Original article

Alteration of cholinergic and peptidergic neurotransmitters in rat ileum induced by acute stress following transient intestinal infection is mast cell dependent

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Keywords: *mast cells; transient infection; acute cold restriction stress; acetylcholine; substance P*

Background Mast cells are implicated in the development of irritable bowel syndrome (IBS), which is associated with the activation of the "neural-immune" system. The aim of this study was to investigate the role of mast cells in the remodeling of cholinergic and peptidergic neurotransmitters induced by acute cold restriction stress (ACRS) post infection (PI) using mast cell deficient rats (Ws/Ws) and their wild-type controls (+/+).

Methods Transient intestinal infection was initiated by giving 1500 *Trichinella spiralis* (T.S.) larvae by gavage. ACRS was induced for 2 hours at day 100 PI. Samples of terminal ilea were prepared for H&E staining, mast cell counting and activation and assessment of IL-1β and IL-10.

Results When infected, both strains of rats experienced an acute infectious stage followed by a recovery. Histological scores were significantly higher in infected rats compared with those of the non-infected controls at day 10 PI (10 day-PI vs. control: +/+: 2.75±0.17 vs. 0.42±0.09; Ws/Ws: 2.67±0.67 vs. 0.50±0.34; P <0.01). In +/+ rats, post-infection ACRS induced the formation of low-grade inflammation, represented by the imbalance of IL-1β and IL-10 (IL-1β: PI+ACRS vs. control: (1812.24±561.61) vs. (1275.97±410.21) pg/g, P <0.05; IL-10: PI+ACRS vs. control: (251.9±39.8) vs. (255.3±24.7) pg/g, P >0.05), accompanied by hyperplasia and activation of mast cells (PI+ACRS vs. control: 58.8±19.2 vs. 28.0±7.6; P <0.01). The balance between acetylcholine (ACh) and substance P (SP) was also disturbed (ACh: PI+ACRS vs. control: (743.94±238.72) vs. (1065.68±256.46) pg/g, P <0.05; SP: PI+ACRS vs. control: (892.60±231.12) vs. (696.61±148.61) pg/g, P <0.05). Nevertheless, similar changes of IL-1β/IL-10 and ACh/SP were not detected in Ws/Ws rats.

Conclusion The imbalance of ACh/SP, together with the activation of mucosal immunity induced by post-infection ACRS were lacking in mast cell deficient rats, which supports the premise that mast cells play an important role in cholinergic and peptidergic remodeling in the ileum of rats.

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Transient intestinal infections and stressful events are the most common causes in the development of symptoms of irritable bowel syndrome (IBS). During this process, mast cells may be critical in promoting the activation of the "neural immune" system, which results in the appearance of IBS-like symptoms.[1] It has been reported that in IBS patients, mast cell hyperplasia at the level of the colon was observed and the number in close apposition to nerves turned out to be greatly increased.[2,3] Likewise, mast cells in the jejunum of nematode parasitizing rats were also found to form close associations with both cholinergic and peptidergic neurons.[4,5] Sixty-six percent of mast cells were in close proximity to the peptidergic nerve fibers containing substance P (SP) and calcitonin gene related peptide (CGRP), and 4%–8% of them even formed membrane-to-membrane contacts with neural endings. All these studies provided structural basis for the assertion that mast cells may participate in the modulation of intestinal neurotransmission.

Stimuli of intestinal infections, food allergies and stress may elicit an alteration in excitatory neuronal circuitry. It has been found that in jejunal smooth muscle and myenteric plexus preparations of *Trichinella spiralis* (T.S.) infected rats, the production of acetylcholine (ACh) significantly decreased both during the course of infection and post infection (PI).[6,7] In food allergy mice, the cholinergic pathway contributed less to the mechanical contraction of proximal colon induced by electrical field stimulation (EFS) than the non-cholinergic pathway.[8] Stimuli of acute stress also suppressed cholinergic innervation by inhibiting the synthesis of choline acetyltransferase (ChAT) and/or promoting the

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synthesis of acetylcholinesterase (AChE) in intestine and brain.[9-11] Accordingly, though the intestinal motility was usually active in the above situations, the activation of cholinergic excitatory innervation was not enhanced. Considering the close positional association between mast cells and peptidergic neural fibers, we emphasize the importance of peptidergic neurotransmission in the development of IBS-like symptoms. As one of the most important neuropeptides in the intestine, SP exerts its function as both excitatory neurotransmitter and sensory neuropeptide to modulate intestinal motility and visceral pain. The concentration of SP in the colon of the IBS patients was found to be greatly increased.[12] Also, the response of isolated myenteric ganglion to SP in the intestine of T.S. infected guinea-pigs was amplified.[13] Together, these findings indicate the importance of SP in the development of IBS related symptoms.

Though exposure to an attack of acute stress on the basis of transient intestinal infection is the most common pathogenic pattern of PI-IBS, alterations of ACh and SP signaling and their dependence on mast cells remain unclear. In the present study, we established a rat model after 2 hours of acute cold restriction stress (ACRS) following transient intestinal infection with T.S. in mast cell deficient rats and their wild-type controls in parallel. We used this model to clarify the alteration of cholinergic and peptidergic innervation and the involvement of mast cells in this process.

METHODS

Animals

Male mast cell deficient rats (WsRC-Ws/Ws, 7 weeks old) and their wild-type controls (WsRC-+/+, 7 weeks old) were purchased from Japan TGC Inc. (Kanagawa, Japan). They were housed solitarily in polypropylene cages and kept under standard controlled environmental conditions with 12-hour light/dark cycles. All the rats were allowed food and water *ad libitum* while housed. The experiments were conducted when the rats reached approximately 12 weeks of age. All procedures employed in this study were approved by the Animal Care Committee of Peking University.

Overall design

To study the effect of ACRS on the remodeling of intestinal innervation after transient intestinal infection and the role of mast cells, we infected the intestines of the animals with 1500 T.S. at day 0 by gavage to both the +/+ rats and Ws/Ws rats, and allowed them to recover for 100 days. At day 100, all the rats were given ACRS or sham stress for 2 hours. Saline was given to the non-infected controls. Then, the ileum tissue samples were obtained for histological study, mast cell activation evaluation, and the presence of inflammation related cytokines (IL-1β, IL-10) and excitatory neurotransmitters (SP, ACh). Namely, each genotype of rat was divided into four groups (n=6 for each): PI, ACRS, PI+ACRS, and control group. To identify the effect of acute infection, both infected and non-infected Ws/Ws and +/+ rats (n=6) were sacrificed simultaneously at day 10 (10 day-PI, 10 day-control) for histological evaluation.

Transient intestinal infection with T.S.

Rats were infected by oral administration of 1500 T.S. larvae suspended in 1 ml of 0.9% NaCl solution. The corresponding non-infected controls were given 1 ml of saline. The larvae were isolated from skeletal muscle of infected Kunming mice after digestion with the standard 2.5% pepsin-0.5% HCl solution, as described by Duan et al.[14]

Procedure of ACRS

The ACRS and PI+ACRS groups of rats underwent a secondary ACRS attack from 8.00 a.m. to 10.00 a.m. on the 100th day. The rats were restrained into individual polymethyl methacrylate (PMMA) restraint cages and placed in a cold room at 4°C for 2 hours. The sham stressed controls underwent the action of capture in their home cages only and were not restrained.

H&E staining and histological scoring

The terminal ileum (about 3 cm to the cecum) of rats was resected and fixed for 12 hours in 10% neutral buffered formalin. Regular 5 μm-thick paraffin embedded sections were made for conventional H&E staining. The histological changes were evaluated in a blind fashion by histological scores, ranging from 0 to 3 to semi-quantify intestinal inflammation, which was adapted from a previous scoring system:[15] 0: no inflammation; 1: slight infiltration of lymphocytes in mucosal layer; 2: infiltration with eosinophils leading to separation of crypts, mucosal hyperplasia; 3: massive infiltration with inflammatory cells and/or disturbed mucosal architecture.

Evaluating mast cell proliferation and activation

The ileum tissue fixed in Carnoy's solution for 1 hour was applied for mast cell stain with Alcian Blue-Safranin O solution (0.36% and 0.18%, respectively, in 1.0 mol/L HCl-sodium acetate buffer, pH 1.42) for 30 minutes. Five different areas in the mucosal layer at 200× magnification in each slide were randomly selected for mast cell counting, and the average number was calculated. Activation and degranulation of mast cells were assessed using transmission electron microscopy (TEM). The ileum tissues were cut into small tissue pieces (about 1 mm in square) immediately after resection, and placed into 2.5% glutaraldehyde (GA) in 0.1 mol/L phosphate buffer (PB, pH 7.4) for 24 hours. Following fixation, the specimens were washed using 0.1 mol/L PB, and fixed with 1% osmium tetroxide in 0.1 mol/L PB for 1 hour. Specimens were dehydrated in a graded series of ethanol and embedded in an epoxy resin, Quetol-812. After trimming, ultrathin sections were cut and counterstained with uranyl acetate and lead citrate. Mast cells were observed under a Hitachi H-600 electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 75 kV.

Analysis of ileal excitatory neurotransmitters (SP, ACh) and inflammation related cytokines (IL-1β, IL-10)

The content of SP and IL-1β in ileum tissue was measured using sensitive radioimmunoassay kits according to the manufacturer's instructions purchased from Puerweiye Biotech Co. Ltd. (Beijing, China). The content of ACh and IL-10 in ileum tissue was measured by ELISA kits purchased from Cusabio Biotech Co. Ltd. (USA) and R&D systems (USA), respectively.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) or mean ± standard error (SE), and statistical analysis was carried out using one-way analysis of variance (ANOVA) or unpaired Student's *t* test (the rate of weight gain). A *P* value less than 0.05 was considered statistically significant. Statistical analyses were calculated using SPSS 13.0 software.

RESULTS

Determination of transient intestinal infection

After administering the T.S. infection, both the +/+ rats and Ws/Ws rats experienced acute intestinal infection and subsequent recovery. In the acute infectious stage (1–10 days PI), both strains of rats exhibited less activity, piloerection, less food and water consumption, and stopped gaining weight (Figure 1A). Obvious intestinal inflammatory responses were detected by histological study, which presented as epithelial edema, hyperemia, and marked eosinophil infiltration in the ileum (Figure 2A and 2B). The histological scores of 10 day-PI groups in both +/+ rats and Ws/Ws rats were significantly higher than those of the non-infected controls (10 day-PI vs control: +/+: 2.75±0.17 vs. 0.42±0.09; Ws/Ws: 2.67±0.67 vs. 0.50±0.34; P <0.01). After 100 days of recovery, the infected +/+ rats restored their weight, while the infected Ws/Ws rats did not (Figure 1B), indicating that the systemic influence of intestinal T.S. infection on the Ws/Ws rats was more severe than that on the +/+ rats. At day 100 PI, no evident inflammatory cell infiltration was detected, and the histological changes in the ilea were restored to basal levels (Figure 2C and 2D). Inflammatory responses of the intestine of both the +/+ rats and Ws/Ws rats were transient. Either the 2-hour ACRS alone or the ACRS following transient intestinal infection could not induce significant histological changes in either strain of rats (Figure 2E and 2F).

Mast cell counting and TEM study for mast cell degranulation

In both control and PI group of Ws/Ws rats, no mast cells were found in the ileum using microscopy, while in the +/+ rats, mast cells were alcian blue-positive and distributed in both the mucosa and submucosa layer (Figure 3A and 3B). By counting the mast cells in the mucosa, we found that the transient intestinal infection with T.S. triggered the hyperplasia of mast cells in

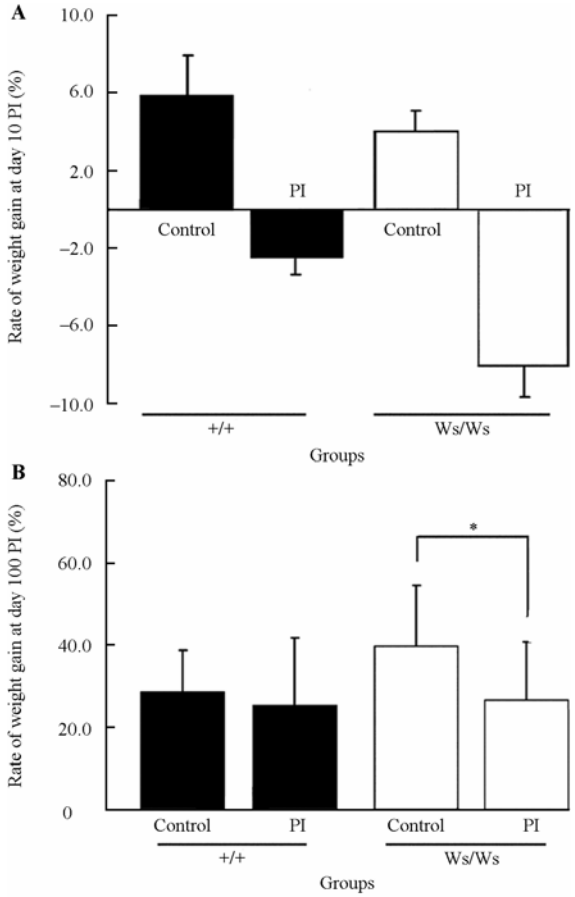

Figure 1. The rate of weight gain at days 10 and 100 post infection. During the acute infectious stage, both the +/+ rats and Ws/Ws exhibited weight loss (**A**). After 100-day recovery, the overall weight gain of infected +/+ rats was almost restored, while that of Ws/Ws rats was not (unpaired Student's *t* test, $^{*}P$ <0.05) (**B**) Data were expressed as mean ± SD.

mucosa, while 2 hours of ACRS did not (Figure 3C). The number of mast cells in the PI group and PI+ACRS group were significantly higher than those of the controls and the ACRS group, respectively (PI vs. control: 58.4±24.8 vs. 28.0±7.6; PI+ACRS vs. ACRS: 58.8±19.2 vs. 31.6±7.6; P <0.01). However, ACRS could not stimulate the hyperplasia of mast cells (ACRS vs. control: 31.6±7.6 vs. 28.0±7.6; PI+ACRS vs. PI: 58.8±19.2 vs. 58.4±24.8). Furthermore, when we observed the mast cells by TEM, we noticed that in the +/+ rats, the mast cells of the control group and PI group retained a full complement of electron-dense secretory granules, while those of the ACRS and PI+ACRS groups underwent piecemeal degranulation.[16] The membranes of granules did not fuse with each other, but exhibited a loss of dense granule content that remained within the cytoplasm of the activated mast cells (Figure 4). Therefore, stimuli of the transient intestinal infection with T.S. and ACRS, respectively, promoted mast cell hyperplasia and activation, and the stimulus of ACRS post infection resulted in the activation of multiple proliferative mast cells.

Analysis of inflammation related cytokines (IL-1β and IL-10)

To thoroughly evaluate the effect of transient intestinal

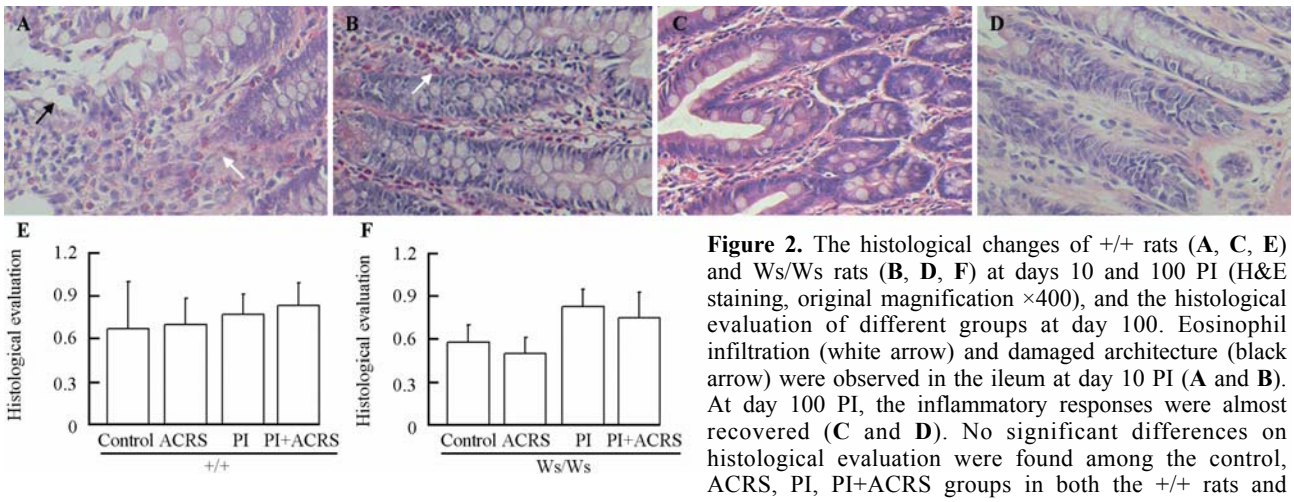

Figure 2. The histological changes of +/+ rats (**A**, **C**, **E**) and Ws/Ws rats (**B**, **D**, **F**) at days 10 and 100 PI (H&E staining, original magnification ×400), and the histological evaluation of different groups at day 100. Eosinophil infiltration (white arrow) and damaged architecture (black arrow) were observed in the ileum at day 10 PI (**A** and **B**). At day 100 PI, the inflammatory responses were almost recovered (**C** and **D**). No significant differences on histological evaluation were found among the control, ACRS, PI, PI+ACRS groups in both the +/+ rats and Ws/Ws rats (**E** and **F**). Data were expressed as mean ± SE, using one-way ANOVA.

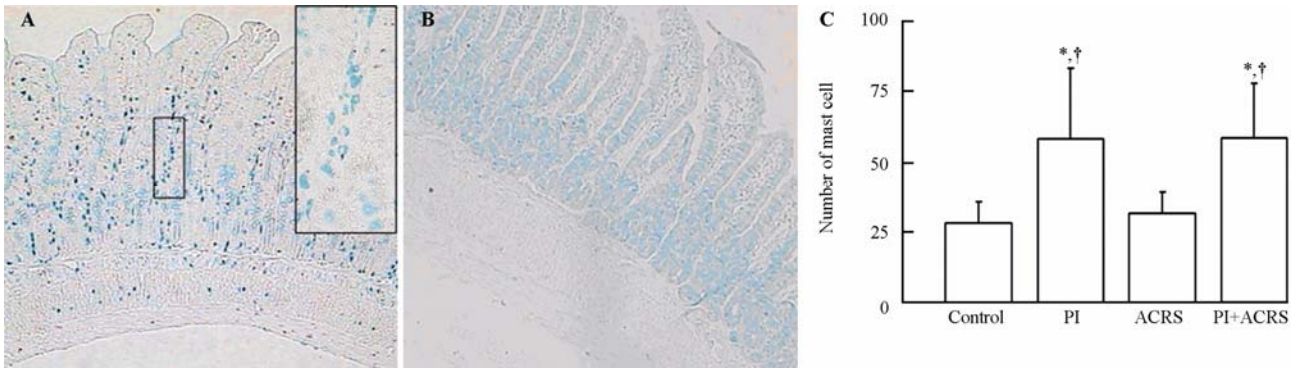

Figure 3. The results of mast cell stain and counting (Alcian Blue-Safranin O staining, original magnification ×100). Alcian blue positive cells were observed in the ileum of +/+ rats (**A**), but not in Ws/Ws rats (**B**). The number of mast cells in PI and PI+ACRS groups was significantly higher than the control and ACRS groups (**C**). Compared with control group, $^{*}P$ <0.01; compared with ACRS group, $^{\dagger}P$ <0.01 (one-way ANOVA analysis). Data were expressed as mean ± SD.

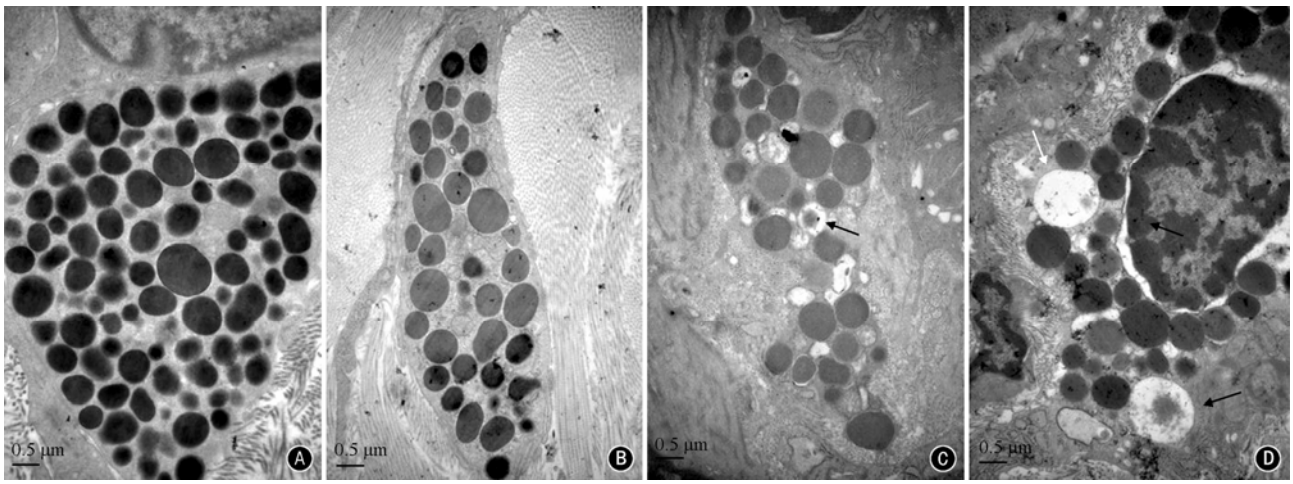

Figure 4. Electron micrograph of mast cell degranulation in different groups of +/+ rats (Original magnification ×20 000). In control group (**A**), the granule content remained electron-dense. In PI group, the granules were slightly enlarged, but the content was still in contact (**B**). In ACRS (**C**) and PI+ACRS (**D**) groups, the electron density diminished. Vesicles of different degranulating degrees were observed. The partially degranulated samples exhibited a dense core and partial loss of granule content (black arrows). The completely degranulated ones exhibited just residual containers without content (white arrows).

infection, 2 hours of ACRS and ACRS post infection on the ileal inflammatory status, we detected the expression level of pro-inflammatory cytokine IL-1β and the anti-inflammatory cytokine IL-10. In +/+ rats, neither the transient intestinal infection nor ACRS resulted in obvious changes in the content of IL-1β and IL-10 in ileal

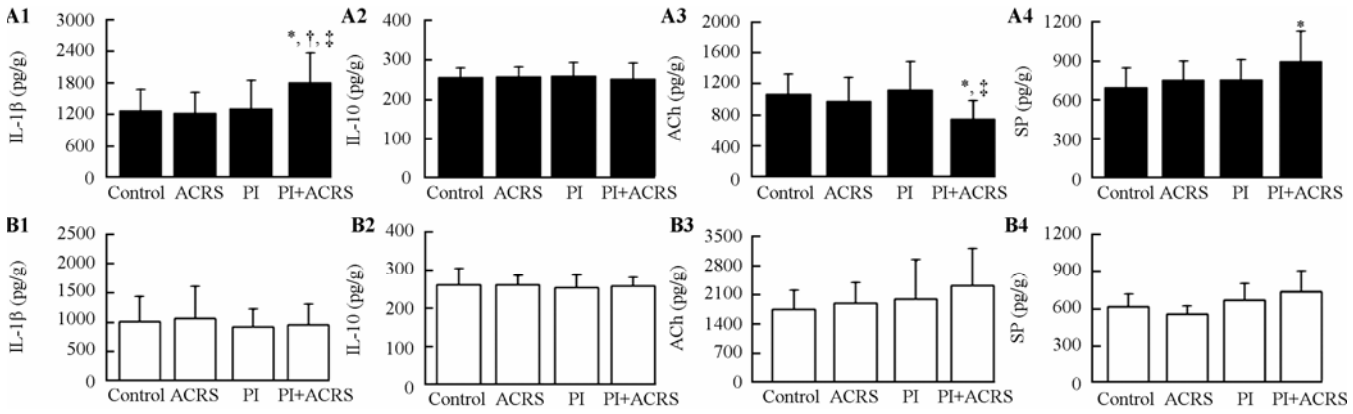

Figure 5. Quantification of IL-1β, IL-10, ACh, and SP in different groups of +/+ rats (**A**1–**A**4) and Ws/Ws rats (**B**1–**B**4). Compared with control group, $^{*}P$ <0.05; compared with ACRS group, $^{\dagger}P$ <0.05; compared with PI group, $^{\ddagger}P$ <0.05 (one-way ANOVA analysis).

tissue. However, on the basis of transient intestinal infection, the following 2 hours of ACRS significantly promoted the expression of IL-1β without influencing IL-10 expression in ileal tissue of +/+ rats (IL-1β: PI+ACRS vs. control: (1812.24±561.61) vs. (1275.97±410.21) pg/g, P <0.05; IL-10: PI+ACRS vs. control: (251.9±39.8) vs. (255.3±24.7) pg/g, P >0.05), accompanied with the activation of multiple mast cells. Namely, the balance between IL-1β and IL-10 was disturbed after the stimulus of post-infection ACRS. Whereas in the Ws/Ws rats, an imbalance between IL-1β and IL-10 induced by ACRS post infection was not detectable. No significant differences of IL-1β or IL-10 were detected among the groups of control, ACRS, PI, and PI+ACRS (Figure 5).

Assessment of expression of ACh and SP in the ileum of rats

In +/+ rats, though the single stimulus of T.S. infection and 2 hours of ACRS had no effect on the expression of ACh and SP in the ileum, after performing the 2 hours of ACRS on the +/+ rats following the transient intestinal infection, the balance between ACh and SP was disrupted, represented by the down-regulation of ACh and up-regulation of SP (ACh: PI+ACRS vs. control: (743.94±238.72) vs. (1065.68±256.46) pg/g, P <0.05; SP: PI+ACRS vs. control: (892.60±231.12) vs. (696.61±148.61) pg/g, P <0.05). In the Ws/Ws rats, a similar alteration of ACh and SP in PI+ACRS group were not detected when compared with wild-type controls, no significant changes of the expression of ACh and SP were found, and the content of ACh even turned out to be slightly elevated (Figure 5).

DISCUSSION

Various animal models induced by transient intestinal infection or stress were used to study the mechanism of IBS-like symptoms,[17-21] in which these stimuli were considered to be important in the promotion of persistent intestinal dysmotility and visceral hypersensitivity.[22] Since consensus on "mast cell involvement in the activation of 'neuro-immune-endocrine' network" has been reached, it will hopefully become the therapeutic target for IBS.[1] The introduction of mast cell deficient animals, like Ws/Ws rats, can provide direct evidence of its critical role. Until now, however, studies using this kind of animal focused on the dysfunction of the intestinal mucosal barrier and ion transport,[23,24] rarely did any of these studies focus on elucidating the role of mast cells in neural remodeling, which contributes significantly to the mediation of intestinal motility and visceral hypersensitivity.

In the present study, to reproduce the neural remodeling of IBS patients, especially those who have a definite prior history of infection and stressful life events, we subjected +/+ and Ws/Ws rats with acute stress following transient intestinal infection, and used non-treated rats and those attacked with either infection or acute stress as controls. We found that acute stress following transient intestinal infection resulted in an increase of ACh and decrease of SP in +/+ rats, accompanied with an imbalance of IL-1β/IL-10 and activation of proliferative mast cells. Whereas in Ws/Ws rats, the alteration of ACh/SP and IL-1β/IL-10 in +/+ rats was lacking, which indicated that the alteration of "neural-immune" system induced by acute stress following transient intestinal infection is dependent on mast cells.

Relationship between mast cells and low-grade inflammatory responses

Mast cells are important effector cells of the innate immune system, which play critical roles in dislodging parasites like T.S. When the hosts become infected, the mast cells recruited from blood may increase the function of epithelial secretion and paracellular epithelial permeability. Meanwhile, increased rates of epithelial turnover induced by mast cell tryptase were important in the expulsion of adult T.S. and inhibitory to larval development.[25] Despite the protective responses mediated by mast cells, undesirable consequences such as diarrhea and histological damage can also be induced,[26] which is consistent with our findings. During the acute infectious stage, the histological damage of +/+ was serious and stools became watery, while in the infected Ws/Ws rats,

though the inflammatory cell infiltration was exhibited, the changes of villus was not as prominent, and no watery stool occurred at all. One hundred days PI, in opposition to that of +/+ rats, the weight gain of Ws/Ws was not completely restored (Figure 1), indicating the critical role of mast cells in host protection.

The mucosal immune activation in IBS patients has received much attention. Although no obvious histological damages can be detected, the immunocytes such as T cells, mast cell, and the pro-inflammatory cytokines such as IL-1β can be significantly increased.[12,27] The close apposition of immunocytes to gut nerves supplying the mucosa provides a basis for neuroimmune cross-talk, which may explain gut sensorimotor dysfunction and related symptoms in patients with IBS.[28] Gwee et al[27] reported that the level of colonic IL-1β was markedly increased in those who had a prior history of infection and were diagnosed as PI-IBS 3 months later, suggesting that the preliminary infection may contribute to mucosal immune activation and development of IBS symptoms. According to the study of Neal et al,[29] depressive emotions were also important in maintaining the IBS symptoms, represented by a lower recovery rate in those who were infected with the presence of depression prior to or after diagnosis than in those without psychological risk factors (12.5% vs. 47.4%). Therefore, both intestinal infection and stress cannot be ignored in the progress of IBS symptoms.

Similarly, in our study we found that a single stimulus of transient intestinal infection or ACRS was not strong enough to elicit changes of IL-1β and IL-10, but they can respectively induce the mast cell hyperplasia or mast cell activation (Figure 3). When these two factors were combined, along with significant mast cell degranulation (Figure 4), the imbalance of IL-1β and IL-10 in ileum of +/+ rats (Figure 5) showed up quickly, with no obvious histological changes (Figure 2E). In this regard, we deduced that mast cell infiltration induced by prior intestinal infection might be the basis of intestinal inflammatory responses, and the mast cell activation and degranulation induced by the following ACRS further triggered mucosal immunity activation. Also, this view was verified in the parallel study using Ws/Ws rats, in which no significant alteration in IL-1β or IL-10 was detected between the control group and the PI+ACRS group (Figure 5), demonstrating the critical role of mast cells in the activation of the mucosal immune system.

Involvement of mast cells in the neural remodeling induced by ACRS post infection

Generally, cholinergic innervation is one of the most important factors in regulating the gastrointestinal motility and mucosal ion transport.[30,31] However, in some conditions, like post infection or in stress, its predominance might be attenuated.[32,33] Moreover, the activation of non-cholinergic ones like peptidergic and purinergic innervation might be elevated.[12,13,34] In the present study, we found that the single stimulus of transient intestinal infection with 1500 T.S. was not strong enough to elicit a persistent decrease of ACh lasting until day 100 PI, which was supported by the previous reports of Wang et al,[35] who demonstrated that in the small intestine of T.S. infected mice, the expression level of vesicular acetylcholine transporter (VAChT) was transiently down-regulated and recovered at day 60 PI. However, we found that the ACRS following transient intestinal infection prominently triggered the down-regulation of ACh and up-regulation of SP in ileum of +/+ rats (Figure 5). Also, the imbalance of ACh/SP was absent in Ws/Ws rats. These results, taken together with those of mast cell counting and activation analysis, lead us to conclude that proliferation and degranulation of mucosal mast cells are prerequisite to the development of ACh/SP remodeling induced by ACRS post infection.

In summary, the present study demonstrated that the imbalance of ACh/SP, together with the activation of mucosal immunity induced by ACRS post infection, was lacking in mast cell deficient rats, which supports the view that mast cells play an important role in the alteration of cholinergic and peptidergic remodeling in the ileum of rats.

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