Spinal Cord NMDA Receptors Modulate Peripheral Immune Responses and Spinal Cord *c-fos* Expression after Immune Challenge in Rats Subjected to Unilateral Mononeuropathy

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To characterize further the neural involvement in local immune reactions, we evaluated the effect of intrathecal NMDA-receptor blocker dizocilpine maleate (MK-801) on the peripheral immune response itself and on spinal cord *c-fos* expression induced by the delayed-type hypersensitivity (DTH) response. Immune challenge took place in the hind paw ipsilateral or contralateral to an injured sciatic nerve in both previously sensitized and immune-naïve animals. An enhanced immune response was observed bilaterally in the hind paws of animals subjected to unilateral mononeuropathy compared with sham-operated controls. In contrast, no such enhancement was observed when neuropathic animals were challenged in the front paws. The increased DTH response was blocked successfully by the intrathecal administration of an analgesic dose of MK-801. Compared with sham-operated animals, an-

imals subjected to unilateral mononeuropathy showed both a differential distribution and an increase in the number of *c-fos*-labeled neurons in the dorsal horn of the L3–L5 spinal cord segments after immune challenge. This was observed irrespective of whether the challenge took place ipsilateral or contralateral to the injured nerve. In addition to reversing the changes in immune response, intrathecal administration of MK-801 reversed the pattern of *c-fos* immunoreactivity in the spinal cord after immune challenge in neuropathic animals. These data suggest that select groups of spinal cord neurons participate in enhancing the peripheral immune response to a specific antigen in neuropathic animals and that this enhancement involves central NMDA receptors.

Key words: *c-fos*; pain; immunity; mononeuropathy; T lymphocytes; NMDA; MK-801

The interaction taking place between the nervous system and the immune system has received considerable attention over the past decade, and the potential immunomodulatory effects of various neuropeptides have been documented in several studies (Croitoru et al., 1990; Hemmick and Bidlack, 1990; Siemion et al., 1990). Although most studies to date have examined the *in vitro* effects of these neuropeptides on various immune cells, there are few studies that have examined the role of these peptides on immune function *in vivo*. Even less is known about the effect of conditions, such as chronic pain, that modulate neuropeptide levels on endpoints associated with immune response. In one recent study of the influence of pain on the immune system, Page et al. (1993) demonstrated that postoperative pain decreased natural killer cell activity and increased tumor metastasis. In another study, changes in plasma cytokines were demonstrated after nerve injury (Wells et al., 1992). In general, however, the potential effects of central nervous system changes induced by persistent pain on local-peripheral immune response have not been addressed.

In a recently developed model of chronic, painful mononeuropathy (Bennett and Xie, 1988), central changes associated with the development of hyperalgesia have been demonstrated. A recent study also has shown that the intrathecal administration of NMDA-receptor antagonists such as dizocilpine maleate (MK-

801) successfully alleviated most of the hyperalgesic behavior associated with this model (Yamamoto et al., 1994). Other work studying metabolic activity has demonstrated bilateral increases in the spinal cord dorsal horn of the L3–L5 segments after the induction of unilateral mononeuropathy (Mao et al., 1992a, 1993), indicating that unilateral neuronal injury is associated with bilateral spinal cord changes. A review of the literature suggests that this model of persistent pain is the best model to study the possible effects of nociception on immune response. The perineural inflammation induced by this model is localized, because control experiments in which the chronic gut is placed at the fascial plane, not touching the nerve trunk, have failed to generate hyperalgesic response in operated animals (Herzberg et al., 1994). Therefore, this model allows for control experiments in which chronic gut-induced inflammation is present without the induced hyperalgesia (unlike the injection of complete Freund's adjuvant). Thus, this model was used in the present study to investigate possible central mechanisms mediating changes in immune responses after the induction of mononeuropathy. Furthermore, to study the role of NMDA receptors in mediating such changes, MK-801 was administered to two groups of animals before challenge.

MATERIALS AND METHODS

The experimental protocol is summarized in Tables 1–3. Three experiments were conducted. The first experiment was designed to assess possible changes in *c-fos* expression in the dorsal horn of the spinal cord after immune challenge in the hind paw of animals subjected to unilateral mononeuropathy or to sham surgery. In the second experiment, we tested the involvement of spinal cord NMDA receptors in mediating peripheral alterations, both in the magnitude of local immune responses and in the T-cell population at the challenge site. In addition, we analyzed the effect

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Table 1. Summary of first experiment

Number	Day				
	0	3	4 and 11	18	19
8 female rats	Left sciatic ligation	Test for hyperalgesia	4 animals; 1 mg KLH in the base of the tail 4 animals; PBS in the base of the tail	Challenge w/2 mg HA-KLH in left hind foot pad	Assess DTH, perfuse, harvest spinal cords
8 female rats	Left sham surgery	Test for hyperalgesia	4 animals; 1 mg KLH in the base of the tail 4 animals; PBS in the base of the tail	Challenge w/2 mg HA-KLH in left hind foot pad	Assess DTH, perfuse, harvest spinal cords
8 female rats	Right sciatic ligation	Test for hyperalgesia	4 animals; 1 mg KLH in the base of the tail 4 animals; PBS in the base of the tail	Challenge w/2 mg HA-KLH in left hind foot pad	Assess DTH, perfuse, harvest spinal cords
8 female rats	Right sham surgery	Test for hyperalgesia	4 animals; 1 mg KLH in the base of the tail 4 animals; PBS in the base of the tail	Challenge w/2 mg HA-KLH in left hind foot pad	Assess DTH, perfuse, harvest spinal cords

Table 2. Summary of second experiment

Number	Day				
	0	3	4 and 11	18	19
10 female rats	Left sciatic ligation	Test for hyperalgesia	1 mg KLH in the base of the tail	5 animals: intrathecal MK-801 followed by 2 mg HA-KLH in the left hind paw 5 animals: intrathecal PBS followed by 2 mg HA-KLH in the left hind paw	Assess DTH, perfuse, harvest spinal cords and hind paws
10 female rats	Left sham surgery	Test for hyperalgesia	1 mg KLH in the base of the tail	5 animals: intrathecal MK-801 followed by 2 mg HA-KLH in the left hind paw 5 animals: intrathecal PBS followed by 2 mg HA-KLH in the left hind paw	Assess DTH, perfuse, harvest spinal cords and hind paws
10 female rats	Right sciatic ligation	Test for hyperalgesia	1 mg KLH in the base of the tail	5 animals: intrathecal MK-801 followed by 2 mg HA-KLH in the left hind paw 5 animals: intrathecal PBS followed by 2 mg HA-KLH in the left hind paw	Assess DTH, perfuse, harvest spinal cords and hind paws
10 female rats	Right sham surgery	Test for hyperalgesia	1 mg KLH in the base of the tail	5 animals: intrathecal MK-801 followed by 2 mg HA-KLH in the left hind paw 5 animals: intrathecal PBS followed by 2 mg HA-KLH in the left hind paw	Assess DTH, perfuse, harvest spinal cords and hind paws

Table 3. Summary of third experiment

Number	Day				
	0	3	4 and 11	18	19
8 female rats	Left sciatic ligation	Test for hyperalgesia	4 animals; 1 mg KLH in the base of the tail 4 animals; PBS in the base of the tail	Challenge w/2 mg HA-KLH in left front foot pad	Assess DTH, perfuse, harvest spinal cords
8 female rats	Left sham surgery	Test for hyperalgesia	4 animals; 1 mg KLH in the base of the tail 4 animals; PBS in the base of the tail	Challenge w/2 mg HA-KLH in left front foot pad	Assess DTH, perfuse, harvest spinal cords

of an NMDA antagonist on spinal cord *c-fos* expression induced by immune challenge in unilateral mononeuropathy animals. The third experiment was designed to test for possible systemic effects of unilateral mononeuropathy on the delayed-type hypersensitivity (DTH) response. In all experiments, freely cycling female Sprague–Dawley rats weighing 175–225 gm were used.

In the first experiment, a total of 32 animals was used. Four groups of eight animals underwent left or right sciatic ligation surgery or a respective sham operation. Each group was subdivided further into two subsets; four animals from each group were sensitized with a specific antigen, and the remaining four animals served as immune-naïve control animals. Local immune response was measured as the increase in paw thickness after challenge with the specific antigen and the accumulation of CD4⁺ and CD8⁺ T cells in the challenged area. Keyhole limpet hemocyanin (KLH) served as the sensitizing and challenging antigen, as reported previously (Exon et al., 1990; Herzberg et al., 1994). A total of 40 animals was used in the second experiment. Four groups of rats were subjected to left (10 rats) or right (10 rats) sciatic ligation or to sham surgery of the left (10 rats) or right (10 rats) hind limb. All animals then were sensitized with KLH. Immediately before immune challenge, five animals from each group received an intrathecal bolus injection of 2.5 nM MK-801 in 10 μ l of PBS, as described previously (Lee et al., 1992). The remaining five animals in each group received 10 μ l of PBS and served as vehicle-injected controls. In the third experiment, 16 animals were used. Two groups of rats (8 rats/group) were subjected to left sciatic ligation or sham surgery. The two groups then were subdivided into two subsets; four animals from each group were sensitized to KLH, and the remaining four animals served as immune-naïve control animals. To test for possible systemic effects of the unilateral mononeuropathy, immune challenge took place in the left front paw. In addition, after assessment of the DTH response, spinal cords from these animals were harvested and both lumbar (L3–L5) and cervical (C7–T1) segments of the spinal cord were processed with immunocytochemistry to demonstrate *c-fos* expression. Finally, to evaluate the possible effects of the stage of estrus on either the hyperalgesia or the DTH response, vaginal smears were taken from all of the animals in the third experiment before immune challenge and were stained with hematoxylin and eosin.

Surgery and hyperalgesia testing

Chronic, constrictive injury (CCI) was induced in female Sprague–Dawley rats weighing 175–225 gm using a modified sciatic ligation method (Bennett and Xie, 1988; Maves et al., 1993). Animals were anesthetized

first with a xylazine/ketamine mixture (1 mg/kg). The common sciatic nerve was exposed by blunt dissection of the biceps femoris muscles. Four loose 4-0 chromic gut sutures were tied, touching the nerve without constricting it or interfering with the epineural circulation. The surgical site was closed using two metal clips. In sham-operated animals, ~2 cm of 4-0 chromic gut was placed in the fascial plane between the biceps femoris muscles, but not touching the nerve. Three days after surgery, the animal's withdrawal response to a thermal stimulus, induced by radiant light source, was assessed to verify induction of hyperalgesia.

Materials

KLH was obtained from Calbiochem (La Jolla, CA). Monoclonal mouse antibodies to the rat CD4 and CD8 cell markers were obtained from Accurate Chemical and Scientific (Westbury, NY). Goat anti-mouse polyclonal antibody conjugated to fluorescein isothiocyanate (FITC) was obtained from Boehringer Mannheim (Indianapolis, IN). *c-Fos* polyclonal rabbit antibody was obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Biotinylated goat anti-rabbit antibody, avidin–biotin complex (ABC), and normal goat serum were obtained from Vector (Burlingame, CA). Diaminobenzidine tetrahydrochloride (DAB) was obtained from Sigma (St. Louis, MO).

Immunization schedule. Induction of DTH to KLH was performed as described by Exon et al. (1990). Briefly, 3 and 10 d after surgery, the animals were injected subcutaneously with 1 mg of KLH in 0.2 ml of PBS at the base of the tail. Preliminary experiments showed no DTH reaction to KLH in immune-naïve rats. Immune-naïve rats were injected with PBS on the same dates.

Measurement of DTH. Two weeks after initial immunization, the animals in the first two experiments were challenged with 2 mg of heat-aggregated KLH (HA-KLH) in 0.1 ml of 0.1 M PBS, pH 7.4, injected into the left hind footpad. The right hind footpad was injected with an equal volume of PBS. Hind paw thickness was measured in both hind legs before and 24 hr after HA-KLH or PBS injection using a tissue caliper with 0.05 mm accuracy. For normalization, any changes in thickness of the right hind paw (PBS-injected) were subtracted from the changes occurring in the left hind footpad. DTH results are reported in millimeters of paw swelling. Animals in the third experiment (see Table 3 for experimental protocol) were challenged similarly in the front left paw, PBS was injected into the right front paw, and the DTH reaction was quantified using a tissue caliper as in the first two experiments.

Assessment of cellular infiltration. In the second experiment, HA-KLH-challenged and PBS-injected paws were harvested after measurement of the DTH response and post-fixed overnight in 4% *p*-formaldehyde. Skin-sample blocks measuring 5 $\times$ 5 $\times$ 5 mm³ from

Figure 1. Graphic summary of paw withdrawal latencies (PWLs) from the first experiment, assessed 3 d after surgery in ligated animals and compared with sham-operated controls. Two-way ANOVA was used to detect significant differences in PWL response across groups. *Lig.*, Ligation.

Figure 2. Summary of DTH response from the first experiment expressed as an increase in paw thickness of the challenged (left hind paw) 24 hr after challenge. Two-way ANOVA was used to detect significant differences in DTH across groups.

Figure 3. Summary of *c-fos* expression from the first experiment after challenge to the hind paw. The laminar distribution and number of *c-fos*-immunoreactive neurons per 10 sections from the dorsal horn of ligated and sham-operated animals after immune challenge are indicated. Two-way ANOVA with the Fisher post hoc test was used to detect significant differences in the number of *c-fos*-immunoreactive neurons in respective laminae across treatment groups. *A*, *c-Fos*-immunoreactive neurons in the dorsal horn of the L3–L5 region of the spinal cord in sham-operated animals after immune challenge. The *small arrow* indicates the side of challenge; the *large broken arrow* indicates the side of sham surgery. *B*, *c-Fos*-immunoreactive neurons in the dorsal horn of the L3–L5 segments of the spinal cord in animals subjected to CCI after immune challenge. The *large solid arrow* indicates the side of ligation.

the plantar surface of the paws were dehydrated and blocked in paraffin. Care was taken to maintain the temperature of the paraffin below 57°C during the embedding procedure to prevent destruction of potential cell-surface antigens. Five micrometer sections were cut using a rotary microtome and mounted on gel-coated glass slides. The sections then were deparaffinized and rehydrated. All antibody incubations were performed while sections were on the glass slides in a humid chamber. All antibodies were diluted in a blocking solution of 2% normal goat serum in PBS. After a 1 hr incubation at room temperature with blocking solution, sections were incubated overnight at 4°C with a 1:500 solution of mouse anti-rat CD4 or CD8 antibodies. After three washes in PBS, sections were incubated at room temperature for 30 min with a 1:1000 solution of goat anti-mouse IgG polyclonal antibody conjugated to FITC. Slides then were washed with PBS and covered with glass coverslips using Fluormount (BDH Laboratory Supplies, Poole, UK). The number of CD4⁺ and CD8⁺ cells within the subcutaneous area surrounding the DTH response in the entire section was counted in five sections from each animal (5 animals in each subgroup as described in Table 2) using computerized image analysis (Universal Imaging Software, West Chester, PA) and quantified as number of cells/0.1 mm². Alternate sections were stained with hematoxylin and eosin to ensure that cells counted as CD4⁺ or CD8⁺ T cells indeed were lymphocytes. This procedure eliminated the counting of autofluorescent striated muscle fibers and fibrous strands as lymphocytes. Control sections were incubated in PBS without the primary antibody and incubated subsequently in goat anti-mouse secondary antibody.

Assessment of *c-fos*-immunoreactive neurons in the spinal cord. Under chloral hydrate anesthesia (1 mg/kg), animals were perfused transcardially with 500 ml of cold saline solution followed by 750 ml of 4% *p*-formaldehyde in PBS. Spinal cords were removed with attached spinal roots to identify the L3–L5 region accurately. Spinal cords were post-fixed overnight in 4% *p*-formaldehyde, washed, and stored in 20% sucrose in PBS. Spinal segments L3–L5 were removed, and 40 μ m sections were cut in a transverse plane using a freezing-sliding microtome. The sections then were processed for *c-fos* immunoreactivity as described previously (Lee and Beitz, 1992). Briefly, after a 1 hr incubation at room temperature in blocking solution (2% normal goat serum in PBS), sections were incubated overnight at 4°C with the primary antibody at 1:2000 dilution in blocking solution. Sections then were processed using the ABC-peroxidase–DAB reaction. Sections were incubated with a 1:200 dilution of ABC in PBS for 1 hr at room temperature, washed with PBS three times, and then placed in 0.05 M Tris buffer, pH 7.6. They were incubated subsequently for 5 min in 0.02% DAB and 0.002% H₂O₂ in Tris buffer. Sections were mounted on gel-coated slides, dehydrated, and coverslipped. Laminar boundaries of the spinal cord gray matter were defined according to the atlas of Paxinos and Watson (1986) and the description of Molander and Grant (1995). The laminae were outlined, and the number of *c-fos*-immunoreactive neurons in laminae I–V was counted in 10 sections from each spinal cord segment at a magnification of 400 $\times$, using a computerized image-analysis system (Image I, Universal Imaging). In the third experiment, sections from both the L3–L5 and the C7–T1 regions were processed for *c-fos* expression. Immunocytochemical controls included omission of primary or secondary antibodies during the procedure.

Statistical analysis

Statistical analysis was performed using both the StatView and Excel statistics packages. ANOVA was used to analyze the data obtained for the DTH response, the number of *c-fos*-immunoreactive cells per laminae, and the number of CD4⁺ or CD8⁺ within the area of subcutaneous tissue across treatment groups. The Fisher post hoc test was used to determine significant differences.

RESULTS

Results obtained from the first experiment are summarized in Figures 1–3. The data obtained from the second experiment are summarized in Figures 4–7. Figures 8 and 9 depict representative photomicrographs of *c-fos*-immunoreactive neurons in the left dorsal horn from these two experiments. The results of the third experiment are summarized in Figures 10–13. Vaginal smears performed in the third experiment showed the same distribution of estrus stage among the animals subjected to unilateral mononeuropathy and sham-

Figure 4. PWL observed in the second experiment 3 d after surgery in ligated animals compared with sham-operated controls. Two-way ANOVA was used to detect significant differences in PWL response across groups. Lig., Ligation.

Figure 5. Effect of MK-801 on the DTH response (second experiment) expressed as an increase in the thickness of the challenged left hind paw 24 hr after challenge. Two-way ANOVA was used to detect significant differences in DTH across groups.

operated animals. Because the treatment groups were composed of four animals in each group, it was not possible to identify a significant relationship between the stage of estrus and the magnitude of the hyperalgesia-like behavior.

Thermal hyperalgesia and DTH

In all experiments, a typical thermal hyperalgesia was induced after sciatic ligation (see Figs. 1, 4, 10). Animals subjected to sham surgery showed no hyperalgesia, and no difference in the response to a thermal stimulus was detected between animals subjected to ligation of the left sciatic nerve and those subjected to ligation of the right sciatic nerve. No significant changes in paw thickness were observed in the right paws (injected with PBS). The DTH reaction was enhanced in neuropathic animals that were challenged in the hind paws as compared with sham-operated controls, both ipsilaterally and contralaterally to the injured nerve (Figs. 2, 5). In contrast,

Figure 6. A summary of *c-fos* expression from the second experiment. The laminar distribution and *c-fos*-immunoreactive (*c-fos* IR) neurons per 10 sections from the left and right dorsal horns of the spinal cord in sham-operated and ligated animals after immune challenge are indicated. Two-way ANOVA with the Fisher post hoc test was used to detect significant differences in the number of *c-fos*-immunoreactive neurons in respective laminae (*lam.*) across treatment groups. Animals were injected intrathecally with PBS or MK-801 before challenge (see Materials and Methods). *A*, *c-Fos*-immunoreactive neurons in the left dorsal horn of L3–L5 segments of the spinal cord in sham-operated and neuropathic animals after immune challenge. *B*, *c-Fos*-immunoreactive neurons in the right dorsal horn of L3–L5 segments of the spinal cord in sham-operated and neuropathic animals after immune challenge.

when challenge occurred in the front paws, there were no significant changes in DTH response in the animals subjected to unilateral mononeuropathy compared with sham-operated animals (Fig. 11). Intrathecal administration of 2.5 nM MK-801 (a dose found to decrease thermal hyperalgesia for at least 24

hr without affecting the behavior of control sham-operated animals) (Mao et al., 1992b) abolished this increase in the DTH response reliably without affecting the DTH response in sham-operated animals (Fig. 5). Immune-naïve animals showed no significant changes in DTH reaction after sciatic ligation (see Fig. 2).

Figure 7. Migration of CD4⁺ and CD8⁺ T cells into the subcutaneous tissue after immune challenge in animals subjected to unilateral mononeuropathy or sham surgery and a specific intrathecal treatment. Immunoreactive cells were counted in five sections from each animal (5 animals/subgroup). Cellular infiltration is expressed as the number of cells per 0.1 mm² per section. Two-way ANOVA was used to detect significant differences in cellular infiltration after immune challenge across groups.

c-Fos expression in the spinal cord

No *c-fos*-immunoreactive neurons were observed in the control sections (Fig. 15*F*). A comparison of spinal cord sections from immune-naïve and sensitized animals after immune challenge in the hind paw indicates that there was a significant increase in the number of *c-fos*-immunoreactive neurons in laminae I and II of the L3–L5 spinal cord segments ipsilateral to immune challenge in sensitized sham-operated animals after induction of the DTH reaction (Fig. 3*A*). Animals subjected to unilateral mononeuropathy, however, showed significant increases in the number of *c-fos*-immunoreactive neurons in laminae III–V of the left dorsal horn when challenge took place ipsilaterally to the injured nerve (Fig. 3*B*). If challenge took place contralateral to the injured nerve, there was a selective increase in laminae III and IV, but not in lamina V. MK-801 reduced the increases in *c-fos*-immunoreactive neurons reliably in the ipsilateral laminae III–V observed in neuropathic animals after immune challenge (Fig. 6*A*). Decreased *c-fos* expression also was observed in lamina III of the contralateral dorsal horn after MK-801 treatment in neuropathic animals (Fig. 6*B*). Sham-operated animals, however, showed a slight bilateral increase in the number of *c-fos*-immunoreactive neurons in laminae III–V after MK-801 treatment before immune challenge (Fig. 6*A,B*). When animals were challenged in the front paws, an increase in *c-fos* immunoreactivity was observed in laminae I and II of the C7–T1 spinal segments of both sham-operated animals and neuropathic animals when comparing immune-naïve with immune-sensitized rats (Fig. 12*A*). More important, there were no significant increases in *c-fos* staining in deeper laminae of C7–T1, and no significant changes were observed in the lumbar spinal cord.

Effect of unilateral mononeuropathy on T-cell populations

An enhanced DTH response in animals subjected to unilateral mononeuropathy, manifested as a significant increase in paw swelling after challenge compared with sham-operated animals, also was apparent when assessing the subcutaneous tissue histologically. The number of immune cells per unit area of subcutaneous tissue was greater in ligated animals subjected to immune challenge compared with sham-operated controls (Figs. 7, 14). Moreover, immune challenge in the hind paw ipsilateral to the ligated nerve resulted in an increase in both CD4⁺ and CD8⁺ T cells in the subcutaneous tissue compared with sham-operated animals. However, when challenge took place contralateral to the ligated nerve, a selective increase in CD4⁺ T cells was observed in the subcutaneous tissue, with no significant change in the number of subcutaneous CD8⁺ T cells in the challenged area as compared with sham-operated animals (Fig. 7). Intrathecal MK-801 blocked these increases in T cells in animals challenged ipsilaterally and contralaterally to the ligated nerve. However, MK-801 had no effect on the population of CD4⁺/CD8⁺ T cells in the subcutaneous tissue of the challenged paws in sham-operated animals. No immunoreactive CD4⁺ or CD8⁺ T cells were observed in control sections in which the primary antibody was omitted.

DISCUSSION

This study provides data that suggest that central changes taking place in the spinal cord under conditions of neuropathic pain mediate alterations in local immune responses. That the spinal cord mediates the increased DTH response is illustrated by the finding that intrathecal MK-801 blocks the increased DTH response in animals subjected to unilateral mononeuropathy without affecting sham-operated animals. Previous work has documented that the dose of MK-801 used in this experiment decreases the hyperalgesia-like behavior successfully in neuropathic animals for at least 24 hr after a single intrathecal injection (Mao et al., 1992b). This is not surprising, because earlier reports have concluded that MK-801 is an irreversible inhibitor of the NMDA receptor (Reid et al., 1990). Furthermore, the increased DTH response appears to be associated with alterations in the lumbosacral spinal cord region rather than with a systemic effect induced by the mononeuropathy. This is based on the fact that there were no changes in DTH response when challenge took place in the front paws of animals subjected to unilateral sciatic ligation (Fig. 11). In addition, when challenged in the front paws, both neuropathic animals and animals subjected to sham surgery showed an increase in *c-fos*-immunoreactive neurons in laminae I and II of cervical spinal cord sections (Fig. 12). However, when challenged in the hind paws, only neuropathic animals exhibited an additional increase in *c-fos*-immunoreactive neurons in laminae III–V of lumbar spinal cord sections (Fig. 3). This increased *c-fos* expression in laminae III–V of animals subjected to unilateral mononeuropathy also was inhibited by intrathecal MK-801 administration, suggesting that both the peripheral increased DTH response and the central increased *c-fos* expression are mediated, in part, by spinal cord NMDA-receptor activation. It is important to note that both the increased DTH response and the increase in *c-fos* immunoreactivity were associated primarily with the side of immune challenge rather than with the side of sciatic ligation. This implies that the unilateral mononeuropathy affects both hind paws, but not the front paws, via an NMDA-sensitive spinal cord circuit that remains to be defined. In contrast to the bilateral effects of the unilateral mononeuropathy, the behavioral

Figure 8. Photomicrographs illustrating *c-fos*-stained neurons in the left dorsal horn from the first experiment. Immune-naïve and sensitized animals were subjected to sham surgery or ligation of left or right sciatic nerve and were challenged with KLH at the plantar surface of the left hind paw (see Materials and Methods, first experiment). *A, B*, Animals subjected to left sham surgery. *C, D*, Animals subjected to loose ligation of left sciatic nerve. *E, F*, Animals subjected to loose ligation of the right sciatic nerve. *A, C, E*, Animals sensitized to KLH before challenge. *B, D, F*, Immune-naïve animals. Scale bar, 200 μ m (all micrographs were taken at 400 $\times$ magnification). Arrowheads depict *c-fos*-immunoreactive neurons in laminae I and II. Arrows indicate *c-fos* neurons in laminae III–V.

Figure 9. Photomicrographs of left dorsal horn *c-fos* immunocytochemistry in the second experiment. Rats sensitized to KLH were subjected to sham surgery or ligation of left or right sciatic nerve and were challenged with KLH at the plantar surface of the left hind paw. Before challenge, rats were administered 10 μ l of PBS or 2.5 nM MK-801, i.t. (see Materials and Methods, second experiments). *A, B*, Animals subjected to left sham surgery. *C, D*, Animals subjected to loose ligation of the left sciatic nerve. *E, F*, Animals subjected to loose ligation of the right sciatic nerve. *A, C, E*, Animals treated with 10 μ l of PBS, i.t., before challenge. *B, D, F*, Animals treated with 2.5 nM MK-801, i.t., before challenge. Scale bar, 200 μ m (all micrographs were taken at 400 $\times$ magnification). Arrowheads depict *c-fos*-immunoreactive neurons in laminae I and II. Arrows indicate *c-fos* neurons in laminae III-V.

Figure 10. Summary of PWLs from the third experiment 3 d after surgery in ligated animals compared with sham-operated animals. Challenge took place in the hind paws. Two-way ANOVA was used to detect significant differences in PWL response across groups.

assessment of paw withdrawal latency (PWL) to a heat stimulus (Figs. 1, 4, 10) demonstrated that although a decrease in PWL was observed in the ligated hindlimb, only a slight and statistically insignificant increase in PWL was observed in the contralateral limb in experimental animals. It is possible that the number of animals evaluated in each group was too small for identification of significant changes in PWL of the unoperated limb. Another explanation for an apparent increase in the PWL of the unoperated limb is that the animals are reluctant to bear weight on the ligated side, causing an apparent increase in PWL on the unoperated side.

Previous research on other pain models have demonstrated that anatomic as well as functional changes occur in the spinal cord dorsal horn after neuronal trauma or chronic peripheral inflammation. For example, Zhang et al. (1993) have shown that after

Figure 11. Summary of the DTH response from the third experiment expressed as the increase in the challenged left front paw thickness 24 hr after challenge. Two-way ANOVA was used to detect significant differences in DTH across groups.

Figure 12. Summary of *c-fos* expression from the third experiment after challenge in the front paw. The laminar (*lam*) distribution and number of *c-fos*-immunoreactive neurons in the left and right dorsal horn per 10 sections from the cervical (C7–T1) spinal cord are indicated. Two-way ANOVA with the Fisher post hoc test was used to detect significant differences in the number of *c-fos*-immunoreactive neurons in respective laminae across treatment groups. *A*, *c-fos*-immunoreactive neurons in the left dorsal horn of C7–T1 segments of the spinal cord in sham-operated and neuropathic animals after immune challenge. *B*, *c-fos*-immunoreactive neurons in the right dorsal horn of C7–T1 segments of the spinal cord in sham-operated and neuropathic animals after immune challenge.

dorsal rhizotomy, substance P (SP)- and serotonin-containing terminals increase in lamina II. In addition, LaMotte and Kapadia (1993) have demonstrated a terminal-field expansion of saphenous afferents into the sciatic nerve territory of the spinal dorsal horn after pronase injection into the sciatic nerve. Induction of arthritis has been reported to increase SP, glutamate, and calci-

Figure 13. Summary of the laminar (*lam*) distribution and number of *c-fos*-immunoreactive (*c-fos* IR) neurons in the left and right dorsal horn per 10 sections from the lumbar (L3–L5) spinal cord from the third experiment. Two-way ANOVA with the Fisher post hoc test was used to detect significant differences in the number of *c-fos*-immunoreactive neurons in respective laminae across treatment groups. *A*, *c-Fos*-immunoreactive neurons in the left dorsal horn of L3–L5 segments of the spinal cord in sham-operated and neuropathic animals after immune challenge. *B*, *c-Fos*-immunoreactive neurons in the right dorsal horn of L3–L5 segments of the spinal cord in sham-operated and neuropathic animals after immune challenge.

tonin gene-related peptide throughout the superficial dorsal horn of the spinal cord (Sluka and Westlund, 1993). Also, after the induction of peripheral inflammation, an expansion of the receptive fields of spinal lamina I projection neurons has been observed and has been accounted for by central changes rather than by peripheral alterations (Hylden et al., 1989). Based on these and other studies indicating anatomical and neurochemical alterations in the spinal cord after peripheral manipulation, it appears feasible that sciatic ligation induces anatomical and functional changes of the sciatic and saphenous nerve terminals in the spinal cord. The altered pattern of *c-fos* expression observed in ligated animals after peripheral immune challenge may be a manifestation of these changes. Such changes, including the possible development of aberrant connectivity, may facilitate the development of hyperalgesia and allodynia and contribute to the activation of spinal cord neurons in laminae III–V in response to peripheral immune

challenge. This may influence further the levels or types of neuropeptides released from nerves innervating the hind paws in response to the immune challenge. Considering that even resting CD4⁺ T lymphocytes express the mRNA coding for the natural killer cell-1 receptor, such changes could be of consequence with respect to local immune response. However, the increase in *c-fos* expression in the spinal cord of ligated animals after peripheral immune challenge could be caused simply by an increased sensitivity or a different sensory input (perhaps a deep pain sensation) induced by the DTH response in these animals.

One factor that may have contributed to our observed results is potential changes in the neuropeptide content of primary afferent fibers in response to the chronic, painful mononeuropathy. Indeed, such changes have been demonstrated by Yamamoto and Yaksh (1993) after sciatic ligation. These investigators showed a decrease in SP and an increase in vasoactive intestinal peptide (VIP) in the ligated sciatic nerve. That changes in skin content of neuropeptides could result in abnormal immune responses was postulated by Giannetti et al. (1992), who demonstrated a decrease in the SP and an increase in VIP contents in skin from atopic dermatitis patients compared with skin from age- and sex-matched healthy volunteers. Thus, changes in neuropeptide content in the ligated nerve could account for our results. Yet, unlike the unilateral change in neuropeptide content in the ligated nerve observed by Yamamoto and Yaksh (1993), our finding that the increased DTH response was bilateral and reversible with the intrathecal administration of an NMDA-receptor blocker suggests bilateral involvement of the spinal cord. This implies that the sciatic ligation actually can affect immune responses in both hind paws via spinal cord circuitry. Recent evidence that unilateral sciatic stimulation causes bilateral release of VIP in the subcutaneous tissue of the rat's hind paws (Herzberg et al., 1995) supports such a hypothesis.

Our data are in contrast with the results of Basile et al. (1993), which demonstrated a decrease in the skin vascular response as measured by vasodilatory responses and plasma extravasation after local injection of SP into the hind paws of animals subjected to unilateral mononeuropathy. Their findings suggest nonspecific local changes in the skin innervated by the ligated nerve. In contrast, the increased DTH response observed in the present study appears to be a specific neuroimmune event mediated by an interaction between circulating immune cells and peripheral nerve fibers, with possible alterations in neuropeptide release in response to the peripheral immune challenge. The latter occurs, in part, because of chemical and functional changes in the spinal cord.

The above data raise the question of how the spinal cord regulates peripheral immune responses in the hind limb. The two likely possibilities are (1) via sympathetic output to the paw, and (2) via alterations in neuroimmune mediators released from primary afferents after the peripheral immune challenge. Previous studies have shown both sympathetic and primary afferent involvement in inflammatory arthritis (Levine et al., 1986). In addition, the release of peripheral neuropeptides that have various immunomodulatory effects has been demonstrated in response to inflammatory mediators (Hua and Yaksh, 1993) (for review, see Daniele et al., 1992). Unfortunately, the data obtained in the present study do not address directly the issue of regulation of peripheral immune responses by sympathetic versus primary afferent fibers. This will be the subject of a future study.

In the present study, we have demonstrated that painful mononeuropathy alters immune response via a mechanism that

Figure 14. Representative skin samples from the hind paws of animals subjected to unilateral mononeuropathy or sham surgery after immune challenge. *A*, Hematoxylin- and eosin-stained skin sample of the right hind paw (injected with PBS) from a sham-operated animal. *B*, Hematoxylin- and eosin-stained skin sample of the left hind paw (challenged with HA-KLH) from sham-operated animal (left hind sham surgery). *Arrowheads* indicate lymphocytes infiltrating the subcutaneous tissue. *C*, Hematoxylin- and eosin-stained skin sample of the left hind paw (challenged with HA-KLH) from animal subjected to right sciatic ligation. *Arrowheads* indicate lymphocytes infiltrating the subcutaneous tissue. *D–F*, Immunofluorescence for rat CD4⁺ T cells. *D*, Skin sample of the left hind paw (challenged with HA-KLH) from a sham-operated animal (left hind sham surgery). *Arrowheads* indicate CD4⁺-immunoreactive lymphocytes. *E*, Skin sample of the left hind paw (challenged with HA-KLH) from an animal subjected to right sciatic ligation. *Arrowheads* indicate CD4⁺-immunoreactive lymphocytes. *F*, Skin sample of the left hind paw of the same animal as in *E*. The primary antibody (rabbit anti-rat CD4⁺) was eliminated from the reaction to test for the specificity of the immunocytochemical procedure. Scale bar, 200 μ m (all micrographs were taken at 400 $\times$ magnification).

Figure 15. *c-Fos* immunoreactivity in the left dorsal horn after immune challenge in the front paws (third experiment). *A*, Representative section from C7-T1 region of an immune-naïve, sham-operated animal (left hind leg) after challenge with KLH. *B*, Representative section from C7-T1 region of an animal sensitized to KLH and sham-operated (left hind leg) after challenge with KLH. *C*, Representative section from C7-T1 region of an immune-naïve, neuropathic animal (left sciatic) after challenge with KLH. *D*, Representative section from C7-T1 region of a neuropathic animal (left sciatic) sensitized to KLH after challenge with KLH. *E*, Representative section from C7-T1 region of an animal sensitized to KLH and sham-operated (left hind leg) after challenge with KLH. *F*, Sample from the L3-L5 region of an animal sensitized to KLH and sham-operated (left hind leg) after challenge with KLH. The primary antibody was eliminated from the immunocytochemistry procedure to test for the specificity of the procedure. Scale bar, 200 μ M (all micrographs were taken at 400 $\times$ magnification). *Arrowheads* depict *c-fos*-immunoreactive neurons in laminae I and II. *Arrows* indicate *c-fos* neurons in laminae III-V.

clearly involves spinal cord neurons. This involvement is mediated by spinal cord NMDA receptors, as evidenced by the ability of MK-801 to reverse the increased DTH response in neuropathic animals without affecting sham-operated animals. Additional studies are necessary to examine whether there is an anatomical rearrangement of primary afferents in the spinal cord after the induction of unilateral mononeuropathy. It also is important to evaluate whether a unique population of spinal neurons is responsible for the increased DTH response in these animals or whether the activation of these neurons merely reflects an altered sensory input generated by the DTH response in combination with the peripheral neuropathy.

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