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Inhibition of Murine Neutrophil Recruitment In Vivo by CXC Chemokine Receptor Antagonists

Shaun R. McColl and Ian Clark-Lewis

In this study, we have examined the ability of chemokine receptor antagonists to prevent neutrophil extravasation in the mouse. Two murine CXC chemokines, macrophage-inflammatory protein (MIP)-2 and KC, stimulated the accumulation of leukocytes into s.c. air pouches, although MIP-2 was considerably more potent. The leukocyte infiltrate was almost exclusively neutrophilic in nature. A human CXC chemokine antagonist, growth-related oncogene (GRO)-α(8–73), inhibited calcium mobilization induced by MIP-2, but not by platelet-activating factor in leukocytes isolated from the bone marrow, indicating that this antagonist inhibits MIP-2 activity toward murine leukocytes. Pretreatment of mice with GROα(8–73) inhibited, in a dose-dependent manner, the MIP-2-induced influx of neutrophils to levels that were not significantly different from control values. Moreover, this antagonist was also effective in inhibiting the leukocyte recruitment induced by TNF-α, LPS, and IL-1β. Leukocyte infiltration into the peritoneal cavity in response to MIP-2 was also inhibited by prior treatment of mice with GROα(8–73) or the analogue of platelet factor 4, PF4(9–70). The results of this study indicate 1) that the murine receptor for MIP-2 and KC, muCXCR2, plays a major role in neutrophil recruitment to s.c. tissue and the peritoneal cavity in response to proinflammatory agents and 2) that CXCR2 receptor antagonists prevent acute inflammation in vivo. The Journal of Immunology, 1999, 163: 2829–2835.

Neutrophil recruitment to inflammatory sites of injury or infection is integral to the inflammatory response and is an essential host defense mechanism. To gain access to such sites, neutrophils must adhere to and migrate through the cells of the endothelium (1, 2). This process, referred to as extravasation, is mediated by a number of molecules, including adhesion molecules, multifunctional inflammatory cytokines, and chemotactic factors (1, 2). Among the latter are lipid mediators (3), complement components (4), members of the S100 family of proteins (5), and members of the chemokine gene superfamily (6, 7).

Members of the chemokine gene superfamily of cytokines share homologous sequences and a highly conserved cysteine motif. Chemokines can be divided into four branches, depending on whether the first two cysteines are separated (CXC) or not (CC) by an intervening amino acid (7), whether the second cysteine is missing (C) (8), or whether the first pair of cysteines are separated by three intervening amino acids (CX3C) (9, 10). Members of the CXC subfamily can be divided into two classes: Glu-Leu-Arg-containing CXC chemokines and non-Glu-Leu-Arg-containing CXC chemokines (Glu-Leu-Arg being the three amino acids before the CXC motif). The former are chemotactic in vitro for neutrophils but not for mononuclear cells, whereas the latter exhibit chemotactic activity toward monocytes and lymphocytes (11).

Collectively, chemokines mediate the recruitment of all known subpopulations of leukocytes. Antagonism of chemokine receptors therefore represents an important therapeutic target for the treatment of a variety of disease processes. N-terminal modification of several chemokines has been shown to antagonize the binding of ligands to the receptor for such chemokines. For instance, N-terminal deletion converts monocyte chemotactant protein-1 (MCP-1) (3) and RANTES to antagonists of native MCP-1 and RANTES, MCP-3 and macrophage-inflammatory protein (MIP)1α, respectively (12, 13), whereas retention of the initiating methionine converts RANTES into a receptor antagonist (14). These antagonists have been shown to prevent entry of HIV into target cells (15, 16) and to prevent development of arthritis in animal models (17, 18). The results of these previous studies have provided proof of concept for the use of chemokine receptor antagonists to block chemokine function in vivo.

Structural studies on CXC chemokines have revealed that the receptor binding and activation domains are also situated at the N terminus of the molecules (19, 20). This has led to the development of truncated analogues of CXC chemokines with the ability to antagonize CXC receptors. Two such antagonists, growth-related oncogene (GRO)-α(8–73), which comprises aa 8–73, and platelet factor (PF)-4(9–70), which comprises aa 9–70 with an arginine substitution at the first amino acid, were recently shown to inhibit the binding of IL-8 and GROα to human CXCR2 receptors as well as the cellular activation induced by these two chemokines (21). Studies investigating the role of CXC chemokine receptors in vivo are rare and to date are limited to examining the effects of genetic deletion (22). Because in vivo studies are essential to our understanding of the biologic relevance of various members of the chemokine gene superfamily, we have investigated the ability of CXC receptor antagonists to modulate neutrophil recruitment to s.c. tissues in the mouse in response to the murine CXC chemokine MIP-2, as well as by other proinflammatory agents. We now report that the truncated GROα and PF4 analogues function effectively as receptor antagonists in vivo and that the receptor antagonized.

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3 Abbreviations used in this paper: MCP-1, monocyte chemotactant protein-1; MIP, macrophage-inflammatory protein; GRO, growth-related oncogene; PF, platelet factor; mu, murine; fura-2-AM, fura-2-acetoxymethyl ester; PAF, platelet activating factor.
muCXCR2, plays a major role in neutrophil recruitment to s.c. tissue and the peritoneal cavity in response to proinflammatory agents.

Materials and Methods

Mice and reagents

Male BALB/c mice 6–8 wk old were obtained from the Central Animal House at the University of Adelaide, South Australia. Air pouches were raised on the dorsum by s.c. injection of 2.5 ml of sterile air on days 0 and 3. All experiments were conducted on day 6 as previously described (23). All other reagents were obtained from the Aldrich Chemical Company (Castle Hill, New South Wales, Australia). Chemokines and chemokine antagonists were synthesized as previously described (20). Recombinant murine TNF-α and IL-1β were purchased from R&D Systems (Minneapolis, MN). TNF-α was produced in a bacterial expression system, whereas IL-1β was produced in a mammalian expression system. LPS was purified from Salmonella enteritidis strain 11RX as previously described (24). The IgG-purified anti-MIP-2 Abs used in this study were raised in rabbits using full-length murine MIP-2 that was chemically synthesized (23). The Abs were tested for cross-reactivity against other chemokines (JE, muMIP-1α, muMIP-1β, muRANTES, C10, TCA-3, and lymphotactin) in direct ELISA and Western blot. No cross-reactivity was observed.

Induction of leukocyte migration

On day 6, 1 ml of the agonists, as indicated in the figure legends, dissolved in endotoxin-free PBS or the equivalent volume of endotoxin-free PBS, was injected in the air pouches. At the indicated times, the mice were euthanized by asphyxiation with CO₂, the air pouches were washed once with 1 ml of PBS and then twice with 2 ml of PBS, and the pooled exudates were centrifuged at 100 × g for 10 min at room temperature. The supernatants were removed, and the cells were resuspended in PBS, stained in Türk’s solution (crystal violet 0.01% w/v in acetic acid 3% v/v), and counted. Two hundred thousand cells were centrifuged onto microscope slides at 500 rpm for 5 min using a cytopsin centrifuge (Shandon, Lab Supply, Adelaide, South Australia). The slides were air dried and then stained with Diff-Quick (Sigma-Aldrich, Castle Hill, New South Wales, Australia) to allow quantitation of the granulocyte and mononuclear leukocyte subpopulations. In air pouch experiments involving use of GROα(8–73), the indicated concentrations of the antagonist were injected into the peritoneal cavities the evening before injection of the agonists.

Induction of peritonitis

One milliliter of either PBS, or GROα(8–73), or PF4(9–70) (both at 250 μg/ml) was injected into the peritoneal cavity of mice. Five minutes later, 1 ml of either PBS or MIP-2 was injected into the peritoneal cavity. The mice were sacrificed, and peritoneal cells were collected and counted 2 h later. Differential analysis was conducted as described above.

Passive immunization with anti-chemokine Abs

Passive immunization was achieved by injecting 200 μg of either protein G-Sepharose-purified rabbit anti-MIP-2 Abs or the equivalent amount of Ig purified from a preimmune rabbit into the peritoneal cavity of mice the evening before injection of the agonists into the air pouch, as previously described (23).

Measurements of intracellular calcium mobilization

Leukocytes were harvested from the bone marrow of BALB/c mice following dissection of the femur. These cells comprised >85% neutrophils. The cells were suspended at 10 × 10⁶ per ml in HBSS and incubated with 2 μM fura-2-acetoxymethyl ester (fura-2-AM; Molecular Probes, Eugene, OR) for 30 min at 37°C. The cells were washed to remove unincorporated fura-2-AM, and transferred to the thermostatted (37°C) cuvette compartment of a fluorometer (Aminco-Bowman AB2, SLM Aminco, Rochester, NY), and the fluorescence was monitored using excitation and emission wavelengths of 340 and 510 nm, respectively. The internal calcium concentrations were calculated as described (25).

Statistical analysis

Numerical values in the figures and tables are means ± SEM. The data for each group were analyzed as indicated in the respective figure legends, either by unpaired, two-way ANOVA with a Bonferroni posttest, or using the Student’s t test (unpaired, two-way). Statistical significance was considered to be achieved at p < 0.05.

Results

Characterization of effect of GROα(8–73) on activation of murine leukocytes by chemokines

The ability of the CXCR2 antagonist GROα(8–73) to inhibit murine leukocyte activation by MIP-2 was examined in vitro using the intracellular calcium mobilization assay. MIP-2 induced a rapid, transient mobilization of intracellular calcium in cells isolated from the bone marrow of BALB/c mice, and prior exposure of these cells to MIP-2 led to homologous desensitization to further treatment with MIP-2, a characteristic typical of the interaction of chemokines with their receptors (Fig. 1A). GROα(8–73) at a concentration 100-fold that of MIP-2 failed to induce an increase in the level of intracellular calcium (Fig. 1B). However, prior exposure of the cells to GROα(8–73) completely inhibited the ability of MIP-2 to induce an increase in the level of intracellular calcium. Platelet-activating factor (PAF) was included as an agonist at the end of this experiment as a positive control to...
indicate that the cells were still responsive to chemotactic factors (Fig. 1B). The effect of PAF without prior exposure of the cells to GROα(8–73) is shown in Fig. 1C. Data collected over a series of experiments are summarized in Table I. In a series of experiments conducted on leukocytes isolated from the spleen of IL-5 transgenic mice, a rich source of eosinophils, GROα(8–73) inhibited intracellular calcium mobilization in response to MIP-2 without modifying the response to eotaxin (data not shown).

Characterization of the cellular infiltrate in the air pouch induced by CXC chemokines

A single s.c. air pouch was formed on the backs of BALB/c mice by injection of sterile air. The effect of MIP-2 on the recruitment of leukocytes into the pouch was then determined. MIP-2 at various concentrations was injected into the pouches, and after 2 h, the cellular exudate was collected and counted (Fig. 2A). MIP-2 induced a dose-dependent increase in the number of cells accumulating in the air pouch.

To determine kinetics of infiltration in response to exogenous chemokine, MIP-2 (0.5 μg/ml) or 1 ml of PBS was injected into the air pouches, and the cellular exudate was collected at different time points postinjection (Fig. 2B). Low numbers of leukocytes were present in the air pouch at time 0. No significant increase in leukocyte accumulation in the air pouches was detected following injection of diluent (PBS) alone. In contrast, injection of MIP-2 resulted in a transient influx of leukocytes, with maximal accumulation at between 2 and 4 h after injection. The levels of the cells in the pouch exudate had returned almost to baseline by 6 h postinjection.

Table I. Inhibition of MIP-2-induced intracellular calcium mobilization in murine leukocytes by GROα(8–73)

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>Increase in Intracellular Calcium (nM) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP-2</td>
<td>24.30 ± 2.5 (n = 5)</td>
</tr>
<tr>
<td>MIP-2 following MIP-2</td>
<td>8.01 ± 2.0 (n = 5)</td>
</tr>
<tr>
<td>MIP-2 following GROα(8-73)</td>
<td>2.15 ± 1.0 (n = 5)</td>
</tr>
<tr>
<td>GROα(8-73)</td>
<td>0.70 ± 0.4 (n = 5)</td>
</tr>
</tbody>
</table>

* Bone marrow leukocytes were isolated from BALB/c mice, incubated with fura-2-AM and assayed for intracellular calcium mobilization as described in Materials and Methods. The following concentrations of agonists and antagonists were used: MIP-2, 500 ng/ml; GROα, 1 mg/ml.

The ability of the functionally similar murine CXC chemokine, KC, and its human homologue GROα, to induce leukocyte recruitment into the pouch exudate was also examined (Fig. 2C). The effect of GROα was consistently similar to that of MIP-2, in terms of the number of leukocytes recruited at the same dose, whereas KC was not as potent. Although KC induced a statistically significant increase in the number of leukocytes accumulating in the air pouch exudate (∼2-fold greater than control levels), this was only observed at 10 μg KC, and a dose of 1 μg KC failed to induce a detectable increase in the number of leukocytes recruited. Over six independent experiments, MIP-2 and GROα, at concentrations of 0.2 and 1.0 μg/ml, consistently induced a ∼5-fold increase in the number of leukocytes accumulating in the air pouch. Because of the lack of potency observed with KC, and the fact that GROα is a human chemokine, MIP-2 was used in subsequent experiments.

To characterize the cellular infiltrate in the air pouches induced by MIP-2, exudate cells collected from air pouches at 2 h postinjection with different concentrations of MIP-2 were stained and counted. The results are expressed as the number of neutrophils, eosinophils, and monocytes per 100 cells counted (Fig. 3A) and the total number of neutrophils, eosinophils, and monocytes in the exudate (Fig. 3B). The majority of the cells in the pouch at 2 h postinjection with PBS were neutrophils, with smaller numbers of eosinophils and monocytes. On stimulation with MIP-2, the relative levels of these cells altered. In terms of percentage of total cells, the number of neutrophils increased, whereas the percentage of monocytes and eosinophils decreased (Fig. 3A). However, the overall number of all three cell types increased significantly in response to MIP-2 (data not shown). Representative photomicrographs of the cell infiltrate obtained are shown in Fig. 4, A and B, indicating the increase in the relative number of neutrophils accumulating in the air pouch on injection of MIP-2. T and B lymphocytes, as identified by morphology, were not observed at any stage, regardless of the dose of MIP-2 or the time point assessed. This was also confirmed by flow cytometric analysis (data not shown).

Inhibition of leukocyte recruitment to the s.c. air pouch by GROα(8–73)

Mice were given a single i.p. dose of GROα(8–73) 16 h before injection of MIP-2 into air pouches. The pouch exudate was collected 2 h after injection of MIP-2, and the number of cells was analyzed. The effect of GROα was consistently similar to that of MIP-2, in terms of the number of leukocytes recruited at the same dose, whereas KC was not as potent. Although KC induced a statistically significant increase in the number of leukocytes accumulating in the air pouch exudate (∼2-fold greater than control levels), this was only observed at 10 μg KC, and a dose of 1 μg KC failed to induce a detectable increase in the number of leukocytes recruited. Over six independent experiments, MIP-2 and GROα, at concentrations of 0.2 and 1.0 μg/ml, consistently induced a ∼5-fold increase in the number of leukocytes accumulating in the air pouch. Because of the lack of potency observed with KC, and the fact that GROα is a human chemokine, MIP-2 was used in subsequent experiments.

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FIGURE 2. Leukocyte recruitment in s.c. air pouches in response to chemokines. Air pouches were formed on the backs of BALB/c mice as described (see Materials and Methods). A, Pouches were injected with 1 ml of the indicated concentrations of MIP-2 and were aspirated with PBS 2 h postinjection. B, Pouches were injected with either 1 ml of endotoxin-free PBS or MIP-2 (0.5 μg/ml), and the pouches were washed out after increasing periods of time. C, Air pouches were injected with 1 ml of endotoxin-free PBS, MIP-2, GROα, or KC at the indicated concentrations, and the pouches were washed out at 2 h postinjection. The number of leukocytes accumulating was assessed with a hemocytometer. Data points depicted in this figure represent means ± SEM obtained from at least five mice. * Significantly different from control values at p < 0.05 (unpaired two-way Student’s t test)
counted. The results of these experiments indicate that prior treatment of mice with the GRO α(8–73) inhibits leukocyte recruitment into the air pouch in a dose-dependent manner (Fig. 5). There was no significant inhibitory effect of GRO α(8–73) at a dose of 50 μg/mouse; however, significant inhibition was observed when mice were pretreated with 125 and 250 μg of the antagonist.

Experiments were then conducted to determine whether the antagonist was capable of inhibiting leukocyte recruitment in response to other inflammatory agents. Preliminary studies were therefore conducted to assess the effects of TNF-α, IL-1β, or LPS on leukocyte recruitment into the s.c. air pouch. TNF-α, IL-1β, and LPS were therefore injected into the air pouch, the exudate was collected 2 h later, and the number of leukocytes present was determined as described above. All three inflammatory agonists stimulated the influx of leukocytes into the air pouch (Table II). The cells were also differentially stained and enumerated. The results are expressed as the number of neutrophils, eosinophils, and monocytes per 100 cells counted (Fig. 6A) and the total number of neutrophils, eosinophils, and monocytes in the exudate (Fig. 6B).

As observed with MIP-2, the major cell type entering the air pouch on stimulation was the neutrophil, although this varied between agonists: LPS exclusively induced neutrophil recruitment, whereas TNF-α also increased the recruitment of monocytes. In contrast, compared with the control values, IL-1β induced recruitment of neutrophils and monocytes to approximately the same extent.

To determine whether GROα(8–73) inhibits leukocyte recruitment induced by TNF-α, LPS, and IL-1β, mice were pretreated with 250 μg of GROα(8–73), and the following day, TNF-α, LPS, IL-1β, MIP-2, or GROα (the latter as positive controls) was injected into the air pouches. The exudates were collected 2 h later, and the number of cells accumulating was determined. Prior treatment of the mice with GROα(8–73) significantly inhibited leukocyte recruitment in response to all of the agonists tested (Fig. 7A).

As a further control, the effect of a synthetic CC chemokine receptor antagonist on acute inflammation induced by TNF-α was examined. Prior treatment of mice with RANTES(9–68) at a dose shown to inhibit development of arthritis in the MRL/lpr mouse (I.

FIGURE 3. Relative levels of neutrophils, eosinophils, and monocytes accumulating in the pouch exudate in response to MIP-2. Air pouches were raised on the backs of 6- to 8-wk-old male BALB/c mice. One milliliter of either endotoxin-free PBS or different concentrations of MIP-2 was injected into the pouches, and the exudate was collected 2 h later. A, Cells were stained using Diff-Quick, and the number of neutrophils, eosinophils, and monocytes per 100 cells was determined. B, Total number of neutrophils, eosinophils, and monocytes in the exudate. Data represent mean ± SEM from at least five mice (SE bars are too small to see in this figure).

FIGURE 4. Effect of MIP-2 on the nature of the cellular exudate in the s.c. air pouch. Air pouches were raised on the backs of 6- to 8-wk-old male BALB/c mice. One milliliter of either endotoxin-free PBS (A) or MIP-2 (1 μg/ml; B) was injected into the pouches, and the exudate was collected 2 h later. These photographs are from two separate mice and are representative of at least 20 other results.

FIGURE 5. Effect of dose of GROα(8–73) on leukocyte recruitment. Air pouches were raised on the backs of 6- to 8-wk-old male BALB/c mice. GROα(8–73) was injected into the peritoneal cavity at the indicated concentrations 16 h before the injection of 1 ml of MIP-2 (1 μg/ml) into the air pouch. The pouches were washed out at 2 h postinjection, and the number of leukocytes accumulating was assessed with a hemocytometer. The data points depicted in this figure represent mean ± SEM obtained from at least three mice. * and **, Significantly different from control values at p < 0.05 and 0.01, respectively (unpaired two-way ANOVA).
Clark-Lewis, unpublished observation) failed to inhibit leukocyte recruitment into air pouches in response to TNF-α (Fig. 7B).

**Inhibition of leukocyte recruitment to the s.c. air pouch by passive immunization with anti-MIP-2 Abs**

The results obtained using GROα(8–73) suggest that activation of chemokine receptors including the receptor for MIP-2 and KC is responsible for the infiltration observed following injection of TNF-α, IL-1β, and LPS. Thus, it is likely that these are inducing the production of MIP-2 or related chemokines that also bind to the same receptors as the GROα(8–73). Abs to MIP-2 were therefore used to further evaluate the role of MIP-2 in the neutrophil recruitment in response to TNF-α, IL-1, and LPS. Mice were pretreated with polyclonal Abs directed against MIP-2 or Abs isolated from a preimmune rabbit 16 h before induction of inflammation (Fig. 8). Mice were also injected with MIP-2 as a positive control. The exudate cells were collected 2 h postinjection and counted. The response to all four agonists was significantly inhibited by the anti-MIP-2 Abs, whereas the preimmune serum had no detectable effect on the induction leukocyte recruitment. The inhibition of leukocyte recruitment was observed equally at the level of neutrophil, eosinophil, and mononuclear phagocyte recruitment (data not shown).

### Table II. Ability of proinflammatory agonists to induce leukocyte accumulation in murine s.c. air pouches

<table>
<thead>
<tr>
<th>Condition</th>
<th>Leukocytes in Exudate ($\times 10^6$)</th>
<th>Mean ± SEM</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0.61 ± 0.08</td>
<td></td>
<td>36</td>
</tr>
<tr>
<td>TNF-α (10 ng/ml)</td>
<td>3.92 ± 0.55*</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>LPS (1 ng/ml)</td>
<td>4.70 ± 0.64*</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>IL-1β (0.1 ng/ml)</td>
<td>2.50 ± 0.73*</td>
<td></td>
<td>5</td>
</tr>
</tbody>
</table>

*Air pouches were raised on the backs of 6- to 8-wk-old male BALB/c mice as described in Materials and Methods. PBS, TNF-α (50 ng/ml), IL-1β (0.1 ng/ml), or LPS (1 ng/ml) were then injected into the air pouch. The pouches were washed out at 2 h postinjection, and the number of leukocytes accumulating was assessed using a hemocytometer. The data points depicted in this figure represent mean ± SEM obtained from the number of mice indicated.

显著性不同于对照值，p < 0.05（不匹配的两组ANOVA）

### FIGURE 6. Relative levels of neutrophils, eosinophils, and monocytes accumulating in the pouch exudate in response to TNF-α, LPS, or IL-1β. Air pouches were raised on the backs of 6- to 8-wk-old male BALB/c mice. One milliliter of endotoxin-free PBS, TNF-α (50 ng/ml), LPS (1 ng/ml), or IL-1β (0.1 ng/ml) was injected into the pouches, and the exudate was collected 2 h later. A, Cells were stained using Diff-Quick, and the number of neutrophils, eosinophils, and monocytes per 100 cells was determined. B, Total number of neutrophils, eosinophils, and monocytes in the exudate. Data are means ± SEM from at least five mice.

### FIGURE 7. Effect of GROα(8–73) on leukocyte recruitment into the air pouch in response to inflammatory agonists. Air pouches were raised on the backs of 6- to 8-wk-old male BALB/c mice. A, GROα(8–73) was injected into the peritoneal cavity at a dose of 250 μg/mouse 16 h before the injection of TNF-α (50 ng/ml), IL-1β (0.1 ng/ml), LPS (1 ng/ml), MIP-2 (1 μg/ml), or GROα (1 μg/ml) into the air pouch. B, Alternatively, RANTES(9–68) was injected into the peritoneal cavity at a dose of 50 μg/mouse 16 h before the injection of TNF-α (50 ng/ml) into the air pouch. The pouches were washed out 2 h postinjection, and the number of leukocytes accumulating was assessed with a hemocytometer. The data points depicted in this figure represent mean ± SEM obtained from at least three mice. *, Significantly different from control values at p < 0.05 (unpaired two-way ANOVA).

### FIGURE 8. Effect of anti-MIP-2 Abs on leukocyte accumulation induced by TNF-α, IL-1β, or LPS. Mice were injected with 200 μg i.p. of MIP-2 polyclonal Abs or control IgG Abs 16 h before injection of 1 ml of TNF-α (50 ng/ml), IL-1β (0.1 ng/ml), LPS (1 ng/ml), MIP-2 (1 μg/ml), or sterile PBS into s.c. air pouches. The exudate cells were harvested 2 h later and counted. Results are mean ± SEM of the results from at least five different mice. *, Significantly different from control values at p < 0.05 (unpaired two-way ANOVA).
Inhibition of murine peritonitis by GROα(8–73) and PF4(9–70)

In select experiments, the effect of the CXCR2 antagonist on leukocyte recruitment to the peritoneal cavity was also assessed. In addition, the ability of a related truncated chemokine antagonist, PF4(9–70), was examined. Mice were treated i.p. with 250 μg of GROα(8–73) or PF4(9–70) 5 min before injection of either PBS or MIP-2 into the peritoneal cavity. The peritoneal cavities were then washed as described in Materials and Methods, and the number of leukocytes accumulating was assessed. Treatment with either of the antagonists effectively inhibited neutrophil recruitment in response to MIP-2 (Fig. 9).

Discussion

In this study, we have examined the ability of recently described human CXC chemokine antagonists (21) to inhibit inflammatory responses in the murine system in vivo. In vitro, these antagonists inhibit the biologic action of the subgroup of CXC chemokines that contain the Glu-Leu-Arg sequence motif and stimulate predominantly neutrophils through activation of CXCR2 (6, 11, 21). To examine the effects of the antagonists in vivo, two mouse models of acute inflammation were used, the s.c. air pouch and a model of acute peritonitis. These models were chosen because the cellular exudates can be collected and assessed ex vivo. We observed inhibition by the CXCR2 antagonists of neutrophil recruitment in s.c. tissue and in the peritoneal cavity in mice in response either to direct injection of exogenous muMIP-2, a CXCR2 agonist, or to other acute proinflammatory agents including muTNF-α, muIL-1β, and LPS. These results demonstrate that chemokine receptor antagonists block neutrophil recruitment in vivo and suggest the potential usefulness of CXCR2 antagonists in control of acute inflammation.

The two antagonists used in the present study, PF4(9–70) and GROα(8–73), have previously been characterized with human neutrophils (21). Both molecules inhibited functions mediated by CXCR2, which is activated by IL-8, GROα, and all of the other Glu-Leu-Arg-containing CXC chemokines (11, 19). In the human system, IL-8 binds a second private receptor, CXCR1, and the two receptors, CXCR1 and CXCR2, appear to be coexpressed on neutrophils and elicit similar functions, although functional differences have been reported (26–28). The GROα(8–73) antagonist is a truncation analogue of human GROα, whereas the PF4(9–70) antagonist is a truncation analogue of human PF4 that contains an N-terminal arginine substitution (21). The mouse system differs significantly from the human, as no murine homologue of IL-8 or of CXCR1 has been identified (22). It is therefore possible that the functions attributed to IL-8 and CXCR1 in humans are accomplished in the mouse by other Glu-Leu-Arg-containing CXC chemokines acting exclusively on CXCR2. Like humans, other primates, sheep, pigs, and rabbits also have IL-8. Thus, the mouse system provides a useful model for the in vivo study of antagonists for CXCR2. The previous results demonstrating receptor antagonism by truncation mutants suggest that such antagonists may prove useful in determining the importance of CXCR2 in acute inflammatory responses (12, 13).

A mouse generated by gene targeting that lacks muCXCR2 (22) had defective neutrophil recruitment to the peritoneal cavity in response to thioglycolate. However, this mouse is characterized by pronounced neutrophilia, an abnormal production of myeloid stem cells, and B lymphocytosis. These primary defects therefore make it difficult to interpret the precise role of CXCR2 in inflammatory responses. Moreover, chemokines normally act in inflammatory conditions that are localized to specific tissues and that usually follow a progressive disease pattern resulting in either chronic disease or eventual resolution. Thus, whereas the CXCR2−/− mouse demonstrates the effect of chronic deficiency in responses mediated by this receptor, it does not indicate the effects of blocking responses under acute inflammatory conditions. The present study takes a different approach of inhibiting CXCR2 function by competition with endogenous or exogenously administered ligands. CXC chemokines have also previously been implicated in acute inflammatory responses in vivo in the mouse using neutralizing Abs (23). However, whereas the Ab approach targets one ligand, the antagonists used in the present study target the receptor, which effectively blocks activity of all ligands on that receptor (12, 13, 21). The results of the present study suggest that direct antagonism of the receptor utilized by MIP-2 in the murine system, muCXCR2, effectively inhibits neutrophil recruitment induced by MIP-2 as well as by TNF-α, IL-1β, and LPS, and therefore provide the clearest evidence to date of the importance of CXCR2 in neutrophil recruitment in the murine system under acute inflammatory conditions.

Previous data have demonstrated the relative importance of both KC and MIP-2, two CXCR2 ligands, in the mouse in terms of neutrophil recruitment into s.c. air pouches in response to TNF-α (23). Passive immunization of mice with neutralizing Abs directed against either KC or MIP-2 effectively inhibited neutrophil accumulation in response to TNF-α as well as by IL-1β and LPS (the present study), supporting the notion that neutrophil recruitment in response to the latter inflammatory agents is due in large part to the production of these molecules. In the present study, it was observed that MIP-2 was far more potent than KC in inducing leukocyte accumulation in the air pouch. Although recombinant KC has been shown to be active as a chemoattractant for murine neutrophils in vitro, a similar low potency was reported for neutrophil accumulation in the peritoneal cavity in response to KC (29). Assuming that the muKC used in both of these studies was fully active, these combined data suggest that KC may not play as important a role in acute neutrophil recruitment to s.c. tissue as MIP-2. Indeed, in that previous study, KC was reported to be constitutively expressed in s.c. tissue, leading to the hypothesis that it may play an important role in basal trafficking of neutrophils (29).

In contrast to the human situation, in which neutrophil migration is induced by ligands for CXCR1 or CXCR2, in the mouse, the CC chemokine MIP-1α also induces neutrophil migration through interaction with its receptor muCCR1 (23, 30–32). We found in the present study that the CXCR2 antagonist did not inhibit the ability...
of muMIP-1α to induce activation of murine leukocytes as determined in the calcium mobilization assay (data not shown), indicating that this antagonist does not interfere with the binding of muMIP-1α to its receptor(s). The fact that all of the neutrophil responses could be substantially (indeed, almost completely) blocked by CXCR2 antagonists, despite the known involvement of MIP-1α in neutrophil recruitment, may be explained in several ways: first, that muCXCR2 ligands are dominant in the models of acute inflammatory responses used in the present study; second, that muCXCR2 and muCCR1 are both required for neutrophil recruitment in these models, and blockade of one of these receptors effectively inhibits all recruitment; and third, that because MIP-1α is produced upon mitogenic stimulation of T cells (6, 33), it could be more important as a neutrophil chemoattractant in inflammatory responses involving T cells, rather than in the acute inflammatory responses investigated in the present study. In a previous study, we showed that inhibition by neutralizing Abs of the activity of endogenously synthesized MIP-1α inhibits neutrophil recruitment into s.c. air pouches during acute inflammation induced by exogenous TNF-α (23). Thus, the second theory appears to be correct. Although these previous data point to a role for MIP-1α in neutrophil recruitment into the s.c. air pouch, it is important to note that direct injection of this CC chemokine does not induce accumulation of neutrophils (23). These combined observations imply the existence of a chemokine network in acute inflammation, involving several chemokines of which the roles appear to be regulated temporally. Inhibiting the biologic activity of one of several chemokines in the network therefore inhibits the biologic response. Such a theory appears to explain the consistent ability of Abs directed against single chemokines to significantly inhibit biologic effects in vivo when other chemokines are known or suspected to play a similar role.

In summary, our data indicate that CXCR2 antagonists, which had been previously characterized in vitro, also function effectively in vivo. The results of this study provide several important pieces of information. First, they indicate that the receptor antagonized by GRO(8–73) and PF(49–70), muCXCR2, is a major receptor mediating neutrophil recruitment to s.c. tissue in response to direct injection of MIP-2, as well as following injection of IL-1β, TNF-α, and LPS, agonists that act via the induction of MIP-2. Second, while it is clear that, because of a lack of potency, the CXC truncation mutants studied in this report will not be useful therapeutically, our data provide proof of concept that small molecule CXCR antagonists may be useful in controlling acute inflammation by blocking neutrophil recruitment.

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References