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Research Article

Localized inflammation of a rat's hindpaw elicits an accumulation of beta-endorphin-(END) containing immune cells. We investigated the production, release, and antinociceptive effects of lymphocyte-derived END in relation to cell trafficking. In normal animals, END and proopiomelanocortin mRNA were less abundant in circulating lymphocytes than in those residing in lymph nodes (LN), suggesting that a finite cell population produces END and homes to LN. Inflammation increased proopiomelanocortin mRNA in cells from noninflamed and inflamed LN. However, END content was increased only in inflamed paw tissue and noninflamed LN-immune cells. Accordingly, corticotropin-releasing factor and IL-1beta released significantly more END from noninflamed than from inflamed LN-immune cells. This secretion was receptor specific, calcium dependent, and mimicked by potassium, consistent with vesicular release. Finally, both agents, injected into the inflamed paw, induced analgesia which was blocked by the co-administration of antiserum against END. Together, these findings suggest that END-producing lymphocytes home to inflamed tissue where they secrete END to reduce pain. Afterwards they migrate to the regional LN, depleted of the peptide. Consistent with this notion, immunofluorescence studies of cell suspensions revealed that END is contained predominantly within memory-type T cells. Thus, the immune system is important for the control of inflammatory pain. This has implications for the understanding of pain in immunosuppressed conditions like cancer or AIDS.

Immune Cell–derived β-endorphin

Production, Release, and Control of Inflammatory Pain in Rats

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Abstract

Localized inflammation of a rat's hindpaw elicits an accumulation of β-endorphin–(END) containing immune cells. We investigated the production, release, and antinociceptive effects of lymphocyte-derived END in relation to cell trafficking. In normal animals, END and proopiomelanocortin mRNA were less abundant in circulating lymphocytes than in those residing in lymph nodes (LN), suggesting that a finite cell population produces END and homes to LN. Inflammation increased proopiomelanocortin mRNA in cells from noninflamed and inflamed LN. However, END content was increased only in inflamed paw tissue and noninflamed LN-immune cells. Accordingly, corticotropin-releasing factor and IL-1β released significantly more END from noninflamed than from inflamed LN-immune cells. This secretion was receptor specific, calcium dependent, and mimicked by potassium, consistent with vesicular release. Finally, both agents, injected into the inflamed paw, induced analgesia which was blocked by the co-administration of antiserum against END. Together, these findings suggest that END-producing lymphocytes home to inflamed tissue where they secrete END to reduce pain. Afterwards they migrate to the regional LN, depleted of the peptide. Consistent with this notion, immunofluorescence studies of cell suspensions revealed that END is contained predominantly within memory-type T cells. Thus, the immune system is important for the control of inflammatory pain. This has implications for the understanding of pain in immunosuppressed conditions like cancer or AIDS. (*J. Clin. Invest.* 1997. 100:142–148.) Key words: corticotropin-releasing factor • IL-1 • analgesia • lymphocytes • opioid

Introduction

Recent findings strongly suggest an involvement of the immune system in the control of pain. Immunosuppression in animals by cyclosporine A (CsA)[1] (1) or whole-body irradiation (2) inhibits the generation of endogenous analgesia and, thus, exacerbates pain. Conversely, the local stimulation of immune cells in subcutaneous inflamed tissue by various cytokines or by corticotropin-releasing factor (CRF) decreases pain (3–5). These phenomena are based on interactions between immune cells and peripheral sensory nerves. Lymphocytes and other immunocytes residing in inflamed tissue contain opioid peptides (1, 2, 6, 7). Apparently, these peptides can be secreted and activate opioid receptors on peripheral terminals of sensory neurons (1, 4). This decreases the excitability of those neurons and the release of proinflammatory neuropeptides (i.e., substance P [8]) and eventually leads to the inhibition of pain (for review see reference 9).

A prominent opioid peptide involved in pain is β-endorphin (END) (1, 4, 10). This peptide is derived from proopiomelanocortin (POMC), which is synthesized and processed within various types of immunocytes, particularly under pathological conditions (11–13). In inflamed subcutaneous tissue, mRNA-encoding POMC is upregulated and END is readily detectable within lymphocytes and monocytic cells (2).

This study sought to examine these mechanisms at the cellular level in the context of lymphocyte migration. Under normal circumstances, the majority of lymphocytes recirculate by passing from the blood to lymph nodes (LN) and are then returned to the blood via the efferent lymphatic ducts and the thoracic duct. In peripheral inflammation, large numbers of lymphocytes (particularly memory T cells) and other cells enter the injured tissue and eventually accumulate in the afferent lymphatic ducts, which serve to drain cells from the inflamed site to the local LN (14). Since an important function of these cells appears to be the control of pain at the site of inflammation, we hypothesized that synthesis, content, and release of END from lymphocytes will change in a manner reflecting their migration from blood through the inflamed subcutis and LN. Therefore, we assessed circulating lymphocytes, subcutaneous tissue, and cells residing in noninflamed and inflamed LN, and we compared these distinct populations between normal animals and animals subjected to chronic inflammatory pain.

Methods

Subjects. Experiments were conducted in male Wistar rats (Charles River Laboratories, Wilmington, MA) (180–225 g) housed individually in cages lined with ground corn cob bedding. Standard laboratory rodent chow and tap water were available ad libitum. Room temperature was maintained at 22±0.5°C and a relative humidity between 40 and 60%. A 12/12 h (7 a.m./7 p.m.) light–dark cycle was used. Behav-

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1. *Abbreviations used in this paper:* α-helical CRF, CRF receptor-agonist; CRF, corticotropin-releasing factor; CsA, cyclosporine A; END, β-endorphin; FCA, Freund's complete adjuvant; IL-1ra, IL-1 receptor antagonist; ir-END, immunoreactive β-endorphin; i.pl., intraplantar; LN, lymph nodes; POMC, proopiomelanocortin; PPT, paw pressure threshold.

ioral testing was performed in the light phase. The guidelines on ethical standards of the International Association for the Study of Pain were followed. Animal facilities were accredited by the American Association for Accreditation of Laboratory Animal Care and experiments were approved by the Institutional Animal Care and Use Committee of the Intramural Research Program/National Institute on Drug Abuse/NIH in accordance with Institute for Laboratory Animal Research, National Research Council, Department of Health, Education and Welfare, Publication (NIH) 85-23, revised 1985.

Reagents. Human and rat CRF, CRF antagonist (α-helical CRF), and EGTA were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant human interleukin-1β (IL-1) and recombinant IL-1 receptor antagonist (IL-1ra) were obtained from R & D Systems, Inc. (Minneapolis, MN). END and rabbit anti-END were purchased from Peninsula Laboratories Inc. (Belmont, CA). CsA was purchased from Sandoz, (Nürnberg, Germany). Freund's complete adjuvant (FCA) was obtained from Calbiochem Corp. (La Jolla, CA) and halothane from Halocarbon Laboratories, (North Augusta, SC). Doses were calculated as the free base and drugs were dissolved in the following vehicles: sterile isotonic saline (CsA), sterile water (IL-1, CRF, IL-1ra, and α-helical CRF). Routes and volumes of drug administration were intraplantar (i.pl.) (0.1 ml) or intraperitoneal (i.p.) (1 ml).

Pretreatment of animals. To induce inflammation, rats received an i.pl. injection of 0.15 ml FCA into the right hindpaw under brief halothane anesthesia. Control animals were anesthetized but not injected. The paw volume was monitored using a plethysmometer (Ugo Basile, Comerio, Italy). The inflammation remained confined to the treated paw throughout the observation period. Some experiments were conducted in animals pretreated (at 48, 24, and 4 h before testing) with i.p. CsA (3 mg/injection) or vehicle (1 ml).

Preparation of cell suspensions. 4–5 d after treatment with FCA, rats were killed by CO_2 inhalation. Popliteal (from inflamed and noninflamed hind limbs) and axillary LN were removed and ground using a cell dissociation sieve (size 60 mesh; Sigma Chemical Co.). Cells were reconstituted in 5–15 ml HBSS and centrifuged at 700 *g* for 10 min at 20°C using a swinging bucket rotor. Each cell suspension was prepared from 1 inflamed, 6–8 axillary, or 12–15 noninflamed popliteal LN.

To harvest circulating lymphocytes, rats ($n = 2$ per experiment) were anesthetized with halothane, decapitated, and 3–4 ml of blood was collected in heparin-coated tubes. HBSS (3–4 ml) was added and 4 ml of this suspension was layered over 3 ml of Accu-paque for rats (Accurate Chemical and Science Corp., Westbury, NY). After centrifugation (20 min, 2,800 *g*) the lymphocyte layer was removed, suspended in HBSS (1:1) and again centrifuged (10 min, 900 *g*). The cell pellets were washed and centrifuged twice in HBSS (5 min, 2,800 *g*).

Cell pellets were reconstituted in HBSS aiming at a concentration of 0.05–0.15 $\times$ 10^6 cells/ml. This concentration was chosen based on pilot experiments that had shown that both content and release of END were negatively correlated with the concentration of cells, which may be a result of END's degradation by ectoenzymes on the surface of immune cells (15, 16). Cell viability, as determined by the Trypan blue exclusion method, was > 95%.

Extraction and quantification of POMC mRNA. For each experiment, suspensions containing $\sim 2.5 \pm 0.5 \times 10^6$ cells (corresponding to $\sim$ 5 inflamed LN, 50 noninflamed LN, or 8 ml blood, respectively) were centrifuged (700 *g*, 10 min at room temperature), decanted and spun again in a microcentrifuge (2,800 *g*, 5 min). The supernatant was removed, cell pellets were frozen in liquid nitrogen and stored at −70°C. Total RNA was extracted by applying a Trizol solution method (GIBCO BRL, Gaithersburg, MD). A 470-bp cDNA encoding rat POMC sequences corresponding to 30 bp of intron A, 150 bp of exon 2, and 270 bp of intron B, and an 117 bp Pst1/Xmal digested fragment of the cyclophilin cDNA were used to generate antisense cRNA probes with [^{32}P]UTP as a label (specific activity 5 $\times$ 10^4 Ci/mmol), using a Riboprobe kit (Promega Corp., Madison, WI). 10 μg of each total RNA sample was hybridized in solution to a POMC/cyclophilin probe mixture for 16 h at 42°C. The reaction mixture was subjected to digestion by RNase A and RNase T1 for 45 min in 37°C. Protected fragments were precipitated with a RNase Inactivation/Precipitation mixture (RPA II kit; Ambion, Inc., Austin, TX), dissolved, denatured, and electrophoresed on 5% polyacrylamide gels. The gels were dried and exposed to a film. Signals corresponding to POMC and cyclophilin mRNA were cut out and their radioactivity was counted. Each POMC mRNA value was normalized to the value of cyclophilin mRNA from the same lane and the mean was calculated of two to three blots per RNA sample. For each type of LN (inflamed, noninflamed, untreated), nine separate RNA samples were prepared, and the mean was calculated. Data are expressed as pg of POMC mRNA/10 μg of total RNA as determined by a sense POMC mRNA standard curve.

Extraction of END from immune cells. 300-μl aliquots from four to six individual cell suspensions were incubated with 0.5 μl of 1 M acetic acid, heated in a boiling water bath for 10 min, cooled on ice, and homogenized by ultrasound. After centrifugation (2,800 *g*, 5 min), a 250-μl aliquot of the supernatant was lyophilized and stored at −20°C until further processing.

Extraction of END from paw tissue. Subcutaneous paw tissue ($\sim$ 0.5 g) was removed from noninflamed and inflamed paws at 1, 2, and 4 d after FCA treatment ($n = 6$). Paw tissue samples were weighed and placed in boiling 0.1 M HCl (10 $\times$ vol) for 10 min, cooled on ice, and homogenized using a polytron tissue homogenizer (1 min). Samples were centrifuged (3,000 *g*, 15 min at 4°C), and a 300-μl aliquot was removed, lyophilized, and stored at −20°C until further processing.

Release experiments. A 300-μl vol of the cell suspension was incubated with 100 μl of either HBSS, α-helical CRF (25–100 ng), or IL-1ra (5–500 ng) at 37°C in a shaking water bath. After 5 min 100 μl of either HBSS, CRF (25–100 ng), or IL-1 (25–100 ng) was added. Another 5 min later, the suspension (total volume, 500 μl) was centrifuged (700 *g*, 10 min) using a swinging bucket rotor. 300-μl aliquots of the supernatants were lyophilized and stored at −20°C until further processing. To determine whether the END release was calcium-dependent, analogous experiments were performed in a similar buffer, except $CaCl_2$ was replaced by $MgCl_2$, and intracellular calcium was removed by addition of 0.1 mM EGTA. Potassium-evoked release experiments were performed by raising KCl concentrations from 6 to 50 mM and lowering NaCl concentrations to maintain isotonicity. The concentrations of cells and agents were chosen based on pilot experiments; release was determined from four to eight individual cell suspensions per data point.

Radioimmunoassay. Samples were reconstituted in 0.3 ml HBSS and assays were performed using an RIA kit (Peninsula Laboratories Inc.). Tubes were prepared in duplicate containing 100 μl of standard concentrations of END or unknown samples (except total count, nonspecific binding, and total binding tubes) dissolved in RIA buffer (containing 0.1 M sodium phosphate, 0.05 M NaCl, 0.01% NaN_3, 0.1% BSA, and 0.1% Triton X-100). Samples and standards were sealed and incubated with rabbit anti-END (100 μl) overnight at 4°C. On day 2, ^{125}I-END (100 μl, 12,000–15,000 cpm) was added and tubes were incubated overnight at 4°C. On day 3, 100 μl of goat anti–rabbit IgG and 100 μl of normal rabbit serum were added and tubes were vortexed and incubated for 90 min at room temperature. Subsequently, 0.5 ml RIA buffer was added and tubes were spun (3,000 *g*, 20 min at 4°C). After aspiration of supernatants (except total count tubes), radioactivity in the pellets was counted.

Algesiometry. Nociceptive thresholds were evaluated using an Analgesy-Meter (Ugo Basile) (17). Rats (seven per group) were handled twice before testing, then gently restrained under paper wadding and incremental pressure (maximum 250 *g*) applied onto the dorsal surface of the hindpaw. The pressure required to elicit paw withdrawal, the paw pressure threshold (PPT), was determined. The mean of three consecutive measurements, separated by 10 s, was determined. The same procedure was then performed on the contralateral side; the sequence of sides was alternated between subjects to preclude order effects. Baseline PPT were tested at 0, 6, 12, 24, and 96 h

after treatment with FCA. In separate experiments (96-h FCA), rats received an i.pl. injection of either vehicle or different doses of CRF (0.1–1.5 ng) or IL-1 (0.2–2 ng). PPT were determined at the time of the maximal effect (5 min after injection). Finally, the attenuation of the CRF- and IL-1–induced effects by simultaneous i.pl. injection of an END-specific antibody (0.025–0.2 μg; no cross-reactivity to other related peptides) was examined.

Immunofluorescence studies for END and cell markers. To discriminate between END-containing memory versus naive T cells, cells from LN and the circulation were collected as described previously. T-helper cells (CD4) are comprised of two subsets: naive and memory cells (18). Since antibodies against rat memory T cells were not available, we first stained these cells with an antibody against END and then with an antibody against all T-helper cells ($CD4^+$). In another experiment, we stained for END and for naive T cells ($CD45RC^+$). We hypothesized that if END-containing cells stain for CD4 but infrequently stain for CD45RC, then $END^+/CD4^+$ cells would be predominantly END-containing memory-type T cells (18). Immune cells (5×10^6 cells/tube) were examined for coexistence of CD4 and END by double staining with FITC-preconjugated anti-CD4 (W3/25) and a rabbit antibody to END, followed by an IgG secondary goat anti–rabbit antibody preconjugated with Texas red. The coexistence of a marker for naive cells and END was examined using FITC-preconjugated anti-CD45RC (OX-22) and the END antibody as described above. Cells were permeabilized with Triton X-100 and fixed with 2% paraformaldehyde after incubation with cell marker antibodies. The cells were washed in PBS between each additional antibody step by spinning at 1,500 *g* (20 min). They were incubated for 45 min with the END primary antibody and for 30 min with all other antibodies. Finally, the cell pellet was resuspended in PBS and placed on slides for visual analysis and photography using a Zeiss Overtskop 80. Control experiments for specific staining were performed by preadsorption of anti-END with END (100 μM).

Data analysis. PPT are given as raw values (mean±SEM). For dose–response curves, an ANOVA and a subsequent linear regression ANOVA was performed to test the zero slope hypothesis. Comparisons were made using the Wilcoxon paired-sample test for dependent data and the Mann-Whitney U test for independent data. Differences were considered significant if $P < 0.05$ (two-tailed).

Results

POMC mRNA content of immune cells. In all types of cells, POMC mRNA was readily detectable at a hybridization density ratio of at least 4.38±0.48% POMC/cyclophilin (mean±SEM). Cyclophilin mRNA did not change significantly within each gel ($P > 0.05$, Friedman test). POMC mRNA levels were lowest and similar in circulating lymphocytes of untreated and FCA-treated animals (Fig. 1). The highest levels were found in cells from LN of FCA-treated rats ($P < 0.001$, Mann-Whitney U test) whereas the values in LN of untreated rats were intermediate and significantly different ($P < 0.05$, Mann-Whitney U test) from each of the aforementioned (Fig. 1).

END content of immune cells. The content of immunoreactive (ir) END was significantly lower ($P < 0.05$, Mann-Whitney U test) in cells from inflamed LN compared to those from noninflamed popliteal or axillary LN of FCA-treated rats, and was similar to LN from untreated rats ($P > 0.05$, Mann-Whitney U test) (Fig. 2). Values from noninflamed popliteal and axillary LN were pooled subsequently since they were not significantly different from each other ($P > 0.05$, Mann-Whitney U test, data not shown). Circulating lymphocytes of untreated animals contained the lowest amounts of ir-END (Fig. 2). Values in circulating lymphocytes of FCA-treated rats were intermediate and significantly different ($P < 0.05$, Mann-Whitney U test) from each of the aforementioned (Fig. 2).

END content of paw tissue. The content of END in the inflamed paws increased linearly with the duration of inflammation ($P < 0.05$, regression ANOVA) (Fig. 3). 4 d after FCA-treatment, the content of END was significantly higher in inflamed than noninflamed paw tissue ($P < 0.05$, Mann-Whitney U test).

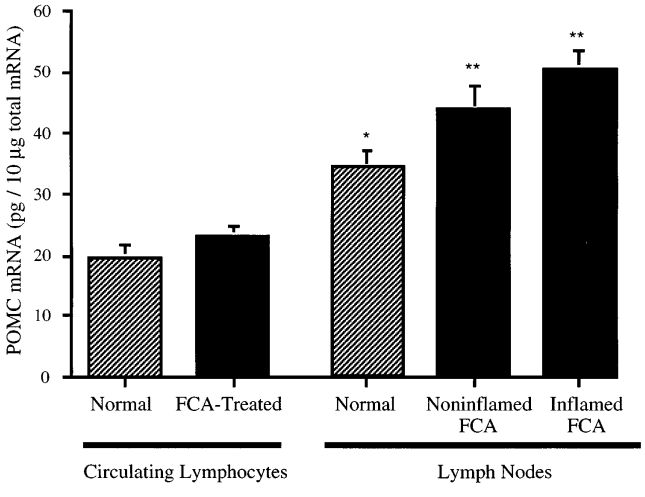

Figure 1. Levels of POMC mRNA in circulating lymphocytes and in cells derived from LN. Values represent mean±SEM of three independent experiments. Asterisks denote significant differences between circulating lymphocytes and LN from normal (untreated) (*$P < 0.05$, Mann-Whitney U test) and FCA-treated rats (**$P < 0.01$, Mann-Whitney U test). Values are not significantly different between circulating lymphocytes of normal and FCA-treated rats and between noninflamed and inflamed LN of FCA-treated animals ($P > 0.05$, Mann-Whitney U test).

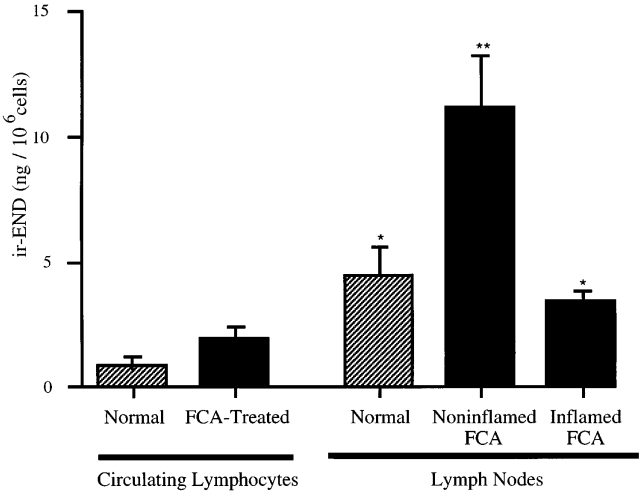

Figure 2. Levels of ir-END in circulating lymphocytes and in cells derived from LN. Values represent mean±SEM of at least four independent experiments. Significant differences were found between circulating lymphocytes from normal or FCA-treated rats and cells from LN (*$P < 0.05$, Mann-Whitney U test). END content was highest in noninflamed LN (**$P < 0.01$, Mann-Whitney U test). The values within normal and inflamed LN were similar ($P > 0.05$, Mann-Whitney U test).

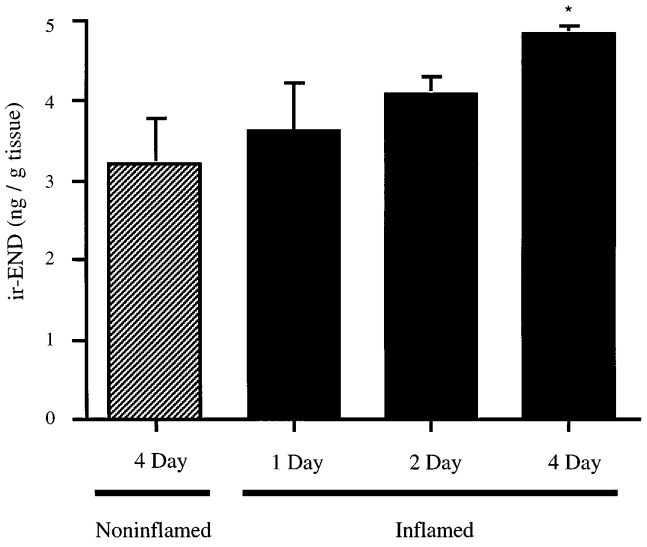

Figure 3. Content of ir-END in inflamed and noninflamed paws. Values increased in the inflamed paw over the 4 d after FCA treatment ($P < 0.05$, regression ANOVA) and were significantly higher in inflamed compared with noninflamed paws at 4 d (*$P < 0.05$, Mann-Whitney U test). Values are expressed as ir-END (ng/g tissue±SEM; six experiments for each time point).

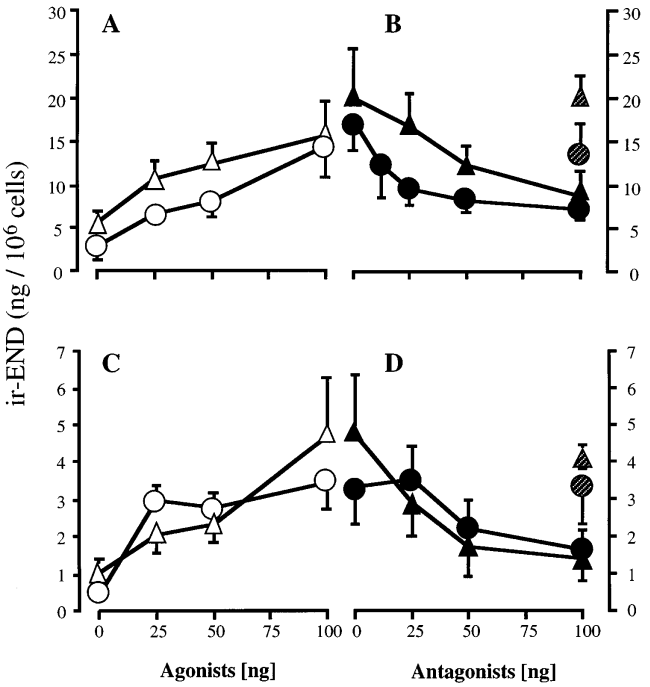

Figure 4. IL-1-(○) and CRF-(△) induced release of ir-END (*A* and *C*) and antagonism by IL-1ra (●) and α-helical-CRF (▲) (*B* and *D*) in cell suspensions from noninflamed (*A* and *B*) and inflamed (*C* and *D*) popliteal LN. Each point represents the mean±SEM of four to eight experiments. Data on inflamed LN (*C* and *D*) are from reference 4. IL-1–(100 ng) induced release was inhibited dose dependently by IL-1ra ($P < 0.05$, regression ANOVA), but not by α-helical CRF (*hatched circle*) ($P > 0.05$, Mann- Whitney U test) (*B* and *D*). CRF-(100 ng) induced release was attenuated dose dependently by α-helical CRF ($P < 0.05$, regression ANOVA), but not by IL-1ra (*hatched triangle*) ($P > 0.05$, Mann-Whitney U test) (*B* and *D*).

END release. In untreated control animals, release of ir-END from cells of popliteal or axillary LN was highly variable and without consistent pattern (data not shown). Neither IL-1– nor CRF-induced END release was dose dependent ($P > 0.05$, ANOVA). Similarly, in circulating lymphocytes of normal or FCA-treated rats, neither IL-1 nor CRF induced release of ir-END at any dose ($P > 0.05$, ANOVA) (data not shown).

Both IL-1 and CRF produced dose-dependent release of ir-END in cell suspensions prepared from LN of FCA-treated animals (IL-1: $P < 0.05$, CRF: $P < 0.005$; regression ANOVA) (Fig. 4, *A* and *C*). END release was similar in suspensions from noninflamed popliteal and from axillary LN ($P > 0.05$, Mann-Whitney U test, for all doses of IL-1 and CRF, data not shown). END release was significantly higher from noninflamed (Fig. 4 *A*) than from inflamed (Fig. 4 *C*) popliteal LN suspensions ($P < 0.05$, Mann-Whitney U test, for all doses of IL-1 and CRF). In both inflamed and noninflamed LN suspensions, IL-1–(100 ng) induced release was inhibited dose dependently by IL-1ra ($P < 0.05$, regression ANOVA), but not by α-helical CRF ($P > 0.05$, Mann-Whitney U test). CRF-(100 ng) induced release was attenuated dose dependently by α-helical CRF ($P < 0.05$, regression ANOVA), but not by IL-1ra ($P > 0.05$, Mann-Whitney U test) (Fig. 4 *B*). END release by IL-1 or CRF was reduced to that of basal levels when calcium was replaced by magnesium and intracellular calcium was removed by the addition of 0.1 mM EGTA (Fig. 5). Increasing K^+ concentrations from 6 to 50 mM, evoked the release of END similar to that produced by CRF and IL-1 ($P < 0.05$, Wilcoxon test) (Fig. 5).

In animals that had been immunosuppressed by treatment with CsA neither basal release (0.85±1.25 versus 1.56±0.61 ng/10^6 cells) nor stimulated END release were significantly different from vehicle-pretreated rats in any type of LN ($P > 0.05$, Mann-Whitney U test) (Table I). However, in inflamed LN the number of cells per node was significantly lower in CsA- ($1.69\pm0.24 \times 10^6$) than in vehicle-treated animals ($2.91\pm0.34 \times 10^6$) ($P < 0.05$, Mann-Whitney U test). In noninflamed LN, the number of cells per node was not different between treatments (data not shown).

Algesiometry. Baseline PPT were similar in both hindpaws (Table II). PPT began to decrease in treated paws as soon as 6 h after FCA injection, reached a minimum at 12 h, and did not change significantly thereafter. In noninflamed paws, PPT remained significantly higher throughout the observation period. Similar to our previous studies (4), i.pl. injection of CRF and IL-1 significantly elevated PPT above baseline in inflamed but not in noninflamed paws (Table II). These effects were dose dependent (CRF: ED_{50} = 1.59 ng; IL-1: ED_{50} = 4.0 ng; $P < 0.05$, regression ANOVA) and inhibited significantly by simultaneous i.pl. injection of the antibody against END (CRF: IC_{50} = 12.8 ng; IL-1: IC_{50} = 18.1 ng; $P < 0.05$, regression ANOVA).

Immunofluorescence. In all of the cell preparations examined most of the cells immunoreactive to END also stained with anti-CD4 ($CD4^+/END^+$) (Fig. 6, *A* and *C*; yellow). This was seen in both circulating lymphocytes from treated (Fig. 6 *A*) and normal animals (data not shown) and in cells from inflamed (Fig. 6 *C*) and noninflamed LN (data not shown). A number of $CD4^+$ cells did not stain for END ($CD4^+/END^-$)

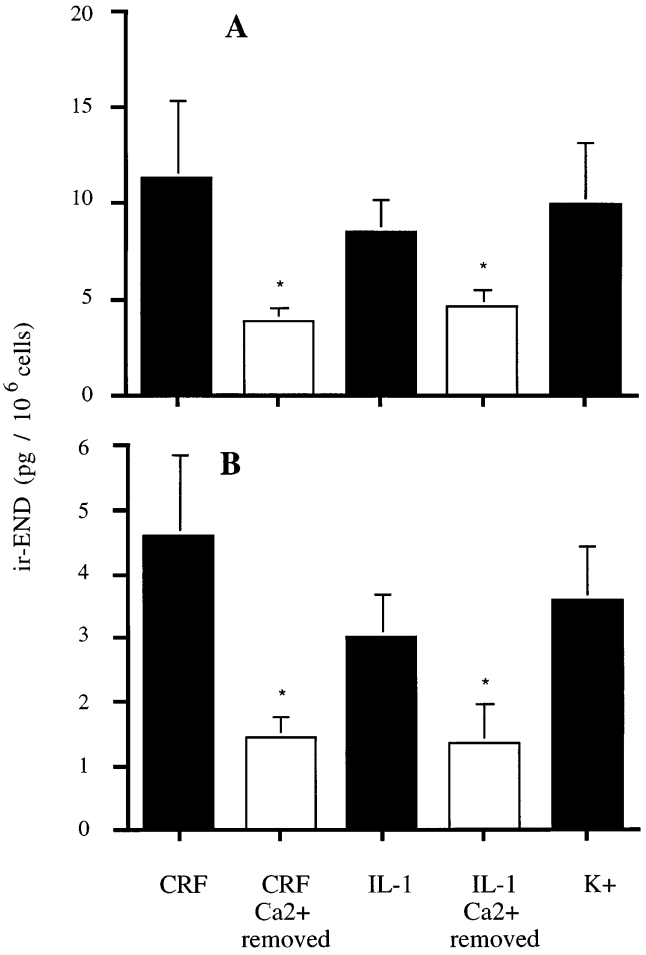

Figure 5. Calcium-dependent and K^+-evoked release of ir-END from noninflamed (*A*) and inflamed LN (*B*). Release of END by K^+, CRF, or IL-1 was not significantly different within inflamed or noninflamed LN ($P > 0.05$, Mann-Whitney U test). Replacement of calcium by magnesium in the incubation buffer and addition of 0.1 mM EGTA significantly reduced IL-1– and CRF-induced release ($*P < 0.05$, Mann-Whitney U test). Values are expressed as ir-END (ng/10^6 cells±SEM) from at least four experiments per condition.

(Fig. 6 *A*; green). Coexistence of the naive cell marker, CD45RC and END were rarely seen in any of the cell preparations examined (Fig. 6, *B* and *D*).

Discussion

Immune cell–derived opioid peptides can interact with opioid receptors on peripheral sensory nerve terminals to inhibit inflammatory pain. In this study we have examined immune cells that contain POMC mRNA and END and their role in a putative site-directed delivery of END to inflamed subcutaneous tissue.

Under normal circumstances, lymphocytes (mostly naive-type T cells) migrate very efficiently through LN, but only limited numbers migrate through nonlymphoid, e.g., peripheral subcutaneous tissues. However, after challenge by an inflammatory pathogen the lymphocyte traffic through the peripheral inflamed tissue increases markedly and consists mostly of memory-type and some naive-type T cells (19, 20). This also induces a response in the regional LN, which are strategically placed throughout the body to receive antigen draining from local sites of challenge. This response includes an increase in blood flow and a marked increase in the number of lymphocytes (mostly memory cells) migrating through the node and leaving through the efferent lymphatics (20, 21). Consistent with this notion, in our model of localized inflammation of one hindpaw, lymphocytes aggregated in the inflamed LN in much greater numbers than in the contralateral noninflamed LN. In terms of immune cell traffic, the inflamed LN and chronically inflamed peripheral tissue resemble each other (14). In fact, chronic inflammation may lead to the development of organized lymphoid tissue structures, and the traffic of immune cells through this ectopic lymphoid tissue can be quantitatively similar to that occurring through LN (20).

To examine whether END production changes during the migration of lymphocytes from the circulation through peripheral inflamed tissue and to the regional LN, we investigated END content and precursor mRNA, determined by RNA protection assay. Although only a fragment of the POMC messenger sequence is measured here, earlier studies have demonstrated the expression of a full-length POMC message within immune cells, particularly under pathological conditions (11, 13). Our study shows that circulating lymphocytes have small amounts of POMC mRNA and END, compared to cells in LN.

Table II. Analgesic Effect of IL-1 and CRF Injected i.pl. in Inflamed and Noninflamed Paws of FCA-treated Rats

Treatment	Noninflamed (g)	Inflamed (g)
Baseline	76±2.8	74±3.9
Baseline (4 d after FCA)	78±3.2	40±2.8
CRF i.pl. (4 d after FCA)	74±3.0	153±21.4
IL-1 i.pl. (4 d after FCA)	75±2.5	151±17.5

Values are expressed as PPT in grams±SEM. CRF and IL-1 produced significant PPT elevations in inflamed paws (Mann-Whitney U test, six experiments per treatment).

Table I. END Release from Popliteal and Axillary Lymph Nodes of FCA-treated Rats Pretreated with CSA or Vehicle

Incubation	Inflamed		Popliteal		Axillary	
100 ng	CsA	Vehicle	CsA	Vehicle	CsA	Vehicle
IL-1	5.71±0.51	3.93±0.45	9.76±1.42	6.95±1.63	4.67±0.79	5.31±0.71
CRF	2.36±0.71	2.33±0.74	12.41±3.14	12.71±5.13	11.15±4.15	7.77±2.09

Values are expressed as ir-END (ng/10^6 cells±SEM). No significant differences were detectable between CsA and vehicle pretreatments (Mann-Whitney U test, three experiments per treatment).

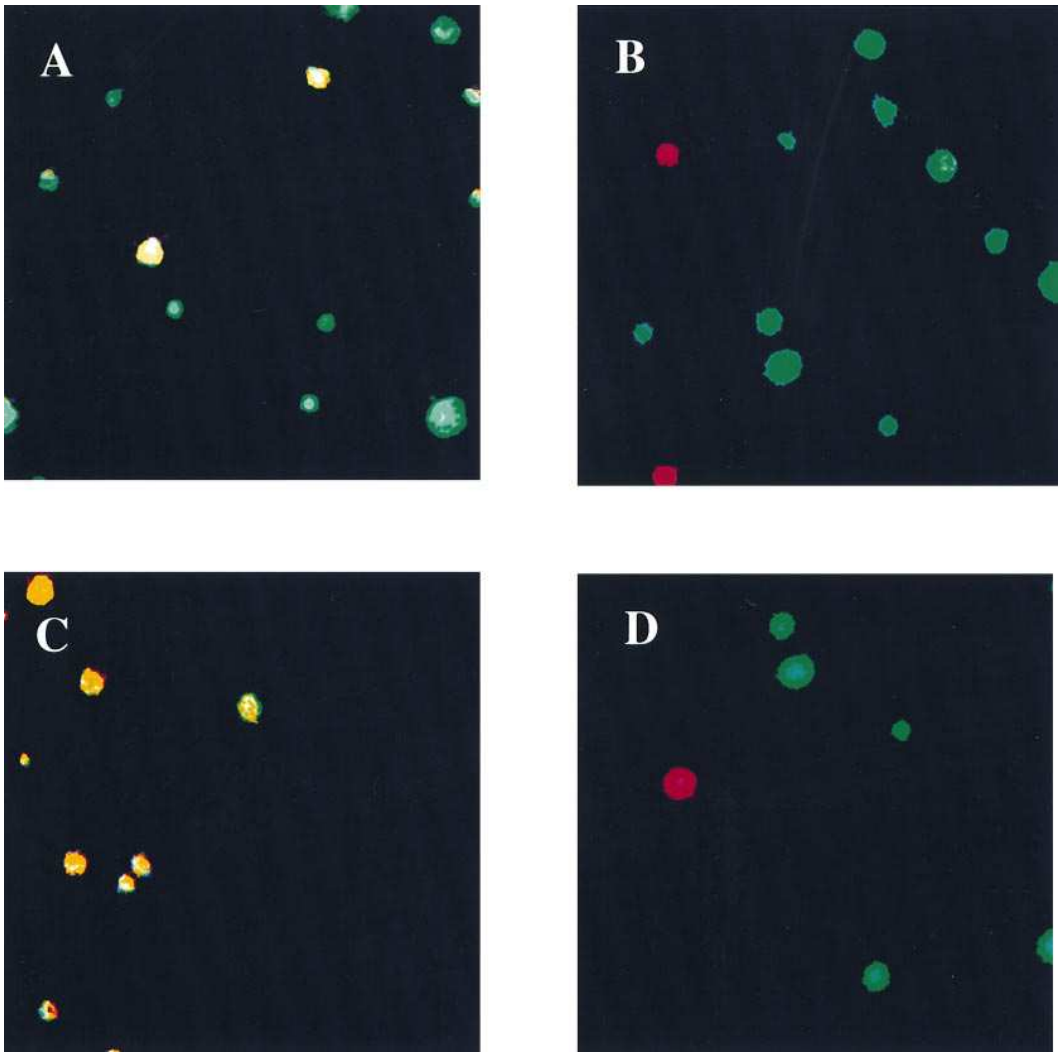

Figure 6. Cells stained with antibodies against T-helper cells ($CD4^+$FITC) (*A* and *C*; green), naive cells ($CD45RC^+$FITC) (*B* and *D*; green), and END (red). *A* and *C* show a high proportion of coexistence of CD4 and END (yellow) in cells from blood (*A*) and lymph node (*C*). Few cells stained for END only (not shown) indicating that most END-containing cells are a subpopulation of CD4 cells. Little double staining was seen for the naive cell marker (CD45RC; green) and END (red) in cells harvested either from blood or lymph node (*B* and *D*), indicating that the majority of $END^+/CD4^+$ cells are END-containing memory T cells (18).

This suggests that only a small proportion of the total population of circulating lymphocytes contain POMC mRNA and END, and that these cells preferentially home to LN (20). Interestingly, this is independent of whether the animal is FCA-treated or not. Inflammation led to an increase in the expression of POMC mRNA in the immune cells of both inflamed and noninflamed LN, which suggests that a generalized response to inflammation had occurred. The cellular content of END, however, did not follow this pattern since END was increased in lymphocytes of the noninflamed, but not of the inflamed, LN. This may be explained by the fact that cells from the noninflamed LN have not migrated through inflamed tissue, whereas cells in the inflamed LN have released END in the inflamed subcutaneous tissue before they migrate to the regional LN, depleted of the peptide. Consistent with this notion, the END content of the inflamed paw increased over the 4 d after FCA treatment. It is likely that this increase is both representative of the accumulation of END-containing immune cells and of the END released by these cells.

To corroborate the above hypothesis we examined the release of END and a possible functional role for this release in pain inhibition. CRF and IL-1 have been studied extensively in END release from immune cells in various models (4, 12, 22). We have demonstrated previously that CRF and IL-1 receptors are upregulated within the inflamed paw and LN, and that these receptors are localized primarily on immune cells (5). In agreement with these observations, and with the END content mentioned above, lymphocytes from animals with inflammation, but not from normal animals, exhibited dose-dependent release of END by CRF and IL-1. This was dose dependently antagonized by the specific antagonists α-helical CRF and IL-1ra, respectively. Together these findings strongly suggest that CRF and IL-1 act by activation of their respective receptors. END release was higher from noninflamed LN, in accord with the higher content of END. Circulating lymphocytes did not release significant amounts of END, which is consistent with the notion that only a small portion of these cells contains END. Interestingly, despite the fact that END content was similar in LN of untreated and in inflamed LN of treated rats, CRF and IL-1 were only capable of releasing END in treated animals, suggesting that CRF and IL-1 receptors on immune cells are only functional in treated animals (5). Importantly, CRF and IL-1 release of END from immune cells was calcium dependent and was evoked by increasing potassium concentrations. Together, these findings strongly suggest that END is released from vesicles, similar to the situation in neurons (23).

The functional role of END released in the inflamed subcutaneous tissue is evident from the fact that injection of CRF

and IL-1 into the inflamed paw produced analgesic effects that were inhibited by a locally applied specific antibody to END. Similarly, exposing the animal to stress can elicit analgesia in inflamed paws (1, 2, 10, 22). This effect is blocked by specific antibodies, selective antagonists, and antisense oligodeoxynucleotides for CRF (22). This indicates that endogenous CRF expressed in inflamed tissue is a prominent agent to trigger opioid peptide release and to inhibit pain.

Previously, we have shown that immunosuppression by CsA inhibits the analgesia elicited by stress, IL-1, and CRF (1, 4). In this study treatment with CsA resulted in a significant reduction of cell numbers within inflamed LN but not of END content in lymphocytes. Thus the inhibition of the analgesic effects by CsA apparently is not a direct result of the amount of END contained within immune cells. It is more likely that the total amount of END released within inflamed tissue is diminished by the reduction in the number of cells migrating through the tissue.

Finally, the double-staining experiments with antibodies to END and different cell phenotypes demonstrate that END is indeed mostly present in memory-type T cells and not in naive cells. This is consistent with the notion that activation of lymphocytes is a prerequisite for END production. Although this is the first demonstration of this phenomenon for opioid peptides, it is in agreement with the increased production of other proteins like cytokines in memory-type T cells (24).

In summary, this study has shown that the production and release of END from lymphocytes varies as a reflection of cell migration from the circulation through inflamed subcutaneous tissue to the LN, and as a reflection of these cells' function to control pain at the injured site. Consistent with such a site-directed mechanism for pain control, memory T cells are the predominant lymphocytes containing END. Immune cells release END in FCA-treated animals but not in normal animals, which is consistent with the upregulation of END synthesis and content and suggestive of a generalized immune system response. CRF and IL-1 receptors on lymphocytes can mediate the release of END, which is apparently contained in vesicles within these cells. These immune cells (apparently memory cells) (20) migrate into inflamed tissue where they release END to inhibit pain. Thereafter they travel through afferent lymphatic ducts to the regional LN, depleted of the peptide.

Our findings show that the immune system is a source for opioid peptides and plays an important role in pain control within peripheral inflammation. The delivery of opioids to injured tissue appears to occur in a deliberate, site-directed manner, such that inhibition of nociceptive stimuli can be achieved at the earliest possible time, i.e., within the damaged tissue. Thus, in addition to the well-known central sites involved in endogenous opioid antinociception (25), the immune system contributes a novel mechanism of intrinsic pain inhibition that prevents the excitation of sensory neurons at their peripheral terminals before nociceptive stimuli can even reach more central sites.

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