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The Chemokine RANTES Is a Crucial Mediator of the Progression from Acute to Chronic Colitis in the Rat

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Chemokines have well characterized proinflammatory actions, including the ability to induce extravasation of leukocytes that participate in chronic inflammation. In this study, we evaluated the role of a C-C chemokine, RANTES, in the chronic phase of a rat model of colitis. Colitis was induced by intracolonic administration of trinitrobenzene sulfonic acid. At various timepoints thereafter (2 h to 14 days), colonic tissue levels of several chemokines were measured. Unlike the expression of monocyte chemoattractant protein-1, macrophage inflammatory protein-2, and cytokine-induced neutrophil chemoattractant, the expression of RANTES was significantly elevated during the chronic phase of colitis (≥7 days after induction). Colonic RANTES mRNA expression was also significantly elevated during the chronic phase of colitis. The numbers of macrophages and monocytes in the colonic mucosa increased substantially during the chronic phase, as did expression of two of the receptors (CCR1 and CCR5) to which RANTES is known to bind. Administration on days 7 through 14 after trinitrobenzene sulfonic acid administration of a CCR1/CCR5 receptor antagonist, Met-RANTES, resulted in a significant reduction of both macroscopic and microscopic colonic damage, as well as reducing the recruitment into the colon of monocytes, mast cells, and neutrophils. In some rats, treatment with Met-RANTES resulted in a near-complete resolution of colonic damage and inflammation. These results suggest a crucial role of RANTES in the progression from acute to chronic inflammation in a rat model of colitis. The Journal of Immunology, 2001, 166: 552–558.

RANTES is a C-C chemokine that promotes the recruitment and activation of inflammatory cells such as monocytes (6), lymphocytes (7), mast cells (8), and eosinophils (9). RANTES is a ligand for the chemokine receptors CCR1, CCR3, and CCR5 (10), and increased expression of RANTES has been observed in vivo in inflammatory diseases such as glomerulonephritis (11, 12), adjuvant-induced arthritis (13), and granulomatous inflammation (14). Of particular relevance to this study is a report of increased expression of RANTES in colonic biopsies from patients with active inflammatory bowel disease (IBD) (15). In that study, the expression of RANTES mRNA was found to be increased in biopsies from patients with Crohn’s disease or ulcerative colitis, particularly in intraepithelial lymphocytes and in the subepithelial lamina propria. RANTES expression in animal models of colitis has not been described. Moreover, no interventional studies have been performed to determine whether RANTES makes an important contribution to inflammation and/or injury in human IBD or in experimental colitis.

The purpose of this study was to assess the contribution of RANTES to the pathogenesis of colitis induced in the rat by trinitrobenzene sulfonic acid (TNBS). In particular, we were interested in the possibility that RANTES, through its ability to recruit monocytes and mast cells, may play a role specifically in the chronic phase of colonic inflammation following TNBS administration. Previous studies using this model have documented important roles for a number of inflammatory mediators (e.g., leukotrienes, tachyrkins, prostaglandins, IL-1, TNF) in the inflammation and injury occurring during the first week after TNBS administration. Indeed, inflammation during the acute phase could be markedly reduced by a number of drugs that targeted these inflammatory mediators (16–21). However, modifying the inflammatory response once it has progressed to a more chronic phase has proven to be much more difficult.

Materials and Methods

Animals

Male Wistar rats (175–200 g) were purchased from Charles River Breeding Farms Limited (Montreal, Canada). The rats were fed a standard chow pellet diet, had free access to water, and were maintained on a 12-h light/dark cycle. All procedures in this study were approved by the Animal Care Committee of the University of Calgary and conformed to the guidelines established by the Canadian Council on Animal Care.

Induction of colitis

Rats were lightly anesthetized with halothane, and the hapten TNBS (60 mg/ml in 0.5 ml of 50% ethanol) was administered into the distal colon via a cannula (22). At selected times thereafter (2 h to 14 days), the rats were...
killed by cervical dislocation. The colon was excised and examined for macroscopic damage by an observer unaware of the treatments. The colonic damage was scored using the criteria outlined in Table I. After examination, tissue samples were taken for determination of tissue chemokine levels and for measurement of RANTES mRNA expression. Age-matched, untreated rats served as controls.

**Colonic chemokine expression**

Samples of distal colonic tissues were homogenized in 1.5 ml of lysis buffer containing 0.1% Triton X-100, 500 mM NaCl, 50 mM HEPES, 0.1 mg/ml leupeptin, and 10 mg/ml PMSF. The homogenates were incubated on ice for 30 min and then centrifuged (400 × g for 10 min). The supernatants were collected and stored at −80°C until used for determination of chemokine protein levels by ELISA (23) and total protein concentration by colorimetric protein assay (Bio-Rad, Richmond, CA). The chemokines that were measured were RANTES, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-2, and cytokine-induced neutrophil chemoattractant (CINC).

**mRNA expression**

RNA was extracted from colonic tissue using Trizol reagent according to the manufacturer’s instructions (Life Technologies, Burlington, Canada). The yield and purity of the RNA was determined spectrophotometrically at 260 and 280 nm. Isolated RNA (2 μg in diethyl pyrocarbonate-treated water) was reverse transcribed to cDNA and amplified by PCR as previously detailed (24). Briefly, DNA amplification was performed under the following conditions: denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The RANTES/GAPDH genes were amplified for 30 and 22 cycles, respectively. The sequences for the RANTES and GAPDH primers used have been described elsewhere (9, 24, 25). To ensure that the amplified PCR product was measured only during the exponential phase of the amplification reaction, the optimum number of PCR cycles was determined for each primer pair. In addition, to exclude the presence of contamination, amplification was always performed in which cDNA was omitted from the PCR mixture. Amplified products were visualized on a 2% agarose gel stained with ethidium bromide, and the size of the PCR product was confirmed with m.w. markers (Life Technologies). The relative quantity of mRNA was estimated by densitometric analysis using Quantity One software (Bio-Rad), normalized to the respective GAPDH, and expressed as a ratio. Data are presented as the mean normalized ratio ± SE.

Expression of mRNA for the chemokine receptors CCR1 and CCR5 was also examined by RT-PCR at various times after induction of colitis, as described above. The primers used for this purpose have been described previously (26).

**Effects of Met-RANTES**

Met-RANTES is a modified chemokine that binds with high affinity, but does not activate, the chemokine receptors CCR1 and CCR5 (27, 28). Rats were treated with Met-RANTES (40 or 200 μg/rat; i.v.) on day 7 after the administration of TNBS and every 24 h thereafter for 1 wk. Control rats received the vehicle (sterile saline) at the same times. All rats were killed 14 days after TNBS administration for assessment of colonic damage. Additional tissues were taken for analysis of myeloperoxidase (MPO) activity as an index of granulocyte infiltration (17) and for histological examination. Slides were coded to prevent observer bias, and histological sections were then evaluated and scored by an observer unaware of the treatments. The 200-μg dose of Met-RANTES used in this study has been shown to be effective in reducing inflammation in a rodent model of glomerulonephritis (12).

**Morphological studies**

Colonic tissues were fixed overnight in 10% neutral buffered formalin, dehydrated in graded concentrations of ethanol, embedded in paraffin, and sectioned. Sections (5 μm thick) were stained with hematoxylin and eosin according to standard protocols for histological examination. RANTES has been shown to play a role in recruitment of mast cells (8) and monocytes (12) into tissues; thus, the effect of treatment with Met-RANTES on the recruitment of these cells into the colon after TNBS administration was determined. For monocyte enumeration, sections were pretreated with 3% hydrogen peroxide in methanol for 10 min to inactivate endogenous peroxides, followed by permeabilization in PBS containing normal horse serum and Triton X-100 at room temperature for 60 min. Specific labeling of monocytes was performed by incubation of sections overnight at 4°C with anti-ED1 Ab (clone I1C7, 1:10 dilution; Pharmingen, Mississauga, Ontario, Canada) to detect mononuclear phagocytes. Sections were then treated with HRP-conjugated secondary Ab (rat anti-mouse IgG; Jackson ImmunoResearch, West Grove, PA) for 90 min at room temperature. The slides were treated with diaminobenzidine for color development and counterstained with hematoxylin. As a negative control, mouse IgG was used.

For mast cell staining, colonic tissues were fixed for 48 h in Carnoy’s fixative (ethanol/chloroform/acetic acid, 6:3:1), processed, and then stained with Alcian blue/safranine (29). Slides were coded to prevent observer bias, and histological sections were then evaluated and scored by an observer unaware of the treatments the rats had received.

**Materials**

TNBS was obtained from Fluka Chimica (Buchs, Switzerland). RT-PCR reagents were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Met-RANTES was synthesized by Serono Pharmaceutical Institute (Geneva, Switzerland). All other chemicals were obtained from Sigma (St. Louis, MO) or VWR Scientific (Edmonton, Canada).

**Statistical analysis**

All data are shown as mean ± SE. Comparisons between two experimental groups of data were performed using Student’s unpaired t test, except in the case of the colonic damage scores, in which a Mann-Whitney U test was used. Comparisons among three or more experimental groups were performed using a one-way ANOVA followed by a Dunnett’s multiple comparison test. Probability values of <5% (p < 0.05) were considered significant.

**Results**

Consistent with previous reports (17, 30), intracolonic administration of TNBS caused severe ulceration and damage to the distal colon of the rat. The macroscopic damage scores, MPO activities, and expression of chemokines at various times after TNBS administration are summarized in Fig. 1. Over the course of the 2-wk study, there was a gradual decline in the colonic damage score. MPO activity reached a peak at 72 h post-TNBS, and gradually declined thereafter. RANTES expression was not significantly different from controls (i.e., rats without colitis) during the acute phase of colitis (2–72 h post-TNBS). However, RANTES expression was significantly elevated during the period 7–14 days after administration of TNBS, a time when the expression of the other chemokines did not differ significantly from controls. In addition to the changes in chemokine protein expression shown in Fig. 1, we observed that expression of CINC was markedly up-regulated during the acute phase of colitis (2–72 h post-TNBS; peak increase of 20-fold at 4 h), but was not significantly different from controls during the 7- to 14-day post-TNBS period.

In parallel with the changes in RANTES protein expression, significant increases in RANTES mRNA expression in the colon were seen 7 days (2.6-fold) and 14 days (3.5-fold) after administration of TNBS (Fig. 2).
Time course of changes in colonic monocyte and mast cell numbers

As summarized in Fig. 3, significant increases in colonic monocyte and mast cell numbers occurred during the period 7–14 days after TNBS administration (i.e., the numbers were significantly elevated at day 14, but not at day 7).

Time course of changes in chemokine receptor expression

The expression of mRNA for the chemokine receptors CCR1 and CCR5 increased significantly following induction of colitis. The ratio of CCR1 to GAPDH mRNA increased from 0.23 ± 0.14 in healthy controls to 1.55 ± 0.19, 1.98 ± 0.07, and 2.32 ± 0.20 at 3, 7, and 14 days after TNBS administration, respectively (p < 0.01 at each of these times). In the case of CCR5, the expression of mRNA as a ratio of GAPDH mRNA expression was not significantly changed at day 3 post-TNBS (1.78 ± 0.09) vs the healthy controls (1.25 ± 0.04). However, at both day 7 and day 14 there was a significant (p < 0.01) increase in expression (2.28 ± 0.10 and 2.81 ± 0.27, respectively).

Effects of Met-RANTES on macroscopic severity of colitis

Treatment with Met-RANTES (200 μg) after the onset of colitis significantly reduced the macroscopic scores for colonic damage (Fig. 4). All rats in the vehicle-treated group exhibited severe damage. In the group treated with Met-RANTES, four of the six rats exhibited little or no colonic damage, whereas the remaining two rats exhibited damage of similar severity to that seen in the controls (Fig. 4).

Treatment with a lower dose of Met-RANTES (40 μg/day; n = 5) did not significantly affect the colonic damage score (5.8 ± 0.2 vs 5.6 ± 0.3 in saline-treated controls) or colonic MPO activity (37.7 ± 7.9 vs 32.6 ± 5.0 U/mg in saline-treated controls).

Discussion

In recent years, a number of studies have provided evidence consistent with a role for various chemokines (e.g., IL-8, MCP-1, RANTES) in IBD (15, 30–33). Specifically, increased expression of these chemokines was observed in colonic tissue from IBD patients vs controls. The extent to which these chemokines contribute to the pathogenesis of IBD is not clear, as interventional studies using inhibitors of chemokine synthesis or antagonists of chemokine receptors have not yet been performed. However, in this study the use of an animal model of colitis has permitted us to determine the time course of expression of a number of chemokines and chemokine receptors, and then to examine the impact on...
the course of colitis of treatment with a chemokine receptor antagonist. Of four chemokines examined, only the tissue levels of RANTES were significantly elevated during the chronic phase of colitis (i.e., 1 wk after TNBS administration). This was the same period of time during which there were significant increases in the numbers of monocytes and mast cells within the colonic mucosa. Expression of mRNA for the RANTES receptors, CCR1 and CCR5, was also significantly increased during the chronic phase of colitis. Treatment during this period with Met-RANTES (200 μg/day), an antagonist of the CCR1 and CCR5 receptors (27, 28), resulted in a significant reduction of the severity of colitis and a significant reduction of the numbers of granulocytes, monocytes, and mast cells within the colonic mucosa. Particularly noteworthy was the observation that in four of the six rats treated with the 200 μg/day dose of Met-RANTES, the mucosal ulceration had completely resolved, and there was only mild inflammation limited to the mucosal layer.

Met-RANTES is one of a number of chemokine analogs that act as RANTES antagonists (27, 28, 34). The ability of Met-RANTES to reduce tissue injury in this study is consistent with its effects in models of arthritis (35) and renal inflammation (12). Moreover, granulocytes have been shown to contribute significantly to acute tissue injury in experimental colitis in rats and rabbits (38–40). Treatment with Met-RANTES produced a substantial (~63%) reduction in tissue MPO activity, an index of tissue granulocyte numbers.

The effectiveness of Met-RANTES in abrogating colitis suggests that the CCR1 and/or CCR5 are key receptors in mediating the effects of RANTES in this model. Therefore, it is noteworthy that CCR1 has been implicated in chronic inflammation in other tissues. For example, CCR1 was reported to mediate the gross pulmonary fibrotic response to bleomycin treatment (41). The role of CCR5 in colitis has been examined in a mouse model. The colitis induced by oral administration of dextran sodium sulfate was found to be significantly reduced in mice lacking the gene for CCR5 than in wild-type controls, although there was not any evidence of a reduction in macrophage infiltration into the colon (42).

It is also important to note that CCR1 and CCR5 are expressed to differing degrees by various leukocyte subsets. CCR1 has been reported to be expressed on activated T cells, monocytes, eosinophils, dendritic cells, and neutrophils (4, 41, 43). In contrast, CCR5 has been shown to be expressed on activated T cells, monocytes, macrophages, and dendritic cells, but not on neutrophils (41, 43, 44). The expression of CCR1 on neutrophils may account for the earlier increase in expression of this receptor than of CCR5 after induction of colitis because neutrophil recruitment is an early event in the TNBS model.

Although Met-RANTES at a dose of 200 μg/day produced a statistically significant reduction of colonic damage and MPO activity as compared with vehicle-treated controls, it was apparent that this antagonist did not reduce the severity of tissue injury or inflammation in all of the rats that received this agent. The reasons for the apparent failure of response in two of the six rats are not clear. It is possible that these rats had more severe colonic injury at the onset of treatment than the other rats treated with
Met-RANTES. Alternatively, it is possible that in some rats, other mediators of inflammation play a more predominant role than RANTES in mediating the progression from acute to chronic colitis. It is noteworthy that heterogeneous responses to therapies are very common in patients with IBD, with 20–40% of patients failing to show a response to the most widely used therapeutic agents (44–47). Of course, one cannot rule out that treatment with higher doses of Met-RANTES or treatment over a longer period of time may produce beneficial effects in a higher percentage of rats.

In summary, the results presented herein demonstrate that RANTES expression is markedly elevated in the chronic phase of TNBS-induced colitis in the rat, a time when the expression of a number of other chemokines (MIP-1, MCP-2, CINC) was not significantly elevated above control levels. Expression of two of the key receptors for RANTES was also elevated during the chronic phase of colitis. Moreover, treatment with a CCR1/CCR5 receptor antagonist significantly accelerated the healing of colonic injury, most likely by antagonizing the actions of RANTES. Our results provide novel evidence of a crucial role for RANTES in the progression from acute to chronic colitis in this model. The beneficial effects of RANTES antagonism in experimental colitis suggest that chemokine receptor antagonists are potentially useful therapeutic approaches to the treatment of IBD.

**FIGURE 5.** Representative histological appearance after Met-RANTES or vehicle treatment in rats with colitis. A, Appearance of colonic tissue from a rat treated with vehicle on days 7 through 14 after induction of colitis with TNBS. Note the extensive inflammatory infiltrate extending transmurally. There is massive thickening of the bowel wall. B–D, Examples of the appearance of colonic tissue from rats treated with Met-RANTES on days 7 through 14 after induction of colitis. B and D, Examples in which the Met-RANTES treatment resulted in almost complete resolution of colitis. The epithelium is intact, and there is only mild inflammation of the mucosa and submucosa. The thickness of the bowel wall is substantially reduced compared with that seen in rats treated with vehicle. C, An example of the appearance of the colon in a rat that did not exhibit resolution of colitis after treatment with Met-RANTES. Transmural inflammation is evident, as well as disruption of the mucosal layer.
FIGURE 6. Effects of Met-RANTES on colonic MPO activity (top), monocyte numbers (middle), and mucosal mast cell numbers (bottom) in rats with colitis. Met-RANTES (200 μg/rat; iv) or vehicle was adminis-
tered to rats on days 7 through 14. Dotted lines represents mean levels in

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