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

MI-RAN KI1, IL-HWA HONG1, JIN-KYU PARK1, KYUNG-SOOK HONG1, OK-KYUNG HWANG1, JUNG-YUAN HAN1, AE-RI JI1, SE-IL PARK1, SEUNG-KEUN LEE1, SUNG-EUN YOO2 and KYU-SHIK JEONG1

1College of Veterinary Medicine, Kyungpook National University, Daegu; 2Korea 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 within signal peptide which has two 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...
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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{H. pylori} whole cell lysate in murine models.

**Materials and Methods**

\textit{H. pylori} strains and culture conditions. \textit{H. pylori} ATCC 49503 (strain 60190) and ATCC43504, wild-type, cytotoxic, \textit{cagA}-positive strains with the \textit{vacA} genotype s1/m1; ATCC 51932 (Tx30a), a wild type, \textit{cagA}-negative with the \textit{vacA} genotype s2/m2 (24) were purchased from American Type Culture Collection (Manassas, VA, USA). \textit{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$_2$ incubator.

Cell line culture conditions. AGS human gastric epithelial cells (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$_2$ incubator.

**Preparation of DNA template.** Isolation of genomic DNA of \textit{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 \textit{vacA} fragment encoding the mature VacA toxin (30) from \textit{H. pylori} strain ATCC 49503 (GeneBank accession No.: U05676). The sequences of sense primer with an \textit{Ndel} site and antisense primer with a \textit{Xhol} site were 5’-GGAAATTCATAT GTTTTTTCACACCCTGATCA-3’ and 5’-CGGCTCGAGAGCGTC GTAGCGAAA-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, 120; 68˚C for 7 min, 1: The resulting PCR product was digested with \textit{Ndel} and \textit{Xhol} 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 \textit{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 \textit{E. coli} DH5α’ (ECOS™ 101, Genetix Ltd., UK). The insert DNA was transformed by 1 ng of pVAC953 and kanamycine-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 kanamycine 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$_2$PO$_4$ and 12.54 g of K$_2$HPO$_4$ per liter) supplemented with 0.5% glucose and 30 μg/mL of kanamycine, 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. \textit{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$_2$ 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 × g for 10 min at 4°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 (pH 7.9) 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 μg 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°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 2.5×10^4 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 tetramethylbenzidine (tetrathiazoline) 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, Tecxan 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 sire-mesh cages at 20°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 μL of a 0.2 M NaHCO₃ solution via intragastric gavage (i.g.). Animals (n=5/group) were inoculated i.g. three times on days 0, 3 and 6 with 200 μL suspension containing 1x10^8 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°C until used for oral immunization.

Immunization: Mice were immunized on days 15, 22 and 27 after inoculation of H. pylori with 100 μg of ATCC 43503 lysate or 20 μg of recombinant VacA suspended in PBS in the absence or presence of 5 nmol of thioldiated 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 nmol 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 μL 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 μL of the homogenized gastric tissue into 100 μL 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 μm, 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.

ELISA. A 96-well plate (Nunc MaxiSorp, Denmark) was coated by incubation overnight at 4°C or for 2h at 37°C with recombinant VacA protein (100 ng/mL) or supernatant or lystate 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°C. Serial dilutions of serum (1:1) were added to the wells and incubated for 2h at 37°C or overnight at 4°C. The plates were washed and incubated with horseradish peroxidase-conjugated rabbit anti-rabbit immunoglobulin antibodies (Promega Co. USA) for 1h at 37°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, Tecxan Group, Ltd., Switzerland).
using microtiter plates coated with \textit{H. pylori} lysates (1 \mu g/ 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\degree C. Horseradish peroxidase-conjugated anti-mouse IgG polyclonal antibody (Promega, USA) was used as a secondary antibody.

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

\section*{Results}

\textit{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\degree C for 20 h in \textit{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 anti-His antibody and bands with 90 and 83 kDa 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.

\textit{Detection of VacA among crude \textit{H. pylori} lysate using anti-rVacA antibody.} The rVacA containing its truncated forms induced comparable amounts of specific antibodies in serum after immunization of rabbit. Immunoglobulins raised...
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 NH₄Cl. 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.\(^a\)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Urease activity (A550±S.D.)</th>
<th>Mucosal antibody(^b)</th>
<th>Serum antibody(^b)</th>
<th>Gastritis grade(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Anti-lysate</td>
<td>Anti-VacA</td>
<td>Anti-lysate</td>
</tr>
<tr>
<td>PBS</td>
<td>0.47±0.51</td>
<td>0.45±0.17</td>
<td>0.40±0.01</td>
<td>2.00±0.51</td>
</tr>
<tr>
<td>PBS+CpG</td>
<td>0.35±0.13</td>
<td>0.42±0.09</td>
<td>0.46±0.02</td>
<td>2.30±0.39</td>
</tr>
<tr>
<td>Lysate</td>
<td>0.28±0.04</td>
<td>0.85±0.61</td>
<td>0.57±0.13</td>
<td>2.68±0.47</td>
</tr>
<tr>
<td>Lysate+CpG</td>
<td>0.45±0.25</td>
<td>0.66±0.29</td>
<td>0.57±0.06</td>
<td>2.83±0.30</td>
</tr>
<tr>
<td>rVacA</td>
<td>0.46±0.42</td>
<td>0.48±0.16</td>
<td>0.63±0.07</td>
<td>2.47±0.36</td>
</tr>
<tr>
<td>rVacA+CpG</td>
<td>0.23±0.03</td>
<td>0.92±0.41</td>
<td>0.76±0.07</td>
<td>1.24±1.06</td>
</tr>
<tr>
<td>Mean±S.D.</td>
<td>0.37±0.28</td>
<td>0.63±0.37</td>
<td>0.57±0.14</td>
<td>2.26±0.75</td>
</tr>
<tr>
<td>p-valued</td>
<td>0.082</td>
<td>0.012</td>
<td>0.001</td>
<td>0.025</td>
</tr>
</tbody>
</table>

Data are expressed as means±S.D. \(^a\)Mice were infected with ATCC 43504 (cagA+/s1m1 vacA+) and, 3 weeks later, received three intra-gastric inoculations with lysate or rVacA plus the adjuvant CpG-ODN. Control groups consisted of mice that received PBS alone or PBS plus CpG-ODN; \(^b\)IgG against *H. pylori* lysate or rVacA was detected using ELISA in mucus and sera, respectively; \(^c\)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); \(^d\)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* 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+ 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.

Acknowledgements
This research was supported by a grant (CBM31-B3003-01-01-00) from the Center for Biological Modulators of the 21st Century Frontier R & D Program, the Ministry of Education, Science and Technology, Korea.

References


Received January 7, 2009
Revised March 9, 2009
Accepted April 21, 2009