Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Mast Cells Play a Crucial Role in *Staphylococcus aureus* Peptidoglycan-Induced Diarrhea

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Bacterium-induced diarrhea results in 2 to 2.5 million deaths in the world each year. The mechanism needs to be further understood. *Staphylococcus aureus* infection has a close relation with diarrhea; its cell wall component peptidoglycan (PGN) has strong biological activity on immune cells and possibly plays a role in *S. aureus*-induced diarrhea. The present study showed that oral PGN-induced diarrhea in mice in a dose-dependent manner. Intestinal epithelial cells absorbed PGN via the intracellular pathway. Intestinal mast cells were activated after PGN gavage. Toll-like receptor (TLR)2 expression was detected in mast cells in the intestine as well as in the murine mast cell line p815 cells. Blocking TLR2 or nucleotide-binding oligomerization domain (NOD)1 with related antibodies or RNA interference abolished PGN-induced p815 cell activation. The mast cell mediator histamine and serotonin had synergistic effects in PGN-induced diarrhea. In summary, oral PGN can induce diarrhea in mice, and TLR2 and NOD1 mediate the PGN-induced mast cell activation that plays a critical role in diarrhea induction. Blockade of TLR2 or NOD1 or treating mice with a mast cell stabilizer can efficiently inhibit PGN-induced-diarrhea, providing potential therapeutic significance. (*Am J Pathol* 2007, 171:537–547; DOI: 10.2353/ajpath.2007.061274)

Diarrhea is a condition characterized by frequent watery or loose stools. Viral infection- and bacterial toxin-induced diarrhea are the most frequent cases, which result in 2 to 2.5 million deaths annually worldwide,¹ mainly due to severe dehydration and electrolyte im-

balance.² Chronic diarrhea occurs in chronic intestinal diseases such as chronic infection, Crohn's disease, and ulcerative colitis,^{3,4} which causes malnutrition and chronic intestinal tissue damage, etc. The mechanisms of diarrhea have been well discussed, such as in osmotic diarrhea or secretory diarrhea. Bacterial toxin-induced diarrhea is categorized as secretory diarrhea.^{5,6} In fact, intestinal epithelial cells are thought to be tolerant to commensal bacterial toxins and components^{7,8}; however, how these microbial products break down the established "tolerance" and induce diarrhea and other inflammatory reactions remains unclear.

Mast cells are strategically localized at the host-environment interfaces such as the skin, the airway, and the intestine.⁹ The proinflammatory cytokine, tumor necrosis factor- α , which is presynthesized and stored in mast cells, is capable of killing invaded microbes to prevent infection.¹⁰ Mast cells thus are regarded as a critical component of host defense against bacterial infections. However, besides tumor necrosis factor- α , mast cells also release histamine, serotonin, and an array of other mediators in response to bacterial stimulation.¹¹ Histamine and serotonin-induced diarrhea has been well documented.^{12,13} Bacterial-derived histamine is one of the factors causing diarrhea following ingestion of infected fish,¹⁴ with histamine release from food hypersensitivity reactions being well described.¹⁵ However, the mechanism of diarrhea caused by microbial product-induced mast cell mediator release remains largely unknown.

Staphylococcus aureus (*S. aureus*) is one of the pathogens associated with infectious diarrhea.¹⁶ Peptidoglycan (PGN) is a cell wall component of almost all gram-positive (including *S. aureus*) and gram-negative bacteria that has strong immune activity. It is capable of modifying the functions of some immune cells¹⁷ and may play a critical role in bacterial diarrhea. Mast cells express Toll-like receptors (TLRs) including TLR2 and can respond to

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the stimulation of PGN to release chemical mediators leading to inflammatory reactions in tissue.^{18,19}

The hypothesis underlying the present study is that microbial products activate mast cells in the intestine to release mediators such as histamine and serotonin to induce diarrhea. The objectives of this study involved testing i) whether oral PGN induces diarrhea in mice, ii) whether TLR2 and nucleotide-binding oligomerization domain (NOD)1 mediate PGN-induced mast cell activation in the intestine, and iii) whether mast cell activation is required in PGN-induced diarrhea. The results show that PGN induces mouse diarrhea via mast cell activation that can be inhibited by mast cell stabilizers. PGN-induced mast cell activation can be inhibited by anti-TLR2 antibody, TLR2 RNA interference, or NOD1 RNA interference.

Materials and Methods

Mice

BALB/c mice were purchased from The Charles River Laboratory (St. Constant, QC, Canada), and mast cell-deficient WBB6F1-*W/W^c* (*W/W^c*) mice and their normal littermates WBB6F1^{+/+} (*+/+*), TLR2^{-/-}, and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were 8 to 10 weeks of age and housed under pathogen-free conditions with free access to food and water ad libitum. All experiments were approved by the animal research ethics board of McMaster University.

A Murine Model of Diarrhea Elicited by PGN Administration

Diarrhea was induced by oral administration of purified PGN (in short, oral PGN, graded doses in 0.25 ml of saline per mouse, ultrapure; InvivoGen, Wiltshire, UK; Figure 1) to randomly grouped mice that were fasted overnight. In a period of 3 hours after the oral PGN delivery, the individual mouse cages were inspected (by a blinded observer) for the presence of feces that were categorized into four types (hard pellet, soft pellet, soft watery, and watery). To collect the feces, mice were placed in cages equipped with water-resistant sheets on the bottom. All of the feces in the inspecting period were enumerated, collected, and weighed before and after drying in an oven for 1 hour at 80°C. The percentage of water in stool was calculated for each mouse. The wet weight of the small and large intestines as well as the body weight were recorded. The ratios of the small and large intestine/body weight were calculated to assess the watery fluid accumulation in the intestinal tract.

The following experimental groups were designed: i) naïve control, mice treated with oral saline; ii) PGN group, mice treated with oral PGN at graded doses; iii) anti-TLR2 group, mice treated with anti-TLR2 antibody (5 µg/mouse, i.p.) 30 minutes before oral PGN; iv) anti-NOD1 group, mice treated with anti-NOD1 antibody (5 µg/mouse, i.p.) 30 minutes before oral PGN; v) mast cell-deficient mouse group, *W/W^c* mice and the *+/+* littermate mice treated with oral PGN; vi) mast cell stabilizer

Figure 1. Oral PGN induces diarrhea in mice. Mice were treated with oral PGN at a given dose in 0.25 ml of saline. Each group consisted of 10 mice. **A:** Mouse feces were inspected for 3 hours after the oral PGN. Bars represent percentage of mice with particular stool types. **B:** Bars represent ratios of water in the feces. **C:** Bars represent fecal frequency in the 3-hour inspection period. **D:** Bars represent ratios of the intestine/body weight. Data are expressed as the means $\pm$ SD. **P* < 0.05 compared with group 0. *W/W^c*, mast cell-deficient mice. B6, C57BL/6 mice. TLR^{-/-}, TLR2-deficient mice. *W/W^c* *+/+* 1 to 3, *W/W^c* mice that have been reconstituted with graded bone marrow-derived mast cells from *+/+* mice (in cells/mouse); *+/+* 1 = 30,000, *+/+* 2 = 40,000, and *+/+* 3 = 50,000. SA, *S. aureus*. H-SA, heat-killed *S. aureus*. An untreated group of mice was also designed; the results of this group showed no significant difference from the group with dose 0 (with 0.25 ml of saline) (data not shown).

group, mice treated with graded doses of ketotifen 30 minutes (i.p.) before oral PGN; and vii) mast cell mediator antagonist group, mice treated with mast cell mediator antagonists (Table 1) 30 minutes before oral PGN.

Groups of mice were treated with oral *S. aureus* (American Type Culture Collection no. 10390; American Type Culture Collection, Manassas, VA) or heat-killed *S. aureus* (prepared by heating a suspension of the *S. aureus* in saline at 60°C for 1 hour) at a dose of 10⁹ cfu/mouse in 0.25 ml of saline; the live oral *S. aureus* was repeated once at 12 hours after the first treatment. In addition to measuring the same set of parameters in the mice treated with oral PGN, PGN contents in ileal contents were also evaluated.

Detection of PGN Absorption

PGN absorption in intestinal mucosa was detected using immuno electron microscopy and enzyme-linked immunosorbent assay (ELISA). The procedures of immunoelectron microscopy were performed as we described previously with some modifications.²⁰ In brief, segments of jejunum were fixed with 4% paraformaldehyde mixed with 0.75% glutaraldehyde for 2 hours. After washing with cacodylate buffer (0.1 mol/L), tissue was dehydrated with a

Table 1. Diarrhea Inhibition with Mast Cell Mediator Antagonists

Mediators	Receptors	Drugs	Dosage (per mouse)	Diarrhea inhibition	
				Used alone	Combined with
Histamine	H1	Pyrimidine	1 mg	—	
	H2	Cimetidine	0.5 mg	—	
	5-HT _{1c/2}	Ketanserin	0.025 mg	—	H1; 8/10
	5-HT ₃	Azasetron	0.1 mg	—	H1; 8/10
	5-HT ₄	RS 39604	0.1 mg	—	H1; 8/10

—, no effect; 8/10 (or 0/10), diarrhea was inhibited in 8 (or 0) out of 10 mice.

series of graded ethanol and embedded with LR White (Electron Microscopy Sciences, Hatfield, PA) at -20°C under a UV light for 24 hours. Ultrathin sections were prepared and incubated with anti-PGN antibody (Gene Tex, San Antonio, TX), followed by horseradish peroxidase-conjugated second antibody staining (Sigma, Oakville, ON, Canada). Sections were then postfixed with osmium tetroxide vapor for 1 minute and stained with uranyl acetate and lead citrate. The sections were observed with an electron microscope (JEOL JEM 1200; Tokyo, Japan). The absorbed PGN in epithelial cells was quantified using an image analysis technique. Twenty equal-sized electron photomicrographs were taken from each group. The area of PGN-positive products was determined with a computerized image process system (MOP-Videoplan, Kontorn, Germany). Total protein was extracted from another set of intestinal segments. The PGN content in the samples was determined using ELISA with the same antibody described above.

Immunostaining

Segments of intestine were snap-frozen in liquid nitrogen. Cryosections were prepared and fixed with ice-cold acetone for 20 minutes. Sections were incubated with goat anti-mouse mast cell protease-1 antibody (1 $\mu\text{g}/\text{ml}$; Santa Cruz Biotechnology, Santa Cruz, CA) and fluorescein isothiocyanate-anti-TLR2 antibody (1 $\mu\text{g}/\text{ml}$; eBiosciences; San Diego, CA) for 1 hour at room temperature. After washing, sections were incubated with Cy5-anti-goat secondary antibody (50 ng/ml; DAKO, Oakville, ON, Canada) mixed with propidium iodide (10 $\mu\text{g}/\text{ml}$; Sigma) for 1 hour. Sections were observed with a confocal microscope (Carl Zeiss Micro Imaging Inc., Thornwood, NY).

Murine mast cell line p815 cells were purchased from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were incubated with fluorescein isothiocyanate-anti-TLR2 antibody for 1 hour at room temperature. After washing, the stained cells were smeared on a slide and observed under a confocal microscope.

Reverse Transcription-Polymerase Chain Reaction

The total RNA was extracted from p815 cells using an RNeasy Mini kit (Qiagen, Mississauga, ON, Canada), and 1 μg of RNA was reverse transcribed using the iScriptcDNA Synthesis Kit (Bio-Rad, Mississauga, ON,

Canada). The resulting complementary DNA (1 μg) was then subjected to polymerase chain reaction using the HotStar Taq Master Mix Kit (Qiagen) with the annealing temperature at 60°C for 30 seconds and ran for 35 cycles. The following primers were used in the experiments: TLR2 sense, 5'-TTGCTCCTGCGAACTCCTAT-3'; and TLR2 antisense, 5'-AATGGGAATCCTGCTCACTG-3' (GenBank accession no. AF185284; 352 bp).

Western Blotting

The total proteins were extracted from p815 cells in protein extracting buffer [20 mmol/L Tris-Cl buffer, pH 7.5, containing 1 mmol/L ethylenediamine tetraacetic acid, a protease inhibitor cocktail (complete, Mini, ethylenediamine tetraacetic acid-free, 1 tablet of 10-ml buffer; Sigma), 1% sodium dodecyl sulfate, 10% Triton X-100, and 2 mol/L dithiothreitol]. After 30 minutes on ice, the samples were centrifuged ($17,600 \times g$, 10 minutes, 4°C), and protein concentration of resulting supernatant was measured using the Bradford method²¹ with bovine serum albumin as standard. Sample proteins were denatured in a 250 mmol/L Tris-Cl loading buffer, pH 6.8, containing 100 mmol/L ethylenediamine tetraacetic acid, 2% sodium dodecyl sulfate, 10% glycerol, 1% β -mercaptoethanol, and bromophenol blue, heated at 100°C for 10 minutes. Each aliquot was loaded in duplicate onto a sodium dodecyl sulfate-polyacrylamide gel. Proteins were separated by electrophoresis using 10% acrylamide gel for TLR2 and β -actin and were then transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline, pH 8, for 1 hour at room temperature and then incubated overnight at 4°C with primary antibody: anti-TLR2 (1:5000), anti-NOD1 (1:5000), anti-I κ B (1:3000), or anti-phosphorylated I κ B (1:3000) (Cell Signaling Technology, Inc., Beverly, MA) for 1 hour. Incubation with secondary antibody conjugated to horseradish peroxidase (1:10,000; DAKO) was performed for 1 hour at room temperature. After washing, the hybridized bands were detected using enhanced chemiluminescence detection kits and Hyperfilm ECL reagents (Amersham, Piscataway, NJ).

RNA Interference

For the silence of TLR2 RNA or NOD1 RNA, p815 cells were transfected with specific small interfering RNA (siRNA) duplexes. The sequences of target mRNAs used in this study

were: TLR2, 5'-AAUCCGGAGGCUGCAUAUCC-3'²²; and NOD1, 5'-AAGAGCCUCUUUGUCUUCACC-3',²³ at a final concentration of 300 nmol/L using OligoFectamine (Invitrogen, Mississauga, ON, Canada) in serum-free Dulbecco's modified Eagle's medium for 4 hours. Control siRNA 5'-AACGAAGCAACUAAGCUCG-3'²⁴ did not target any genes. The maximal effect of RNA interference in p815 cells occurred at 48 to 72 hours after the transfection. The cells were then subjected to further experiments.

Measurement of 5-HT and Histamine Release

Jejunal segments from naïve mice were cut into $2 \times 2 \times 2$ -mm³ pieces and maintained at 37°C in individual tubes for 5 minutes under gentle vibration. After centrifugation for 5 minutes at 500 rpm, the tissue pellets were resuspended in RPMI 1640 media in the presence or absence of PGN (1 µg/ml) for 30 minutes at 37°C. The levels of serotonin and histamine in culture media were determined by Immuno-tech ELISA (Beckman Coulter, Fullerton, CA) according to the instructions provided by the manufacturer. The tissues were fixed with 2.5% glutaraldehyde and processed for electron microscopy to visualize the mast cell degranulation. Histamine and 5-hydroxytryptamine (5-HT) release was also measured in the supernatants of p815 cell culture after the addition of PGN (1 µg/ml); some p815 cells were pretreated with ketotifen (20 µg/ml), anti-TLR2 (2 µg/ml), anti-NOD1 (2 µg/ml), siRNA-TLR2 (300 nmol/L), siRNA-NOD1 (300 nmol/L), or control siRNA (300 nmol/L).

Mast Cell Reconstitution

Mast cells derived from bone marrow of $+/+$ or TLR2^{-/-} mice were generated according to previous report.²⁵ Cultured mast cells (>95% purity) were injected to W/W^c mice via the tail vein at a dose of 30,000, 40,000, and 50,000 cells/mouse in 0.25 saline; the transfer was executed once more a month later. The mice were used for oral PGN experiments 2 months after the first mast cell reconstitution. As examined with Carnoy staining and electron microscopy, mast cells were distributed in the intestine of W/W^c mice with mast cell reconstitution at a similar density of $+/+$ mice (data not shown).

Statistical Analysis

Data were expressed as means $\pm$ SD. Differences between two groups were analyzed with the Student's *t*-test; three or more groups were analyzed with the analysis of variance. Differences between means at a level of $P < 0.05$ were considered significant.

Results

Oral PGN Induces Diarrhea

Mice were exposed to graded doses of PGN in saline via intragastric gavage. One to 2 hours after the gavage, mice began to have diarrhea with soft stool to

watery stool at PGN doses from 50 to 200 µg/mouse (mice did not show diarrhea in the doses of PGN less than 50 µg/mouse; data not shown). The severity of diarrhea appeared in a dose-dependent manner. The percentage of mice with certain stool types with different treatments is summarized in Figure 1A. Fecal water contents were determined and calculated for each mouse that showed significantly more water in the feces of PGN-recipient mice than control mice (Figure 1B). In the inspection period of 3 hours, naïve control mice had 6 to 10 pellets; PGN-recipient mice had significantly more pellets than the controls (Figure 1C). The results demonstrate that oral PGN is able to induce diarrhea in mice. Considering that TLR2 is the pattern recognition receptor for PGN, TLR2-deficient (TLR2^{-/-}) mice or mice pretreated with anti-TLR2 antibody were treated with PGN. The results showed that TLR2^{-/-} mice or mice pretreated with anti-TLR2 did not have diarrhea, whereas the wild-type C57BL/6 mice showed the same degree of diarrhea as BALB/c mice did (Figure 1, A–D). Mice pretreated with anti-NOD1 also did not show diarrhea (data not shown).

To clarify the role of mast cell in PGN-induced diarrhea in mouse, mast cell-deficient mice, W/W^c mice, and the $+/+$ littermate mice were treated with PGN, resulting in diarrhea in $+/+$ mice but not in W/W^c mice. To confirm further the role of mast cell in PGN-induced diarrhea, W/W^c mice were passively transferred with bone marrow-derived mast cells derived from $+/+$ mice at doses of 30,000, 40,000, and 50,000 cells/mouse or TLR2^{-/-} mice (50,000 cells/mouse) and exposed to PGN. As expected, mice receiving bone marrow mast cells from $+/+$ mice showed diarrhea in a cell number-dependent manner, whereas those receiving bone marrow mast cells from TLR2^{-/-} did not show diarrhea (Figure 1, A–D). After sacrifice, considerable watery fluids pooled in the jejunum as well as in the ileum and colon of the PGN-recipient mice with competent mast cells. The intestinal weight (including the watery fluids inside) increased in PGN-treated mice in a dose-dependent manner (Figure 1D).

After the first gavage with live *S. aureus*, mice did not show diarrhea. Thus, the gavage with live *S. aureus* was repeated 12 hours after the first gavage. The mice showed mild diarrhea 8 to 10 hours after the second gavage (Figure 1A). Mice treated with heat-killed *S. aureus* showed diarrhea 6 to 8 hours after the first gavage that lasted for 10 to 24 hours and ceased automatically (Figure 1A). The other pathogenic phenomenon such as water in stool, ratio of intestine/body weight and stool frequency also observed in mice treated with *S. aureus* gavage were close to those mice treated with PGN gavage (200 µg/mouse) (Figure 1, A–D).

A portion of ileal contents was collected for PGN analysis. As shown by ELISA, the contents of PGN were below detectable levels in naïve mice (in ng/100 µg of ileal contents): 20.5 ± 3.6 in mice treated with oral PGN (200 µg/mouse), 14.8 ± 3.1 in mice treated with live *S. aureus* and 32.1 ± 4.3 in mice treated with heat-killed *S. aureus*.

Figure 2. PGN absorption. Representative electron-immune photomicrographs (peroxidase method) show PGN-positive staining in intestinal mucosa (10 mice per group). Jejunal segments were excised 3 hours after the oral PGN. **A:** Tissue from naïve mice. **B:** Tissue from mice treated with the oral PGN (100 μg/mouse). PGN immunopositive products present as dark staining in endosome shapes (solid arrows). **C:** Tissue from mice treated with oral PGN (100 μg/mouse). PGN-positive products appear in the lamina propria (arrows); some PGN products contact mast cells; the mast cell shows extensive degranulation (open arrows). Bar = 2 μm. **D:** PGN-immunopositive staining product area in intestinal epithelial cells that was measured with an image process system (MOP-Videoplan). Each bar represents the average area (mean ± SD) of PGN products from 20 electron photomicrographs of each group. **E:** PGN contents in intestinal tissue were determined with ELISA. Bars represent PGN contents (mean ± SD).

Intestinal Epithelial Cells Absorb PGN via An Intracellular Pathway

To understand the mechanism of PGN-induced diarrhea, we next clarified if the administered PGN could be absorbed by intestinal epithelial cells to contact mast cells at the lamina propria. As shown by immunoelectron microscopy, the PGN was absorbed by intestinal epithelial cells mainly via an intracellular pathway (Figure 2B). We did not see PGN in the paracellular spaces. Some PGN was transported to the lamina propria to contact mast cells (84 of 256, 29.4%; Figure 2C). These mast cells showed extensive degranulation (Figure 2C). The

content of PGN in intestinal tissue was also evaluated by ELISA. The absorption of PGN appeared in a dose-dependent manner (Figure 2, D and E).

PGN Administration Induces Mast Cell Activation in the Intestine

Mast cell degranulation, serotonin, and histamine release were selected as markers of the mast cell activation in this study. Carnoy solution-fixed intestinal segments were processed for counting mast cell number with light microscopy. Mast cells were counted in 30 villus-crypt units

Figure 3. PGN activates mast cells in the intestine. Mice were treated with graded PGN doses (0 to 200 $\mu\text{g}/\text{mouse}$), the highest PGN dose plus anti-TLR2 (or anti-NOD1) antibody, or pretreated with mast cell stabilizer. **A:** Intestinal mast cell degranulation was observed under an electron microscope. Degranulation was defined as decreased density of granules or lost contents of granules. Sixty mast cells were analyzed for each group. Bars represent the ratio of mast cell degranulation (mean $\pm$ SD, averaged from 20 mast cells of each group). Keto, mice were pretreated with ketotifen at a dose of 500 $\mu\text{g}/\text{mouse}$; αTLR2 , treated with anti-TLR2 antibody at a dose of 5 $\mu\text{g}/\text{mouse}$; αNOD1 , treated with anti-NOD1 antibody at a dose of 5 $\mu\text{g}/\text{mouse}$. **B** and **C:** histamine and serotonin levels released from excised intestinal segments incubated in the presence of PGN *ex vivo*. Bars represent the levels of histamine (**B**) and serotonin (**C**) in culture media (Mean $\pm$ SD, averaged from 10 mice of each group; measured with ELISA. The results were normalized with tissue weight). * $P < 0.05$ compared with the mice treated with dose 0 of PGN. #Compound with PGN dose 200 (**A**) or 20 (**B** and **C**). **D–G:** A representative confocal image of immunostaining for mast cells and TLR2 in the intestine. **D** shows propidium iodide staining (red) on nuclei; **E** shows murine mast cell protease 1-positive staining (green); **F** shows anti-TLR2 staining (blue), and **G** is a merged image of **D**, **E**, and **F**; the light blue color is merged by green and blue, indicating these mast cells express TLR2.

Figure 4. PGN activates murine mast cell line p815 cells. p815 cells were cultured in the presence or absence of PGN (10 μ g/ml) and pretreatment with or without anti-TLR2 (or NOD1) or TLR2 siRNA (or NOD1 siRNA). TLR2 was positively stained in p815 cells. **A1**, negative control; **A2**, TLR2-positive staining; **A3**, p815 cells were pretreated with TLR2 siRNA and then stained with anti-TLR2 antibody. The green color indicates TLR2-positive products. **B**: p815 cells absorb PGN. **B1**: p815 cells show positively stained PGN inside cells (PGN products were stained in green color); **B2**, p815 cells were treated with anti-TLR2 antibody before the exposure to PGN; **B3**, p815 cells were treated with siRNA-TLR2 before the exposure to PGN. **C**: TLR2 expression in p815 cells. Negative control lanes, samples not reverse transcribed (mRNA) or human serum (protein) added. **D**: Western blots of I κ B and phosphorylated I κ B in p815 cells. Nil, no PGN was added; PGN10, PGN was added at 10 μ g/ml; α TLR2/PGN10, p815 cells were pretreated with anti-TLR2 antibody (2 μ g/ml) for 30 minutes before exposure to PGN (10 μ g/ml). **E**: Western blots of NOD1 in p815 cells. **F** and **G**: p815 cells were cultured in the presence or absence of PGN. The levels of histamine and serotonin were determined with ELISA. Bars represent the levels of histamine (**E**) and serotonin (**F**) (mean $\pm$ SD, averaged from six separate experiments). * P < 0.05 compared with the group "0." Ketotifen (20 μ g/ml). α TLR, anti-TLR2 antibody (2 μ g/ml); α NOD1, anti-NOD1 antibody (2 μ g/ml); siTLR2, siRNA-TLR2; siNOD1, siRNA-NOD1; con siRNA, control siRNA.

that were randomly selected from each intestinal segment (including jejunum and ileum; recorded as per field in colon at a magnification $\times 200$). The numbers of mast cells in the intestine were counted (in mean $\pm$ SD) as 4.6 ± 2.1 (jejunum), 5.8 ± 2.5 (ileum), and 1.7 ± 1.2 (colon). As determined by electron microscopy, mast cell degranulation was defined as lost granular contents (with an empty ring surrounding the remainder of granular matrices) or decreases in density of granules that was clearly observed in intestinal tissue of mice treated with PGN (Figure 2C). The ratio of degranulation in mast cells increased significantly after treatment with PGN in a dose-dependent manner (Figure 3A) that was higher in the jejunum than in the ileum. In an *ex vivo* study, excised jejunal segments were incubated at 37°C with or without PGN. As shown by ELISA, PGN administration resulted in significantly higher levels of serotonin and histamine in culture media as compared with controls (Figure 3, B and C). Mast cell degranulation in the cultured jejunal tissue

was also observed using electron microscopy after PGN stimulation (data not shown). Treatment with the mast cell stabilizer ketotifen or antibodies against TLR2 or NOD1 efficiently inhibited the release of serotonin and histamine (Figure 3, A–C) as well as mast cell degranulation (data not shown).

Toll-Like Receptor 2 and NOD1 Mediate PGN-Induced Mast Cell Activation

It is reported that bone marrow-derived mast cells express TLR2,^{18,26} of which PGN is one of the ligands.^{27,28} To determine whether intestinal mast cells express TLR2, immunostaining was performed in both intestinal tissue and mast cell line p815 cells. Cells in the lamina propria were stained positively for both mouse mast cell protease-1 (Figure 3E) and TLR2 (Figure 3F); the immunopositive staining for mouse mast cell protease-1 and TLR2

colocalized in some cells (Figure 3G), demonstrating that these mast cells were mast cells expressing TLR2. To clarify the role of TLR2 in mediating PGN-induced mast cell activation, murine mast cell line p815 cells were cultured in the presence or absence of PGN. Confocal images showed the positive staining for TLR2 on the surface of p815 cells (Figure 4A2). Pretreatment with TLR2 siRNA abolished the expression of TLR2 in p815 cells (Figure 4A3). To understand whether p815 cells absorb PGN, PGN was added to the culture; the p815 cells were subsequently stained with anti-PGN antibody and observed with confocal microscopy. PGN-positive products were observed inside of the cytoplasm of p815 cells (Figure 4B1). To see whether TLR2 plays any role in the absorption of PGN, some p815 cells were pretreated with anti-TLR2 antibody or transfected with TLR2 siRNA before the exposure to PGN. The results showed that the PGN absorption was abrogated by the pretreatment with either anti-TLR2 antibody (Figure 4B2) or TLR2 siRNA (Figure 4B3). The expression of TLR2 in p815 cells was further confirmed by reverse transcription-polymerase chain reaction and Western blotting (Figure 4C).

To probe the pathways involved in PGN-induced mast cell activation, we assessed the effects of exposure to PGN on cytoplasmic I κ B levels by immunoblotting. The decrease in I κ B levels indicates the release of nuclear factor- κ B and its translocation to the nucleus. p815 cells were treated with PGN (1 and 10 μ g/ml) for 30 minutes and tested for the phosphorylation and expression of the unphosphorylated I κ B- α (a 37-kd protein) and I κ B- β (a 49-kd protein) using Western blot assays. PGN caused a significant decrease in both I κ B- α and I κ B- β levels that could be blocked by pretreatment with either anti-TLR2 or anti-NOD1 antibodies (Figure 4D), suggesting that both I κ B- α and I κ B- β are involved in releasing nuclear factor- κ B from the cytosolic complex to be translocated to the nucleus. To clarify whether PGN binds NOD1 in the cytoplasm of mast cells, expression of NOD1 (a 95-kd protein) was tested using Western blotting. Apart from the localization of a 95-kd band was stained, an extra band was localized about 140 kd, which might be a band of the combined complex of NOD1 and PGN (Figure 4Ea). To verify whether the extra band was a complex of both NOD1 and PGN, the membrane was stripped and restained with anti-NOD1 antibody, resulting in approximately the same bands as the staining with both anti-NOD1 and anti-PGN (Figure 4Eb). The membrane was then stripped one more time and stained with anti-PGN antibody. A single band was stained at the same location of the extra band (Figure 4Ec); this indicated that the extra band was a complex of PGN and NOD1.

To determine whether the absorbed PGN activated p815 cells, the levels of histamine and serotonin in p815 cell culture media were measured by ELISA. As depicted in Figure 4, levels of histamine (Figure 4F) and serotonin (Figure 4G) in the culture were significantly increased in response to PGN stimulation that was inhibited by pretreatment with antibodies against TLR2 or transfection with siRNA of TLR2.

Nucleotide-binding oligomerization domain protein 1 and NOD2 are pathogen-recognition receptors in the cell that sense breakdown products of PGN (muropeptides).²³ In

Figure 5. Mast cell stabilizer inhibits PGN-induced diarrhea. Mice were treated with mast cell stabilizer ketotifen before the oral PGN (10 mice per group). The stool types were inspected and recorded in a period of 3 hours after PGN administration. Bars represent the percentage of mice with particular types of stools. The doses of ketotifen per mouse: **A**, 0 mg; **B**, 0.1 mg; **C**, 0.2 mg; and **D**, 0.5 mg. Another group of mice was treated with ketotifen alone (0.5 mg/mouse) without PGN; the mice showed 100% hard pelleting during the observing period.

preliminary studies, we observed that antibodies against NOD1 were more efficient at inhibiting PGN-induced mast cell activation than was the anti-NOD2 antibody (data not shown); we thus focused on observing the blocking effect of the anti-NOD1 antibody on the action of PGN. To clarify whether NOD1 was involved in the effect of PGN activating mast cells, RNA interference of NOD1 and anti-NOD1 antibody pretreatment was performed, which resulted in attenuation of the PGN-induced release of histamine (Figure 4F) and serotonin (Figure 4G) from p815 cells. The pretreatment with control siRNA did not affect the effect of PGN in inducing mast cell activation.

Mast Cell Stabilizers Inhibit PGN-Induced Diarrhea

Mice were pretreated with graded doses of mast cell stabilizer ketotifen (administered via peritoneal injection 30 minutes before the oral PGN) and then treated with oral PGN. Diarrhea was inhibited by the pretreatment with ketotifen in a dose-dependent manner (Figure 5). A group of mice was treated with oral PGN first after the diarrhea symptoms occurred; a single dose (0.5 mg/mouse, i.p.) of ketotifen also resulted in stopping diarrhea in 90% of mice (9 of 10 mice).

Synergistic Effect of Antihistamine and Antiserotonin Attenuates PGN-Induced Diarrhea

Mast cells have an array of chemical mediators that can be released on activation and induce inflammatory

reaction in tissue. To identify which mast cell-associated mediators were responsible for oral PGN-induced diarrhea, several mediator antagonists were introduced individually to mice before the oral PGN administration. We observed the effect of histamine and serotonin receptor antagonists on PGN-induced diarrhea (Table 1). No significant effect was observed in response to one drug alone. However, a combination of histamine H1 antagonist and serotonin (5-HT₃) antagonist significantly suppressed PGN-induced diarrhea (8 out of 10 mice did not show diarrhea; two mice showed soft feces). Groups of mice were treated with the antagonists of histamine or serotonin alone (without PGN) but showed no diarrhea.

Discussion

The present article reports a bacterial diarrhea animal model that can be induced by oral PGN, live *S. aureus*, or heat-killed *S. aureus*. The data demonstrate that the increase in number of *S. aureus* in the intestine by *S. aureus* gavage significantly increased PGN levels in intestinal contents; PGN can be absorbed by intestinal epithelial cells to transport it to the lamina propria; intestinal mast cells can be activated by the absorbed PGN to release mediators such as histamine and serotonin. TLR2 is the receptor of PGN on mast cells that mediates PGN absorption; both TLR2 and NOD1 play a critical role in PGN-induced mast cell activation. PGN-induced diarrhea requires the presence of mast cells.

It is reported that PGN can be absorbed in the small intestine in rats.²⁹ Our results are in line with these studies. Immunoelectron microscopy revealed that the PGN was absorbed by intestinal epithelial cells mainly via an intracellular pathway to arrive at the lamina propria. The absorbed PGN thus has the opportunity to contact the immune cells in the intestine. Indeed, we observed about 1/3 mast cells accompanying with the absorbed PGN, a scenario implicating that the degranulation in these mast cells may be induced by the absorbed PGN. Although the amount of PGN in the intestine of naive mice (without oral PGN) was below detectable levels, a large quantity of PGN can be absorbed from increased luminal PGN concentrations. Thus, it is conceivable that when bacterial overgrowth occurs in the intestine, more microbial products, including PGN, may be absorbed and activate mast cells to release proinflammatory mediators, leading to inflammatory reactions in the intestine. The morphological data provide physical evidence to confirm that the intestinal epithelial cell is able to absorb PGN to induce mast cell degranulation, which supports the previous findings.^{18,19} This is slightly different from some previous reports on this topic. Ikeda et al³⁰ proposed that PGN did not induce murine mast cell degranulation. The difference may be that Ikeda et al³⁰ performed their experiments *in vitro* with bone marrow-derived mast cells; the experimental conditions are essentially different from our *in vivo* study.

Previous reports indicate that mast cells express TLR2,^{18,19} and PGN activates mast cells via TLR2 liga-

tion.^{23,26} Our results are in agreement with these studies. On the other hand, contrasting results were also reported by Jawdat et al,³¹ who showed that mast cells in TLR2-deficient mice are still able to respond to PGN and thus drew a conclusion that TLR2 was not required in response to the stimulation of PGN in mast cells. In fact, TLR2 is not the sole molecule mediating the effect of PGN; some other molecules such as NOD1, NOD2, peptidoglycan recognition proteins, and complement systems are also able to respond to PGN stimulation.^{32–35} It is thus possible that TLR2-deficient mast cells can still respond to the stimulation of PGN via other pathways. Here, we provide evidence that TLR2 acts as a carrier to transport the absorbed PGN into mast cells in the lamina propria and then activates mast cells. We have verified that mouse intestinal mast cells express TLR2 and that mast cells absorb PGN depending on the presence of TLR2 on the cell surface. Blocking TLR2 with anti-TLR2 antibody or TLR2 RNA interference inhibited PGN absorption into mast cells (Figures 3 and 4); this demonstrates that TLR2 is required as a carrier to transport PGN into mast cells.

The immunostaining showed that PGN-positive products were localized in the cytoplasm of p815 cells. The positive staining could be the decomposed components of absorbed PGN.

To identify the role of mast cell in PGN-induced diarrhea, experiments with mast cell-deficient mice or mast cell-depleted mice were preferred. The *W/W*^o mice are an ideal strain of mast cell-deficient animals. The present data show that mast cell-deficient mice do not develop diarrhea after oral PGN administration in contrast to mice with normal mast cells that develop diarrhea in response to oral PGN, thus demonstrating that PGN-induced diarrhea is mast cell-dependent. Furthermore, pretreatment with the mast cell stabilizer ketotifen significantly inhibited PGN-induced diarrhea. Using ketotifen inhibited the established PGN-induced diarrhea, implying that administration with mast cell stabilizer has potential therapeutic value in the treatment of diarrhea caused by *S. aureus* infection in the intestine.

The present data indicate that mast cells play a crucial role in PGN-induced diarrhea. We were interested to know whether the antagonists of mast cell mediators have any effect on inhibiting PGN-induced diarrhea. Because the single antagonist did not show any significant effect on PGN-induced diarrhea, we combined the antagonists of mast cell mediators in experiments. The results showed that the combination of histamine H1 antagonist and 5-HT₃ antagonist significantly blocked PGN-induced diarrhea. It is reported that histamine can excite enteric nerves³⁶ and increase epithelial ion secretion.³⁷ Serotonin also acts on enteric nerves^{38,39} and epithelial ion secretion.⁴⁰ Thus, by blocking histamine alone, the effect of serotonin still induces enteric nerve excitation and epithelial ion secretion to contribute to the induction of diarrhea and vice versa. It is reported that mice deficient in the serotonin transporter exhibit accumulation of serotonin in the intestinal mucosa, leading to severe diarrhea.⁴¹ These studies together with the present data indicate an important role for histamine and serotonin in PGN-induced diarrhea. Because there

is an array of mediators in mast cells, the present study does not exclude the possibility that other mediators of mast cells are also involved in PGN-induced diarrhea.

The results show that plenty of watery fluids accumulated in the jejunum after oral PGN; that is a factor apparently contributing to the PGN-induced diarrhea. The source of watery fluids in the jejunum could be increased ion secretion from the epithelium⁴² or increased permeability of intestinal blood vessels.⁴³ We can eliminate the possibility that the increase in ion secretion by intestinal epithelial cells was induced by PGN directly because the ion secretion in T84 monolayers did not increase the response to PGN stimulation as we observed recently (data not shown). Other investigators⁸ also noticed that human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands including PGN. However, intestinal epithelial cells are capable of transporting the oral PGN to the lamina propria as shown by the present data (Figure 2). The absorbed PGN may activate mast cells in the lamina propria to release mediators and further activate epithelial cells to increase ion secretion. This postulation is supported by the fact that the PGN-induced mouse diarrhea was inhibited by pretreatment with mast cell stabilizer ketotifen, as well as our recent observation that T84 monolayer-transported PGN activated the cocultured human mast cells in the basal chambers of a transwell system, which caused a significant drop in the resistance of T84 monolayers as well as increased the permeability (data not shown). Others have also reported that airway epithelium-absorbed PGN activated mast cells in airway mucosa and exacerbated the allergic inflammation.⁴⁴

In summary, the present study demonstrates that intestinal mast cells play a critical role in PGN-induced diarrhea, that TLR2 mediates PGN absorption into mast cells, that PGN activates NOD1 inside the cytoplasm to activate mast cells, and that there is a synergistic effect of histamine and serotonin from mast cells on PGN-induced diarrhea.

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