NEUROHORMONES REGULATE T CELL FUNCTION

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An intricate link exists between the neuroendocrine and immune systems; however, the mechanisms responsible for this linkage are not understood. β-Endorphin (β-END)[1] and the related pentapeptides methionine-enkephalin (MET-ENK) and leucine-enkephalin (LEU-ENK) belong to the opioid family of neurohormones, which were originally identified in brain tissues for their analgesic effects but are now recognized to have activity in the periphery, as well as the central nervous system (CNS) (1–3). Endogenous secretion of these neuropeptides correlates with autonomous rhythms, physical activities, and emotional states (2, 4). Circulating levels of β-END are increased with certain kinds of stress, and although speculative, it is possible that the stress-induced release of this or other opioids is related to the ability of stress to alter lymphocyte function (2, 4).

Evidence from both in vivo pharmacological and in vitro biochemical studies suggests that opioids bind to opiate/opioid receptors and that such receptors exist in multiple forms (1, 5). Opioid binding sites have been best described for nervous tissues prepared from rodents or other animals and for cultured neuroblastoma or neuroblastoma-hybrid cells (1, 2, 5–7). Radiolabeled ligands and synthetic peptides with high affinity for specific types of receptors have been used to characterize opioid binding sites (1, 5). It is generally accepted that β-END, MET-ENK, or LEU-ENK bind to opiate/opioid receptors of the δ and μ types (1, 2, 5). Analgesic activity of these neuropeptides resides in their common NH_2-terminal amino acid sequences (H-Tyr-Gly-Gly-Phe-Met-OH or -Leu-OH) and is reversed by opiate receptor antagonists such as naloxone (1, 2). Opioids also bind to other (nonclassical) sites and such binding, unlike that to the opiate/opioid receptors, is naloxone irreversible and not strictly dependent on the NH_2 terminus of the peptides (1–3).

The first evidence that indicated that opioids exerted effects on the immune system was the observation in 1979 by Wybran et al. (8) that rosetting between human T cells and sheep erythrocytes was increased by MET-ENK. Since then there have been conflicting reports concerning the effects of opioids on lymphocytes (2, 3) and the significance of these peptides in relation to lymphocyte function has remained a matter for speculation (2, 3). Some investigators have reported that lectin-induced

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proliferation and lymphokine secretion were enhanced by β-END whereas others have found this opioid inhibitory (reviewed in references 2 and 3). Lymphocyte responsiveness to mitogens including blastogenesis and release of lymphokines must, by necessity, be measured in the presence of accessory cells (9). The previous studies (2, 3) have relied on mixed populations (i.e., cultures of PBMC), which may account, at least in part, for the difficulty in clearly defining the effects of opioids on lymphocyte function. There is also confusion in the literature concerning the mechanism(s) by which opioids act on immune cells (3). The effects of β-END on proliferation and lymphokine secretion have been ascribed to naloxone irreversible (non-opiate receptor) mechanisms; however, the enhancement of T cell rosetting caused by MET-ENK was abolished by this opiate receptor antagonist (reviewed in references 2–4).

To gain a better understanding of the actions of opioids on immune function we chose to focus on T lymphocytes because these cells are crucial for both cellular and humoral immunity (10). Previously, β-END and other opioids have been shown to affect the motility of leukocytes derived from myeloid precursors (11–14). We have used a cell migration assay to measure the effects of endogenous and synthetic opioids on highly purified human peripheral blood T cells. Since migration is a lymphocyte behavior mediated by direct interaction of soluble factors with cell surface receptors (15), these assays allowed us to characterize the opioid binding sites on T cells and compare our findings with known properties of the opiate/opioid receptors on nerve cells. In this communication we show that T cell migration is affected by direct interaction with physiological concentrations of β-END or the enkephalins. Synthetic peptides with a high degree of specificity for classical δ or μ opiate/opioid receptors were, like the endogenous opioids, stimulatory. Moreover, activity was dependent on the integrity of the opioid NH_2 terminus and inhibited by naloxone. We propose that human peripheral blood T cells have opiate/opioid receptors analogous to the δ and μ types previously described for neurons (1, 5) and that endogenous opioids may be signaling agents that play a significant role in directing T cell movement.

Materials and Methods

Preparation of Human Peripheral Blood T Cells. T lymphocytes were prepared from buffy coats (American Red Cross, Boston, MA) by a three-step protocol. First, human PBMC were prepared on a Ficoll-Hypaque gradient. The PBMC were then passaged over nylon wool to eliminate B cells and macrophages. In the third step, $CD16^+$ (natural killer) cells were coated with a saturating amount of B73.1 (mouse anti-human CD16 mAb) (16) and then absorbed on goat anti-mouse coated beads (BioMag beads; Advanced Magnetics, Inc., Cambridge, MA). Cells recovered in a typical preparation were ≥97% $CD3^+$ as assessed by flow cytometry.

Opioids and Synthetic Peptides. The preparations of purified human β-END used in these studies were from Calbiochem-Behring Corp. (La Jolla, CA). The naturally occurring pentapeptides LEU-ENK (Tyr-Gly-Gly-Phe-Leu) and MET-ENK (Tyr-Gly-Gly-Phe-Met), and the synthetic peptides [D-Ala-^{2}-N-Me-Phe^{4}Met(o)^4-ol]-enkephalin (FK 33-824), (D-Ala^2, D-Leu^5]-enkephalin (DADLE), (D-Ala^2,Me-Phe^4, Gly(ol)^5]-enkephalin (DAGO), [D-Ser^{2}Leu^5]-enkephalin Th^6 (DSLET), cyclic [D-Pen^2,D-Pen^5]-enkephalin (DPDPE), and H-Gly-Gly-Phe-Leu-OH (Des-Tyr-LEU-ENK) were purchased from Sigma Chemical Co. (St. Louis, MO). Naloxone (Narcan) was obtained from Dupont Pharmaceuticals (Manati, Puerto Rico).

Migration Assays. A microchemotaxis chamber (Neuroprobe, Inc., Cabin John, MD) was

used to measure T cell migration. Lymphocytes (5×10^6/ml) within the upper reservoirs were separated from peptides in the lower wells by a nitrocellular filter (Sartorious, 8 μm pore; Neuroprobe, Inc.) and were incubated for 1.5 h at 37°C. The filter was then fixed, stained with Congo red dye (Sigma Chemical Co.), and mounted between glass slides, as previously described (17). Cells which migrated into the filter were identified by fluorescence microscopy and then enumerated using an optical image analyzer (Optomax V image analyzing system; Analytical Instruments, Hollis, NH).

Statistical Analysis. Data analyses were performed on the PROPHET system, a national computer system sponsored by the Chemical/Biological Information Handling Program, National Institutes of Health, Bethesda, MD. Values measured for stimulated and control cells (spontaneous locomotion) were compared for each distance measured through the filter. The effects of the opioids on T cell migration were evaluated by using appropriate comparison procedures (paired *t*-test or Dunnet's test).

Results

Endogenous Opioids Are Chemotactic for Human T Cells. Endogenous opioids were potent stimulators of human T cell migration (Figs. 1-3). The response measured for β-END, LEU-ENK, and MET-ENK was greater ($p < 0.001$; paired *t*-test; $p < 0.01$, Dunnett's test) than the spontaneous locomotion (i.e., response measured for assay buffer alone) (Figs. 1-3). The effect exerted by the opioids was dose dependent, peaking at 10^{-5} M; the half-maximal response was measured at dilutions of 10^{-10} M and the activity titered to opioid doses as low as 10^{-13} M (Fig. 1 *B*).

T cell migration may, in theory, result from an enhancement in random motion (chemokinesis) or directed movement in response to a concentration gradient (chemotaxis) (15). To distinguish between these two types of locomotion we carried out experiments where in some test wells the stimulus was added in equal concentrations to both the upper and lower chambers, thereby abolishing the concentration gradient between the wells. By comparing the responses measured in the presence or absence of the gradient we were able to distinguish between chemotaxis and chemokinesis. When β-END was added to only the lower wells, and thus a gradient existed between the upper and lower chambers, T cells moved into the filters ($p < 0.001$; paired *t*-test; $p < 0.01$; Dunnetts test) (Fig. 2). The response, on the other hand, was equivalent to the spontaneous locomotion ($p > 0.05$, NS; paired *t*-test) when β-END was added to both upper and lower chambers (i.e., in the absence of a gradient). These findings indicate that T cell migration to β-END is dependent upon a concentration gradient between the upper and lower chambers, and therefore, the activity is chemotactic.

When T cells were challenged with MET-ENK or LEU-ENK at doses between 10^{-13} and 10^{-9} M, migration was measured only when a gradient existed between the upper and lower wells, and therefore, the activity was chemotactic. At very high concentrations (10^{-7} to 10^{-5} M) locomotion was measured in the presence or absence of a gradient; therefore, at nonphysiological concentrations (10^{-7} to 10^{-5} M) enkephalins stimulate chemokinesis.

Naloxone Blocked T Cell Locomotion to Opioids. T cell migration in response to MET-ENK or LEU-ENK was inhibited by naloxone (Table I). As expected, this opiate receptor antagonist had no effect on locomotion caused by the lymphokine IL-2; the response to IL-2 was equivalent for naloxone-treated or nontreated cells (with or without Naloxone; Table I), indicating that naloxone was not nonspecifically toxic for the lymphocytes.

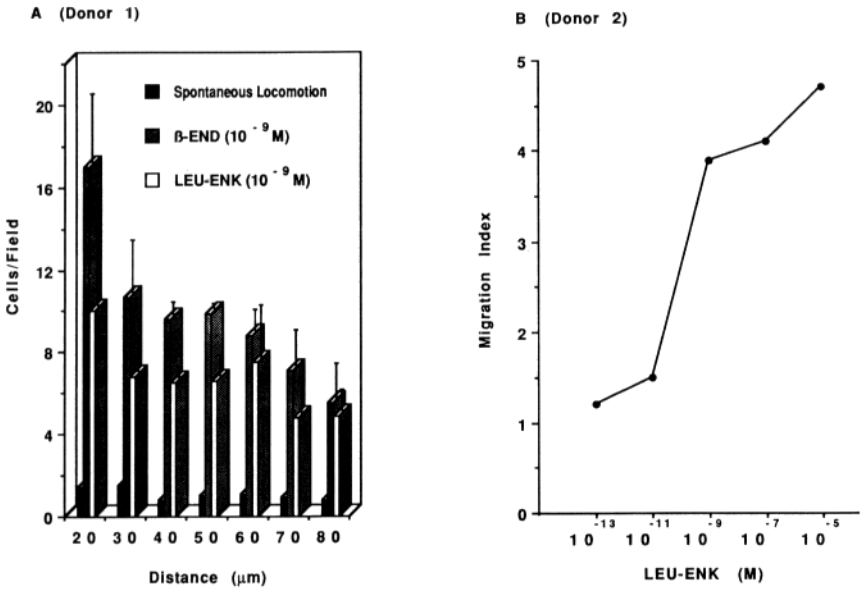

FIGURE 1. Human peripheral blood T lymphocytes migrate to β-endorphin and leucine-enkephalin. β-END and LEU-ENK were prepared in Dulbecco's modified PBS with 0.1% chick egg albumin (assay buffer) and then added in 35 μl volumes to the lower wells of the chemotaxis chamber. A nitrocellulose filter was placed between the upper and lower reservoirs of the chamber and the lymphocytes (5×10^6/ml) were then added to the upper wells in 60-μl portions of assay buffer. After a 1.5-h incubation at 37°C the filters were removed, fixed, and stained with Congo red dye (13). Migration was scored for triplicate wells utilizing an optical image analyzer; 10 high power fields (hpf) were measured at 10 μm distances through the filter. Data are presented as the means ± SD for 30 values (i.e., 10 readings × triplicate assays). (*A*) β-END and LEU-ENK were added to the lower reservoirs as 10^{-9} M solutions. (*B*) LEU-ENK was added to the lower wells at the dilutions indicated. Migration was scored as described for *A*. Total counts were determined by adding the values measured at 10 μm intervals through the filter. The data are presented as the Migration Index (the number of cells measured for LEU-ENK/the number measured for the spontaneous locomotion).

Enkephalin Analogues with Specificity for μ or δ Receptors Enhanced T Cell Locomotion. Synthetic peptides that bind to μ or δ, or both types, of opiate/opioid receptors stimulated T cell migration, albeit, to a lesser extent than the endogenous opioids MET-ENK or LEU-ENK (Fig. 3, *A–C*). The response measured for enkephalin analogues including: FK 33-824, DSLET, DPDPE, DADLE, or DAGO was greater then spontaneous locomotion ($p < 0.01$, Dunnetts test). It is noteworthy that the response was enhanced by the analogue DAGO (Fig. 3 *C*) in as much as this peptide shows specificity for the μ type opioid/opiate receptor (1, 5, 18, 19). Peptides with high affinity for δ receptors were also stimulatory as indicated by the fact that DPDPE or DADLE were stimulatory ($p < 0.01$; Dunnetts test)) (Fig. 3, *B* and *C*) (1).

To further characterize the T cell sites we measured the response to a nonanalgesic peptide, Des-Tyr-LEU-ENK which is identical to LEU-ENK except that the NH_2-terminal tyrosyl of the opioid is absent from this analogue (20, 21). Whereas LEU-ENK was stimulatory, Des-Tyr-LEU-ENK had no effect on T cell migration ($p > 0.05$) (Fig. 3 *A*), indicating that the NH_2 terminus of the peptides must be critical to their activity.

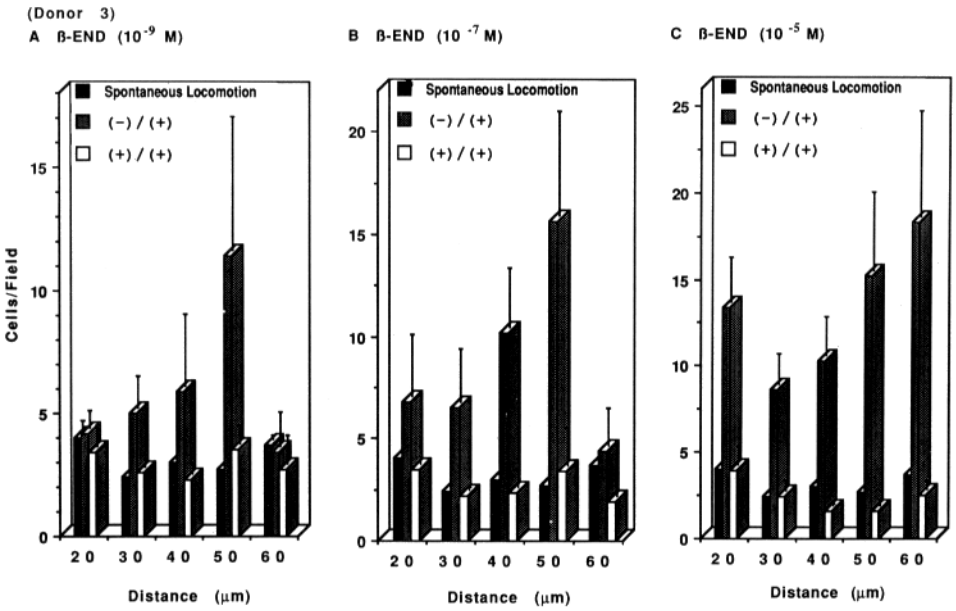

FIGURE 2. β-endorphin stimulates human T cell chemotaxis. Assays to distinguish chemotaxis and chemokinesis were as described for Fig. 1 except that preparations of β-END were dispensed to the lower [(−)/(+)] or the upper and lower [(+)/(+)] wells as indicated. The data of Fig. 2 were obtained by measuring the response of T cells from a single donor to β-END: (*A*) 10^{-9} M; (*B*) 10^{-7} M; and (*C*) 10^{-5} M. Migration was measured as described for Fig. 1 *A*.

Discussion

Our studies show that the naturally occurring opioids β-END, MET-ENK, or LEU-ENK and enkephalin analogue with a high degree of specificity for classical δ or μ opiate/opioid receptors caused an enhancement in the directed locomotion of human peripheral blood T cells. This response was dependent on the integrity of the opioid NH_2 terminus (Fig. 3 *A*) and inhibited by naloxone (Table 1). Previously, [^{3}H]naloxone (22) and a synthetic ligand specific for the δ receptors (23) have been shown to bind to T cells; however, the physiological significance of these opiate/opioid binding sites was unclear. Our studies indicate that T cell function is affected by direct interaction with opioids and that these cells have classical μ, as well as δ type opiate/opioid receptors.

In our assays a greater number of cells responded to MET-ENK or LEU-ENK than to their analogues (Fig. 3). The synthetic peptides, on the other hand, are more potent analgesics than MET-ENK or LEU-ENK (1). Endogenous opioid pentapeptides are rapidly hydrolyzed in vivo by aminopeptidases (1, 20, 21); however, their analogs are more resistant to such enzymatic activity (1, 20, 21). Possibly, the level of degradation in our in vitro assays is less than that in vivo and differences between the rates at which the natural pentapeptides are inactivated may account, at least partially, for this apparent dissimilarity.

T lymphocytes are highly motile immune response cells which probably accumulate in inflammatory sites by recruitment from the blood (15, 24). Evidence from

TABLE I

Naloxone Inhibits T Cell Migration to Methionine-Enkephalin and Leucine-Enkephalin

Donor‡	Stimulus	Treatments*				Effect of naloxone on specific migration¶ (percent response)
		Without naloxone		With naloxone		
		Total count§	Specific migration‖	Total count	Specific migration	
4	MET-ENK (10^{-11} M)	1,460	830	582	2	0.2
	MET-ENK (10^{-9} M)	3,250	2,620	915	335	12.8
	IL-2 (100 U/ml)	2,070	1,440	2,048	1,468	102.0
	Assay Buffer (Spontaneous locomotion)	630	0	580	0	
5	MET-ENK (10^{-5} M)	4,998	3,093	2,550	768	24.8
	LEU-ENK (10^{-5} M)	5,076	3,171	2,364	582	18.4
	Assay Buffer (Spontaneous locomotion)	1,905	0	1,782	0	

* T cells (prepared as described in Materials and Methods) were cultured in RPMI 1640 medium supplemented with 2% FCS, 100 U/ml of penicillin/streptomycin and 2 mM glutamine, or in the culture medium containing 10^{-5} M naloxone for 15 h at 37°C in a 5% CO_2 atmosphere. Viability of the cells incubated with or without naloxone was equivalent (≥97%), as judged from vital staining with trypan blue. Lymphocytes pretreated with naloxone were added to the upper wells of the migration chamber in assay buffer (Dulbecco's modified PBS with 0.1% chick egg albumin that contained naloxone (10^{-5} M).

‡ The data were obtained in two experiments with lymphocytes from different donors.

§ Migration was scored as described for Fig. 1 *A*. Total counts were obtained by adding the values measured at 10-μm intervals through the filter.

‖ Specific migration was determined by subtracting the spontaneous response to assay buffer (spontaneous locomotion) from the stimulated responses.

¶ The effect of naloxone on specific migration is shown as percent response = [specific migration for cells treated with naloxone/specific migration for nontreated cells] × 100%.

in vitro studies suggests that cytokines released by immune cells play a role in recruitment (15, 24). Murine T helper lines have been shown to secrete enkephalins and enkephalin-related peptides (10, 25) and human PBMC have been reported to release endorphins (4). Our studies show the opioids to be potent T cell chemoattractants (Figs. 1 and 2). Possibly, local release of opioids may stimulate T cell movement from the blood to immune response sites.

Under normal, nonstressful conditions circulating levels of β-END are between 1 and 100 × 10^{-12} M (26–28). The basal level is increased 3–10-fold with exercise (28), certain kinds of stress (29), and during pregnancy, labor and delivery (30). Studies carried out in animal models have shown that the levels of β-END within the brain may be as much as 100 times that in the periphery (31). Physiological concentrations of β-END and other opioids have been reported to have effects on the motility of phagocytes including human neutrophils and monocytes (11–14). Our studies show opioids stimulate T cell chemotaxis at concentrations detectable in peripheral blood (Figs. 1–2). Taken together these studies suggest that opioids have potent effects on the motility of immune response cells. Within the body immune cells move in and out of the blood and the circulating level of opioids may control both entry and exiting of such cells. Interestingly, Stefano et al. (32) have shown that opioids affect the motility of immunocytes (amebocytes) harvested from animals

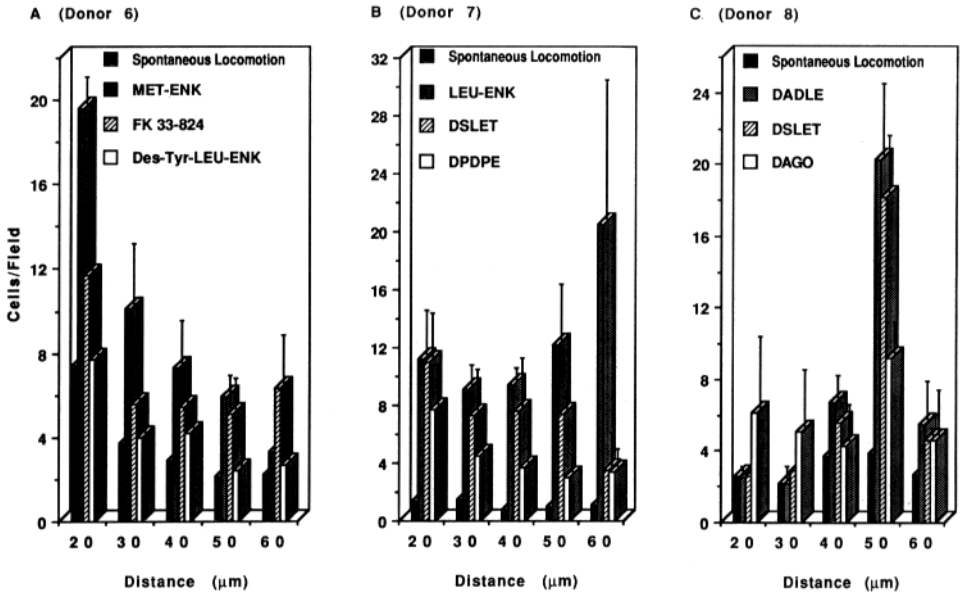

FIGURE 3. Synthetic enkephalin analogs selective for μ or δ opioid/opiate receptors stimulate T lymphocyte migration. Assays were as described for Fig. 1 *A* except that the peptides were diluted to 10^{-5} M in assay buffer and then added to the lower wells of the chemotaxis chamber. Data are presented as for Fig. 1 *A*. (*A*) FK 33-824, [D-Ala-^{2}-N-Me-Phe^{4}Met(o)^4-ol]-enkephalin; Des-Tyr-LEU-ENK, (H-Gly-Gly-Phe-Leu-OH). (*B*) DSLET, [D-Ser^{2}Leu^5]-enkephalin-Th^6; DPDPE, cyclic (D-Pen^2,D-Pen^5]-enkephalin. (*C*) DADLE, [D-ala^2,D-Leu^5]-enkephalin; DSLET, [D-Ser^{2}Leu^5]-enkephalin-Th^6; DAGO, [D-Ala^2,MePhe^4,Gly(ol)^5]-enkephalin. (*A*, *B*, and *C*) Three different experiments performed with cells from three different donors.

belonging to two phyla of invertebrates, the mollusc *Mytilis edulis* and the insect *Leucophaea maderae*. The studies by Stefano and coworkers show that opioid effects are not restricted to mammals and suggest that opiate/opioid receptors developed early in the course of evolution (32). To date, no other cytokines or microbial products have been shown to affect the locomotion of such diverse kinds of immune cells. Our studies show that opioids affect human T cell movement and since T cells are crucial for cellular and humoral immunity our findings provide evidence that these neurohormones are immunoregulatory agents which probably exert significant control on immune function.

Summary

In this communication we show that T cell locomotion is affected by direct interaction with neurohormones. Opioid peptides, including β-END, MET-ENK, LEU-ENK, and related enkephalin analogues enhanced migration of human peripheral blood T lymphocytes. Activity was dependent on the peptide NH_2-terminal sequence, stimulated by enkephalin analogues with specificity for classical δ or μ types of opiate receptor, and inhibited by the opiate receptor antagonist naloxone. Our studies suggest that such neuropeptides stimulate T cell chemotaxis by interaction with sites analogous to classical opiate receptors. We propose that the endogenous

opioids β-END, MET-ENK, and LEU-ENK are potent immunomodulating signals that regulate the trafficking of immune response cells.

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