Leukocyte Recruitment to Peritoneal Cavity of Rats Following Formalin Injection: Role of Tachykinin Receptors

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Received August 6, 2003; Accepted February 3, 2004

Abstract. The aim of this work was to verify whether formalin would induce leukocyte recruitment following intraperitoneal (i.p.) injection in rats. Formalin (1.25 – 2.5%) induced cell recruitment, which was concentration- and time-dependent (0 – 24 h). Two peaks of leukocyte recruitment were observed. The first peak (from 2 to 4 h) was characterized by a mixed polymorphonuclear and lymphocyte cell population (representing an increase of 100 – 220% and 55 – 60%, respectively), whereas the second peak was characterized by a marked increase in lymphocytes at 24 h (representing an increase of 230%). Pretreatment of animals with specific antagonists for neurokinin NK₁, NK₂, and NK₃ receptors (SR140333, SR48968, and SR142801 compounds, respectively) reduced the early leukocyte increase (representing a significant reduction of 65%, 51%, and 46%, respectively), whereas only the treatment with NK₂-specific antagonist reduced the late cell increase induced by formalin injection (amounting to a significant reduction of 48%). These results suggested that substance P, neurokinin A, and neurokinin B release accounted for formalin-induced cell migratory activity. The anti-inflammatory drug dexamethasone also reduced cell recruitment, which was mainly related to a reduction in 79% of the neutrophils at 4 h following 1.25% formalin injection, suggesting also a release of lipid mediators (eicosanoids and/or platelet-activating factor) and/or cytokines/chemokines by the formalin injection.

Keywords: formalin, leukocyte, cell migration, tachykinin receptor, neurokinin

Introduction

Injection of formalin into the paws of small animals has been used as an experimental tool to understand the role of sensory terminal nerve endings in the genesis of pain sensation since its description by Dubuisson and Dennis (1). One of the drawbacks of this test, however, as reported in the initial work, was a prominent accompanying edematogenic response noticeable following 20 min of injection into the paw, which remained inflamed for several days. The edematogenic response following formalin injection into rat paws has more intensively been studied since the beginning of the last decade (2 – 4). One such study (4), demonstrated that the early edematogenic effect was due to an initial component, derived from stimulation of sensory nerve endings, definitively establishing formalin as a neurogenic type of inflammation inducing agent (5).

In the late seventies and early eighties, a definite participation of leukocytes in edema formation using experimental models of inflammation was established (6 – 8). More recently, neuropeptides such as the tachykinin substance P (SP) and calcitonin-gene related peptide (CGRP) were reported to induce leukocyte recruitment in murine models of inflammation (9, 10). In addition, neurokinin NK₂-receptor activation was shown to mediate cell (neutrophil and eosinophil) adhesion to large venules following SP, capsaicin, or hypertonic saline administration to rats, but not seem to be associated with it (11).

The aim of the present study was to verify whether formalin would induce leukocyte recruitment as part
of its inflammatory responses, using the rat peritoneal cavity as the site of inflammation (12). In addition, participation of tachykinins, also called neurokinins, was assessed through the use of specific antagonists at neurokinin NK₁, NK₂, and NK₃ receptors.

**Material and Methods**

**Animals**

Female Holtzman rats, weighing 140–170 g and supplied locally by Bioterism Center of our university, were used. Animals were left for 24 h, 4 to 6 animals per cage, to adapt to the new environment with food and water ad libitum until the beginning of the experiments. Ethical guidelines of the International Association for the Study of Pain in conscious animals were followed all through the experiments (13).

**Formalin dilution and administration**

To prepare the various formalin concentrations used in the present study, an aliquot from a formaldehyde solution (37%) was diluted with sterile physiological saline (NaCl 0.9%) to obtain 0.625%, 1.25%, or 2.5% (v/v) formalin solutions. A volume of 0.9 ml sterile saline was added to 0.1 ml of either formalin concentrations (0.625%, 1.25%, and 2.5%) for intraperitoneal (i.p.) injections (final volume: 1.0 ml) to maximize formalin contact with tissues inside the cavity while maintaining the total amount of formalin administered when compared with studies in rat paws (1, 4). The pH of final formalin solutions (6.5–7.0) was adjusted to 7.2 prior to the injections, as it has been shown that this procedure did not significantly affect leukocyte recruitment (data not shown). These concentrations of formalin were chosen for their ability to induce nociception and edema formation following the injection in rat paws, as previously shown (1, 4). The time of formalin injection was called time zero. Animals that were i.p.-injected with 1 ml of sterile physiological saline served as the control group. A group of animals that was not injected with either formalin or saline was also studied (naïve animals).

**Cell counts in peritoneal lavage fluid**

Peritoneal lavage fluid was collected following neck dislocation and exsanguination from the control and injected animals with the various formalin concentrations according to the time course protocols (0, 2, 4, and 24 h). For cell collection, 10 ml of phosphate-buffered saline (PBS, 0.15 M, pH = 7.4) added with heparin (5 U/ml) was intraperitoneally injected and the external peritoneal wall gently massaged. The lavage fluid was collected, through means of a plastic Pasteur pipette, by a abdominal incision. In general, more than 9.0 ml were collected/animal in conic 50-ml centrifuge tubes. The tubes were centrifuged at 200 × g for 10 min and the supernatant discarded. The cell pellet was resuspended with 1.0 ml of PBS and two aliquots of 0.1 ml content were separated for total cell counts (10⁶ cells/ml exudate) in Neubauer chambers and specific (%) counts from cytospin-prepared smears stained by May-Grunwald-Giemsa. A 400- and 1000-fold magnification under optical microscopy were, respectively, used.

**Drug treatment**

Some groups of formalin-injected animals were previously (15 min before) injected by the intramuscular (i.m.) route with specific antagonists for NK₁, NK₂, and NK₃ receptors (SR140333, SR48968, and SR142801, respectively; Sanofi-Synthelabo, Montpellier, France) diluted in a saline-ethanol solution (20:1), following trademark specifications. Animals injected with the same amount of vehicle (saline plus ethanol solution = SR vehicle) constituted the control group. SR140333 and SR48968 doses and route of administration were chosen according to data from the literature (14). For the NK₁ antagonist, a unique dose of 2 mg/kg has been used. Standard anti-inflammatory drugs such as dexamethasone (Decadron; Prodome Química e Farmacêutica, Ltd., Campinas, Brazil) or pizotifen (Novartis, Basel, Switzerland) diluted in saline were also used by the subcutaneous (s.c.) route 30 min before formalin. Controls for the latter groups were composed of animals injected with s.c. saline 30 min before formalin injection. Drugs were administered in a volume of 0.1 ml/100 g animal. Mean number of animals used per group varied from 3–15.

**Statistical analyses**

Mean number of leukocytes (± S.E.M.) following different times of formalin injection and drug-treated animals were compared with their respective controls using one-way ANOVA for multiple comparisons. The least significant difference between means accepted was P<0.05, given by a software program (Sigma Stat 1.0).

**Results**

**Leukocyte recruitment following intraperitoneal formalin administration**

Formalin induced a significant increase in total leukocyte number present in rat peritoneal cavity in comparison with control (saline-injected) animals (P<0.05), which was dose- and time-dependent (Fig. 1:...
Total cell number was significantly increased following 4 h of 1.25% formalin, which was specifically due to an increase in neutrophil and lymphocyte number (Fig. 1a). Unexpectedly, the highest concentration of formalin (2.5%) did not induce significant change in total leukocyte counts following 4 h of injection, although specific counts revealed that eosinophils, usually low, were significantly raised in relation to control animals at this time-point (Fig. 1a). Total cell number increased following 24 h of 2.5% formalin administration, as shown in Fig. 1b, which was essentially due to a marked lymphocyte increase and a significant decrease in mast cell number ($P<0.002$). Counts from an earlier time-point studied (2 h) with formalin-injected animals, revealed that neutrophils (1.25% and 2.5% formalin) and lymphocytes (2.5% formalin) were both already significantly increased in relation to control animals (Table 1). An injection of a lower concentration of formalin (0.625%) did not induce a significant change in recovered leukocyte composition of rat peritoneal cavity as compared with control (saline)-injected animals at any time-point studied (data not shown).

![Fig. 1](image-url)  
Kinetics of leukocytes recruited to rat peritoneal cavity following intraperitoneal injection of formalin. Animals were either injected with 1 ml of 1.25% formalin, 2.5% formalin, or sterile physiological saline (Control) at zero time. Leukocytes in the lavage fluid were counted in a Neubauer chamber (total count) at 4 h (a) or 24 h (b) after injection. Stained smears of peritoneal lavage fluid were analyzed under optical microscopy (1000-fold magnification). *$P<0.05$ and **$P<0.01$, significantly different from the respective control group by one-way ANOVA (mean ± S.E.M., n = 4 – 11/group).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total count (10^6 cells/ml exudate)</th>
<th>Eosinophil</th>
<th>Neutrophil</th>
<th>Lymphocyte</th>
<th>Mast cell</th>
<th>Macrophage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>12.5 ± 0.72</td>
<td>0.2 ± 0.06</td>
<td>2.5 ± 0.70</td>
<td>3.0 ± 0.50</td>
<td>0.4 ± 0.10</td>
<td>6.5 ± 0.56</td>
</tr>
<tr>
<td>Saline</td>
<td>6.9 ± 0.41</td>
<td>1.3 ± 0.38</td>
<td>1.0 ± 0.34</td>
<td>1.8 ± 0.21</td>
<td>0.3 ± 0.08</td>
<td>2.5 ± 0.35</td>
</tr>
<tr>
<td>1.25% Formalin</td>
<td>7.5 ± 1.00</td>
<td>0.9 ± 0.18</td>
<td>2.0 ± 0.54*</td>
<td>2.6 ± 0.57</td>
<td>0.2 ± 0.08</td>
<td>1.8 ± 0.05</td>
</tr>
<tr>
<td>2.5% Formalin</td>
<td>12.2 ± 2.15*</td>
<td>1.7 ± 0.37</td>
<td>3.5 ± 1.30*</td>
<td>2.8 ± 0.38*</td>
<td>0.3 ± 0.14</td>
<td>3.9 ± 1.00</td>
</tr>
</tbody>
</table>

Formalin (1.25% or 2.5%) was prepared from a dilution of formaldehyde solution with physiological saline (NaCl 0.9%). Following 2 h, peritoneal cavities were lavaged and leukocytes counted in Neubauer chambers (total count). Cell differentiation was made in stained smears under optical microscopy (1000-fold magnification). *$P<0.05$; significantly different from the respective saline-injected group by one-way ANOVA (mean ± S.E.M., n = 4 – 7/group).
Neurokinin receptor antagonists in formalin-induced responses

Animals which were previously treated with specific antagonists for NK₁ (SR140333, 2 mg/kg), NK₂ (SR48968, 2 mg/kg), and NK₃ (SR142801, 2 mg/kg) receptors had a significantly reduced total cell number in rat peritoneal cavity following 4 h of the 1.25% formalin-injection (Fig. 2a). The cell types reduced, however, varied depending on the antagonist and dose used, although lymphocytes had been significantly reduced by NK₁ and NK₂ antagonists (Fig. 2b). Strikingly, a lower dose of NK₁ and NK₂ antagonist (0.1 mg/kg) was enough to significantly inhibit lymphocyte recruitment to the rat peritoneal cavity (Table 2). Reduction of total cell number by the treatment of the 1.25% formalin-injected animals with the NK₁ antagonist was also related to a significant reduction in eosinophil, neutrophil, and mast cell number (Table 2) at 4 h after the injection. Comparable amount of the NK₁ antagonist did not significantly affect the cell number of the control animals, at the same time-point (data not shown). Eosinophil, neutrophil, and macrophage numbers were also reduced by NK₂-antagonist (Table 2). In contrast to the acute inhibitory effect presented by the animals treated with all three NK antagonists on 1.25% formalin-induced leukocyte recruitment at 4 h of observation, the antagonists were not so effective in reducing total and specific cell counts following 24 h of 2.5% formalin injection (Fig. 3a). In this case, only animals treated with the specific NK₂ antagonist presented a reduced total cell number, which was due to a significant reduction in lymphocyte number (P<0.05, Fig. 3b). Surprisingly, animals treated with the NK₂ and NK₁ antagonist showed a twofold increase in neutrophil number relative to control animals at this time-point (P<0.001, data not shown). In addition, none of the NK-antagonists significantly affected the cell number present in lavage fluid collected 4 h following the 2.5% formalin administration, with the exception of mast cell number which was significantly increased in relation to control values in SR140333-treated animals (P<0.001, data not shown).

Effect of dexamethasone and pizotifen on formalin responses

Treatment of the animals with dexamethasone (Dexa, 1 mg/kg) reduced total cell number obtained following 1.25% formalin administration (data not shown), which was mainly due to a significant reduction in neutrophils that migrated to the peritoneal cavity of the animals (P<0.02, Table 3). Dexamethasone pre-treatment also reduced total cell counts obtained 24 h following 2.5% formalin administration, which was related to a decrease

![Image](62x159 to 533x391)
in specific number of lymphocytes as shown in Table 3, although eosinophils and macrophages were also reduced (data not shown). Total and specific cell counts, following 4 h of the 2.5% formalin administration were not significantly affected by previous treatment with 1 mg/kg dexamethasone (Table 3). Pizotifen, at an effective inhibitory dose (2 mg/kg) in serotonin (5-HT)-induced paw edema (data not shown), did not alter specific cell counts in the either 1.25% formalin-injected animals (at 4 h) or 2.5% formalin-injected animals (at 4 and 24 h), also shown in Table 3.
Discussion

Formalin injection into the paws of small animals is a well-known model used in the study of pain (15). In this model, formalin is able to acutely affect peripheral sensory nerve endings (5), with subsequent release of mediators, which give rise to a biphasic painful response (3, 16). However, when Dubuisson and Dennis (1) first described this response, they had also observed that inflammation (edema) was a prominent feature accompanying the painful response, later confirmed by others (4, 17). In general, leukocyte migration is an usual component of inflammation and contributes to it (18). In this sense, it has been our interest in this study to verify whether leukocyte recruitment would occur following formalin administration. For this, a simple model to assess cell recruitment, which consists of counting the total and specific leukocyte number present in rat peritoneal lavage fluid, following different times of formalin injections, was conducted. We have observed that, indeed, formalin induced a dose-and-time-dependent leukocyte recruitment to rat peritoneum in doses often used either in pain or edema studies (15, 19). The leukocyte recruitment induced by formalin was always characterized by a mixed polymorphonuclear (neutrophils) and mononuclear (predominantly lymphocytes) cell population as early as 2 h of observation, of shorter latency than that induced by other usual pro-inflammatory stimuli such as carrageenan or endotoxin from gram-negative bacteria (6, 20). As the cell recruitment only starts from 2 h after formalin injection, recruited cells do not seem to be implicated in the initial phase of pain (first 15 min) observed in rat paws following the formalin administration nor the early edematogenic response seen at 30 min of the injection, as previously described (21), suggesting that the edema resulted from a vascular more than a cellular (leukocyte) component at this time-point. However, the cell recruitment observed from 2 to 4 h after the formalin administration in our model certainly may indicate that the cells do contribute to mount an edematogenic response from 2 h observation in the rat paw model. Differences between inflammatory mechanisms in rat paws and peritoneum may be considered, but our model proved to be a valuable model to detect such response. In addition, increase in cell recruitment occurred biphasically, depending on the concentration of formalin used, is similar to the responses observed in pain and edema studies. Particularly, with the higher formalin (2.5%) used, a first increase in cell recruitment (lymphocytes) was observed within 2 h of injection, which was reverted to control levels by 4 h after injection. A second increase in lymphocyte number was observed by 24 h after 2.5% formalin administration. In our model, as no increase in cell number was observed at 4 h following 2.5% formalin treatment, it suggests that a modulatory component is acting at this time-point. Thus, both a stimulatory followed by an inhibitory component acting on cell recruitment are triggered by 2.5% formalin concentration. Release of inhibitory cytokines, such as interleukin (IL)-4 (22) or IL-10 (23), could both account for the inhibitory effect triggered by the highest concentration of formalin.

The main mediators released by peripheral sensory nerve endings so far described are known as tachykinins or NK and CGRP (24). Tachykinins include SP, NK A, and NK B (24). In rats, SP is known to be the principal agonist at NK₁ receptors, whereas NK A and NK B are

<table>
<thead>
<tr>
<th>Drug/stimulus</th>
<th>Cell number (10⁶ cells/ml exudate)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neutrophil</td>
<td>Lymphocyte</td>
<td>Neutrophil</td>
</tr>
<tr>
<td></td>
<td>4 h</td>
<td>24 h</td>
<td>4 h</td>
</tr>
<tr>
<td>1.25% Formalin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.5 ± 1.6</td>
<td>4.6 ± 0.8</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>1.5 ± 0.5*</td>
<td>4.3 ± 1.1</td>
<td>ND</td>
</tr>
<tr>
<td>Pizotifen</td>
<td>7.7 ± 4.1</td>
<td>4.6 ± 0.7</td>
<td>ND</td>
</tr>
<tr>
<td>2.5% Formalin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.3 ± 1.0</td>
<td>4.0 ± 1.2</td>
<td>4.3 ± 0.6</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>3.0 ± 0.4</td>
<td>2.0 ± 0.1</td>
<td>2.6 ± 0.6</td>
</tr>
<tr>
<td>Pizotifen</td>
<td>2.9 ± 0.5</td>
<td>2.5 ± 0.4</td>
<td>3.3 ± 1.2</td>
</tr>
</tbody>
</table>

Dexamethasone (1 mg/kg) or pizotifen (2 mg/kg) was subcutaneously injected 0.5 h before formalin. Leukocytes present in aliquots from peritoneal lavage fluid were counted by microscopy (10⁶ cells/ml exudate) and further identified in stained smears. *P<0.05, significantly different from the respective control group by one-way ANOVA (mean ± S.E.M., n = 4 – 9/group). ND = not determined.
the main agonists at NK₂ and NK₃ receptors, respectively, although NK₁ can also be an agonist at NK₃ receptors, with lesser affinity (25). In addition, peripheral sensory nerve endings which innervate blood vessels also may contain CGRP (26). Although CGRP contribution in our study was not addressed, SP, NK A, and NK B could have been released by formalin administration in the rat peritoneum. In fact, we have demonstrated that the treatment with specific antagonists of all three tachykinin receptors (NK₁, NK₂, and NK₃) led to a reduced cell number (polymorphonuclear and mononuclear cells) in the rat peritoneal cavity when the formalin concentration used was 1.25%. Data in the literature showed that SP and related peptides can stimulate chemotaxis in rabbit neutrophils (27) and chemoluminescent activity in rat polymorphonuclear cells in vitro (28). More recent data from DeRose et al. (29) have suggested mediation by NK₁ receptors of neutrophil adhesion to bronchial epithelial cells following SP administration, as it was inhibited by a specific NK₁ antagonist (CP-96,345). In addition, SP alone or in combination with other inflammatory mediators can induce both neutrophil and mononuclear recruitment as shown in various studies (9, 30); in particular, NK₁ receptors were already described in lymphocytes (31) and mast cells (32). From our data, it can be suggested that all three neurokinins SP, NK A, and NK B were released following formalin injection under our conditions. In addition, our data suggested that NK₁ receptors were present in all leukocytes recruited by 2.5% formalin, mainly neutrophils and lymphocytes, since their recruitment was significantly inhibited by the specific NK₁ antagonist used, confirming data from the literature (31 – 33). Considering the effectiveness of NK₁ and NK₂ antagonists in our hands, which inhibited cell recruitment induced by formalin at a very low dose (0.1 mg/kg), our data further suggest that cell migration is a quite sensitive response to these drugs. We can also suggest that lymphocytes expressed, besides NK₁, both NK₂ and NK₃ receptors, depending on time and concentration of formalin administered. It is tempting to speculate that release of NK₁ and/or SP was an early crucial event that accounted for the migratory response seen later (24 h), since only animals treated with NK₂ antagonist showed a reduced lymphocyte migration at this time-point. How this early release of neuropeptides could explain a later increase in cell number at 24 h following formalin administration is, at the present, unknown. Use of specific antagonists at NK receptors also suggested that lymphocytes, which

![Fig. 4. Proposed mechanisms involved in inflammation induced by formalin injection in rat peritoneal cavity. DRG, dorsal root ganglia; SC, spinal cord; PMN, polymorphonuclear cells (neutrophils and eosinophils); Mn, mononuclear cells (lymphocytes and macrophage-monocyte).](image-url)
were present at in the early stage (4 h), presented predominantly NK1 receptors. In contrast, lymphocytes presented mainly NK2 receptors at a later time (24 h). Taking together, these data suggested that subsets from the same lymphocyte lineage (NK1 or NK2 set) can be mobilized, depending on initial stimulus intensity (formalin 1.25% or 2.5%) or time of observation (4 or 24 h).

Mechanisms other than NK release (34) may be operating under formalin-treatment of the animals. For instance, release of chemotactic factors as leukotriene (LT) B4, platelet-activating factor (PAF), and cytokine /chemokines could be involved since steroids are known inhibitors of their expression (35) such as that seen by dexamethasone in our study. In fact, dexamethasone significantly inhibited neutrophil recruitment, whereas pizotifen was without effect in the same model. As serotonin is an important inflammatory mediator in rats (36 – 38), and treatment of the animals with a specific antagonist (pizotifen) did not modify formalin-induced responses, we could conclude that serotonin was not an important mediator under this condition.

In conclusion, we present evidence for a biphasic leukocytic response following formalin injection into rat peritoneal cavity characterized by a mixed polymorphonuclear (neutrophil) and mononuclear (mainly lymphocytes) cell population. This response was evident as early as 2 h of the injection, which was followed by a remarkable later increase in lymphocytes (at 24 h). Mediation of such a response by SP, NK A, and NK B of leukocyte recruitment induced by formalin administration was suggested by the use of specific antagonists of these NK receptors. NK release, following the formalin injection, would be derived from an alteration in the peritoneal cavities of actively sensitized rats. Agents Actions. 1985;17:499–505.

Acknowledgments

We are much grateful to Mr. X. Elmonds-Alt from Sanofi Recherche for supplying the specific neurokinin /tachykinin antagonists (SR compounds) used in the present study and to Webster Gleyser dos Reis for technical assistance. We also want to thank to FAPEMIG, CNPq, and CAPES for financial support.

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