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Tissue Monocytes/Macrophages in Inflammation

Hyperalgesia versus *Opioid-mediated Peripheral Antinociception*

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Background: **Opioid-containing leukocytes migrate to peripheral sites of inflammation. On exposure to stress, opioid peptides are released, bind to opioid receptors on peripheral sensory neurons, and induce endogenous antinociception. In later stages of Freund's complete adjuvant–induced local inflammation, monocytes/macrophages are a major opioid-containing leukocyte subpopulation, but these cells also produce proalgesic cytokines. In this study, the role of tissue monocytes/macrophages in hyperalgesia and in peripheral opioid-mediated antinociception was investigated.**

Methods: **After intraplantar injection of Freund's adjuvant, leukocyte subpopulations and opioid-containing leukocytes were analyzed by flow cytometry in the inflamed paw in the presence or absence of monocyte/macrophage depletion by intraplantar injection of clodronate-containing liposomes (phosphate-buffered saline and empty liposomes served as controls). Paw volume was measured with a plethysmometer. Hyperalgesia was determined by measuring heat-induced paw withdrawal latency and paw pressure threshold. Paw pressure threshold was also measured after swim stress and injection of fentanyl.**

Results: **At 48 and 96 h of inflammation, it was found that (1) monocytes/macrophages were the largest leukocyte subpopulation (> 55% of all leukocytes) and the predominant producers of opioid peptides (71–77% of all opioid-containing leukocytes in the paw), (2) clodronate-containing liposomes depleted monocytes/macrophages by 30–35% ($P < 0.05$), (3) hyperalgesia was unaltered by liposome injection ($P > 0.05$), and (4) opioid-containing leukocytes and swim stress but not fentanyl-induced antinociception were significantly decreased by clodronate-containing liposomes ($P < 0.05$, $P > 0.05$, all by t test; opioid-containing cells and swim stress–induced increase of paw pressure threshold were reduced by 35–42% and 20%, respectively).**

Conclusion: **Partial depletion of tissue monocytes/macrophages impairs peripheral endogenous opioid-mediated antinociception without affecting hyperalgesia.**

SEVERAL peripheral endogenous antinociceptive mechanisms are involved in counteracting inflammatory hyperalgesia. Most of these involve the release of opioid peptides, endocannabinoids, somatostatin, or antiinflammatory cytokines.[1,2] Endogenous opioid-mediated antinociception has been most extensively studied, and its physiologic and clinical relevance has been established in postoperative pain in humans and in animal models.[1] Local inflammation can be experimentally induced in rats by intraplantar injection of Freund's complete adjuvant (FCA), and this leads to the recruitment of opioid-containing leukocytes. On exposure to stress, opioid peptides are released, bind to opioid receptors on peripheral sensory neurons, and elicit antinociception. This antinociception is fully antagonized by previous local injection of the opioid receptor antagonist naloxone.[3,4] Infiltrating leukocytes are the essential source of endogenous opioid peptides because opioid-mediated antinociception is abolished by immunosuppression or by blockage of adhesion molecule–mediated leukocyte recruitment.[5–7] With increasing duration of inflammation, more opioid-containing leukocytes are recruited, and peripheral endogenous antinociception is more intense.[8] At late stages of inflammation (96 h after FCA), monocytes/macrophages seem to constitute a major fraction of opioid-containing leukocytes.[8] Furthermore, monocytes/macrophages have been shown to contain β endorphin,[9,10] and local injection of anti-β endorphin antibody fully blocks endogenous peripheral opioid-mediated antinociception in early and late inflammation[3,4]

Monocytes/macrophages have also been linked to the development of hyperalgesia because they produce nociceptive mediators such as tumor necrosis factor α and interleukins 1 and 6.[11,12] In carrageenan-induced inflammation, the infiltrate is mostly formed by monocytes/macrophages at the time of the highest intensity of hyperalgesia.[13] In neuropathic models, mechanical allodynia is related to the magnitude of monocyte/macrophage infiltration.[11,14] Furthermore, depletion of monocytes/macrophages ameliorates hyperalgesia in chemically induced peritonitis (acid-induced writhing)[15] and in neuropathic pain models.[14]

To study the role of monocytes/macrophages in a model of chronic inflammatory pain, we depleted these cells by clodronate-containing liposomes.[16] After liposomal uptake, monocytes/macrophages metabolize clodronate to adenosine 5'(β,γ-dichloromethylene) triphosphate,[17] which blocks the mitochondrial adenosine diphosphate/adenosine triphosphate translocase, resulting in the collapse of the mitochondrial membrane potential[18] and the induction of apoptosis.[19] Specifically, we studied (1) the time point during the first 96 h after

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intraplantar injection of FCA, when monocytes/macrophages form a major part of the inflammatory infiltrate and whether they are a predominant opioid-containing leukocyte subpopulation; (2) whether intraplantar injection of clodronate-containing liposomes affects paw volume and whether it reduces the number of monocytes/macrophages as well as opioid-containing leukocytes; and (3) whether injection of clodronate-containing liposome alters hyperalgesia and endogenous or exogenous opioid-mediated antinociception.

Materials and Methods

Animals and Induction of Inflammation

Male Wistar rats weighing 170–220 g were used. All rats were intraplantarly (right hind paw) injected with 150 μl FCA (Calbiochem, La Jolla, CA). Animal experiments were approved by the animal care committee of the State of Berlin (Landesamt für Arbeitsschutz, Gesundheitsschutz und Technische Sicherheit, Berlin, Germany) and strictly followed the guidelines of the International Association for the Study of Pain.[20] All procedures were performed during brief halothane anesthesia. All behavioral experiments were conducted by an examiner blinded to the treatment protocol.

Preparation of Liposomes

Liposomes were prepared as previously described.[16,21] All incubations were performed at room temperature unless specifically mentioned. Phosphatidylcholine (86 mg; Lipoid KG, Ludwigshafen, Germany) and cholesterol (8 mg; Merck, Darmstadt, Germany) were suspended in 10 ml chloroform in a round-bottom flask. The mixture was vacuum dried (vacuum pump PC510; Vacubrand GmbH, Wertheim, Germany; and Rotationsevaporator R-124; Büchi Labortechnik AG, Favil, Switzerland). The lipid film that formed on the interior of the flask was dispersed by gentle rotation in 20 ml phosphate-buffered saline (PBS) to obtain empty liposomes. Otherwise, 5 g clodronate (dichloromethylene-disphosphonate; a gift from Roche Diagnostics, Mannheim, Germany) was added to the 20 ml PBS to obtain clodronate-containing liposomes. The liposomes were washed twice by centrifugation for 30 min (15,000*g*, 4°C) and resuspended in 10 ml PBS (PBS was also used as a control). The diameter of the liposomes was determined by light scattering (3.5–4 μm; particle size analyzer LS; Beckman Coulter GmbH, Krefeld, Germany). The multilayered liposomes were stored at 4°C for up to 7 days.

Flow Cytometry

Rats were killed by halothane inhalation. Inflamed paw tissue was removed from the plantar surface by sharp dissection and processed as previously described.[7,8] Briefly, paw tissue was enzymatically digested, and single-cell suspensions were prepared. All aliquots of single-cell suspensions were stained with the monoclonal antibody mouse anti-rat CD45 conjugated with CyChrome (4 μg/ml, identifies all hematopoietic cells; antibodies were obtained from BD Biosciences, Heidelberg, Germany, unless specifically stated). T cells (subpopulation of lymphocytes) were identified by coincubation with the mouse anti-rat CD3 phycoerythrin (4 μg/ml). For intracellular staining, cell suspensions were fixed with 1% paraformaldehyde and then permeabilized with saponin buffer. All cell suspensions were incubated with CD45 CyChrome monoclonal antibody and with one of the following combinations of monoclonal antibodies: (1) mouse anti-rat RP-1 phycoerythrin (identifies polymorphonuclear cells; 12 μg/ml) and mouse anti-rat ED1 fluorescein isothiocyanate (identifies monocytes/macrophages; 2 μg/ml; Serotec, Oxford, Great Britain); or (2) mouse 3E7 (isotype immunoglobulin [Ig] G_{2a}; identifies all opioid peptides; 20 μg/ml; Gramsch Laboratories, Schwabhausen, Germany) followed by rat anti-mouse IgG_{2a} phycoerythrin. The specificity of the staining was verified by incubation with appropriate isotype-matched control antibodies.

Quantification was performed by mixing the antibody-stained single cell suspension with fluorescent TruCOUNT® beads. A total of 70,000 events were acquired per paw and cell numbers were calculated in relation to the known number of TruCOUNT® beads. Data were analyzed using CellQuest Pro software (all from BD Biosciences).

Measurement of Paw Volume, Hyperalgesia, and Antinociception

Paw Volume. The paw volume of the FCA-injected and the noninjected contralateral hind paw were determined using a plethysmometer (Ugo Basile, Comerio, Italy). Two consecutive measurements were averaged.[3,7]

Thermal Hyperalgesia. Paw withdrawal latency (PWL) was measured by the Hargreaves test as previously described.[22] Briefly, rats were placed in a clear plastic chamber that was positioned on a glass surface (model 336 Analgesia Meter; IITC Life Science, Woodland Hills, CA). Radiant heat was applied to the plantar surface of the hind paw from underneath the glass floor with a high-intensity projector lamp bulb, and PWL was measured using an electronic timer. Latencies were measured twice per paw, and the mean values were calculated. Measurements were separated by at least 30 s. The heat intensity was adjusted to obtain a basal PWL of approximately 9–10 s in the noninflamed paw, and a 20-s cutoff time was used to prevent tissue damage.

Mechanical Hyperalgesia. Paw pressure thresholds (PPTs) were determined using the modified Randall-Selitto test as previously described (Ugo Basile).[5–7] Briefly, increasing pressure was applied to the dorsal surface of paw until the rat withdrew its paw (= PPT;

cutoff at 250 g). Measurements were performed three times (with 10-s intervals) on both the inflamed and the noninflamed contralateral hind paw, and averages were calculated.

Antinociception Induced by Cold-water Swim Stress and by Intraplantar Fentanyl Injection. PPTs were measured before (baseline) and 1 min after cold-water swimming (CWS; swimming in water at 2–4°C for 1 min) or 5 min after intraplantar injection of fentanyl (Janssen-Cilag, Neuss, Germany; 1 μg in 100 μl H_2O). Previous studies and pilot experiments have demonstrated that CWS and fentanyl injection induced peak effects on PPT at the above-mentioned time points and that these were fully antagonized by intraplantar but not by subcutaneous naloxone (injected at the most effective intraplantar dose), indicating a peripheral site of action.[3,23] Data are presented as percent of maximum possible effect (%MPE = $[PPT_{treated} - PPT_{pretreated}]/[250 - PPT_{pretreated}]$).

Experimental Protocols

Leukocyte Subpopulations and Opioid-containing Leukocytes in the Inflamed Paw. Rats (n = 30) were intraplantarly injected with FCA. Groups of rats (n = 5) were killed at 2, 12, 24, 48, 72, and 96 h after FCA injection, and leukocyte subpopulations in the inflamed paw were quantified by flow cytometry. Two separate sets of rats (n = 3 per group) were injected with FCA and killed after 48 and 96 h, respectively. Single-cell suspensions of the inflamed paw were triple stained with anti-CD45 CyChrome, anti-ED1 fluorescein isothiocyanate, and 3E7 plus rat anti-mouse IgG_{2a} phycoerythrin. Controls were stained with isotype-matched antibodies.

Clodronate-containing Liposomes: Effect on Leukocyte Subpopulations. Rats (total n = 63) were inoculated with FCA and were killed after different durations of inflammation (12, 48, and 96 h after intraplantar injection of FCA). At each stage of inflammation, rats were randomly assigned to three treatment groups (n = 7/group): clodronate-containing liposomes, empty liposomes, or PBS (solvent control). Liposomes and PBS were intraplantarly injected in a volume of 150 μl. Injections were performed at 1 h after FCA inoculation (for all rats), at 24 h after FCA (for rats with 48 h of inflammation), and at 48 h after FCA (for rats with 96 h of inflammation). Animals were killed at 12, 48, or 96 h after FCA, respectively. Leukocyte subpopulations and opioid-containing leukocytes were quantified by flow cytometry.

Clodronate-containing Liposomes: Effect on Paw Volume, Hyperalgesia, and CWS-induced Antinociception. Rats (total n = 36) were injected as described in Clodronate-containing Liposomes: Effect on Leukocyte Subpopulations. Paw volume and PWL were determined at 48 and 96 h of inflammation in all three groups (n = 6/group). In an additional set of rats (total n = 54) undergoing identical treatment, PPT was measured before and after CWS at 12, 48, and 96 h of inflammation in all three groups (n = 6/group).

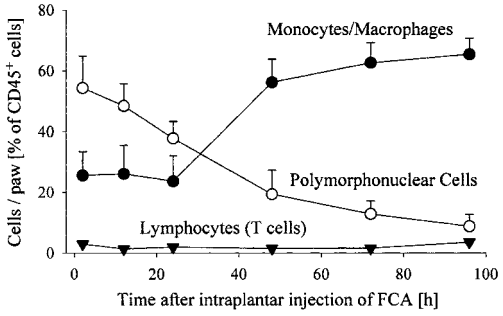

Fig. 1. Time course of leukocyte subpopulations in the inflamed paw: Rats were intraplantarly injected with Freund's complete adjuvant (FCA) and were killed at different stages of inflammation (n = 5 per time point). Flow cytometric analysis of $CD45^+$ leukocytes of the inflamed paw demonstrated that $ED1^+$ monocytes/macrophages were the predominant leukocyte subpopulation during later stages of inflammation (*open circle* = $RP\text{-}1^+$ polymorphonuclear cells; *filled circle* = $ED1^+$ monocytes/macrophages; *filled inverted triangle* = $CD3^+$ lymphocytes; data are presented as mean ± SEM).

Clodronate-containing Liposomes: Antinociception to Exogenous Opioids. Rats (total n = 36) were injected as described in Clodronate-containing Liposomes: Effect on Leukocyte Subpopulations. PPT was measured before and 5 min after intraplantar injection of fentanyl (1 μg) in all three groups at 48 and 96 h of inflammation (n = 6/group).

Statistics

Data were analyzed for equal variance and for normal distribution. If data were normally distributed and had an equal variance, a *t* test was used. Otherwise, the Mann–Whitney test was chosen. The null hypothesis was that clodronate-containing liposomes had no effect on leukocyte subpopulations in the inflamed paw or on hyperalgesia or antinociception, in comparison with PBS injection. A *P* value less than 0.05 was considered significant. All data are presented as mean ± SEM.

Results

Leukocyte Subpopulations and Opioid-containing Leukocytes

Flow cytometric analysis of leukocytes in the inflamed paw demonstrated that polymorphonuclear cells were the predominant leukocyte subset (38 ± 6 to 54 ± 11% of $CD45^+$ cells) during early inflammation (less then 24 h after FCA; fig. 1). At later time points (beyond 24 h after FCA), the major leukocyte subpopulation was monocytes/macrophages (56 ± 8 to 66 ± 5% of $CD45^+$ cells; fig. 1). Triple-color flow cytometry at 48 h further showed that a subset of $CD45^+$ leukocytes contained

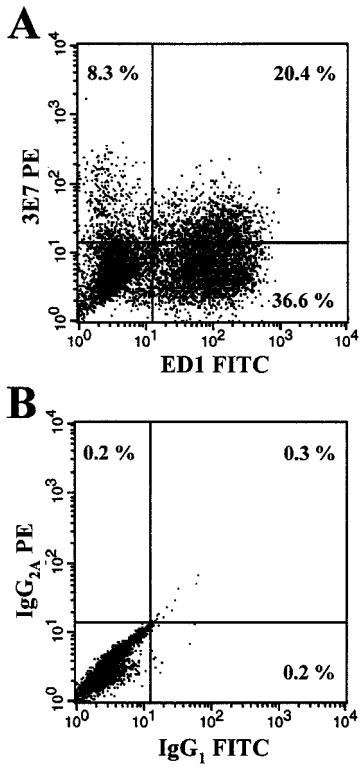

Fig. 2. Opioid-containing leukocytes in the inflamed paw during late inflammation: Rats were intraplantarly injected with Freund's complete adjuvant, and 48 h later, triple-color flow cytometry was performed on cell suspensions of the inflamed paw. A gate was set on $CD45^+$ leukocytes, and these were analyzed for expression of opioid peptides ($3E7^+$ cells, *y-axis*) and of the monocyte/macrophage marker (*A*, $ED1^+$ cells, *x-axis*, percentage of total $CD45^+$ leukocytes in each quadrant). The majority of all opioid-containing leukocytes were monocytes/macrophages (*upper right vs. upper left quadrant*, representative results of three independent experiments). Staining with isotype matched control antibodies is shown in *B*. FITC = fluorescein isocyanate; Ig = immunoglobulin; PE = phycoerythrin.

opioid peptides (fig. 2A; 29% [upper left plus upper right quadrant] of all $CD45^+$ cells are $3E7^+$ $CD45^+$ cells; 36% at 96 h after FCA, data not shown). The majority of these opioid-containing leukocytes were $ED1^+$ monocytes/macrophages at both time points (fig. 2A; 71% [upper right *vs.* upper right plus upper left quadrant]; 77% at 96 h after FCA, data not shown). Specificity of staining was checked by incubation with isotype-matched control antibodies (fig. 2B).

Effect of Liposome Injection on Inflammation and on Leukocyte Subpopulations

Paw volume was significantly increased in the inflamed in comparison with the noninflamed paw at 48 and 96 h after FCA ($P < 0.001$, *t* test; table 1). Intraplantar injection of clodronate-containing liposomes resulted in a small but statistically significant increase in paw volume at 48 h but not at 96 h after FCA ($P < 0.05$, *t* test). No significant changes in paw volume were detected in the noninflamed paw ($P > 0.05$, *t* test).

Table 1. Effect of Liposome Injection on Paw Volume

Paw	Time after FCA, h	Phosphate-buffered Saline	Clodronate Liposomes	Empty Liposomes
Inflamed	48	2.1 ± 0.05	2.2 ± 0.03*	2.0 ± 0.08
	96	2.0 ± 0.07	2.2 ± 0.08	2.1 ± 0.06
Noninflamed	48	1.0 ± 0.02	1.0 ± 0.03	1.0 ± 0.02
	96	1.0 ± 0.03	1.0 ± 0.04	1.1 ± 0.05

Rats (n = 6/group) were intraplantarly injected with Freund's complete adjuvant (FCA) together with phosphate-buffered saline, clodronate-containing liposomes, or empty liposomes. Paw volume was measured at 48 and 96 h after injection of FCA into the inflamed and noninflamed contralateral paws. Minor increases in paw volume were detected in inflamed paws of rats inoculated with clodronate-containing liposomes. (*$P < 0.05$, *t* test at 48 h after intraplantar injection of FCA, clodronate liposomes *vs.* phosphate-buffered saline, all other $P > 0.05$, *t* test). Data are presented as mean ± SEM.

Analysis of leukocyte subpopulations in the inflamed paw by flow cytometry showed that intraplantar injection of clodronate-containing resulted in significant 30 and 35% decreases of monocytes/macrophages at 48 and 96 h after FCA, respectively ($P < 0.05$, *t* test; fig. 3A). In contrast, clodronate-containing liposomes did not significantly affect the number of polymorphonuclear cells at either time point ($P > 0.05$, *t* test; fig. 3B). Lymphocytes constituted only a small leukocyte subpopulation (< 5% of $CD45^+$ cells) at both time points, and injection of clodronate-containing liposomes resulted in minor alterations (fig. 3C). At 12 h after FCA, clodronate-containing liposomes did not significantly alter any leukocyte subpopulation (PBS *vs.* clodronate-containing liposomes: monocytes/macrophages: 149 ± 66 *vs.* 153 ± 56 × 10^3 cells/paw, polymorphonuclear cells: 179 ± 32 *vs.* 228 ± 74 × 10^3 cells/paw, lymphocytes: 8 ± 2 *vs.* 10 ± 2 × 10^3 cells/paw; all $P > 0.05$, *t* test).

Effect of Liposome Injection on Thermal and Mechanical Hyperalgesia

Paw withdrawal latency and PPT were significantly lower in the inflamed in comparison with the noninflamed paw at 48 and 96 h after FCA ($P < 0.001$, *t* test; table 2). Injection of clodronate-containing liposomes did not result in significant alterations of PWL or PPT in the inflamed paw ($P > 0.05$, *t* test). Similarly, PPT values at 12 h after FCA were unchanged (PBS *vs.* clodronate-containing liposomes: 35.0 ± 0.9 *vs.* 35.0 ± 1.1 g; $P > 0.05$, *t* test; empty liposomes: data not shown).

Effect of Liposome Injection on Opioid-containing Leukocytes and on Antinociception

Intraplantar injection of clodronate-containing liposomes resulted in significant 35 and 42% decreases of opioid-containing leukocytes at 48 and 96 h after FCA, respectively ($P < 0.05$, *t* test; fig. 4A). CWS-induced PPT increase was significantly decreased by 20% after intraplantar injection of clodronate-containing liposomes at both 48 and 96 h after FCA ($P < 0.05$, *t* test; fig. 4B). At 12 h after FCA, injection of clodronate-containing

Table 2. Effect of Liposome Injection on Thermal and Mechanical Hyperalgesia

Paw	Time after FCA, h	Phosphate-buffered Saline	Clodronate Liposomes	Empty Liposomes
		Paw Withdrawal Latency, s		
Inflamed	48	5.1 ± 0.3	5.5 ± 1.0	5.4 ± 0.4
	96	5.7 ± 0.3	5.3 ± 0.4	5.0 ± 0.4
Noninflamed	48	10.6 ± 0.5	11.9 ± 0.3*	11.1 ± 0.5
	96	11.1 ± 0.8	9.7 ± 0.6	9.8 ± 0.4
		Paw Pressure Threshold, g		
Inflamed	48	33.3 ± 0.9	34.8 ± 1.3	34.3 ± 1.2
	96	34.7 ± 1.2	32.5 ± 0.9	33.9 ± 1.3
Noninflamed	48	75.0 ± 2.2	73.9 ± 1.4	77.2 ± 1.4
	96	76.7 ± 3.6	75.6 ± 2.0	76.6 ± 2.3

Rats (n = 6/group) were intraplantarly injected with Freund's complete adjuvant (FCA) together with phosphate-buffered saline, clodronate-containing liposomes, or empty liposomes. Paw withdrawal latency and paw pressure threshold were measured at 48 and 96 h after FCA in the inflamed and noninflamed contralateral paws. Liposome injection did not significantly alter paw withdrawal latency or paw pressure threshold in the inflamed paw at either time point ($P > 0.05$, *t* test; $^*P < 0.05$, *t* test, paw withdrawal latency, noninflamed paw). Data are presented as mean ± SEM.

liposomes had no effect on the number of opioid-containing cells or on CWS-induced PPT increase (PBS *vs.* clodronate-containing liposomes: opioid-containing leukocytes 172 ± 42 *vs.* 170 ± 32 × 10^3 cells/paw; 68 ± 5 *vs.* 60 ± 6% MPE, both $P > 0.05$, *t* test).

Paw pressure threshold increases after intraplantar injection of fentanyl were not significantly altered by previous intraplantar injection of clodronate-containing liposomes at any time point ($P > 0.05$, *t* test; table 3). Differences in PPT at baseline were not observed among the different groups (48 h after FCA: PBS 34.2 ± 0.7 g, empty liposomes 35.0 ± 1.4 g, and clodronate-containing liposomes 32.0 ± 1.4 g; 96 h after FCA: PBS 34.2 ± 1.1 g, empty liposomes 35.8 ± 1.1 g, and clodronate-containing liposomes 32.0 ± 0.7 g; $P > 0.05$, *t* test) or in comparison with the PPT at baseline described in table 2. Intraplantar injection of the solvent (100 μl H_2O) in the absence of fentanyl did not significantly increase the PPT in comparison to baseline (data not shown).

Discussion

In the current study, we demonstrate in a model of FCA-induced inflammation (1) that tissue monocytes/macrophages are the predominant leukocyte subpopulation and the major producers of opioid peptides at later stages of inflammation (*i.e.*, 48 and 96 h after FCA), (2) that clodronate-containing liposomes significantly and selectively reduce the number of monocytes/macrophages and opioid-containing leukocytes in the inflamed paw, and (3) that depletion of monocytes/macrophages does not affect mechanical and thermal hyperalgesia but results in a significant reduction of CWS-induced endogenous opioid antinociception without an effect on exogenous opioid-induced antinociception.

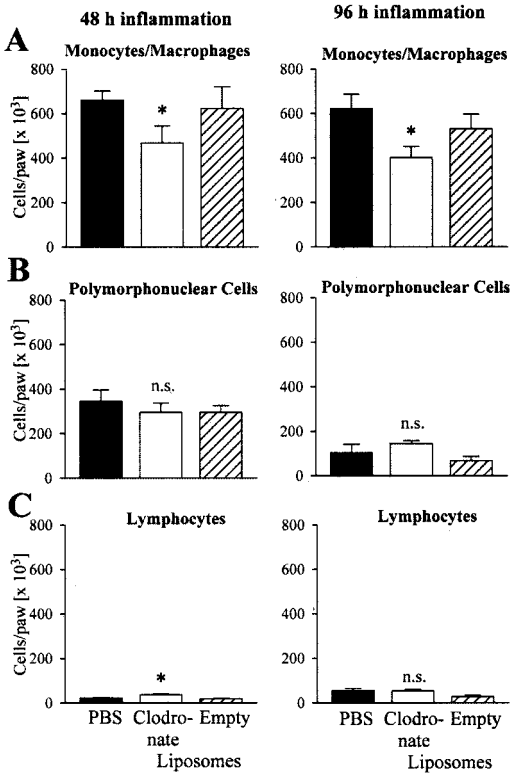

Fig. 3. Effect of liposome injection on leukocyte subsets in the inflamed paw: Rats (n = 7/group) were intraplantarly injected with Freund's complete adjuvant followed by two intraplantar injections of phosphate-buffered saline (PBS; solvent as control), clodronate-containing liposomes, or empty liposomes. Clodronate-containing liposomes induced a significant depletion of ED1$^+$ monocytes/macrophages at both time points of inflammation (*A*, $^*P < 0.05$, *t* test; *black bar* = PBS; *white bar* = clodronate-containing liposomes; *dashed bar* = empty liposomes). Clodronate-containing liposomes did not significantly decrease the number of RP-1$^+$ polymorphonuclear cells or of CD3$^+$ lymphocytes (*B* and *C*, respectively). All data are presented as mean ± SEM. n.s. = not significant.

To evaluate the role of monocytes/macrophages, we systematically analyzed the composition of the inflammatory infiltrate during the first 96 h of inflammation. Extending our previous results,[8] we now demonstrate that polymorphonuclear cells are the predominant leukocyte subpopulation during the first 24 h after inoculation with FCA (fig. 1). In later stages (beyond 24 h after FCA), the majority of infiltrating leukocytes are tissue monocytes/macrophages (fig. 1). Similar findings were recently reported in mice at 48 h after FCA inoculation[24] and in rats after intramuscular carrageenan injection,[13] indicating that such a composition of infiltrate is a common feature in models of inflammatory hyperalgesia. Earlier, nonquantitative studies in rats using immunohistochemistry showed that leukocytes in late inflammation (96 h after FCA) express various opioid peptides, such as β endorphin, metenkephalin, dynorphin, and endomorphins.[25,26] Using a quantitative technique (flow cytom-

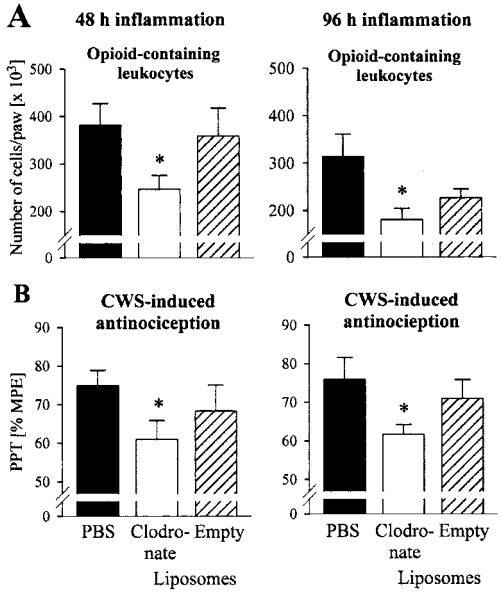

Fig. 4. Effect of liposome injection on opioid-containing leukocytes and on cold-water swim stress (CWS)–induced antinociception: Rats (n = 7/group) were injected according to the schedule described in figure 3. Clodronate-containing liposomes induced a significant reduction of 3E7$^+$ opioid-containing leukocytes at both time points (*A*, *$P < 0.05$, *t* test; *black bar* = phosphate-buffered saline [PBS]; *white bar* =clodronate-containing liposomes; *dashed bar* = empty liposomes). A separate set of rats (n = 6/group) was injected as described above. Paw pressure threshold (PPT) increases after CWS were significantly lower in rats injected with clodronate-containing liposomes (*B*, *$P < 0.05$, *t* test; MPE = maximum possible effect). All data are presented as mean ± SEM.

etry), we now demonstrate that 70–80% of opioid-containing leukocytes in late inflammation are monocytes/macrophages (fig. 2).

To characterize the functional role of monocytes/macrophages, we used clodronate-containing liposomes for the depletion of this leukocyte subpopulation as previously described.[16] Because we did not achieve a significant reduction of monocytes/macrophages in the inflamed paw by systemic treatment (data not shown), we injected clodronate-containing liposomes directly into the inflamed hind paw. Such local injection has been shown to effectively deplete tissue monocytes/macrophages in the synovium, in the testis, in the lung, and in the cornea.[27–30] Using this approach, we selectively depleted tissue monocytes/macrophages in the inflamed paw by 30–35% without reducing other leukocyte subpopulations (such as polymorphonuclear cells or lymphocytes) or paw edema (fig. 3 and table 1). Our findings are in line with numerous studies that demonstrated a selective toxicity of clodronate-containing liposomes for monocytes/macrophages both *in vitro* and *in vivo*.[31–33] Although the selectivity of clodronate treatment is widely accepted, the efficacy (*i.e.*, the decrease in cell numbers) is considerably more variable, probably because of differences in liposomal tissue penetration and the heterogeneity of the monocyte/macrophage population.[16] Clodronate-containing liposomes are highly efficacious (*i.e.*, reduction by > 70%) in depleting circulating monocytes and some subsets of monocyte-derived tissue macrophages (*e.g.*, spleen or liver)[32–34] and moderately efficacious (*i.e.*, 15–60% depletion of tissue monocytes/macrophages) in the inflamed synovium (FCA-induced arthritis;[34–36]) or at the site of a peripheral nerve injury (Wallerian degeneration after nerve transection[14,37]). In line with these studies, tissue monocytes/macrophages were depleted by 30–35% during late stages of inflammation (48 and 96 h after FCA) in the current experiments. Because solubility of clodronate in aqueous solution is limited and because this solubility determines liposomal incorporation, liposomes were prepared with an optimal concentration of clodronate following the directions originally described by Van Rooijen.[16,38] Because liposomal uptake by monocytes/macrophages is dependent on the surface charge of the liposomes,[16] we tested liposomes with positive and negative surface charges, but the efficiency of depletion was similar (data not shown). However, in accord with *in vitro* studies demonstrating that induction of apoptosis is a time-dependent process requiring at least 12 h,[18,31] we found that, in contrast to late stages of inflammation, depletion was not successful in early inflammation (12 h after FCA).

Table 3. Effect of Liposome Injection on Fentanyl-induced Antinociception

	Maximum Possible Effect, %		
Time after FCA, h	Phosphate-buffered Saline	Clodronate Liposomes	Empty Liposomes
48	56.9 ± 8.6	56.1 ± 7.6	61.9 ± 11.5
96	55.9 ± 6.2	55.1 ± 8.3	53.4 ± 6.6

Rats (n = 6/group) were intraplantarly injected with Freund's complete adjuvant (FCA) together with phosphate-buffered saline, clodronate-containing liposomes, or empty liposomes. At 48 and 96 h after FCA, fentanyl was injected into the inflamed paw, and paw pressure threshold was determined 5 min after injection. No significant changes in paw pressure threshold were detected between groups at either time point of inflammation ($P > 0.05$, *t* test). Data are presented as mean ± SEM.

Despite a 30–35% reduction in the number of tissue monocytes/macrophages by clodronate-containing liposomes, thermal and mechanical hyperalgesia in the inflamed paw was unaltered (table 2). In accord with this observation, the role of tissue monocytes/macrophages in the development of hyperalgesia is less well-documented in inflammatory than in neuropathic pain: In neuropathic animal models, a correlation has been shown between monocyte/macrophage infiltration and the degree of mechanical hypersensitivity.[11,39,40] Monocyte depletion or blockage of monocyte recruitment resulted in decreased hyperalgesia in most[14,24] but not all studies.[41] Recently, the role of tissue monocytes/macrophages in hyperalgesia was analyzed in mice lacking the chemokine receptor CCR2 that is responsible for

the chemokine-mediated recruitment of monocytes to peripheral tissue: Although formalin-evoked pain and mechanical allodynia in a neuropathic model were reduced by 70 and 100%, respectively, only a modest (20–30%) and nonsignificant reduction of mechanical allodynia was observed in FCA-induced inflammation.[24] Our previous studies also support the notion that hyperalgesia in FCA-induced inflammation is not closely linked to the degree of leukocyte infiltration: Neither cyclosporin A–induced immunosuppression nor leukocyte depletion by irradiation influenced mechanical hyperalgesia in late inflammation (96 h after FCA).[5,25] Similarly, in early inflammation (6 h after FCA), adhesion molecule blockage by antibody against intercellular adhesion molecule 1 resulted in a 50% reduction of infiltrating leukocytes without an effect on mechanical hyperalgesia.[7] Taken together, a moderate reduction of tissue monocytes/macrophages does not alter hyperalgesia in FCA-induced inflammation.

Clodronate-containing liposomes reduced both monocytes/macrophages and opioid-containing leukocytes to the same extent (30–40%; figs. 3 and 4A), and this depletion resulted in a significant reduction of CWS-induced antinociception (fig. 4B). To exclude adverse effects of clodronate-containing liposomes on opioid receptors on peripheral sensory neurons, we demonstrated that antinociception to a locally injected opioid agonist (*i.e.*, fentanyl) is unaltered (table 3). We previously reported that peripheral endogenous opioid-mediated antinociception in late inflammation (96 h after FCA) is abolished in rats systemically immunosuppressed by irradiation or by cyclosporin A treatment.[5,25] In contrast to these earlier studies, we now observed that endogenous peripheral opioid-mediated antinociception was diminished but not abolished by selectively targeting monocytes/macrophages. This might be because of the fact that 60–70% of opioid-containing leukocytes are still present after partial depletion of monocytes/macrophages (fig. 4B). In addition, peripheral opioid-mediated antinociception might also involve β-endorphin release from other cells, such as keratinocytes.[42] Taken together, these facts indicate that peripheral endogenous opioid-mediated antinociception is affected by moderate decreases in opioid-containing leukocytes.

In summary, we describe a novel function of tissue monocytes/macrophages. Tissue monocytes/macrophages contain opioid peptides, and they contribute to endogenous peripheral, stress-induced antinociception during later stages of a chronic inflammation. In addition, leukocyte depletion seems to have more profound effects on opioid-mediated antinociception than on hyperalgesia. We suggest that novel antiinflammatory treatment strategies (*e.g.*, blockage of adhesion molecules in inflammatory bowel disease in humans[43]) should be carefully evaluated to determine whether they might unexpectedly increase pain if endogenous antinociception mediated by monocytic cells is more affected than hyperalgesia.

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