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Systemic changes following carrageenan-induced paw inflammation in rats

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Abstract
Objective and design Carrageenan-induced paw edema has been described as a local and acute inflammatory process. In fact, little is known about the time course and systemic changes following a carrageenan injection. In this study, we examine the systemic changes that follow carrageenan injection in the paw.
Methods Acute inflammation was produced by subplantar injection of carrageenan in a hind paw of Sprague–Dawley rats. Saline was used in control rats. Paw volume was measured with a plethysmometer. The hot plate latency test was used to quantify antinociception. C-reactive protein (CRP) levels were measured with a sandwich enzyme immunoassay. Fibrinogen concentration was measured using the gravimetric method. Lung morphometric analysis was performed using an image processing package. Lungs and paws were also examined for tissue factor (TF) and proinflammatory cytokines expression by immunohistochemistry.
Results We found diverse systemic changes including increased levels of acute phase proteins, such as CRP and fibrinogen, and a lung inflammatory process characterized by lung edema, fibrin deposition, and leukocyte infiltration. An elevated expression of TF, IL-6, IL-1β, and TNFα, was observed in paw and lung tissue sections by immunohistochemical methods.
Conclusion This study provides new evidence that a local carrageenan injection induces a systemic response.

Keywords Carrageenan · Paw model · Lung inflammation · Tissue factor expression · Proinflammatory cytokines

Introduction

Paw edema induced by carrageenan, originally described by Winter et al. [1], is a well researched and highly reproducible model of inflammation. Cardinal signs of inflammation, such as edema, hyperalgesia, and erithema, develop immediately following subplantar injection of carrageenan into a hindpaw, as a result of the action of proinflammatory agents, such as bradykinin, histamine, prostaglandins, thromboxanes, reactive oxygen, etc., that can be generated at the site of the insult or by infiltrating cells [2–5].

On the other hand, injection of carrageenan into the pleura induces pleurisy, another well-characterized experimental model of acute inflammation. This acute inflammatory response is characterized by an increase in vascular permeability and cellular infiltration leading to lung edema, as a result of extravasation of fluid and proteins and accumulation of leukocytes at the inflamed site [2, 6, 7].

There is little information about the systemic reaction evoked by subplantar administration of carrageenan. Srivastava and Srimal [8] evaluated platelet function in this model and found enhanced platelet aggregation, while Cicala et al. [9] demonstrated a haemostatic imbalance

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characterized by increased plasma fibrinogen levels, antithrombin III activity, and serum interleukin-6 (IL-6) levels, as well as shortened prothrombin time and an increased platelet responsiveness to ADP.

Here, we used the intraplantar injection of carrageenan to study in detail the systemic consequences of an apparently localized acute inflammation. We found increased levels of C-reactive protein and fibrinogen, as well as a lung inflammatory process characterized by edema, fibrin deposition, leukocyte infiltration, and expression of tissue factor, IL-1β, IL-6, and TNFα.

Materials and methods

Materials

Lambda carrageenan was obtained from Sigma, USA. The C-reactive protein (CRP) kit was obtained from ALPCO Diagnostics, USA. Rabbit polyclonal anti-human tissue factor, fluorescein isothiocyanate (FITC)-labeled goat polyclonal anti-rabbit IgG, and FITC-labeled bovine polyclonal anti-goat IgG antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). Mouse polyclonal anti-rat IL-6, mouse polyclonal anti-rat TNFα, and goat polyclonal anti-rat IL-1β antibodies from R&D Systems (Minneapolis, USA) were used. Tetramethylrhodamine isothiocyanate-conjugated rabbit anti-mouse IgG antibody was acquired from Abcam (Cambridge, UK).

Rat paw edema

Sprague–Dawley rats (250–300 g body weight) were acclimatized to laboratory conditions for 1 week with free access to water and food and observed for any sign of illness. All procedures were carried out in accordance with the Animal Ethics Committee of IVIC.

Rats were divided into two groups ($n = 6$ each group) and lightly anesthetized with thiopenthal. One group received a subplantar injection of 100 µl of carrageenan 2 % (wv^{-1}) diluted in saline in the left hind paw pad, while the control group received the same volume of saline. Paw volume was measured by using a water plethysmometer (Ugo Basile, Italy) immediately before subplantar injection, and 2, 4, 8, 12, 24, 36, and 48 h thereafter. The increase in paw volume was calculated by subtracting the initial paw volume (basal) from the paw volume measured at each time point.

Measure of nociception

The hot plate latency test was used to quantify thermal antinociception. Briefly, animals were placed individually onto a hot plate with temperature of 48–50 °C (Hot Plate 39D, IITC). Heat exposure was continued until the animal showed a withdrawal response in the form of hind paw licking, shaking, or lifting. The latency time of the withdrawal response was determined before carrageenan injection and at 2, 4, 8, 12, 24, 36, and 48 h thereafter. The shortening of the latency time in response to carrageenan was taken as an index of hyperalgesia.

Determination of acute phase proteins

The CRP levels were measured in serum with a sandwich enzyme immunoassay (ALPCO Diagnostics, Salem, USA). Fibrinogen concentration was measured using the gravimetric method of Ingram [10].

Histological methods

Animals were sacrificed at different time intervals after carrageenan injection. Organs were fixed by cardiac perfusion using a fixative solution (4 % wv^{-1} formaldehyde, buffered with 66.66 mM phosphate, pH 7.6), and a sample of tissue from the injected paw was immersed in fixative for 24 h at room temperature.

Lung tissue was prepared as follows: after intracardiac perfusion, the lungs were subjected to tracheal instillation of fixative, the trachea was then ligated and the lungs excised, the fixation was completed at 4 °C for 12 h, and then the lungs were transferred to 15 % sucrose in PBS at 4 °C overnight.

Tissues were dehydrated with ethanol, clarified with xylene, and embedded in Paraplast®. Tissue sections were cut, dewaxed in xylene, rehydrated with distilled water, and stained with haematoxylin and eosin to evaluate tissue architecture. Fibrin was stained using a modification of Carstairs' method as previously described [11]. With this method, fibrin is colored red and red blood cells yellow.

The ImageJ (version 1.44p) public domain Java image processing package (National Institutes of Health, Bethesda, MD, USA) was used to calculate area fractions occupied by nuclei, alveolar space, and fibrin as described previously [12].

Immunohistochemistry

Paraffin-embedded lung and paw sections (5 µm) were used for the immunohistochemical expression of tissue factor (TF), IL-1β, IL-6, and TNFα. After removal from paraffin with xylene, the slides of lung and paw were incubated for 1 h in 70 % ethanol supplemented with 0.25 % NH_4OH, rehydrated with 50 % ethanol for 10 min, and transferred to PBS. The antigen was retrieved by boiling slides in 0.01 M citrate buffer (pH 6.0) in a 500 W

microwave oven twice for 5 min, and extensively washed with PBS. Unspecific binding sites were blocked using 5 % BSA in PBS for 1 h at room temperature before incubation with the primary antibody.

Tissue sections were incubated overnight at 4 °C in a humidified chamber with a 1:1,000 dilution of primary antibodies in the following combinations: a rabbit polyclonal anti-TF with a mouse polyclonal anti-IL-6 or a mouse polyclonal anti-TNFα with a goat polyclonal anti-IL-1β. Omission of the primary antibody served as negative control. After washing with PBS, the slides were incubated for 1 h at room temperature with a 1:500 dilution of two secondary antibodies: rhodamine-labeled anti-mouse IgG with either FITC anti-rabbit IgG or FITC anti-goat IgG. The slides were washed with PBS, and then the nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI; 5 μg ml^{-1}) for 5 min and washed again with PBS. Finally, the slides were mounted with Immunomount® (Biomedia).

Observation and imaging of immunolocalization was carried out using a fluorescent microscope Eclipse E600 (Nikon, Tokyo, Japan) equipped with epifluorescence illumination and appropriate filters; G-2A was used for rhodamine, B-2A for FITC, and UV-2A for DAPI. Photographs of fluorescent images were taken with a digital camera (Coolpix 8700; Nikon). The images were merged using the ImageJ 1.44p software.

The nonparametric Mann–Whitney test was used to determine significance between groups. The program InStat (GraphPad Software) was used for the statistical analysis. The differences were considered significant when $P < 0.05$.

Results

Paw edema

The injection of carrageenan produced a significant ($P < 0.01$) and rapid increase in the volume of the injected paw, reaching its maximum 4 h post-injection (Fig. 1a). This level of inflammation was maintained for about 8 h and subsequently declined to remain around 40 % of the maximum volume until the end of the experiment. No signs of edema were detected in the contralateral hind paw.

Nociceptive measures

Carrageenan promoted a significant and sudden shortening in the withdrawal latency of the inflamed paw which peaked 4 h after the injection ($P < 0.01$), matching the time of maximum inflammation (Fig. 1b). From that moment on, withdrawal latencies gradually returned to baseline levels. Carrageenan injection did not modify the response values of the contralateral hind paw.

Acute phase protein levels

CRP levels increased significantly after 2 h post-injection of carrageenan ($P < 0.05$), and reached maximum values between 4 and 12 h. Thereafter, CRP levels remained high until the end of the experiment. CRP levels were not affected in control rats (Fig. 1c). On the other hand, plasma fibrinogen levels significantly increased from 8 h on ($P < 0.05$) reaching its maximum 36 h following carrageenan-induced edema (Fig. 1d). Fibrinogen levels did not change in control rats.

Histological analysis of lung samples

Normal lung architecture was observed in tissue sections from a rat without carrageenan administration (Fig. 2a). In contrast, clear signs of inflammation were observed, such as edema, fibrin deposition, and cellular infiltration 2 h after subplantar administration of carrageenan (Fig. 2b). These signs were more pronounced 4 h later, resulting in a strong reduction of alveolar space (Fig. 2c). Infiltrating inflammatory cells were predominantly neutrophils and mononuclear cells (Fig. 2f). All inflammatory signs diminished gradually as seen 24 and 36 h after carrageenan injection (Fig. 2d, e).

Table 1 contains the data on lung morphometry after subplantar injection of carrageenan. Data are fractions (as %) of the total lung area occupied by each tissue component (nuclei, alveolar space, or fibrin). At least three photographs per rat were examined and five sections of each photograph were randomly selected. Nuclear fractional area increased gradually from 8.7 ± 2.3 to 23.5 ± 4.9 % ($P < 0.05$) 4 h following carrageenan administration. Thereafter, it decreased slowly but did not reach the basal value even 36 h after the challenge (15.5 ± 5.3 %; $P = 0.09$). The alveolar space fractional area decreased significantly from 76.3 ± 0.5 to 69.5 ± 0.2 % ($P < 0.05$) as early as 1 h after carrageenan injection and to 62.5 ± 4.7 % ($P < 0.05$) 4 h later. The reduction of the alveolar space was statistically significant even 36 h following carrageenan injection (72.4 ± 1.0 %; $P < 0.05$). The fibrin fractional area increased from 2.8 ± 1.1 % to 18.2 ± 3.0 % ($P < 0.05$) of the lung area 4 h after carrageenan injection. Fibrin deposits decreased slowly but did not disappear even 36 h after induction of the inflammatory process.

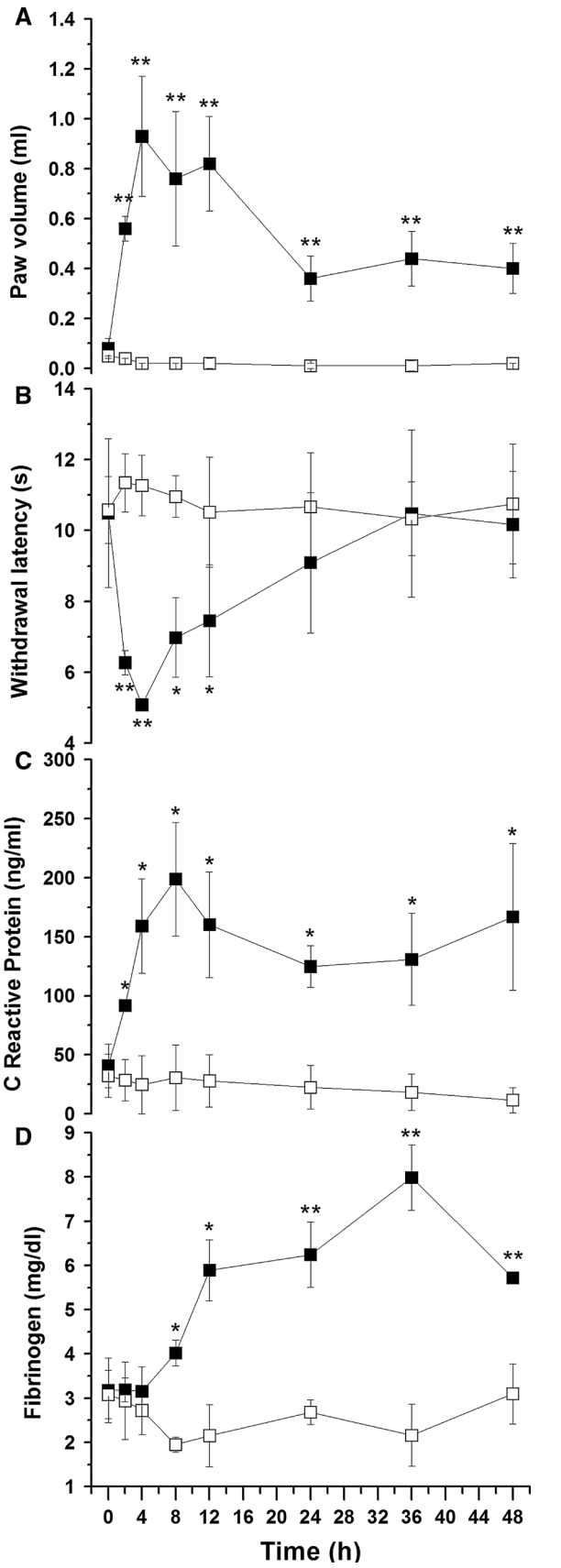


Fig. 1 Effect of carrageenan on the paw edema volume (**a**), withdrawal latency (**b**), CRP (**c**), and fibrinogen levels (**d**). Inflamed rats (*closed squares*) received a subplantar injection of 100 µl of carrageenan 2 % (wv^{-1}), while the control group (*open squares*) received an equal volume of saline. Results are shown as mean ± SEM from six rats. The nonparametric Mann–Whitney test was used to determine significance between groups. $*P < 0.05$; $**P < 0.01$ versus control group

Cytokines and TF immunohistochemical expression

To determine whether the acute lung inflammation observed following carrageenan subplantar injection was associated with a prothrombogenic state and altered cytokines expression in the lung, we visualized the expression of TNFα, IL-1β, IL-6 as well as TF in this organ and in the injected paw using immunohistochemistry (Figs. 3, 4).

Figure 3 shows positive staining for IL-6 (red) and TF (green) in lung tissue sections collected from rats at 4 h (Fig. 3a, b) and 24 h (Fig. 3c, d) after carrageenan injection. Immunostaining was localized mainly in the infiltrating inflammatory cells as well as in vascular walls. Tissue factor was mainly expressed by neutrophils, mononuclear, and endothelial cells, while IL-6 was expressed by mononuclear cells and fibroblasts. Low positive staining was observed in control rats. A substantial increase in immunostaining for TNFα and IL-1β was also found in the lung tissue collected from rats at 4 and 24 h after carrageenan injection. TNFα and IL-1β were mainly expressed by infiltrating cells (data not shown).

Paw tissue sections obtained at 4 h (Fig. 4a, b) and 24 h (Fig. 4c, d) after carrageenan injection showed positive staining for TF and IL-6 localized mainly in the infiltrating inflammatory cells. Again, low positive staining was observed in control rats. The expression of TNFα and IL-1β was also increased in paws collected from rats at 4 and 24 h after carrageenan injection (data not shown).

In general, the percentage of cells expressing TF and the above-mentioned cytokines was always higher in the paw than in the lung. Their expression kinetics was also different in these organs (Fig. 5). In the paw, immunostaining of TF and cytokines increased reaching a peak 4 h after inflammation induction, then it decreased progressively but remained higher than basal levels even 36 h later. In contrast, their expression in the lung reached maximum values 1 h after carrageenan challenge and remained almost constant until the end of the experiment. Low positive staining for TNFα, IL-1β, IL-6, and TF was observed in the paw and lung tissues of control rats (data not shown).

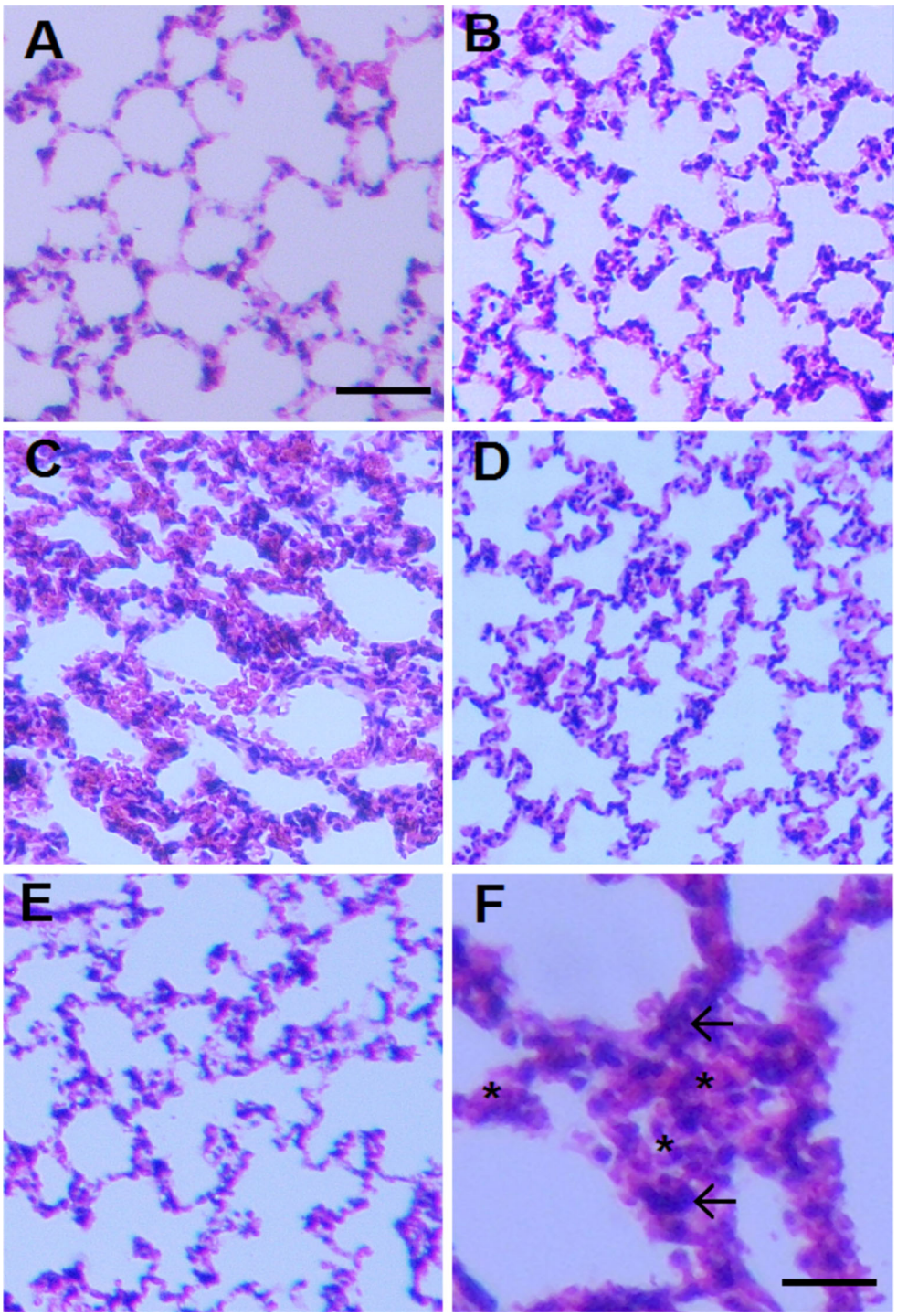


Fig. 2 Lung sections, stained with hematoxylin and eosin, from a rat without carrageenan edema induction (**a**) show no signs of inflammation, while carrageenan-treated rats 2 h (**b**), 4 h (**c**, **f**), 24 h (**d**), and 36 h (**e**) after induction of inflammation show edema, fibrin deposition, and leukocyte infiltration into the alveolar spaces and lung parenchyma. **f** higher magnification of sample shown in **c** indicating neutrophil infiltration (*arrows*) and fibrin deposition (*asterisks*). Representative results of $n = 5$ rats studied in each group are shown. *Scale bar* 30 μm (**a**–**e**), 15 μm (**f**)

Table 1 Effect of carrageenan (2 mg/paw) on area occupied by nuclei, alveolar space, and fibrin deposition in the lung

Fraction (%) of lung area			
Time (h)	Nuclei	Alveolar space	Fibrin
0	8.7 ± 2.3	76.3 ± 0.5	2.8 ± 1.1
1	15.6 ± 2.0*	69.5 ± 0.2*	14.1 ± 3.9*
2	14.8 ± 3.7*	65.9 ± 4.0*	19.3 ± 2.2*
4	23.5 ± 4.9*	62.5 ± 4.7*	18.2 ± 3.0*
24	15.4 ± 2.6*	65.5 ± 5.5*	15.6 ± 2.6*
36	15.5 ± 5.3	72.4 ± 1.0*	13.0 ± 2.6*

Percentages were obtained from five randomly selected fields from lung sections ($n = 5$). The nonparametric Wilcoxon Mann–Whitney test was used to determine significance between groups

* $P < 0.05$

Discussion

Numerous studies have been performed on the inflammatory response induced by carrageenan. One usual method consists of injecting carrageenan under the plantar skin of a rat hindpaw [1]. Also, carrageenan has been injected into the pleural space to induce pleurisy, a preclinical model of lung inflammation [2, 6, 7]. Both of these models have been described as local and acute inflammatory processes.

The carrageenan paw model has been widely employed as an animal model for screening anti-inflammatory drugs, and most studies have used edema as the dependent measure [13, 14]. Comparison of our results with previously published data shows similar time course curve for paw edema development after carrageenan injection [2]. It is also well known that administration of carrageenan results in production of a cutaneous hyperalgesia to thermal stimuli indicated by a decreased withdrawal latency. Hargreaves et al. showed that hyperalgesia begins at 1 h, peaks at 2–3 h, and subsides by 8 h following injection of 1 mg of carrageenan. Our results are similar to those reported by Hargreaves et al. [15]; however, the hyperalgesia lasted until 12 h after the beginning of the inflammatory process, which might be due to the use of a double amount of carrageenan to induce the inflammation.

The possibility that such carrageenan injections induce widespread systemic alterations has been addressed only seldom. Cicala et al. described a hemostatic imbalance following carrageenan-induced rat paw edema demonstrating that a systemic reaction occurs in response to the local inflammation. They found changes in some blood parameters following carrageenan-induced rat paw edema, such as increased platelet reactivity and high fibrinogen levels [9]. In the present study, we also found high fibrinogen levels that were evident 8 h after carrageenan injection and peaked at 36 h.

Inflammatory reactions, infections, or tissue injury trigger acute phase proteins synthesis, such as CRP. It is synthesized mainly in the liver, but it may also be synthesized by local inflammatory cells in the area of tissue damage [16, 17]. We determined the CRP levels following carrageenan injection and found that its levels increased at 2 h, peaked at 8 h, and did not return to baseline even at 48 h. Numerous evidences indicate that CRP has proinflammatory and prothrombotic effects in vivo. For example, an injection of CRP into humans increased the plasma levels of IL-6 and IL-8 and activated the blood coagulation system as reflected by an increase in the levels of prothrombin fragment F1 + 2, a product of prothrombin activation [18]. Furthermore, a prothrombotic effect of CRP has also been demonstrated in experiments in vitro in which CRP significantly increased the expression and activity of tissue factor in monocytes, human umbilical vein endothelial cells, and vascular smooth muscle cells [19–21]. Probably, higher CRP levels following carrageenan administration contribute at least in part to the increased expression of tissue factor and IL-6 observed in lung and paw of carrageenan-treated rats.

Moreover, cytokines could also strongly impair the balance between pro- and anti-coagulant factors. In particular, in vivo expression of TF seems dependent on IL-6, as demonstrated in studies showing that inhibition of IL-6 completely abrogates TF-dependent thrombin generation in experimental endotoxemia, whereas specific inhibition of other proinflammatory cytokines had less or no effect [22–24].

Surprisingly, in this study, a lung inflammatory process was also observed after subplantar injection of carrageenan. This process involved a significant reduction of alveolar space due in part to prominent fibrin deposition and marked leukocyte infiltration which are characteristic of an acute lung inflammation. To our knowledge, this is the first report of a lung inflammatory process induced by subplantar administration of carrageenan. These data strongly suggest that an apparently acute process, such as paw edema, may have systemic implications, as have been also suggested by previous studies [8, 9].

Lung histological micrographs from carrageenan-treated rats showed activated neutrophils and mononuclear cells within the lung parenchyma and in the alveolar space. Neutrophils play a crucial role in the development and full manifestation of acute inflammation. Neutrophil infiltration into inflamed tissue contributes to the inactivation of foreign antigens and to the remodeling of injured tissue. An exaggerated recruitment accounts for tissue destruction via the liberation of granule enzymes, production of cytokines, etc., which amplify the inflammatory response and cause parenchymal lung damage and subsequent lung dysfunctions [3, 25].

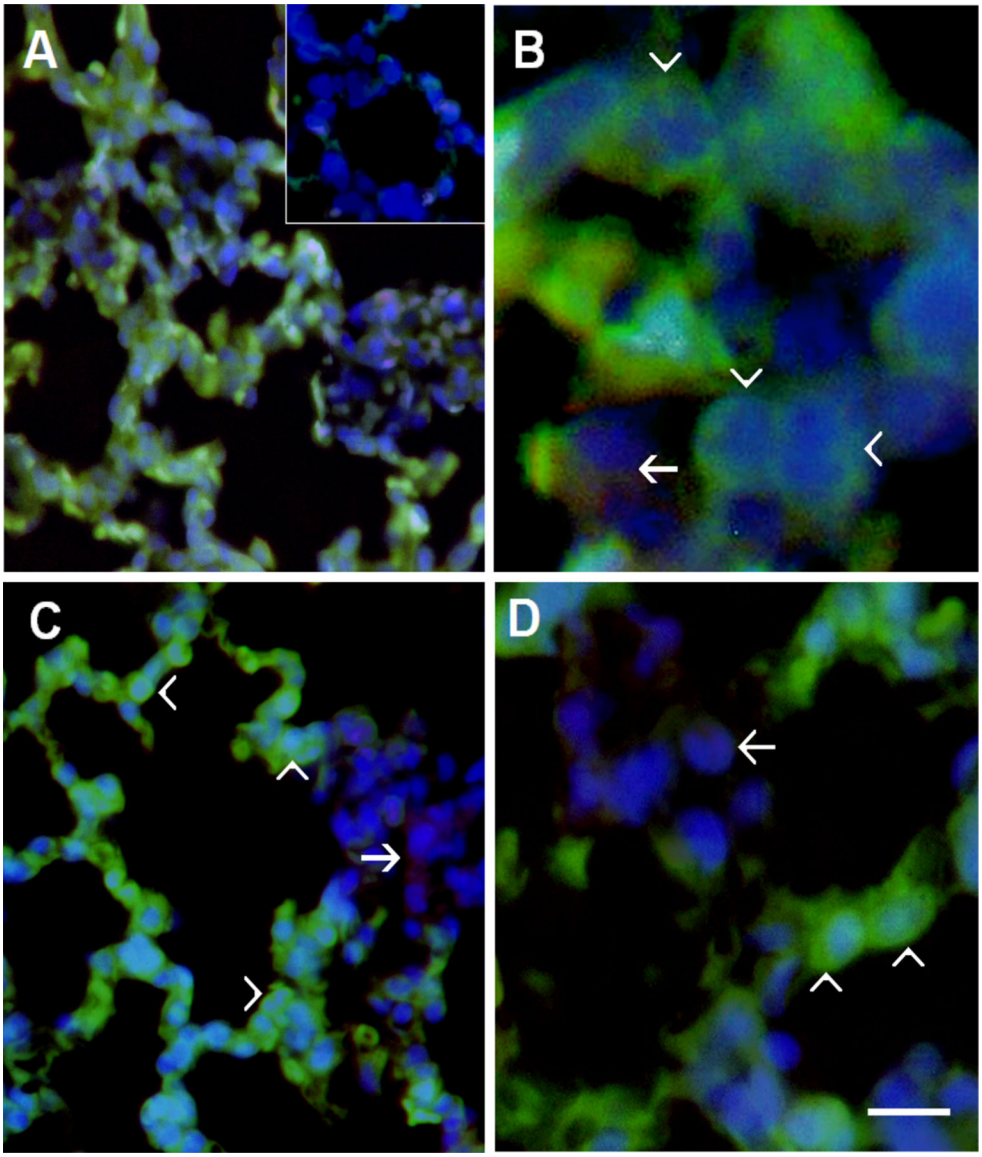


Fig. 3 Immunofluorescence of lung sections from rats 4 h (**a**, **b**) and 24 h (**c**, **d**) after intraplantar injection of carrageenan. Sections were stained for TF (*green*) shown in combination with IL-6 (*red*) and counterstained with nuclear marker DAPI (*blue*). The *inset* shows low immunostaining in the control group. *Arrowheads* indicate clusters of interstitial neutrophils and monocytes expressing TF. *Arrows* indicate IL-6-expressing mononuclear cells. Representative results of $n = 5$ rats studied in each group are shown. *Scale bars* low magnification, 20 μm (**a**, **c** and *inset*); high magnification, 5 μm (**b**) and 8 μm (**d**) (Color figure online)

On the other hand, it is also known that carrageenan induces the synthesis of proinflammatory cytokines as it was demonstrated in experimental animals. Mazzon et al. [26] described that a carrageenan injection causes a significant increase in TNFα and IL-1β in the pleural exudate and lung tissues in a carrageenan-induced pleurisy model in mice. TNFα and IL1β contribute to propagate local or systemic inflammatory processes [27, 28]. Furthermore, an increase in IL-6 serum levels was observed 24 h after carrageenan administration in rat hind paw [9]. Based on these observations, we determined by immunohistochemistry the expression of IL-6, IL-1β, and TNFα in paw and lung tissue of rats at different time interval after carrageenan administration. Our findings showed that (1) the expression of these proinflammatory cytokines was always higher in the paw than in the lung, which might be explained by the nearness to the stimulus injection site; (2) their expression was evident in both tissues 1 h after carrageenan injection but increased continuously until 4 h in the paw; (3) the expression diminished after peaking in paw and lung, but it

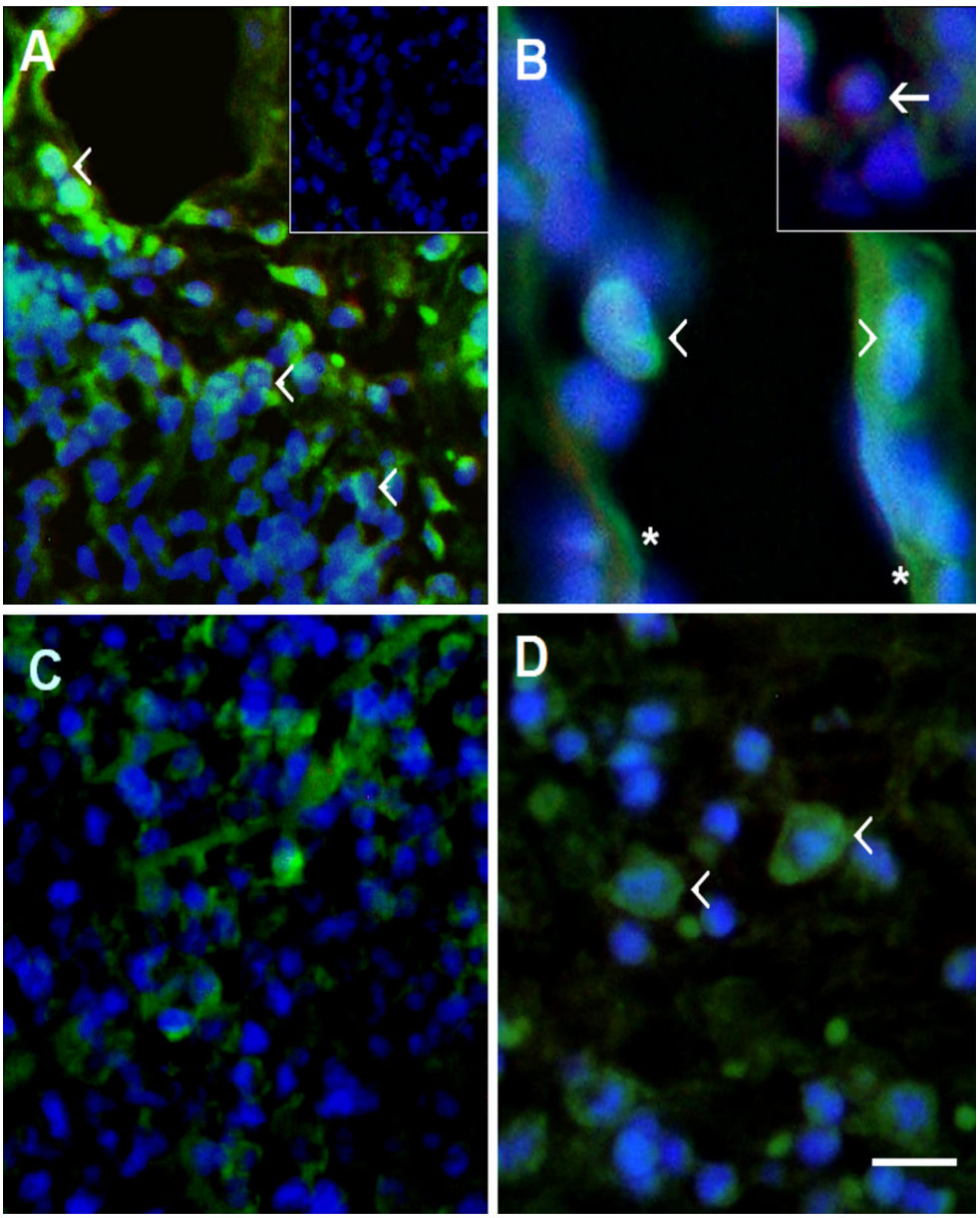
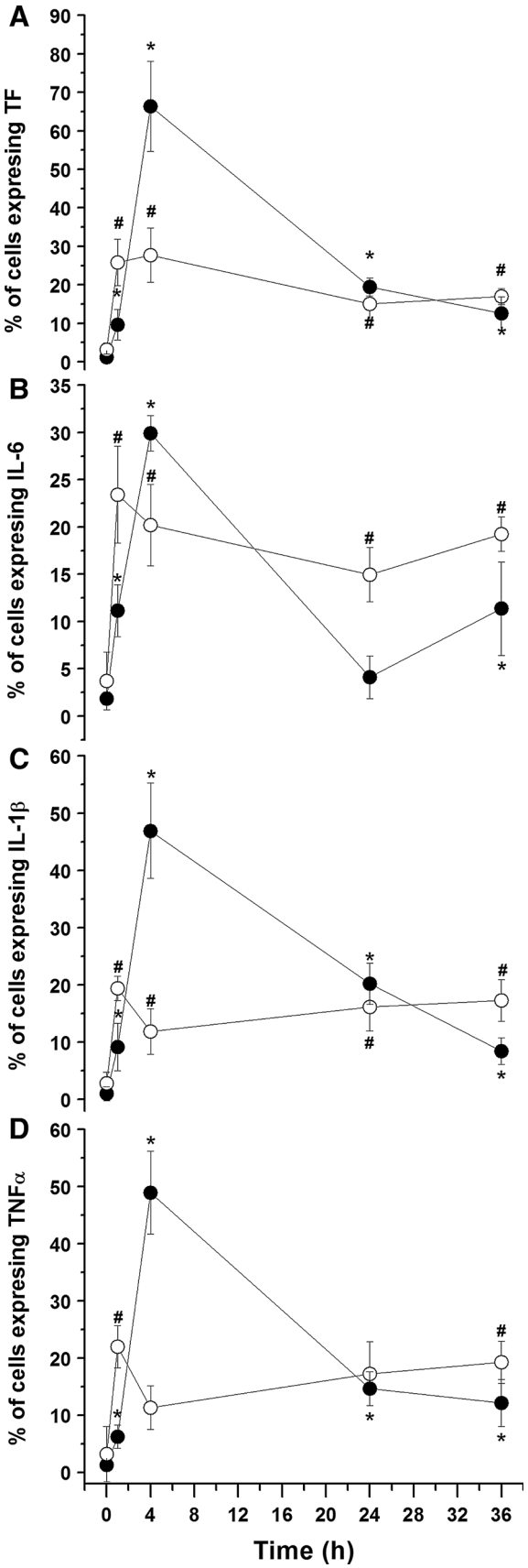


Fig. 4 Immunofluorescence of paraffin-embedded paw sections from rats 4 h (**a**, **b**) and 24 h (**c, d**) after intraplantar injection of carrageenan. Sections were stained for tissue factor (*green*) shown in combination with IL-6 (*red*) and counterstained with nuclear marker 4′,6′-diamidino-2-phenylindole (DAPI; *blue*). The *inset* represents the control sections incubated only with secondary antibodies. *Arrowheads* indicate clusters of interstitial neutrophils and monocytes expressing TF. The *arrow* indicates an IL-6-expressing mononuclear cell. The *asterisks* indicate endothelial cells expressing TF. Representative results of $n = 5$ rats studied in each group are shown. *Scale bars* low magnification, 20 μm (**a** and **c**) and 35 μm (*inset* of **a**); high magnification, 5 μm (**b**) and 8 μm (**d** and *inset* of **b**) (Color figure online)

was persistent in both organs even 36 h after induction of the inflammatory process.

Cicala et al. found a shortening of prothrombin time without any change in activated partial thromboplastin time at 24 h following carrageenan injection in the paw, compared to control and to values obtained at 3 h after carrageenan injection. They concluded that this could only reflect the increase in coagulation factors involved in the extrinsic pathway, such as factor VII and TF [9]. In the present study, we demonstrated by immunohistological analysis an increased expression of TF in paw and lung tissue of rats treated with carrageenan in comparison with control animals. It is well known that TF/FVIIa plays an important role in inflammation, besides its function as an initiator of coagulation. Expression of TF on the cell surface and its appearance as a soluble molecule are



◀**Fig. 5** Expression of tissue factor (**a**), IL-6 (**b**), IL-1β (**c**), and TNFα (**d**) in rat lung (*closed circles*) and paw (*open circles*) following carrageenan-induced paw edema. Data represent mean ± SD ($n = 5$). The nonparametric Mann–Whitney test was used to determine significance between groups. *$P < 0.05$ versus basal value

characteristic features of acute and chronic inflammation in conditions such as sepsis and atherosclerosis [29, 30]. Moreover, blocking TF activity completely inhibits inflammation-induced thrombin generation in models of experimental endotoxemia or bacteraemia, which confirms the narrow link that exists between coagulation and inflammation [24, 31, 32].

The sustained expression of tissue factor in paw and lung tissues favors a procoagulatory state. Immunostaining of TF was observed associated mainly to neutrophils, monocytes, and endothelial cells. It is well known that these cells are able to express TF [19, 33–36]. Expression of TF might lead to thrombin generation with the subsequent fibrin deposition into the lung as was observed in the histological analysis performed in this study. It has been previously shown that fibrin formation was involved in carrageenan-induced rat paw edema [37].

Thrombin and other coagulation proteases, such as FXa, might represent mediators that are generated or released during the carrageenan-induced acute inflammation and could continue stimulating the inflammatory response and its perpetuation as do other substances, such as prostaglandins (PGE2), leukotrienes (LTD4), interleukins (IL-1 and IL-6), nitric oxide (NO), and oxygen reactive species [5].

Preliminary results also showed morphological changes including cellular infiltration in liver, kidney, and spleen of carrageenan-treated rats. Some experiments are being carried out in order to characterize these histological changes.

In summary, our study provides new evidences that a local carrageenan injection induces a systemic response, characterized by (1) increased levels of acute phase proteins, such as CRP and fibrinogen; (2) increased production of proinflammatory cytokines; (3) leukocyte infiltration into different tissues; and (4) a procoagulant state induced by tissue factor expression and evidenced by fibrin deposition. All these changes are responsible for the acute injury reaction in the lung and probably other organs that occur simultaneously to the development of the carrageenan-induced paw edema.

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