed, including no changes in cardiovascular or respiratory activity or alteration of body temperature.

AIDS patients. Clinical studies in AIDS patients were conducted at five centers (Tulsa, Brussels, Denver, New York, and Chicago) (6, 82–84, 86–98). The patients in these studies were HIV-infected persons who could be classified as pre-AIDS or patients suffering from ARC (AIDS-related complex). Treatment with methionine enkephalin ranged in dosage from 10 $\mu\text{g}/\text{kg}$ 3 \times /week up to 100 $\mu\text{g}/\text{kg}$ 3 \times /week in the first month of treatment. Succeeding months of treatment often included a schedule of treatment infusion of 100 $\mu\text{g}/\text{kg}$ of methionine enkephalin once a week. A dose response was apparent, which included minimal influence on T cell subsets observed in the Denver study at a fixed dose of 10 $\mu\text{g}/\text{kg}$ 3 \times /week for 12 weeks, to significant increases in T cells at 20 to 80 $\mu\text{g}/\text{kg}$ 3 \times /week for 1 to 6 months at Brussels and Tulsa. Increases in CD 3, CD 4, and CD 8 T cell subsets, NK cell activity, IL2 production, and blastogenic response to PHA stimulation were observed in 70 to 80% of the patients studied. Increases of T cell subsets were seen to be sustained for periods of 1 to 19 months of treatment, most patients being treated for 3 to 6 months. No significant toxicity was observed in any of these patients.

The most recent clinical study with methionine enkephalin was conducted by Bihari and colleagues (55) who studied the influence of two doses of methionine enkephalin (60 or 125 $\mu\text{g}/\text{kg}$) once a week in 46 ARC patients over a 12-week placebo controlled trial. No significant toxicity was observed in any of the patients. The high-dose group (125 $\mu\text{g}/\text{kg}/\text{week}$) exhibited a significant increase in IL2 receptors, CD56 (NK) cell numbers, blastogenic response to stimulation with PHA, pokeweed mitogen, or CMV, CD3, CD4, and CD8 positive T cell numbers, and a significant reduction in total lymph node size after 8 and/or 12 weeks of treatment.

The low-dose group (60 $\mu\text{g}/\text{kg}$) increased CD 4 and CD 56 cell numbers.

Cancer patients. Patients with Kaposi sarcoma (9 patients), lung cancer (12 patients), melanoma (3 patients), hypernephroma (1 patient), or pancreatic cancer (1 patient) were treated with methionine enkephalin for varying periods of time (1 week to 12 months) over a wide range of doses (10 $\mu\text{g}/\text{kg}$ 3 \times /week up to 80 $\mu\text{g}/\text{kg}$ 3 \times /week) (82–84, 86–92). Increases in T cell subsets were observed after 1 to 2 weeks of treatment. The T cell subsets influenced included CD 3, CD 4, CD8, and CD 2 positive cells. Increases in blastogenic responses to PHA, ConA, and pokeweed mitogen were observed. A major finding was an increase in interleukin 2 receptor expression. NK cell activity was measured in 14 of these patients and an increased NK activity was present in 12 of the 14 patients. No toxicity attributable to the molecule was observed in any of these patients (85, 86, 88, 90, 99).

A variety of normal and malignant human lymphopoietic and nonlymphopoietic cells express a membrane antigen with functional enkephalinase activity (100 and 101). This membrane-associated enkephalinase, which has also been called common acute lymphoblastic leukemia antigen, CD10, melalloendopeptidase, EC 3.4.24.11, and neutral endopeptidase, hydrolyses enkephalins and multiple other bioactive peptides. It is conceivable that one mechanism of downregulating the immunomodulatory effects of enkephalin is accelerated degradation of the peptide in a microenvironment containing normal or malignant cells expressing high levels of enkephalinase.

DISCUSSION AND CONCLUSIONS

As indicated in this review ample evidence exists that enkephalins may exert immunomodulatory influences. While biphasic responses have been demonstrated, it is clear that at physiologic concentrations (10^{-10} and 10^{-12} M) the enkephalins are capable generally of exerting immunoenhancing actions in the majority of subjects. Most reports indicate that the enkephalins have a high affinity for δ -like subreceptors on immune cells. However, it is also clear that these δ subreceptors differ from those found in the central nervous system because known δ ligands have different affinities for receptors in the brain and immune system. The receptor on immune cells may vary not only in kind and number among individuals but also as a function of species, strain, age, disease state, sex, and "set points" of activation. It is very difficult to compare results from different laboratories because of the great diversity of conditions employed in the reported studies (i.e., differences in the enkephalin doses, differences in exposure

times, differences in the subject populations studied, etc.). Certainly *in vivo* human studies indicate that activation states of immune cells can be enhanced by treatment with enkephalins (79–82, 85–90, 92, 96, 97, 102).

In vitro studies of the effects of methionine enkephalin on human immune cells have shown this peptide to cause increases in the blastogenic response to phyto-mitogens, increase NK cell activity, increase the activity of granulocytes, and increase the formation of T cell rosettes. Of great interest is the fact that treatment with methionine enkephalin has led to increased production of the cytokines IL2 and interferon gamma and by human T cells. *In vivo* studies of the effects of treatment with methionine enkephalin in normal volunteers and in patients with cancer, HIV infection (AIDS), or ARC have confirmed the results from the *in vitro* studies. *In vivo* methionine enkephalin treatment was shown to result in an increase in numbers of cells in various T cell subsets, enhanced blastogenesis of lymphocytes following mitogen stimulation, and increased NK cell activity. Finally, methionine enkephalin has been shown to be produced by human T cells (1) and macrophages from its prohormone proenkephalin A (2). We believe that methionine enkephalin may best be considered to be one of the cytokines of the immune system because it is produced by cells of the immune system and influences the numbers and functions of immunocompetent and accessory cells that are normally engaged in bodily defense.

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