Effects of Chronic Intrathecal Infusion of a δ Opioid Agonist in Dogs

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To define the effects of chronic spinal exposure to a highly selective δ opioid agonist c[DPen₂,DPen⁵]enkephalin (DPDPE), adult beagles were prepared with chronic lumbar intrathecal catheters. Groups of dogs received intrathecal infusions (100 μl/h) of saline (vehicle), DPDPE 3 mg/ml or 6 mg/ml for 28 days. Over the 28-day period, saline or 3 mg/ml showed minimal changes in neurological function, whereas in the 6 mg/ml animals, prominent hind limb dysfunction evolved over the 28-day interval. Histopathology in control animals displayed a modest pericatheter reaction considered normal for this model. Dogs receiving DPDPE (three of four at 6 mg/ml and one of four at 3 mg/ml) but not saline (zero of four) developed large inflammatory masses (granulomas) in the intrathecal space located proximal to the catheter tip. In these masses, severe chronic inflammatory changes in combination with necrosis and fibrosis was detected. Occasional focal destruction of neuropil was detected also in the adjacent spinal cord parenchyma. These masses contained extensive accumulation of tumor necrosis factor-α (TNF-α), revealing infiltration of macrophages, granulocytes, and monocytes. In separate animals, prepared with dual intrathecal catheters, lumbar CSF was sampled at specified time points following intrathecal bolus (3 mg/ml) and 24 h DPDPE infusion (3 mg/ml and 6 mg/ml). Steady-state cerebrospinal fluid (CSF) DPDPE levels were 18.6 ± 1.0 and 22.6 ± 4.0 μg/ml for 3 mg/ml and 6 mg/ml infusions respectively. These results indicate that this δ opioid agonist DPDPE produces a concentration and time-dependent formation of an intrathecal inflammatory mass.

Key Words: DPDPE; δ opioid agonist; dog; intrathecal; granuloma.

Animal studies have shown that spinally delivered delta opioid agonists will yield a powerful analgesia (Tung and Yaksh, 1982; Porreca et al., 1987). This effect is mediated by δ opioid receptors present on dorsal horn neurons and the terminals of small primary afferents (Besse et al., 1990; Traynor et al., 1990). This dorsal horn localization is consistent with the ability of δ agonists to block the C fiber transmitter release (Aimone and Yaksh, 1989) and attenuate the small afferent evoked discharge of dorsal horn nociceptors (Dickenson et al., 1987). Previous studies suggested that spinal δ receptors may also modulate pain states in humans. Intrathecal delivery of the δ-prefering peptide [DAla⁵,DLεu³]enkephalin (DADLE) produced significant analgesic efficacy in cancer patients (Moulin et al., 1985; Onofrio and Yaksh, 1983). Though suggestive, this agent has limited selectivity for the μ versus the δ receptor. c[DPen₂,DPen⁵]enkephalin (DPDPE) is a peptide that is highly selective for the δ receptor and is resistant to metabolism (Weber et al., 1992). The intrathecal delivery of this agent in several animal models has demonstrated that DPDPE can produce a potent analgesia that displays a pharmacology consistent with an action at the δ receptor (Drower et al., 1991; Porreca et al., 1987). Such an agent would make it possible to determine whether in humans spinal delta receptors also have an antinociceptive action.

Prior to undertaking delivery of novel drugs spinally in humans, it is necessary to evaluate the spinal kinetics of the agents and the toxicological profile in preclinical experiment (Yaksh et al., 1999b). Accordingly, in the present studies, we examined (1) the maximum usable dose of DPDPE delivered by chronic intrathecal delivery for 28 days in the dog prepared with chronic lumbar intrathecal catheters, and (2) the intrathecal kinetics of DPDPE in that dog model. In these studies, we unexpectedly observed a concentration dependent local toxicity in DPDPE infused dogs.

MATERIALS AND METHODS

These studies were conducted according to protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego. After a minimum of five days of acclimation, baseline data were collected on animals in the dose ranging and safety phases. Dogs were prepared on day –3 with intrathecal catheters that were externalized and connected to infusion pumps (Panomat C-10, T-10 or equivalent, Disetronic Medical Systems, Inc., St. Paul, MN) for continuous infusion. Animals were then randomly assigned to treatment groups. After completing the test article delivery period, dose ranging and safety animals were anesthetized, blood and cisternal CSF were sampled, and the dogs underwent whole body perfusion (saline and fixative) for necropsy and histopathological examination. Animals in the pharmacokinetics phase were euthanized and underwent gross necropsy.

Animals. Male and female beagles were obtained from Marshall Farms (North Rose, NY). The animals were approximately 8 to 24 months old and...
weighed 11 to 13 kg at the initiation of treatment. Dogs were housed in individual runs in an AAALAC accredited vivarium. Water and food (Certified Teklad 25% Lab Dog Diet, Harlan Teklad, Madison, WI) were provided except during food fasting periods prior to surgery.

**Intrathecal Catheter Placement**

To permit continuous infusion in the intrathecal space, test articles were delivered by a chronically placed intrathecal catheter. After receipt in the facility, animals underwent a five to seven day acclimation period and prophylactic treatment with sulfamethoxazole trimethoprim tablets (15–23 mg/kg, po twice daily, 480 mg/tablet from day –5 to day –1). On day –3, after an overnight fast, animals were anesthetized with atropine (0.4 mg/kg, im) followed by xylazine (1.5 mg/kg, im). After intubation, animals were maintained by spontaneous ventilation under 1–2% isoflurane and 60% N\textsubscript{2}O/40% O\textsubscript{2}. Oxygen saturation, inspired and end tidal values of gas, CO\textsubscript{2}, N\textsubscript{2}O, and O\textsubscript{2}, and heart and respiratory rates were continuously monitored. Surgical areas were shaved, surgically prepared and the animal was placed in a stereotactic headholder and draped.

**Safety animals.** To catheterize the intrathecal space, a midline incision was made on the dorsal head extending 1 cm rostral from the occiput to C\textsubscript{1} and the muscles retracted to expose the cisternal membrane. A cisternal CSF sample was then taken by puncture of the cisternal membrane with a 23G needle for chemical analyses. A small opening was then made in the cisternal membrane to access the intrathecal space. The PE10 intrathecal dosing catheter, previously e-beam irradiated, was then inserted through the opening and passed approximately 43 cm to the lumbar enlargement. Placement in the intrathecal space was determined by sampling lumbar CSF from the catheter and confirmation at necropsy.

After catheter placement, dexamethasone sodium phosphate (0.25 mg/kg, im) was given. The externalized end of the catheter was plugged and passed subcutaneously with a trocar to exit the left scapular region. The muscle and skin were sutured. Anesthetic gases were then turned off and the animal was monitored during recovery. Butorphanol tartrate (Torbugesic \textsuperscript{®} 0.04 mg/kg, im) was administered to relieve postoperative pain. The surgery required approximately 1 h to complete.

Following recovery, a nylon vest (Alice King Chatham Medical Arts, Hawthorne, CA) was replaced on the animals (animals having been previously acclimated to the vest prior to surgery) and an infusion pump placed in the vest pocket where it was connected to the externalized end of the intrathecal catheter. The intrathecal catheter was continuously infused until initiation of test article treatment with 0.9% (w/v) sodium chloride for injection, USP (saline), at approximately 100 μl/h. **Pharmacokinetic animals.** For placement of two intrathecal catheters (dosing and sampling), a midline incision was made on the dorsal head extending 1 cm rostral from the occiput to C\textsubscript{1} and the muscles retracted to expose the cisternal membrane. A small opening was then made in the cisternal membrane to access the intrathecal space. The PE50 intrathecal sampling catheter was then inserted through the opening and passed approximately 40 cm. The PE10 intrathecal dosing catheter was then inserted through an adjacent opening and passed approximately 43 cm to the lumbar enlargement. Lumbar CSF was then sampled from both catheters to confirm placement in the intrathecal space. The externalized ends of the catheters were plugged and passed subcutaneously with a trocar to exit the left and right scapular regions respectively. The muscle and skin were sutured with 3-0 Vicryl \textsuperscript{™}. Anesthetic gases were then turned off and the animal was monitored during recovery. Butorphanol tartrate (Torbugesic \textsuperscript{®} 0.04 mg/kg, im) was administered to relieve postoperative pain. The surgery required approximately 1.5 h to complete.

Following recovery, a nylon vest was replaced on the animals (animals having been previously acclimated to the vest prior to surgery) and an infusion pump placed in each of the vest pockets where they were connected to the externalized ends of the intrathecal catheters. The intrathecal catheters were continuously infused until initiation of test article treatment with 0.9% (w/v) sodium chloride for injection, USP (saline), at approximately 100 μl/h. For PE50 sampling catheters, saline infusion was continued during nondosing intervals to maintain catheter sampling patency; the saline infusion was interrupted during intrathecal lumbar sampling intervals. For behavioral function, motor function and arousal assessments were performed daily throughout the in-life phase of the study as described in detail elsewhere (Yaksh et al., 1997). Physiological (heart rate, blood pressure, respiration) and nociception responses were measured at regular intervals. The thermally evoked skin twitch response was measured using a probe (approximately 1 cm surface area) maintained at approximately 62.5 ± 0.5°C. The probe was applied to shaven thoracic-lumbar areas of the back. Typically, this stimulus results in a brisk contraction of the local, underlying musculature within 1–3 s after probe placement. Upon appearance of this response, the probe was removed and the latency recorded. Failure to respond within 6 s was cause to remove the probe and assign that value (6 s) as the latency (Sabbe et al., 1994).

**Clinical laboratory measurements.** Blood and cisternal CSF samples were obtained prior to surgery and on the day of necropsy. Plasma samples were taken at specified time points during the infusion interval for drug analysis. Cisternal CSF samples for drug assays were obtained at sacrifice prior to sacrifice. Blood was obtained by cephalic venipuncture. Cisternal CSF was obtained while under anesthesia by sterile puncture of the cisterna magna with a 23-gauge needle at surgery and a 22-gauge 1.5-inch spinal needle at necropsy. At the end of the infusion interval, animals were euthanized and necropsied with histopathology performed on the spinal cord.

**Pharmacokinetics Study**

**Study paradigm.** Pharmacokinetic animals (n = 3) with dual lumbar catheters were prepared to determine (1) the clearance of DPDPE from lumbar CSF after a bolus intrathecal injection, (2) steady-state lumbar CSF levels produced by continuous infusion of two concentrations of DPDPE, and (3) the time course of clearance in lumbar CSF of DPDPE after termination of a continuous intrathecal infusion of DPDPE. Animals received an intrathecal bolus (1 ml) of the 3 mg/ml DPDPE followed by a 0.5 ml saline flush. Samples were taken periodically over the ensuing 24 h. After the 24-h sampling interval, an intrathecal infusion of 3 mg/ml (2.4 ml/day) DPDPE for 24 h was initiated. Following this infusion/sampling interval, the infusion dose was increased to 6 mg/ml (2.4 ml/day) for the following 24 h. After the 24-h infusion of DPDPE (6 mg/ml), drug infusion was terminated and samples were
collected at intervals over 24 h after termination of the drug during the clearance period.

**Necropsy.** On the scheduled date of necropsy of a safety study animal, each animal was anesthetized with sodium pentobarbital (35 mg/kg or to effect, iv). A percutaneous puncture of the cisterna magna was performed to collect cisternal CSF (approximately 2.5 ml). The chest was then opened, aorta catheterized, and the blood cleared by perfusion at 80–160 mmHg of pressure with approximately 4 l of saline (0.9% sodium chloride) followed by approximately 4 l of 10% formalin. The spinal cord dura was exposed by laminectomy of the spinal canal. Methylene blue dye was injected through the catheter to confirm catheter placement and integrity. For the pharmacokinetic studies, a similar protocol was followed with the principal exceptions being that subjects received an overdose of sodium pentobarbital (50 mg/ml, iv), cardiac perfusions were not performed, and both intrathecal catheters were assessed for patency and location by dye injection.

**Drug assay.** DPDPE was quantified by an atmospheric pressure ionization liquid chromatographic-mass spectrometric assay (see below; Rossi and Yaksh, 2002). Samples (5 μl) containing DADLE as internal standard (IS) were de-salted by reverse phase C18 solid phase extraction using ZipTip micro-cartridges. One μl aliquots of extracted eluate were injected onto an Agilent Zorbax SBC-18 column (30 × 2.2 mm; 3.5 Fm) at a flow rate of 0.4 ml/min. The isocratic mobile phase of methanol and 10 mM ammonium formate (pH 3; 75:25, v/v) was then diverted to waste for 45 s after injection, after which time flow was directed to the single quadrupole mass spectrometer, and DPDPE was detected by positive mode selected ion monitoring. Standard curves were linear (r² > 0.991) over the concentration range 1–1000 ng/ml. The efficiency of extraction recovery was 97% and the intra-assay and inter-assay precision was within 9% relative SD. DPDPE and IS were stable in the injection solvent at 4°C for at least 48 h.

**Histopathology.** For safety animals, four blocks of spinal cord (cervical, thoracic, lumbar [catheter tip region], and distal lumbar) were embedded in paraffin, sectioned, stained with haematoxylin-eosin, and examined by light microscopy by a board-certified veterinary pathologist unaware of the experimental treatment. Semiquantitative evaluations were made as to the degree of inflammation and the pathological changes in the meninges, vessels, nerve roots, and spinal cord parenchyma found in these sections (Yaksh et al., 1997). Observed tissue was assigned an individual pathology score based on the evaluation. The pathology score was on a whole number scale from 0 to 4, where 0 represented no injury or inflammation and 4 represented very severe injury and/or inflammation. Additionally, a single overall pathology ranking was assigned to each animal, using a semiquantitative scale of 1 to 12. This score reflected the ranking of that individual animal as compared with all other animals in the study.

Additional immunohistochemical analysis was performed on paraffin-embedded sections using following primary antibodies: monoclonal mouse GFAP (1:1000), rabbit TNF-α (1:1000, Serotec, Raleigh, NC), mouse antihuman macrophages MAC (1:200, Serotec). Briefly, sections were deparaffinized in xylene and rehydrated through graded alcohols. Nonspecific immunolabeling was blocked using 5% normal goat serum (NGS) in phosphate buffered saline containing 0.2% Triton X-100, pH7.4. Excess blocker was removed and mouse and rabbit primary antibodies were applied (24 h/4°C). Afterwards, sections were washed in PBS-TX 100 and incubated with fluorescence secondary antibodies, goat antimouse IgG Alexa Fluoro-488, or goat antirabbit Alexa Fluoro-594 (Molecular Probes, Eugene, OR). In addition, a nuclear marker for neuronal and nonneuronal cells, Dapi (4’, 6-diamidino-2-phenylindole dihydrochloride, Molecular Probes, OR) was added to the solution and incubated together with secondary antibodies 1 h at room temperature. Finally, sections were rinsed and cover slipped with antifade mounting medium kit (Molecular Probes, OR) and observed under a Leica fluorescence microscope. To confirm specificity of immunolabeling, sections were processed without primary antibodies or by substituting normal mouse or rabbit serum for mouse or rabbit primary sera.

**Data analysis.** Data are expressed as means and standard errors of the respective observations (e.g., mmHg, beat/min, μg/ml). For motor function, the cumulative coordination scores were obtained by adding the positive value of the individual daily motor function score for the 28 days of test article delivery. Continuous normal data were compared using repeated measures and one-way ANOVA with post hoc Bonferroni tests for multigroup comparisons. Distribution free testing of data, such as ranked spinal cord pathology, was performed using the Jonckheere test for comparison of three or more groups with post hoc Mann-Whitney U tests for multigroup comparisons. All statistical comparisons were made at the p < 0.05 level of significance.

Pharmacokinetic data were analyzed using PK Solutions 2.0 (©1999, Summit Research Services, Ashland, OH) statistical software for noncompartmental pharmacokinetic data analysis. Analysis included half-lives, descriptive curve parameters, curve area, statistical moment, and volume of distribution calculations.

**RESULTS**

**General Behavior**

All animals survived surgery and completed the scheduled infusion or dosing intervals.

Assessment of general behavioral indices throughout the study showed no systematic changes in arousal or muscle tone (data not shown) within any of the three treatment groups. No evidence of urinary retention or changes in daily stool were noted at any time during this study.

**Neurological Assessments**

No significant neurological abnormalities or motor dysfunction associated with infusion were observed in three of the four saline animals. One saline animal displayed a mild hind limb ataxia beginning on day 4 that persisted unaltered throughout the in-life phase of the study. There was a concentration-dependent development of motor dysfunction in the DPDPE treatment groups (Fig. 1). This dysfunction evolved over the 28-day interval of infusion with the mean time of onset for motor dysfunction being 11 ± 5 and 6 ± 1 days for the 3 mg/ml and 6 mg/ml DPDPE treatment groups respectively. Neurologically, the abnormalities noted included decreased hind limb reflexes (e.g., wheelchair reflex, tactile placing reflex), decreased righting reflex, and pain and discomfort upon ambulation and manipulation.

**Physiological Parameters**

The baseline (postsurgery day 0) infusion observations for the several measured parameters did not vary between the infusion groups (see Table 1).

**Body temperature and body weight.** Group mean body temperatures and weights did not significantly change compared to baseline measures for any of the treatments (see Table 1).

**Respiratory rate, blood pressure, and heart rate.** Mean respiratory rates ranged from 16 to 32 breaths/min and did not vary from baseline measures over the course of the infusions. Mean arterial pressures ranged from 96 to 127 mmHg and did not vary from baseline over the course of the infusions. Heart rates ranged from 58–162 beats/min and did not vary significantly from baseline over the infusion interval (see Table 1).
Nociceptive response. By the seventh day of infusion, skin twitch latencies in DPDPE-treated animals had increased in a dose-dependent fashion and remained elevated as compared to the vehicle control (see Fig. 2).

Necropsy. At necropsy, all animals’ catheters were confirmed to be in the intrathecal space with the dura intact and catheter tips located in the proximity of T10-L4. Methylene blue dye injected at necropsy confirmed that all catheters were patent and that dye diffused evenly rostrally and caudally in the intrathecal space.

Gross pathology. At necropsy, vehicle and 3 mg/ml DPDPE-treated animals showed no remarkable changes in external spinal cord morphology upon gross examination. Two of four 6 mg/ml DPDPE treated animals’ spinal cords showed some discoloration and erosion of the dura in the vicinity of the catheter tip.

Histopathology
Overview. In vehicle-treated animals, a modest pericatheter reaction was observed at all levels of the catheter with no significant masses or changes in spinal cord morphology (Fig. 3). This experimental group showed minimal reaction as defined by glial reaction (GFAP) or macrophage infiltration. In contrast, in the high dose DPDPE-infused animals, significant localized collections of inflammatory cells (granulomas) occurred with severe multifocal inflammation and mild to moderate damage to the neuropil (Figs. 3 and 4). In the 3 mg/ml DPDPE group, lesions were similar in nature though considerably less severe. Dorsal root ganglia were not affected.

Pathology ranking. Assignment of an overall pathology rank across groups indicated a dose-dependent response and the individual ranking is presented in Table 2. Comparison of the rank-ordered overall pathology using the Jonckheere statistical test for ordered alternatives revealed the results to be proportional to dose ($p < 0.05$).

Parenchyma and granuloma histochemistry. The large well-organized inflammatory masses in the 6 mg/ml DPDPE-infused dogs were always present proximal to the catheter tip.

TABLE 1
Mean Baseline Physiological Parameters (± SEM)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>6 mg/ml</th>
<th>3 mg/ml</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body temp. (°F)</td>
<td>101.3 ± 0.2</td>
<td>101.7 ± 0.1</td>
<td>101.5 ± 0.3</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>12.4 ± 1.0</td>
<td>12.9 ± 0.7</td>
<td>12.9 ± 0.9</td>
</tr>
<tr>
<td>Resp. rate (breaths/min)</td>
<td>19 ± 3</td>
<td>32 ± 1</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>103 ± 11</td>
<td>113 ± 5</td>
<td>105 ± 8</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>110 ± 10</td>
<td>115 ± 14</td>
<td>78 ± 10</td>
</tr>
<tr>
<td>Skin twitch latency (s)</td>
<td>1.3 ± 0.3</td>
<td>1.9 ± 0.1</td>
<td>1.4 ± 0.1</td>
</tr>
</tbody>
</table>

FIG. 1. The individual animal 28-day cumulative coordination score versus spinal pathology ranking and the mean cumulative coordination scores by treatment group. Open circles, saline animals; gray circles, 3 mg/ml DPDPE animals; dark circles, 6 mg/ml DPDPE animals. Rank order was significant and determined to be dose-dependent with the Jonckheere test for ordered alternatives.

FIG. 2. The mean skin twitch latencies (± SEM) for the 6 mg/ml, 3 mg/ml, and saline infusion groups assessed periodically over the 28-day infusion interval.
FIG. 3. The H-E staining of spinal cord tissue for DPDPE and control animals: (A) vehicle-treated animal at the thoracic level; (B) 6 mg/ml DPDPE treated animal, cervical level; (C) 6 mg/ml DPDPE-treated animal, thoracic level; (D) 6 mg/ml DPDPE treated animal, lumbar (above catheter tip) level. Scale bar A–D 300 μm.

FIG. 4. H-E staining, in coronal section taken at the L2 segment of a dog receiving 6 mg/ml DPDPE. (A) Formation of large granulomatous mass (G) in the ventrolateral area. Note the unilateral compression of the ventral white matter (WM) at the site of granuloma. (B) The catheter tip (*) was occupied by many inflammatory cells. Note the penetrating collections of inflammatory cells from the granuloma around the ventral spinal artery and directly through the pia mater to the spinal cord parenchyma (arrows). (C) Appearance of vascular profiles surrounded by inflammatory cells in the gray (arrows) and white matter (double arrows). Note that vessels were limited to the vicinity of the granuloma, in both the white (C′) as well as gray (C″) matter. No changes were found in the dorsolateral area. (D, D′) The granuloma contained mixed infiltrates of inflammatory cells, small to medium sized vessels lined by thin or thick perivascular cuffs, as well as mixture of necrotic (N) or fibrotic (F) tissue. (D′) Detail from D. (E) The thickness of dura at the site of granuloma (ventral) was considerably increased, as compared to the dura on the dorsal aspect. Scale bar 1600 μm in A, 600 μm in B, C, D, 120 μm in C′, C″, 500 μm in D′, 200 μm in E.

TABLE 2
Inflammation Scoring and Ranking by Individual Animal

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Animal ID</th>
<th>Severity (lumbar)</th>
<th>Overall ranking</th>
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<tbody>
<tr>
<td>6 mg/ml DPDPE</td>
<td>3090434</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>2987384</td>
<td>4</td>
<td>12</td>
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<tr>
<td></td>
<td>2990113</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>3 mg/ml DPDPE</td>
<td>2960231</td>
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<td></td>
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<td>2</td>
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<td>1</td>
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<td></td>
<td>2922347</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3000885</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

*Severity: 0, within normal limits; 1, slight inflammation; 2, mild inflammation; 3, moderate inflammation; 4, severe inflammation.

Jonckheere statistical test for ordered significance revealed the pathology ranking to be proportional to dose (p < 0.05).
in the lumbar segments and not in upper thoracic and cervical segments (Fig. 3). As indicated, the granuloma arose largely from the dura-arachnoid and was constituted of a mixture of inflammatory cells, with lymphocytes and macrophages representing the dominant cell population. Histological examination revealed necrosis or fibrosis within the mass formation (Fig. 4). The spinal dura adjacent to the granuloma site displayed an increased thickening (compare Figs. 4A, 4D, and 4E). Vascular profiles surrounded by mono- or multiple layers of inflammatory cells could be detected in the spinal cord parenchyma (Figs. 4C and 4C’). Thus, multifocal inflammatory reaction along with focal neuropil destruction represented the major type of tissue alterations in high dose DPDPE-treated dogs (Figs. 4C, 4C’, 4D, and 4D’).

Additional immunocytochemical staining characterized the cellular composition of granuloma masses with respect to glial elements, pro-inflammatory cytokines, and macrophages. Normal patterns of parenchymal staining, as evidenced by Dapi immunoreactivity intermixed with GFAP positive astrocytes, was observed in spinal cord segments above the catheter tip (Fig. 5C). At the level of the inflammatory mass, GFAP positive cells were observed in adjacent tissue and not in the mass. The perivascular cuffs in the parenchymal white matter (Fig. 5A) were surrounded by normal astrocytic figures (Fig. 5B). In contrast, occasional reactive gliosis limited to the regions of vessels in the spinal cord parenchyma (Figs. 5D and 5E) or to the spinal cord compression could be detected. However, small areas of attenuated GFAP staining could be detected in nearby regions of significant compression indicating significant cell loss (Fig. 5E). No evidence of bacterial or fungal involvement could be detected, based on histological stains (data not shown).

The inflammatory masses typically contained extensive accumulation of inflammatory cells expressing TNF-α (Fig. 6A). Some perivascular cuffs also revealed moderate TNF-α positive infiltrates. The MAC-positivity (Fig. 6B), expressed by a population of granulocytes, monocytes, and by tissue macrophages, was displayed throughout the entire granuloma, and probably represents the majority of cell population synthesizing TNF-α. These intensely stained macrophages were often infiltrating the granulomatous tissue, and invading the inner surface of vessel profiles (Figs. 6B and 6B’). Similar patterns of TNF-α and MAC-immunopositivity occurred in the spinal cord parenchyma and the perivascular cuffs (Figs. 6C and 6D). The co-localization picture (Fig. 6E) confirmed that the TNF-α was present in a majority of the macrophages.

Clinical Chemistries

Hematology. No changes observed in hematology values were considered to represent any systematic changes associated with drug or saline infusion (data not shown).

Cisternal CSF. Cisternal CSF in the control animals displayed a slight increase in protein concentration. DPDPE infused groups displayed marked increase in protein concentrations compared to baseline measures. There were no significant changes in cisternal glucose concentrations or white blood cell counts in any group (see Table 3). Comparison of the rank-ordered increase in cisternal protein concentrations with the Jonckheere statistical test for ordered significance revealed the increases to be proportional to dose (p < 0.05).

Cisternal DPDPE concentrations. DPDPE levels in the cisterna magna at necropsy displayed mean levels of 2.5 ± 2.2 μg/ml in the 6 mg/ml group and substantially more, 19.4 ± 8.5 μg/ml in the 3 mg/ml DPDPE group. No DPDPE activity was detected in the saline group (see Table 3).

Pharmacokinetics

Bolus treatment. The bolus kinetics data for individual animals are presented in Figure 7. The calculated values for the three animals and the mean parameters are presented in Table 4. As indicated, after bolus delivery of 3 mg/ml, there was biphasic clearance of the DPDPE from the lumbar intrathecal space, with τ/2D and T1/2E values of 14.0 ± 10.3 and 100.0 ± 17.2 min, respectively. Examination of the concurrent inulin data and the DPDPE/inulin ratio indicate that DPDPE was cleared more rapidly than the extracellular marker during the initial distribution phase.

Continuous infusion and clearance. The data for continuous infusions and drug clearance for individual animals are presented in Figure 8. With chronic infusion of 3 mg/ml and 6 mg/ml, each for 48 h, steady-state DPDPE concentrations in
FIG. 5

FIG. 6
lumbar CSF were 18.59 ± 1.0 and 22.58 ± 4.02 μg/ml infusions, respectively. After the termination of the 6 mg/ml infusion, lumbar DPDPE levels fell in a monophasic fashion.

Kinetic parameters calculated for this clearance are presented in Table 5. As indicated, the clearance phase, T₁/₂E, was on the order of 65.7 ± 8.1 min, a value that corresponds well with the T₁/₂E calculated for the bolus delivery.

**DISCUSSION**

**Behavioral Effects of Intrathecal DPDPE Infusion**

The intrathecal delivery of DPDPE resulted in a dose-dependent increase in the skin twitch response latency within 24 h of initiating spinal drug infusion. This acute increase is reflective of the increased intrathecal concentration that was noted to occur in lumbar CSF with continuous infusions of the two DPDPE concentrations. The observed change in thermal escape latency occurred in the absence of any early changes in motor function. The skin twitch is a C fiber mediated polysynaptic reflex that reflects opiate sensitive spinal nociceptive processing (Doucette et al., 1987; Houde and Wikler, 1951). Accordingly, the present observations in dogs are consistent with an extensive literature that emphasizes the antinociceptive effects of spinal opioid agonists. Previous studies with a moderately selective versus opioid selective agonist (d-alad2-d-leu 5- enkephalin) showed antinociception in rats (Tung and Yaksh, 1982), dogs (Atchison et al., 1986), primates (Yaksh, 1983), and humans (Onofrio and Yaksh, 1983). DPDPE is a highly δ-selective agent (Mosberg et al., 1983) and has been shown to be analgesically active in rats after intrathecal delivery (Porreca et al., 1987) and now also in a large animal model.

Over the period of infusion there was a progressive loss of the inhibition of the skin twitch response latency. Previous work has shown that such extended exposure to μ and δ opioid agonists leads to a pharmacological tolerance wherein the antinociceptive effects are diminished (Russell and Chang, 1989; Sabbe et al., 1994; Stevens and Yaksh, 1992). While pharmacological tolerance is a likely mechanism of the observed changes in antinociception, the cisternal levels of DPDPE (and perhaps in the lumbar intrathecal space) were substantially lower than expected in those safety animals receiving 28-day high concentration infusions. As will be noted below, all of these high concentration animals displayed sig-
significant inflammatory masses and we hypothesize that this lower DPDPE concentration might reflect a rapid clearance of the drug from the lumbar CSF. If so, this increased clearance might also contribute to the loss of antinociception observed here.

In addition to the antinociceptive action, over the 28-day infusion interval, dogs receiving the higher concentrations of DPDPE showed a reliable and progressive onset of hind limb dysfunction. The increased motor tone and time course suggested a gradually developing upper motor neuron type lesion.

**Histological Effects of Intrathecal DPDPE Infusion**

After the 28-day intrathecal infusions, histology revealed modest pericatheter reactions in vehicle infused dogs, consistent with a typical local intrathecal meningeal reaction to a foreign body, such as the catheter (Weller, 1999). In contrast, in animals receiving the δ opioid agonist DPDPE, we observed a concentration-dependent development of a compressive intrathecal mass proximal to the catheter tip. Inspection of serial sections suggested that the inflammatory mass appeared to take origin from the dura-arachnoid layer. Cellular constitution of the mass consisted of multifocal accumulations of neutrophils, monocytes, macrophages, and plasma cells, resembling a granuloma (Adams and Hamilton, 1989; Sheffield, 1990).

No evidence of bacterial or fungal involvement could be detected, based on histological stains or CSF cultures. This inflammatory response was associated with increased cisternal CSF protein and the progressive development of neurological signs consistent with a compressive spinal cord lesion.

These effects were most evident in the groups receiving 6 mg/ml. Given the restricted distributional kinetics of the intrathecal space, the current thinking points to the importance of local drug concentration as an important variable in the tissue toxicity produced by intrathecally infused drugs (Yaksh et al., 1999b). The steady state CSF concentration of DPDPE adjacent to the catheter tip observed in the present study at 6 mg/ml was on the order of 25 μg/ml.

**Intrathecal DPDPE Kinetics**

The intrathecal delivery of DPDPE resulted in a biphasic decline in concentration, reflecting an initial redistribution and a second slower clearance that reflect the movement for the injection site by bulk redistribution in the CSF and into the tissue from the CSF (Shafer and Shafer, 1999). Systematic examination for the presence of DPDPE metabolites was not undertaken in the present study. However, previous work has indicated it to be remarkably stable after incubation in blood or brain tissue, a finding consistent with its cyclic structure (Weber et al., 1992). The T_{1/2E} calculated after bolus delivery was comparable to that observed when the clearance from steady state was determined in the continuous infusion experiments, suggesting that the kinetics of he agent after bolus or contin-

### TABLE 4

<table>
<thead>
<tr>
<th>3 mg/ml DPDPE Bolus Kinetics Summary</th>
<th>Dog 3066002</th>
<th>Dog 3018644</th>
<th>Dog SBX-8</th>
<th>Mean ± SEM</th>
<th>Dog population</th>
</tr>
</thead>
<tbody>
<tr>
<td>t_{1/2D} (min)</td>
<td>4.2</td>
<td>30.7</td>
<td>7.0</td>
<td>14.0 ± 10.3</td>
<td>15.4</td>
</tr>
<tr>
<td>t_{1/2E} (min)</td>
<td>109.4</td>
<td>118.1</td>
<td>72.4</td>
<td>100.0 ± 17.2</td>
<td>95.9</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>187.4</td>
<td>207.1</td>
<td>124.4</td>
<td>173.0 ± 30.5</td>
<td>184.2</td>
</tr>
<tr>
<td>AUC, (μg/ml/min)</td>
<td>1695.5</td>
<td>3202.5</td>
<td>1565.8</td>
<td>2154.5 ± 643.4</td>
<td>2082.5</td>
</tr>
<tr>
<td>V_d (obs. area)</td>
<td>284.3</td>
<td>160.7</td>
<td>200.9</td>
<td>215.3 ± 44.6</td>
<td>200.3</td>
</tr>
</tbody>
</table>

*Note.* Bolus volume = 1 ml. t_{1/2D}, distribution half-life; t_{1/2E}, elimination half-life; MRT, mean residence time; AUC, area under the curve; V_d, volume of distribution.

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**FIG. 8.** This figure presents the drug kinetics following 3 mg/ml and 6 mg/ml DPDPE infusions and washout. The clearance interval began at the conclusion of the 24-h 6 mg/ml infusion. The 6 mg/ml infusion was terminated and the time was recorded as time 0 for the clearance phase. Infusion and clearance interval was approximately 24 h. Low levels of drug at time 0 are due to prior 3 mg/ml DPDPE bolus treatment approximately 24 h prior to infusion initiation.
uous infusion were similar. The half life of DPDPE resembles that previously reported in this model for molecules such as inulin, brain derived nerve growth factor, and ziconotide, an N-type calcium channel blocker (Yaksh et al., 1997, 1999a). With continuous infusion, steady states were achieved by 8 h, a finding consistent with the estimated lumbar CSF elimination half-life of DPDPE.

In the safety studies, cisternal CSF was sampled at the time of sacrifice. As indicated, the levels of cisternal DPDPE in the high-dose animals were considerably less than those observed in animals receiving lower doses. This unexpected finding has been similarly observed in recent work with intrathecal morphine where a similar granuloma formation in dogs showing high levels of granuloma formation supraspinal redistribution was attenuated as evidenced by reduced cisternal concentrations (Yaksh et al., in press). While lumbar CSF levels in animals displaying inflammatory masses have not yet been assessed, this reduced cisternal redistribution may reflect an increased local clearance and/or metabolism. It is currently appreciated that intrathecal drug can diffuse though the meninges (Ummenhofer et al., 2000; Zenker et al., 1994). To the degree that the inflammatory mass damages local meningeal integrity in the vicinity of the catheter tip, a more pronounced clearance of local drug may occur.

**Nonspecific Mechanisms Initiating Intrathecal Inflammatory Reactions**

Several variables contributing to the triggering of this intrathecal granuloma may be considered. (1) Absence of positive CSF or injectate cultures or histological signs of fungal or bacterial infection suggests that the granuloma is not an infectious process. (2) The DPDPE solutions delivered spinally in a concentration-dependent fashion (Heagy et al., 1995; Sharp et al., 2000; Wick et al., 1995), as well as on vascular endothelial cells (Stefano, 1998). In recent preliminary work we have identified significant binding of δ opioid receptor targeted antibody in dog granulomas (Sommers, Cizkova, and Yaksh, unpublished observations).

While the role played by a δ opioid receptor in the development of a granuloma with intrathecal DPDPE is not certain, it is clear that δ opioid agonists have potent effects upon thymocyte activation and T-cell proliferation (Bidlack, 2000). Thus, T-lymphocyte chemotaxis is activated by DPDPE in a concentration-dependent fashion (Heagy et al., 1990). Endothelial cells also display δ opioid binding, and local application of δ opiate agonists resulted in enhanced granulocyte adherence (Stefano, 1998). Consistent with this activating influence on inflammatory cells, δ agonists can evoke the formation of pro-inflammatory cytokines (TNF-α and IL1β) from macrophages and free radical formation by neutrophils (Gomez-Flores et al., 2001; Haberstock and Marotti, 1995; Nelson and Lysle, 2001). As noted in the present studies, significant expression of TNF-α was observed in the granuloma and in macrophages in the perivascular cuffs. Accordingly, given the observed origin of the mass, we suggest that the inflammatory cells derive from this dural microvasculature, which lies in proximity to the catheter tip (Kerber and Newton, 1973). Given the above body of observations, we are inclined to speculate that with persistent exposure to high concentrations of a δ (or

**TABLE 5**

<table>
<thead>
<tr>
<th>Dog population</th>
<th>Dog 3066002</th>
<th>Dog 3018644</th>
<th>Dog SBX-8</th>
<th>Mean ± SEM</th>
<th>Dog population</th>
</tr>
</thead>
<tbody>
<tr>
<td>t½D (min)</td>
<td>78.9</td>
<td>58.6</td>
<td>59.5</td>
<td>65.7 ± 8.1</td>
<td>75.3</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>177.7</td>
<td>331.4</td>
<td>418.4</td>
<td>309.2 ± 86.2</td>
<td>238.2</td>
</tr>
<tr>
<td>AUC (μg/ml/min)</td>
<td>5425.8</td>
<td>1683.6</td>
<td>1746.8</td>
<td>2952.1 ± 1515.0</td>
<td>3118.6</td>
</tr>
<tr>
<td>Vd (obs. area)</td>
<td>126.6</td>
<td>307.6</td>
<td>304.4</td>
<td>246.2 ± 73.2</td>
<td>213.7</td>
</tr>
</tbody>
</table>

*Note.* t½D, distribution half-life; t½E, elimination half-life; MRT, mean residence time; AUC, area under the curve; Vd, volume of distribution.
μ) opioid agonist, a cascade is initiated that is mediated in part by an action upon local endothelial cells that serve to increase cytokine release. This then leads to a local increase in T cell and granulocyte migration through the dural capillary network. We recognize that in the chronic implant model there is a modest ongoing stimulus that is provided by the catheter. This low level stimulation may, on occasion, also provide moderate levels of cytokines that become sufficient to initiate a local aseptic reaction that leads to enhanced inflammatory cell migration. These inflammatory cells lead to an additional local release of cytokines (TNF-α) that can initiate additional inflammatory responses, including the activation of local dendritic cells (McMenamin, 1999). Mu and delta opioid agonists have been shown to facilitate the stimulatory effect of dendritic cells on T-cell proliferation (Makarenkova et al., 2001). We again emphasize that additional studies are required to reliably implicate the role of δ opioid receptors in this proposed cascade.

Clinical Relevance of Intrathecal Granuloma Formation

We believe that the present findings indicating a concentration-dependent granulomatous reaction to continuous infusions of intrathecal DPDPE and recent parallel findings with chronic intrathecal morphine have particular relevance to the clinical utilization of these agents. While DPDPE has not been administered in humans, morphine has been widely used as a continuously infused agent since 1981 (Onofrio et al., 1981). Until the 1990’s, no untoward events as noted here were reported (Wallace and Yaksh, 2000). At that time, the concentrations of morphine placed in pumps were increased to obtain longer pump refill intervals. Since that time, six clinical case reports describe 13 catheter patients in whom a local compressive lesion was initially manifested as a progressive motor or sensory dysfunction (Anderson et al., 2001; Bejjani et al., 1997; Blount et al., 1996; Cabbell et al., 1998; Coombs et al., 1985; Langsam, 1999; North et al., 1991). Recently, a comprehensive retrospective review has been published that reflects experiences with an additional 45 instances of human chronic morphine infusion patients with comparable symptomatology (Coffey and Burchiel, 2002). In the published case reports, the mass was identified by imaging and/or by subsequent resection. There is no evidence of infection (Anderson et al., 2001; Bejjani et al., 1997; Cabbell et al., 1998). Histology has emphasized the presence of macrophages, neutrophils, and monocytes, with a necrotic center, a description closely paralleling that which we have noted with intrathecal DPDPE and morphine in dogs (present study; Allen et al., 2002). Importantly, as with the previous work with dog infusions, this problem has not been reported in patients receiving bactofen for spasticity, suggesting a specific drug effect (Coffey and Burchiel, 2002; Penn, 1992).

The importance of the present studies is that they reveal the potential of granuloma formation as an important component of potential toxicity in the use of spinal delivery. The intrathecal space has become increasingly appreciated as an important route of delivery for drugs to manage a variety of syndromes including pain, spasticity, and obesity for the treatment of meningeocarcinomatosis, spinal infections, and neuropathologies, such as degenerative disorders with growth factors (see Yaksh, 1999). In many cases, the proposed therapy requires a persistent drug exposure. Aside from the potential direct tissue toxicity that could arise from such a local drug action, the current evidence suggests that an important factor may well be the evolution of space occupying granulomas. Our understanding of how this collection of inflammatory cells develops will be important in further implementing this route of drug delivery. The observations here suggest that the canine model will provide an important vehicle for assessing those mechanisms.

ACKNOWLEDGMENT

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REFERENCES


