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Critical Role for Mast Cells in the Pathogenesis of 2,4-Dinitrobenzene-Induced Murine Colonic Hypersensitivity Reaction

Anneke Rijnierse, Andries S. Koster, Frans P. Nijkamp, and Aletta D. Kraneveld

The immunological mechanisms underlying the role of mast cells in the pathogenesis of inflammatory bowel disease (IBD) are poorly defined. In this study, non-IgE mediated colonic hypersensitivity responses in BALB/c mice induced by skin sensitization with dinitrofluorobenzene (DNFB) followed by an intrarectal challenge with dinitrobenzene sulfonic acid featured as a model to study the role of mast cells in the development of IBD. Vehicle- or DNFB-sensitized mice were monitored for clinical symptoms and inflammation 72 h after dinitrobenzene sulfonic acid challenge. DNFB-sensitized mice developed diarrheic stools, increased colonic vascular permeability, hypertrophy of colonic lymphoid follicles (colonic patches), and showed cellular infiltration at the microscopic level. Increased numbers of mast cells were found in the colon of DNFB-sensitized mice located in and around colonic patches associated with elevated levels of mouse mast cell protease-1 in plasma indicating mast cell activation. Colonic patches of DNFB mice, stimulated in vitro with stem cell factor indicated that an increase in TNF-\(\alpha\) levels in the colon is mainly mast cell originated. Finally, neutrophil infiltration was observed in the colon of DNFB-sensitized mice. Induction of this model in mast cell-deficient WBB6F1/\(W^w\) mice shows a profound reduction of characteristics of the colonic hypersensitivity reaction. Reconstitution with bone marrow-derived mast cells in WBB6F1/\(W^w\) mice fully restored the inflammatory response. This study demonstrates the importance of mast cells in the development of clinical symptoms and inflammation in the presented murine model for IBD. The Journal of Immunology, 2006, 176: 4375–4384.

Inflammatory bowel disease (IBD) is attended by severe abdominal pain, diarrhea, weight loss, rectal bleeding, and increased presence of inflammatory cells and inflammatory products in the gastrointestinal tract (1–3). The major representatives of chronic IBD are Crohn’s disease and ulcerative colitis. The pathogenesis of IBD is thought to be a complex of interactions between environmental, genetic, and immunological factors. The exact etiology and pathophysiology still remains unknown, but an inadequate or prolonged activation of the intestinal immune system plays a crucial role (3). Several pathophysiological features of IBD resemble hypersensitivity-like responses in the gastrointestinal tract (4, 5).

Delayed-type hypersensitivity (DTH) is classically characterized by T cell-driven Ag-specific inflammatory cell infiltration within 12–72 h after a second encounter with the Ag (6). Originally, B cell-driven and IgE-mediated mast cell degranulation was only associated with immediate-type hypersensitivity reactions (7). However, mast cell degranulation is described to be required for the elicitation of DTH reactions (8). In addition, mast cell degranulation was shown to be involved in a wide range of non-IgE-mediated inflammatory processes like multiple sclerosis, tuberculosis, contact dermatitis, nonatopic asthma, and also IBD (9). In the gastrointestinal tract, mast cells are distributed throughout all layers of the intestinal wall and numbers increase in intestinal disorders like IBD (10). Not only was there an increased number of mast cells observed in patients with Crohn’s disease and ulcerative colitis (11, 12), but also activation in the inflamed area was found (12). Furthermore, intestinal mast cells in IBD bear a different content compared with normal subjects (10). For example, they contain a higher density of the multifunctional, proinflammatory cytokine TNF-\(\alpha\) (13). Now, one of the most promising therapies for IBD is directed against TNF-\(\alpha\) using mAbs (14). These studies suggest a pivotal role for mast cells and its mediators in the pathogenesis of IBD.

The trinitrobenzene sulfonic acid (TNBS)-colitis model is generally accepted as a hapten-induced T cell-mediated immunological model for IBD (15). However, the role of mast cells in this model is controversial and contradictory (16, 17). In this study, we present a consistent novel chemically induced immunological model for colonic IBD associated with mast cell activation. Our non-IgE-mediated hypersensitivity model is useful in studying immune-related processes in the course of IBD and resembles several pathophysiological features of IBD.

A hypersensitivity reaction in the colon was elicited with the low m.w. compound dinitrofluorobenzene (DNFB) upon skin sensitization followed by a local intrarectal challenge with the corresponding dinitrobenzene sulfonic acid (DNS). The effect of DNFB sensitization and local intrarectal challenge on mast cell activation and infiltration was examined in the hypersensitivity reaction. Furthermore, the influence of mast cells on subsequent reactions like clinical symptoms, tissue damage, and cellular infiltration was monitored. Proof of principle of the crucial role for mast cells in this model is demonstrated by the use of genetic mast cell-deficient mice (WBB6F1/\(W^w\)) and congenic normal mice in this murine model for IBD.
Materials and Methods

Animals

Male BALB/c mice were obtained from Charles River Laboratories. These mice were 6–8 wk of age and weighed 20–25 g by time of use. Male mast cell-deficient mice (WBB6F1/Wv) and their respective normal littermates (WBB6F1+/+) were purchased from The Jackson Laboratory. These mast cell-deficient mice were used at 32 wk of age and the control littermates (+/+) were age-matched. All the animals were housed in groups not exceeding eight mice per cage. Tap water and chow food were allowed ad libitum at a 12-h day-night cycle. All experiments were conducted in accordance with the Animal Care Committee of Utrecht University (Utrecht, The Netherlands).

Mast cell reconstitution

Selective reconstitution of mast cells in mast cell-deficient W/Wv mice was conducted by the methods earlier described by Kraneveld et al. (18) with slight modifications. In brief, bone marrow-derived mast cells (BMMC) were obtained from +/- mice. Bone marrow was aseptically flushed from femurs of +/- mice and cultured for 4 wk in RPMI 1640 medium containing 10% FCS, 4 mM L-glutamine, 0.5 mM 2-ME, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM nonessential amino acids. Reconstituent mouse IL-3 (10 ng/ml) and stem cell factor (SCF; 10 ng/ml) was added to the medium to drive bone marrow cell development to mucosal type mast cells. Medium was refreshed once a week and fresh IL-3 and SCF were added to the medium. Purity of the BMMC population was determined by flow cytometry (c-kit-specific). The culture contained a uniform cell population (>90%). Furthermore, staining cells with toluidin blue indicated that nearly 99% of the viable cells were mast cells after 4 wk culture (data not shown). Mast-cell-deficient W/Wv mice were injected via the tail vein with 5 x 10^6 cultured BMMC cells and the recipients were studied 26 wk later. Age-matched mast-cell-deficient W/Wv mice and congenic normal mice were used when examining the BMMC-reconstituted W/Wv mice.

Induction of colonic hypersensitivity

Mice were sensitized on day 0 by application of either DNFB (0.6% in acetone:olive oil, 4:1) or vehicle (acetone:olive oil, 4:1) epicutaneously on the shaved abdomen (50 μl) and paws (50 μl divided over 4 paws). On day 1, the mice received a boost of DNFB or vehicle on the abdomen only (50 μl). All the animals were challenged intracutaneously with DNFB (0.6%) dissolved in 10% ethanol on day 5. The sensitization, boost and challenge took place under light inhalation anesthesia (halothane 3%). The mice were refreshed once a week and fresh IL-3 and SCF were added to the medium. Purity of the BMMC population was determined by flow cytometry (c-kit-specific). The culture contained a uniform cell population (>90%). Furthermore, staining cells with toluidin blue indicated that nearly 99% of the viable cells were mast cells after 4 wk culture (data not shown). Mast-cell-deficient W/Wv mice were injected via the tail vein with 5 x 10^6 cultured BMMC cells and the recipients were studied 26 wk later. Age-matched mast-cell-deficient W/Wv mice and congenic normal mice were used when examining the BMMC-reconstituted W/Wv mice.

Clinical characteristics of the inflammatory response were obtained by assessing stool consistency. The stool consistency was scored every day at the same time point by placing the mice separately in cages without bedding. They were left in the cages until they relieved enough feces to enable the production of semithin sections of high quality with preserved morphological characteristics, colonic tissue was embedded in glycol methacrylate (GMA). After careful dissection of the colon, it was placed in saline. The colon was then opened longitudinally over the mesenteric line and feces were removed by gently washing in saline. The colon then was placed with the serosal side up and dissected free from fat. The making of Swiss rolls was accomplished by rolling the colon from the distal to the proximal end. The roll was immediately placed in ice-cold 4% paraformaldehyde in PBS (pH 7.4) and routinely embedded in GMA for further use (20). Serial sections of 3 μm were cut using a microtome (Leica) and routinely stained with H&E to observe damage and cellular infiltration. One observer evaluated all sections and scored according to Hartmann et al. (21) for cellular infiltration, rare inflammatory cells in the lamina propria were counted as 0; increased numbers of inflammatory cells in the lamina propria as 1; confluence of inflammatory cells, extending into the submucosa as 2; and a score of 3 was given to transmural extension of the infiltrate. For tissue damage, no mucosal damage was counted as 0; discrete lymphoepithelial lesions were counted as 1; surface mucosal erosion as 2; and a score of 3 was given to extensive mucosal damage and extension through deeper structures of the bowel wall. The combined histological score ranged from 0 (no changes) to 6 (extensive cell infiltration and tissue damage). Per mouse, three different longitudinal sections of the colon tissue were observed and a score was given to the total of all sections. A chloroacetate esterase (CAE) staining was used to detect mast cells (22). However, CAE staining also detects some neutrophils. Serial sections stained with a subsequent peroxidase staining, representative for neutrophils, revealed no overlap between CAE- and peroxidase-positive staining indicating that CAE did not stain neutrophils under our conditions. Per mouse, three different longitudinal sections of the colon tissue were stained and analyzed. The number of CAE-positive cells was quantified by microscopic visualization and manually counting. Results are expressed as median number of cells (minimum-maximum) per colon section. Infiltration of neutrophils was scored as follows: 0, no infiltration; 1, infiltration between the mucosa and submucosa, not around colonic patches; 2, extensive infiltration, especially around colonic patches; cluster formation. Results are expressed as median neutrophils score (minimum-maximum).

Histology and immunohistochemistry

To enable the production of semithin sections of high quality with preserved morphological characteristics, colonic tissue was embedded in glycol methacrylate (GMA). After careful dissection of the colon, it was placed in saline. The colon was dissected free from fat and placed in formamide (500 μl). Evans blue dye was extracted from the colon at 40°C for 24 h. The dry weight was determined after drying the colons for 4 wk at 40°C. The extravasation of Evans blue dye-labeled macromolecules from the blood circulation into the colonic tissues was quantified by measuring the OD of the plasma samples and formamide extracts on a Benchmark microplate reader (Bio-Rad) at a wavelength of 595 nm. Vascular permeability was determined by dividing the total amount of Evans blue extracted from the colon by the concentration of Evans blue in the plasma. The vascular permeability was expressed as microliters of extravasated plasma per milligram dry weight of the colon.

Vascular permeability changes were determined as described previously with slight modifications for colon tissue (19). Evans blue (1.25% in sterile saline, 50 μl) was injected i.v. in the tail vein 2 h before the end of an experiment. After 2 h, the mice were killed with an overdose of sodium pentobarbital and blood samples were taken via cardiac puncture. Four percent EDTA (10% v/v) was added to whole blood to obtain plasma. The colon was excised carefully and opened longitudinally. Feces were re-
Preparation of single-cell suspension of colonic patch and FACS analysis

The preparation of a single-cell suspension was adapted from Dohi et al. (23). In brief, colonic patch was excised from the intestinal wall and washed once with RPMI 1640. All colonic patches originating from one mouse were pooled. Repeated cell dissociation took place with collagenase at 0.1 mg/ml in RPMI 1640 with 100 U/ml penicillin, 100 μg/ml streptomycin, 40 μg/ml gentamicin for 20 min at 37°C using fresh collagenase solution each time. After the dissociation, the cells were washed twice with RPMI 1640. Of each single-cell suspension, 1 × 10^6 cells were incubated with 1 μg of designated Ab in PBS + 10% FCS for 60 min on ice. After incubation, the cells were washed three times with PBS. The pellet was resuspended in 200 μl of PBS + 10% FCS and analyzed using CellQuest (BD Biosciences). PE-conjugated hamster anti-CD3 was used to detect T cells, allophycocyanin-conjugated rat anti-CD11c was used for the detection of c-kit-positive cells (mast cells), PE-conjugated rat anti-B220/CD45 was used to detect B cells, and, finally, PE-conjugated rat anti-CD11c was used to detect dendritic cells.

Preparations of tissue homogenates

To determine mast cell infiltration into the tissue, whole colon homogenates were made. After sacrificing of the mice, the colon was excised carefully and opened longitudinally. Feces were removed by gently washing in saline. The colon was placed in ice-cold PBS enriched with protease inhibitors (Complete Mini; Roche) in flat-bottom tubes. The tissue was dispersed on ice for 10 s according to the rotor-stator principle (Ystral). The homogenates were centrifuged (14,000 rpm, 4°C, 10 min) and the supernatant was frozen until further use to assess mouse mast cell protease-1 and TNF-α levels.

Mast cell activation and infiltration in vivo

To monitor mast cell activation in time, blood samples of DNFB- and vehicle-sensitized mice were taken 30 min, 24 h, and/or 72 h after intracolonic DNS challenge. Blood samples were collected via heart puncture and washed once with RPMI 1640. Of each single-cell suspension, 1 × 10^5 cells were incubated with 1 μg of designated Abs in PBS + 10% FCS for 60 min on ice. After incubation, the cells were washed three times with PBS. The pellet was resuspended in 200 μl of PBS + 10% FCS and analyzed using CellQuest (BD Biosciences). PE-conjugated hamster anti-CD3 was used to detect T cells, allophycocyanin-conjugated rat anti-CD11c was used for the detection of c-kit-positive cells (mast cells), PE-conjugated rat anti-B220/CD45 was used to detect B cells, and, finally, PE-conjugated rat anti-CD11c was used to detect dendritic cells.

Statistical analysis

Stool consistency data were analyzed from raw scoring data using a distribution-free Kruskal-Wallis ANOVA followed by a Dunn’s multiple comparison test. Mast cell numbers, colonic patch count, tissue damage score, and neutrophil scoring were all expressed as median (range) and analyzed with the use of a nonparametric test (Mann-Whitney U). The following data were analyzed by one-way ANOVA: mouse mast cell protease-1 content in plasma and colon tissue, and TNF-α production in colonic patches. Vascular leakage was tested with a Student’s t test. In figures, group means ± SEM are given. A value of p < 0.05 was considered to be significant. All data manipulations and statistical analyses were conducted by the usage of GraphPad Prism (version 3.0).

Results

DNFB induces a colonic hypersensitivity reaction characterized by development of diarrhea, lymphoid structure hypertrophy, and increased vascular permeability

The presence of loose stool and diarrhea is indicative for the presence of a damaged colon. DNFB-sensitized animals significantly develop loose stool and diarrhea 72 h after DNS challenge compared with normal well-formed pellets in vehicle-treated animals 72 h after challenge (Fig. 1A). Twenty-four hours after challenge, no significant difference between the two treatment groups could be found. This can be attributed to the irritant effect of the 10% ethanol used as a mucosal barrier breaker along with the DNS challenge. Stool consistency improves again in vehicle-sensitized mice after 72 h, whereas the stool of DNFB-sensitized animals deteriorates in time after challenge.

Colonic patches are small lymphoid follicles that appear at the mucosal side of the colon. They consist predominantly of B cell zones, but also T cell areas were found (23). Colonic patches are spread nonconsistently and differ in size and shape in each single animal. The number of colonic patches differed significantly between the two treatment groups 72 h after challenge (Table I). DNFB-sensitized animals have an increased number of colonic patches present in the colon compared with vehicle-sensitized animals. In addition, enlarged sizes of colonic patches in DNFB-sensitized mice indicated hypertrophy of these lymphoid structures compared with vehicle-sensitized mice. The distribution of leukocyte population of colonic patch is presented in Table II. Even though the distribution of inflammatory cells does not differ between vehicle- and DNFB-sensitized mice 72 h after DNS challenge, indicated by percentages in Table II, the total cell yield in DNFB-sensitized mice was significantly higher than in vehicle-sensitized mice. This demonstrates that the total amount of inflammatory cells (i.e., T, B, and dendritic cells) in colonic patches is significantly increased in DNFB-sensitized mice 72 h after challenge. c-kit expression of the leukocyte population in the colonic patch was below detection limit (1%).

Plasma extravasation quantified by the accumulation of the Evans blue dye in the colonic tissue is a measurement for changes in vascular permeability. Seventy-two hours after challenge, there was a significant increase in vascular exudation from the blood all purchased from Sigma-Aldrich. DNS was purchased from Eastman Kodak. Tween 20 was purchased from Janssen Pharmaceutica. Sodium pentobarbital was purchased from Sanofi. RPMI 1640 medium was purchased from Invitrogen Life Technologies. Evans blue dye was obtained from Fluka Chemie. The rat Ig anti-mouse mast cell protease-1 Ab was a gift of Dr. H. R. Miller, Royal (Dick) School of Veterinary Studies (University of Edinburgh, Edinburgh, United Kingdom) and the biotin-conjugated goat anti-rat Ig-specific polyclonal Ab was purchased by BD Pharmingen. The BCA protein assay was purchased at Pierce. Collagenase IV and complete mini protease inhibitors were from Roche Diagnostics. The TNF-α ELISA kit, IL-3, and SCF were all purchased from BioSource International. The mouse mast cell protease-1 ELISA was from Morexden Scientific. Maxisorp surface 96-well plates were purchased from Nunc Immunoplate. All Abs for FACS analysis are obtained from BD Pharmingen.

Materials

DNFB, olive oil, o-phenylenediamine dihydrochloride, the AEC chromogen staining kit, normal goat serum, and napthol AS-D CAE, H&E were stored at 20°C until further use. TNF-α levels in supernatants of colonic homogenates and tissue culture samples were determined with a commercially available TNF-α ELISA kit (BioSource International).
into colonic tissue of DNFB-sensitized animals compared with vehicle-treated animals (Fig. 1B).

**DNFB-induced colonic hypersensitivity changes colon morphology and induces cellular infiltration**

To study cellular infiltration and damage of the colon, serial sections of 3 μm of GMA-embedded Swiss rolls were made at three different depths of the tissue leaving at least 100 μm between two series that were stained with a standard H&E staining. DNFB-sensitized mice showed a significantly higher damage score compared with vehicle-sensitized mice 72 h after DNS challenge (damage score: vehicle: 0 (0–1) and DNFB: 2.5 (1–3); results are expressed as median (minimum-maximum), p < 0.05 (Mann-Whitney), n = 5–6 mice/group, 3 sections/mouse). Fig. 2A demonstrates a neatly packed mucosal lining of a vehicle-sensitized mouse 72 h after DNS challenge. Mucosal swelling and infiltration of inflammatory cells can be seen in DNFB-sensitized mice 72 h after challenge (Fig. 2, B and C; indicated by arrows). This infiltration was particularly seen between the mucosa and the submucosa around colonic patches at the distal end of the colon (Fig. 2C). Due to this infiltration, the colonic tissue is damaged at some locations rupturing the mucosa from the submucosa (Fig. 2B). Hypertrophy of colonic patches can repulse lamina propria cells, but leaves the epithelial lining, which separates the mucosa from the lumen, intact (arrowhead in Fig. 2C). Furthermore, epithelial cells are enlarged and swollen in DNFB-sensitized mice 72 h after challenge suggesting increased mucus production (small arrows in Fig. 2, B and C).

The peroxidase-staining method was used to detect neutrophils. Colonic tissue of DNFB-sensitized animals showed a significantly increased infiltration of these polymorphonuclear cells compared with vehicle-sensitized animals. Results are expressed as median number of colonic mast cells (24). To assess mast cell activation, mouse mast cell peroxidase (25) was used. Mayer’s hematoxylin counterstaining was performed to detect the infiltrating cells.

**Table I. Number of colonic patches in BALB/c, mast cell-deficient W/Wv mice, mast cell-reconstituted W/Wv mice, and control littermates after vehicle or DNFB sensitization 72 h after DNS challenge**

<table>
<thead>
<tr>
<th>DNFB Sensitization</th>
<th>Colonic Patches/Colon</th>
<th>n</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>−</td>
<td>1.0 (0–3)</td>
<td>6</td>
</tr>
<tr>
<td>+</td>
<td>3.0 (3–6)</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>+/+b</td>
<td>−</td>
<td>2.0 (1–4)</td>
<td>17</td>
</tr>
<tr>
<td>−</td>
<td>4.0 (1–5)</td>
<td>15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>W/Wv</td>
<td>−</td>
<td>2.0 (0–2)</td>
<td>20</td>
</tr>
<tr>
<td>+</td>
<td>0.0 (0–1)</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>BMMC→W/Wv</td>
<td>−</td>
<td>2.0 (0–3)</td>
<td>21</td>
</tr>
<tr>
<td>+</td>
<td>2.0 (0–3)</td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>

*Colonic patches were counted at the mucosal side of colon of vehicle (−) or DNFB (+) sensitized mice. Results are expressed as median number of colonic patches per colon (minimum-maximum); n, number of mice; significant differences between the two treatments within one strain are indicated (Wilcoxon rank sum test).

* +/+, Control littermates; W/Wv, mast cell-deficient mice; and BMMC→W/Wv, BMMC-reconstituted W/Wv mice.

**Table II. Cell yield of colonic patch and distribution of leukocyte population in colonic patch obtained with FACS analysis**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle/DNS</th>
<th>DNFB/DNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell yield (10⁶ cells/mouse)</td>
<td>1.3 ± 0.1*</td>
<td>2.1 ± 0.2*</td>
</tr>
<tr>
<td>T cells (CD3)</td>
<td>8 ± 2 b</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>Mast cells (CD117)</td>
<td>&lt;1⁻</td>
<td>&lt;1⁻</td>
</tr>
<tr>
<td>B cells (B220)</td>
<td>23 ± 4</td>
<td>19 ± 4</td>
</tr>
<tr>
<td>Dendritic cells (CD11c)</td>
<td>2 ± 0.2</td>
<td>2 ± 0.2</td>
</tr>
</tbody>
</table>

* Values are shown as mean ± SEM, n = 4 mice. *p < 0.05.

b Values are shown as mean percentage of total cells ± SEM, n = 4 mice.
A significant increase in mouse mast cell protease-1 in colon tissue of DNFB-sensitized animals, measured in supernatant of colon homogenates, was found, suggesting infiltration and/or proliferation of mast cells into the colon has taken place 72 h after challenge (Fig. 3B). An increase in mast number can be found throughout the whole colon, but predominantly at the distal end of the colon. In addition, clusters of mast cells are located around colonic patches as pictured by CAE staining in Fig. 4. Also the number of CAE-positive mast cells was increased after DNFB sensitization and DNS challenge compared with vehiclesensitized and DNS-challenged mice (mast cell number per colon: vehicle/DNS 16 (6–22), DNFB/DNS 28 (22–36); results are expressed as median (range); \( p < 0.05, n = 6 \)).

TNF-\( \alpha \) is one of the major cytokines released upon mast cell activation. Therefore, we have determined TNF-\( \alpha \) levels in colon of vehicle and DNFB-sensitized mice 72 h after challenge. Colonic
tissue TNF-α levels were significantly increased in DNFB-sensitized mice in vivo compared with vehicle-sensitized mice 72 h after challenge (vehicle: 22.849 ± 2.023 and DNFB: 39.867 ± 4.399 pg/mg; results are expressed as mean TNF-α (picograms)/total protein (milligrams) ± SEM; n = 10–11 mice; p < 0.01). To investigate whether colonic mast cells are the major source for TNF-α, colonic tissue specimens and colonic patches were collected and cultured in the presence of the specific mast cell activator SCF for 48 h. SCF is known as the c-kit ligand. On mature myeloid cells, c-kit is only expressed by mast cells (25). Previously, Wershil et al. (26) demonstrated that stimulation of the c-kit receptor by SCF leads to activation and degranulation of the mast cell. Fig. 5 demonstrates that SCF-stimulated colonic patches obtained from DNFB-sensitized mice 72 h after DNFB challenge produce significantly more TNF-α in vitro than colonic patches from vehicle-sensitized animals. However, mast cells are not the only source for TNF-α in hypersensitivity-like responses. TNF-α is also produced and secreted by T lymphocytes. Therefore, a general T cell stimulus, anti-CD3, was used to activate T cells in colonic patches and colon tissue in vitro. As shown in Fig. 5, TNF-α levels were significantly lower compared with the SCF-stimulated colonic patch for both treatment groups. In comparison, supernatants of cultures from colon tissue deprived of colonic patches stimulated with either SCF or anti-CD3 did not show significant differences in TNF-α production between the two treatment groups and both stimuli (data not shown).

**Mas** cells play a key role in the development of DNFB-induced colonic hypersensitivity

To assess the functional role of mast cells, several features of this hapten-induced model were studied in mast-cell-deficient mice and their normal littermates. Reconstitution of bone marrow-derived mast cells in W/W’ served as control to confirm that the lack of functional mast cells was responsible for the failure to induce colonic hypersensitivity. The +/- littermates and W/W’ mice were age-matched with the BMMC→W/W’ mice.

DNFB-sensitized mast cell-deficient animals do not develop increased levels of loose stool and diarrhea whereas +/- and BMMC→W/W’ do (Fig. 6). Vehicle-sensitized W/W’ mice have deteriorated stool consistency compared with DNFB-sensitized W/W’ animals. The number of colonic patches was significantly increased in DNFB-sensitized +/- and BMMC→W/W’ mice compared with W/W’. Damage and cellular infiltration studied at the histological level was scored on GMA-embedded colon tissue (Table III). Leukocyte infiltration in DNFB-sensitized +/- was observed comparable to previously seen in BALB/c mice (Fig. 7A). W/W’ mice failed to establish such an inflammatory feature upon DNS challenge after DNFB sensitization (Fig. 7B). This response however, could be restored after BMMC reconstitution (Fig. 7C).

In addition, in +/- mice, a significant rise in plasma mouse mast cell protease-1 levels was observed in DNFB-sensitized mice 72 h after challenge (Fig. 8A). No significant mouse mast cell protease-1 levels in plasma could be detected in W/W’ mice confirming the fact that these animals do not have functional mast cells. The ability to detect mouse mast cell protease-1 in vehicle-sensitized BMMC→W/W’ indicates that reconstitution of W/W’ with BMMC was successful. However, a difference between the two treatment groups was absent in BMMC→W/W’ mice. As is

![FIGURE 4. Localization of mast cells in and around colonic patches 72 h after DNS challenge in a DNFB-sensitized mouse. CAE staining on 3-μm sections of GMA-embedded colon tissue.](image)

![FIGURE 5. TNF-α production in colonic patches obtained from vehicle- or DNFB-sensitized mice 72 h after DNS challenge. Colonic patches were stimulated ex vivo with SCF or anti-CD3 for 48 h. Colonic patches from vehicle-sensitized mice, colonic patches from DNFB-sensitized mice after DNS challenge. Results are expressed as mean TNF-α (picograms)/total protein (milligrams) ± SEM; n = 4–6 mice/group (one-way ANOVA followed by a Bonferroni multiple comparison test).](image)

![FIGURE 6. Stool consistency in vehicle- and DNFB-sensitized mast cell-deficient mice, control littermates and mast cell-reconstituted mast cell-deficient mice 72 h after DNS challenge. Score 0, normal well formed pellets; score 1, loose stool/easy to smear; score 2, diarrhea/watery stool; +/-, control littermates; W/W’, mast cell-deficient mice; BMMC→W/W’, BMMC-reconstituted W/W’ mice. Results are expressed as cumulative percentage of total scored stool (score 0 not shown); *p < 0.05 compared with vehicle-sensitized mice within the same strain, n = 12 mice/group; #, p < 0.1 compared with DNFB-sensitized +/- and W/W’ mice, n = 12 mice/group; $, p < 0.05 compared with vehicle-sensitized +/- mice (Kruskal-Wallis followed by a Dunn’s test).](image)
shown in Fig. 8B, minor levels of mouse mast cell protease-1 in the supernatant of colon homogenates could be detected in either vehicle- or DNFB-sensitized W/Wv and BMMC→W/Wv mice. In +/+ mice, however, a significant difference could be detected between vehicle- and DNFB-sensitized mice. The results observed in the supernatant of colon homogenates of either vehicle- or DNFB-sensitized BMMC→W/Wv mice correspond with the finding of no significant differences in mouse mast cell protease-1 levels in plasma in these animals.

Immunohistochemistry demonstrates a significant increase in the number of CAE-positive mast cells in +/+ DNFB-sensitized animals 72 h after challenge. Moreover, the number of CAE-positive mast cells is enhanced in BMMC→W/Wv DNFB-sensitized mice 72 h after DNS challenge (Table IV). However, this increase is not significant compared with vehicle-sensitized BMMC→W/Wv mice due to a large variability. An immunohistochemical staining for mouse mast cell protease-1 was performed for in-depth investigation of mast cells after reconstitution. This staining specifically stains mucosal mast cells. As can be seen in Table IV, this immunohistochemical staining shows a similar profile as the mouse mast cell protease-1 plasma levels of +/+, W/Wv, and BMMC→W/Wv mice. A statistically significant difference in mouse mast cell protease-1-positive mast cells could only be detected in DNFB-sensitized +/+ mice compared with vehicle-sensitized mice 72 h after challenge. After mast cell reconstitution, similar numbers of mouse mast cell protease-1-positive cells were found in both vehicle- and DNFB-sensitized BMMC→W/Wv mice compared with vehicle-sensitized +/+ mice. However, no differences between the two treatment groups were present.

Even though the mouse mast cell protease-1 and the CAE staining show the same profile in mast cell numbers, the mouse mast cell protease-1 staining was capable of detecting more mast cells in the mouse colon. This discrepancy in mast cell numbers between the mouse mast cell protease-1 and CAE staining can be explained by differences in staining methods. Lower numbers of mast cells in the CAE staining could be a result of the loss of enzymatic activity as a result of the process of embedding and staining. Moreover, it could be possible that the mast cell contains less CAE than mouse mast cell protease-1 resulting in a less intense staining.

**Discussion**

Several lines of evidence strongly suggest that mast cells are involved in the pathogenesis of IBD (10). The presence of increased numbers of mast cells and its mediators can be found in the mucosa of patients with IBD (13, 27). Because the gastrointestinal tract is exposed to exogenous agents entering the body by intestinal bacteria and via food intake, a constant activation of the immune system occurs. Mast cells are cells involved in recognition of the parasitic nematodes, food allergens, enterotoxigenic bacteria, and invading pathogens and can therefore be found in a constitutively activated state in the gastrointestinal tract (28). Breaking
In the present experiments, we describe a novel murine model for IBD and examine the putative role of mast cells in this model. Cutaneous sensitization with the low m.w. hapten DNFB, followed by an intrarectal challenge with DNS resulted in hypersensitivity responses in the colon. This DTH-like reaction is classically known as a non-IgE-mediated reaction. Induction of DTH reactions with DNFB has been shown to lead to successful models for contact dermatitis, nonatopic asthma, and small intestinal inflammations (18, 29, 30). An acute response occurs within 6 h after challenge and is a result of direct mast cell activation and degranulation. The cell-mediated response takes place 24–72 h after challenge. Increased plasma levels of mouse mast cell protease-1 of DNFB-sensitized compared with vehicle-sensitized mice were present at 30 min, 24 h, and 72 h after the challenge, indicating that mast cell activation occurs in this model.

The lack of acute effect (<6 h) on stool consistency could be a result of a masking effect of ethanol, used here to break the mucosal barrier of epithelial cells. Ethanol is known to cause local irritation. Although often used in the TNBS-colitis model as vehicle, it was recently shown that 50% ethanol can induce colitis by itself and prevent oral tolerance (31). In our model, 10% ethanol is used as a mucosal barrier breaker. However, histological examination and stool consistency prove that an irritant effect of ethanol is still present at 24 h (previous observations by authors; data not shown). The irritant effect of ethanol seems to have faded 72 h after challenge and DNFB-sensitized mice suffered from diarrhea. Therefore, this time point was chosen to study colonic hypersensitivity reactions.

A prominent role in the pathogenesis of IBD can be attributed to the proinflammatory cytokine TNF-α because increased levels can be found in inflamed tissue of IBD patients (13, 32). Significantly increased TNF-α levels could be found in colon of DNFB-sensitized mice 72 h after DNS challenge. SCF-induced TNF-α production in vitro by colonic patches obtained from DNFB-sensitized mice 72 h after DNS challenge, together with the observation of increased mast cell infiltration in colonic patches, strongly suggests that activated mast cells located in and around colonic patches may be an important source of TNF-α. Furthermore, hypertrophy of colonic patches was previously established by Dohi et al. (23). They showed that after induction of TNBS-colitis, those lymphoid follicles were thicker and contained an increased number of T and B lymphocytes. This is in correspondence with the hypertrophy of colonic patches after DNFB sensitization and DNS challenge observed in our study (Table I).

### Table IV. Number of CAE-positive and mouse mast cell protease-1-positive mast cells in mast cell-deficient W/W′ mice, mast cell-reconstituted W/W′ mice, and control littermates after DNFB or vehicle sensitization 72 h after DNS challenge

<table>
<thead>
<tr>
<th>CAE Staining</th>
<th>DNFB Sensitization</th>
<th>Mast cells (number/colon)</th>
<th>Mouse Mast Cell Protease 1 Staining</th>
<th>n</th>
<th>p</th>
<th>n</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n, p</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+</td>
<td>−</td>
<td>9 (5–16)</td>
<td>4</td>
<td></td>
<td>&lt;0.05</td>
<td>50 (44–71)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>19 (17–24)</td>
<td>3</td>
<td>&lt;0.05</td>
<td>156 (120–306)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>W/W′</td>
<td>−</td>
<td>4 (3–5)</td>
<td>4</td>
<td></td>
<td>&lt;0.05</td>
<td>21 (15–27)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5 (3–7)</td>
<td>4</td>
<td>NS</td>
<td>27 (23–37)</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>BMMC→W/W′</td>
<td>−</td>
<td>6 (2–15)</td>
<td>4</td>
<td>NS</td>
<td>56 (24–97)</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>6 (4–43)</td>
<td>4</td>
<td>NS</td>
<td>52 (21–145)</td>
<td>5</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Mast cells are counted after CAE or mouse mast cell protease 1 staining. Per mouse, three different longitudinal sections were counted and averaged. Results are expressed as median average number of CAE or mouse mast cell protease 1-positive cells per colon section (minimum-maximum); n, number of mice; significant differences between the two treatments within one strain are indicated (Wilcoxon rank sum test).

b +/+. Control littermates; W/W′, mast cell-deficient mice; and BMMC→W/W′, BMMC-reconstituted W/W′ mice.
Besides mast cells, c-kit is also expressed by interstitial cells of Cajal (ICC) in the gastrointestinal tract (33). These cells can be addressed as the pacemaker cells of the gut and are responsible for the peristaltic movement. Until today, no reports have been published indicating cytokine production by ICC. Therefore, these cells are not likely to be a source for TNF-α. Mast cells, however, are not the only immune cells responsible for the release of TNF-α and this proinflammatory cytokine is also produced by T lymphocytes (34). To investigate the origin of TNF-α further, we also in vitro-stimulated colonic patches from DNFB-sensitized mice 72 h after DNS challenge with the general T cell activator anti-CD3 and measured TNF-α in supernatant. And although T cells are more abundantly present than mast cells in the colonic patch, the observed significantly higher TNF-α levels in colonic patch after in vitro SCF stimulation compared with the anti-CD3-stimulated colonic patch suggests that TNF-α produced by mast cells is quantitatively more important than T cell-derived TNF-α in this model. Furthermore, the observation of mast cell infiltration and activation in and around colonic patches by immunohistochemistry suggests communication and/or interaction between mast cells and lymphocytes, both cell types involved in hypersensitivity responses. The interaction between mast cells and T cells has been shown to be bidirectional accomplishing regulatory and modulatory roles (35). Activated T cells are capable of inducing mast cell activation and degranulation (36) and of inducing cytokine and chemokine production (37), both as a result of physical cell-to-cell contact. This suggests that TNF-α measured in supernatant of anti-CD3-stimulated colonic patches could also be derived from mast cells, which are indirectly activated by T lymphocytes.

More definite evidence for the functional role of mast cells in this murine model for colonic hypersensitivity was obtained by studies in mast cell-deficient W/Wv mice. The characteristic features for colonic hypersensitivity failed to establish in DNFB-sensitized mast cell-deficient W/Wv mice after DNS challenge. Reconstitution with in vitro-cultured bone marrow-derived mast cells in W/Wv mice resulted in the appearance of a number of features like diarrhea, hypertrophy of colonic patches, mast cell infiltration, and damage of colon tissue, similar to results obtained in control +/+ mice after DNFB sensitization and DNS challenge. However, even though a rise in mouse mast cell protease-1 levels was present in BMMC→W/Wv, indicating that the reconstitution was successful, there was no significant difference between DNFB- and vehicle-sensitized mice. Furthermore, only minor mouse mast cell protease-1 levels could be detected in supernatant of colon homogenates in both treatment groups of BMMC→W/Wv mice as well as W/Wv mice. A plausible explanation could be that after reconstitution a different type of mast cell appears in the intestinal mucosa and that mouse mast cell protease-1 is not a correct marker for mast cell activation in reconstituted animals. In addition, histological examination of mast cells present in the mucosa showed difference in morphology, size, and granular density between +/+ and BMMC-reconstituted W/Wv mice (our unpublished observation). Galli (38) stressed that appropriate studies should be done to assess the number, phenotype, and distribution of mast cells in BMMC recipients. Therefore, an additional mouse mast cell protease-1 immunohistochemical staining was performed. This staining showed an equivalent profile of mast cell numbers as the mouse mast cell protease-1 levels in plasma. This supports the observation that mouse mast cell protease-1 might not be the correct marker for mast cell activation and infiltration in reconstituted mast cell-deficient mice. It is beyond the scope of this study to further investigate and identify the content of reconstituted mast cells in vivo.

Although significant cellular infiltration was seen in the colon of both DNFB-sensitized +/+ mice as BMMC-reconstituted W/Wv mice (Fig. 7, A and C), a small lymphoid structure was also observed in the mucosa of W/Wv (Fig. 7B). This can be addressed as a cryptopatch, which appears in the mucosa throughout the whole gastrointestinal tract (39, 40). These cryptopatches do not exhibit organized lymphoid tissue like Peyer’s patches and colonic patches, but contain progenitors cells for intraepithelial lymphocytes (41). Cryptopatches are known to be the extrathymic source for the almost the whole intraepithelial lymphocyte population in the murine intestine (39). It could be suggested that these structures develop into colonic patches or serve as a source for the inflammatory cell infiltration and the hypertrophy of colonic patches.

Stool consistency of vehicle-sensitized mast cell-deficient W/Wv mice is deteriorated compared with vehicle-sensitized +/+ animals. This is probably due to the fact that W/Wv mice also have a mutation in ICC (42). It is evident that alterations in the amount or function of these pacemaker cells could lead to differences in stool consistency. In BMMC→W/Wv mice, the absence of ICC could contribute to the observation that the stool consistency of DNFB-sensitized animals is even more deteriorated compared with DNFB-sensitized +/+ controls. In BALB/c mice, the presence of diarrhea and the number of colonic patches is of higher prevalence than in +/+ mice. These differences can be attributed to strain differences.

Conflicting studies exist about the role of mast cells in low m.w. molecule-induced models for experimental colitis. Fukumoto et al. (43) demonstrated that mast cells are not essential in the development of the TNBS-induced rat model. Rectal installment of TNBS in 50% ethanol in mast cell-deficient W/Wv rats led to the same inflammatory conditions of the colon as seen in control W/Wv littermates. Similar studies of TNBS in 50% ethanol-induced colitis in mast-cell-deficient W/Wv mice showed the same results (16). Contradictorily, Xu et al. (17) describes an important role for mast cells in intestinal inflammation and fibrosis in TNBS in 50% ethanol-induced colitis in mice (44). Furthermore, ketotifen, an antihistaminic drug with mast cell-stabilizing properties, can attenuate the severity of TNBS-colitis (45). An explanation for these conflicting results can be found in the use of 50% ethanol, used as vehicle in the TNBS-colitis model to break the mucosal barrier. As mentioned before, 50% ethanol is capable of inducing an inflammation by itself (31). This damage-induced inflammation by ethanol is mast cell-independent and most likely masks the hapten-specific response induced by TNBS. This makes the TNBS model an unreliable model to study the role of mast cells in IBD.

The mechanism by which mast cells are activated in our model is currently under investigation. It has been suggested that upon sensitization with a hapten or other causative agents, B lymphocytes are stimulated to produce an Ag-specific lymphocyte factor. This factor has recently been demonstrated to be Ig-L chain (IgLC) (29). Free IgLC can bind to mast cells and a subsequent second contact with the corresponding Ag (challenge) can lead to mast cell activation (29, 46, 47). This recent finding sheds a new light on the involvement of mast cells in nonatopic diseases, like IBD. Purified Ag-specific IgLC are capable of passively immunizing naive mice. Intranasal challenge resulted in acute bronchoconstriction associated with plasma leakage and mast activation (47), whereas epicutaneous challenge on the ears lead to a profound ear swelling typical for a dermal hypersensitivity reaction (29). These earlier observations in both lung and skin suggest similar mechanisms in this newly described colonic hypersensitivity model. Current investigations have started to prove this hypothesis and to establish a role for IgLC in this model.
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In conclusion, although the role for mast cells is controversial in animal models for IBD, this is, to our knowledge, the first study to show an important consistent role for mast cells in colonic hypersensitivity. Our findings are confirmatory for the hypothesis of the essential function for mast cells in the cellular cascade leading to an Ag-induced hypersensitive response as postulated earlier (18, 48). The presented chemically induced immunological model for IBD can be used to develop potential therapies targeting the mast cell as an important source for the IBD-cytokine TNF-α.

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Disclosures

The authors have no financial conflict of interest.

References


