Neutrophils and the calcium-binding protein MRP-14 mediate carrageenan-induced antinociception in mice

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Background: We have previously shown that the calcium-binding protein MRP-14 secreted by neutrophils mediates the antinociceptive response in an acute inflammatory model induced by the intraperitoneal injection of glycogen in mice.
Aim: In an attempt to broaden the concept that neutrophils and MRP-14 controls inflammatory pain induced by different type of irritants, in the present study, after demonstrating that carrageenan (Cg) also induces atinociception in mice, we investigated the participation of both neutrophils and MRP-14 in the phenomenon.
Methods: Male Swiss mice were injected intraperitoneally with Cg and after different time intervals, the pattern of cell migration of the peritoneal exudate and the nociceptive response of animals submitted to the writhing test were evaluated. The participation of neutrophils and of the MRP-14 on the Cg effect was evaluated by systemic inoculation of monoclonal antibodies anti-granulocyte and anti-MRP-14.
Results: Our results demonstrate that the acute neutrophilic peritonitis evoked by Cg induced antinociception 2, 4 and 8 h after inoculation of the irritant. Monoclonal antibodies anti-granulocyte or anti-MRP-14 reverts the antinociceptive response only 2 and 8 h after Cg injection. The antibody anti-MRP-14 partially reverts the antinociception observed after 4 h of Cg injection while the anti-granulocyte antibody enhances this effect. This effect is reverted by simultaneous treatment of the animals with both antibodies. After 4 h of Cg injection in neutrophil-depleted mice a significant expression of the calcium-binding protein MRP-14 was detected in the cytoplasm of peritoneal macrophages. This suggests that the enhancement of the effect observed after treatment with the anti-neutrophil antibody may be due to secretion of MRP-14 by macrophages. It has also been demonstrated that endogenous opioids and glucocorticoids are not involved in the antinociception observed at the 4th hour after Cg injection.
Conclusion: These data support the hypothesis that neutrophils and the calcium-binding protein MRP-14 are participants of the endogenous control of inflammatory pain in mice despite the model of acute inflammation used.

Key words: MRP-14; neutrophils; antinociception; carrageenan; mice.

Introduction

Inflammation is initiated by release of pharmacological agents and consequent recruitment of leukocytes from the blood stream to the injured tissues. These events involve expression of adhesion molecules on the vascular endothelium and adherence, diapedesis, and emigration of leukocytes to the extra vascular compartments.[1,2]

In most of the animal models of inflammation, a high proportion of neutrophils and a low proportion of monocytes promptly arrive at the focus of injury. Besides its typical morphology, neutrophils can be characterized by the high expression of the calcium-binding proteins MRP-8 and MRP-14.[3] These proteins were isolated, sequenced, and their genes cloned in an attempt to characterize the migration inhibitory factor (MIF) that accounts for their denomination as MIF-related proteins.[4,5] These proteins belong to the S-100 protein family and form heterodimeric complexes called calprotectin.[6,7]

MRP-8 and MRP-14 proteins are also named L1 light and heavy chain,[8,9] calgranulin A and B,[10,11] p8 and p14[12] respectively, and cystic fibrosis antigen.[13] The expression of these proteins is restricted to a specific stage of myeloid differentiation, since both proteins are expressed in circulating neutrophils and monocytes but are absent in normal tissue macrophages and lymphocytes.[14,15] MRP-8 and MRP-14 comprise 45 and 1% of the total proteins in the cytosol of neutrophils and monocytes respectively.[16] Under inflammatory conditions and/or upon calcium mobilization they are translocated from the cytosol to the cytoskeleton and to cell plasma membrane.[17,18] It has been demonstrated that both proteins are secreted by activated monocytes via a tubulin and Protein Kinase C-dependent pathway.[19] Another relevant characteristic of these proteins is that they are highly conserved, presenting 60–80% homology between humans, rat, and mouse.[16,20] Recently, it has been demonstrated that the heterodimer of these proteins binds arachidonic acid with high affinity.[21,22]

A novel function recently described for MRP-14 is its ability to regulate neutrophil adhesion to fibrinogen via β_2 integrin (CD11b/CD18, Mac-1).[23] MRP-8/14 complex is found in high concentrations in body fluids of patients with acute and chronic diseases such as chronic bronchitis, cystic fibrosis and rheumatoid arthritis,[24-26] suggesting a possible extracellular role for these proteins. In this context, it has been reported a marked anti-inflammatory effect of MRP-8/14 complex in a model of adjuvant-induced arthritis in the rat.[27] Additionally, it was demonstrated that the calcium-binding protein MRP-14 but not the MRP-8 deactivates activated peritoneal macrophages.[28]

It is generally accepted that neutrophils are at the forefront of an inflammatory response. These cells act not only as effector cells but also as regulatory cells in inflammatory reactions through the production of a variety of protein factors including proteolytic enzymes and cytokines.[29-31] On the other hand macrophages, considered as the body's alarm cell,[32] also secrete a large variety of chemical mediators released during the inflammatory response.[33] It is also well established that mediators released by these cells can interfere in the pathophysiology of inflammatory pain.[34,35]

Among these chemical mediators witch regulate the inflammatory nociceptive response are TNF-α, prostaglandins, interleukin (IL)-1, IL-6, IL-8 and others.[36-37] Otherwise, it has also been demonstrated the participation of interleukins such as IL-4, IL-10 and IL-13 on inhibitory mechanisms of inflammatory hyperalgesia.[38-40] In this context, results obtained in our laboratory demonstrated that the calcium-binding protein MRP-14 induces an antinociceptive effect when evaluated in a model of inflammatory pain in the mouse.[41] We have also demonstrated that MRP-14 released by neutrophil mediates the inhibition of pain response in a model of neutrophilic peritonitis induced by glycogen in mice.[41] In this paper we demonstrate that the intraperitoneal inoculation of a carrageenan (Cg) solution in mice also induces antinociception. Thus, the aim of this work was to investigate the participation of both neutrophils and MRP-14 on Cg-induced antinociception, in an attempt to broaden the hypothesis that neutrophils, via MRP-14, is an endogenous antinociceptive component of the inflammatory response as previously suggested.[41]

Materials and methods

Animals

Outbreed male mice from the Swiss strain, weighing 20–25 g, were used throughout this study. The animals were maintained under controlled light (12/12 hours) and temperature (22±2°C) with free access to food and water. Throughout the experiments, the animals were managed using the principles and guidelines for the care of laboratory animals according to Zimmermann.[42]

Carrageenan-induced peritonitis

Carrageenan (Sigma, MO, USA) was prepared in sterile saline (300 μg/0.2 ml per animal). Mice were injected intraperitoneally with 0.5 ml of this solution and killed in an ether chamber after 0.5, 2, 4, 8, 12, 24 and 48 h. The peritoneal exudate was collected and the total and differential counts of leukocytes determined. Controls were injected with sterile saline. Animals injected with Cg solution were submitted, after different time intervals, to the nociceptive test.

Nociception evaluation by the writhing test

The mouse writhing test used was based on the method of Koster *et al.*[43] The abdominal contortions resulting from intraperitoneal (i.p.) injection of acetic acid (0,6%; Merck, Darmstadt, Germany) at a dosage of 60 mg/Kg (v/v) consist of a contraction of the abdominal muscles together with the stretching of the hind limb. The number of abdominal contortions was counted cumulatively over a period of 20 min after acetic acid injection. The antinociceptive activity was expressed as the reduction in number of abdominal contortions in treated as compared to control animals (injected only with acetic acid).

Monoclonal antibodies (mAbs)

Rat mAb (IgG2b) anti-granulocyte (anti-GR-1), an antibody that depletes mice of mature granulocytes,[44] and rat mAb (IgG) anti-murine MRP-14 (EPM–1B4D3) were used. The antibody anti-Gr-1 (0.25 mg of protein

in 500 μl of saline) was injected i.p. 36 h before the i.p. injection of Cg. The mAb anti-MRP-14 (0.4 mg of protein in 100 μl of saline) was injected intravenously 5 min before the phlogistic agent injection.

Immunohistochemistry

The animals injected or not with anti-granulocyte antibody were killed in an ether chamber 4 h after Cg injection. The peritoneal exudate was collected with 5 ml of RPMI media and the peritoneal cells counted and concentrated to 2×10^6 cell/150 μl in RPMI media. Experiments were performed using 24-well plates (Costar, NY, USA) with sterile round glass coverslips in the bottom. The plates were incubated for 1 h at 37°C followed by lavage with phosphate-buffered saline (PBS) and fixation in Millonig fixative for 24 h. In the moment of the assay, plates were washed for three times with PBS and incubated with glycine buffer (50 mM) for 30 min at room temperature. Subsequently, the buffer was discarded, the wells washed with PBS and the cell membranes permeabilized with a Triton X-100 solution (0.02%) for 5 min. All incubations were done at room temperature and PBS was used for washing the wells after each step. Endogenous peroxidase was quenched with 0.3% H_2O_2 in PBS for 50 min. The cells were then incubated with normal horse serum in PBS for 1 h and overlaid with the primary antibody (mAb anti-MRP-14) at 4°C overnight. Thereafter they were incubated with biotinylated goat anti-rat IgG secondary antibody (Sigma, MO, USA), followed by streptoavidin peroxidase complex (Sigma, MO, USA) for 30 min. The material was stained with 10 mg of diaminobenzidine tethahydrochlorid (DAB; Sigma, MO, USA) with 0.03% H_2O_2 in Tris-buffered saline (0,05 M; pH 7,6). After staining, the cells were washed in tap water and counterstained with Harry's hematoxilin (Merck, Darmstadt, Germany) for 40 sec. Finally, the cells were dehydrated and mounted in Entellan (Merck, Darmstadt, Germany).

Participation of opioids

Mice were injected with naloxone chloridate (Rhodia, São Paulo, Brasil), a universal opioid antagonist, prepared in sterile saline (1 mg/Kg per animal). Animals were injected with 200 μl of this solution by subcutaneous route 15 min before the i.p. injection of acetic acid in animals pretreated with Cg.

Participation of glucocorticoids

Mice were injected with metyrapone (Ciba-Geigy, São Paulo, Brazil), dissolved in sterile saline. Metyrapone blocks the synthesis of glucocorticoids without causing a typical deficiency of mineralocorticoids.[45] Two daily doses of 30 mg/Kg each were given intraperitoneally at 12 h intervals for 5 days.[46] Control
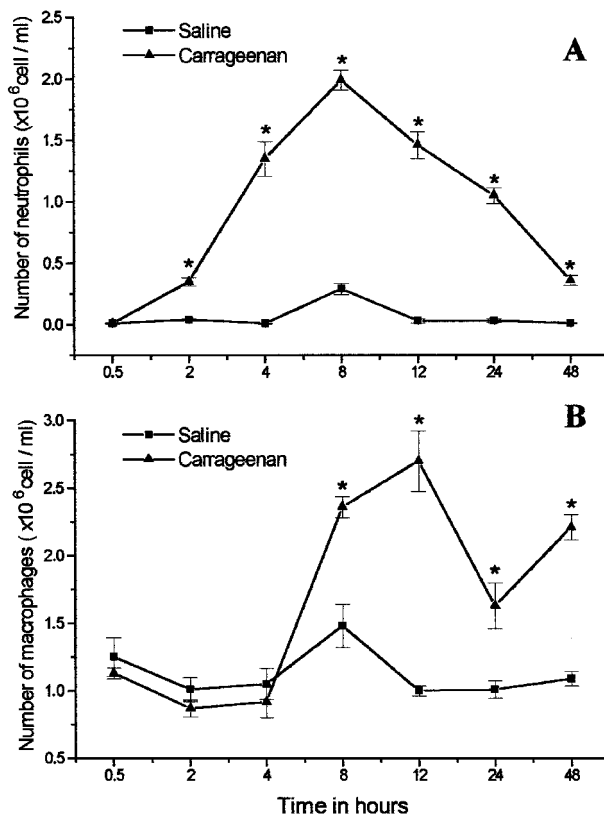

FIG. 1. Kinetic of leukocyte influx to peritoneal cavity of mice after i.p. injection of a carrageenan (Cg) solution (300 μg/0,2 ml per animal). Number of neutrophils in the peritoneal cavity after the irritant injection (A). Number of mononuclear cells after Cg injection (B). The results are expresses in absolute numbers obtained in relation to the total number of collected cells. Data represent mean values ±S.E.M. for 8–16 animals for each experimental time. *Significantly different from mean values of saline group ($p < 0.05$).

animals received the same amount of saline. Cg injection was made 2 h after the administration of the last dose metyrapone and the writhing test evaluated 4 h after injection of the phlogistic agent.

Statistical analysis

Results are expressed as mean ± standard errors of means (S.E.M.) and were compared by the Student's t test or by analysis of variance[47] followed by Duncan's test.[48] A probability level of less than 0.05 was taken as significant ($p < 0.05$).

Results

Leukocyte migration induced by carrageenan

Considering that the protein MRP-14 is expressed in large amounts in the cytosol of neutrophils[16] and that this protein mediates an antinociceptive effect in a neutrophilic peritonitis induced by glycogen,[41] we decided to investigate if this effect was restricted to the glycogen model or if it could be evidenced in an
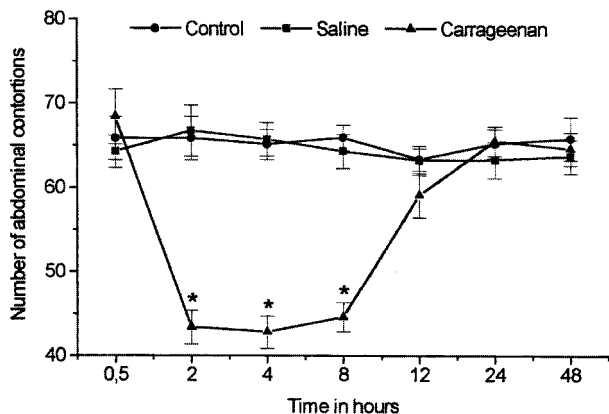
FIG. 2. Effect of carrageenan (Cg) on painful sensibility of mice evalued in the writhing test. The test was applied in different times after the i.p. injection of Cg (300 μg/0,2 ml per animal). Controls were injected only with acetic acid. Values represent the mean ±S.E.M. of 8–16 animals for each group. *$p < 0.05$ in comparison with values obtained in control animals.

FIG. 3. Effect of mAb anti-granulocytes (anti-Gr–1) on antinociception induced by carrageenan (Cg) in animals submitted to the writhing test. The mAb anti-Gr–1 was injected 36 h before the i.p. injection of Cg and 2, 4 or 8 h later submitted to the writhing test. Animals injected with non related mAb were also evaluated. Controls were injected only with acetic acid. Values represent the mean ±S.E.M. of 8–16 animals for each group. *$p < 0.05$ in comparison with values obtained in control animals. **$p < 0.05$ in comparison with the group treated with non related mAb + Cg.

another model of neutrophilic peritonitis. For this purpose a model of neutrophilc peritonitis was established using Cg as the phlogistic agent.

Results show that the i.p. injection of this irritant evokes significant alterations in the pattern of circulating leukocytes. The total number of neutrophils in the peritoneal cavity of animals injected with Cg was significantly higher than controls. The maximal number of polymorphonuclear cells in the peritoneal cavity was observed 8 h after the irritant injection (Fig. 1A). On the other hand, a marked increase in the number of mononuclear cells 8 h after the phlogistic agent injection was observed (Fig. 1B).

Carrageenan-induced neutrophilic peritonitis and antinociception evaluated by the writhing test

To investigate a possible correlation between the influx of neutrophils to the peritoneal cavity and a possible antinociceptive effect of the irritant, animals were submitted to the writhing test after Cg injection. As show in Figure 2, mice injected with Cg and tested after 2, 4 or 8 h in the pain test showed a significant decrease in the number of abdominal contortions when compared with animals previously injected with saline or with acetic acid alone. (±30% inhibition in all the times tested).

Effect of mAb anti-granulocyte on antinociception in the carrageenan-induced peritonitis

To investigate whether the analgesic effect observed was due to the presence of neutrophils in the inflammatory site, depletion of these cells with a specific mAb was used. Animals pretreated with mAb
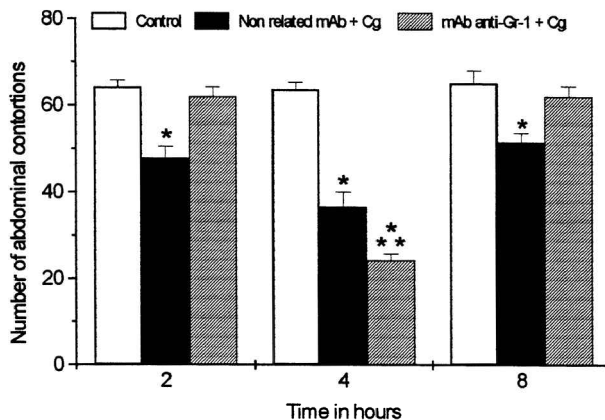
anti-granulocyte (anti-Gr-1, 0.25 mg, i.p.), 36 h before the i.p. injection of Cg, showed a 99% reduction in the total number of neutrophils in the peritoneal exudate (data not shown). Depletion of neutrophils, totally reverted the antinociceptive response only after 2 and 8 h of the irritant injection (Fig. 3). In contrast, this treatment enhanced the antinociceptive effect at 4 h after Cg injection. The treatment of animals with non-related rat IgG2b did not influence the antinociceptive effect observed in any times after the irritant injection (Fig. 3).

Effect of mAb anti-MRP-14 on the antinociceptive response induced by carrageenan

To investigate whether MRP-14 was implicated in the antinociception observed, mice were treated with mAb anti-MRP-14. The animals were treated with 40 μg of protein in 50 μl saline, i.v., 5 min before the i.p. injection of Cg. This treatment completely abolished antinociception detected 2 and 8 h after the irritant injection (Fig. 4). However, this treatment only partially reverted antinociception observed after 4 h of Cg injection. Treatment of animals with non-related rat IgG had no effect in the antinociceptive effect induced by this irritant (Fig. 4).

Simultaneous treatment of the animals with anti-granulocyte and anti-MRP-14 mAbs

Once the depletion of neutrophils enhances the antinociceptive response when the test were applied 4 h after Cg injection, we decided to evaluate the response of the animals in the absence of neutrophils
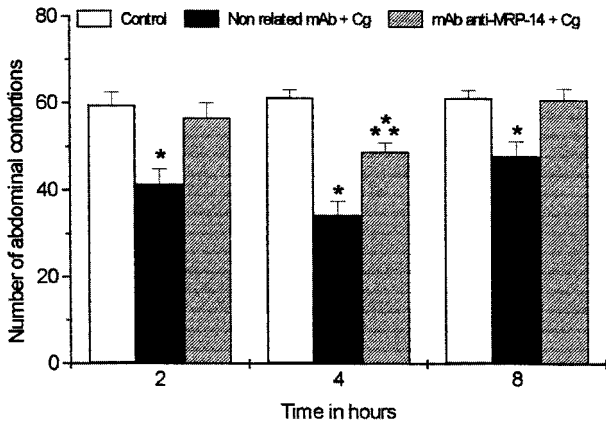
FIG. 4. Effect of mAb anti-MRP-14 on antinociception induced by carrageenan (Cg) in animals submitted to the writhing test. The mAb anti-MRP-14 was injected 5 min before the i.p. injection of Cg and 2, 4 or 8 h later submitted to the writhing test. Animals injected with non-related mAb were also evaluated. Controls were injected only with acetic acid. Values represent the mean ±S.E.M. of 8–16 animals for each group. *$p < 0.05$ in comparison with values obtained in control animals. **$p < 0.05$ in comparison with the group treated with non related mAb + Cg.

and MRP-14. Mice were inoculated with mAb anti-granulocyte 36 h before treatment with the mAb anti-MRP-14. The Cg solution was injected 5 min after the last treatment and the writhing test applied 4 h after the phlogistic agent injection. Results show that treatment with both antibodies abolished the potentialization of antinociceptive effect induced by Cg in animals pretreated with antibody anti-granulocyte (Fig. 5). Treatment of animals with non-related mAbs
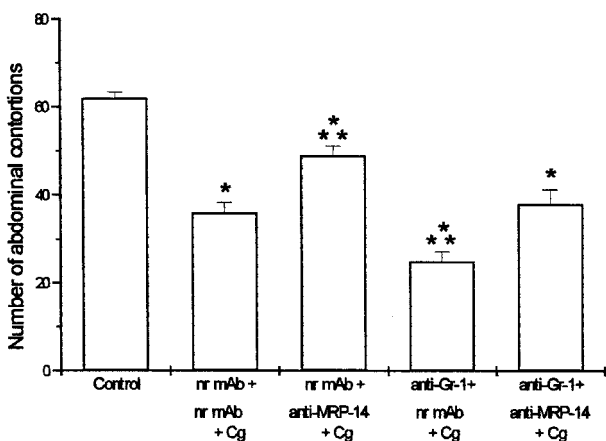
did not alter the antinociceptive response evaluated 4 h after the irritant injection (Fig.5).

Detection of MRP-14 in peritoneal cells of animals inoculated with carrageenan

Considering that the treatment with mAb anti-MRP-14 was able to revert the potentialization of anti-nociceptive effect observed 4 h after the Cg injection in animals depleted of neutrophils, the expression of MRP-14 in other inflammatory cells was evaluated by imunocitochemistry. Cells were obtained from the abdominal cavity of animals 4 h after the irritant injection in mice pretreated or not with anti-granulo-cyte mAb. Cells obtained from the peritoneal exudate 4 h after Cg injection were positive for MRP-14 protein (Fig. 6A). However, when the test was made with cells obtained from the peritoneal exudate 4 h after the irritant injection in mice depleted of neutrophils, the expression of MRP-14 was significantly more intense. A larger number of cells expressing the protein in their cytoplasm was observed (Fig. 6B).
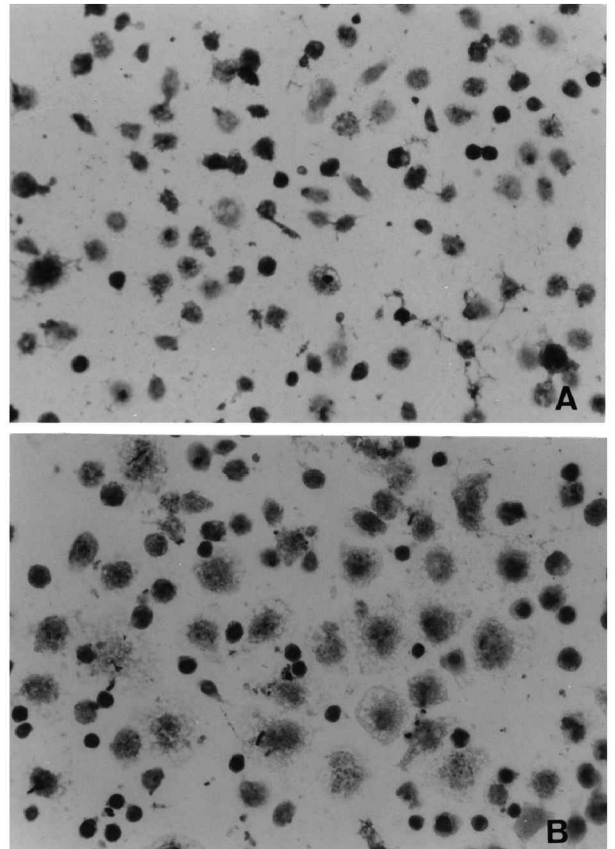

FIG. 5. Effect of simultaneous treatment of the animals with anti-granulocyte (anti-Gr-1) and anti-MRP-14 mAbs on anti-nociception induced by carrageenan (Cg) in animals sub-mitted to the writhing test. The mAb anti-Gr-1 was injected 36 h before the irritant and the anti-MRP-14 was injected 5 min before the injection of Cg. The nociceptive test was applied 4 h after Cg injection. Animals injected with non related mAbs (nr mAbs) + Cg, nr mAb + anti-MRP-14 + Cg or nr mAb + anti-Gr-1 + Cg were also evaluated. Controls were injected with acetic acid. Values represent the mean ±S.E.M. of 8–16 animals for each group. *$p < 0.05$ in comparison with values obtained in control animals. **$p < 0.05$ in comparison with animals treated with nr mAbs + Cg.

FIG. 6. Photomicrography of cells obtained from the peri-toneal cavity of animals injected with Cg 4 h before and immunostained for the calcium-binding protein MRP-14 (A). In B, cells obtained from the peritoneal exudate induced by Cg injection in an animal previously depleted of neutrophils. The expression of MRP-14 is more intense in this prepara-tion. Optic microscopy ($\times$ 100).

Participation of endogenous opioids on antinociception induced by carrageenan

Once the mAbs anti-granulocyte and anti-MRP-14 did not totally revert the antinociceptive effect induced 4 h after Cg injection, the possible participation of endogenous opioids in the phenomenon was evaluated. Animals were treated with naloxone chloridate 15 min before the nociceptive test in mice pretreated 2 or 4 h with Cg. Results demonstrated that opioid peptides did not influence the antinociception induced by Cg (data not shown).

Participation of glucocorticoids in the antinociceptive effect evoked by carrageenan

The participation of glucocorticoids, other possible endogenous factor important on the control of inflammatory pain, was evaluated during antinociception induced 4 h after Cg injection. Pretreatment of animals with metyrapone, an inhibitor of glucocorticoid synthesis, given intraperitoneally in two daily doses (12/12h) for 5 days, did not alter the antinociceptive effect induced by Cg (data not shown).

Discussion

As reported in the literature, chemical mediators released during the inflammatory process are endowed with the property of inhibiting pain response. It has been shown that interleukins such IL-4, IL-10 and IL-13 have the propriety to limit inflammatory hiperalgesia by blocking the release of pro-inflammatory cytokines by inflammatory cells.[38-40] Furthermore, it was demonstrated by Giorgi *et al.*[41] that the calcium-binding protein MRP-14 has a marked antinociceptive activity in an inflammatory pain model in mice. These authors brought evidences that neutrophils participate in the control of inflammatory pain by secreting this protein to the inflammatory milieu.[41] Reports made by these authors however, were limited to a model of neutrophilic peritonitis induced by glycogen. It remains then to be investigated whether neutrophils and MRP-14 also control inflammatory pain in other models of neutrophilic peritonitis. Here we demonstrate that similarly to the glycogen model, the intraperitoneal inoculation of carrageenan (Cg) in mice not only induces a typical neutrophilic peritonitis but also a marked antinociceptive response when animals were tested in a model of inflammatory pain. The intraperitoneal injection of Cg solution 2, 4 or 8 h before the acetic acid inoculation induces a significant antinociceptive response. Interesting to note that at these times a large number of neutrophils were found in the peritoneal cavity of the animals.

Based on these observations we investigated the possible participation of neutrophils and of the calcium-binding protein MRP-14 in the antinociception induced by Cg. Results showed that treatment of animals with mAbs anti-granulocyte or anti-MRP-14 before the phlogistc agent administration were able to totally revert antinociception observed 2 and 8 h after Cg injection. Unexpectedly, pretreatment of animals with anti-granulocyte mAb induced an increase in the antinociceptive effect observed 4 h after the irritant injection. Further, the treatment of animals with anti-MRP-14 mAb partially reverted antinociception observed 4 h after the irritant injection. Based on these observations, animals were treated with both antibodies previously to the evaluation of the antinociceptive response. The association of antibodies anti-granulocyte and anti-MRP-14 induced the reversion of increase in antinociceptive effect observed when the animals were treated only with the anti-granulocyte mAb followed by Cg injection.

Amorim Dias (personal communication, 2002) have demonstrated that the intraperitoneal inoculation glycogen in granulocyte-depleted mice induces the expression of MRP-14 in the cytoplasm of mononuclear cells. Similarly, mononuclear cells obtained from the peritoneal cavity of neutropenic mice injected with Cg also express the calcium-binding protein MRP-14. Thus, these data suggest that the enhancement of the antinociceptive effect observed after treatment of the animals with anti-granulocyte antibody might be due to secretion of MRP-14 by macrophages. However, the mechanisms by which neutrophil depletion induces macrophages to express the protein MRP-14 remains to be investigated.

Since antinociception observed 4 h after Cg injection was not totally reverted by treatment of animals with anti-MRP-14 antibody, other endogenous factors could be involved in the inhibition of pain sensibility. The liberation of endogenous opioids after Cg injection might be one of these factors. It has been largely demonstrated that endogenous opioids participate in control of inflammatory pain.[49-50] Our results however, showed that these mediators seems not to be involved in the antinociception observed at the 4th hour after Cg injection since treatment of the animals with naloxone did not alter this effect. As has been demonstrated, Cg can activate the hypothalamus-adrenal axis inducing the release of glucocorticoids by the adrenal gland.[51] As reported in the literature, this hormone is endowed with a potent anti-inflammatory activity and consequently, an analgesic property.[52-54] Our data demonstrate that an exacerbation in the release of glucocorticoids by adrenal glands, seems not to influence antinociception in this model since the inhibition of glucocorticoid synthesis by metyrapone administration did not interfere with the antinociceptive

response. Thus, besides the partial mediation of MRP-14 in the antinociceptive effect observed after 4 h of Cg injection other mechanisms involved in this effect remains to be elucidated. In summary, results obtained in this work demonstrate that neutrophils and the calcium-binding protein MRP-14 participate in the endogenous control of the inflammatory pain in mice despite the model of acute inflammation used.

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