Effects of Bupivacaine and Tetrodotoxin on Carrageenan-induced Hind Paw Inflammation in Rats (Part 1)

Hyperalgesia, Edema, and Systemic Cytokines

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Background: Local anesthetics exert antiinflammatory actions. To elucidate potential mechanisms, the authors examined effects of bupivacaine or tetrodotoxin, administered to rats by ipsilateral or contralateral sciatic blockade or systemically, on carrageenan-induced hind paw hyperalgesia, edema, and stimulated cytokine production in circulating blood cells.

Methods: Twelve groups of rats (n = 9–12) received injections in three sites: (1) right or left hind paw (carrageenan or saline), (2) left sciatic block, and (3) systemically (subcutaneously in the upper back). Sciatic and systemic injections were performed with epinephrine plus bupivacaine, tetrodotoxin, or saline; injections were repeated 6 h later. Fifteen hours later, hyperalgesia and/or sensory and motor block were assessed behaviorally, and paw edema was quantified by magnetic resonance imaging. Stimulated production of tumor necrosis factor α, interleukin 10, and interleukin 1β in whole blood cultures was measured by enzyme-linked immunosorbent assay.

Results: Either ipsilateral or contralateral sciatic blocks using either bupivacaine or tetrodotoxin reduced carrageenan-induced edema and hyperalgesia. Systemic bupivacaine and tetrodotoxin were ineffective in preventing edema and hyperalgesia. Bupivacaine was effective in suppressing systemic tumor necrosis factor α and interleukin 1β by all three routes, whereas tetrodotoxin was ineffective by all three routes.

Conclusion: Bupivacaine and tetrodotoxin, via a contralateral or ipsilateral sciatic block, attenuate local inflammatory edema and hyperalgesia induced by hind paw injection of carrageenan in rats. Mechanisms underlying contralateral effects of sciatic blockade remain unexplained. Bupivacaine inhibits carrageenan-evoked systemic cytokine production by a mechanism not shared by tetrodotoxin; this action may involve tetrodotoxin-resistant sodium channels or a variety of non–sodium-channel targets.

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Received from the Department of Anesthesiology, Perioperative and Pain Medicine, Children’s Hospital, Boston, Massachusetts. Submitted for publication July 5, 2005. Accepted for publication February 23, 2006. Supported by the Anesthesia Pain Research Fund, Children’s Hospital, Boston, Massachusetts, the French Society of Anesthesiologists, Paris, France; and the Department of Anesthesiology and Reanimation, Hopital Bicêtre, Le Kremlin-Bicêtre, Paris, France. Dr. Berde is coholder of US patent 6,326,020 B1, which concerns potential clinical uses of tetrodotoxin combination formulations as long-acting local anesthetics. In the event of possible future commercial development, Dr. Berde and Children’s Hospital, Boston, could potentially receive royalties.

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CARRAGEENAN-INDUCED hind paw inflammation has been extensively used as an experimental inflammatory pain model.1 In rats with carrageenan-induced hind paw inflammation, prolonged administration of bupivacaine via an ipsilateral, but not contralateral, sciatic nerve block decreased both inflammation and hyperalgesia.2 Remarkably, Bileviciute-Ljungard and Lundberg3 reported that contralateral, but not remote systemic, administration of bupivacaine could reduce rat hind paw edema and pain behaviors. Carrageenan induces production of inflammatory cytokines.4,5 In mice receiving hind paw injections of carrageenan, inflammation-induced increases in Staphylococcus aureus Cowan (SAC) and lipopolysaccharide-stimulated production of tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) in whole blood cultures were prevented by either contralateral quadriceps injection of bupivacaine microspheres or sciatic nerve neurolysis.6,7 Changes in cytokine production after lipopolysaccharide or SAC challenge in vitro have been used to assess blood cell reactivity.8

Previous studies of effects of local anesthetics (LAs) on inflammation have therefore used different species (rats and mice), several different LA formulations (i.e., sustained-release microspheres vs. aqueous LAs), different routes of administration (intramuscular, sciatic blockade, systemic), and different endpoints for measurement of local and systemic inflammatory responses; these studies have yielded conflicting results.1–3,6,7,9 To further explore the action of LAs on carrageenan-induced inflammation, we investigated effects of bupivacaine via different routes (ipsilateral or contralateral sciatic block or systemic) on behavioral measures of nerve blockade and hyperalgesia in both hind paws and on indices of systemic inflammation as measured by lipopolysaccharide and SAC-stimulated production of TNF-α and IL-1β in whole blood cultures. A nerve-blocking paradigm was adapted to provide continuous sensory and motor blockade for the necessary 15-h time period (See Materials and Methods). Inflammation-induced hind paw edema was quantified both by measurement of paw circumference and by using a novel magnetic resonance imaging technique.10

The commonly used amino-amide and amino-ester LAs exert their conduction blocking and analgesic activity by blocking voltage-gated sodium (Na+) channels, but they also have actions on a wide variety of other cellular targets that could modulate inflammation.11 The inhibi-
tion of inflammatory responses elicited by immunocompetent cells was suggested to be caused by LA targets different from Na⁺ channel blockade in a recent in vitro study.¹² To explore whether actions of LAs on the local and systemic inflammatory response depend on their ability to block sodium channels, similar experiments were conducted using the site 1 sodium channel-blocking toxin tetrodotoxin. Unlike LAs, tetrodotoxin seems to act specifically on sodium channels¹³ and not on either calcium or potassium channels.

**Materials and Methods**

All procedures were conducted in accordance with the Children’s Hospital Animal Care and Use Committee (Boston, Massachusetts). Young adult male Sprague-Dawley rats weighing 250–300 g were used. The animals were kept on a 12-h light–dark cycle with free access to food and water. The rats were handled repeatedly over at least 3 days before experiments to habituate them to the investigators and the testing paradigm.

**Solutions**

Carrageenan was prepared fresh before each experiment (0.2 ml of 2% wt/vol solution of lambda carrageenan in saline (0.15 M, pH 7.4; Sigma Chemical Co., St. Louis, MO). Tetrodotoxin (50 μM) stock solutions were made by dissolving 1 mg tetrodotoxin (Sigma Chemical) in 10 ml of 20 mM sodium citrate buffer.¹⁴ Bupivacaine, 0.5% wt/vol (5 g/l), with epinephrine was used as the LA (Sigma Chemical). Epinephrine from a commercial 0.5% and the volume of the injection to 0.2 ml, (2) addition of epinephrine, and (3) using a second injection 6 h later. Epinephrine was necessary to prolong block durations sufficiently and to limit systemic toxicity of tetrodotoxin.¹⁵ All sciatic block injections and systemic (subcutaneous interscapular) injections were performed with solutions containing epinephrine at 55 μM (1:100,000) final concentration, so that each animal received the same total dose of epinephrine. Pilot experiments (data not shown) showed that rats receiving two sciatic block injections of either tetrodotoxin with epinephrine or bupivacaine with epinephrine in these doses at 6-h intervals maintained dense sensory and motor blockade (using von Frey filaments, thermal withdrawal latencies, and extensor postural thrust maneuvers) over the course of 15 h, as required for this paradigm.

**Experimental Groups**

Animals were assigned to 1 of 12 experimental groups (n = 9–12/group) as shown in table 1. Solutions for injections were prepared and then coded by coinvestigators so that the primary investigator (H.B.) was blinded to the contents of the injectates.

Each animal received three injections at time = 0: (1) a right (contralateral) or left (ipsilateral) subcutaneous hind paw injection (0.2 ml with 2% wt/vol carrageenan or saline), (2) a left sciatic block (0.2 ml with 0.5% bupivacaine, 50 μM tetrodotoxin, or 9 g/l saline), and (3) a systemic injection (subcutaneous interscapular with bupivacaine, tetrodotoxin, or saline). Tetrodotoxin or bupivacaine was only injected at one site (sciatic or back) in each animal. The animals received the same medications for a second left periscatic injection and a second injection subcutaneously in the back 6 h later, to provide a prolonged effect of bupivacaine, tetrodotoxin, or saline (table 1 and fig. 1).

**Magnetic Resonance Imaging**

All experiments were performed on a Bruker Biospec (Bruker Instruments, Inc., Billerica, MA) operating at 4.7 T with maximum gradient strength of 400 mT/m. A volume coil was used as a transmitter to ensure homogeneous excitation, while a surface coil of diameter 4 cm was used as a receiver to image both hind paws of anesthetized rats.

After matching, tuning, and acquisition of scout images, the hind paws were imaged in the coronal plane with a multislice T2-weighted rapid acquisition with relaxation enhancement imaging sequence (2,000-ms repetition time, 28-ms effective echo time, 1-mm slice thickness, and four signal averages). The matrix size was 128 × 128, and field of view was 4 × 4 cm², resulting in a spatial resolution of 312 × 312 μm². Injection of carrageenan solution into the hind paw resulted in ob-
Table 1. Summary of Treatment Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Left Sciatic Area</th>
<th>Left Hind Paw (SC)</th>
<th>Right Hind Paw (SC)</th>
<th>Back</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>Saline + epi</td>
<td>Saline</td>
<td>Saline + epi</td>
</tr>
<tr>
<td>2</td>
<td>Carr</td>
<td>Saline + epi</td>
<td>Carr</td>
<td>Saline + epi</td>
</tr>
<tr>
<td>3</td>
<td>Contra bupi block</td>
<td>Bupi + epi</td>
<td>Carr</td>
<td>Saline + epi</td>
</tr>
<tr>
<td>4</td>
<td>Systemic bupi</td>
<td>Saline + epi</td>
<td>Saline</td>
<td>Bupi + epi</td>
</tr>
<tr>
<td>5</td>
<td>Contra bupi block + carr</td>
<td>Bupi + epi</td>
<td>Carr</td>
<td>Saline + epi</td>
</tr>
<tr>
<td>6</td>
<td>Systemic bupi + carr</td>
<td>Saline + epi</td>
<td>Carr</td>
<td>Bupi + epi</td>
</tr>
<tr>
<td>7</td>
<td>Ipsi bupi block + carr</td>
<td>Bupi + epi</td>
<td>Saline</td>
<td>Saline + epi</td>
</tr>
<tr>
<td>8</td>
<td>Contra TTX block</td>
<td>TTX + epi</td>
<td>Carr</td>
<td>Saline + epi</td>
</tr>
<tr>
<td>9</td>
<td>Systemic TTX</td>
<td>Saline + epi</td>
<td>Saline</td>
<td>TTX + epi</td>
</tr>
<tr>
<td>10</td>
<td>Contra TTX block + carr</td>
<td>TTX + epi</td>
<td>Carr</td>
<td>TTX + epi</td>
</tr>
<tr>
<td>11</td>
<td>Systemic TTX + carr</td>
<td>Saline + epi</td>
<td>Carr</td>
<td>TTX + epi</td>
</tr>
<tr>
<td>12</td>
<td>Ipsi TTX block + carr</td>
<td>TTX + epi</td>
<td>Carr</td>
<td>Saline + epi</td>
</tr>
</tbody>
</table>

Treatments given in the 12 groups. Proximal sciatic blocks were performed on the left sides, hind paw injections were performed on either the right (contralateral) or the left (ipsilateral) side as indicated, and systemic injections were subcutaneously administered in the upper back between the scapulae.

Bupi = bupivacaine; carr = carrageenan; contra = contralateral (non-carr side); epi = epinephrine; ipsis = ipsilateral (carr side); SC = subcutaneous; TTX = tetrodotoxin.

Observables increases of signal intensity within the muscle in the T2-weighted images. The edema signal was approximately as bright as the signal from bone marrow and subcutaneous fat. To perform quantitative assessments of the edema signal, the four or five slices containing edema from each study were thresholded such that only bright pixels containing edema, marrow, and fat remained (fig. 2), eliminating the darker signal from muscle and cortical bone. The total number of pixels containing edema, marrow, and subcutaneous fat was thus estimated for each edematous paw from the thresholded image. This number was divided by the total number of pixels in the image and multiplied by 100 to obtain an estimate of the percentage of tissue within each image containing edema, marrow, and subcutaneous fat. This approach allowed us to calculate and compare the relative percentage of edema signals between the different groups of rats, assuming similar volumes of marrow and subcutaneous fat and rough equivalence of paw volumes. We refer to the measurement performed in this way as a relative edema volume because the measurement also contains lipid signal.

Behavioral Measurements

All behavioral tests were performed by a single investigator (H.B.), who was blinded to the study groups. Behavioral tests were performed 15 h after initial injections, as outlined in figure 1. Thermal nociception was measured by a modified hot-plate test.19 The time that a rat would leave its hind paw on a hot plate (model 39D Hot Plate Analgesia Meter; IITC Inc., Woodland Hills, CA) at 52°C was measured using a stopwatch (this time is called thermal latency). The paw was removed from the hot plate after 12 s by the investigator to avoid thermal injury and thermal hyperalgesia.19 Failure to remove the hind paw after 12 s is regarded as dense thermal nocifensive blockade. This test was repeated three times on each hind paw for each rat.

The extensor postural thrust is a measure of motor strength in normal righting movements.20 The rat was held above a digital balance and allowed to bear weight on one hind paw at a time. The maximum mass (in grams) that the rat could bear without its ankle touching the balance was measured.

The development of mechanical hypersensitivity after hind paw inflammation was assessed by the application of calibrated von Frey filaments. Animals were placed on a plastic mesh floor in individual plastic boxes and allowed to accommodate to their environment. Then, von Frey filaments were applied vertically to the plantar surface of both hind paws. Filaments were applied three times over 2 s. If no response was elicited, a larger diameter filament was applied in the same manner. The filaments were applied in increasing order until the brisk withdrawal or paw flinching was elicited, which was considered as a positive response. This withdrawal threshold was determined twice, with testing separated by 10 min, and the mean withdrawal threshold was used for data analysis. When no response is observed using the largest diameter, the animal is regarded to have a dense mechanical nocifensive sensory blockade.

Fig. 1. Time line for interventions. GA = general anesthesia; MRI = magnetic resonance imaging.

Anesthesiology, V 105, No 1, Jul 2006.
Paw Circumference

As a secondary method to evaluate the edema, we used a technique previously described. The paw circumference was measured by a thread, to the nearest mm, at the metatarsal level.

Assay for Stimulated TNF-α, IL-10, and IL-1β Production in Whole Blood Cultures

To reduce the confounding factors associated with the isolation of monocytes or neutrophils, such as adherence-induced activation or altered expression of cell-surface receptors, we induced cytokines production in whole blood cultures. Blood was collected twice by tail venipuncture, immediately before drug injections (after induction of general anesthesia) and 15 h after drug injections, as outlined in figure 1. The collected blood was placed into a heparinized syringe (5 U heparin) from which whole blood cultures were performed. A blood sample (0.5 ml) was diluted 1:5 in RPMI-1640 medium (VWR, West Chester, PA) supplemented with antibiotics (penicillin–streptomycin; Sigma, St. Louis, MO). Five hundred–microliter aliquots of diluted blood were cultured in 20 four-well plates according to three different conditions: (1) without lipopolysaccharide or SAC (baseline), (2) with lipopolysaccharide (Escherichia coli O111:B4, 10 μg/ml; Sigma), or (3) with SAC (100 μg/ml; Pansorbin Calbiochem, San Diego, CA) in a 5% CO₂ incubator for 24 h at 37°C. The supernatant was then harvested, centrifuged at 300g at 4°C for 10 min, and stored at −70°C until assayed.

The amounts of TNF-α, interleukin 10 (IL-10), and IL-1β in the supernatants were measured with a commercial enzyme-linked immunosorbent assay kit (DuoSet; R&D systems, Minneapolis, MN) according to the manufacturer’s instructions. The assay detection limits were 30 pg/ml for TNF-α and IL-10, and 15 pg/ml for IL-1β.

At the end of each experiment, rats were killed with an overdose of pentobarbital (100 mg/kg administered intravenously; Nembutal, Abbott Laboratories, Chicago, IL).

Statistical Analysis

Because the behavioral and the paw circumference data were not normally distributed, differences between groups were assessed using nonparametric tests (Kruskal-Wallis and Dunn tests). A stringent criterion (P < 0.01) was applied to protect against false positives (type I error) and account for multiple comparisons. The results are expressed as median with 25th and 75th percentiles or as median with interval range for the von Frey data. Group differences in magnetic resonance imaging measurements of relative edema volumes (REVs) were assessed using a one-way analysis of variance with post hoc analysis via Fisher protected least significant difference test. The results are expressed as mean ± SD. A P value below 0.01 was considered as the minimum level of statistical significance. The distribution of cytokine concentrations in each group was checked for normality using the Shapiro-Wilk test. Differences between groups were assessed using a one-way analysis of variance with post hoc analysis via Fisher protected least significant difference test. The results are expressed as mean ± SD. A P value below 0.01 was considered as the minimum level of statistical significance.

Results

Magnetic Resonance Imaging Measurements of Hind Paw Edema

Fifteen hours after the injection of carrageenan, a significant edema was observed in the hind paw carrageenan group (hind paw carrageenan, systemic saline, and contralateral saline) compared with the control group (hind paw saline, contralateral perisciotic saline, and systemic saline) as characterized by the REV measurements (table 2). In the groups receiving hind paw
injection of saline along with either contralateral bupivacaine block or systemic bupivacaine, the REV was not significantly different from that of the control group receiving contralateral saline and systemic saline. In the groups receiving hind paw carrageenan plus bupivacaine as a contralateral block or as an ipsilateral block, the REV was significantly less than in the hind paw carrageenan group but still significantly greater than in the control group. In the group receiving hind paw carrageenan and systemic bupivacaine, the REV was not significantly different from that of the hind paw carrageenan group. For the non-hind paw–injected sides, there were no significant differences in the mean REVs in the different treatment groups. Figures 2A–D show coronal spin echo T2-weighted images before (black background) and after (blue background) application of a threshold criterion. Images show bilateral rat paws for the carrageenan group (A), the carrageenan and contralateral bupivacaine block group (B), the carrageenan and ipsilateral bupivacaine block group (C), and the carrageenan and systemic bupivacaine group (D).

### Evaluation of Hind Paw Edema by Paw Circumference

**Hind Paw Injection Side (Carrageenan or Saline).** Fifteen hours after the injection of carrageenan, a significantly increased paw circumference was observed in the hind paw carrageenan group compared with the control group (fig. 3). In the groups receiving hind paw carrageenan and an ipsilateral or contralateral sciatic block with either bupivacaine or tetrodotoxin, mean paw circumferences were significantly less than in the hind paw carrageenan group. Groups receiving hind paw carrageenan plus systemic tetrodotoxin or bupivacaine had mean paw circumferences that were not significantly different from those of the hind paw carrageenan group (fig. 3).

**Non-Hind Paw Injection Side.** No significant differences were observed between the groups (data not shown).

### Behavioral Tests

**Thermal Nociceptive Withdrawal Latencies.**

**Hind Paw Injection Side (Carrageenan or Saline).** Fifteen hours after injection of carrageenan, a significant decrease in heat withdrawal latency was observed in the carrageenan group compared with the control group, indicating thermal hyperalgesia in the carrageenan group (fig. 4). Contralateral (left) sciatic injection of bupivacaine or tetrodotoxin had no effect on right hind paw withdrawal latencies in groups receiving right hind paw saline. In groups receiving right hind paw carrageenan and a contralateral block with either bupivacaine or tetrodotoxin, withdrawal latencies were significantly greater than in the carrageenan group, indicating partial prevention of carrageenan-induced thermal hyperalgesia. Systemic bupivacaine and tetrodotoxin were ineffective in prevention of carrageenan-induced thermal hyperalgesia. In groups receiving left hind paw carrageenan and an ipsilateral sciatic block with either bupivacaine or tetrodotoxin, withdrawal latencies were at the cutoff value of 12 s, indicating dense thermal

### Table 2. MRI Measurements of Hind Paw Relative Edema Volumes

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Relative Edema Volume, %</th>
<th>P vs. Control Group</th>
<th>P vs. Carrageenan Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>2.2 ± 0.6</td>
<td>—</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Carrageenan</td>
<td>6</td>
<td>9.4 ± 0.96</td>
<td>&lt; 0.0001</td>
<td>—</td>
</tr>
<tr>
<td>Contra bupi block</td>
<td>5</td>
<td>2.0 ± 0.4</td>
<td>NS</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Carr + contra bupi block</td>
<td>5</td>
<td>6.54 ± 0.78</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Carr + ipsi bupi block</td>
<td>4</td>
<td>7.32 ± 0.5</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Carr + systemic bupi</td>
<td>4</td>
<td>10.5 ± 1.2</td>
<td>&lt; 0.0001</td>
<td>NS</td>
</tr>
</tbody>
</table>

Relative edema volumes (%) obtained by magnetic resonance imaging.

Bupi = bupivacaine; carr = carrageenan; contra = contralateral (non-carr side); ipsi = ipsilateral (carr side); NS = not significant.

Fig. 3. Paw circumference (mm) assessing the edema 15 h after the injection in the hind paw injection side assessed using nonparametric tests (Kruskal-Wallis and Dunn tests). Results are expressed as median with 25th and 75th percentiles. Bupi = bupivacaine; carr = carrageenan; contra = contralateral (non-hind paw injection side); ipsi = ipsilateral (hind paw injection side); syst = systemic; TTX = tetrodotoxin. * P < 0.01 versus control group. § P < 0.01 versus carrageenan group.
nocifensive blockade even under conditions of hind paw inflammation (fig. 4).

**Non–Hind Paw Injection Side.** In the groups receiving a sciatic block with either bupivacaine or tetrodotoxin, the animals showed dense thermal nocifensive blockade; otherwise, there were no differences between groups in mean thermal latencies (data not shown). Therefore, carrageenan-induced inflammation produced ipsilateral, but not contralateral, hind paw thermal hyperalgesia.

**Von Frey Filament Mechanical Withdrawal Thresholds.**

**Hind Paw Injection Side (Carrageenan or Saline).** Fifteen hours after the injection of carrageenan, a significant decrease in the withdrawal threshold was observed in the carrageenan group compared with the control group, indicating mechanical hyperalgesia (fig. 5). Contralateral (left) sciatic injection of bupivacaine or tetrodotoxin had no effect on the right hind paw withdrawal thresholds in groups receiving right hind paw saline. In the groups receiving right hind paw carrageenan and a contralateral sciatic block with either bupivacaine or tetrodotoxin, withdrawal thresholds were significantly greater than in the carrageenan group, indicating partial prevention of carrageenan-induced mechanical hyperalgesia. Systemic bupivacaine and tetrodotoxin were ineffective in prevention of carrageenan-induced mechanical hyperalgesia. In the groups receiving left hind paw carrageenan and an ipsilateral block with either bupivacaine or tetrodotoxin, mean withdrawal thresholds were 60 g or greater, indicating dense mechanical nocifensive blockade, even under conditions of hind paw inflammation (fig. 5).

**Non–Hind Paw Injection Side.** For groups receiving right-sided carrageenan or saline injections, animals receiving left-sided sciatic blocks with either bupivacaine or tetrodotoxin all showed dense mechanical nocifensive blockade. For the other groups, there were no differences in mean withdrawal thresholds (data not shown). Therefore, carrageenan-induced inflammation produced ipsilateral, but not contralateral, hind paw mechanical hyperalgesia.

**Weight Bearing (Extensor Postural Thrust Maneuver).**

**Hind Paw Injection Side (Carrageenan or Saline).** Fifteen hours after the injection of carrageenan, a significant decrease in the weight an animal could bear on the injected (right) paw was observed in the carrageenan group compared with the control group (fig. 6). Sciatic injection of either bupivacaine or tetrodotoxin had no effect on contralateral (right) weight bearing compared with controls. In the groups receiving carrageenan and a contralateral block with either bupivacaine or tetrodotoxin, the weight an animal could bear in the right paw was significantly greater than in the carrageenan group but still significantly lower than in the control group. Ipsilateral sciatic blockade with either tetrodotoxin or bupivacaine in animals receiving left hind paw injections produced marked impairment of weight bearing (fig. 6).

**Non–Hind Paw Injection Side.** For all treatment conditions, weight bearing was markedly impaired in the limbs that had received a sciatic block with either bupivacaine or tetrodotoxin, and unimpaired in all other groups (data not shown).
lipopolysaccharide- or SAC-stimulated incubation conditions were also statistically indistinguishable from concentrations of the same cytokines evoked in blood cultures in the control groups obtained by venipuncture at H15 (see previous section and figs. 7–9; data not shown). The observation of lower amounts of evoked production of cytokines in the carrageenan-treated H0 treatment groups compared with the carrageenan-treated H15 groups is consistent with the view that the additional increases in stimulated cytokine production in the H15 groups reflect a systemic leukocyte activation response to carrageenan that evolves over time.

Production of TNF-α, IL-10, and IL-1β in Cultures of Circulating Blood Cells Obtained at H0 (Simultaneous with Hind Paw Injections and LA Injections)

Concentrations of TNF-α, IL-1β, and IL-10 in cultured blood in the absence of either lipopolysaccharide or SAC stimulation were always low in all of the study groups obtained by venipuncture at H0, indicating that incubation in the culture plates per se did not significantly stimulate cytokine production in cultured cells from these groups.

Lipopolysaccharide or SAC stimulation of blood cultures obtained by venipuncture at H0 produced concentrations of each of the three cytokines that were statistically indistinguishable among all treatment conditions (hind paw carrageenan or saline; tetrodotoxin, bupivacaine; carrageenan group). In the groups receiving hind paw saline and either bupivacaine or carrageenan and bupivacaine by all three routes, lipopolysaccharide-stimulated TNF-α production was not significantly different from the production observed in the control group. In the groups receiving hind paw carrageenan and bupivacaine by all three routes, lipopolysaccharide-stimulated TNF-α production was not significantly different from the production observed in the control group, and it was significantly less than in the carrageenan group, showing that bupivacaine by all three routes markedly inhibited the increased production of TNF-α produced by hind paw carrageenan. After SAC stimulation, in the groups receiving carrageenan

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Production of TNF-α, IL-10, and IL-1β in Cultures of Circulating Blood Cells Obtained at 15 h after Hind Paw Injections

Concentrations of TNF-α, IL-1β, and IL-10 in cultured blood obtained at H15 in the absence of either lipopolysaccharide or SAC stimulation were low in all groups, indicating that the culture plates per se did not significantly stimulate cytokine production in cultured cells from these groups.

TNF-α. Twenty-four hours after stimulation with lipopolysaccharide (fig. 7A) or SAC (fig. 7B), TNF-α production was enhanced in blood cultures obtained 15 h after hind paw saline (control group). A significant further increase in TNF-α production after lipopolysaccharide or SAC stimulation was observed in blood cultures obtained 15 h after hind paw carrageenan injection. In the groups receiving hind paw saline and either bupivacaine or tetrodotoxin by all three routes, TNF-α production was not significantly different from the production observed in the control group. In the groups receiving hind paw carrageenan and bupivacaine by all three routes, lipopolysaccharide-stimulated TNF-α production was not significantly different from the production observed in the control group, and it was significantly less than in the carrageenan group, showing that bupivacaine by all three routes markedly inhibited the increased production of TNF-α produced by hind paw carrageenan. After SAC stimulation, in the groups receiving carrageenan

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Fig. 6. Extensor postural thrust (EPT) (g) assessing the motor strength in the hind paw injection side, 15 h after the injection assessed using nonparametric tests (Kruskal-Wallis and Dunn tests). Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as median with 25th and 75th percentiles. Bupivacaine; carrageenan; tetrodotoxin. Results are expressed as mean ± SD. *P < 0.01 versus control group. §P < 0.01 versus carrageenan group.

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and bupivacaine. TNF-α production was significantly higher than in the control group and significantly lower than in the carrageenan group, showing that bupivacaine by all three routes had a statistically significant, but incomplete, suppressive effect on SAC-stimulated TNF-α production (fig. 7B). Tetrodotoxin by all three routes did not have any effect on the carrageenan-induced TNF-α production after lipopolysaccharide or SAC stimulation.

**IL-1β.** Twenty-four hours after stimulation with lipopolysaccharide (fig. 8A) or SAC (fig. 8B), IL-1β production was enhanced in the hind paw saline (control) group. A significant further increase in IL-1β production after lipopolysaccharide or SAC stimulation was observed 15 h after hind paw carrageenan injection. In the groups receiving hind paw saline and bupivacaine or tetrodotoxin by all three routes, lipopolysaccharide- or SAC-stimulated IL-1β production was not significantly different from the production observed in the control group. In the groups receiving hind paw carrageenan and bupivacaine by all three routes, lipopolysaccharide- or SAC-stimulated IL-1β production was not significantly different from the production observed in the control group and was significantly lower than in the carrageenan group, showing that bupivacaine by all three routes markedly inhibits carrageenan-induced increased production of IL-1β after lipopolysaccharide stimulation (fig. 8A). In blood cultures from these same treatment groups receiving hind paw carrageenan, SAC-stimulated IL-1β production was significantly higher than in the control group, and there were no significant differences between all of the groups receiving hind paw carrageenan (fig. 8B). Tetrodotoxin by all three routes did not have any effect on the carrageenan-induced increases in IL-1β production after lipopolysaccharide (fig. 8A) or SAC (fig. 8B) stimulation.

**IL-10.** Twenty-four hours after stimulation with lipopolysaccharide (fig. 9A) or SAC (fig. 9B), IL-10 production was enhanced (control group). Lipopolysaccharide or SAC induced further increases in IL-10 production in all of the groups receiving hind paw carrageenan compared with all of the groups receiving hind paw saline. No differences were observed between the groups receiving carrageenan. Therefore, tetrodotoxin and bupivacaine, by any route, did not prevent carrageenan-induced increases in IL-10 production after either lipopolysaccharide or SAC stimulation.

**Discussion**

In the current study, both contralateral and ipsilateral sciatic blockade using bupivacaine significantly attenuated carrageenan-induced unilateral hind paw inflammatory edema. Contralateral bupivacaine sciatic blocks significantly attenuated carrageenan-induced mechanical and thermal hyperalgesia, whereas ipsilateral sciatic

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Fig. 8. Interleukin-1β (IL-1β) production at 15 h in whole blood cell cultures: after lipopolysaccharide (LPS) stimulation (A) or *Staphylococcus aureus* Cowan (SAC) stimulation (B). Bupi = bupivacaine; carr = carrageenan; contra = contralateral (non–hind paw injection side); ipsi = ipsilateral (hind paw injection side); syst = systemic; TTX = tetrodotoxin. Results are expressed as mean ± SD. * P < 0.01 versus control group. § P < 0.01 versus carrageenan group.

Fig. 9. Interleukin-10 (IL-10) production at 15 h in whole blood cell cultures: after lipopolysaccharide (LPS) stimulation (A) or *Staphylococcus aureus* Cowan (SAC) stimulation (B). Bupi = bupivacaine; carr = carrageenan; contra = contralateral (non–hind paw injection side); ipsi = ipsilateral (hind paw injection side); syst = systemic; TTX = tetrodotoxin. Results are expressed as mean ± SD. * P < 0.01 versus control group. § P < 0.01 versus carrageenan group.
blocks produced dense sensory and motor blockade even in the presence of this inflammatory stimulus. Systemic administration of bupivacaine did not affect either hyperalgesia or edema. In view of the multiple molecular targets of amino-amide LAs such as bupivacaine, the same experiments were also performed with tetrodotoxin, which seems to act much more selectively on sodium channels. In the current study, bupivacaine and tetrodotoxin showed similar impact in suppressing local hind paw edema when administered by ipsilateral or contralateral sciatic block, similar suppression of hyperalgesia when administered by contralateral sciatic block, dense sensory and motor block when administered by ipsilateral block (even in the presence of inflammation), and similar lack of impact on edema and hyperalgesia when administered by remote systemic injection. These results suggest that ipsilateral or contralateral sciatic injection of bupivacaine or tetrodotoxin, acting by regional rather than systemic mechanisms, inhibit carrageenan-induced local hind paw edema and hyperalgesia. In contrast, bupivacaine and tetrodotoxin showed markedly different effects on a systemic inflammatory marker, namely lipopolysaccharide-stimulated release of the cytokines TNF-α and IL-1β in cultures of circulating blood cells. Bupivacaine was effective in suppressing these systemic inflammatory markers by all three routes, whereas tetrodotoxin was ineffective by all three routes. Table 3 summarizes these differences in actions of bupivacaine and tetrodotoxin on local and systemic responses to carrageenan-induced inflammation.

Previous studies of effects of LAs on carrageenan-induced hyperalgesia, edema, and inflammation have yielded some conflicting results.1–36,7,9 Some of these differences may result from the different sites of LA delivery, different methods used to achieve prolonged effect, and the confounding proinflammatory and antinflammatory effects of microspheres and coincorporated dexamethasone.17 As detailed in the Materials and Methods, to avoid some of these confounding factors, we used three modifications to provide continuous blockade for the required 15-h period for both bupivacaine and tetrodotoxin: (1) increasing the bupivacaine concentration to 0.5% and the volume of the injection to 0.2 ml, (2) addition of epinephrine, and (3) using a second injection 6 h later.

In accordance with previous studies,9 we observed that carrageenan induced ipsilateral edema and hyperalgesia without inducing either edema or hyperalgesia in the contralateral hind paw. Conversely, one article3 had shown a bilateral decrease in hind paw withdrawal thresholds after unilateral carrageenan injection. In the current study, bupivacaine or tetrodotoxin via a sciatic block did not influence the contralateral hind paw behavioral measures, and systemic bupivacaine or tetrodotoxin had no effect on hind paw edema or behavioral measures in noninjected paws.

In accordance with previous studies,1,2 the local hyperalgesia and edema induced by carrageenan was partly inhibited by a bupivacaine block. Gentili et al.2 described a significant decrease of carrageenan-induced edema in rats related to a prolonged (> 6 h) peripheral nerve block. This antiinflammatory effect was associated with a persistent analgesic effect. Of interest, in accordance with the results of Bileviciute-Ljungar and Lundberg,3 an injection in the leg contralateral to the carrageenan injection had the same effect as an ipsilateral injection. Moreover, systemic bupivacaine did not affect the pain-related behavior and the edema. The impact of contralateral LA administration in our study and the study of Bileviciute-Ljungar and Lundberg was remarkable and not readily explained.

In addition to local, unilateral inflammation, the carrageenan model evokes a systemic inflammatory response, as evidenced by increased TNF-α, IL-1β, and IL-10 production in cultures of circulating leukocytes after lipopolysaccharide or SAC stimulation.25 LAs have been shown in previous studies using carrageenan-induced inflammation to influence systemic as well as local inflammatory responses6,7,24,25 In our previous study, contralateral intramuscular administration of bupivacaine microspheres in mice inhibited evoked cytokine (TNF-α and IL-1β) release in circulating leukocytes.6 In the current study, increased production of TNF-α and IL-1β in cultures of circulating blood cells after lipopolysaccharide stimulation in rats receiving hind paw carrageenan injections was completely prevented by bupivacaine treatment, via ipsilateral or contralateral sciatic block or systemically. In the case of SAC-stimulated blood cultures, evoked increases in TNF-α production were partially pre-

Table 3. Summary of Effects of Bupivacaine and Tetrodotoxin on Local and Systemic Inflammatory Responses

<table>
<thead>
<tr>
<th></th>
<th>Local Edema and Hyperalgesia</th>
<th>Systemic Inflammation (Cytokine Activation in Cultures of Circulating Blood Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ipsi or contra bupivacaine sciatic block</td>
<td>Inhibition</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Systemic bupivacaine</td>
<td>No effect</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Ipsi or contra tetrodotoxin sciatic block</td>
<td>Inhibition</td>
<td>No effect</td>
</tr>
<tr>
<td>Systemic tetrodotoxin</td>
<td>No effect</td>
<td>No effect</td>
</tr>
</tbody>
</table>

Summary table: Effects of bupivacaine and tetrodotoxin on local hyperalgesia and edema and on a systemic marker of inflammation, priming of proinflammatory cytokines in cultures of circulating blood cells.

Contra = contralateral; ipsi = ipsilateral.
vented by bupivacaine by any of these three routes, and evoked increases in IL-1β production were not prevented by bupivacaine by any route. The administration of bupivacaine had no effect on the carrageenan-induced IL-10 production after either lipopolysaccharide or SAC stimulation. In the absence of carrageenan-induced hind paw inflammation, bupivacaine by any route did not influence systemic cytokine production. The actions of lipopolysaccharide and SAC on mononuclear cells leading to TNF-α and IL-1β release involve different Toll-like receptors and different signaling pathways.20 Differential actions of bupivacaine on different Toll-like receptors and signaling pathways may be a possible explanation of our observations of differential effects of bupivacaine on the inflammatory profiles elicited by lipopolysaccharide or SAC in the animals receiving carrageenan.

Tetrodotoxin had no effect on systemic cytokine production when administered by any of the three routes. These differences in actions of bupivacaine and tetrodotoxin on these markers of systemic inflammation were unexpected, because bupivacaine and tetrodotoxin had similar effects on amelioration of hind paw carrageenan-induced local inflammatory edema and hyperalgesia. Taken together, these results would suggest that LAs may influence local and systemic inflammatory responses by different mechanisms.

The local tissue neurogenic inflammatory response to injury is generally attributed to an efferent function of primary afferent terminals mediated through axon reflexes. It can also be initiated in the spinal cord by primary afferent depolarization large enough to trigger dorsal root reflexes. Activation of dorsal root reflexes was shown to play a role in the development of neurogenic cutaneous inflammation.27 The different local and systemic consequences of ipsilateral, contralateral, and systemically applied bupivacaine and tetrodotoxin could possibly relate to an effect on dorsal root reflex activation. Duarte et al.28 recently reported additional evidence for the effectiveness of bupivacaine for suppressing postoperative pain through a mechanism other than a direct blockade of afferent discharges at the incision site. In this study, alldynia, particularly in the secondary areas, was selectively suppressed for at least a week by preincisional bupivacaine given subcutaneously at the incision site and elsewhere. Differential effects of drug and site of administration on expression of cytokines and mitogen-activated protein kinases in dorsal root ganglia and in the spinal cord could also be involved.29,30 A unilateral nerve injury can produce bilateral loss of distal innervation,31 and ipsilateral and mirror-image inflammatory neuopathic pain can be created acutely and chronically through glial and proinflammatory cytokine actions.32 Additional studies to investigate the effects of LAs on spinal mechanisms involved in carrageenan-induced hind paw inflammation will be reported separately.

The clinical implications of local, systemic, and spinal inflammatory effects of LAs require further study. In a recent study, preemptive epidural analgesia (mixture of bupivacaine and fentanyl) reduced postoperative pain and production of proinflammatory cytokines33 but did not alter production of IL-10. IL-10 inhibits lipopolysaccharide-induced production of proinflammatory cytokines by mononuclear cells under in vitro and in vivo conditions.34 Production of IL-10 has therefore been considered to a part of a host-protective mechanism during excessive inflammatory processes. In our experiments, both challenges (lipopolysaccharide and SAC) stimulated the production of IL-10 by the cultured cells, and carrageenan enhanced this production, but neither bupivacaine nor tetrodotoxin by any of the three routes influenced evoked production of IL-10.

A potential trivial mechanism for the effects of contralateral sciatic blocks would be if drugs spread proximally from injection sites in the buttocks back into the epidural space. Two observations argue against this possibility. First, previous studies using block injections labeled with fluorescent dyes showed very little rostral spread, never close to the spine.35 Second, sciatic blocks using this technique, either in the current study or in previous studies using larger volumes,15 have never demonstrated significant degrees of contralateral sensory or motor blockade by behavioral measures.

Another potentially confounding feature of this model is that co-injection of epinephrine is required to prolong block durations sufficiently and to limit systemic toxicity of tetrodotoxin.18,35 Pilot experiments showed that rats receiving two sciatic block injections of either tetrodotoxin with epinephrine or bupivacaine with epinephrine at 6 h intervals maintained dense sensory and motor blockade (using von Frey filaments, thermal withdrawal latencies, and extensor postural thrust maneuvers) over the course of 15 h, as required for this paradigm. Because epinephrine can decrease proinflammatory cytokine expression,36 we cannot entirely exclude a possible effect of epinephrine on cytokine production or a cooperative interaction between epinephrine and bupivacaine. However, tetrodotoxin injected with epinephrine showed no effect on cytokine production. Moreover, control (saline-injected) groups received the same doses of epinephrine as the bupivacaine and tetrodotoxin groups, to account for both local and systemic actions of epinephrine.

In conclusion, bupivacaine and tetrodotoxin via a contralateral or ipsilateral sciatic block attenuate local inflammatory edema and hyperalgesia induced by carrageenan in rats. Bupivacaine, by all three routes, inhibits carrageenan-induced stimulated activation of systemic cytokines, whereas tetrodotoxin by all three routes is...


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The authors would like to thank Dan Benhamou and Jean-Xavier Mazoit (Professors, Hopital de Bicetre, Paris, France) for their encouragement and support of this work, which is a portion of Dr. Beloeil’s Ph.D. thesis at the University of Paris.

Jennifer Jaskiewicz and Michelle Stein (Research Assistants, Department of Anesthesiology, Perioperative and Pain Medicine, Children’s Hospital, Boston, Massachusetts) provided excellent technical assistance in preparation of figures.