Identification of immunodominant *Helicobacter pylori* proteins with reactivity to *H. pylori*-specific egg-yolk immunoglobulin

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The importance of hens eggs as a source of specific antibodies (IgY) is well recognized. The protective effect of IgY obtained from hens immunized with *Helicobacter pylori* whole-cell lysate has been reported for the control of *H. pylori* infection. However, IgY produced by whole-cell lysates presents the possibility of cross-reactivity with other bacteria, including the normal human flora, and this could decrease the efficiency of IgY. In the present study, the immunodominant proteins of *H. pylori* with reactivity to *H. pylori*-specific IgY (IgY-Hp) were identified. IgY obtained from hens immunized with various fractions of *H. pylori* proteins was isolated and purified, titres of IgY-Hp against *H. pylori* were determined and cross-reactivity between IgY-Hp and normal human bacteria was examined by Western blot analysis. Finally, immunodominant *H. pylori* proteins were identified by LC/MS analysis. IgY obtained 2 months after immunization with *H. pylori* whole-cell lysate showed the highest antibody titre. Five immunodominant proteins were identified that were strongly reactive to IgY-Hp: urease α-subunit (62 kDa), heat-shock protein 60 (60 kDa), urease β-subunit (26 kDa), probable peroxiredoxin (22 kDa) and probable thiol peroxidase (18 kDa). Immunization of hens with the immunodominant proteins identified would produce a more specific IgY against *H. pylori*.

INTRODUCTION

Eradication therapy of *Helicobacter pylori* is positively recommended worldwide for peptic ulcer disease (Anonymous, 1997; Korean *H. pylori* Study Group, 1998). Although *H. pylori*-associated gastritis is frequently observed, eradication of *H. pylori* is not generally recommended in this case. However, persistence of *H. pylori*-associated gastritis can induce atrophic gastritis (Satoh et al., 1996), which can then progress to gastric cancer by multiple pathways, although *H. pylori* itself is not concerned directly with cancer. *H. pylori*-associated gastritis rarely progresses to gastric cancer, though *H. pylori* is well known as a carcinogen (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 1994) or a risk factor (Korean *H. pylori* Study Group, 1998). The majority of the population with *H. pylori* infection shows only the gastritis state.

Eradication therapy for *H. pylori* employs two or more antibiotics and a proton-pump inhibitor; this therapy has undesirable side effects, including increasing the prevalence of antibiotic-resistant strains and increasing medical cost. Vaccine development is the preferred approach for *H. pylori* treatment, but an effective vaccine has not yet been developed (Michetti et al., 1999; Chakravarti et al., 2000). Recent studies have shown a variety of antibacterial activities against *H. pylori*. Non-antibiotic substances are easily obtained from edible sources that inhibit proliferation of *H. pylori* and prevent adherence of *H. pylori* to gastric epithelial cells. Thus, anti-adhesion and mucosal protective agents could represent potential targets for *H. pylori* treatment (Kim et al., 1997; Mysore et al., 1999; Simon et al., 1997; Mabe et al., 1999).

In a previous study, we demonstrated that egg-yolk immunoglobulin (IgY) against *H. pylori* whole-cell lysate is able to inhibit growth of *H. pylori* and that it reduces gastric inflammation in *H. pylori*-infected Mongolian gerbils (Roe et al., 2002). These facts suggest that IgY could be used as a novel modality against *H. pylori*-associated gastric diseases. However, IgY produced by whole-cell lysates may cross-react with other bacteria, including the normal human flora, and this could decrease the efficiency of IgY. Therefore, immu-
nization using a selective antigen is required. In an attempt to produce a more effective \textit{H. pylori}-specific IgY (IgY-Hp), we have elucidated in this study the immunodominant \textit{H. pylori} proteins that are strongly reactive to IgY-Hp.

\textbf{METHODS}

\textbf{Preparation of \textit{H. pylori} proteins.} \textit{H. pylori} ATCC 43504 was cultured in PBS (pH 7.2) and incubated for 1 h before adding 5 \% (v/v) bovine calf serum (PAA Laboratories Inc.) and antibiotics (2.5 \% amphotericin B, 10 \% vancomycin, 5 \% trimethoprim and 2.5 \% IU polymixin B ml$^{-1}$, all from Sigma) at 37$^\circ$C under 10 \% CO$_2$ at 200 rpm. \textit{H. pylori} cells were harvested by centrifugation at 12,000 g for 10 min to a concentration of 0.5 g wet weight ml$^{-1}$ PBS (pH 7.4) and disrupted by sonication at 20,000 Hz for 45 s; this process was performed a total of five times. Cellular material was first removed by centrifugation and the supernatant (800 \mu l) was then collected (\textit{H. pylori} whole-cell lysate). The \textit{H. pylori} whole-cell lysate was further separated into three fractions of >60, 60–25 and <25 kDa; the lysate was subjected to SDS-10 \% PAGE and the gel fractions were electrophoresed at 100 V for 3 h using an electrolute (Bio-Rad Laboratories). Protein concentrations were determined by the BCA method (Pierce).

\textbf{Immunization.} Brown Leghorn hens (25 weeks old, n = 30) were immunized intramuscularly with various \textit{H. pylori} proteins (200 \mu g ml$^{-1}$) using Freund’s complete adjuvant (Difco). Three booster injections in Freund’s incomplete adjuvant were given at 2-week intervals following the first injection. After the final immunization, the eggs laid were collected daily for 6 months and stored at 4$^\circ$C.

\textbf{Isolation and purification of IgY.} Isolation of IgY was carried out as described by Akita & Nakai (1993) with modifications. Briefly, the egg yolks were separated, pooled and frozen prior to purifying the IgY.

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\textbf{ELISA.} To assess the antibody activity of IgY-Hp, we used the ELISA described by Akita & Nakai (1992) with modifications. Briefly, 96-well plates were coated with \textit{H. pylori} whole-cell lysate (300 ng per well). After blocking with 1 \% (v/v) BSA, IgY-Hp (1 mg ml$^{-1}$) was diluted in a tenfold serial dilution in PBS (pH 7.2) and 100 \mu l of each dilution was added to plates. Plates were then washed with PBS-T (0.05 \% (v/v) Tween 20 in PBS, pH 7.2) and incubated for 1 h after adding alkaline phosphatase-conjugated goat anti-chicken IgY (Promega), diluted 1 : 1000 in blocking buffer, washed and incubated with alkaline phosphatase-conjugated anti chicken IgY (Promega) diluted 1 : 2000 in blocking buffer and then washed.

\textbf{Capillary liquid chromatography/mass spectrometry (LC-MS).} Coomassie brilliant blue-stained protein bands were excised from the gel. Gel slices were dried using a Speedvac concentrator, rehydrated in 30 \mu l 25 \% ammonium bicarbonate, pH 7.8, containing 0.2 \mu g trypsin and incubated at 37$^\circ$C for 20 h. The supernatants were evaporated and dissolved in 5 \mu l of an aqueous solution containing 0.1 \% (v/v) aqueous formic acid and 2 \% (v/v) acetonitrile for MS analysis. The HPLC system used was an Agilent 1100 series and this was coupled with a Finnigan LCQ DECA ion-trap mass spectrometer (Thermo Quest) equipped with a nanospray ionization source for LC-MS analysis of the digests. For LC separation, we used a reverse-phase capillary column (Vydac 218MS C$\alpha_1$, 5 \mu m, 100 \times 0.2 mm id.).

\textbf{RESULTS}

\textbf{Western blot analysis.} Electrophoretically transferred protein (2.5 \mu g protein per well) from unstained 10 \% SDS-polyacrylamide gels was used for Western blot. Proteins were transferred onto PVDF membranes (Bio-Rad). After transfer, membranes were blocked with blocking buffer (5 \% (v/v) non-fat milk milk powder, 10 mM Tris/HCL pH 7.5, 100 mM NaCl, 0.1 \% (v/v) Tween 20) and rinsed with wash buffer (blocking buffer without milk powder). The membranes were then incubated with IgY (1 mg ml$^{-1}$) diluted 1 : 100 in blocking buffer, washed with washing buffer, incubated with alkaline phosphatase-conjugated anti chicken IgY (Promega) diluted 1 : 2000 in blocking buffer and then washed.

\textbf{Cross-reactivity.} To determine the cross-reactivity of IgY-Hp against other bacteria, Western blot analysis was performed as described previously against the following members of the normal human gut flora: \textit{Escherichia coli} KCTC 1116, \textit{Streptococcus mutans} KCTC 3065, \textit{Lactobacillus salivarius} KCTC 3500 and \textit{Staphylococcus aureus} aureus KCTC 1916. E. coli and \textit{Staphylococcus aureus} were cultured in Luria–Bertani medium (Difco) at 37$^\circ$C for 24 h and \textit{Streptococcus mutans} and \textit{Lactobacillus salivarius} were cultured anaerobically for 2 days at 37$^\circ$C in brain heart infusion and Lactobacillus de Man–Rogosa–Sharpe (both from Difco) media, respectively.
fraction (lane 4) might include HSP60 and urease α-subunit and the low-molecular-mass fraction might include peroxiredoxin and thiol peroxidase.

Purification and characterization of IgY from egg yolk

IgY was isolated effectively using the λ-carrageenan and ultrafiltration method and the purity of IgY was characterized by 8 % SDS-PAGE analysis. The mean purity of IgY obtained was 90 %. The titres of IgY produced by various fractions of H. pylori protein were determined by ELISA. We found that a 1 : 1000 IgY dilution (1 mg ml⁻¹) was optimal for the measurement of IgY titres against various protein fractions, because this dilution rate gave an optimal absorbance range. IgY obtained from immunization with H. pylori whole-cell lysate showed a higher titre than that produced by immunization with other proteins. Specifically, IgY obtained 2 months after immunization with H. pylori whole-cell lysate showed the highest antibody titre (1·152) (Table 1); the titre of the control (non-immunization) IgY was 0·202.

Cross-reactivity of IgY-Hp against normal human bacteria

IgY obtained 2 months after immunization with H. pylori whole-cell lysate was used as the first antibody. In immunoblotting analysis, several dominant bands with reactivity to IgY-Hp were observed against H. pylori (Fig. 2). Immunoblotting revealed a few bands when E. coli, L. salivarius, Staphylococcus aureus and Streptococcus mutans were tested against IgY-Hp. Control IgY reacted with an H. pylori protein of approximately 22 kDa and rarely reacted with other H. pylori proteins; the band was definitely reduced in intensity with control IgY in the immunoblot compared with IgY-Hp (compare lanes 1 in Figs 2a and 2b). In contrast, the reactions of IgY-Hp and control IgY against E. coli were similar (compare lanes 2 in Figs 2a and 2b). These results indicate that IgY-Hp is very specific against H. pylori, but that the reaction against E. coli is not specific.

Identification of immunodominant H. pylori proteins with reactivity to IgY-Hp

Peptide mixtures resulting from in-gel tryptic digestion of each slice were fractionated by capillary column reverse phase-HPLC and analysed on-line using ESI-IT LC/MS. The resulting uninterpreted CID spectra were analysed using the SEQUEST database search algorithm. Five proteins were identified as urease α (26 kDa) and β (62 kDa) subunits, HSP60 (60 kDa), peroxiredoxin (22 kDa) and thiol peroxidase (18 kDa) of H. pylori. These results are shown in more detail in Table 2.

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**Table 1. Titres of IgY-Hp obtained by immunizing with various H. pylori protein fractions**

A 1 : 1000 dilution of IgY-Hp solution was used for ELISA; values are A₄05. ND, Not determined; p.i., post-immunization; WCL, whole-cell lysate.

<table>
<thead>
<tr>
<th>Time p.i. (months)</th>
<th>Protein fraction</th>
<th>WCL</th>
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<tbody>
<tr>
<td></td>
<td>&gt;60 kDa</td>
<td>60–25 kDa</td>
</tr>
<tr>
<td>2</td>
<td>0·397</td>
<td>0·570</td>
</tr>
<tr>
<td>3</td>
<td>0·363</td>
<td>0·471</td>
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<tr>
<td>4</td>
<td>0·333</td>
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<tr>
<td>5</td>
<td>0·225</td>
<td>0·223</td>
</tr>
<tr>
<td>6</td>
<td>0·208</td>
<td>0·224</td>
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</tbody>
</table>

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**Fig. 1.** Profile of H. pylori protein fractions. SDS-12 % PAGE was performed with each H. pylori protein fraction obtained using an electrophor. Lanes: 1, molecular size marker; 2, whole-cell lysate; 3, >60 kDa; 4, 60–25 kDa; 5, <25 kDa.

**Fig. 2.** Western blot analysis. H. pylori and normal human bacteria were reacted with IgY-Hp (a) and control IgY (non-immunization) (b). Each lane contained 2·5 μg bacterial protein. IgY-Hp and control IgY (both 1 mg ml⁻¹) were diluted 1 : 100. Lanes: 1, H. pylori; 2, E. coli; 3, L. salivarius; 4, Staphylococcus aureus; 5, Streptococcus mutans.
data that show a range of antibacterial activity against gastritis therapy. Therefore, recent studies have presented *H. pylori* with high effectiveness and safety, since antibiotic gastritis related to *H. pylori* indicates that cross-reactivity exists between IgY-

### DISCUSSION

In this study, we identified that the immunodominant proteins recognized by IgY-*Hp* are related to the urease β-subunit (62 kDa), HSP60 (60 kDa), urease α-subunit (26 kDa), probable peroxiredoxin (22 kDa) and probable thiol peroxidase (18 kDa) of *H. pylori*. Among the proteins identified, urease and HSP60 have been studied as vaccine antigens against *H. pylori* infection (Yamaguchi *et al*, 2000). IgY has recently attracted considerable attention as an alternative therapeutic agent and some of the suggested potential of IgY includes protective effects against dental caries (Shon *et al*, 1998; Smith *et al*, 2001), human rotavirus (Hatta *et al*, 1993), enterotoxigenic *E. coli* (Yokoyama *et al*, 1992) and *Salmonella typhimurium* (Sunwoo *et al*, 1996). The basic reasons for using the specific IgY include the replacement effect of antibiotics for humans and therapeutic effects against diarroheal disease in pigs and calves in order to reduce sensitivity for antibiotics efficiently and safely. Antibiotics are strongly recommended for use against *H. pylori*-associated gastric ulcer disease (Anonymous, 1997; Korean *H. pylori* Study Group, 1998), but many people with gastritis related to *H. pylori* are treated by conservative anti-gastritis therapy. Therefore, recent studies have presented data that show a range of antibacterial activity against *H. pylori* with high effectiveness and safety, since antibiotic therapy has several undesirable side-effects including high medical costs, low eradication effect of 85 % (Kim *et al*, 1999) and the emergence of mutant strains (Goddard & Logan, 1996; Nam *et al*, 2000).

In the previous study, we confirmed that growth of *H. pylori* was inhibited *in vitro* and *in vivo* by IgY-*Hp*, but IgY-*Hp* obtained after hyperimmunization with *H. pylori* whole-cell lysate may cross-react with *E. coli* and other members of the normal bacterial flora. In this study, IgY was prepared from the egg yolks of hens immunized with various protein fractions of *H. pylori*. Among the various fractions, IgY obtained from whole-cell lysate immunization of *H. pylori* showed the highest titre. Interestingly, IgY obtained from the protein fraction with low molecular mass (<25 kDa) was found to have a relatively higher titre than the high or intermediate protein fractions. By Western blot analysis, although IgY-*Hp* was strongly reactive to *H. pylori* proteins, it also reacted with other bacteria (*E. coli*, *L. salivarius*, *Staphylococcus aureus* and *Streptococcus mutans*). This indicates that cross-reactivity exists between IgY-*Hp* and other bacteria. Therefore, a study on the screening of selective antigens with high immunocompetence from *H. pylori* proteins is required.

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### Table 2. Identified immunodominant *H. pylori* proteins with reactivity to IgY-*Hp*

<table>
<thead>
<tr>
<th><em>H. pylori</em> protein</th>
<th>Molecular mass (kDa)</th>
<th>Accession no.</th>
<th>Observed mass</th>
<th>Xcorr*</th>
<th>ΔCn†</th>
<th>Peptide sequence</th>
</tr>
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<tbody>
<tr>
<td>Urease β-subunit</td>
<td>62</td>
<td>P14917</td>
<td>1394·7</td>
<td>3·6749</td>
<td>0·437</td>
<td>K.SIKEVDVFADS.R.I</td>
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<tr>
<td>HSP60</td>
<td>60</td>
<td>P42383</td>
<td>1109·6</td>
<td>1·5651</td>
<td>0·301</td>
<td>K.DRVDHALSAT.K.A</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>802·4</td>
<td>1·5838</td>
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<td></td>
<td>1536·8</td>
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<td>0·429</td>
<td>R.IVDKINNTIVDGK.G</td>
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<td></td>
<td></td>
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<td>980·5</td>
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<td>884·6</td>
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<td>26</td>
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<td>1025·3</td>
<td>2·1825</td>
<td>0·231</td>
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<td>Probable thiol peroxidase</td>
<td>18</td>
<td>O25151</td>
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<td>1·5006</td>
<td>0·292</td>
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*Cross-correlation: similarity between the mass-to-charge ratios of the fragment ions predicted from the amino acid sequence obtained from the database and the fragment ions observed in the LC/MS spectrum.
†Delta-normalized correlation: scored by normalizing the Xcorr values to 1·0 and observing the difference between the first- and second-ranked amino acid sequences.
‡Trypsin recognition sites are indicated by full stops (.).
BabA (Mizushima et al., 2001) and flagella (Porwollik et al., 1999) are well-known pathogenic and virulence factors of *H. pylori*. Most studies have focused on urease-based vaccines in animals or humans because of the essentiality of urease. Although immunization with urease has been successful in animal models (Michetti et al., 1994), clinical trials in humans with recombinant urease are not likely to protect fully against *H. pylori* challenge (Keller & Michetti, 2001). *H. pylori* HSP60 has also been studied as a vaccine candidate. This protein is a chaperonin for urease and is associated with adhesion to human gastric epithelial cells (Sharma et al., 1997). It is reported that immunization with the sequence of amino acids 189–203 of *H. pylori* HSP60 significantly reduced *H. pylori* colonization in mice, and this suggested that it might be a target for bacterial elimination by the immunity raised by *H. pylori* infection (Yamaguchi et al., 2000).

In the present study, the urease β- and α-subunits and HSP60 were identified as immunodominant proteins with reactivity to IgY-Hp. Interestingly, we found other immunodominant proteins, probable peroxiredoxin and probable thiol peroxidase. We don’t know the functions of these new proteins, and the mechanisms by which *H. pylori* persists in the gastric mucosal gel layer should be elucidated in further studies.

In conclusion, five immunodominant proteins strongly reactive to IgY-Hp were identified. We believe that the immunization of hens with selective antigens with high immunocompetence will enable the production of highly specific IgY against *H. pylori*. In addition, the proteins identified may serve as potential targets for antimicrobial agents for the prevention of *H. pylori* infection.

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