Activated Mast Cells in Proximity to Colonic Nerves Correlate With Abdominal Pain in Irritable Bowel Syndrome

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Background & Aims: **The mechanisms underlying abdominal pain perception in irritable bowel syndrome (IBS) are poorly understood. Intestinal mast cell infiltration may perturb nerve function leading to symptom perception. We assessed colonic mast cell infiltration, mediator release, and spatial interactions with mucosal innervation and their correlation with abdominal pain in IBS patients.** ***Methods:*** **IBS patients were diagnosed according to Rome II criteria and abdominal pain quantified according to a validated questionnaire. Colonic mucosal mast cells were identified immunohistochemically and quantified with a computer-assisted counting method. Mast cell tryptase and histamine release were analyzed immunoenzymatically. Intestinal nerve to mast cell distance was assessed with electron microscopy.** ***Results:*** **Thirty-four out of 44 IBS patients (77%) showed an increased area of mucosa occupied by mast cells as compared with controls (9.2% ± 2.5% vs. 3.3 ± 0.8%, respectively; $P < 0.001$). There was a 150% increase in the number of degranulating mast cells (4.76 ± 3.18/field vs. 2.42 ± 2.26/field, respectively; $P = 0.026$). Mucosal content of tryptase was increased in IBS and mast cells spontaneously released more tryptase (3.22 ± 3.48 pmol/min/mg vs. 0.87 ± 0.65 pmol/min/mg, respectively; $P = 0.015$) and histamine (339.7 ± 59.0 ng/g vs. 169.3 ± 130.6 ng/g, respectively; $P = 0.015$). Mast cells located within 5 μm of nerve fibers were 7.14 ± 3.87/field vs. 2.27 ± 1.63/field in IBS vs. controls ($P < 0.001$). Only mast cells in close proximity to nerves were significantly correlated with severity and frequency of abdominal pain/discomfort ($P < 0.001$ and $P = 0.003$, respectively).** ***Conclusions:*** **Colonic mast cell infiltration and mediator release in proximity to mucosal innervation may contribute to abdominal pain perception in IBS patients.**

Irritable bowel syndrome (IBS) is one of the most common gastrointestinal disorders seen in primary care and specialist practice characterized by abdominal discomfort or pain and disturbed defecation that cannot be explained by structural or biochemical abnormalities.[1]

Abdominal pain/discomfort is the hallmark symptom of IBS,[1] which correlates with the overall severity of the disease.[2] The pathogenesis of IBS symptoms is loosely understood. Although gut motor abnormalities may account for alterations in gut transit, they are poorly correlated with patients' abdominal pain/discomfort.[3] There is general agreement that increased sensitivity to stimuli arising from the gut wall (i.e., visceral hypersensitivity) contributes to abdominal pain/discomfort in at least a subset of IBS patients.[4] Several mechanisms are thought to be involved in the visceral hypersensitivity of IBS patients,[5] including (1) modulatory effect of the central nervous system in response to information conveyed from the gut, (2) hyperexcitability of dorsal horn neurons in the central limb of the visceral afferent pathway, and (3) sensitization of sensory neural endings at the end-organ level.

The causes underlying the end-organ hypersensitivity in IBS are only partially understood. It has been hypothesized that a low-grade inflammatory response at the level of the gut wall could be involved.[5,6] Consistent with this hypothesis is evidence that at least certain subsets of IBS patients have an increased number of inflammatory cells in the colonic[7] and ileal[8] mucosa as well as in *muscularis externa* of jejunum.[9] Also, IBS may develop after a bout of acute infectious gastroenteritis[6,10,11] and during periods of remission from inflammatory bowel disease.[12] However, these previous studies do

Abbreviations used in this paper: BABIM, bis(5-amidino-2-benzimidazolyl)methane; BSA, bovine serum albumin; IBS, irritable bowel syndrome; PAR, proteinase-activated receptors; SBTI, soybean trypsin inhibitor.

0016-5085/04/$30.00
doi:10.1053/j.gastro.2003.11.055

not provide insights into the possible mechanisms involved in the altered sensory-motor function of IBS.

A number of reasons raise the hypothesis that mast cells could be involved in the disturbed sensory-motor function of IBS. First, an increased number of these cells has been detected in the colonic[7,13] and ileal[8] mucosa of IBS patients. Second, in the human intestine, mast cells lie in close proximity to mucosal innervation.[14,15] Third, animal studies demonstrated that, upon activation, mast cells release mediators that cause increased excitability of enteric[16,17] and primary afferent neurons,[18] leading to visceral hypersensitivity[19] and abnormal gut motor function.[20]

Tryptase and histamine are two major inflammatory mediators released during mast cell degranulation.[21] Tryptase, like other proteases, signals to cells through proteinase activated receptors (PARs).[22] Proteases cleave within the extracellular N-terminal tails of PARs to expose tethered ligand domains that bind and activate cleaved receptors.[22] Of the 4 cloned PARs, tryptase selectively activates PAR-2.[17,23] Mast cell tryptase induces activation of PAR-2 located on enteric nerves and visceral afferents causing long-lasting neuronal hyperexcitability.[16,17,24] Histamine can also activate visceral afferents[18] and enteric neurons[25] via interaction with H_1 or H_2 receptors.[26,27] Thus, both tryptase and histamine are candidate mediators for disturbed gut sensory-motor function.

The aims of the present study were to assess the impact of colonic mast cell infiltration and activation, as well as mast cell-to-mucosal innervation relationship on the severity and frequency of abdominal pain/discomfort of IBS patients.

Materials and Methods

Patients

IBS patients were all seen in the Department of Internal Medicine and Gastroenterology of the University of Bologna and met the Rome II criteria.[1] Healthy controls were recruited by public advertisement and included in the study after thorough exclusion of gastrointestinal complaints. None of the study participants were taking nonsteroidal anti-inflammatory drugs or other anti-inflammatory drugs (including mast cell stabilizers, immunosuppressants, and steroids); had undergone major abdominal surgery; or had any organic syndrome, asthma, celiac disease (excluded by detection of anti-transglutaminase and anti-endomysial antibodies), allergic diseases (including exclusion of a history of food allergies and, by presence of food specific IgE antibodies), intestinal bacterial or mycotic contamination, metabolic or psychiatric disease as diagnosed by history taking, consultations, and appropriate laboratory tests. They all gave written informed consent, and the study was approved by the local Ethical Committee and conducted in accordance with the Declaration of Helsinki.

All participants underwent left colonoscopy after cleansing of distal colon with two 500-mL water enemas performed the evening before and the morning of the procedure. In all cases, 4 mucosal biopsy specimens were obtained from the proximal descending colon. Two biopsy specimens were used for routine H&E histology and immunohistochemistry and 2 for electron microscopy. In 36 unselected cases, 4 additional biopsy specimens could be obtained for histamine and tryptase release assays and tryptase Western blotting.

Symptom Questionnaire

Each patient completed a modified version of the Bowel Disease Questionnaire (BDQ) to evaluate symptoms.[28] An Italian version of the questionnaire has been previously used in IBS studies in our country.[29]

Patients were asked to score frequency and severity of their symptoms over the last 2 weeks before interview. In fact, recollection is poor beyond this limit, and shorter periods might not appropriately reflect the usual clinical picture and this time frame has been previously shown to be a reliable predictor of average pain intensity.[30] The severity of abdominal pain/discomfort was graded 0–4 according to its influence on patients' usual activities: 0, absent; 1, mild (not influencing usual activities); 2, relevant (diverting from, but not urging modification of, usual activities); 3, severe (influencing usual activities markedly enough to urge modifications); 4, extremely severe (precluding daily activities). The frequency of abdominal pain/discomfort was graded 0–4 according to the following scale: 0, absent; 1, up to 1 day/week; 2, 2 or 3 days/week; 3, 4–6 days/week; 4, daily. Furthermore, the following symptoms were assessed as present/absent: diarrhea, constipation, bloating/distention, flatulence, relief of abdominal pain/discomfort by defecation or passage of air per rectum, mucus in stool, onset of symptoms associated with a change in frequency (or form) of stool, hard or lumpy stool, loose or watery stool, straining during a bowel movement, urgency, and feeling of incomplete bowel movement.

Histology and Immunohistochemistry

Biopsy specimens were fixed in buffered 10% formalin and processed for either H&E histology or immunohistochemistry. For the latter, paraffin-embedded specimens were cut with a microtome at 3–4 μm, mounted on to gelatin-coated slides, and processed either for immunohistochemistry or indirect immunofluorescence using widely applied methods. Briefly, for single-labeling immunohistochemistry, following antigen unmasking, randomly selected sections were incubated with mouse monoclonal antibodies directed against tryptase (mast cell marker; diluted at 1:2000; Dakopatts Glostrup, Denmark) overnight at 4°C. Following thorough washing with PBS, slides were reincubated for 2 hours at room temperature with secondary biotinylated anti-mouse-rabbit antibodies (1:200; Dakopatts Glostrup, Denmark) followed by streptavidin-horseradish peroxidase conjugate. For immuno-

fluorescence, slides were processed as described above with the only difference that sections were reincubated for 2 hours at room temperature with rhodamine or fluoresceine-conjugated secondary antibodies (1:100; Dakopatts Glostrup).

Mast Cell Counts

All histologic sections were evaluated by an expert pathologist (D.S.), who was unaware of the diagnosis, for exclusion of overt mucosal inflammation or microscopic colitis. Quantification of inflammatory cells was performed on sections immunostained for mast cells with a Leitz Dialux microscope (equipped with a Ploem epi-illumination system with "N2" filter cube to detect rhodamine fluorescence) in blind fashion using a computer-assisted analysis system (Cytometrica@software; C&V, Bologna, Italy). Measurements were performed using a modification of a previously published method.[31] Briefly, microscopic fields were digitized and randomly sampled with the aid of a grid (0.5 mm^2) located below the slide. A stereological grid containing cross-shaped points was overlaid on the digitized sampled fields by the computer software and used to determine the area occupied by mast cells over that occupied by lamina propria (i.e., number of points hitting the cells divided by the total number of cross-shaped points in the lamina propria).

Electron Microscopy

Biopsy specimens were fixed in cacodylate-buffered 2.5% glutaraldehyde for 4 to 6 hours at 4°C and postfixed in 1% osmium tetroxide for 1 hour at 4°C. After dehydration and embedding in araldite, semithin sections were stained with toluidine blue and examined under a light microscope. A pathologist (G.P.) performed a blind analysis of all sections. Representative portions of the intestinal mucosa (i.e., absence of artifacts) were selected for ultrathin sectioning. Thin sections were collected on 200 mesh grids, stained with uranyl acetate and lead citrate, and observed with a Philips 410 T transmission electron microscope. After exclusion of fields exclusively comprising epithelial structures, 10 randomly selected fields of the intestinal mucosa measuring 90 μm^2 were examined for each sample. In each field (×9100 magnification), the following parameters were recorded: total number of mast cells, number of mast cells with degranulation features (i.e., labyrinthic membranous arrays or clearing of individual granules), and number of mast cells located within 5 μm of nerve fibers.

Tryptase Western Blotting

Mucosal biopsy specimens were frozen in liquid nitrogen and then rinsed in PBS. Biopsy specimens were then homogenized in Camiolo buffer (75 mmol/L potassium acetate, 300 mmol/L NaCl, 100 mmol/L L-arginine, 10 mmol/L EDTA, 0.25% Triton X-100, pH 7.4) for 1 minute at 4°C. The homogenized sample was then centrifuged at 3000 rpm for 15 minutes at 4°C. The supernatant was then transferred to a fresh tube and assayed for protein content. Protein concentrations were determined using the bicinchoninic acid assay,[32] with bovine serum albumin (BSA) as standard. The method was adapted for use in 96-well microtiter plates. Equal amounts of protein (15 μg) from each patient were separated using a 7%–17% PAGE. After electrophoresis, proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA) and then blocked (1X PBS, 2% BSA, 5% milk powder, 0.1% Tween 20) for 1 hour at room temperature. The membrane was then incubated overnight at 4°C with an anti-human mast cell tryptase antibody (1:5000; Chemicon, Temecula, CA). After washing (1X PBS, 0.1% Tween 20), the membrane was incubated for 1 hour at room temperature with a goat anti-mouse-horseradish peroxidase antibody (1:10,000; Jackson Laboratories, West Grove, PA). Following a second wash, immunoreactive proteins were visualized by chemiluminescence (ECL kit, Amersham-Pharmacia, Piscataway, NJ). Results are representative of 3 separate blots.

Tryptase and Histamine Release

Spontaneous release of tryptase and histamine from mucosal biopsy specimens was evaluated using a modification of a previously described method.[33] Briefly, upon removal, mucosal biopsy specimens were rapidly immersed in hard plastic tubes containing 2 mL of Hanks' solution (37°C) and continuously oxygenated (95% O_2/5% CO_2). After 25-minute incubation, 200 μL of the bathing solution was removed. Samples to be processed for histamine assay only were immediately heated at 95°C to avoid rapid denaturation of histamine by degrading enzymes (histaminases). All samples were centrifuged at 200*g* for 10 minutes, and 150 μL of supernatant were aliquoted and stored at −70°C until the assay. At the end of the release experiment, biopsy specimens were blotted and weighed. For tryptase activity triplicate aliquots (10 μL) of the supernatants were added to 200 μL of buffer (50 mmol/L Tris/HCl, pH 7.6, 120 mmol/L NaCl, 20 μg/mL heparin) containing 0.5 mmol/L substrate (tosyl-Glycine-Proline-Arginine-pNitroanilide) and incubated at room temperature for 17 hours (± inhibitors as indicated). Cleavage of the substrate was measured using a microtiter plate reader (absorbance 405 nm) and normalized to the weight of the biopsy specimens used in each case. Histamine was detected from triplicate aliquots (50 μL) of the supernatants using a highly selective enzyme-linked immunoassay (Immunotech, Marseille, France) according to the manufacturer's directions. Histamine was measured using a microtiter plate reader (absorbance 405 nm) and normalized to the weight of the biopsy specimens.

Data Expression and Statistical Analysis

Unless otherwise stated, data are reported as mean values ± standard deviation (SD). Data relative to electron microscopy studies are presented as mean values ± SD relative to 10 electron microscopic fields. Data were analyzed by means of the Mann–Whitney *U* and the Yates' corrected χ^2 tests. Correlations were analyzed using the Spearman rank correlation test. Analyses were done by running the SPSS/PC+ (SPSS, Inc, Chicago, IL) statistical package on a personal computer. Two-tailed *P* values less than 0.05 were considered statistically significant.

Table 1. Mucosal Mast Cell Counts

	All	Female	Male	*P* value (male vs. female)	Diarrhea	Constipation	*P* value (vs. diarrhea)
Controls (n = 22)	3.3 ± 0.8	3.4 ± 0.5	3.1 ± 0.9	NS	—	—	NA
IBS (n = 44)	9.2 ± 2.5[a]	10.0 ± 2.3[b]	7.0 ± 1.6[c]	<0.001	8.6 ± 2.7	9.7 ± 2.1	NS

NS, not significant, $P > 0.05$; NA, not applicable.
[a]Significant difference vs. controls ($P < 0.001$).
[b]Significant difference vs. controls ($P < 0.001$).
[c]Significant difference vs. controls ($P < 0.001$).

Results

Patients

Forty-four consecutive patients with IBS (aged 22–75 years; mean, 40.1 years; 31 females, 13 males) as well as 22 healthy controls (aged 20–71 years; mean, 32.5 years; 12 females, 10 males) participated in the study. All patients complained of abdominal pain/discomfort (severity score: 1.78 ± 1.15; frequency score: 2.20 ± 1.49; mean ± SD); 50% had diarrhea and 50% constipation; furthermore, the most frequently associated symptom was bloating (97.8%).

Mast Cell Counts

Thirty-four (77.3%) of the 44 IBS patients had an increased mucosal cell area occupied by mast cells (i.e., greater than the mean ± 2SD of controls: 4.8%). As shown in Table 1 and Figure 1, the mean area of mucosa occupied by tryptase positive mast cells was 181% greater in IBS patients with respect to healthy controls. The mucosal area occupied by mast cells was not different in diarrhea and constipation predominant subgroups ($P = 0.096$). A significant increase in the mean area occupied by mast cells was identified in both male and female IBS patients, compared with the respective control groups (Table 1). However, the mean area occupied by mast cells in female IBS patients was 43% greater than that observed in male IBS patients (Table 1).

Mast Cell Degranulation

To assess the state of activation of mast cells, we quantified the number of mast cells showing ultrastructural features of degranulation, including labyrinthic membranous arrays and clearing of individual granules. Degranulating mast cells could be observed in samples from both IBS patients and controls. However, quantitative analysis of degranulating mast cells showed a 150% significant increase in IBS patients with respect to controls (4.76 ± 3.18 cells/10 fields vs. 2.42 ± 2.26 cells/10 fields, respectively, $P = 0.026$).

Tryptase Western Blots

We quantified the total amount of tryptase, the major protease contained in mast cell secretory granules. Using an antibody directed against human mast cell tryptase, we found a 35-kilodalton broad band on Western blots (Figure 2). All controls had the same molecular weight band, which is the expected size for tryptase (Figure 2). Only 1 out of 6 controls had an elevated level of tryptase (Figure 2). In sharp contrast, 5 out of 6 biopsy

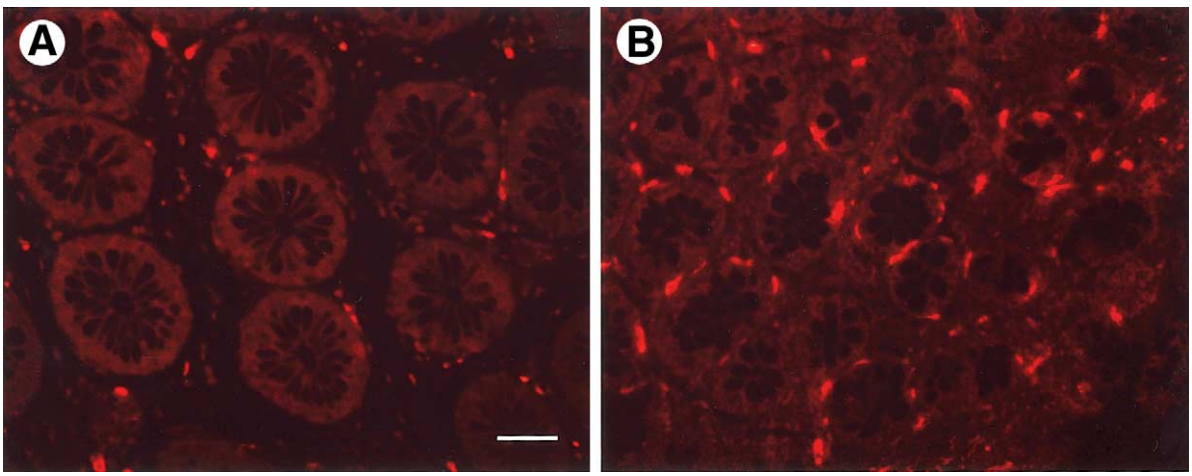

Figure 1. Representative photomicrographs showing tryptase positive mast cells in the colonic mucosa of a healthy control (*A*) and an IBS patient (*B*). Note the higher number of positive mast cells in the IBS patient as compared with the control. (*bar* = 25 μm.)

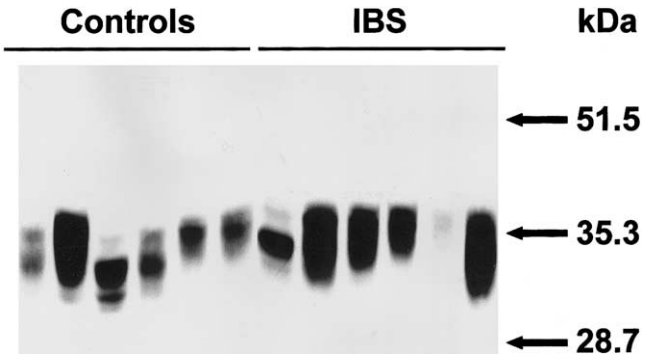

Figure 2. Representative immunoelectrophoretic analysis of tryptase in human colonic mucosal biopsies. Protein was isolated from human mucosal biopsies, and 15 μg was subjected to SDS-PAGE followed by incubation with an anti-human mast cell tryptase antibody. Note the elevated level of tryptase in 5 out of 6 biopsy specimens obtained from IBS patients as compared with the low level of tryptase obtained in the majority (5 out of 6) of controls.

specimens obtained from patients with IBS contained much higher levels of immunoreactive tryptase (Figure 2). Because of the broad range of molecular weight, we did not quantify the increase in intensity.

Tryptase Release

The level of tryptase activity that was spontaneously released from mucosal biopsy specimens was quantified in the cleared supernatants obtained by a 25-minute incubation of biopsy specimens in oxygenated buffer using an enzymatic assay. As shown in Figure 3, the level of tryptase activity in IBS patients was 3.7 times higher than that found in healthy controls ($P = 0.015$). Both in control and IBS samples, this activity was significantly inhibited by the addition of the tryptase inhibitor bis(5-amidino-2-benzimidazolyl)methane (BABIM; 100 μmol/L) ($P = 0.013$ and $P = 0.003$, respectively), but not by the trypsin inhibitor soybean trypsin inhibitor (SBTI; 50 μg/mL) ($P = 0.794$ and $P = 0.594$, respectively), thus showing that the activity measured in the assay was specific for tryptase.

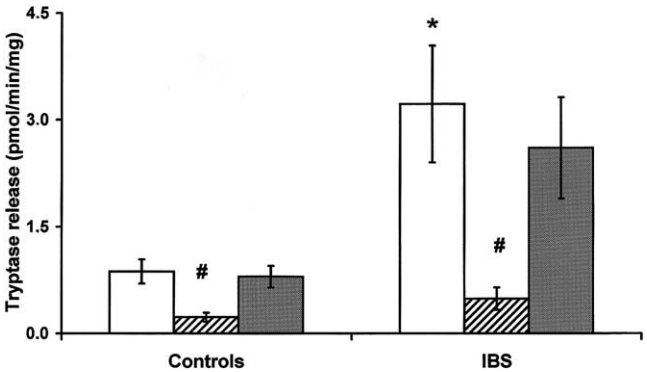

Figure 3. Tryptase release from colonic mucosal biopsy specimens in healthy controls and IBS patients. Tryptase activity was measured in the cleared supernatants obtained by a 25-minute incubation of biopsy specimens in oxygenated buffer using an enzymatic assay. Tryptase activity was measured in the absence (*open bars*) or presence of the tryptase inhibitor BABIM (100 μmol/L) (*hatched bars*) or the trypsin inhibitor SBTI (*shaded bars*) (50 μg/mL). Note the significant increased release of tryptase from IBS samples as compared with controls and the significant inhibition of tryptase activity in the presence of BABIM but not SBTI. *Significant difference from controls ($P = 0.015$). #Significant difference from samples not exposed to BABIM. Values represent means ± standard error.

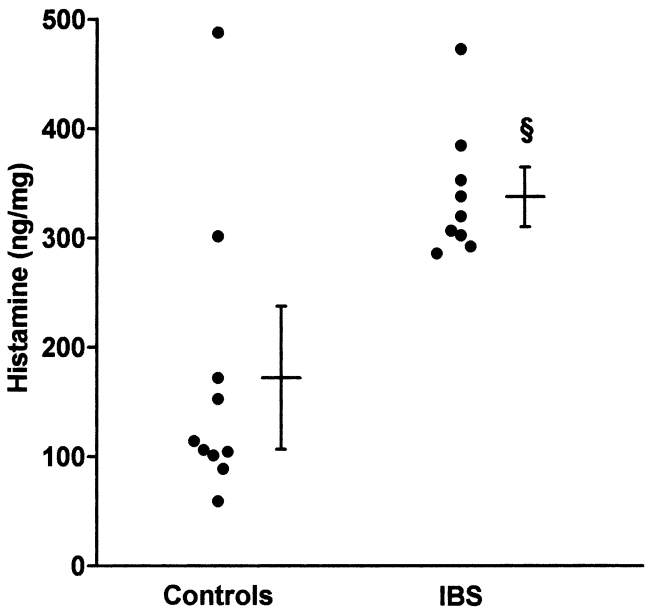

Figure 4. Spontaneous histamine release from colonic mucosal biopsies in healthy controls and IBS patients. Histamine activity was measured in the cleared supernatants obtained by a 25-minute incubation of biopsy specimens in oxygenated buffer using an enzymatic assay. §Significant difference from controls ($P = 0.015$).

Histamine Release

The spontaneous release of histamine from mucosal biopsy specimens of IBS patients and controls was measured in the cleared supernatants obtained by a 25-minute incubation of biopsy specimens in oxygenated buffer using an enzymatic assay. As shown in Figure 4, histamine activity in IBS patients was significantly increased by 101% over the activity found in controls ($P = 0.015$).

Mast Cell—Nerve Interactions

In the colonic mucosa of both healthy controls and IBS patients, mast cells were frequently found in close proximity to intestinal nerves (Figure 5*A–D*) and membrane-membrane contacts were occasionally observed in both groups (Figure 5*B*). Mast cells undergoing active degranulation in proximity to nerve trunks were also occasionally identified (Figure 5*B–D*). Because inflammatory mediators (i.e., histamine and tryptase) re-

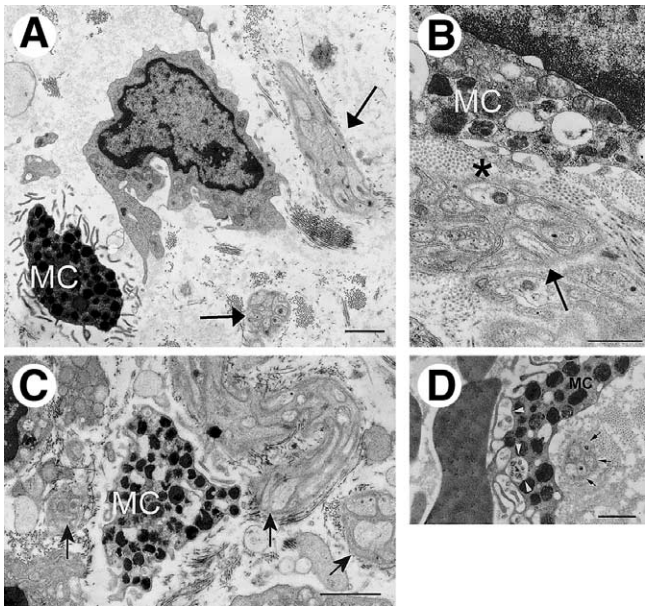

Figure 5. Electron micrographs showing association between nerve fibers (*arrows*) and mast cells (MC) in the colonic mucosa of healthy controls (*A*) and IBS patients (*B–D*). (*A*) Nerve fibers (*arrows*) and a resting mast cell (MC) showing its characteristic granules in a healthy control. (*B*) Electron micrograph obtained from an IBS patient showing membrane-membrane contacts (*asterisk*) between a degranulating mast cell (MC) and a nerve fiber (*arrow*). (*C*) Electron micrograph obtained from an IBS patient showing multiple nerve fibers (*arrows*) located <5 μm from a degranulating mast cell (MC). (*D*) The typical appearance of a degranulating (*arrowheads*) mast cell (MC) in close vicinity (<5 μm) to a nerve fiber. (*bar* = 5 μm in A,C,D; 2 μm in B.)

leased by activated mast cells are more likely to affect neural function if they are in close proximity to the nerves, we investigated the spatial relationships occurring between mast cells and nerves. The number of mast cells per 10 fields <5 μm from nerves was 223% greater in IBS patients compared with healthy controls (7.14 ± 3.87 cells/10 fields vs. 2.27 ± 1.63 cells/10 fields, respectively, $P < 0.001$). Interestingly, a positive significant correlation was found between nerve-to-mast cell proximity and rate of degranulation of mast cells ($r = 0.72$, $P = 0.002$) (Figure 6).

Mast Cell and Symptom Correlation

The correlation between mast cell parameters and severity and frequency of abdominal pain/discomfort is reported in Table 2. A significant correlation was found between vicinity of mast cells to nerves and both severity and frequency of abdominal pain/discomfort ($r = 0.75$, $P = 0.001$ and $r = 0.70$, $P = 0.003$, respectively) (Table 2 and Figure 7). There was no correlation between severity or frequency of abdominal pain/discomfort and lamina propria area occupied by mast cells, release of tryptase and histamine, and number of degranulated mast cells per field (Table 2).

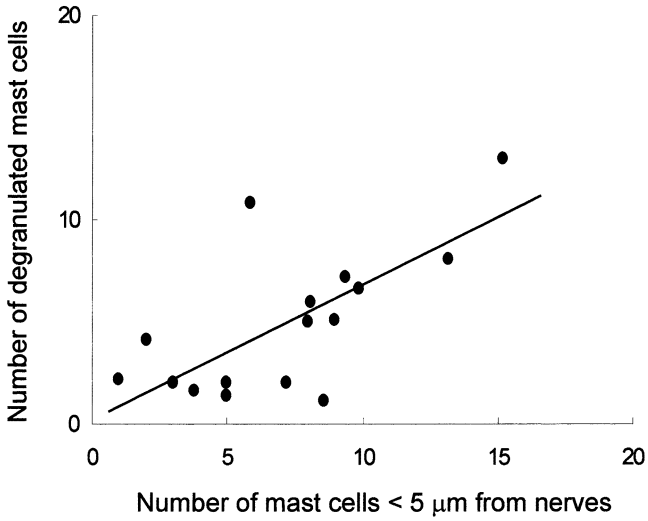

Figure 6. Correlation between the number of degranulated mast cells and the number of mast cells located within 5 μm of nerves in the colonic mucosa of IBS patients ($r = 0.72$; $P = 0.002$). The number of mast cells is relative to 10 electron microscopy fields as described in the Materials and Methods section.

Discussion

In this study, we demonstrated that severity and frequency of perceived abdominal painful sensations are correlated with the presence of activated mast cells in proximity of nerve endings in the gut wall. Furthermore, we showed increased histamine and tryptase release by the colonic mucosa of IBS patients. Because these mast cell mediators are known to alter enteric nervous system physiology and induce visceral hypersensitivity,[19,34] their increased release in close proximity to colonic innervation suggests that these mediators contribute to the disturbed sensory-motor function of IBS.

The role of mucosal inflammation in symptom perception in gastrointestinal diseases remains far from being fully understood. The rationale for testing the impact of intestinal inflammation on the development of visceral hypersensitivity and symptom generation in humans

Table 2. Correlation Between Mast Cell Parameters and Abdominal Pain/Discomfort in IBS Patients

	Abdominal pain/discomfort			
	Severity		Frequency	
	r	*P*	*r*	*P*
Mucosal area occupied by mast cells	0.07	0.64	0.11	0.48
Histamine release	0.12	0.16	0.35	0.35
Tryptase release	0.08	0.74	0.13	0.59
Degranulated mast cells	0.42	0.11	0.29	0.27
Mast cells <5 μm from nerves	0.75	0.001	0.70	0.003

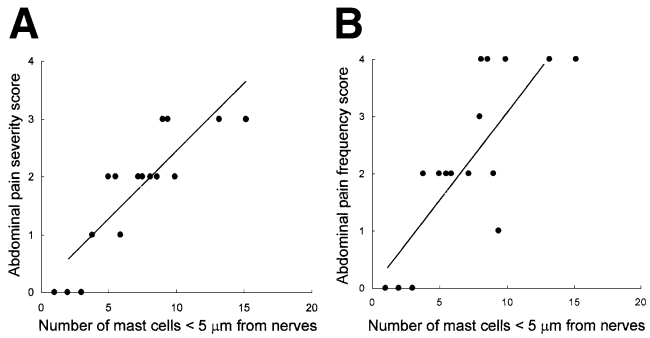

Figure 7. Correlation between severity (*A*) and frequency (*B*) of abdominal pain and the number of mast cells located within 5 μm of nerves in the colonic mucosa of IBS patients ($r = 0.75$, $P = 0.001$ and $r = 0.70$, $P = 0.003$, respectively). The number of mast cells is relative to 10 electron microscopy fields as described in the Materials and Methods section.

originates from abundant data in animal models.[34] Visceral hypersensitivity has been shown to be associated with colonic mucosal inflammation in different gut conditions, although conflicting results have also been published. Gwee et al. demonstrated a chronic inflammatory infiltrate in the rectal mucosa of patients with postinfectious IBS who also experienced urgency and discomfort at lower volumes of rectal distention than matched controls.[35] In keeping with these findings, patients with active ulcerative colitis reported non-noxious and noxious sensations at lower rectal distention volumes than those applied to controls.[36] In contrast, Chang et al. showed a decreased rectal sensitivity in patients with mild to moderate ulcerative colitis.[37] These previous findings are difficult to compare and reconcile with those obtained in the present study. Indeed, the inflammatory response of inflammatory bowel disease and gastrointestinal infection (i.e., postinfectious IBS) could be substantially different, in terms of type and severity of immune activation, from that observed in our patients with nonspecific IBS. Furthermore, these previous studies provided no information on the relationship between the immune response and mucosal neural supply.

We showed a marked (~180%) increase in colonic mucosal area occupied by mast cells in as many as three quarters of IBS patients, regardless of bowel habit. These data are in keeping with the general assumption that mast cells are increased in the mucosa of small[8] and large intestine[6,7,13] of IBS patients. However, we found substantial differences with these previous studies. For example, O'Sullivan et al. found that increased mast cells could be detected in the cecum but not in the left colon[13] and Chadwick et al. found that a significant mast cell infiltration could be detected only in IBS patients with constipation but not with diarrhea.[7] We believe that differences in study design make it difficult to compare our findings with those of these previous studies. We tried to avoid possible confounding factors: first, we included a specifically selected healthy control population, which, unlike previous studies, was not recruited from patients undergoing colonoscopy for "other" intestinal diseases (i.e., polyps, bleeding); second, we used a reliable computerized method[31] to quantify immunolabeled mast cells; and, third, we obtained colonic biopsy specimens from unprepared colons to avoid the known proinflammatory effects of large bowel preparations.[38]

A similar degree of mast cell infiltration was found in IBS patients with diarrhea and constipation. A mechanistic interpretation as to how increased mast cells are involved in such contrasting bowel habit patterns is currently lacking. Evidence indicates that mastocytosis may be responsible for diarrhea caused by increased neuronal secretomotor function[39]; however, we hypothesize that mast cells may also lead to altered enteric neuron function in constipated patients as a result of excessive segmental contractile colonic motor activity. These motor changes would ultimately induce a slow, rather than an accelerated, colonic transit. In line with this hypothesis, it is interesting to note that both diarrhea and constipation can occur in patients with ulcerative colitis, particularly when in remission,[40] and with postinfectious IBS,[6,10] which are both associated with an intestinal inflammatory component.

Our data show that mast cell increase was gender dependent, which is in keeping with the knowledge that the immune system differs between males and females.[41] For example, a greater number of mast cells has been described in the colonic mucosa of female as compared with male rats.[42] Several lines of evidence indicate that gonadal steroids are involved in gender-related differences in immune responses[41] and, particularly, in tissue mast cell infiltration.[43] Taken together, these data raise the hypothesis that gender-dependent differences in immune responses are involved in the observed higher prevalence of IBS in females, in the described gender-related differences in IBS pathophysiology, and in the known effects of the menstrual cycle in the modulation of rectal sensitivity.[44]

Although previous studies have demonstrated an increased number of mast cells in the colonic[13] and ileal[8] mucosa of IBS patients, the state of mast cell activation and mediator release has never been assessed. Studies in animals have provided evidence that mast cell activation triggers visceral hypersensitivity and gastrointestinal motor dysfunction.[4,19,20] Mast cell degranulation in the rat rectum is associated with a delayed (6–12 hours)

occurrence of a lowered threshold for induction of pain following rectal mechanical distention.[45] This is likely due to mast cell-induced increased firing of sensory nerves.[18] Our ultrastructural data are in keeping with this paradigm because we showed that mast cells of IBS patients display a significantly greater rate of activation, as demonstrated by an increased number of cells showing labyrinthic membranous arrays and clearing of individual granules and by greater release of histamine and tryptase.

To support further a role for mast cells in IBS pathophysiology, we measured the content and release of mast cell mediators by colonic mucosal biopsy specimens. Our tryptase Western blotting experiments showed that colonic biopsy specimens of IBS patients contained a larger amount of tryptase in comparison with controls, which well correlates with the increased total number of mast cells. Furthermore, our functional data demonstrated an enhanced release of tryptase in IBS patients. The specificity of the tryptase activity detected by our assay was confirmed by the experiments showing that preincubation of samples with the tryptase inhibitor BABIM significantly suppressed tryptase activity, whereas the trypsin inhibitor SBTI had no effect. The increased release of tryptase in IBS patients suggests that this mediator may affect both extrinsic afferent and enteric neurons with long-lasting effects on secretomotor function and visceral hypersensitivity, as supported by evidence in experimental models.[24,46,47] Furthermore, mast cells are by far the largest storage site of histamine in the human body, and its release has been reported to be a reliable indicator of mast cell degranulation.[48] Histamine is known to activate enteric and extrinsic neurons.[19] For example, prolonged exposure of guinea pig submucosal neurons to histamine resulted in a marked neuronal activation.[25] Similarly, histamine has been demonstrated to cause activation of abdominal visceral C-fiber afferents,[26] an effect likely mediated by H_1 or H_2 receptors expressed on primary sensory neurons.[49] These data, taken in conjunction with our demonstration of $>80\%$ increase in the spontaneous release of histamine from mucosal biopsy specimens, suggest that histamine, in addition to tryptase, could be involved in sensory-motor dysfunction in IBS.

In the human intestine, mast cells lie in proximity to nerves supplying the gut mucosa.[15] This close spatial association has been indicated to be of functional relevance for the crosstalk between the immune and nervous system of the gut.[14] In the present study, we found that mast cells of IBS patients were located in closer vicinity to mucosal innervation (with occasional membrane-membrane contacts), suggesting that mediators released by mast cells, including tryptase and histamine, have an increased potential to affect neural function. In keeping with this hypothesis, we found a significant correlation between abdominal pain/discomfort (both severity and frequency) and vicinity of mast cells to nerves supplying the colonic mucosal. In addition to these findings, previous studies indicate that mast cell infiltration of the human gut wall is associated with abdominal pain and that therapy targeting mast cells can improve abdominal pain perception both in systemic mastocytosis[50] and in IBS.[51] Taken together, these data strongly support a mechanistic role for mast cell involvement in visceral nociception in IBS. On the other hand, no statistical correlation was found between tryptase and histamine release and abdominal pain/discomfort. Different factors may account for this lack of correlation. First, although we primarily assessed histamine and tryptase, mast cells are known to release a wide variety of biologic substances that sensitize sensory nerves.[19,21] This raises the possibility that combinations of these substances are necessary to trigger sensory nerve firing up to the level of abdominal pain/discomfort perception. Second, because mast cells that lie in close proximity to nerves correlate with abdominal pain/discomfort and show an increased rate of degranulation, it is conceivable that only the release of histamine and tryptase in close proximity to nerves is relevant for abdominal pain/discomfort development. However, in this study, we detected the amount of each mediator released from the pool of the mast cells rather than that in close proximity to nerves. Third, in keeping with the Rome II criteria, the symptom questionnaire used in the present study did not discriminate between pain and discomfort, which were considered as a single symptom. Previous studies demonstrated that repetitive noxious sigmoid balloon distention evoked visceral hypersensitivity only in patients with abdominal pain but not in those with nonpainful symptoms (e.g., discomfort, bloating, and sensation of gas).[52] Thus, the possibility that the increased release of histamine and tryptase observed in our patients correlates with abdominal pain, but not with discomfort, needs further evaluation.

The causes of a prominent colonic mast cell infiltrate and activation observed in IBS patients are unknown. Data suggest that factors including previous episodes of infectious enteritis[35] and undiagnosed food allergies[53] may contribute either individually or in combination. In this study, we discarded the possibility to investigate postinfectious IBS to avoid the bias related to retrospective analyses. A prospective assessment of such a correlation is needed. The classical type of activation of mucosal mast cells is through an IgE-dependent (allergy)

mechanism.[21] In our patients, we excluded the influence of food allergies by means of history taking and food-specific IgE antibodies. However, food allergy diagnosis remains elusive, and we cannot exclude that at least some of our patients suffered from a subclinical form of allergic gut disease.[53] On the other hand, it is now widely accepted that mast cells can also respond to a non–IgE-dependent stimulation.[21] The best-known triggers of this alternative way of activation include bacterial toxins, neurotransmitters, and stress,[54] all of which may be involved in IBS pathophysiology.[5]

In conclusion, these results provide the rationale for considering nerve-mast cell interactions as a mechanism for abdominal pain/discomfort generation in IBS and, hence, as a target for therapy.

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Received April 15, 2003. Accepted November 20, 2003.

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Supported by the Italian Ministry of University, Research, Science and Technology (to G.B., V.S., R.D.G., and R.C.), by National Institutes of Health grants DK43207 and 57840 (to N.W.B.), by the Canadian Institutes for Health Research (to S.M.C.), and by an educational grant from Janssen (to V.S.).

The authors thank Dr. P. Chieco for his help with morphometric studies, Dr. S. Guerrini for her contribution to immunohistochemical studies, Dr. S. Pileri for the generous gift of anti-tryptase antibodies used in the study, and Drs. R. and L. Cogliandro for their contribution to patient selection and management.