Safety Evaluation of Intrathecal Substance P-Saporin, a Targeted Neurotoxin, in Dogs


*Department of Anesthesiology, University of California, San Diego, La Jolla, California 92093; †Neurosystems Center, Department of Preventive Sciences, University of Minnesota, Minneapolis, Minnesota 55455; ‡VA Medical Center, Minneapolis, Minnesota 55417; §Department of Pathology, Oregon Health Science Center, Portland, Oregon 97239; ¶Campus Veterinary Medicine, University of California, San Diego, La Jolla, California 92093; and ¶Advanced Targeting Systems Inc., San Diego, California 92121

Received November 3, 2005; accepted January 10, 2006

Intrathecal (IT) substance P-Saporin (SP-SAP), a 33-kDa–targeted neurotoxin, produces selective destruction of superficial neurokinin 1 receptor (NK1r)–bearing cells in the spinal dorsal horn. In rats, SP-SAP prevents the formation of hyperalgesia and can reverse established neuropathic pain behavior in rodents. To determine the safety of this therapeutic modality in a large animal model, beagles received bolus IT lumbar injections of vehicle, SP-SAP (1.5, 15, 45, or 150 µg), or a nontargeted preparation of saporin (SAP, 150 µg) for immunohistological analysis of spinal cords. Doses of 15 µg SP-SAP and above produced a significant and equivalent loss of NK1r-bearing cells and dendrites in lumbar laminae II and I compared to vehicle- or SAP-treated animals. Cervical regions in all animals displayed no loss of NK1r immunoreactivity as compared to controls. Total numbers of neurons in the lumbar dorsal horn or alpha-motor neurons in the ventral horn demonstrated no significant changes. No increases in Fos expression were noted in the astrocytic marker glial fibrillary acidic protein were noted following treatment with SP-SAP, suggesting a lack of generalized neurotoxicity. Additional dogs received doses of 1.5–150 µg SP-SAP or SAP and were sacrificed after 28 or 90 days to assess behavioral and physiological parameters. Although some acute motor signs were observed with both SP-SAP and SAP, no long-lasting significant events were noted in any of these animals. These data indicate no adverse toxicity at doses up to 10 times those necessary for producing loss of superficial NK1r-bearing neurons in a large animal model.

Key Words: spinal; neurotoxin; neurokinin 1 receptor; substance P; saporin.

Pain, secondary to tissue injury, results from the activation of small primary afferents that contain and release several neurotransmitters in the spinal dorsal horn including the peptide substance P (SP). SP is known to evoke excitation in second-order dorsal horn neurons by an interaction with the neurokinin 1 receptor (NK1r). Following high-intensity stimuli, but not low-intensity stimuli, SP is released from primary afferents activating NK1r on second-order neurons (Dionne et al., 1998; Honore et al., 1999, 2002; Mantyh, 2002; Mantyh et al., 1995). These neurons then project rostrally to brain stem and higher centers, which integrate nociceptive input. Thus, these NK1r-bearing superficial laminae I and II neurons are believed to play a crucial role in spinal pain processing (Todd, 2002). Studies in rodents with NK1r antagonists (Gonzalez et al., 1998; Rupniak et al., 1993; Seguin et al., 1995; Yaksh et al., 1997; Yamamoto and Yaksh, 1991) or NK1r-directed antisense (Hua et al., 1998) and clinical trials with oral NK1r antagonists (Dionne et al., 1998) have shown only moderate analgesic efficacy. Similarly, NK1r knockout mice have demonstrated only modest changes in the maintenance of hyperalgesia (Mansikka et al., 1999). These small changes in pain behavior following spinal NK1r blockade or knockout are likely due to the fact that the spinal NK1r–bearing neurons receive multiple excitatory input from primary afferents via a variety of neurotransmitters, most notably glutamate.

An alternate strategy to simply blocking the SP input has been to lesion specifically this subset of NK1r neurons by the use of a targeted neurotoxin substance P-saporin (SP-SAP). This agent consists of a conjugate of the 11-amino acid SP peptide and the 30-kDa ribosome-inactivating protein saporin (SAP). The SP-SAP conjugate binds to the G-protein–linked NK1r with an affinity approximately equal to that of SP alone, and the receptors, along with the conjugate are internalized. Once internalized, the complex is sequestered into endosomes. SAP escapes the endosomes and prevents protein synthesis by nicking ribosomal RNA (Stirpe et al., 1987). This intracellular SAP produces a loss of function within 4 days and cell death within approximately 7 days, suggesting a lack of acute cellular lysis (Mantyh et al., 1997). The conjugate has an effective dose₅₀ potency in the low nanomolar range for the NK1r-containing cells, and the selectivity of lesions produced by SP-SAP is due...
to the fact that SAP does not cross cellular membranes at concentrations below 100nM (Wiley and Lappi, 1997).

In previous works in rodents, bolus intrathecal (IT) delivery of SP-SAP produced a significant reduction in the number of superficial NK1r-positive neurons. This depletion had no effect upon acute nociceptive thresholds but blocked the facilitated state initiated by persistent small afferent input and resulted in a potent and persistent loss of hyperalgesia in rats (Mantyh et al., 1997; Nichols et al., 1999; Vierck et al., 2003). Studies in rats with nerve injury–induced hyperalgesia also displayed reversal of hyperalgesia after IT SP-SAP (Nichols et al., 1999). Thus, SP-SAP apparently prevents the development of hyperalgesia without producing anesthesia or acute analgesia. These properties have led to an interest in the utility of SP-SAP for treatment-resistant pain states in humans.

The internalization of the conjugate via the NK1r depends on the presence of SP. As noted above, extracellular cleavage of the conjugate will render the molecule nonfunctional. This property is advantageous in that it is suspected that little rostral-caudal diffusion of the active conjugate will occur, thus allowing regional localization of its effects and reducing the likelihood of action in nontargeted regions, such as the brain stem or cerebral cortex. However, this susceptibility to degradation, likely by peptidases, brings into question the ability of the intact conjugate to diffuse along the radial axis of the spinal cord to reach the target dorsal horn neurons in larger species, including humans.

The potential clinical implementation of this molecule requires an initial assessment of its efficacy in a larger spinal cord such as the dog and characterization of the long-term effects over a wide range of doses. In the present studies, we sought to (1) determine the minimum dose of IT SP-SAP which leads to a loss of NK1r-bearing cells in the superficial dorsal horn; (2) define the effects upon physiology and spinal morphology in multiples of the minimally effective dose at intervals out to 3 months; and (3) define the time course of clearance of SP-SAP in lumbar cerebrospinal fluid (CSF) and spinal tissue after lumbar IT delivery.

**MATERIALS AND METHODS**

**SP-SAP and SAP**

Sterile solutions of SP-SAP (LOT no. 21–33), SAP (LOT no. 22–32), and phosphate-buffered saline (PBS) were provided by Advanced Targeting Systems, Inc. (San Diego, CA) and stored at –70°C until use. Pyrogenicity of SP-SAP was assessed by an limulus amebocyte lysate assay (Associates of Cape Cod, Cape Cod, MA). SAP Lot no. 22–32 contained 0.22 EU/mg, and SP-SAP lot no. 21–33 contained 0.8 EU/mg. SAP in these experiments is a recombinant cysteine-containing form of native SAP.

**Animals Preparation and Dosing**

These studies were conducted following approval from the University of California San Diego Institutional Animal Care and Use Committee.

Male beagle dogs (10–15 kg, Marshall Farms, North Rose, NY, or Covance, Princeton, NJ) aged 12–16 months were obtained and allowed a minimum 5-day period of acclimation. After a normal neurological examination and assessment of normal clinical chemistry, the animals were entered into the study.

**IT Catheter Placement**

In brief, after an overnight fast, animals received atropine (0.4 mg/kg, im) prior to sedation with xylazine (1.5 mg/kg, im). Anesthesia was induced by mask using isoflurane, and the animals were intubated and maintained under spontaneous ventilation with approximately 1–2% isoflurane and 50% N2O/50% O2. Animals were continuously monitored for oxygen saturation and for the inspired and end tidal values of isoflurane CO2, N2O, and oxygen. Heart and respiratory rates were also continuously monitored. Surgical areas were shaved and prepared with chlorhexidine scrub and solution.

Surgery was performed in an Association for Assessment and Accreditation of Laboratory Animal Care- accredited sterile operating suite. Rigid aseptic precautions were followed. The IT catheter was constructed from polyethylene tubing (PE-10, outer diameter = 0.61 mm or PE-50, outer diameter = 0.965 mm, Clay Adams), which was sterilized by E-beam irradiation (Sterigenics, San Diego, CA).

To catheterize the lumbar IT space, the dog was placed in a head holder, a midline incision was made on the lower dorsal skull, and the cisternal membrane was exposed with a combination of blunt and sharp dissection. A small incision was made in the cisternal membrane, and the PE-10 infusion catheter was passed approximately 40 cm to the L2-3 level. The catheter was externalized percutaneously on the back of the neck. At the end of surgery, all wound layers were closed with Vicryl. The animals were allowed to recover from anesthesia and then given butorphanol (0.04 mg/kg, im) for postoperative pain relief.

Animals were allowed to recover from surgery 48–72 h at which time they received a single IT bolus injection of PBS, SP-SAP, or SAP. At approximately 24 h postdosing, dogs were briefly anesthetized as noted above, and the catheters were removed. This was performed to prevent the confounding variable of the extended presence of the IT catheter potentially masking mild neurotoxic effects due to dosing.

**CSF Pharmacokinetic Studies**

Dogs for these nonrecovery studies were sedated, anesthetized, and implanted as above using aseptic techniques. In addition to the PE-10 dosing catheter, a CSF-sampling catheter made of PE-50 was implanted to terminate approximately 5 cm distal to the tip of the injection catheter. Animals remained under isoflurane anesthesia and received a single dose of 150 μg SP-SAP or SAP in a 0.5 ml bolus through the PE-10 catheter followed by a 0.3-ml saline flush to clear the catheter of residual SP-SAP or SAP. In addition to the physiological parameters listed above, blood pressure was monitored via a tail cuff (Critikon DynaMap 8100, GE Healthcare, Waukesha, WI). Lactated ringers, diazepam, or xylazine were given as necessary to maintain physiological parameters within normal ranges.

**Drug Delivery**

SP-SAP was diluted in PBS, 0.1M, pH 7.4, and kept at ~20°C until use. SAP was used as a control for nonspecific SAP effects and was also diluted in PBS. For the control group, PBS alone was injected. All injections were performed using a 0.5-ml bolus delivered over approximately 30 s through the PE-10 catheter. The drug injection was followed by a 0.3-ml saline flush to clear the catheter of the residual SP-SAP.

**Dosing Groups**

Table 1 summarizes the animals prepared for each treatment group. The parameters of each group are presented below.


**TABLE 1**

<table>
<thead>
<tr>
<th>Study Treatment Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group no.</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>

*Pharmacokinetic animals.

**Behavioral Indices**

Specific behavioral indices chosen to assess were state of arousal, muscle tone, and motor coordination. These assessments were observed and recorded twice daily. Scores of 0–3 were assigned for motor coordination and a score of −3 to 3 for arousal and muscle tone, with 0 being normal function and −3 or 3 being severely abnormal as described in detail elsewhere (Yaksh et al., 1997). Motor dysfunction scores were calculated by adding the absolute values of the motor coordination and muscle tone scores for the morning evaluations, producing a minimum score of 0 (normal) and a maximal score of 6. Any animal displaying agitation or signs of pain was treated with butorphanol or buprenorphine.

**Sacrifice and Tissue Collection**

On the designated day of sacrifice, blood samples were collected for clinical chemistries and hematology. At the designated time, animals were deeply anesthetized with sodium pentobarbital iv (35 mg/kg), intubated, and ventilated with an ambulatory bag using room air. A cisternal CSF sample for clinical chemistries was obtained by a percutaneous puncture of the cisternal membrane with a 22-gauge spinal needle. After CSF collection, the chest was opened, the aorta catheterized via a left ventriculotomy, and the animal was perfused with approximately 4 l of 0.9% saline followed by approximately 4 l of 10% neutral buffered formalin. The dura of the spinal cord was exposed by laminectomy of the spinal canal and lower brain stem. The macroscopic appearance of the spinal cord and dura was noted. The cord was blocked (taking care to keep the dura intact) into four sections: cervical, thoracic, high lumbar (catheter tip region), and lower lumbar/sacral for subsequent analysis. In randomly chosen animals across doses, spinal cords, occipital, and frontal lobe, samples were set aside for analysis and measurements of NK1r histochemistry. For immunohistochemistry, spinal blocks were obtained and placed in formalin for 24 h at which time they were transferred to 30% (wt/vol) sucrose in PBS. For histopathology, tissue was immersed in formalin and then submitted for paraffin embedding and H&E staining.

For SP-SAP spinal distribution studies, animals were heparinized (500 U/kg, iv) and sacrificed with Euthasol (iv) while under isoflurane anesthesia. Blood was cleared by incision of the internal and external jugular veins. The spinal cord was removed and the localization of the dosing catheter tip noted. One-centimeter blocks were obtained, five rostral and five caudal to the catheter tip. Blocks were also obtained in the cervical and lower lumbar/sacral region, which were approximately 18 cm rostral and 10 cm caudal, respectively, from the catheter tip. The dura was removed, and spinal cord segments were frozen in isopentane on dry ice for the analysis of SAP content.

**Clinical Chemistry**

As noted above, blood was collected for clinical chemistries and hematology prior to surgery, and CSF for clinical chemistries was collected at the time of surgery to obtain baseline data. At necropsy prior to sacrifice, blood and cisternal CSF were again collected and submitted for the analysis of clinical chemistries.

**Histology**

**Intravenous administration.** A single dog received an intravenous bolus of 150 µg SP-SAP, and blood samples were obtained at 0, 5, 15, 45, 90, 180, 360, and 480 min.
TOXICOLOGY OF SPINAL SUBSTANCE P-SAPORIN 289

RESULTS

Spinal Immunohistochemistry

IT Control Animals

In control dogs (IT PBS), (Fig. 1A), NK1r immunoreactivity was found to be densely present in superficial dorsal horn neurons with both large and small cell bodies. Frequently, these cells displayed dendrites, which arborized widely in a dorsal direction. In laminae IV and V (Fig. 1B) fewer cells were noted, and these were typically magnocellular in character. Identification of these cells was confirmed by demonstrating colocalization of NK1r immunoreactivity with CGRP in the ventral motor horn and with NeuN in the dorsal horn (data not shown). Absorption of the primary antibodies with the respective epitopes abolished immunoreactivity. As indicated in Figures 1C and 1D, IT injection of SAP (150 µg) had no effects upon the appearance of NK1r-positive cells as compared to the vehicle. The presence of NK1r was also noted in a small population of ventral motor horn cells.

IT SP-SAP

Effects upon NK1r-containing cells. In contrast to the effects observed with either PBS or SAP, SP-SAP (150 µg) resulted in a near total loss of NK1r-positive cell bodies or dendrites in the superficial layers with little effect upon NK1r-positive motor horn cells (Fig. 1E). Qualitatively, the administration of 15, 45, or 150 µg SP-SAP resulted in an essentially equivalent loss of NK1r immunoreactivity (Fig. 2). However, when 1.5 µg SP-SAP was used, there appeared to be no differences between this group and the control animals. Independent systematic counts of NK1r-positive cell bodies

anti-NK1r (1:1000, gift from Dr. Shigemoto) was diluted in TSA-blocking solution, and tissue sections were incubated overnight at room temperature. Following the overnight incubation, tissue sections were washed (3X 10 min) in wash buffer (PBS + 0.3% Triton X-100, pH 7.4). This secondary antibody, biotin-conjugated donkey anti-rabbit IgG (1:500, Jackson ImmunoResearch Labs, West Grove, PA), was diluted in TSA-blocking solution, and sections were incubated for 2 h at room temperature. Sections were washed and then incubated for 30 min in TSA-blocking solution plus strepavidin-horse radish peroxidase (1:1500, TSA kit, NEN). Sections were again washed 3X 10 min in wash buffer and then incubated for 10 min in a solution of tyramide-Cy3 (1:75, TSA kit, NEN). Finally, the sections were washed 3X 10 min in PBS, mounted on gelatin-coated slides, air-dried, dehydrated via an alcohol gradient (70%, 90%, and 100%), cleared in xylene, and coverslipped.

For immunohistochemistry involving antibodies specific for all neurons (NeuN), unmyelinated sensory afferents (calcitonin gene–related peptide [CGRP], SP, and IB4), or astrocytes (glial fibrillary acidic protein, GFAP), 60-µm free-floating tissue sections were washed in PBS then incubated for 30 min at room temperature in a blocking solution of 1% normal donkey serum in PBS with 0.3% Triton X-100. This was followed by an overnight incubation at room temperature in either mouse anti-NeuN (1:75, Chemicon, Temecula, CA), rabbit anti-CGRP (1:10,000, Sigma, St. Louis, MO), rabbit anti-IB4 (1:150), or mouse anti-GFAP (1:500, Sigma). Following incubation, tissue sections were washed 3X 10 min in PBS and incubated in the secondary antibody solution for 2 h at room temperature. Secondary antibodies were conjugated to fluorescent markers Cy3 (1:600) and fluorescein isothiocyanate (1:150). Secondary antibodies were obtained from Jackson ImmunoResearch Labs.

To confirm the specificity of the primary antibody, controls included preabsorption with the corresponding synthetic peptide or omission of the primary antibody. To control for the possibility that staining intensities might vary between experiments, control sections were included in each run of staining and served as a standard for immunofluorescence measurements. Using an MRC-1024 Confocal Imaging System (Bio-Rad, Hercules, CA) and an Olympus BH-2 microscope equipped for epifluorescence, spinal cord sections were analyzed by an observer unaware as to treatment by conventional fluorescence and confocal microscopy to characterize the number of immunoreactive cells and fibers. Quantification of NK1r or NeuN expression involved counting the number of NeuN- or NK1r-expressing neurons or NK1r-expressing neuronal fibers (at least 100 µm in length) in the lumbar, thoracic, and cervical levels of the dog spinal cord (see Abbadie et al. [1997] and Mantyh et al. [1997] for details of quantification methodology), and the results were then expressed as total number of cells or fibers. For the quantification of GFAP and CGRP staining, intensity of immunofluorescence was measured using Image Pro Plus software (Media Cybernetics, Silverspring, MD).

Histochemistry

Tissues embedded in paraffin were sectioned at 3–5 µm and stained with H&E. In addition, immunohistochemical staining for GFAP (DAKO, Carpenteria, CA; Kit 20334) was undertaken in the tissue obtained from the vicinity of the catheter tip. Upon completion of the histology, sections from each of the spinal cord sections were examined by light microscopy by a board-certified pathologist (M.R.G.) without knowledge as to treatment. Cross sections of spinal cord stained with H&E and for GFAP were examined for evidence of inflammation, reactive gliosis, and other signs of injury. Inflammatory infiltrates were scored by anatomical location (arachnoid/subarachnoid space, nerve root, or spinal cord) and for severity on a scale of 0–3. The scores for inflammation at each of these sites and the degree of reactive gliosis on GFAP stains were added to give a total pathology score (possible score from 0–12). Distribution-free testing of the ranked spinal cord pathology scores was performed using the Jonckheere test for comparison of dose-ordered effects (Siegel et al., 1988).

Occipital Cortex

To assess the possibility of neurotoxic damage induced by SP-SAP following rostral movement, occipital cortex was harvested 90 days following either PBS or 45 µg SP-SAP treatment. Tissue was either embedded in paraffin, sectioned to 3–5 µm and stained with H&E or was placed in 30% sucrose, sectioned to 20 µm and underwent immunohistopathology for NeuN and GFAP.

SP-SAP and SAP Analysis of Serum, CSF, and Tissue

SP-SAP Enzyme-Linked Immunosorbent Assay

Nunc Immuno-Sorb plates were coated overnight with 100 µl of goat anti-SAP (Advanced Targeting Systems, 1:1000 in 0.1M carbonate-bicarbonate, pH 9.6 binding buffer). Wells were washed with 10mM Tris, 0.14M NaCl, and 0.5% Tween, pH 8.0 (washing buffer), and blocked with Superblock (Pierce, Rockford, IL) and sample or standard applied and incubated for 1 h at room temperature on a BioMek 2000 robot. Wells were washed with washing buffer, and anti-SP (Advanced Targeting Systems) was applied at 1:1000 and incubated for 1 h at room temperature. Wells were washed with washing buffer and goat anti-rabbit horseshadish peroxidase (Chemicon) was added at 1:125 dilutions and incubated for 1 h at room temperature. After washing, TMB/E (Chemicon) was added for color change; the reaction was stopped with the addition of 1M sulfuric acid and analyzed on a SpectraMax plate reader and analyzed with SoftMax (Molecular Devices).
and dendrites in the superficial dorsal horns in these animals demonstrated a large decrease in NK1r-positive cells. Thus, it was evident that IT SP-SAP in doses of 15–150 μg produced a pronounced decrease in the number of NK1r-bearing cells (Fig. 2) and dendrites (data not shown) in the dorsal, but not ventral laminae. In contrast, the number of NK1r-positive cells observed in the SP-SAP (1.5 μg)-treated cord was comparable to that observed with PBS or SAP treatment.

To determine the specificity of the SP-SAP effects, sections were stained with NeuN, to obtain counts of the total neuron population. As shown in Figure 3, the administration of the highest dose (150 μg) of SP-SAP produced no detectable decrease in the total neuron number as compared to 150 μg SAP or PBS alone. There were no differences between treatment groups in total number of cells with NeuN immunoreactivity in the superficial or intermediate dorsal horn or in the motor horn.
Examination of the presence of IB4, SP, and CGRP, markers of primary afferents in the dorsal horn, revealed no evident difference between dogs dosed with PBS or 150 μg SP-SAP (Fig. 4).

GFAP staining was performed to determine if there was any astrocytic reaction. No change in GFAP expression was noted at the spinal site even at the highest dose of SP-SAP (Fig. 5).

Localization of effects. To determine the rostrocaudal localization of the effects of SP-SAP, NK1r-bearing cells were counted in the cervical and thoracic spinal segments of the same animals examined above. There were no differences among PBS-, SAP-, or SP-SAP–treated animals, emphasizing that the local toxicity was limited to the region proximal to the injection site at the doses and volume used (Fig. 2).

Time course of effect. Two dogs were injected with 45 μg SP-SAP and sacrificed at 7 days. Systematic counts of NK1r-bearing cells proximal to the tip in the lumbar spinal cord revealed no changes as compared to PBS-treated animals at 28 days (data not shown). This is in contrast to the large decrease in NK1r-bearing cells seen at 28 days in not only the dogs receiving 45 μg SP-SAP but also those receiving 15 μg SP-SAP.

Spinal Toxicology

Dogs received a single IT injection of vehicle (PBS), 1.5, 15, 45, or 150 μg of SP-SAP, or 150 μg of SAP and sacrificed at time points of approximately 7, 28, and 90 days. All animals survived until the intended date of sacrifice and displayed no apparent long-lasting effects as a result of the dosing regimes.

Behavioral Observations

Behavioral and physiological parameters. Administration of PBS, SAP, or SP-SAP produced no long-term changes in any of the parameters measured. However, following dosing with SP-SAP or SAP, animals displayed transient increases in heart rate, blood pressure, mean arterial pressure, and body temperature (Table 2). In association with these changes, mild repetitive contractions of the cleidocervicalis, sternoephahlicus, sternothyroideus, and/or sternohyoideus muscles with truncal rigidity was observed. Light tactile stroking of the flanks would evoke evident signs of agitation (e.g., allodynia). Animals were given im butorphanol or buprenorphine as deemed necessary when allodynia was present. Bradykinesia was also present in some animals. The time of onset and severity of these signs was generally dose dependent, with animals in the highest dose groups (150 μg SP-SAP or SAP)
displaying signs within 3–4 h after injection and those in the lowest dose group (1.5 µg) displaying only minor signs at 8–12 h. These behaviors lasted for between 1 and 3 days, and by 7 days the behavior of SP-SAP–treated and SAP-treated animals was indistinguishable from the control animals (Table 3). Neurological examinations conducted less than 24 h prior to sacrifice demonstrated no significant changes in any clinical signs.

Clinical pathology. Cisternal CSF protein was similar for all animals at the time of surgery and was increased to varying extents in all dosing groups at the time of necropsy, as would be expected following instrumentation of the IT space (Table 3). Blood clinical chemistries and hematology values were generally within normal limits prior to surgery and at necropsy for all animals. At necropsy, most animals displayed a mild decrease in hematocrit which was likely attributable to aggressive whole-blood sample collection prior to sacrifice (Table 3).

Necropsy. Examination of the spinal cord after laminectomy revealed no remarkable signs along its rostrocaudal extent. Examination of the spinal cord proximate to the intended level of the catheter placement revealed no particular abnormalities as related to the appearance or coloration of the dura.

Histopathology. Histology from sections taken in the vicinity of the catheter tip typically showed no remarkable signs (Fig. 5). Animals receiving even the highest doses of SP-SAP were indistinguishable from animals receiving saline or the inactive SAP. With one exception, all had total pathology scores of 0 or 1, indicating minimal or no morphological evidence of injury. The scores of 1 were almost entirely due to very mild, mononuclear inflammatory infiltrates in the arachnoid or subarachnoid space. These were often perivascular and in some animals consisted only of small numbers of hemosiderin-laden macrophages. Reactive gliosis was not seen in any of the animals. Table 4 summarizes the histopathology scores assessed from these tissues. Rank-order comparisons for ordered means (Jonckheere) revealed no significant trends across treatment groups on any single index or the overall score.

In selected animals, occipital cortex was removed for histopathological analysis by H&E and for immunohistochemistry of NeuN and GFAP. As seen in Figure 6, at 3 months after 45 µg SP-SAP delivery, there were no detectable morphological changes as compared to a PBS-treated animal.

Pharmacokinetics

CSF

As shown in Figure 7A, within the CSF, levels of SP-SAP near the catheter tip reached a maximum average of 4223 ng/ml at 5 min postinjection. There was a rapid clearance with a half-life of approximately 15 min.

Systemic

In a single beagle, an intravenous injection of 150 µg SP-SAP produced a serum maximal concentration of 0.140 ng/ml of the SP-SAP conjugate. The serum levels rapidly declined with a half-life of approximately 5 min (data not shown).
Spinal

At 360 min postdosing, SP-SAP activity in spinal tissue was localized to a region approximately 5–7 cm in length distributed around the catheter tip (Fig. 7B). This distribution is in agreement with previous studies from this laboratory demonstrating a rostral-caudal distribution of 5–10 cm for a bolus injection of this volume (data not shown). These data, along with Figures 1 and 2 emphasize the regional specificity of the SP-SAP distribution.

DISCUSSION

The present studies examined the effects of a single IT bolus dose of up to 150 µg SP-SAP on behavior, basic physiological parameters, and spinal cord histopathology of the dog at intervals of up to 90 days.

IT Effects of SP-SAP

This work demonstrates that the lumbar IT delivery of SP-SAP results in a dose-dependent reduction in superficial NK1r-bearing lumbar neurons and dendrites in a large non-rodent spinal cord. The pharmacological specificity of this action is emphasized by the absence of any effect on NK1r-bearing cells following an equivalent dose of unconjugated SAP. This observation is consistent with the inability of SAP to be internalized into neurons without being attached to the associated ligand (SP). Near-maximal reduction in NK1r-positive cells and dendrites occurred over the dose range of 15–150 µg, but essentially no change from the control was observed with the 1.5-µg dose. An important issue is that of specificity. As noted, counting of neuronal profiles in the dorsal horn showed no systematic differences across treatment. This emphasizes that the loss of neurons was limited to those which were NK1r-bearing and this finding is consistent with the assertion that NK1r-expressing neurons comprise only a small fraction of the total neuronal population of the dorsal horn.

Distribution of Drug Action

Importantly, these effects produced by the IT delivery of SP-SAP were clearly anatomically delimited. Examination of the change in NK1r-bearing membranes even after the highest dose of SP-SAP revealed that these changes were most evident in the superficial laminae (I–II) of the lumbar cord with only minor reduction in laminae III–V as compared to the SAP or vehicle groups. These results are comparable to those previously reported in rats with this toxin complex (Mantyh et al., 1997; Nichols et al., 1999; Vierck et al., 2003). Unlike in the rat, the dog spinal cord reliably displayed a small population of NK1r-bearing cells in the motor horn, and these were identified to be motor neurons by virtue of the colocalization with CGRP. Systematic counts of these NK1r-bearing motor horn cells indicated no reductions in the SP-SAP–treated dogs. Systematic counting of dorsal horn neurons in the cervical cord revealed no differences across treatment groups. These data
support two observations. First, it emphasizes that the reduction in the NK1r-bearing cells of the SP-SAP–treated animals observed proximal to the catheter site was not the result of a generalized lack of NK1r in these animals. Secondly, in combination with data concerning analysis of the rostral-caudal tissue distribution of SAP (Fig. 7B), it provides an important confirmation that the effects of the toxin delivered IT to the lumbar cord exerted its actions only proximal to the catheter tip, even over a 10-fold range of doses.

**Clearance of SP-SAP**

The assessment of SP-SAP spinal kinetics indicates that the conjugated agent is cleared relatively rapidly, with clearance of over 50% of the SP-SAP occurring within 30–60 min. This rapid
loss of the conjugate suggests that there is a rapid cleavage of the SP from the SAP, most likely by peptidases and possibly proteases localized within the CSF and spinal tissue (Katayama et al., 1991; Wang et al., 1991) as well as by uptake into tissue. This rapid inactivation along with the evident failure of SAP alone to have any neurotoxic effects, even at the highest dose employed, likely accounts for the apparent high degree of localization of the NK1r-bearing cell loss. This property emphasizes the likelihood that spread of drug exposure will be limited even in the face of an unintended overdose.

**IT SP-SAP and Physiological Effects**

Although the primary focus of this work was to define the effects of IT SP-SAP on NK1r-bearing spinal neurons, these

### TABLE 3

CSF and Blood Clinical Chemistry Values after IT Vehicle, SP-SAP, or SAP

<table>
<thead>
<tr>
<th>Protein (mg/dl)</th>
<th>Glucose (mg/dl)</th>
<th>WBC (no./mm³)</th>
<th>RBC (no./mm³)</th>
<th>Hematocrit (%)</th>
<th>WBC (no./μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All animals</td>
<td>11 (10–15)</td>
<td>70 (68–72)</td>
<td>11 (2–4)</td>
<td>4 (1–8)</td>
<td>47 (45–50)</td>
</tr>
<tr>
<td>PBS, 28 day</td>
<td>66 (41–92)</td>
<td>71 (71–71)</td>
<td>100 (54–146)</td>
<td>740 (740–740)</td>
<td>40 (39–40)</td>
</tr>
<tr>
<td>1.5 μg SP-SAP, 28 day</td>
<td>22 (18–24)</td>
<td>68 (68–72)</td>
<td>4 (3–5)</td>
<td>283 (238–442)</td>
<td>43 (41–46)</td>
</tr>
<tr>
<td>15 μg SP-SAP, 28 day</td>
<td>20 (18–26)</td>
<td>65 (65–72)</td>
<td>3 (3–4)</td>
<td>485 (591–717)</td>
<td>40 (40–47)</td>
</tr>
<tr>
<td>45 μg SP-SAP, 28 day</td>
<td>12 (12–32)</td>
<td>71 (65–71)</td>
<td>6 (4–17)</td>
<td>80 (45–114)</td>
<td>40 (39–45)</td>
</tr>
<tr>
<td>45 μg SP-SAP, 90 day</td>
<td>68 (62–99)</td>
<td>37 (21–54)</td>
<td>78 (41–145)</td>
<td>3 (3–3)</td>
<td>36 (35–37)</td>
</tr>
<tr>
<td>150 μg SP-SAP, 28 day</td>
<td>45 (37–146)</td>
<td>70 (66–72)</td>
<td>13 (11–17)</td>
<td>—</td>
<td>44 (42–47)</td>
</tr>
<tr>
<td>150 μg SAP, 28 day</td>
<td>34 (29–63)</td>
<td>65 (65–68)</td>
<td>34 (21–39)</td>
<td>—</td>
<td>42 (41–45)</td>
</tr>
</tbody>
</table>

**Note.** hlm: hemosiderin-laden macrophages; pvm: perivenular mononuclear; spinal compr: spinal compression; V-R: Virchow-Robin spaces.

### TABLE 4

Summary of Spinal Cord Histopathology Following Single Bolus Intrathecal Dose of Vehicle, SP-SAP, or SAP

<table>
<thead>
<tr>
<th>IT treatment</th>
<th>Survival time (months)</th>
<th>Animal no.</th>
<th>Spinal compr.</th>
<th>Arachnoid infiltrate</th>
<th>Nerve root infiltrate</th>
<th>Spinal cord infiltrate</th>
<th>GFAP</th>
<th>Pathology score total (sum of all)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1 hlm</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Saline</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Saline</td>
<td>3</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>150 μg SAP</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>1.5 μg SP-SAP</td>
<td>1</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15 μg SP-SAP</td>
<td>1</td>
<td>10</td>
<td>0</td>
<td>1 pvm</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>45 μg SP-SAP</td>
<td>1</td>
<td>13</td>
<td>0</td>
<td>1 pvm</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>45 μg SP-SAP</td>
<td>3</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>150 μg SP-SAP</td>
<td>1</td>
<td>20</td>
<td>0</td>
<td>—</td>
<td>—</td>
<td>1 V-R</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>0</td>
<td>1 pvm</td>
<td>0</td>
<td>pvm</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Note.** hlm: hemosiderin-laden macrophages; pvm: perivenular mononuclear; spinal compr: spinal compression; V-R: Virchow-Robin spaces.

*Sample missing.*
studies emphasize that over the 1- to 3-month survival no adverse effects upon behavior were identified. Motor function was not chronically disturbed, and neurological examination at sacrifice revealed no persistent drug-induced deficits in any indices, including reflex functions. The absence of change in blood pressure or heart rate is important given the possibility that NK1r is present on preganglionic sympathetic neurons (Shapiro and Hille, 1993) and on vascular smooth muscle cells (Kummer et al., 1999; Regoli et al., 1989). A loss of such cells would result in a progressive sympathectomy and a corresponding hypotension or a compensatory increase in heart rate. These effects were not seen. Similarly, bowel and bladder function, although not systematically studied, were judged to be normal, and no evidence of urinary incontinence or retention was noted. Again, the loss of NK1r on sacral parasympathetic neurons might lead to a corresponding loss of such reflex functions (Kawatani et al., 1993; Tiseo and Yaksh, 1990). These effects were also not observed.

Although various studies, mostly in rats, have demonstrated that localized injections of SP-SAP into the medulla can

FIG. 6. Occipital cortex immunohistochemistry for neurons (NeuN) and astrocytes (GFAP) at 90 days after treatment with PBS (Perez-Buendia et al., 1993) or 45 μg SP-SAP (E–H).
produce changes, such as loss of baroreceptor reflex (Helke and Seagard, 2004) and diminished chemosensory-induced ventilation (Nattie and Li, 2002), no evidence of such changes were seen in this study. When taken with the lack of change in cervical and thoracic NK1r–bearing neurons (Fig. 2), these again point to the localization of effect following lumbar injection of SP-SAP.

It should be stressed that these findings cannot be taken as an absolute indication of safety for IT delivery of SP-SAP, as higher doses or administration to other regions may in fact lead to concentrations that might be sufficient to induce cell death in these regions. For example, the radius of the thoracic spinal cord is significantly less than that of the lumbar or cervical cord and administration of SP-SAP into that region may produce a different dose-response curve with respect to potency and toxicity. As noted, at the highest dose, there was an initial transient increase in heart rate, mean arterial pressure, and body temperature and mild truncal rigidity with allodynia. The effects were typically delayed and lasted for anywhere from 4 to 48 h. Within approximately 5 days these signs completely resolved, and the treatment groups were indistinguishable from PBS-treated control animals. The mechanism of this effect is not known, but occurred in both the SAP and SP-SAP groups. The occurrence of this effect with SAP indicates that it is not due to an NK1r-mediated mechanism. The lack of any cell loss in the SAP-treated animals as compared to PBS-treated animals suggests it was not a generalized irreversible neurotoxic event induced by SAP. The delay in time of onset also suggests that the effects were not mediated by an acute local spinal action. These effects have not been described in other species, and their significance is unknown at present.

An additional important aspect of these studies is the long-term follow up out to 90 days. Over this interval, there was no progression of the injury or evidence of additional loss of cells or function. This extended observation is important given the possibility that loss of neurons might lead to long-term reorganization of dorsal horn function which might lead to additional dysfunction or an unusual pain state.

CONCLUSION

The present work emphasizes that in a large animal model, the IT delivery of SP-SAP produces a reduction in the number of dorsal horn NK1r–bearing neurons. These neurons are believed to play an important role in nociceptive processing, a finding consistent with the antinociceptive effects observed after treatment with SP-SAP in rodents. The observations that even the highest dose of SP-SAP had no effects upon the overall cell counts in the dorsal horn and the failure to see changes in CGRP-positive neurons in the ventral horn argues that the effects are anatomically delimited to specific populations of neurons that are characterized by the presence of NK1r. The present studies thus provide data, suggesting the appropriateness of considering the further development of this class of agents for IT delivery to manage chronic pain states. The use of IT agents such as alcohol- or phenol-producing neuronal injury to control a variety of chronic conditions such as pain and spasticity have been described (Ferrer-Brechner, 1989; Flanigan and Boop, 1974; Goldstein, 2001). In contrast to the nonspecificity of the lesion produced by such agents, here the injury and the effect upon function is limited not only by the discrete spread of the intrathecal agent but also by the metabolism of the conjugate to an inactive form and by the limited distribution of its intermediary target (the NK1r-bearing dorsal horn nociceptor). It provides a novel approach for specifically removing a very specific link in the spinal pain pathways.

ACKNOWLEDGMENTS

MH56368 (D.A.L.), DA02110 (T.L.Y.), NS23970, NS048021, and a VA Merit Review (P.W.M.) supported this work. We thank the National Institute
for Mental Health for their continued support of this project. D.A.L. has proprietary interest in the company Advanced Targeting Systems Inc. Advanced Targeting Systems Inc. supplied drug support and funding. SP-SAP is protected under U.S. Patent 6,063,758.

REFERENCES


