Therapeutic Effect of Blocking CXCR2 on Neutrophil Recruitment and Dextran Sodium Sulfate-Induced Colitis

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ABSTRACT

Dextran sodium sulfate (DSS)-induced colitis in mice is characterized by polymorphonuclear neutrophil (PMN) infiltration into the colonic mucosa and lumen. The mechanism by which this occurs is unclear. To begin to understand the mechanism, we determined the role of the PMN chemokine receptor, CXCR2, in DSS-induced colitis by using CXCR2(−/−) mice or by neutralizing CXCR2. DSS was administered through drinking water to CXCR2(−/−) and BALB/c mice for 5 days followed by regular water for 1 day. In the neutralization study, mice were injected with control serum or goat anti-CXCR2 antiserum. BALB/c mice receiving DSS and control serum-injected mice receiving DSS lost weight and showed considerable clinical illness. Histological observation revealed submucosal edema, PMN infiltration into the submucosa and mucosa, extensive crypt damage with abscesses, and ulceration. In contrast, both the CXCR2(−/−) and anti-CXCR2 antiserum-treated mice gained weight and had significantly lower symptom scores. Histology of these mice showed submucosal edema but relatively intact crypt architecture and very few ulcers. Significantly fewer PMNs were found in the mucosa in anti-CXCR2 antiserum compared with control serum-injected inflamed mice, but no significant difference in eosinophil infiltration was observed between the groups. Our experiments identify a role for CXCR2 in DSS-induced colitis and suggest that antagonizing CXCR2 provides some therapeutic efficacy, possibly by impeding PMN recruitment into the mucosa. Antagonizing CXCR2 may form the basis for therapeutic drugs directed at controlling colitis.

Polymorphonuclear neutrophil (PMN) migration into the colon mucosa is a hallmark of the inflammatory bowel diseases (IBDs), Crohn’s disease and ulcerative colitis. PMN accumulation in stool of patients (measured using radiolabeled cells or PMN products such as calprotectin) positively correlates with active disease (Costa et al., 2005; Diamanti et al., 2008). Yet, the mechanisms by which PMNs migrate into the mucosa are incompletely understood, and a better understanding of this process may provide insights into treatment strategies for IBD.

Once PMNs reach the mucosa, their cytotoxic armamentarium is important for a successful first line defense against enteric pathogens. Although the initial infiltration by PMNs is beneficial for killing bacteria, it is presumed that persistent infiltration causes tissue damage. This presumption that PMNs necessarily damage tissue has been challenged recently by experiments with mice modified to express human CXCL8 in their intestinal epithelium and in which PMNs infiltrate the mucosa in large numbers with no inflammatory sequelae (Kucharzik et al., 2005). Moreover, other reports suggest that the depletion of PMNs during active colitis can lead to exacerbated inflammation because of the loss of protective effects, possibly including wound healing (Buell and Berin, 1994; Kühl et al., 2007). Thus, there is uncertainty over the role(s) infiltrating PMNs play in colitis, and reconciling the contradictory concepts that PMNs are harmful versus helpful requires further investigation.

PMN infiltration is mediated by specific chemoattractants generated in the mucosa (Agace et al., 1993), where epithelial cells, including in the intestine, have been shown to secrete ELR (Glu-Leu-Arg) motif-containing CXC chemokines (for review, see Godaly et al., 2001; Stadnyk, 2002). ELR+ CXC chemokines in the mouse include CXCL5/LIX and the functional homologs of human CXCL8, CXCL1/KC, and CXCL2/3 (macrophage inflammatory protein-2). Epithelial cells may

ABBREVIATIONS: PMN, polymorphonuclear neutrophil; IBD, inflammatory bowel disease; DSS, dextran sodium sulfate; TNBS, trinitrobenzene sulfonic acid; HBSS, Hanks’ balanced salt solution.
also express receptors for this family of chemokines (Fren-
deus et al., 2000). The murine ELR\(^+\) CXC chemokines bind to
CXCR2, a seven-transmembrane-spanning G-protein-coupled
receptor, which in addition to mediating chemotaxis causes PMN activation (Matityahu et al., 2002).

The role of PMNs in colitis has been examined by antago-
nizing the ELR\(^+\) CXC chemokines or CXCR2. Depletion of
CXCL5 with antisense oligonucleotides reduced epithelial
CXCL5 expression concomitant with a reduction in PMN infil-
tration and severity of DSS colitis (Kwon et al., 2005).

Transgenic mice overexpressing intestinal epithelial CXCL2
showed heightened PMN recruitment into the DSS-inflamed
colon, which was inhibitable with anti-chemokine antibodies
(Ohtsuka and Sanderson, 2003). The interpretation of these
studies is confounded by other redundant CXC chemokines;
therefore an alternative to antagonizing each CXC chemo-
kine is to use mice deficient in the receptor for this family of
molecules. One recent study using CXCR2\(\text{(-/-)}\) mice showed
reduced PMN infiltration into the mucosa and reduced tissue
damage, also in DSS-induced chronic colitis (Buanne et al.,
2007). This was partly unexpected because studies in the
murine kidney infection model showed a drastic increase in
submucosal neutrophil entrapment and chronic tissue pa-
thyology in receptor knockout mice (Frendéus et al., 2000).
The result using chronic colitis is also contrasted by an ear-
lier study neutralizing CXCR2 in TNBS colitic rats by spe-
cific antibody, which showed that the acute phase of colonic
PMN accumulation was CXCR2-dependent, whereas accumu-
lation in the chronic phase was CXCR2-independent (Ajuebor et al.,
2004). Therefore, the purpose of the present study was to reassess the effect of CXCR2 inhibition on
neutrophil recruitment and acute colitis severity and, specifi-
cally, to compare the CXCR2-deficient mouse with wild-type
mice in which the effects of CXCR2 were antagonized using a
specific antisera applied after the initiation of DSS colitis.

Materials and Methods

Mice. Specific pathogen-free male BALB/c (wild-type) mice (6–8
weeks of age) were obtained from Charles River Canada (Montreal,
QC, Canada). CXCR2\(\text{(-/-)}\) mice (on the BALB/c background) were
purchased from The Jackson Laboratory (Bar Harbor, ME) and
were bred and maintained in the IWK Health Centre animal care facility
under the approval of the University Committee on Laboratory An-
imals. Mice had free access to food and water before their use in the
experiments. In addition, experiments using CXCR2\(\text{(-/-)}\) mice were
conducted in the facilities at the Department of Microbiology, Immun-
ology, and Glycobiology (University of Lund, Sweden).

Induction of Colitis and in Vivo CXCR2 Antiserum Admin-
istration. CXCR2\(\text{(-/-)}\) and wild-type mice were administered 5%
(\text{w/v}) DSS (36,000–50,000 mol. wt.; MP Biomedicals, Irvine, CA) in
their drinking water for 5 days, followed by regular facility water for
1 day. In the neutralization experiment, wild-type mice were given
0.5-ml i.p. injections of goat anti-CXCR2 antiserum (Moore et al.,
2000; Keane et al., 2004) or normal goat serum (control serum) on
days 2 and 4 during the DSS period. At the end of the experiment,
blood was collected from anesthetized mice by cardiac puncture, and
blood smears were prepared for determining the leukocyte differen-
tial. All of the mice were then euthanized, and their colons were
excised, measured, and prepared for histology.

Clinical Illness Scale. Clinical illness severity was scored in
each mouse during the experimental period based on unique combi-
nations of weight loss, stool consistency, and presence of occult or
gross blood. The maximal score of 10 represents weight loss of 20%
combined with diarrhea and gross bleeding. Animals exhibiting this
level of clinical illness were euthanized. No mice died given DSS
treatment directly.

Isolation of Bone Marrow PMNs. The femurs and tibia were
removed from the euthanized mice and flushed through the bone
with HBSS using a 25-gauge needle. After dispersing cell clumps and removing debris, the bone marrow cells
were centrifuged, and the pellet was resuspended in HBSS. Bone
marrow cells suspended in 2 ml of HBSS were laid on top of a
three-step discontinuous Percoll (Amersham Biosciences, Chalfont
St. Giles, UK) density gradient prepared in a 15-ml polystyrene tube
by layering 2 ml each of 75, 67, and 52% Percoll solutions. After
centrifugation at 1647g for 30 min at 24°C, the lowest band (the
75/67% interface) was collected as the PMN fraction. After washing
with HBSS, any remaining red blood cells were eliminated by hypo-
tonic lysis. The purity of PMNs was typically $\approx$90%, assessed by
crystal violet staining.

In Vitro PMN Migration Assay. PMN migration was deter-
mined using 24-well Transwell chambers (Corning Life Sciences,
Acton, MA) with 3-\text{pm} pore size membranes. Membranes were
soaked with migration media (RPMI 1640 medium with 0.6% heat-
inactivated fetal bovine serum) overnight at 4°C and incubated for
3 h at 37°C before use in a migration assay. Chromium labeling was
performed by incubating PMNs (5 x 10^6 cells/ml) with 15 \mu l of
Na\(_2\)CrO\(_4\) (2.5 \text{mC/m}; Amersham Biosciences) for 30 min at 37°C.
Subsequently, CXCR2 neutralization was carried by incubating the
labeled PMNs with anti-CXCR2 antiserum or control serum (1:50
dilution) in migration media for 30 min at 37°C. The different che-
moattractants, including human CXCL8 (PeproTech, Rocky Hill,
NJ), mouse CXCL2 (R&D Systems, Minneapolis, MN), and human
C5a (Sigma-Aldrich, St. Louis, MO) were diluted in migration media
at an optimized concentration of 10^{-8} M and applied (600 \mu l) to the
lower chambers. Antiserum-treated radiolabeled PMNs (100,000
cells/100 \mu l) were loaded on the top of the filter above each well, and
the chamber was incubated for 60 min at 37°C and 5% CO\(_2\). After incubation, the nonmigrated (top), migrated (bottom), and adherent
(filter associated) PMNs were collected separately and counted using
a gamma radiation counter (Wizard 1480; PerkinElmer Wallac,
Turku, Finland). The migrated fraction is expressed as a percentage
of total PMNs applied to the top of the filter.

Histopathology and Scoring. The colons of the animals (exclud-
ing cecum) were prepared as “Swiss rolls.” In brief, the entire colon
was cut open longitudinally along the mesenteric axis and again cut
longitudinally into two full-length colon halves, one of which was
rolled and fixed. After fixation, the Swill rolls were paraffin-embedded,
sectioned, and stained with hematoxylin and eosin. The
inflammatory features were assessed on blinded slides using four
easily identifiable pathological criteria, including the extent of cel-
ular infiltration (0–5), declining crypt architecture (crypt damage,
0–5), size and relative extent of ulceration (0–3), and absence or
presence of edema (0 and 1). Congo red dye (Sigma-Aldrich) was used
to stain colon sections to differentiate between PMNs and eosinophil
granulocytes. The number of PMNs and eosinophils in the submu-
cosa and mucosa were counted in sections representing the different
stages of crypt damage (scale, 0–5), with cells in at least five differ-
ent field areas of each crypt damage score counted within high-power
fields (400 x).

Statistical Analyses. Data are expressed as mean $\pm$ S.E.M. All
animal group comparisons were evaluated by the nonparametric
Mann-Whitney U test, using SPSS version 14.0 (SPSS Inc., Chicago,
IL). Statistical significance was designated as a p value $<$ 0.01.

Results

Response to DSS-Induced Colitis in CXCR2\(\text{(-/-)}\) Mice

In a previous study of DSS-induced colitis in CXCR2\(\text{(-/-)}\) mice, the investigators required two exposures of DSS to
detect a difference compared with wild-type mice (Buanne et al., 2007). We exposed CXCR2(−/−) and BALB/c mice to 5% DSS in their drinking water for 5 days and euthanized the mice 1 day later. The maximal body weight loss of BALB/c mice in these experiments was 5%, and the maximal weight loss of CXCR2(−/−) mice was 1%. Colon histology on sections from BALB/c mice showed submucosal edema, extensive ulcers, cellular infiltration (PMNs, eosinophils, and mononuclear cells), and crypt abscesses, with patches showing complete loss of crypt architecture (scored 5) (Fig. 1). In contrast, colon sections from the CXCR2(−/−) mice showed no ulcers, less extensive crypt damage, and a cellular infiltrate consisting mostly of eosinophils and mononuclear cells (Fig. 1). The same findings were made in mice from two facilities (Fig. 1). Our results using CXCR2(−/−) mice thus suggest that CXCR2 is an important determinant of the extent of colitis and that a single DSS cycle establishes an acute inflammation sufficient to make this conclusion.

Characterization of the Goat Anti-CXCR2 Antiserum

In Vitro. The goat anti-CXCR2 antiserum used here has been shown previously by flow cytometry to detect surface-expressed CXCR2 on mouse PMNs (Echchannaoui et al., 2005). To test whether the anti-CXCR2 antiserum would impair PMN migration in response to chemoattractants, an in vitro neutralization assay was performed. PMNs incubated with control serum migrated in the presence of human CXCL8 and mouse CXCL2, but migration was significantly inhibited in the presence of the anti-CXCR2 antiserum (Fig. 2), suggesting that the antibody blocks CXCR2 or induces its internalization, thus preventing an interaction with chemokine ligands. The antibodies did not inhibit PMN migration in response to C5a, which indicates that blockade of CXCR2 was specific (Fig. 2).

In Vivo. To examine whether the antibodies were toxic, particularly for the colon, healthy mice were injected with the control serum or anti-CXCR2 antiserum. There was no evidence of weight loss, clinical illness, or colon pathology (data not shown).

The depletion of PMNs using antibodies is associated with reduced inflammation in a number of colitis models. Therefore, we tested whether the anti-CXCR2 antiserum caused PMN depletion in DSS-inflamed mice. The blood PMN differential count did not significantly differ between anti-CXCR2 antiserum-injected mice (15.6 ± 6.9%) versus control serum-injected mice (19.6 ± 7.3%), assayed on the last day of the experiment (n = 11). The total bone marrow cell count from the control serum-treated mice was 31 ± 14.5 × 10⁶ and 24 ± 10.5 × 10⁶ cells/bone from the anti-CXCR2 antiserum-injected mice with no significant difference between the groups. These results indicate that the antiserum did not affect the hematopoietic cell pool in injected mice during DSS colitis.

CXCL1, CXCL2, CXCL5, CCL5, and CCL11 mRNA levels were quantified on day 6 by reverse transcriptase-polymerase chain reaction. Chemokine mRNA levels were not grossly altered in the anti-CXCR2 antiserum-treated compared with the control serum-treated group (data not shown). The results suggest that the antibodies have no significant impact on the overall level of chemokine gene expression and that chemokines important for PMN recruitment can be expressed under these conditions.

Effect of Anti-CXCR2 Antiserum on DSS Colitis

We observed a gradual decrease in body weight during DSS treatment in the control serum group (Fig. 3a). On day...
controls ($p < 0.001$, Fig. 4b). The Swiss roll technique differs from cross sections in that it permits histopathological analysis of the entire colon. Control serum-injected mice showed light but diffuse cellular infiltration along much of the colon length but with a maximum in the midcolon. The cellular infiltrate included mononuclear and granulocytic cells and extensive ulcers. On the other hand, anti-CXCR2 antiseraum-treated mice showed patchy infiltrates in the midcolon, composed mainly of mononuclear cells and eosinophils, with few PMNs. In control serum-treated mice, the muscularis externa was periodically interrupted by dilated blood vessels containing extravasating leukocytes and a stream of migrating cells through the smooth muscle into the submucosa. This infiltration also appeared in adventitia of the colon. These types of inflammatory features were rare in the anti-CXCR2 antiseraum-injected mice fed DSS (data not shown).

There was a positive correlation ($r = 0.6248$) between the total clinical scores and total inflammation scores in control serum-treated mice on day 6, which suggests the clinical illness is directly related to events in the colon. On the other hand, anti-CXCR2 antiseraum-injected mice showed a negative correlation ($r = -0.3105$) between the total clinical score and total inflammation score (Fig. 4c), which suggests the anti-CXCR2 antiseraum has a greater effect on preventing clinical illness than on colonic inflammation.

PMNs are presumably the most abundant CXCR2$^+$ cell type in the inflammatory infiltrate. To assess whether the anti-CXCR2 antiseraum inhibited PMN infiltration, we used Congo red staining, which distinguishes eosinophils from PMNs (Fig. 4). To compensate for differences in overall inflammation, we determined PMN numbers in regions with similar crypt damage rather than the total myeloperoxidase content. PMN infiltration was closely associated with the degree of crypt damage in the control serum-treated group, but the anti-CXCR2 antiseraum-injected mice had significantly fewer PMNs in the mucosa, including the animals with high crypt damage scores (3 and 4; Fig. 5a). The eosinophil numbers did not differ between the groups, suggesting that the eosinophil recruitment into the colon is independent of CXCR2 in DSS-induced colitis (Fig. 5b).

**Discussion**

Defining the mechanisms that recruit PMNs into the intestinal mucosa may provide insights into understanding the tissue injury in IBD. One factor important in recruitment is epithelial chemokine secretion, which is typically initiated by infection and generates a chemotactic gradient, and CXC chemokines such as CXCL8 and CXCL5 are up-regulated in mucosal biopsies from IBD patients (Z’Graggen et al., 1997; McCormack et al., 2001). There is considerable evidence that mouse colonic epithelial cells similarly make CXC chemokines during experimental colitis, including CXCL1 and CXCL5 (Ohtsuka and Sanderson, 2003; Kwon et al., 2005). Evidence that the ELR$^+$ CXC chemokines play a role in various inflammatory diseases is derived from studies manipulating the chemokines in animal models. For example, antisense oligonucleotides made to antagonize expression of CXCL5 in mice with DSS colitis reduced mucosal PMN infiltration, and this reduction was associated with lower pathology scores (Kwon et al., 2005). This finding demonstrates that CXC chemokines play a pathophysiological role in ex-

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**Fig. 3.** Daily body weight change and daily clinical illness scores of BALB/c mice administered DSS and treated with control goat (G) serum or anti-CXCR2 antiseraum. The arrows indicate the days mice were injected with their respective serum. a, daily animal body weight changes during the experimental period. The weight difference between the groups is significant on days 3 and 6; *p < 0.01 versus DSS + anti-CXCR2 antiseraum. b, clinical disease score in each mouse was monitored with the hypothesis that receptor neutralization provides a therapeutic. Injections of control serum in mice on days 2 and 4 used to examine whether CXCR2 neutralization can be therapeautic. Injections of control serum in mice on days 2 and 4 were found to be associated with significantly increased illness scores on days 3 and 4 compared with anti-CXCR2 antiseraum-injected mice ($p < 0.01$, Fig. 3b). Reduced clinical illness in anti-CXCR2 antiseraum-injected mice is consistent with the hypothesis that receptor neutralization provides a therapeutic benefit in this model of colitis. Whether mice were protected against tissue pathology was examined next.

As expected, colon sections from mice injected with control serum revealed severe pathology (Fig. 4a), with submucosal edema, ulceration, extensive inflammatory cell infiltration, and complete loss of crypts at multiple sites of the midcolon (damage score up to 5). It is intriguing that with the exception of one mouse with an ulcer, the anti-CXCR2 antiseraum-treated mice showed less evidence of pathology (Fig. 4a). In addition to fewer ulcers, the crypt architecture was maintained, and the infiltrate was diffuse and appeared to have few PMNs. The average histopathology score was significantly lower in anti-CXCR2 antiseraum-treated mice than in...
CXCR2 is the receptor for the ELR<sup>+</sup> family of PMN chemokines in mice, and targeting this receptor should overcome the redundancy among the CXC ligands. Targeted disruption of the CXCR2 gene in models of lung injury resulted in reduced PMN adhesion (Morgan et al., 1997) and thioglycolate-mediated PMN influx into the peritoneal cavity (Cacalano et al., 1994). In the kidney infection model, receptor-deficient mice developed more severe disease, however, with bacteremia and tissue damage due to subepithelial neutrophil entrapment (Frendéus et al., 2000). Entrapment of PMN does not seem to be a decisive factor in the colitis model because PMN infiltration into the mucosa is significantly reduced in receptor-deficient mice. This was also the finding in chronic DSS-induced colitis, that receptor-deficient mice had reduced PMN infiltration combined with less colitis (Buanne et al., 2007). To overcome the ELR<sup>+</sup> CXC chemokine redundancy and any compensatory mechanisms that might develop in CXCR2-deficient mice, we chose to antagonize CXCR2 in wild-type mice using specific antibodies. Antibody neutralization was specific, shown by blocking PMN migration to CXCR2 ligands but not another chemoattractant in vitro. Moreover, inhibition of PMN recruitment and activation through CXCR2 antagonism has been shown to be therapeutic in a number of inflammatory diseases. Ness et al. (2003) demonstrated that CXCR2 neutralization protects against septic injury by delaying inflammatory cell recruitment. Additional studies using monoclonal antibodies demonstrated potent inhibition of PMN recruitment in a rabbit model of endotoxin-induced pleurisy (Broaddus et al., 1994) and in rabbit lung reperfusion injury (Sekido et al., 1993) and that CXCR2/ligand signaling plays a pivotal role in mediating PMN recruitment in ventilator-induced lung injury (Belperio et al., 2002). Receptor blockade using a competitive antagonist led to a reduction in PMN influx and activation in vulnerable tissues (White et al., 1998).

DSS-induced colitis is a popular rodent model that histologically resembles ulcerative colitis, with PMN, eosinophil, and mononuclear cell infiltrates in the mucosa and a therapeutically similar to IBD (Melgar et al., 2008). The anti-CXCR2 antiserum treatment significantly reduced acute clinical illness and acute colon pathology. The most remarkable differences were the lack of ulcers, retained crypt architecture, and low PMN infiltration into the mucosa, further implicating PMNs in the clinical illness and pathology and confirming and extending previous findings showing that antagonizing CXCR2 during TNBS-induced colitis blocks PMN recruitment into the mucosa (Ajuebor et al., 2004; Bento et al., 2008). Antagonizing CXCR2 potentially inhibits infiltration by other hematopoietic cells also expressing CXCR2; for example, eosinophils (Borchers et al., 2002). Yet, in contrast to PMN infiltration, eosinophil infiltration into the colon was intact in anti-CXCR2 antiserum-treated mice. This supports the report antagonizing CXCR2 in mice with TNBS-induced colitis (Bento et al., 2008), which indicated that eosinophil migration was CXCR2-independent. This result suggests that eosinophils may not contribute to the tissue pathology as is presumed for PMNs.

CXCR2 is reportedly expressed on other mature hematopoietic cell types including monocytes (Thomas et al., 1991), ...
hematopoietic cells that interact with the PMNs and influence their infiltration and the ensuing pathology.

Blocking CXCR2 resulted in early significant improvements in clinical illness scores and an overall reduction in PMN infiltration, suggesting that PMNs in the colon tissue contribute to the generation of systemic clinical disease. This is an encouraging outcome relevant to treating human colitis. Although antagonizing CXCR2 may hold promise as a therapy for colitis, if mucosal PMNs are critical to the antibacterial defenses in the colon, then blocking PMN infiltration may introduce new risks such as the dissemination of transmissible commensal bacteria or increased susceptibility to pathogenic bacteria. Still, our findings build on a growing body of evidence showing that CXCR2 is involved in the pathogenesis of colitis and that it plays a role in recruiting PMNs into the diseased colon.

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References


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