Methotrexate Inhibits Integrin Adhesion Molecules in the Mouse Model of Pleurisy Induced by Carrageenan

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ABSTRACT

The aim of this work was to analyze the effect of methotrexate (MTX) upon leukocyte migration and expression of adhesion molecules CD11a/CD18 in the lung, 4 and 48 h after inflammation induction by carrageenan in mice. The results showed that MTX significantly decreased leukocyte influx and CD11a expression in the lung at 4 and 48 h of pleurisy (P < 0.01). MTX also inhibited CD18 expression at 4 h but not 48 h of pleurisy (P < 0.01). These results proved that MTX at the studied doses had important anti-inflammatory properties, acting primarily on leukocyte migration from the pleural cavity to the lung via inhibition of CD11a/CD18 expression in the mouse model of inflammation.

Key words: Methotrexate, CD11a/CD18 adhesion molecules, leukocyte migration, pleurisy, mice

INTRODUCTION

Methotrexate (MTX) was synthesized 50 years ago to obtain the competitive folic acid antagonist drug (Schnabel, 2001). This drug was initially used in tumor treatment and leukemia (Farber et al., 1956) and later as an anti-inflammatory or immunosuppressor drug (Seitz, 1999). MTX is also used in the treatment of refractory rheumatoid arthritis (RA) (Wakabayashi et al., 2003) and other chronic inflammatory diseases such as psoriasis, biliary cirrhosis, Crohn’s disease or intrinsic asthma (Weinblatt et al., 1998; Genestier et al., 2000). Methotrexate is effective in the prophylaxis of acute graft-vs.-host disease (GVHD) when used either alone or with cyclosporin A and/or prednisone (Storb et al., 1986; Sullivan et al., 1989; Nash et al., 1992; Chao et al., 1993) or FK506 (Nash et al., 1996). MTX has also been used as an adjunct therapy for persistent mild cardiac allograft rejection (Olsen et al., 1990).

The mechanism through which MTX exerts its inhibitory effect on leukocyte migration is not known. Studies have revealed that MTX is taken up by cells and tissues and immediately converted to metabolites linked to glutamate (MTX-polyglutamate). The resulting complex is responsible for most of the biochemical and biological activities of methotrexate (Panetta et al., 2002). MTX-polyglutamate inhibits enzymes such as dihydrofolate-reductase (DHFR) and others that are dependent on folate. In addition, the drug appears to act on the biosynthesis of nucleotides (purine and pyrimidine) (Budzik et al., 2000). Many studies have addressed the anti-inflammatory effects of methotrexate, such as apoptosis and inhibition of T cell clones (Genestier et al., 2000), suppression of purine biosynthesis in mitogen-stimulated human T-lymphocytes...
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(Fairbanks et al., 1999), increase of anti-inflammatory cytokines release in vitro (Constantin et al., 1998; Seitz and Dayer, 2000; Sung et al., 2000), adenosine release in vivo and in vitro (Morabito et al., 1998; Montesinos et al., 2002) and inhibition of neutrophil migration in patients with rheumatoid arthritis (Kraan et al., 2000).

In this study the mouse model of pleurisy induced by carrageenan was studied. This model presents a biphasic inflammatory response in the mouse pleural cavity. In the early phase, a significant enhancement of leukocyte influx occurs due to neutrophils 4 h after carrageenan treatment. In the late phase, a significant increase of leukocytes is observed due to mononuclear cells 48 h after carrageenan administration (Saleh et al., 1996). These early and late responses are associated with a marked inflammatory reaction in the airways similar to that which occurs in human asthma.

Thus, the aim of this study was to analyze the anti-inflammatory effect of methotrexate upon leukocyte influx as well as expression of the CD11a/CD18 adhesion molecules in the lung in the experimental inflammation model induced by carrageenan in mice.

MATERIAL AND METHODS

Animals
Non-fasted adult Swiss mice of both sexes (18-25 g), aged 2 months were used throughout the experiments. They were housed in accordance with institutional animal care requirements (temperature 21±2°C, under a light/dark cycle of 12 h) and fed freely on standard rodent chow and water. The following groups of animals were studied: 1) pretreated and 2) untreated with methotrexate prior to the induction of pleurisy with carrageenan. In parallel, two or three animals that had received an injection of either sterile saline (NaCl, 0.9%) by intrapleural (i.pl.) route or methotrexate by intraperitoneal (i.p.) route were included in all experimental groups.

Induction and analysis of pleurisy
As previously described (Saleh et al., 1996), pleurisy was induced by a single intrapleural injection of 0.1 mL of sterile saline plus carrageenan (1%). Since the pleurisy caused by carrageenan exhibits a biphasic nature (4 h and 48 h), both interval-points were chosen to analyze the studied parameters.

Several samples of the lung were collected for further determination of both lung leukocyte influx and lung CD11a/CD18 adhesion molecule expression.

Thus, the doses chosen to analyze lung leukocyte influx as well as lung CD11a/CD18 adhesion molecule expression were 20 mg/kg, i.p. administered 0.5 and 24 h before pleurisy (4 h) and 40 mg/kg, i.p. 0.5 h before pleurisy (48 h) (Dalmarco et al., 2002).

Lung Histology
Following pleurisy induction, the lungs were removed, washed in phosphate buffered saline (PBS – pH 7.6: NaCl 137 mM, KCl 2 mM and phosphate buffer 10 mM) and placed in 2 ml of buffered formalin (10%) for 48 h. Then, the tissues were passed through serial concentrations of alcohol followed by xylene, and then embedded in paraffin and sectioned at 3 microns using a microtome (LEICA-Instruments, Nussloch, Germany). Hematoxylin-eosin sections were examined in a blinded fashion by a pathologist.

Immunohistochemical analysis
Immunostaining of lung sections obtained at 4 and 48 h after pleurisy induction was conducted using the DAKO Envision System (Dako, Carpinteria, CA, USA) according to the manufacturer’s instructions. In brief, endogenous peroxidase activity was blocked with distilled water and the sections and slides were incubated for 1 h with primary antibody against rat anti-mouse CD11a or CD18 (Sigma-Aldrich, St. Louis, Missouri, USA) diluted 1:50 in TRIS-HCL buffer (composition: TRIS 13.9 g; TRIS-HCL 60.6g, NaCl 87.66 g, pH 7.6) at 36-37°C in a humid chamber. After two washes in automation buffer, the sections were incubated with secondary antibody IgG/IgM (DAKO, Carpinteria, California, USA) for 25 min at room temperature in a humid chamber. The slides were incubated with peroxidase - conjugate (Dako, Carpinteria, CA, USA) for 25 min at room temperature in a humid chamber. Following another wash in automation buffer, sections were incubated in the chromogen DAB(diaminobenzidine; Dako). Counterstaining was performed with hematoxylin and examined in a blind fashion by a pathologist.
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Drugs and reagents
The following drugs and reagents were used: methotrexate (Wyent, São Paulo, São Paulo, Brazil); ethyl alcohol (70°, 80°, 95° and PA), methyl alcohol, yellow eosin, paraffin, formol (Synth, Barueri, São Paulo, Brazil); carrageenan lambda (grade IV), monoclonal rat anti-mouse CD11a and CD18 (Sigma-Aldrich, St. Louis, Missouri, USA); TRIS-HCL buffer (TRIS – pH 7.6: 13.9 g; TRIS-HCL 60.6g, NaCl 87.66 g), phosphate buffered saline (PBS – pH 7.6: NaCl 137 mM, KCl 2 mM and phosphate buffer 10 mM), hematoxylin, xylene, Entellan (Merck, Armstard, Germany); dianinobenzidine (DAB) (ACROS-Organics, New Jersey, USA); ethyl ether (Dinamica, São Paulo, São Paulo, Brazil); saline (NaCl 0.9%) (Sanoclear, Pouso Alegre, Minas Gerais, Brazil); IgG/IgM secondary antibodies, streptavidin-biotin-peroxidase (DAKO, Carpinteria, California, USA).

Statistical Analysis
Statistical differences between groups (control and inflamed animals) were determined by Kruskal-Wallis and Dunn tests. Differences were considered significant at P < 0.05.

RESULTS
In all figures, panels A and B represent control groups: A = Saline-treated animals and B = Carrageenan-treated animals.

Effect of methotrexate upon lung cell influx in animals with pleurisy
In the early phase (4 h), methotrexate (MTX; 20 mg/kg, i.p.) administered 0.5 and 24 h before carrageenan injection significantly decreased the congestion between the septal wall in the lung alveolar (P < 0.01). Furthermore, this treatment reduced lung neutrophilic infiltration (P < 0.01) (Table 1 and Fig. 1, panels C and D).

Table 1 - Lung Histological Injury Scores in methotrexate-treated animals (20 mg/kg, i.p.) in the early phase (4 h) of the inflammatory response induced by carrageenan.

<table>
<thead>
<tr>
<th>Histological Analysis</th>
<th>Control</th>
<th>Methotrexate</th>
<th>Significance</th>
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</thead>
<tbody>
<tr>
<td>Lung alveolar septal wall congestion</td>
<td>3</td>
<td>2</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Neutrophils sequestration</td>
<td>3</td>
<td>1</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Mononuclears sequestration</td>
<td>3</td>
<td>1</td>
<td>P &lt; 0.01</td>
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</tbody>
</table>

Controls are results obtained in carrageenan-treated animals in the early phase (4 h) of inflammation. Scores are on a scale of 0 to 3, with 0 = none, 1 = mild, 2 = moderate, and 3 = severe in accordance with Motohiro et al. (1986); Lossos et al. (2000). At least 3–4 slides of a pool of experiments were analyzed.

In the late phase (48 h), MTX (40 mg/kg, i.p.) administered 0.5 h before pleurisy induction significantly decreased neutrophils and mononuclears in the lung (P < 0.01). MTX also decreased the lung alveolar septal wall...
congestion 48 h after (P < 0.01) (Table 2 and Fig. 2, panel C).

Table 2 - Lung Histological Scores in methotrexate-treated animals (40 mg/kg, i.p.) in the late phase (48 h) of the inflammatory response induced by carrageenan.

<table>
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Effect of methotrexate upon lung CD11a/CD18 adhesion molecules expression in animals with pleurisy
In the early phase (4 h), MTX (20 mg/kg, i.p.) administered 0.5 or 24 h before carrageenan, significantly decreased lung CD11a expression (P < 0.01) (Fig. 3, panels C) and CD18 (Fig. 4, panels C and D).

Figure 2 - Comparative analysis of mouse lung sections in the late phase (48 h) of the inflammatory response induced by carrageenan (Cg 1%/cav.) A– Saline-treated animals. B– Carrageenan-treated animals. Effect of methotrexate (40 mg/kg, i.p.), administered 0.5 h (C) before carrageenan, upon inflammatory parameters observed in table 2. Positive staining is indicated by circle (magnification of 200 x)

Figure 3 - Comparative analysis of CD11a expression in lung sections in the early phase (4 h) of the inflammatory response induced by carrageenan (Cg 1%/cav.) A– Saline-treated animals. B– Carrageenan-treated animals. Effect of methotrexate (20 mg/kg, i.p.), administered 0.5 h (C) or 24 h (D) before carrageenan, upon CD11a expression in lung. Positive staining is indicated by circle (magnification of 400 x)
**DISCUSSION**

The present data demonstrated that methotrexate was able to reduce the two pools of leukocyte populations that migrated from the pleural cavity to the lung at distinct periods of time (4 h and 48 h) after induction of mouse pleurisy with carrageenan. This inhibition of lung cell influx was associated with a marked reduction of both CD11a and CD18 adhesion molecules expression. These results were in agreement with other studies that have shown that immunosuppressor drugs such as cyclosporin A inhibited the leukocyte influx in a number of experimental models, including rat arthritis induced by collagen (Oliver et al., 1995), rat cardiac allografts (Ciesielski et al., 1998), and mouse pneumonia induced by *Escherichia coli* (Wang et al., 2002). Furthermore, immunosuppressor drugs caused a significant inhibition of the lymphocytes sensitized by tuberculin in the guinea-pig (Winkelstein, 1975). Other authors have reported that methotrexate inhibited neutrophil chemotaxis in rheumatoid arthritis patients (O’callaghan et al., 1988; Kraan et al., 2000). This drug has also been found to present important antileukemic effects in *in vivo* studies (Dervieux et al., 2002). There are few studies demonstrating the inhibitory effect of immunosuppressor drugs upon leukocyte migration. *In vitro* studies have shown that cyclosporin A (CLPA) inhibited intercellular adhesion molecule-1 (ICAM-1), vascular adhesion...
molecule-1, (VCAM-1), E-selectin, P-selectin, platelet/endothelial cell adhesion molecule-1 (PECAM-1) and the L-selectin ligand CD34 in human endothelial cells (Markovic et al., 2002; Rafiee et al., 2002; Zhou et al., 2004). CLPA reduced also endothelial and keratinocyte ICAM-1 expression in patients with psoriasis (Servitje et al., 1996).

The present model, methotrexate inhibited CD11a expression at 4 and 48 h after pleurisy induction and CD18 at 4 h after. Yamasaki et al. (2003) showed that methotrexate inhibited ICAM-1 and VCAM-1 in cultured human umbilical vein endothelial cells. Other studies have reported that methotrexate significantly decreased the E-selectin, ICAM-1 and VCAM-1 levels in bullous pemphigoid patients (Dahlman-Ghozlan et al., 2000). The findings showed that methotrexate caused a significant reduction in CD11a in both phases (4 and 48 h) and CD18 in the early phase (4 h) of this inflammatory reaction added support to the hypothesis that the anti-inflammatory effects of methotrexate upon cell-cell adhesion were due to inhibition of CD11a molecules function.

The fact that CD18 was not inhibited by methotrexate in the late phase (48 h) suggested that this molecule did not have a central role in the chemotaxis of mononuclear cells in the late phase (48 h) of the inflammatory process induced by carrageenan in mice. According to these results, the hypothesis regarding the participation of CD18 in this inflammatory response could not be discarded, because MTX decreased the CD18 expression in the early phase (4 h) of the inflammatory process induced by carrageenan.

In summary, this work presented evidence that the inhibitory effect of methotrexate upon both neutrophil and mononuclear cells was associated with an inhibition of integrin adhesion molecules priory CD11a. The results regarding the expression of adhesion molecule CD18 indirectly contributed to this anti-inflammatory effect. Taken together, these data indicated that the anti-inflammatory effects of methotrexate upon leukocyte migration occurred via different mechanisms and were possibly dependent on the model of inflammation.

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