activation by PGN leads to degranulation and IL-4 and IL-5 cytokine production, in contrast to TLR4-mediated mast cell activation, in which TNF-α, IL-1β, IL-6, and IL-13 are the major cytokines produced. Finally, we provide new insights into the different roles played by mast cells in vivo in a certain environment. TLR4-mediated activation of peritoneal mast cells is crucial for host protection from Gram-negative bacterial infection, whereas TLR2-mediated activation of skin mast cells causes acute and late reactions by PGN application and may exacerbate the inflammatory lesions of atopic dermatitis, in which *S. aureus* infection is common.

Methods

Mice. TLR2-deficient (TLR2$^{-/-}$), TLR4-deficient (TLR4$^{-/-}$), and corresponding wild-type (TLR2$^{+/+}$ or TLR4$^{+/+}$) mice were kindly provided by S. Akira (Osaka University) (9, 12). WBB6F$_1$-*W/W^v* mice were purchased from Japan SLC (Hamamatsu, Japan). All animal experiments were performed according to the approved manual of the Institutional Review Board of Juntendo University.

Generation of bone marrow–derived mast cells. Bone marrow–derived mast cells (BMMCs) were generated from the femoral bone marrow cells of mice and maintained in the presence of 10% pokeweed mitogen–stimulated spleen-conditioned medium as a source of mast cell growth factors as previously described (18, 21). After 4 weeks of culture, more than 99% of the cells were identifiable as mast cells as determined by Toluidine blue staining and FACS analysis of cell surface expression of *c-kit* and FcεRI.

β-Hexosaminidase release assay. A total of 5 × 10^5 BMMCs/ml in Tyrode's buffer (10 mM HEPES buffer [pH 7.4], 130 mM NaCl, 5 mM KCl, and 5.6 mM glucose) containing 10% FCS (as a source of soluble CD14), 1 mM $CaCl_2$, and 0.6 mM $MgCl_2$ were stimulated with the indicated concentrations of PGN from *S. aureus* (Sigma-Aldrich, Tokyo, Japan) or LPS from *Escherichia coli* (serotype 0111:B4; Sigma-Aldrich) for 1 hour at 37°C. The β-hexosaminidase in the supernatants and cell lysate was quantified by hydrolysis of *p*-nitrophenyl-*N*-acetyl-β-D-glucopyranoside (Sigma-Aldrich) in 0.1 M sodium citrate buffer (pH 4.5) for 90 minutes at 37°C. The percentage of β-hexosaminidase release was calculated as previously described (18).

Intracellular Ca^{2+} mobilization. BMMCs (1 × 10^6 cells/200 μl) were incubated under condition of darkness with 5 μM of fura-2 AM (Dojindo Laboratories, Kumamoto, Japan) for 30 minutes, at 37°C in complete culture medium. Cells were washed with excess amounts of cold Tyrode's buffer and then resuspended in Tyrode's buffer containing 1 mM $CaCl_2$ and 0.6 mM $MgCl_2$ at the concentration of 5 × 10^5 cells/ml. Cells were stimulated with 100 μg/ml of PGN at 37°C. BMMCs stimulated with IgE and anti-IgE were used as positive control. Intracellular Ca^{2+} mobilization was monitored at a 510 nm emission wavelength excited by 340 and 360 nm using a fluorescence spectrophotometer model F2000 (Hitachi Ltd., Tokyo, Japan).

Measurement of cytokine concentrations. BMMCs (1 × 10^6 cells/ml) in complete culture medium were stimulated with the indicated concentration of PGN or LPS. Cells were incubated at 37°C, 3 hours for TNF-α or 6 hours for IL-1β, IL-4, IL-5, IL-6, and IL-13 (a preliminary experiment showed that these time points were optimal for the production of each cytokine from mast cells). The level of each cytokine in supernatant was measured by ELISA kit (Genzyme Techne Corp., Minneapolis, Minnesota, USA).

Western blot analysis. A total of 5 × 10^6 BMMCs/ml were stimulated with PGN (100 μg/ml) or LPS (50 ng/ml) for the indicated time period. The reaction was stopped with cold Tyrode's (CA^{2+}, MG^{2+} free) buffer. The cells were lysed with 20 μl of lysis buffer (1% Triton X-100, 150 mM NaCl, 25 mM Tris-HCl [pH 7.5], 1 mM EDTA, containing 1 μM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin-A, 50 μg/ml aprotinin, and 2 mM sodium orthovanadate), and the lysates were subjected to 10% SDS-PAGE (Bis Tris; Novex, San Diego, California, USA). The immunoblotting using polyclonal antibody to tyrosine-phosphorylated IκB-α (New England Biolabs Inc., Beverly, Massachusetts, USA) and IκB-α (New England Biolabs Inc.) was done according to the manufacturer's instructions (18). The membrane was developed with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA).

Reconstitution of W/W^v mice with BMMCs. Mast cell deficiency of *W/W^v* mice in the peritoneal cavity was selectively reconstituted by the injection, 4 weeks after starting the culture, of 2 × 10^6 BMMCs from TLR4$^{-/-}$, TLR2$^{-/-}$, or respective wild-type mice into the peritoneal cavity as previously described (18, 22). For the reconstitution of skin mast cells of *W/W^v* mice, 1 × 10^6 BMMCs from TLR4$^{-/-}$, TLR2$^{-/-}$, or respective wild-type mice, in 20 μl of normal saline, were injected intradermally into the ears (22, 23) 4 weeks after starting the culture. Five weeks after injection of BMMCs, the mice were used for experiments. Reconstitution of mast cells was confirmed by Toluidine blue or Alcian blue and Safranin staining of the cytospun preparation of peritoneal cells or formalin-fixed tissue section of the skin.

Cecal ligation and puncture. Cecal ligation and puncture (CLP) was performed as previously described (18). Briefly, mice were anesthetized with sodium pentobarbital and the cecum was isolated, ligated with 4-0 silk, and punctured once with a 21-gauge needle. After CLP, mice were observed for mortality at least five times daily over a period of 10 days. Before CLP was performed, the mice were coded so that the CLP was done without notifying individual groups.

Differential cell counts and estimation of cytokine concentrations in peritoneal exudates. Peritoneal exudates were collected from CLP-induced mice at the indicated time points, and total cell numbers were counted. Cytospun preparations were made from the exudates of each mouse, and differential cell counts of infiltrating leukocytes were done by counting 500 leukocytes under oil immersion fields after staining with Diff-Quik (International Reagents, Kobe, Japan). The percentage of

mast cells in the exudates was determined by Toluidine blue (pH 4.0) or Alcian blue and Safranin staining of cytospun preparation. The levels of cytokines in peritoneal fluids were determined by ELISA kits according to the manufacturer's instruction (Genzyme Techne).

Induction of skin inflammation. Mice were lightly anesthetized with ether, and 20 μl of PGN (100 μg/ml in saline) or LPS (100 ng/ml in saline) was injected intradermally into the left ear. The vehicle saline was injected into the right ear as a control. To visualize vascular leakage, 250 μl of 0.5% Evans blue in saline was injected intravenously, 5 minutes before PGN or LPS application. After 15 minutes of application, mice were sacrificed and ears were removed. The amount of dye in the ear was measured as previously described with slight modification (24). An ear was dissolved in 200 μl of 1 N KOH solution overnight at 37°C, and 1.8 ml of a mixture of 0.6 N H_3PO_4 solution and acetone (5:13) was added. After vigorous shaking, the supernatant was collected by centrifugation at 1920 *g* for 10 minutes and the optical density was measured at 620 nm. The amount of dye was calculated according to the standard curve of known concentration of Evans blue. For histological examination, ears were removed 15 minutes or 4 hours after PGN or LPS application and fixed in buffered formalin and then stained with hematoxylin and eosin (H&E) or Toluidine blue. The number of dermal mast cells and neutrophils present at the reaction sites was determined as previously described (25, 26) and expressed as the number of cells per square millimeter of dermis. The mast cells were classified into three categories: extensively degranulated (50% of the cytoplasmic granules exhibiting fusion, staining alterations, and extrusion from the cell), slightly to moderately degranulated (10–15% of the granules exhibiting fusion or discharge), or normal (25, 26).

Statistical analysis. Statistical analysis was performed using the Student *t* test. Statistical analysis of survival data in the CLP experiment was performed using the Logrank test.

Results

PGN but not LPS induces mast cell degranulation and Ca^{2+} mobilization via TLR2. To investigate the involvement of TLR2 and TLR4 in bacterial microorganism-induced mast cell activation, first we investigated the direct effects of these microbial ligands on mast cell degranulation, a major function of mast cells. When evaluated by β-hexosaminidase release, PGN induced degranulation of BMMCs from TLR2-bearing mice, which was almost comparable to that of IgE receptor cross-linking (Figure 1a). In contrast, the same concentration of PGN did not induce any release of β-hexosaminidase from $TLR2^{-/-}$ BMMCs, even though these BMMCs released a similar amount of β-hexosaminidase with TLR2-bearing BMMCs upon IgE receptor cross-linking and calcium ionophore stimulation. This reaction was mediated by TLR2, since $TLR4^{-/-}$ and $TLR4^{+/+}$ mice showed responses similar to that of $TLR2^{+/+}$ mice (Figure 1a). Consistent with previous data, LPS stimulation did not cause degranulation of mast cells from any strains of mice ($TLR2^{+/+}$, $TLR2^{-/-}$, $TLR4^{+/+}$, $TLR4^{-/-}$, C57BL/6, BALB/c, C3H/HeN, C3H/HeJ, and NC/Nga; Figure 1a and data not shown). Degranulation of BMMCs was well correlated with intracellular Ca^{2+} mobilization. Upon PGN stimulation, intracellular increase of Ca^{2+} was observed in $TLR2^{+/+}$ but not in $TLR2^{-/-}$ BMMCs. The concentration of intracellular Ca^{2+} reached a maximum level within 50 seconds after PGN stimulation, and no significant difference was observed in the pattern of Ca^{2+} mobilization compared with FcεRI cross-linking (Figure 1b). The effect of PGN on mast cells was not because of the cytotoxic effect, since these concentrations of PGN did not show any cytotoxicity to BMMCs as evaluated by lactate dehydrogenase release assay.

Different pattern of cytokine production by mast cells upon activation via TLR2 or TLR4. Next we investigated whether PGN or LPS could activate mast cells to secrete cytokines via respective TLRs, since it has been reported that activation of mast cells leads to the secretion of preformed and newly synthesized cytokines (27). The BMMCs from $TLR2^{+/+}$, $TLR4^{+/+}$, and $TLR4^{-/-}$ mice did produce similar levels of IL-4, IL-5, IL-6, IL-13, and TNF-α in response to PGN in a dose-dependent manner (Figure 2a). In contrast, BMMCs from $TLR2^{-/-}$ mice did not produce any of these cytokines upon PGN stimulation. Since BMMCs from $TLR4^{+/+}$ or $TLR4^{-/-}$ mice could produce similar levels of cytokines, the absence of TLR4 did not influence the responses of BMMCs to PGN. Also, the TLR4-bearing BMMCs could produce TNF-α, IL-1β, IL-6, and IL-13 in response to LPS in a dose-dependent manner (Figure 2b). These responses were completely deficient in $TLR4^{-/-}$ BMMCs. The pattern and levels of these cytokines released by $TLR2^{+/+}$ or $TLR2^{-/-}$ BMMCs were not significantly different from those of $TLR4^{+/+}$ BMMCs, suggesting that the absence of TLR2 does not influence the function of TLR4 and that the TLR4 molecule is, at least, a principal LPS signal transducer in mast cells. These results were consistent with our previous work, which used BMMCs from C3H/HeJ mice and showed that intact TLR4 is required for full activation of mast cells by LPS (18).

Stimulation of mast cells via both TLR2 and TLR4 leads to activation of NF-κB signaling. The cytoplasmic domain of members of the TLR family is homologous to that of the IL-1 receptor (IL-1R), which subsequently drives the transcriptional induction of several cytokine genes after activation of NF-κB (28). We determined whether NF-κB activation was also triggered in mast cells upon PGN or LPS stimulation. The phosphorylation of IκB-α at Ser32, essential for release of active NF-κB, is a marker of NF-κB activation. PGN (100 μg/ml) and LPS (50 ng/ml) induced the phosphorylated IκB-α in a TLR2- or TLR4-dependent manner, respectively. The activity of NF-κB in mast cells was at maximum 15 minutes after both stimulations and then gradually decreased within 30 minutes (Figure 3). These results

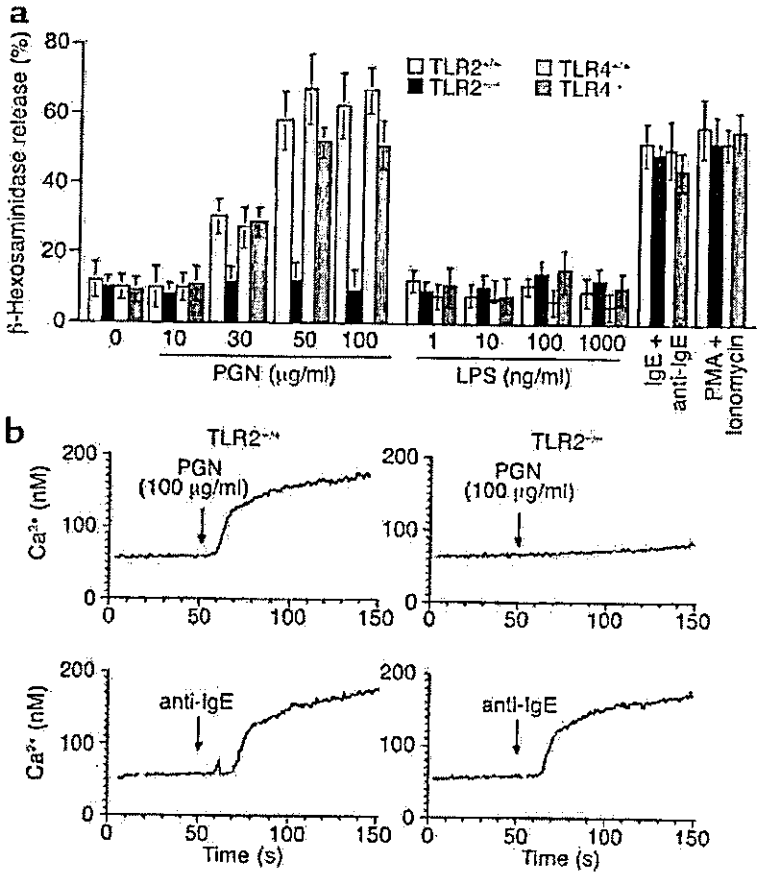

Figure 1
PGN but not LPS induces mast cell degranulation and Ca^{2+} mobilization through TLR2. (a) BMMCs from TLR2$^{+/+}$ (white bars), TLR2$^{-/-}$ (black bars), TLR4$^{-/-}$ (light gray bars), and TLR4$^{+/+}$ (dark gray bars) were incubated with indicated concentrations of PGN or LPS. β-Hexosaminidase in the supernatant was measured as described in Methods. Data shown are mean ± SD of three experiments conducted with different BMMC preparations. BMMCs stimulated with IgE and anti-IgE or PMA and ionomycin were used as positive control. (b) TLR2$^{+/+}$ and TLR2$^{-/-}$ BMMCs were stimulated with 100 μg/ml of PGN; then Ca^{2+} mobilization was evaluated as described in Methods. BMMCs stimulated with IgE and anti-IgE were used as positive control. Data shown is one representative result from several experiments that had similar results.

suggest that both PGN-TLR2 and LPS-TLR4 signaling in mast cells is followed by activation of NF-κB.

Mast cell TLR4 is required for protection of mice from CLP-induced acute bacterial infection. Using the mast cell–dependent acute septic peritonitis model, we clarified the importance of mast cell TLR4 for bacterial infection in vivo. Peritoneal mast cells of mast cell–deficient *W/W^v* mice were reconstituted with BMMCs from TLR4$^{+/+}$, TLR4$^{-/-}$, TLR2$^{+/+}$, or TLR2$^{-/-}$ mice and subjected to CLP. Some mast cell–deficient *W/W^v* mice died on the first day after CLP; 100% died within 3 days (Figure 4a). Some of the *W/W^v* mice reconstituted with TLR4-deficient BMMCs (*W/W^v*/TLR4$^{-/-}$) died within 5 days; only 30% survived 5 days after CLP. In contrast, 80–100% of *W/W^v* mice reconstituted with TLR4$^{+/+}$ BMMCs (*W/W^v*/TLR4$^{+/+}$), TLR2$^{+/+}$ BMMCs (*W/W^v*/TLR2$^{+/+}$), or TLR2$^{-/-}$ BMMCs (*W/W^v*/TLR2$^{-/-}$) survived during the experimental periods. Three days after CLP, *W/W^v*/TLR4$^{+/+}$, *W/W^v*/TLR2$^{+/+}$, and *W/W^v*/TLR2$^{-/-}$ mice showed a significantly higher survival rate than *W/W^v* and *W/W^v*/TLR4$^{-/-}$ mice ($P < 0.05$). This result is similar to the previous result of experiments using C3H/HeJ-derived BMMCs for reconstitution of *W/W^v* mice (18), and there were no significant differences in the survival rate between *W/W^v*/TLR2$^{+/+}$ and *W/W^v*/TLR2$^{-/-}$, indicating again that mast cells play an important role in host protection from early enterobacterial infection via the TLR4 molecule but not the TLR2. Five weeks after reconstitution, *W/W^v*/TLR4$^{+/+}$, *W/W^v*/TLR4$^{-/-}$, *W/W^v*/TLR2$^{+/+}$, and *W/W^v*/TLR2$^{-/-}$ mice had no significant differences in the number of peritoneal mast cells ($4.56 \times 10^4 \pm 0.50 \times 10^4$, $4.77 \times 10^4 \pm 0.37 \times 10^4$, $4.61 \times 10^4 \pm 0.37 \times 10^4$, and $3.57 \times 10^4 \pm 0.95 \times 10^4$ cells per mouse, respectively). More than 95% of these mast cells showed positive staining with Alcian blue and Safranin, which was the property of connective tissue mast cells. Their function evaluated by β-hexosaminidase release after FcεRI cross-linking was the same (data not shown).

Deficiency of mast cell TLR4 results in defective neutrophil recruitment and proinflammatory cytokine production in the peritoneal cavity after CLP. Since it has been reported that early leukocyte, especially neutrophil, recruitment into the peritoneal cavity after CLP is crucial for protection of mice from acute septic peritonitis–induced death, and that this step is highly dependent on mast cell activation by enterobacteria, we examined whether the defective leukocyte recruitment in the peritoneal cavity after CLP was associated with the deficiency of mast cell TLR4. Although there was no significant difference in the number of leukocytes in peritoneal fluid in *W/W^v*, *W/W^v*/TLR4$^{+/+}$, *W/W^v*/TLR4$^{-/-}$, *W/W^v*/TLR2$^{+/+}$, and *W/W^v*/TLR2$^{-/-}$ mice at 5 weeks after reconstitution, the number of leukocytes (most of them were neutrophils) in peritoneal exudates was significantly higher in *W/W^v*/TLR4$^{+/+}$, *W/W^v*/TLR2$^{+/+}$, and *W/W^v*/TLR2$^{-/-}$ mice than in *W/W^v*/TLR4$^{-/-}$ and *W/W^v* mice 6 hours after CLP ($P < 0.01$) (Figure 4b). These results were correlated with

the proinflammatory cytokine production in the peritoneal cavity. The levels of TNF-α, IL-1β, IL-6, and IL-13 in peritoneal cavities of W/W^v/TLR4$^{-/-}$, W/W^v/TLR2$^{-/-}$, and W/W^v/TLR2$^{-/-}$ mice 6 hours after CLP were significantly higher than those of W/W^v and W/W^v/TLR4$^{-/-}$ mice ($P < 0.01$ in each cytokine) (Figure 4c).

TLR2 on mast cell- dependent acute skin responses to PGN. To study whether mast cell TLR2 is a physiologically relevant receptor for PGN in vivo, we compared the ability of intradermal injection of PGN to cause acute and late skin reactions in W/W^v mice and W/W^v mice reconstituted with TLR2$^{-/-}$ or TLR4$^{-/-}$ BMMCs. The vascular permeability, evaluated by extravasation of Evans blue dye, was increased significantly by PGN ($P < 0.001$) but not LPS application in wild-type mice compared with vehicle control (Figure 5a). This reaction was dependent on the skin mast cells, since mast cell-deficient W/W^v mice showed significantly less reaction ($P < 0.001$) (Figure 5b) and reconstitution of skin mast cells with BMMCs from wild-type mice increased the extravasation of the dye almost to the level of normal mice. This reaction was also mediated by TLR2, since TLR2$^{-/-}$ mice and W/W^v mice whose skin mast cells were reconstituted with intradermal injections of TLR2$^{-/-}$ BMMCs did not show significant response to PGN ($P < 0.001$). The reactions of TLR4$^{-/-}$ mice and TLR4$^{-/-}$ BMMC-reconstituted mice were not significantly different from those of wild-type mice and mice reconstituted with wild-type BMMCs (Figure 5b).

The mast cells in the skin 5 weeks after reconstitution showed no differences in number between W/W^v/wild-type (W/W^v/WT), W/W^v/TLR4$^{-/-}$, and W/W^v/TLR2$^{-/-}$ mice (15.8 ± 4.4, 15.2 ± 4.9, and 17.8 ± 8.0 per linear millimeter of ear skin, respectively), and they were both Alcian blue- and Safranin-positive, suggesting that they were connective tissue-type mast cells (Figure 5c). The histopathologic changes of the skin were investigated at 15 minutes and 4 hours after PGN or LPS injection. PGN challenge to W/W^v/WT and W/W^v/TLR4$^{-/-}$ but not W/W^v/TLR2$^{-/-}$ mice resulted in degranulation of skin mast cells (Figure 5d, left column). The local vasodilatation began at 5 minutes after PGN injection in W/W^v/WT and W/W^v/TLR4$^{-/-}$ but not W/W^v/TLR2$^{-/-}$ or W/W^v mice, and vasodilatation gradually increased,

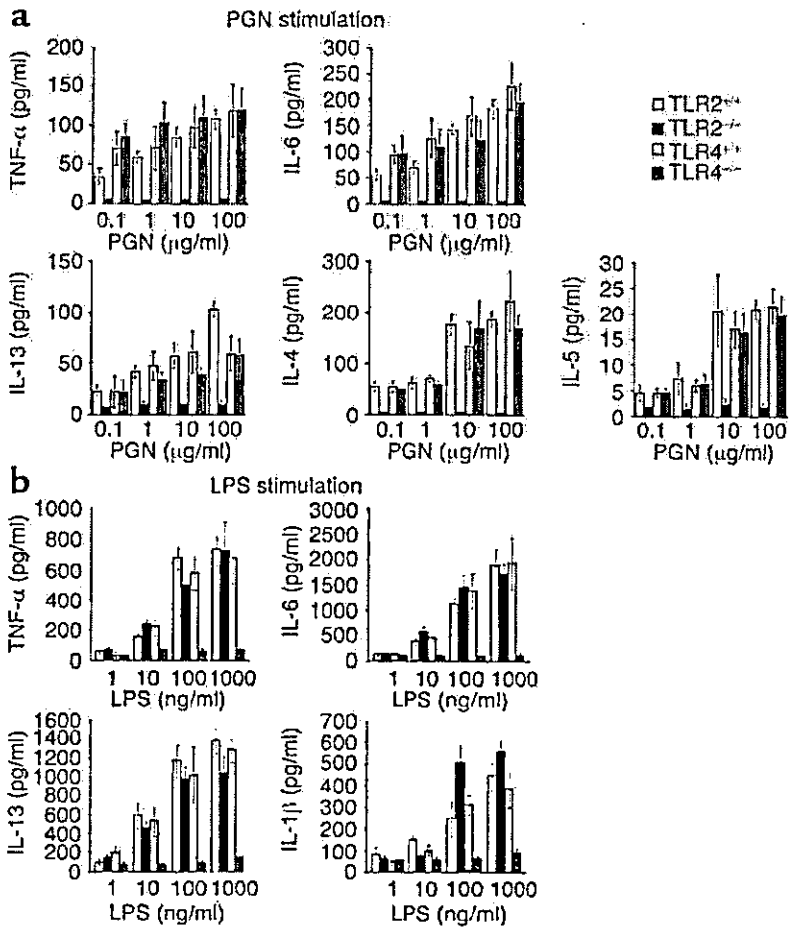

Figure 2
PGN-TLR2 stimulation and LPS-TLR4 stimulation lead to different cytokine production by BMMCs. BMMCs from TLR2$^{+/+}$ (white bars), TLR2$^{-/-}$ (black bars), TLR4$^{+/+}$ (light gray bars), and TLR4$^{-/-}$ (dark gray bars) were stimulated with indicated concentrations of PGN (a) or LPS (b), 3 hours for TNF-α and 6 hours for other cytokines. The concentrations of cytokine in the supernatant were determined as described in Methods. Data shown are mean ± SD of three experiments conducted with different BMMC preparations.

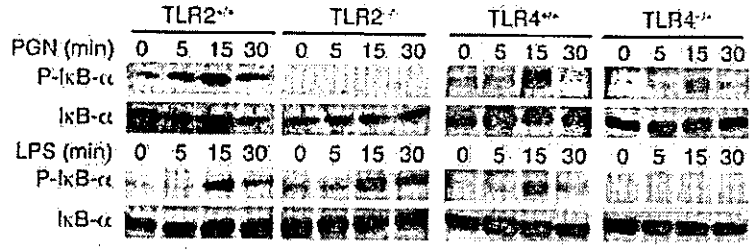

Figure 3
Stimulation of mast cells via both TLR2 and TLR4 leads to activation of NF-κB signaling. BMMCs were stimulated with 100 μg/ml of PGN or 50 ng/ml of LPS for 5, 15, and 30 minutes. Then lysates were subjected to SDS-PAGE and immunoblotted with antibody to phosphorylated IκB-α (P-IκB-α). The same blots were stripped and reblotted with antibody to IκB-α. The result shown is a representative of three experiments conducted with different BMMC preparations that had similar results.

becoming marked at 15 minutes, accompanied by granulocyte infiltration in which the predominant cells were neutrophils (Figure 5d, middle column). Four hours after injection, wheal reaction was decreased but persistent accumulation of neutrophils was still obvious (Figure 5d, right column). In contrast, saline-injected skin had intact mast cells without degranulation in all mice (Figure 5c, bottom row). These reactions, especially degranulation and vasodilatation, were specific to PGN challenge, since LPS did not cause degranulation of mast cells nor vasodilatation of the skin at 15 minutes (Figure 5d, bottom row). Interestingly, LPS caused some accumulation of neutrophils 4 hours after application, but the number of accumulating neutrophils was much less than after PGN application (Figure 5d and Table 1). These histopathological changes (infiltration of neutrophils and mast cell degranulation) were quantitatively evaluated and are shown in Table 1.

Discussion

This study clearly shows that mast cells can function as effector cells in both host defense and pathogenesis of allergic disease through the direct activation of TLR4 and TLR2 on mast cells by microorganisms.

Mast cells represent a potential source of multifunctional cytokines that may participate in the recruitment and activation of other cells in the inflammatory microenvironment (27). Mast cells responded to PGN or LPS via TLR2 or TLR4, respectively. Mast cell responses to LPS are completely impaired in the absence of the TLR4 but not the TLR2 molecule, suggesting that TLR2 is not involved in LPS response in mast cells and that the LPS-induced TLR4 responsiveness was not affected by the existence of the TLR2 molecule. A reciprocal result was obtained in PGN-TLR2 stimulation of mast cells.

Interestingly, upon PGN stimulation, mast cells released more IL-4 and IL-5, less TNF-α, and no IL-1β, even though TNF-α and IL-1β were predominant cytokines produced by mast cells upon LPS stimulation via TLR4 (18). In addition, PGN could induce degranulation of mast cells via TLR2 accompanied by Ca^{2+} influx, whereas the activation of mast cells by LPS via TLR4 did not lead to degranulation (18). So far, we still do not know how TLR2 is different from TLR4 in the signaling pathways of mast cell activation. To the extent that we analyzed the phosphorylation pattern of IκB-α and mitogen-activated protein kinases, we did not observe clear differences in TLR2 and TLR4 signaling pathways in mast cells (Figure 3 and data not shown), even though the severely impaired proinflammatory

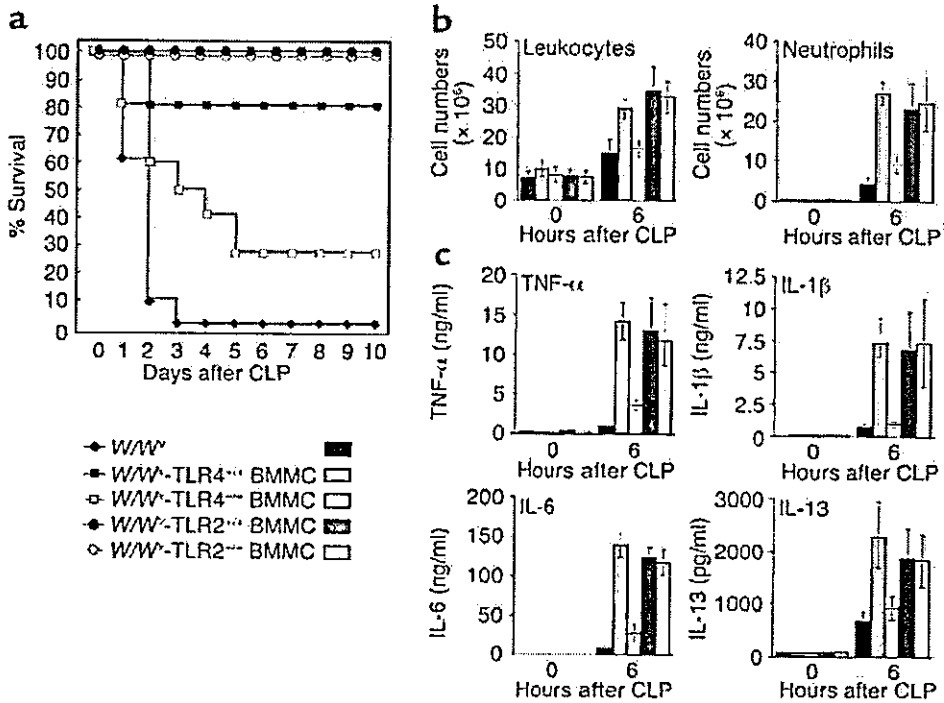

Figure 4
Mast cell TLR4 but not mast cell TLR2 is required for the full expression of innate immunity in a mast cell-dependent sepsis model. (a) *W/Wv* mice without reconstitution or 5 weeks after reconstitution with BMMCs from TLR4$^{+/+}$, TLR4$^{-/-}$, TLR2$^{+/+}$, or TLR2$^{-/-}$ mice (6–16 mice per group) underwent CLP. The mortality was observed and expressed as percent survival. *W/Wv* mice without BMMC reconstitution (injected with saline) were the control group. (b) Six hours after CLP, the mice were killed and peritoneal fluid was investigated. The total numbers of leukocytes and neutrophils were estimated by differential count and expressed as total cells per mouse. (c) The levels of indicated cytokines were measured by ELISA kit. Data shown are mean ± SD of three mice.

cytokine production of TLR2- or TLR4-deficient mast cells was correlated with the loss of NF-κB activation. Although the intracellular domain of TLRs is highly conserved in each TLR (29), recent findings suggest that downstream events are not always mediated by common components such as MyD88, an adaptor protein that links to the IL-1R–associated protein kinase and to the TNF receptor-activated factor 6 (30, 31). As TLR4 can activate both MyD88-dependent and -independent pathways that lead to different induction of genes in macrophages upon LPS stimulation (32–34), it is possible that TLR2 and TLR4 use different pathways for activation of mast cells. While TLR4 recognizes relatively few ligands, TLR2 recognizes a wide variety of infectious pathogens (34). Since it is reported that ligand specificity and signal-transducing ability of TLR2 are determined by heterodimeric interactions with other TLRs, such as TLR6 or TLR1 (35, 36), it would be useful to determine whether heterodimeric interaction of TLR2 and TLR6 is also required in mast cells. In particular, degranulation is a quite specific biological reaction confined to mast cells; it would be interesting to determine the TLR2-mediated specific signaling pathway that leads to this biological response. A recent report

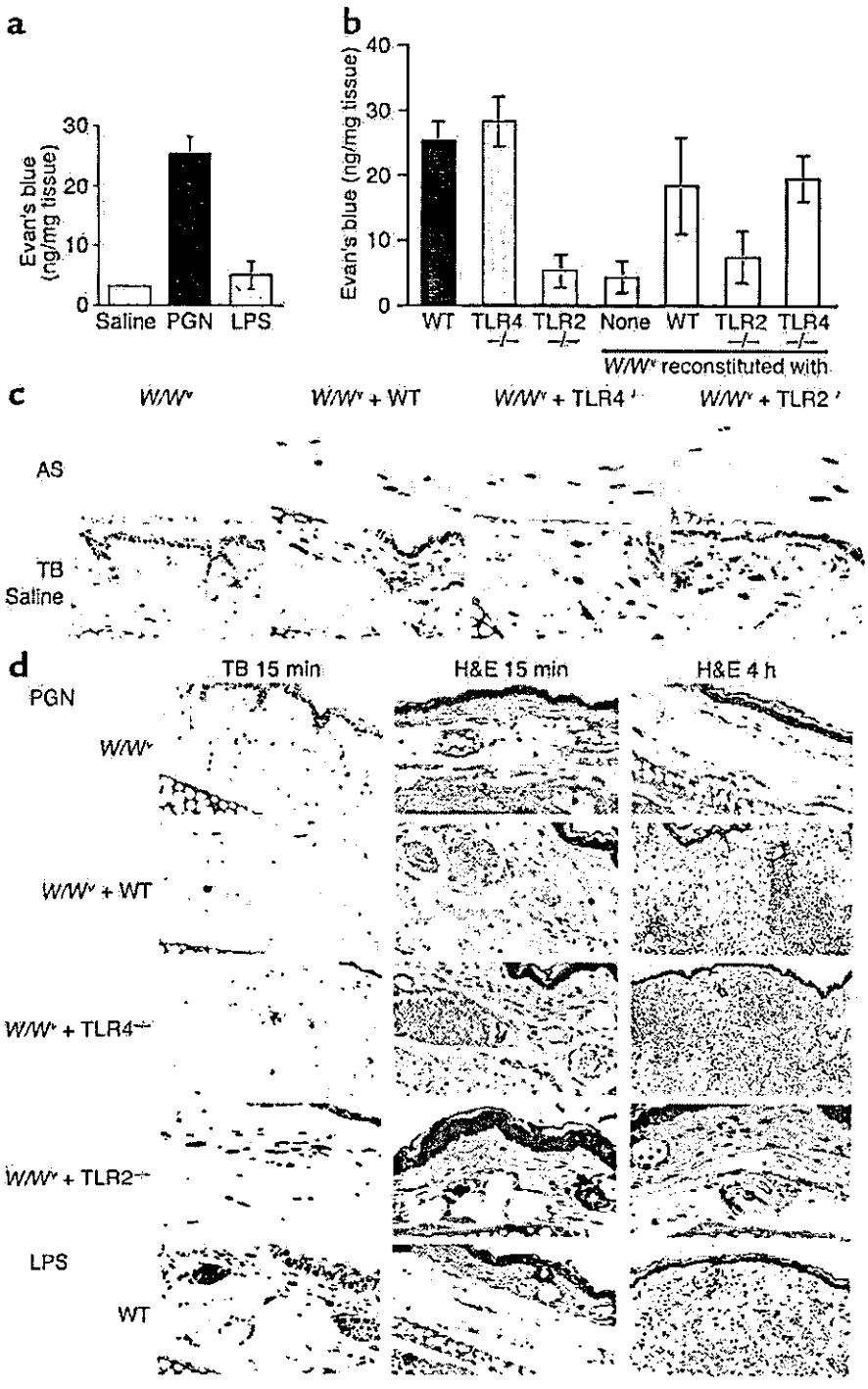

Figure 5
TLR2 on mast cell-dependent skin inflammation upon application of PGN. Reconstitution of skin mast cells of *W/Wᵛ* mice by TLR2 $^{-/-}$, TLR4 $^{-/-}$, or wild-type BMMCs was done as described in Methods. Wild-type mice (**a**) or indicated mice (**b**) were intravenously injected with 0.5% Evans blue 5 minutes before intradermal application and 20 μl of PGN (100 μg/ml) or LPS (100 ng/ml) into the left ear or saline into the right ear. The mice were sacrificed 15 minutes after PGN or LPS injection, and dye contents in the tissues were measured as described in Methods. Data shown are mean ± SD of three mice. (**c**) Reconstitution of skin mast cells of *W/Wᵛ* mice by TLR2 $^{-/-}$, TLR4 $^{-/-}$, or wild-type BMMCs was histologically confirmed by Alcian blue and Safranin staining (AS; without any treatment; upper panels) and Toluidine blue staining (TB; saline-injected; lower panels). (**d**) Mice were intradermally injected with PGN (upper four rows) or LPS (bottom row) as described above. Mice were sacrificed 15 minutes (left and middle columns) or 4 hours later (right column), and ear sections from *W/Wᵛ* (first row), *W/Wᵛ*/wild-type (*W/Wᵛ*/WT; second row), *W/Wᵛ*/TLR4 $^{-/-}$ (third row), or *W/Wᵛ*/TLR2 $^{-/-}$ mice (fourth row) were stained with H&E (middle and right columns) or Toluidine blue (TB; left column) for histological evaluations. All magnification is ×600, except the figures at 4 hours after PGN application from *W/Wᵛ*/WT and *W/Wᵛ*/TLR4 $^{-/-}$ and at 4 hours after LPS application (all ×200).

Table 1
TLR2-dependent activation of mast cells and infiltration of neutrophils in the skin upon PGN application

Reagent applied		PGN				LPS
Mice		W/W^v	W/W^v + WT	W/W^v + TLR4$^{-/-}$	W/W^v + TLR2$^{-/-}$	WT
Mast cell degranulation (%)[A]	Extensive	0	83.3 ± 3.1	86.3 ± 4.7	3.7 ± 1.5	2.3 ± 0.6
	Slight-Moderate	0	11.0 ± 4.6	9.3 ± 4.9	16.3 ± 3.1	8.3 ± 5.1
	None	0	5.7 ± 4.0	4.3 ± 0.6	80.0 ± 3.6	89.3 ± 4.7
Neutrophils (no./mm^2)[B]	0 h	1.3 ± 1.2	1.3 ± 0.6	1.7 ± 0.6	2.3 ± 1.5	2.7 ± 1.2
	4 h	3.7 ± 0.6	219.3 ± 73.9[C]	198.7 ± 57.3[C]	6.3 ± 1.5	36.3 ± 8.5[D]

[A]Mast cells were classified into three categories: extensively degranulated (50% of the cytoplasmic granules exhibiting fusion, staining alterations, and extrusion from the cell), slightly to moderately degranulated (10–15% of the granules exhibiting fusion or discharge), or normal. [B]The number of dermal neutrophils present at the reaction sites was determined as described in Methods and expressed as the number of cells per square millimeter of dermis. Data shown are mean ± SD of three mice. [C]$P < 0.01$ compared with the number of neutrophils in PGN-applied skin of W/W^v and W/W^v/TLR2$^{-/-}$. [D]$P < 0.01$ compared with the number of neutrophils in PGN-applied skin of W/W^v/WT, and W/W^v/TLR4$^{-/-}$.

suggests that JAK3 is a key regulator of mast cell–mediated innate immunity, especially TNF-α production from mast cells after *E. coli* stimulation and clearance of GNB (37). Thus, it would also be helpful to know the relationship of JAK3 activation and TLR signaling in mast cells upon stimulation with GNB.

Mast cell–deficient WBB6F$_1$-W/W^v mice and the model of "mast cell knock-in" mice could be used to analyze the roles of mast cells in biological response in vivo. Consistent with previous work (18), WBB6F$_1$-W/W^v mice were successfully reconstituted with BMMCs from genetically unmatched TLR4$^{+/+}$ and TLR4$^{-/-}$, or TLR2$^{+/+}$ and TLR2$^{-/-}$ mice that are a mixture of C57BL/6 and 129SvJ strains. During the study period, the reconstituted mice did not show any abnormality. The mast cells in both peritoneal cavity and skin 5 weeks after reconstitution were no different in number, ability to develop, and ability to degranulate in vitro. Therefore, the differences in the mortality of mice after CLP and in the acute skin reaction after PGN injection were not due to the difference in development of BMMCs in the W/W^v environments. To determine why mast cells can survive and differentiate in genetically unmatched environments, further investigations are required.

We have previously demonstrated that BMMCs from C3H/HeJ mice, which had constitutive mutation of the intracellular domain of TLR4, show impaired response to LPS by means of cytokine production and NF-κB activation. Our present study using TLR4$^{-/-}$ mice, which lack whole molecules of TLR4, had results similar to those of C3H/HeJ mice. This again clearly indicates that TLR4 but not TLR2 is the critical receptor for activation of mast cells by LPS both in vitro and in vivo. Compared with in vitro results where BMMCs from TLR4$^{-/-}$ mice completely lacked the LPS responses, determined by proinflammatory cytokine production and NF-κB activation, it seemed that W/W^v/TLR4$^{-/-}$ mice showed a better survival rate than unreconstituted W/W^v mice in the CLP model. These differences may reflect the involvement of other TLRs or other molecules, such as CD48 and C3 receptors, in activation of mast cells upon whole-microorganism stimulation in vivo (38, 39).

Since PGN caused degranulation and cytokine production of mast cells in vitro, the role of mast cells in PGN-induced cutaneous acute response was investigated. Wheal reaction was observed within 5 minutes after PGN challenge, but not after vehicle injection, and was gradually enhanced after 15 minutes. We used a mast cell knock-in system with W/W^v mice to exclude the possibility that mediator released from other endogenous cells that express TLR2 can cause increased vascular permeability. The skin reactions were observed within 5 minutes and were not observed in TLR2$^{-/-}$ mice, W/W^v mice, or W/W^v mice reconstituted with TLR2$^{-/-}$ BMMCs, strongly suggesting that degranulation of mast cells through TLR2 activation was responsible for these reactions. In fact, we could observe the highly degranulated mast cells 15 minutes after PGN application in the skin of wild-type, W/W^v/WT, and W/W^v/TLR4$^{-/-}$ mice, but not in the skin of TLR2$^{-/-}$ and W/W^v/TLR2$^{-/-}$ mice, in the histological examination (Table 1). Furthermore, TLR4$^{-/-}$ mice and W/W^v/TLR4$^{-/-}$ mice showed responses similar to those of wild-type mice, suggesting that TLR4 (on mast cells or other cells) in the skin is not involved in PGN-induced vasodilatation. In addition, application of LPS in the skin did not lead to degranulation of mast cells or vasodilatation similar to that induced by PGN, suggesting that response to LPS in the skin has a biologically different character from that of PGN-induced response. Although it has been reported that mast cells can be activated by GPB through IgE bound on cells (40), this is, to our knowledge, the first evidence that shows the direct activation of mast cells via a specific pathogen recognition receptor, TLR2.

In summary, this study clearly shows that TLR4 but not TLR2 is the important molecule for mast cells in response to LPS challenge and that it functions in innate immunity against enterobacterial infection. In contrast, TLR2 but not TLR4 is responsible for activation of mast cells by *S. aureus* that might be involved in the pathogenesis of atopic dermatitis. Finding the microbial ligands and relevant receptors on mast cells, as well as signaling pathways that can activate mast cells, will elucidate the complexity of innate immunity and the roles of mast cells in conditions other than allergy.

Also, the clarification of the mechanisms of mast cell activation by microorganisms through these TLRs may offer new insight into the treatment of allergic disease or inflammation in which bacterial infections and mast cell activations are common.

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CUTTING EDGE

Cutting Edge: VacA, a Vacuolating Cytotoxin of *Helicobacter pylori*, Directly Activates Mast Cells for Migration and Production of Proinflammatory Cytokines[1]

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Mucosal mast cells strategically located at the optimal site interact with invading bacteria. Presence of VacA, the virulent *Helicobacter pylori* cytotoxin, is correlated with the severity of *H. pylori*-induced gastritis. To examine the mechanisms of inflammation in *H. pylori*-induced gastritis, we administered VacA to the mice. Inoculation of VacA resulted in epithelium vacuolization and marked infiltrations of mast cells and mononuclear cells into the mucosal epithelium within 24 h. In an in vitro study using bone marrow-derived mast cells, VacA directly bound and showed a chemotactic activity to the mast cell. In addition, VacA induced bone marrow-derived mast cells to produce proinflammatory cytokines, TNF-α, macrophage-inflammatory protein-1α, IL-1β, IL-6, IL-10, and IL-13 in a dose-dependent manner without causing degranulation. The present study suggests that early activation of mast cells by VacA may be the host early response to clear the bacteria and also may contribute to the pathogenesis of *H. pylori*-induced gastritis. *The Journal of Immunology,* 2002, 168: 2603–2607.

Helicobacter pylori infection is a worldwide problem, and it is now widely accepted that it is one cause of gastric inflammation. The ongoing state of infection might eventually result in the development of atrophic changes and carcinoma (1). VacA, the virulent cytotoxin of *H. pylori*, has been found mainly in the gastric mucosa and its ability to cause vacuolization has been widely documented in many cell types. The similar vacuoles have also been observed in the gastric epithelia of patients with active chronic gastritis associated with *H. pylori* infection (2, 3), and the colonization of those strains expressing higher levels of toxin is correlated with the severity of the gastritis lesion, indicating that this toxin plays significant roles in the pathogenesis of *H. pylori*-induced gastritis (4–6). Oral treatment of mice with either crude extracts of *H. pylori* or the purified cytotoxin induces gastric injury that resembles the pathology observed in humans (7,8). Not only cells of the specific immune system, but also the cells of the innate system such as mast cells, monocytes, and neutrophils are involved in the ongoing state of inflammatory response against this bacterium (9–11).

Mast cells are known as the main effector cells in IgE-mediated allergic responses, but they also play important roles in innate immune responses against bacteria by releasing cytokines and by recruitment of polymorphonuclear leukocytes (12, 13). Although several lines of evidence support the hypothesis that mast cells participate in the gastric inflammation in *H. pylori*-infected peptic ulcer (9), it remained to be clarified the exact mechanisms of mast cell activation at the site of *H. pylori* infection.

In this study, we demonstrated that oral treatment of mice with VacA caused acute inflammation of gastric mucosa with mast cell accumulation. Moreover, we demonstrated direct activation of mast cells by VacA in vitro by showing the chemotactic activity, cytokine production through the binding of VacA to mast cells. Thus, this study has explored the role of this virulence factor in the activation of mast cells, which might be the initial host response to *H. pylori* infection, and discussed the possible involvement of mast cell activation by VacA in the pathogenesis of *H. pylori*-induced gastritis.

Materials and Methods

Animals

BALB/c, C3H/HeN, and C3H/HeJ mice were purchased from Japan SLC (Hamamatsu, Japan). All animal experiments were performed under approved manual of the Institutional Review Board of Juntendo University (Tokyo, Japan).

Preparation of purified VacA and Ab to VacA

The purified VacA from toxin-producing strain *H. pylori* (ATCC 49503; American Type Culture Collection, Manassas, VA) and polyclonal Ab to VacA (14) were kindly provided by Dr. K. Yahiro and Dr. A. Wada (Nagasaki University, Nagasaki, Japan). The preparation of VacA was done as

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previously described (14). In vitro vacuolating activity of VacA was tested using rapid neutral red uptake assay (15). Because acidic treatment was associated with activation and increased binding of VacA to the receptor of susceptible cells (16), for in vitro experiment VacA was activated by bringing the pH to 1.5 with 1 N HCl for 10 min, and then neutralized to pH 7 with 1 N NaOH.

In vivo administration of VacA to mice

Six-week-old BALB/c mice were deprived of food, but allowed free access to water. After 24 h, the mice received oral administration of saline (0.3 ml) or 20 μg of purified VacA in saline. BSA was used as a high protein control. The administration was repeated after 24 h. After a further 24 or 72 h, the mice were killed and the stomach was excised. The frozen sections were stained by H&E or Alcian blue-Safranin for histological evaluation.

Generation of bone marrow-derived mast cells (BMMCs)[3]

BMMCs were generated from the femoral bone marrow of mice and maintained in the completed medium in the presence of 10% PWM-stimulated spleen-conditioned medium as a source of mast cell growth factors, as previously described (13). After 4 wk of culture, >99% of cells were identifiable as mast cells as determined by toluidine blue staining and flow cytometric analysis of cell surface expression of c-*kit* and FcεRI.

Flow cytometric analysis of VacA binding to mast cells

BMMCs (1×10^6 cells/ml) were incubated with 10 μg/ml of activated VacA in balanced salt solution containing 2% BSA for 30 min on ice. After the cells were washed with balanced salt solution twice, the cells were incubated with 1 μg/ml of rabbit anti-VacA Ab, and then with 1 μg/ml of FITC-conjugated goat anti-rabbit IgG (Wako Pure Chemical, Osaka, Japan). The 2.4G2 (BD PharMingen, San Diego, CA) was used as IgG receptor-blocking Ab. The stained cells were analyzed by FACSCaliber (BD Immunocytometry Systems, Mountain View, CA).

Chemotaxis assay of mast cells

Chemotaxis assay of BMMCs was performed as previously described with slight modifications using membrane (5 μM pore size; NeuroProbe, Gaithersburg, MD) coated with human fibronectin (17). VacA in RPMI 1640 containing 0.5% BSA was added into the lower chamber (25 μl). A total of 50 μl of BMMCs (4×10^6 cells/ml) in the same medium were loaded in the upper chamber, and then incubated for 4 h at 37°C. The number of cells adherent to the underside of the filter was counted after staining the membrane with DiffQuick (Kokusai Shiyaku, Kobe, Japan). Stem cell factor (SCF) was used as a positive control.

Measurement of cytokine production from BMMCs

BMMCs (1×10^6 cell/ml) in complete cultured media were stimulated with the indicated concentration of purified VacA at 37°C. According to the preliminary study, the optimal time for stimulation of BMMCs by VacA was 3 h for TNF-α and 6 h for IL-1β, IL-6, IL-10, IL-13, and macrophage-inflammatory protein-1α. The levels of each cytokine in the culture supernatants were determined by ELISA according to the manufacturer's instructions (Genzyme Techne, Minneapolis, MN).

Statistical analysis

Statistical analysis of data was performed using the Student *t* test.

Results and Discussion

In vivo effects of oral administration of VacA

Oral administration of VacA in mice caused several aspects of the histological lesions in epithelium of gastric mucosa, namely loss of gastric gland architecture including epithelial vacuolization, edema, erosion, necrosis, and exfoliation 24 h after inoculation (Fig. 1*B*). The marked infiltrations of mast cells and mononuclear cells and a few eosinophils were observed 24 h after inoculation (Fig. 1, *D* and *F*). The mast cells located in the mucosal layer had spindle shape and less granules (Fig. 1, *G* and *H*). In contrast, the epithelium of gastric mucosa in control mice 24 h after inoculation of saline did not show any lesion, with rare mucosal mast cells (MMC) and no inflammatory cells (Fig. 1, *A*, *C*, and *E*). Administration of BSA as a high protein control did not show any epithelial lesions nor histological changes, as were seen in VacA-treated mice (data not shown). These pathological changes were not observed 72 h after inoculation of VacA with no mononuclear cell and less mast cell infiltrations (data not shown). Interestingly,

[3] Abbreviations used in this paper: BMMC, bone marrow-derived mast cell; SCF, stem cell factor; MMC, mucosal mast cell.

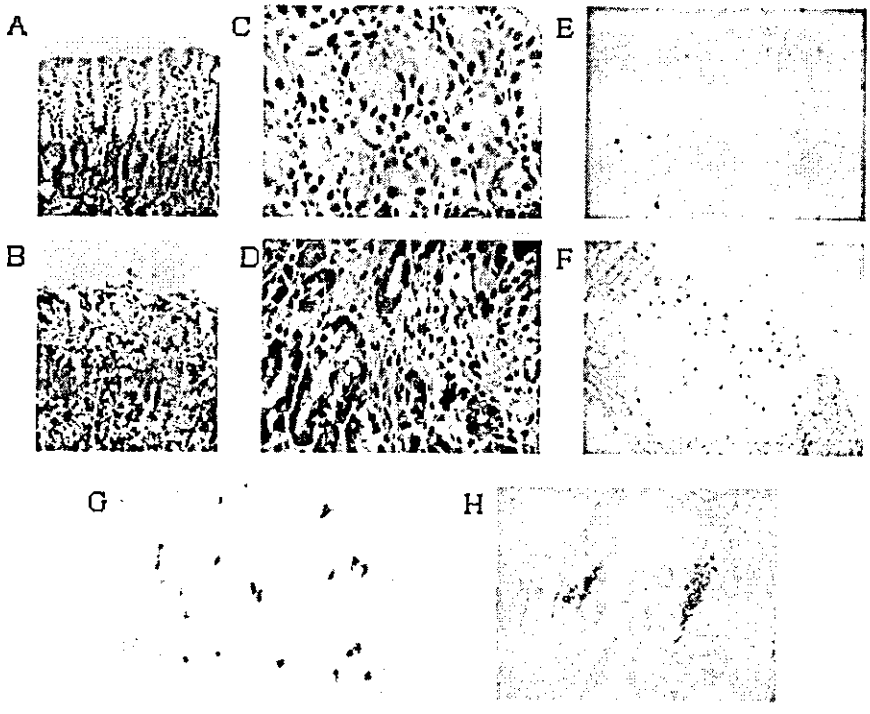

FIGURE 1. VacA causes cell vacuolization and damage to the gastric mucosa with infiltration of mast cells. Gastric tissues of BALB/c mice obtained 24 h after the second inoculation of VacA or saline were stained with H&E (*A* and *B*, ×200, and *C* and *D*, ×400) or Alcian blue/Safranin (*E* and *F*, ×100; *G*, ×400; and *H*, ×1000). Epithelium of the gastric mucosa of control mouse treated with saline was normal with no evidence of inflammation (*A* and *C*) or mast cell infiltration (*E*), whereas that of VacA-treated mouse showed evidence of various epithelial damages including marked vacuolization, edema, necrosis, intercellular mononuclear cell infiltration (*B* and *D*), and mast cell infiltration (*F–H*). Sections shown were representative of five mice in a control and VacA-treated group with similar results.

there was no significant neutrophil infiltration in the epithelium during the course of experiment. This result was consistent with the report that VacA itself did not cause accumulation of neutrophils (7). In the more controlled mouse model infected with *H. pylori*, the first signs of gastric inflammation with mononuclear cell infiltration occurred 3 wk after inoculation (4, 18). It is likely that bacteria should colonize and multiply before the onset of disease, and that simultaneously the VacA is gradually accumulated to the levels which are enough for the development of the mucosal damage as observed in this study. In humans, *H. pylori* infection produces a predominant infiltration of polymorphonuclear leukocytes, little evidence of mast cell infiltration, and usually no evidence of epithelial vacuolization. The differences between this animal model and human *H. pylori* infection may be partly explained by the fact that we were observing very acute changes occurring within hours of exposure to the toxin and not to whole bacteria, which has more various effects on immune cells. Because *H. pylori* infection in humans is a slow progression and the gastric disease becomes manifest after prolonged infection, the early inflammatory changes are difficult to recognize. Also, the production of neutralizing Abs to the 87-kDa protein (VacA) in sera of *H. pylori*-infected patients (2) may help to neutralize the toxin; and therefore, remove its effects of the chronic infection on gastric mucosa. Thus, this model may be relevant to know some aspects of the immune responses in initial acute phase of *H. pylori* infection.

VacA is a chemoattractant for mast cells

Using BMMCs, we next investigated whether the effects of VacA on the accumulation of MMC in epithelium were a direct effect of VacA to the mast cell. As shown in Fig. 2*A*, BMMCs showed a migratory response to VacA in a dose-dependent manner. Compared with the potent-positive control, SCF, the chemotactic activity of VacA was weaker. This effect of VacA was specific, because the treatment of neutralizing polyclonal Ab to VacA abolished the migratory responses induced by VacA (Fig. 2*B*). To determine whether the migratory responses induced by VacA were due to directional (chemotaxis) or random (chemokinesis) activation of mast cells, we performed checkerboard analysis. As shown in Table I, in addition to the gradient-dependent migration of BMMCs to various concentrations of VacA in upper and lower wells, increasing concentrations of VacA in the upper wells led to slight dose-dependent migration of mast cells to the lower wells, suggesting that VacA has predominant chemotaxis activity and slight chemokinesis activity. Thus, the accumulation of mast cells in vivo after administration of VacA seems to be a direct effect of VacA to the mast cells. Nevertheless, we cannot exclude the possibility that there are indirect effects of VacA on MMC in vivo via the activation of cells located in the gastric epithelium. Although we do not know where so many mast cells migrated from to the epithelium within a day, the vacuolization and disruption of gastric epithelial cell by VacA may increase the penetration of VacA and the chance to interact with mast cells in the lamina propria.

Table I. *Checkerboard analysis of the number of migrating mast cells in response to VacA*[a]

VacA in Lower Chamber (μg/ml)	VacA in Upper Chamber (μg/ml)			
	0	5	10	20
0	28.8 ± 7.4	57.2 ± 17.3	110.5 ± 23.0	140.4 ± 37.7
5	112.4 ± 20.7	72.7 ± 19.5	108.0 ± 23.2	160.6 ± 23.0
10	189.9 ± 54.1	119.8 ± 20.8	111.6 ± 22.6	154.8 ± 20.4
20	452.7 ± 271.6	374.4 ± 51.2	325.6 ± 58.5	164.8 ± 24.0

[a] VacA at various concentrations was added to the upper and lower chambers, and the number of migratory cells to the lower compartment was counted 4 h later. The average number of migrated cells in the five high power fields was shown as the mean ± SD of three independent experiments. Each condition was done in triplicate.

VacA directly binds to BMMCs

Because BMMCs did show migratory responses to VacA, we also determined whether VacA could directly bind to the BMMCs. As shown in Fig. 3, immunofluorescence FACS analysis demonstrated that VacA could bind directly to BMMCs of BALB/c and C3H/HeJ mice. We have previously demonstrated that VacA susceptible cells expressed a VacA receptor (16), the receptor protein-tyrosine phosphatase β. Although we have not investigated in this study whether the direct binding of VacA to BMMCs was mediated by the same receptor, receptor protein-tyrosine phosphatase β, it is interesting to know that the activation of mast cells by VacA is mediated by this receptor.

VacA induces cytokine production from BMMCs but not degranulation

Because activated mast cells have ability to produce various cytokines that play important roles in recruitment and activation of inflammatory cells, we next examined whether VacA enabled the stimulated mast cells to produce and secrete proinflammatory cytokines (19). As shown in Fig. 4, BMMCs could respond to produce TNF-α, macrophage-inflammatory protein-1α, IL-1β, IL-6,

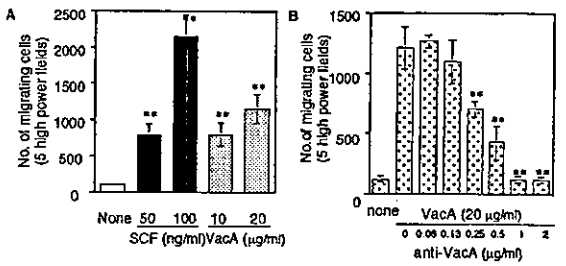

FIGURE 2. VacA stimulates mast cell chemotaxis in vitro. BALB/c BMMCs were used in microchemotaxis assays. *A*, VacA at indicated concentrations was added to the lower chambers of the 48-well chemotaxis chamber, and incubated for 4 h. SCF was used as positive control. *B*, VacA (20 μg/ml) stimulated chemotaxis of BMMCs was blocked by indicated concentrations of anti-VacA polyclonal Ab. The total number of migratory cells in the five high power fields was counted. Data shown was the mean ± SD of three independent experiments. **, $p < 0.01$, significantly different from the mean value of the corresponding control.

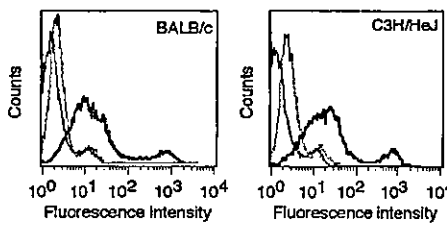

FIGURE 3. Direct binding of VacA to mast cells. VacA binding to BALB/c and C3H/HeJ BMMCs was investigated by flow cytometry using BMMCs alone (solid line) or with FITC-conjugate (dotted line), or with anti-VacA and FITC-conjugate (bold line). Nonspecific binding by FcγR on BMMCs was blocked by 2.4G2 (anti-FcγRII/III) Ab. Data shown were representatives of two independent experiments with similar results.

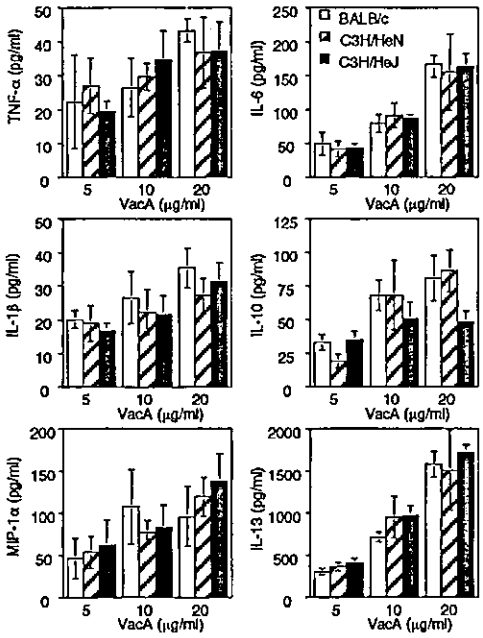

FIGURE 4. Cytokine production from mast cells upon VacA stimulation. BALB/c, C3H/HeN, and C3H/HeJ BMMCs were incubated with VacA at the concentrations as condition indicated in *Material and Methods*. The levels of cytokines in the supernatants were measured by ELISA. Data shown were mean ± SD of three independent experiments. Viability of the cells under each condition was >98%, as assessed by trypan blue dye exclusion test.

IL-10, and IL-13 upon VacA stimulation in a dose-dependent manner. There were no significant differences in the production of cytokines from BMMCs of three strains of mice, BALB/c, C3H/HeN, and C3H/HeJ ($p > 0.05$), suggesting that unlike the responses of LPS from *H. pylori* (20), VacA-induced activation of mast cells was not mediated by Toll-like receptor 4. The viability of BMMCs at 3 or 6 h after stimulation with VacA was >98%, indicating cytokine release was not due to cytotoxic effect of VacA. The mucosal biopsy specimens from patients with *H. pylori* infection contain significantly elevated levels of TNF-α, IL-1β, IL-6, and IL-8 compared with those in specimens from uninfected individuals (21–23). However, it has been still unclear what products of bacteria do stimulate host cells or which cells produce these cytokines in response to bacterial products. Our results suggest that mast cell is the one candidate for producing cells of these cytokines upon VacA stimulation. Interestingly, the activation of mast cells did not lead to the infiltration of neutrophil in vivo (24 and 72 h after administration). We do not know the reason for that. VacA may not be a proper stimulant for mast cells to produce TNF-α, which is thought to be an important cytokine for recruitment of neutrophils (12), because the levels of TNF-α produced by mast cells upon VacA stimulation were not so high as those by other stimulant (13). Furthermore, VacA itself failed to trigger the release of β-hexosaminidase from BMMCs (data not shown). Because it has been reported that *H. pylori* or *H. pylori* extracts potentiate histamine release from rat serosal mast cells in vitro and can induce mast cell degranulation in vivo (24), molecules other than VacA in *H. pylori* extracts might have degranulating activity for mast cells.

When we examined the effects of VacA on BMMCs after longer incubation, BMMCs were susceptible to VacA for vacuolization. After 8 h of incubation, BMMCs did not show vacuolization evaluated by measurement of neutral red uptake (OD value of control vs VacA (20 μg/ml): 0.084 ± 0.015 vs 0.083 ± 0.018, $p > 0.05$). The number of vacuolating mast cells significantly started to increase after 15 h of incubation (OD value of control vs VacA (20 μg/ml): 0.078 ± 0.017 vs 0.237 ± 0.032, $p < 0.01$), which might also explain the in vivo phenomenon that the infiltration of mast cells was prominent at 24 h but not at 72 h. After the accumulation of mast cells in the epithelium of gastric mucosa, they might die due to vacuolization.

In this study, we demonstrated that VacA, a toxin derived from *H. pylori*, could bind and directly activate mast cells for migration and production of proinflammatory cytokines. Although we still do not know what kinds of roles were played by these mast cells during *H. pylori* infection, it is possible that this might be a host early innate immune response to clear bacteria, and also this activation of mast cells by VacA may contribute to the pathogenesis of *H. pylori*-infected gastritis.

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Detergent-induced epidermal barrier dysfunction and its prevention

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Abstract

Various detergents are used as skin cleansing products. In some cases, skin cleanser removes not only dirt but also valuable skin lipids. Therefore, detergents may disrupt epidermal barrier function despite that using of detergents are required for good skin hygiene. Lipid supplements can reverse detergent-induced dysfunction of the skin barrier. Elevated transepidermal water loss (TEWL) and riboflavin penetration in 5% SLS-treated rat and human skin were reversed by supplementation of monoglyceride (MG), squalene (SQ), cholesterol ester (CE) and pseudo-ceramide (Cer2). MG and Cer2 achieved the best results. MG appears to inhibit elution of intercellular ceramides, since more ceramides remained when the detergent was supplemented with MG. Topical application of Cer2 is not effective for recovery from artificially induced barrier disruption, but supplemented Cer2 into skin cleanser has a beneficial effect for prevention of detergent-induced barrier disruption. In conclusion, the prevention of barrier disruption is most important matter for maintaining skin health and barrier function. Therefore, we think that Cer2-supplemented skin cleanser is useful for conservation of skin barrier function.

Keywords: Detergent; Barrier dysfunction; Ceramide; Supplement

1. Introduction

Washing and maintenance of cleanness are very important to skin hygiene. For these purposes, various detergents are used as skin cleansing products.

Often these products are claimed to be mild to the skin. Skin irritancy, evaluated with patch test [1,2], is regarded as important in claiming mildness of a detergent. Recently, the correlation of some skin diseases, such as atopic dermatitis, with epidermal barrier function [3–7] has demonstrated the importance of detergents. Detergent-induced barrier dysfunction [8–10] has become an important parameter to evaluate. The main performance

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of a skin cleanser is removing dirt and grime from skin surface. However, it is unavoidable to remove useful skin lipid with dirt [10]. If a skin cleanser has strong performance to clean skin, it may not only remove various dirt and grime but also extract excessive intercellular lipid. In this case, the skin cleanser disrupts not only skin moisturizing function but also skin barrier function [11,12].

In this study, we want to demonstrate that lipid supplements, especially pseudo-ceramide, in detergent solution are useful in reducing detergent-induced skin barrier dysfunction.

2. Materials and methods

2.1. Test materials

Sodium lauryl sulfate (SLS) (Wako Chemical, Osaka, Japan) solution dissolved in distilled water was used as the detergent solution. C18-monoglyceride (MG) (Sigma, St. Louis, MO), squalene (SQ) (Sigma), cholesterol ester (CE) (Sigma) and pseudo-ceramide type II (Cer2) (described previously [13] as BRS000) were used as lipid supplements in the detergent solution. Treatment solutions contained 0–2.5% lipid supplements in 5% SLS solution.

Skin cream formulas containing 5% Cer2 or Cer1 (described previously [13] as BRS001) and liquid body shampoo formulas containing 1 or 0% Cer2 were prepared by Kao Corporation.

2.2. Washing treatment

Treatment solutions were warmed to 40 °C in water bath before each treatment.

HWY/Slc hairless rat (SLC, Shizuoka, Japan) back skin was gently rubbed 20 times with a cotton ball soaked in 1 ml treatment solution, and then rinsed off with lukewarm tap water after remaining on the skin for 90 s. The skin surface was gently dried with a paper towel.

Glass cylinders, open at both ends, were fixed on healthy human forearms with adjustable belts and then treatment solution was poured into the cylinders. Volunteers held a bar connected to a reciprocal shaker for a set time. The treated arms were rinsed with lukewarm tap water and dried with paper towels.

2.3. Measurement of epidermal barrier function of rat skin

Transepidermal water loss (TEWL) of rat was measured at 22–24 °C (housing condition) with an Evaporimeter EP1 (Servo med, Stockholm, Sweden) before the daily washing treatment.

Measurement of riboflavin penetration (RP) was described previously [14]. A riboflavin-soaked cotton ball was attached to the treated skin for 1 min. This skin was wiped quickly and stripped with 2 cm × 2 cm (4 cm^2) cellophane tape (Nichiban, Tokyo, Japan). Each stripped tape was put into a test tube, and 1% SLS solution was poured into the tube to extract the riboflavin from the tape. Extraction solution was filtrated with a 0.45 μm micropore filter and measured with a fluorometer (Hitachi, Tokyo, Japan). Control tape, which was used to strip on non-treated skin, was used as background.

2.4. Measurement of epidermal function of human skin

Volunteers rested in the air-conditioned room (20 °C, 40%) for at least 30 min. Forearm or lower leg skin was measured for TEWL and skin conductance with Skicon-200 (IBS, Hamamatsu, Japan), and was evaluated for visual dryness score (0 (none) to 4 (severe)) with an expert grader.

2.5. Statistics

The one-way factorial analysis of variance and the Student–Newman–Keuls tests were used for data analysis.

3. Results

3.1. Effect of additional MG to relieve detergent-induced barrier disruption

We previously described [15] that dishwashing cleanser containing Mg was able to reduce deter-

gent-induced dry skin. In this study, we investigated the barrier-conservation effect of MG.

Five hairless rats were treated with each solution. Each dorsal abdomen was rubbed 20 times during 15 s washing period at each treatment. Treated area was rinsed off well with lukewarm water and dried with paper towel. This treatment was done once per day, 5 days per week for 3 weeks. Water-treated control used only deionized water.

Five percent SLS treatment caused significant increase in RP and TEWL values compared with water-treated control. On the other hand, 5% SLS containing 2.5% MG (SLS/MG) treatment did not cause significant increase of these barrier-related indexes. Both indexes of SLS/MG treatment were significantly lower than that of SLS treatment (Fig. 1).

Similar results were demonstrated in the human forearm washing test. We measured ceramide content of treated skin to confirm a relationship between effects of SLS/MG treatment and intercellular lipids.

Forearms of five healthy human volunteers were treated with cup-shake method. After 15 min washing treatment, fresh cylinders were fixed on treated areas and then extraction solvent was poured into them. Remaining skin ceramide was extracted and measured as previously described [15].

In this study, skin surface lipids were similarly extracted regardless of the supplementation or non-supplementation of MG. But extracted ceramides were increased by MG supplementation ($P < 0.05$; Fig. 2).

These results demonstrated that MG prevented ceramide elution during washing treatments.

3.2. Effects of other lipids

We examined the ability of other lipids to prevent detergent-induced barrier disruption. SQ (2.5%), CE (2.5%) or Cer2 (0.5%) were dissolved into 5% SLS solutions. Five animals were treated with each sample for 3 weeks. Supplemented SQ and CE only had lower preventative efficacy than MG. But 0.5% Cer2 had equal efficacy as 2.5% MG (Fig. 3).

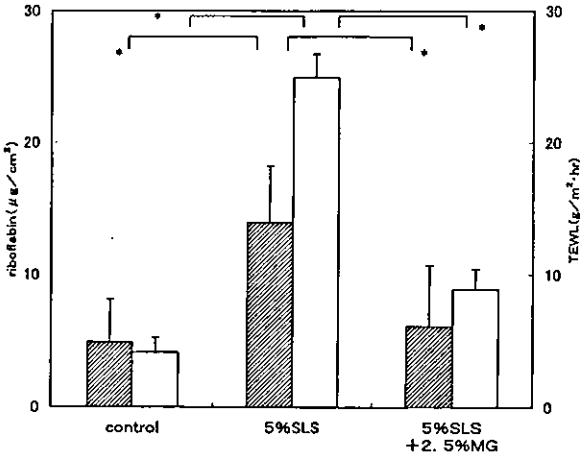

Fig. 1. MG supplementation effect for epidermal barrier function on rat back. Hairless rat back skin was treated with 5% SLS solution containing 2.5% MG. Five percent SLS treatment caused significant increase ($P < 0.05$ vs. control) of RP and TEWL rate. MG solution induced lower TEWL value and RP than that SLS alone treatment during the test period. RP (shadow bar) and TEWL (open bar) were measured (mean+S.D., $n = 5$).

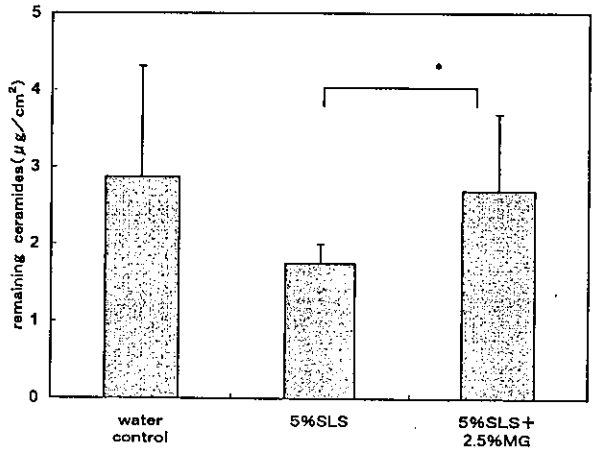

Fig. 2. Remaining ceramides amount after washing treatment with water, 5% SLS and 2.5% MG containing SLS solution. Glass cylinders were fixed on healthy human and then treatment solution was poured into the cylinders. The treated arms were rinsed with lukewarm tap water and dried with paper towels. Fresh cylinders were fixed on treated areas and extraction solvent poured into them. Extracted ceramides was decreased by MG supplementation ($P < 0.05$ vs. 5% SLS) (mean+S.D., $n = 5$).

3.3. Effect of ceramide

We examined the efficacy of Cer2 to prevent detergent-induced barrier disruption for human skin.

Forearms of 10 healthy volunteers were treated with cup-shake method once per day for 4 days. Supplemented 0.2% Cer2 in 2% soap solution had preventative effect as 1% MG. The washing treatment with 2% soap significantly increased the visual skin roughness score and the TEWL value, and significantly decreased the skin conductance value (Fig. 4).

The treatment with 2% soap containing 1% MG or 0.2% Cer2 did not cause significant increase in the visual scores and the TEWL values, and decrease in the skin conductance values. Supplemented MG and Cer2 were significantly effective in preventing detergent-induced deterioration of skin condition (Fig. 4).

3.4. Compare ceramide-supplemented SLS with topical application of ceramide

We compared ceramide-supplemented SLS treatment with topical application of ceramide after SLS treatment for their ability to reduce detergent-induced barrier disruption.

Topical application of Cer1 cream significantly reduced the TEWL value, but Cer2 did not reduce the TEWL value (Fig. 5). Cer1 cream had beneficial effect on recovery from detergent-induced barrier disruption.

Cer2 cream did not show the beneficial effect on the barrier recovery. But supplemented Cer2 was significantly effective in preventing detergent-induced barrier disruption.

3.5. Effect of Cer2-supplemented body shampoo formula

Twenty healthy volunteers were divided into two groups by visual scores of lower leg skin. Ten persons belonging to test group used Cer2-supplemented body shampoo, and remaining 10 persons belonging to control group used placebo (without Cer2) body shampoo at home once per day for 1 month.

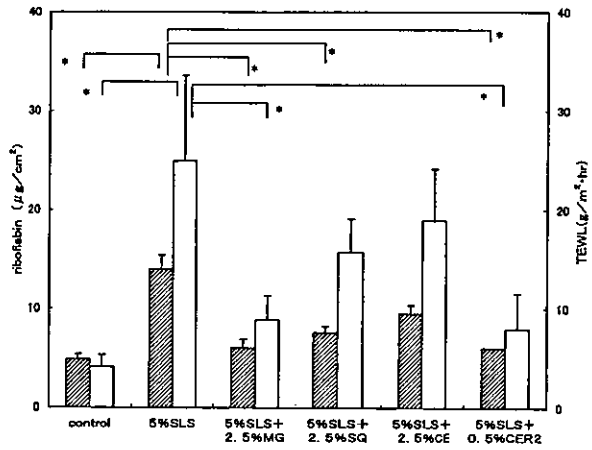

Fig. 3. Skin barrier prevention effect of lipid supplementation. MG (2.5%), SQ (2.5%), CE (2.5%) and Cer2 (0.5%) were dissolved into 5% SLS solution. Hairless rat back skin was treated with each treatment solution. Lipid supplementation caused significant decrease ($P < 0.05$ vs. 5% SLS) of RP alone or RP and TEWL rate. RP (shadow bar) and TEWL (open bar) were measured (mean + S.D., $n = 5$).

Visual scores of skin dryness (scaly skin) decreased in both groups. But a mean score of test group significantly decreased in comparison with a mean score of starting points (Fig. 6).

4. Discussion

Washing and maintenance of cleanness are very important to skin hygiene. For this purpose, various detergents are used as skin cleansing products. In some cases, skin barrier disruption is caused by inappropriate washing manner. So selection of suitable washing products is very important, especially in atopic dermatitis patients, because incorrect selection of washing products causes irritation. So now, many mild detergents are selected to make skin cleansing products [9,16]. If skin cleanser can be used without barrier disruption, it is very important and useful for many persons who have disrupted skin barrier function such as atopic dermatitis patients.

In this study, we wanted to assess such cleansing products in daily use, and to know whether usual washing condition could disrupt skin barrier

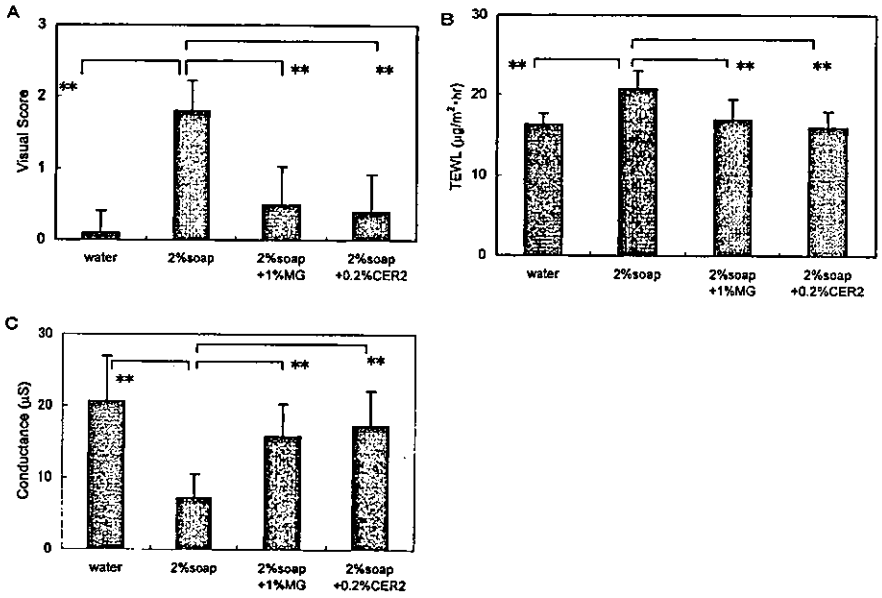

Fig. 4. Synthetic Cer2 supplementation effect for human forearm skin. Human forearm skin was treated with each treatment solution. Lipid supplementation caused significant decrease ($P < 0.01$ vs. 2% soap) of visual score (A) and TEWL (B), and increase ($P < 0.01$ vs. 2% soap) of conductance (C) (mean+S.D., $n = 10$).

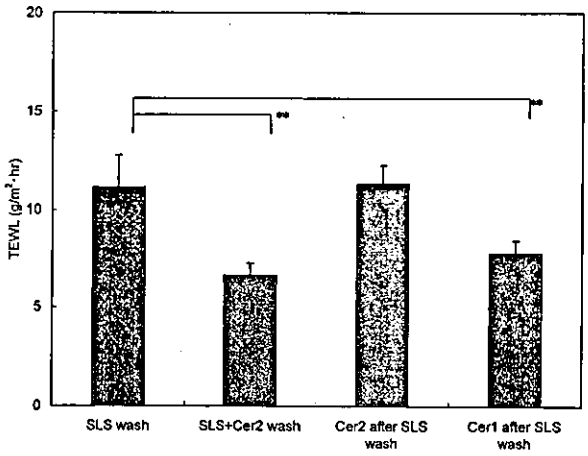

Fig. 5. Efficacy of topical application and supplementation in detergent of Cer2. Hairless rat back skin was treated in SLS solution or Cer2 containing SLS solution. Cer1 or Cer2 containing cream formula was topically allocated on SLS treated back skin just after SLS treatment. Supplemented Cer2 caused significant decrease ($P < 0.05$ vs. 5% SLS) of TEWL value. Topical applied Cer2 was not effective for barrier recovery, but Cer1 caused significant decrease of TEWL value (mean+S.D., $n = 5$).

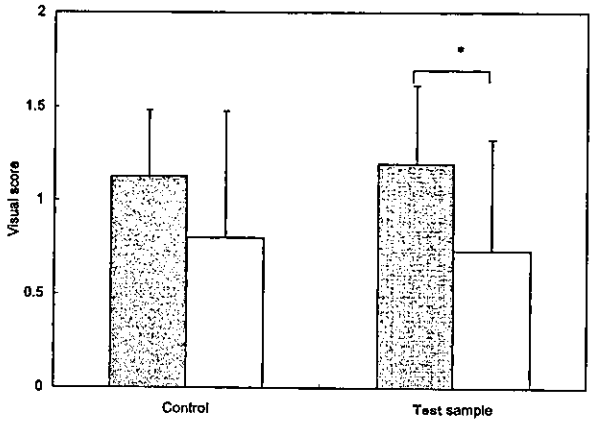

Fig. 6. Efficacy of Cer2-supplemented body shampoo for human leg skin. Control (without Cer2) and test (with Cer2) formulas were compared with visual scaling (dryness) score of initial (open bar) and final (shadow bar). Mean scaling score was decreased at both groups, but a mean score of Cer2-supplemented shampoo group was significantly decreased ($P < 0.05$, mean+S.D., $n = 10$).

function or not. Therefore, we selected a more gentle treatment procedure than that of Ertel et al. [17]. However, barrier disruption was still induced by such gentle method (Fig. 1); so, we think that

prevention of such barrier disruption is very important to skin health.

Previously we described that a dishwashing cleanser containing MG prevented dry skin without depression of cleansing efficacy [15]. In this report, MG mitigated barrier disruption caused by a detergent. These effects may be caused by suppression of ceramide extraction. Intercellular lipids had important roles in skin barrier function. We tried to measure the amount of ceramide left after washing. MG supplementation prevented ceramide elution during washing treatment [15].

Supplementing a Cer2 into the treatment solution was more effective to barrier keeping property than adding SQ or CE (Fig. 3). Supplemented 0.25% Cer2 worked equally as 2.5% MG on prevention of detergent-induced barrier disruption. We tried to measure the left amount of ceramide similar to MG treatment. However, supplemental ceramides left on skin surface after treatment interfered with the measurements of remaining ceramides. Therefore, residual ceramide was more than control. Topical application of Cer2 did not show the beneficial effect on the barrier recovery, and in electromicrographs, intercellular lipids maintain normal structure after skin washing with a Cer2 containing SLS solution. Thus, we think that supplemental ceramides reduce intercellular lipid extraction (Fig. 4).

In this report, we reaffirmed that supplementation detergent solution with lipids is useful not only for the reduction of irritancy but also for maintaining the barrier function.

In particular, we recognized that MG was a useful supplementation agent in detergent to suppress intercellular lipid extraction. Cer2 had equal effects but at lower dosages than MG. We think that ceramide analogs are useful for supplementing cleansing products, and will continue investigating that effect.

Currently treating skin with moisturizers after washing is the most usual procedure to prevent skin barrier disruption and irritation.

Cer1 has a potency of recovering efficacy for acetone/ether-induced artificial barrier disruption. Cer2 is a very useful moisturizer but is not a useful barrier stiffener [13].

In this study, we affirmed these potencies for detergent-induced barrier disruption. Topical application of Cer1 was useful for barrier function recovery, but topically applied Cer2 did not play as a barrier stiffener. However, supplemented Cer2 in skin cleanser was very useful for the prevention of detergent-induced barrier disruption (Fig. 5). To reaffirm this efficacy of Cer2, home use test of Cer2-supplemented body shampoo was employed. Cer2-supplemented body shampoo was useful for the improvement of skin condition (Fig. 6).

We think that skin barrier function can be enhanced not only by using skin moisturizer but also by preventing extraction of intercellular lipid or supplying a barrier enhancer like ceramide. Topical application of barrier stiffener on barrier-disrupted skin is useful for the improvement of skin barrier function. However, we think that a skin cleanser, which can reduce extraction of intercellular lipid, is most useful for skin hygiene and barrier function.

We think that the prevention of barrier disruption is the most important matter for maintaining skin health and barrier function. Secondly, supplementation of intercellular lipids to enhance skin barrier function is useful for maintaining skin health and barrier function.

Therefore, we think that ceramide-supplemented skin cleanser is useful for conservation of skin barrier function.

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