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Cutting Edge: VacA, a Vacuolating Cytotoxin of *Helicobacter pylori*, Directly Activates Mast Cells for Migration and Production of Proinflammatory Cytokines¹

Volaluck Supajatura,^{*‡} Hiroko Ushio,[‡] Akihiro Wada,[§]
Kinnoosuke Yahiro,^{§||} Ko Okumura,^{†‡} Hideoki Ogawa,^{*‡}
Toshiya Hirayama,^{§¶} and Chisei Ra^{2#}

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-1 α , IL-1 β , 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.

H*elicobacter 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

Departments of *Dermatology, †Immunology, and ‡Atopy (Allergy) Research Center, Juntendo University School of Medicine, Tokyo, Japan; Departments of §Bacteriology and ¶Biochemistry, Institute of Tropical Medicine, and ||Department of Applied Chemistry, Faculty of Engineering, Nagasaki University, Nagasaki, Japan; and #Department of Molecular Cell Immunology and Allergology, Advanced Medical Research Center, Nihon University School of Medicine, Tokyo, Japan

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² Address correspondence and reprint requests to Dr. Chisei Ra, Department of Molecular Cell Immunology and Allergology, Advanced Medical Research Center, Nihon University School of Medicine, Tokyo 173-8610, Japan. E-mail address: FceRICRA@med.nihon-u.ac.jp

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

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 μ g 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 Fc ϵ RI.

Flow cytometric analysis of VacA binding to mast cells

BMMCs (1×10^6 cells/ml) were incubated with 10 μ g/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 μ g/ml of rabbit anti-VacA Ab, and then with 1 μ g/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 μ m pore size; NeuroProbe, Gaith-

ersburg, MD) coated with human fibronectin (17). VacA in RPMI 1640 containing 0.5% BSA was added into the lower chamber (25 μ l). A total of 50 μ l 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-1 β , IL-6, IL-10, IL-13, and macrophage-inflammatory protein-1 α . The levels of each cytokine in the culture supernatants were determined by ELISA according to the manufacturer's instructions (Genzyme Techno, 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. 1B). 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.

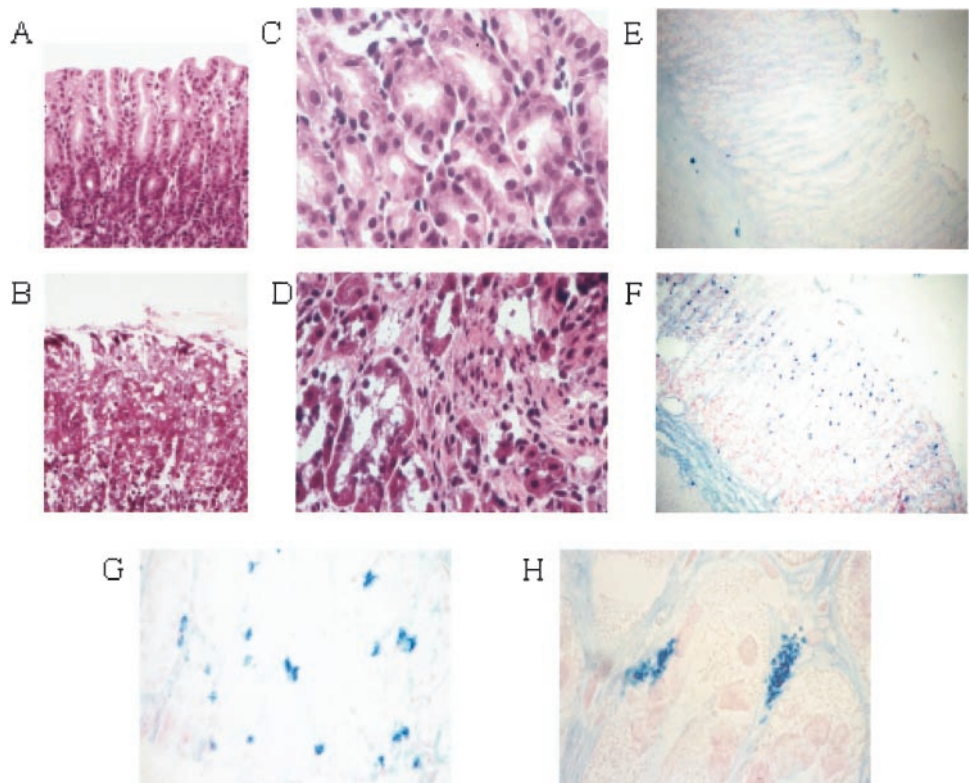


FIGURE 1. VacA causes cell vacuolization and damage to the gastric mucosa with infiltration of mast cells. Gastric tissues of BALB/c mice obtained 24 h after the second inoculation of VacA or saline were stained with H&E (A and B, $\times 200$, and C and D, $\times 400$) or Alcian blue/Safranin (E and F, $\times 100$; G, $\times 400$; and H, $\times 1000$). 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. 2A, 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. 2B). 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

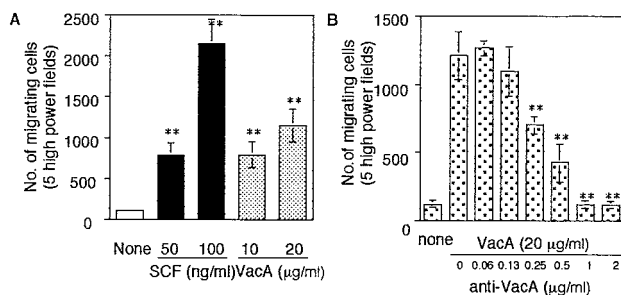


FIGURE 2. VacA stimulates mast cell chemotaxis in vitro. BALB/c BMMCs were used in microchemotaxis assays. *A*, VacA at indicated concentrations was added to the lower chambers of the 48-well chemotaxis chamber, and incubated for 4 h. SCF was used as positive control. *B*, VacA (20 µg/ml) stimulated 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 \pm SD of three independent experiments. **, $p < 0.01$, significantly different from the mean value of the corresponding control.

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 \pm 7.4	57.2 \pm 17.3	110.5 \pm 23.0	140.4 \pm 37.7
5	112.4 \pm 20.7	72.7 \pm 19.5	108.0 \pm 23.2	160.6 \pm 23.0
10	189.9 \pm 54.1	119.8 \pm 20.8	111.6 \pm 22.6	154.8 \pm 20.4
20	452.7 \pm 271.6	374.4 \pm 51.2	325.6 \pm 58.5	164.8 \pm 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 \pm SD of three independent experiments. Each condition was done in triplicate.

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.

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-1 α , IL-1 β , IL-6,

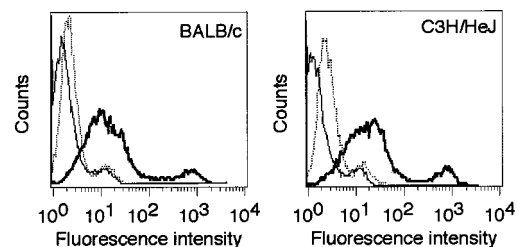


FIGURE 3. Direct binding of VacA to mast cells. VacA binding to BALB/c and C3H/HeJ BMMCs was investigated by flow cytometry using BMMCs alone (solid line) or with FITC-conjugate (dotted line), or with anti-VacA and FITC-conjugate (bold line). Nonspecific binding by Fc γ R on BMMCs was blocked by 2.4G2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

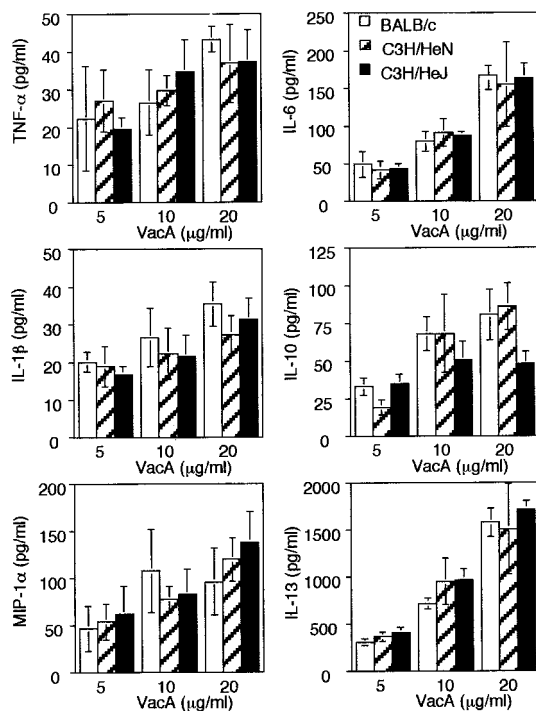


FIGURE 4. Cytokine production from mast cells upon VacA stimulation. BALB/c, C3H/HeN, and C3H/HeJ BMMCs were incubated with VacA at the concentrations as condition indicated in *Material and Methods*. The levels of cytokines in the supernatants were measured by ELISA. Data shown were mean \pm SD of three 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-1 β , 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 eval-

uated by measurement of neutral red uptake (OD value of control vs VacA (20 $\mu\text{g/ml}$): 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 $\mu\text{g/ml}$): 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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