

## Potent Neutralization of Vacuolating Cytotoxin (VacA) of *Helicobacter pylori* by Immunoglobulins Against the Soluble Recombinant VacA

MI-RAN KI<sup>1</sup>, IL-HWA HONG<sup>1</sup>, JIN-KYU PARK<sup>1</sup>, KYUNG-SOOK HONG<sup>1</sup>, OK-KYUNG HWANG<sup>1</sup>, JUNG-YUAN HAN<sup>1</sup>, AE-RI JI<sup>1</sup>, SE-IL PARK<sup>1</sup>, SEUNG-KEUN LEE<sup>1</sup>, SUNG-EUN YOO<sup>2</sup> and KYU-SHIK JEONG<sup>1</sup>

<sup>1</sup>College of Veterinary Medicine, Kyungpook National University, Daegu;

<sup>2</sup>Korea Research Institute of Chemical Technology, Daejeon, Republic of Korea

**Abstract.** *Background:* The recombinant vacuolating cytotoxin (rVacA) of *Helicobacter pylori* that retains native conformational epitopes was evaluated as a vaccine antigen for anti-*H. pylori* treatment. *Methods:* s1m1 vacA gene fraction encoding the mature VacA protein was expressed as a soluble protein in *E. coli* at low temperature. The efficacy of anti-rVacA antibody against VacA or *H. pylori* was assessed in vitro using AGS cells and in vivo using a murine model. *Results:* The rabbit antisera against rVacA completely neutralized the vacuolating activity and partially inhibited the cell death induced by VacA in AGS cells. Oral immunization of C57BL/6 mice with rVacA plus CpG-oligodeoxynucleotide (ODN) as an adjuvant stimulated specific anti-VacA antibody and mucosal immune responses which correlated with decreased systemic immune responses and gastric urease activities ( $p > 0.05$ ). *Conclusion:* The rVacA antigen possessing conformational epitopes may have potential as a vaccine component and may be useful in serological and histopathological analysis.

*Helicobacter pylori* is a spiral-shaped, gram-negative and microaerophilic bacterium that has been recognized as a causative agent of most gastroduodenal diseases including chronic gastritis, peptic ulcer, duodenal ulcer and gastric adenocarcinoma (1, 2). Therefore the eradication of *H. pylori* has been accepted as therapy for gastric diseases (3).

The prevalence of *H. pylori* infection in adults has been estimated to be around 50% of the world's population and

prevalence patterns of the infection are different between developing and developed countries (4, 5). Most *H. pylori* infections are acquired early in life, leading to the subsequent development of gastroduodenal disease unless suitable treatment is received (6, 7). Triple therapy using a proton pump inhibitor in conjunction with two antibiotics has had a high eradication rate of 75% -90% , implying that treatment failure rate is nearly 25% , and is substantially higher in some countries (8, 9). Moreover, reinfection in some patients after successful *H. pylori* eradication has occurred (10, 11). As drug-resistant strains have emerged, the development of a vaccine to eradicate and prevent reinfection has been accepted as an attractive alternative (12).

Various studies on the development of vaccines against *H. pylori* with animal models have been performed, most of which have shown significant protection against *H. pylori* infection after oral vaccination with the use of whole cell lysate or selected antigens known to be involved in the pathogenesis of the infection, such as urease, vacuolating cytotoxin (VacA) and cytotoxin-associated antigen (CagA) (13-17).

Among the antigens, the vacuolating cytotoxin (VacA) induces large cytoplasmic vacuoles and apoptosis which are partly responsible for *H. pylori* induced epithelial cell damage (18, 19). VacA also may play a critical role in chronicity of *H. pylori* infection by suppressing T-cell proliferation (20, 21). The highly purified VacA caused lesions similar to those observed by whole cell extracts (22). Generally, there are two variable regions within the vacA gene among clinically isolated *H. pylori* strains (23). One of these is the s-region within a signal peptide which has two families (s1 and s2) at the 5' end of the vacA gene and the other is the m-region which has two additional families (m1 and m2) on mid-regions of the vacA gene. Strains encoding s1/m1 vacA gene typically produce m1VacA with vacuolating activity on AGS and HeLa cells, whereas m2VacA, produced by strains with the s2/m2 vacA gene, cannot induce vacuoles in those cultured cells. In addition, colonization with vacA s1/cagA-positive strains of *H. pylori* is associated with inflammation

*Correspondence to:* Kyu-Shik Jeong, D.V.M., Ph.D., Department of Pathology, College of Veterinary Medicine, Kyungpook National University, 702-701, #1370 Sangyeok-dong, Buk-ku, Daegu City, Republic of Korea. Tel: +82 539505975, Fax: +82 539505955, e-mail: jeongks@knu.ac.kr

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and epithelial degeneration in gastric mucosa and an increased risk for peptic ulcer disease (PUD), whereas colonization with s2/m2 *vacA/cagA*-negative strains is associated with mild gastric histopathology and is not associated with any significant risk for PUD (24, 25). Thus the studies on VacA are very important in the understanding of the pathophysiology of *H. pylori* associated-diseases. VacA can therefore be considered as a key candidate for vaccine development and as a diagnostic tool.

The expression of proteins using the *Escherichia coli* expression system is the most effective method to produce the target proteins necessary for studies of function and for the assessment of potential vaccines. Previous studies expressing a VacA toxin in *E. coli* have revealed that the recombinant VacA lacks any cytotoxic activity due to the purification under denaturing conditions and the genetic manipulation of this toxin with a tag for efficient purification (26, 27). The antisera to this recombinant protein also failed to neutralize the activity of VacA (26). However, despite the defective functional activity, some studies have shown that protection against *H. pylori* has been achieved to some degree in the murine model of infection using this recombinant antigen plus mucosal adjuvants (15). In some cases, it inhibited the vacuolating activity induced by the native toxin *in vitro* (27, 28). These recombinant proteins containing misfolded conformations may induce insufficient immune responses leading to failure of complete eradication of the pathogen. Therefore the production of recombinant VacA toxin with conformational epitopes may lead to the development of an effective vaccine which has high immunogenicity and may be useful for clinical and laboratory diagnostic applications (26, 29).

While the production of functional recombinant VacA has been thoroughly demonstrated (30, 31), the development of a vaccine using the recombinant VacA toxin with a well-preserved antigenic structure remains to be elucidated. In this study, the conformational recombinant VacA toxin was produced and its immunogenicity was assessed by comparing that of *H. pylori* whole cell lysate in murine models.

## Materials and Methods

***H. pylori* strains and culture conditions.** *H. pylori* ATCC 49503 (strain 60190) and ATCC43504, wild-type, cytotoxic, *cagA*-positive strains with the *vacA* genotype s1/m1; ATCC 51932 (Tx30a), a wild type, *cagA*-negative with the *vacA* genotype s2/m2 (24) were purchased from American Type Culture Collection (Manassas, VA, USA). *H. pylori* was inoculated onto Mueller Hinton agar plates supplemented with 5% horse serum. The plates were incubated at 37°C for 2~3 days under microaerobic conditions by using the BD GasPak EZ Campy Container system (Becton, Dickinson and Company, USA) or 5% CO<sub>2</sub> incubator.

**Cell line culture conditions.** AGS human gastric epithelial cells (KCLB 21739) were purchased from Korean Cell Line Bank

(KCLB, Seoul, Korea) and grown in RPMI 1640 medium supplemented with 10% FBS (Invitrogen Co. USA), 1% penicillin-streptomycin and 1% antibiotic-antimycotic at 37°C in 5% CO<sub>2</sub> incubator.

**Preparation of DNA template.** Isolation of genomic DNA of *H. pylori* was carried out with GeneClean II kit (Q-Biogene, Irvine, CA, USA) according to the manufacturer's instruction. The DNA-containing supernatant was used for DNA template for PCR.

**Polymerase chain reaction and plasmid construction.** Primers were designed to amplify a *vacA* fragment encoding the mature VacA toxin (30) from *H. pylori* strain ATCC 49503 (GeneBank accession No.: U05676). The sequences of sense primer with an *NdeI* site and antisense primer with a *XhoI* site were 5'-GGAATTCATATGTTTTTACAACCGTGATCA-3' and 5'-CCGCTCGAGAGCGTAGCTAGCGAAA-3', respectively. The parameters for PCR were 94°C for 2 min, ×1; 94°C for 30 s, 45°C for 30 s, 68°C for 2 min, ×10; 94°C for 30 s, 45°C for 30 s, 68°C for 2 min + 5 s increase per cycle, ×20; 68°C for 7 min, ×1. The resulting PCR product was digested with *NdeI* and *XhoI* and ligated to the pET 41b (+) vector (Novagen Inc., Madison, WI, USA) digested with the above mentioned enzymes to create plasmid pVAC953. The recombinant plasmid contains the *vacA* fragment encoding the mature VacA cytotoxin (amino acids 34 to 854, including the A34M substitution) with an 8-His tag at the carboxyl terminus amplified in *E. coli* DH5α' (ECOS™ 101, Genetix Ltd., UK). The insert DNA was analyzed with T7 promoter (5'-TAATACGACTCACTATAGGG-3') or T7 terminator (5'-GCTAGTTATTGCTCAGCGG-3') by a sequencing analyzer (Genotech. co., Daejeon, Korea).

**Expressing the target gene.** *E. Coli* BL21 (DE3) was transformed by 1 ng of pVAC953 and kanamycin-resistant clones were selected. One colony bearing pVAC953 was inoculated into 5 mL of Luria-Bertani broth (LB; 10 g of tryptone, 5 g of yeast extract and 5 g of sodium chloride) containing 30 µg/mL of kanamycin using a rotary shaker (200 rpm) at 37°C overnight. The overnight cultures were diluted 1 to 100 into modified Terrific broth (TB; 12 g of tryptone, 12 g of yeast extract, 4 mL of glycerol, 2.31 g of KH<sub>2</sub>PO<sub>4</sub> and 12.54 g of K<sub>2</sub>HPO<sub>4</sub> per liter) supplemented with 0.5% glucose and 30 µg/mL of kanamycin, and incubated at 24°C until they reached an optical density of 0.4-0.6 at 600 nm. These cultures were induced with 0.1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated at 20°C for another 20 h. *E. coli* soluble extracts were prepared as follows. Around 100 mL volume of IPTG-induced cultures were pelleted by centrifugation at 5000 rpm for 10 min and resuspended in a 20 mL of lysis buffer containing 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 5% glycerol, 0.5% NP40, protease inhibitor cocktail (Complete mini, Roche Co. Germany), 1 mM PMSF (Roche Co. USA) and 0.5 mg/mL egg white lysozyme (Sigma Co. USA). The bacterial suspensions were incubated for 30 min at room temperature and then treated with freezing at -70°C and thawing at 37°C three times. After three successive rounds of freeze-thaw, DNase, RNase and MgCl<sub>2</sub> were added to a final concentration of 20 µg/mL, 10 µg/mL and 10 mM, respectively. The bacterial suspensions were incubated on ice for 30 min and then sonicated using Sonics Vibra-cell™ for 1 minute at an amplitude of 40 and a 1-second pulse. The bacterial lysates were

centrifuged at  $12000 \times g$  for 10 min at  $4^{\circ}\text{C}$  to remove insoluble cell debris. The supernatant was used for purification using His-Bind affinity chromatography (Novagen Co. USA). The purification of rVacA toxin was performed according to the manufacturer's protocol. Briefly, the first pool of fractions eluted with 0.5 M imidazole by His-Bind column chromatography were reloaded into another His-Bind column equilibrated with 20 mM Tris-HCl (pH7.9) supplemented with 0.5 M NaCl, 0.02% NP-40, 0.2% glycerol and 0.1M imidazole. The 0.5 M imidazole eluate through the second chromatography was dialyzed against PBS and used in this study as a recombinant vacuolating cytotoxin.

*Production of the polyclonal anti-recombinant VacA (rVacA) antibodies raised in rabbit.* New Zealand White rabbits approximately 2.5 kg in size were immunized by intradermal injections of 50  $\mu\text{g}$  of rVacA toxin mixed with a little truncated form of this toxin in Freund's adjuvant on days 1, 21 and 35. Immunoglobulins were purified using recombinant Protein G-Agarose (GibcoBRL, Gaithersburg, MD, USA) according to the manufacturer's protocol. The yield and quality of the antibodies in serum or the purified immunoglobulins were monitored by indirect ELISA.

*Immunoblot analysis of VacA.* *E. coli* soluble extracts or eluted fractions from His-bind affinity column were separated by SDS-PAGE, transferred to Immobilon-P membrane (Milipore Co. USA). The membranes were incubated with TBST (10 mM Tris-HCl, pH8.0, 150 mM NaCl, 0.05% Tween 20) containing 3% BSA for 1h followed by washing three times with TBST for 10 min each and then incubated with a mouse monoclonal anti-His antibody (Novagen Co. USA) or a rabbit polyclonal anti-rVacA antibody at a dilution of 1:2000 in TBST containing 5% non-fat milk overnight at  $4^{\circ}\text{C}$ . After washing three times with TBST for 10 min each, the membranes were incubated with anti-mouse or anti-rabbit IgG antibodies conjugated with horseradish peroxidase at a dilution of 1: 10,000 (Promega Co. USA). Signals were amplified by the enhanced chemiluminescence system (Pierce Co. USA) and exposed to X-ray film (Kodak Co. USA).

*Vacuolating assay and Viability assay.* Vacuolating activity was assessed according to Cover *et al.* (32). Briefly, AGS cells were seeded at  $2.5 \times 10^4$  cells per well into 96-well plates and were grown overnight. The grown cells were washed with PBS and overlaid with serum-free culture medium in the presence or absence of 10 mM  $\text{NH}_4\text{Cl}$  to which the cell-free supernatants (CFS) obtained from *H. pylori* vacA wild-type, s1/m1 type were added. Then cells were incubated for 5 h at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  incubator. After incubation, cell vacuolation was examined by inverted light microscopy and quantified by a neutral red uptake assay. Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Roche Co. Germany) according to the manufacturer's protocol.

*Neutralization of the native VacA toxin activity by anti-rVacA antibody.* Purified anti-rVacA antibodies were tested for their ability to neutralize VacA cytotoxic activity and vacuolating activity. Briefly, prior to adding the AGS monolayer, ATCC 49503 CFS was pretreated with the purified immunoglobulins for 30 min at  $4^{\circ}\text{C}$ . The neutralizing activity of immunoglobulins was assessed in an *in vitro* MTT assay (595 nm) and neutral red uptake assay (550 nm), respectively, as described above.

*ELISA.* A 96-well plate (Nunc MaxiSorp, Denmark) was coated by incubation overnight at  $4^{\circ}\text{C}$  or for 2h at  $37^{\circ}\text{C}$  with recombinant VacA protein (100 ng/mL) or supernatant or lysate of *H. pylori* in PBS. Plates were washed 3 times in TBST and blocked with the same buffer containing 1% BSA for 1h at  $37^{\circ}\text{C}$ . Serial dilutions of serum (1:1) were added to the wells and incubated for 2h at  $37^{\circ}\text{C}$  or overnight at  $4^{\circ}\text{C}$ . The plates were washed and incubated with horseradish peroxidase-conjugated rabbit anti-rabbit immunoglobulin antibodies (Promega Co. USA) for 1h at  $37^{\circ}\text{C}$ . After washing 3 times with TBST, plates were incubated with One-step ultra TMB (tetramethylbenzidine) substrate (Pierce Co. USA) for 5-15 min at room temperature, depending on the rate of color development which was stopped with 2M sulfuric acid. The intensity of color was measured at 450 nm in an ELISA reader (Tecan Sunrise ELISA Reader, Tecan Group, Ltd., Switzerland).

*Animal experiment for therapeutic vaccination.* *Mice:* Specific-pathogen free female 7-week-old C57BL/6 mice were purchased from Charles River Laboratories (Japan). Mice were housed in stainless-steel wire-mesh cages at  $20^{\circ}\text{C}$  in a temperature and humidity-controlled room, they were kept on a 12-h light-dark cycle and provided unrestricted amounts of standard mouse chow and freely available tap water. Animal procedures were conducted in accordance with NIH guidelines.

*Challenge procedure:* Mice were initially fasted for 8 h and prior to inoculation, administered 150  $\mu\text{L}$  of a 0.2 M  $\text{NaHCO}_3$  solution via intragastric gavage (*i.g.*). Animals ( $n=5/\text{group}$ ) were inoculated *i.g.* three times on days 0, 3 and 6 with 200  $\mu\text{L}$  suspension containing  $1 \times 10^8$  CFU *H. pylori* (ATCC 43504) and fed after 4 h.

*Preparation of H. pylori whole cell lysate:* *H. pylori* whole cell lysate (WCL) was prepared as follows. Briefly, the organisms grown on blood agar plate were harvested and resuspended in PBS. The resulting suspension was washed by centrifugation and resuspended in PBS followed by disruption using ultrasonic waves on ice. The cellular debris was removed by centrifugation and the supernatant was stored at  $-70^{\circ}\text{C}$  until used for oral immunization.

*Immunization:* Mice were immunized on days 15, 22 and 27 after inoculation of *H. pylori* with 100  $\mu\text{g}$  of ATCC 43503 lysate or 20  $\mu\text{g}$  of recombinant VacA suspended in PBS in the absence or presence of 5 nmoles of thiolated synthetic oligodeoxynucleotides containing immunostimulatory CpG motifs (CpG-ODN) as adjuvant (5'-TCC ATG ACG TTC CTG ACG TT-3') (33). The control mice were immunized with PBS in the absence or presence of 5 nmoles of CpG-ODN. All the mice were immunized *i.g.*

*Determination of H. pylori infection in gastric tissue specimens:* Mice were sacrificed 10 weeks after challenge with *H. pylori* and the stomachs were excised and divided longitudinally into two halves. One section was placed in 500  $\mu\text{L}$  of sterile saline solution, and homogenized with vigorous shaking incubation for 5 min. For indirect quantitative analysis of *H. pylori* infection, gastric urease activity was measured by adding 10  $\mu\text{L}$  of the homogenized gastric tissue into 100  $\mu\text{L}$  of urea broth containing phenol red indicator. A positive urease activity was defined as a color change from yellow-orange to pink within 24 h.

*Histopathology:* The other section was fixed in 4% buffered formaldehyde and then embedded in paraffin, sectioned at 4  $\mu\text{m}$ , and stained with hematoxylin and eosin (H&E). Sections were scored based on the intensity of the infiltration of inflammatory cells (34).

*Measurement of mucosal and systemic immune response:* Antibodies to *H. pylori* antigens were quantitated by an ELISA

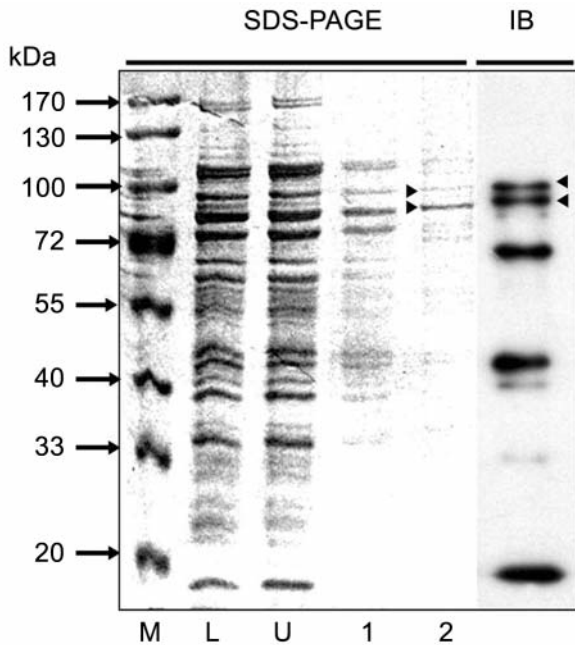


Figure 1. Expression of soluble rVacA fused to an 8-Histidine at C-terminus in *Escherichia coli*: SDS-PAGE analysis of rVacA. M; molecular weight marker, L; crude lysate prior to purification, U; unbound fraction from 1st Ni<sup>2+</sup>-affinity chromatography column, 1; the eluate of 0.5 M imidazole from 1st Ni<sup>2+</sup>-affinity chromatography column and 2; the eluate of 0.5M imidazole from 2nd Ni<sup>2+</sup>-affinity chromatography. The rVacA with a histidine tag shows molecular weight of ~90 kDa and 83 kDa and accompanied by several lower forms detected by immunoblotting with monoclonal anti-Histidine tag antibody in the right end line. The arrowhead indicates the position of 90 kDa and 83 kDa of rVacA, respectively.

using microtiter plates coated with *H. pylori* lysates (1 µg/ well) or recombinant VacA (100 ng/well). After blocking with 1% BSA for 1 h, plates were incubated with mice sera (diluted 1:100) or supernatant of homogenized gastric tissue solution (diluted 1:100) overnight at 4°C. Horseradish peroxidase-conjugated anti-mouse IgG polyclonal antibody (Promega, USA) was used as a secondary antibody.

**Statistical analysis:** The Kruskal-Wallis test for one-way analysis of variance (ANOVA) was used in this study to examine the differences among groups.

## Results

**Expression of recombinant VacA.** Expression of a soluble recombinant VacA fused to an octa-histidine tag at the carboxyl-terminus was induced by adding 0.1 mM IPTG at 20°C for 20 h in *E. coli* BL21 (DE3). The rVacA was partially purified by affinity chromatography with nickel chelating resin. As shown in Figure 1, several bands of apparent molecular weights 90 kDa, 83 kDa, 67 kDa, 40 kDa and 20 kDa were recognized in immunoblot by a monoclonal

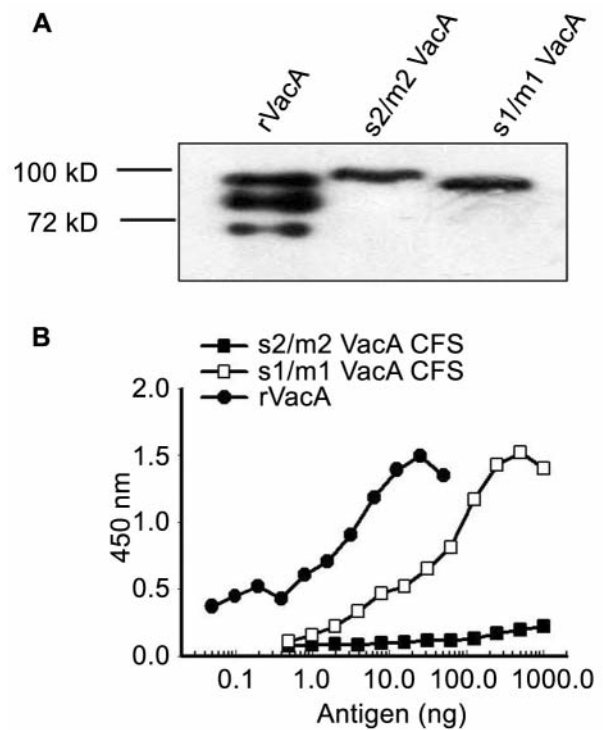


Figure 2. Detection of *H. pylori* VacA with anti-rVacA antibody using immunoblot and ELISA. Immunoblot analysis of VacA. 400 ng of recombinant VacA (lane 1), 40 µg and 8 µg of CFS of ATCC 51932 (lane 2) and ATCC 49503 (lane 3), respectively, were detected by immunoblotting with anti-rVacA antibodies. The rVacA, native s2/m2 VacA and native s1/m1 VacA appeared at about 90 kDa, 92 kDa and 89 kDa, respectively (A). Reactivity of various VacA samples with anti-rVacA antibodies. rVacA (●); CFS of s1/m1 vacA strain, ATCC 49503 (□); CFS of s2/m2 vacA strain, ATCC 51932 (■). 1:1 serial dilutions of rVacA (100 ng), CFS of 51932 (10 µg) or CFS of 49503 (10 µg) was incubated in wells in 96-well microtiter plates at 4°C, overnight, and the 1:1000 diluted antibodies were reacted with antigens (B).

anti-His antibody and bands with 90 and 83kDa were predominant in SDS-PAGE profiles. The 90 kDa band was considered as the complete form of rVacA and other bands as its truncated forms. The recombinant protein was eluted from the column with 0.5 M imidazole as a single peak followed by further purification with another nickel chelating resin. The eluate from the second nickel chelating resin was concentrated using Microcon YM-50 (Millipore Co. USA) whose cut-off molecular weight is less than 50 kDa followed by dialysis against PBS and then it was used as rVacA. Levels of purified rVacA containing its truncated forms were about 0.25 mg of protein per 1 liter of culture.

**Detection of VacA among crude *H. pylori* lysate using anti-recombinant VacA antibody.** The rVacA containing its truncated forms induced comparable amounts of specific antibodies in serum after immunization of rabbit. Immunoglobulins raised

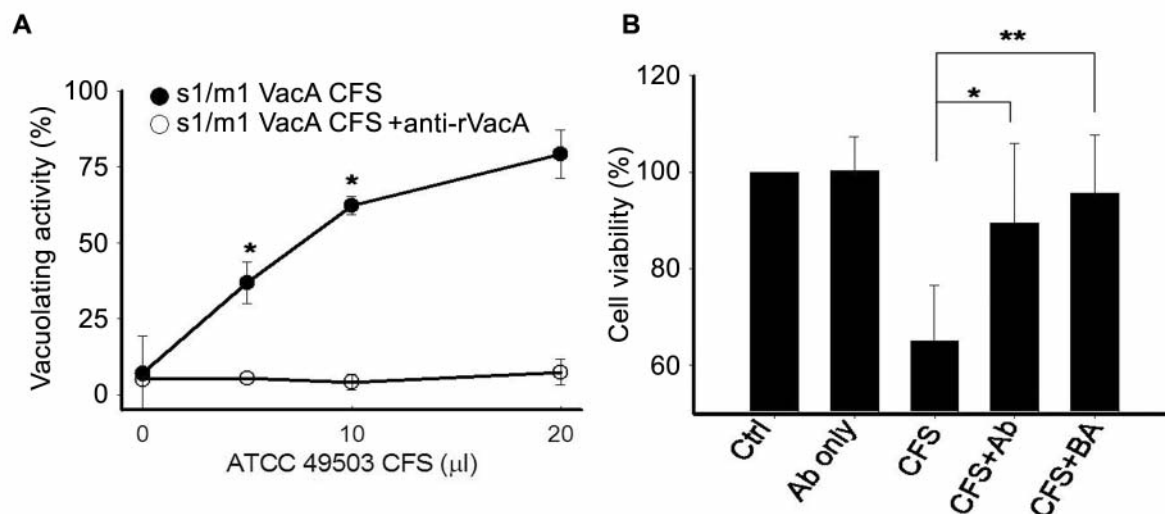


Figure 3. Neutralizing activity of rVacA antibody against s1/m1 VacA-induced cell vacuolation and cell death in AGS cells. (A) AGS cells were co-cultured with the indicated quantities of 49503 CFS in the presence (○) or absence (●) of the 1:100 diluted anti-rVacA antibodies. Vacuolating activity was measured by a neutral red dye uptake assay with ATCC 49503 CFS in the presence of 10 mM NH<sub>4</sub>Cl. Values represent the means and standard deviations from quadruplicate samples and expressed as the percentages of neutral red dye uptake relative to that of maximal values. (B) Inhibitory effect of anti-rVacA antibody against cell death induced by VacA. To assess the cell viability, AGS cells were treated for 20 h with 20 μL of PBS (for control) or CFS of s1/m1 VacA in the presence of or absence of anti-rVacA antibody. For comparison, effect of vacuolation inhibitor on cell viability using bafilomycin A1 was assessed instead of anti-rVacA antibody. Cell viability was measured by an MTT cell proliferation colorimetric assay. Values represent the means and standard deviations from quadruplicate samples and expressed as the percentages of the values of control (no additives). † $p < 0.05$  (*t*-test, for each comparison) and \* $p < 0.05$  and \*\* $p < 0.01$  (pairs *t*-test).

against rVacA recognized the native VacAs in an immunoblot not only with the ~89 kDa s1/m1 type but also with the ~92 kDa s2/m2 type (Figure 2 A). In ELISA, however, s2/m2 VacA was not confirmed (Figure 2 B). Even the s2/m2 VacA in about 20-fold-concentrated CFS did not react with anti-rVacA antibodies. These results imply that immunoblotting is superior to ELISA for the detection of s2/m2 VacA with anti-rVacA antibody. Inversely, ELISA has a significantly better specificity than immunoblotting for discerning one isomer from others with a corresponding antibody.

**Neutralizing effect of anti-recombinant VacA antibody.** In addition, this antibody completely neutralized the vacuolating activity induced by cell free supernatant (CFS) of *H. pylori* (Figure 3 A). To determine whether the antibody capable of neutralizing the vacuolating activity can inhibit cell death induced by VacA, AGS cells were incubated with various VacA preparations for 20 h in the presence of 10 mM NH<sub>4</sub>Cl. Cell viability was quantified by MTT assay. As expected CFS containing s1/m1 VacA capable of extensive vacuolation of AGS cells decreased cell viability and CFS treated with anti-rVacA antibody partially protected cells from cell death induced by VacA (Figure 3 B). The development of vacuolation induced by VacA in cultured cells such as AGS is prevented by bafilomycin A1, a V-type

ATPase inhibitor which also reduced cell death induced by VacA, implying the vacuolation induced by VacA at least partially correlates with VacA-induced cell death.

**Oral immunization of recombinant *H. pylori* VacA with CpG-ODN.** The possibility of recombinant VacA as the oral vaccine with CpG-oligodeoxynucleotide (ODN) adjuvant was assessed in C57BL/6 mice. As shown in Figure 4, while mucosal immune responses against rVacA plus CpG-ODN increased, systemic immune responses decreased, implying that CpG-ODN stimulated mucosal immune responses against rVacA and protection against *H. pylori* infection may be associated with increased mucosal immune responses and decreased serum immune responses in that vaccinated group. In accordance with this postulation, mice immunized with *H. pylori* lysate or rVacA plus CpG-ODN showed low values of urease activity in comparison to the controls (Table I). Histology of the gastric biopsies revealed a mild degree of gastritis especially in the fundus (data not shown). The infiltration of eosinophils rather than neutrophils was prominent in that region. Although the difference was not significant, the gastritis score also decreased slightly in the vaccinated groups (lysate, lysate plus CpG-ODN, rVacA and rVacA plus CpG-ODN) in comparison to that of the control

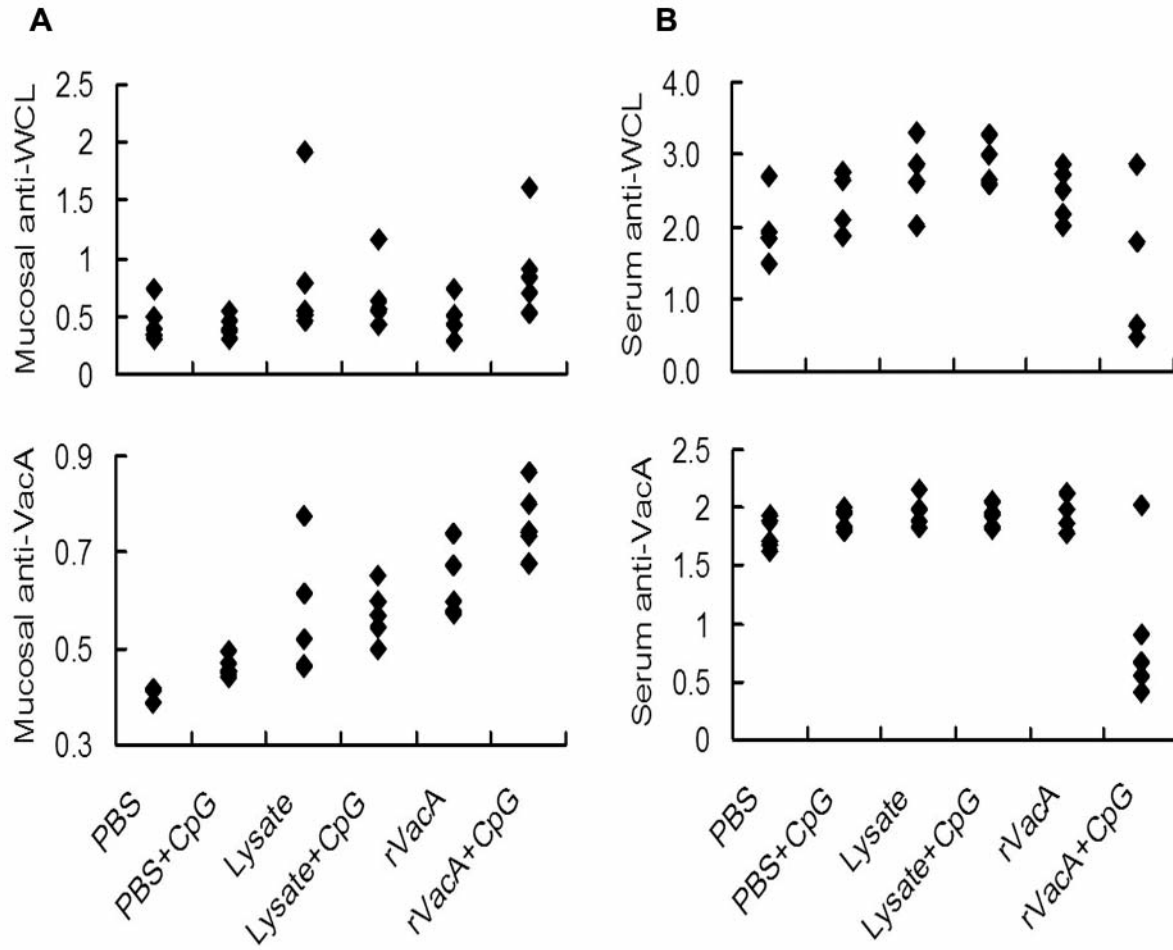


Figure 4. Comparison of systemic and mucosal immune responses following vaccination against *H. pylori*. The gastric mucosal saline extracts and sera were obtained from C57BL/6 mice following 3 therapeutic vaccinations with indicated vaccines over a 3-week period after inoculation of ATCC 43504 i.g. three times a week. *H. pylori* whole cell lysate (WCL) specific IgG responses (upper panel) and VacA specific IgG responses (lower panel) in mucosal saline extracts (A) and in sera (B) were analyzed using indirect ELISA.

Table I. Results of therapeutic vaccination against *H. pylori* infected mice<sup>a</sup>.

Groups	Urease activity (A550±S.D.)	Mucosal antibody <sup>b</sup>		Serum antibody <sup>b</sup>		Gastritis grade <sup>c</sup>
		Anti-lysate	Anti-VacA	Anti-lysate	Anti-VacA	
PBS	0.47 ±0.51	0.45±0.17	0.40±0.01	2.00±0.51	1.77±0.13	2.0±0.0
PBS+CpG	0.35±0.13	0.42±0.09	0.46±0.02	2.30±0.39	1.91±0.09	1.7±1.2
Lysate	0.28±0.04	0.85±0.61	0.57±0.13	2.68±0.47	1.97±0.13	1.2±1.1
Lysate+CpG	0.45±0.25	0.66±0.29	0.57±0.06	2.83±0.30	1.92±0.10	1.3±1.2
rVacA	0.46±0.42	0.48±0.16	0.63±0.07	2.47±0.36	1.91±0.15	1.6±1.3
rVacA+CpG	0.23±0.03	0.92±0.41	0.76±0.07	1.24±1.06	0.91±0.65	1.5±1.2
Mean±S.D.	0.37±0.28	0.63±0.37	0.57±0.14	2.26±0.75	1.73±0.46	1.5±1.0
p-valued	0.082	0.012	0.001	0.025	0.173	0.592

Data are expressed as means±S.D. <sup>a</sup>Mice were infected with ATCC 43504 (cagA+/s1m1 vacA+) and, 3 weeks later, received three intragastrical inoculations with lysate or rVacA plus the adjuvant CpG-ODN. Control groups consisted of mice that received PBS alone or PBS plus CpG-ODN; <sup>b</sup>IgG against *H. pylori* lysate or rVacA was detected using ELISA in mucus and sera, respectively; <sup>c</sup>The grade of gastritis was scored with respect to the density of eosinophils infiltrated in fundus from grade 0 (no gastritis) to grade 3 (severe gastritis); <sup>d</sup>The data were analyzed by means of Kruskal Wallis test with the help of the SPSS 12.0.

groups (PBS and PBS plus CpG-ODN) (Table I). Taken together, rVacA plus CpG-ODN stimulated mucosal immune responses which appeared likely to correlate with decreased systemic immune responses and gastric urease activity.

## Discussion

So far, in contrast to the native VacA, a recombinant protein expressed in *E. coli* has low potential to elicit immune response due to its denatured structure and low binding affinity to anti-VacA antibody in sera of *H. pylori* infected patients (26). In this study, the recombinant VacA produced by means of the modified methodology by MacClain *et al.* (30) retained conformational epitopes so that it elicited neutralizing responses against VacA cytotoxicity. The results suggest that the recombinant VacA may be a potential vaccine candidate and its specific antibody is useful for serological testing and histopathological analysis for diagnosis of *H. pylori* infection. To date, most antibodies capable of neutralizing VacA-induced vacuolation in cultured cells are induced by native VacA. VacA is expressed in *H. pylori* as a 140-kDa (139-kDa) protoxin that undergoes amino- and carboxyl-terminal processing, yielding a mature 88-kDa (87-kDa) secreted VacA toxin (35). The mature secreted 88-kDa VacA toxin yields two fragments that are 33 and 55 kDa in mass (p33 and p55, respectively). A hydrophobic region near the amino terminus of the p33 domain are required for the formation of anion-selective membrane channels (28), and the p55 domain is responsible for VacA binding to mammalian cells, particularly in the carboxyl-terminal approximately 100-residue region (27, 35). Garner *et al.* reported antibodies reacting only with the recombinant p55 VacA fragment neutralized vacuolating cytotoxin activity (36). The antibodies in the present study were raised from partial purified recombinant VacA which contained several fragments *i.e.* ~90 kDa, ~86kDa, ~67kDa and ~40 kDa with an 8-histidine tag at the carboxyl terminus. These recombinant proteins seem to result from proteolytic truncation at the amino-terminal end of the recombinant VacA (~90kDa) produced in *E. coli*. Thus, the neutralizing activity may come from the antibodies reacting with recombinant VacA fragments containing the p55 domain which have the m-region and C-terminus of the VacA fragment.

VacA is characterized by allelic variation within the signal sequence and middle region and have distinct antigenic properties as different VacA isoforms (37). Marchetti *et al.* reported immunization of mice with a type s1/m1 VacA antigen conferred protective immunity against *H. pylori* strains expressing homologous types of VacA but not a type s2/m2 VacA (15). Although VacA is known to be a major virulence factor, the low seroprevalence of

specific anti-VacA antibody in sera of *H. pylori* infected patients has been reported (38). These results may be due to not only antigenic diversity among different VacA proteins but also the misfolded form of the recombinant VacA causing low affinity to the antibody. Actually, rVacA expressed as inclusion bodies produced lower level of antibodies from an immunized rabbit due to its low immunogenicity compared to conformational rVacA (data not shown). Reportedly (37, 39), antibodies to VacA were best detected in assays using homologous VacA antigens. In agreement with this explanation, the antibodies to recombinant s1/m1 VacA strongly reacted with homologous VacA in an immunoblot and ELISA but did not react with s2/m2 VacA in ELISA although it was detected in an immunoblot. Thus, since the antibody against s1/m1 VacA fails to recognize s2/m2 VacA, if VacA is to be included as a vaccine component or diagnosis kit, the mixture of multiple VacA isomers could be reliable. Vinion-Dubiel *et al.* demonstrated the pooled polyclonal antisera against native s1/m1, s1/m2 and s2/m2 VacA yielded a positive signal with each of the 12 different wild-type strains (39). Independently, because *H. pylori* strains producing type s1/m1 VacA are thought to be more frequently associated with peptic ulceration and gastric cancer (24, 40), it is necessary to study the pathophysiological activities of s1/m1 VacA and develop the methods to discern the toxic strains and protect them as well.

Based on the importance of VacA in pathogenesis of *H. pylori*, the possibility of recombinant VacA as the oral vaccine with CpG-ODN adjuvant was assessed in C57BL/6 mice. Successful mucosal vaccination requires strong adjuvants to improve the poor immunogenicity of co-administered antigens. Cholera toxin (CT) and *E. coli* labile enterotoxin (LT) are powerful mucosal adjuvants. Oral immunization with *H. pylori* lysate or recombinant antigen with CT or LT eradicated *H. pylori* infection in mice (15). The inclusion of CT or LT, however, has been a major limitation for their use as vaccines in humans due to factors such as a high incidence of diarrhea (41). Among non-toxic immune enhancers, CpG-ODN has been potent adjuvant against *H. pylori* (33, 42). CpG-ODN contains unmethylated CG dinucleotide in specific base sequence contexts (CpG motif) which has immunostimulatory properties including the induction of a Th1-type response (42, 43). For the usefulness of CpG-ODN, in this study, it was adopted as a mucosal adjuvant. The results showed that VacA vaccine with CpG-ODN stimulated mucosal immune response while *H. pylori* lysate vaccine stimulated both mucosal and systemic immune responses. On the other hand, *H. pylori* lysate with CpG-ODN decreased mucosal immune responses, indicating that whole cell lysate vaccines are highly immunogenic and contain their own built-in immunostimulating molecules (44), and furthermore, may

hold immunosuppressive elements or molecules modulating the immune system in an undesired direction (45). Mucosal immune responses have been related to reduction of bacterial colonization in the stomach after therapeutic *H. pylori* immunization in mice (46). The presented results also showed a mucosal immune response correlated with decreased urease activity of gastric mucosa, a marker of *H. pylori* infection. The protection has been related to specific mucosal CD4<sup>+</sup> T-cell responses with a Th1 profile as well as to mucosal IgA responses locally in the stomach (47, 48). In contrast, Ferrero *et al.* (49) showed that immunization of mice against *H. felis* infection induced the proliferation of large numbers of IgG-secreting cells, as well as the synthesis of local IgG antibodies, in the gastric mucosa of the animals. Protection against *H. felis* infection occurred despite the absence of gastric IgA responses; hence they proposed that locally synthesized specific IgG antibodies contribute to immunity against gastric *Helicobacter* infection. In this study, although mucosal IgA antibodies were not detected, mucosal immune responses determined by IgG antibodies against applied antigens showed that, as mentioned above, VacA vaccine stimulated mucosal immunity rather than systemic immunity which is a requisite for oral vaccines. These findings suggest that VacA is a promising candidate antigen for use in a therapeutic vaccine against *H. pylori*. Typically, *H. pylori* associated-gastritis is characterized by the intensive infiltration of neutrophils and mononuclear cells in gastric mucosa (50). The results of the study, however showed that eosinophilic gastritis increases in the stomach of *H. pylori* treated groups and decreases in the stomach of vaccinated groups. It has been shown that eosinophilic gastritis increases in *H. pylori* infected patients and decreases after *H. pylori* eradication (51, 52). It is likely that eosinophilic infiltration is influenced by the *H. pylori* density but the association between *H. pylori* infection and eosinophilic gastritis remains unclear (53). In this study, *H. pylori* colonization was not determined in the murine models, although VacA vaccine had promising effects such as decreased systemic immune responses and reduced gastric urease activity. Therefore it remains to be investigated whether the recombinant VacA vaccine induces activation of mucosal immune responses with IgA production which in turn triggers bacterial eradication in murine models with chronic *H. pylori* infection. In contrast, as VacA mimics FK506, an immunosuppressive drug *in vitro* (20), it has been speculated that VacA may have efficacy when challenged for prophylactic vaccination purposes in humans (12). However, m1 VacA does not efficiently interact with murine primary T-cells thereby not inducing an immunosuppressive effect (54) in murine models as shown in this study. Therefore the efficacy of VacA vaccination needs to be evaluated in Mongolian gerbils that show similar pathological gastric features to *H. pylori* infection in humans (12).

In conclusion, a 90 kDa recombinant VacA expressed in *E. coli* is able to elicit neutralizing immunoglobulin against VacA which completely inhibits the vacuolation induced by supernatants of homologous VacA-producing *H. pylori* and partially inhibits VacA-induced cell death. The recombinant VacA plus CpG-ODN stimulated mucosal immunity but not systemic immunity in the murine model. Therefore the recombinant VacA with conformational epitopes may be useful for a potential vaccine component against *H. pylori* and clinical and laboratory differential diagnosis of *H. pylori* infection.

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