Effects of vaccination by a recombinant antigen ureB138 (a segment of the β-subunit of urease) against *Helicobacter pylori* infection

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*Helicobacter pylori* has to counteract acidity during colonization in the stomach. The most important region for the enzymic activity of *H. pylori* urease, consisting of 138 aa (ureB138), was determined by a comparison of the homology of amino acid sequences, and a structural analysis, between urease of *H. pylori* and various other species. This region was expressed in *Escherichia coli* as a fusion protein with glutathione S-transferase (GST), which was cleaved by PreScission protease between the GST moiety and ureB138. The ureB138 protein was then purified by gel filtration. The polyclonal antibody (pAb) induced by immunization with the purified ureB138 could suppress urease activity by about 50%, while the pAb against the *H. pylori* urease did not show any inhibitory effect at all. Immunohistochemical analysis indicated that the ureB138-specific pAb specifically recognized the *H. pylori* infecting human gastric tissues. The effects of vaccination of recombinant ureB138 against infection by this organism were also examined. Specific IgG and IgA antibodies against *H. pylori* urease were induced in the serum of mice immunized with ureB138. A reduction in the number of colonizing *H. pylori* was observed in mice treated with ureB138 compared to ones treated with BSA and infection control mice. In the protected mice, severe gastritis characterized by marked infiltration of mononuclear cells was noted compared with the gastritis observed in unprotected mice. Immunohistochemical staining for IgA in gastric mucosa showed that the number of mice positively stained with IgA was significantly higher in ureB138-vaccinated mice than in non-vaccinated mice. This indicates that local IgA antibody and severe post-immunization gastritis correlate well with the protection of mice against *H. pylori* infection.

**INTRODUCTION**

*Helicobacter pylori* was originally isolated from the stomachs of patients with active chronic gastritis and gastric ulcers by Marshall & Warren (1984). It is a Gram-negative spiral bacterium that persistently infects almost 50% of the world’s adult population (Graham et al., 1991). The infection may lead to the occurrence of various gastroduodenal diseases. *H. pylori* produces a large amount of urease (10–15% of total protein by weight), which is essential for the survival and pathogenesis of the bacteria (Eaton et al., 1991). Ammonia generated by the hydrolysis of urea neutralizes gastric acidity and forms a neutral microenvironment surrounding the bacterium within the gastric lumen.

Treatment of patients presenting with peptic ulcer disease consists of acid suppression and antimicrobial therapy. Although current treatments are effective, the emergence of...
antibiotic-resistant strains (Malfertheiner, 1993) and the high cost of therapy are significant problems (Takshima et al., 1988). Recently, many studies of vaccines against *H. pylori* have been reported (Corthesy-Theulaz et al., 1995; Goto et al., 1999; Raghavan et al., 2002). Urease is one of the antigens most commonly studied as a vaccine candidate (Pappo et al., 1995).

The urease of *H. pylori* (Hu & Mobylo, 1990) is a high molecular mass (530 kDa) multimeric enzyme composed of two distinct subunits, \( x \) (26.5 kDa) and \( \beta \) (61.7 kDa). It is different from most other microbial ureases (Jabri et al., 1995; Benini et al., 1999), which contain three different subunits, and from the jack bean urease, which is a single polypeptide (Takshima et al., 1988). The *H. pylori* urease localizes in both the cytoplasm and on the surface of *H. pylori*, and is required to counteract acidity during colonization in the stomach (Hawtin et al., 1990). The structure of the *H. pylori* enzyme has been determined already by Ha et al. (2001). The active site resides on the \( \beta \)-subunit and contains a bi-nickel centre near the active site. According to a structural analysis of the urease, the important amino acid residues are Cys321, His322 and His323, which are implicated in catalysis and substrate binding (Martin & Hausinger, 1992). Furthermore, His248, His274 and Lys219 are determined as the positions of the nickel ligands (Park & Hausinger, 1993). These residues are conserved among the ureases of various bacteria (Jabri et al., 1995; Benini et al., 1999) and plants (Takshima et al., 1988). A characteristic flap, which forms a helix–turn–helix motif, is present at the entrance to the active site cavity (Ha et al., 2001). The amino acid sequence of the flap is strictly conserved in *H. pylori* and *Klebsiella aerogenes*, whose sequence corresponds to aa 313–336 of *H. pylori* urease. The contribution of the flap to the catalytic activity is supported by the drastic reduction in the activity of *K. aerogenes* urease with a mutation of His322 (Park & Hausinger, 1993).

Based on these analyses, we determined a 138 stretch (aa 201–338) that is functionally important for urease activity. This region corresponds to the sequence between aa 472 and aa 609 of jack bean urease, which includes crucial amino acid residues such as cysteine and histidine that interact with the nickel ions. Similarly, many histidine residues are concentrated in the sequence between aa 201 and 338 of \( \beta \)-subunit of *H. pylori* urease. The homology for this region is 63% with jack bean urease.

Based on these facts, in this study, we examined the characteristics of the ureB138 protein. Furthermore, its function as a vaccine was investigated from the perspectives of immunochemistry and histochemistry using mice infected with *H. pylori*.

**METHODS**

**Preparation of recombinant ureB138.** Genomic DNA extracted from *H. pylori* was used as a template for PCR, in which the following primers were employed to amplify the ureB138 region: forward primer (5'-AAGGATCCGCTTCAAGCATGGAGCC-3'), which contains a BamHI site (underlined), and reverse primer (5'-GGGATCCCTTTGAATCAGCGAACTG-3'), which contains an EcoRI site (underlined). An aliquot of the PCR mixture was analysed by agarose gel electrophoresis.

To construct a plasmid for the expression of recombinant ureB138 fused with the glutathione-S-transferase (GST) protein, the PCR-amplified DNA fragment was ligated to the expression vector, pGEX-6P-1 (Pharmacia), which includes the sequence of the PreScission protease (Amersham Biosciences) cleavage site. *E. coli* BL21 was transformed with the construct and the recombinant ureB138 protein induced by addition of a final concentration of 1 mM IPTG. The cultured cells were harvested by centrifugation, washed in PBS and lysed by sonication. The supernatant was directly applied to a GSTPrep FF column (Amersham Biosciences). To remove the GST tag, PreScission protease was added to the column and incubated at 5 °C for 20 h. The flow-through containing the ureB138 protein, which had been cleaved from the GST moiety, was collected. Both the protease and the GST moiety retained by the column were eluted with 10 mM reduced glutathione. Additionally, the ureB138 fractions were purified by size-exclusion chromatography with a Superdex75 column (Pharmacia). The purity of recombinant ureB138 was confirmed by 15% SDS-PAGE.

**Production of polyclonal antibodies (pAbs).** For obtaining pAbs, such as anti-ureB138 pAb, anti-*H. pylori* urease pAb and anti-human copper–zinc superoxide dismutase pAb (as a negative control; Sugino et al., 1996; Suzuki et al., 1999), rabbits were immunized subcutaneously by each antigen with Freund’s adjuvant three times at 2 week intervals, and blood samples were taken from a vein. The resultant pAbs were purified using affinity chromatography (Sepharose 4B coupled with *H. pylori* urease).

**Inhibition assay of *H. pylori* urease activity by antibodies.** In order to investigate the inhibitory effect of pAbs on the enzymic activity of *H. pylori* urease, the urease (1 μg in 50 μl 20 mM sodium phosphate buffer, pH 6.5) was mixed with each purified pAb described above in 96-well microtitre plates. The mixture was then incubated for 90 min at room temperature (pre-incubation). Another mAb (HpU-2) for *H. pylori* urease (Ikeda et al., 1998) was employed as a positive control. After the pre-incubation, 100 μl of the above reaction solution was mixed with 100 μl 20 mM sodium phosphate buffer (pH 6.5) containing 100 mM urea and 0.005% phenol red. Using a microplate reader (Molecular Devices), the time-course of the colour development was monitored by its absorbance at 550 nm at 30 min intervals for 4 h at room temperature. Then, the rate of inhibition (%) was calculated by using the absorbance value at 135 min.

**Bacterial strain and growth conditions.** The Sydney strain (SS1) of *H. pylori* was grown on a Brucella agar plate containing 7% fetal bovine serum (Gibco-BRL Life Technologies) for 4 days at 37 °C under a microaerophilic atmosphere (5% O₂, 10% CO₂, 85% N₂). A colony was suspended into 8 ml Brucella broth (Becton Dickinson) and cultured for 7 h under the same conditions.

**Schedule of immunization of recombinant ureB138 into mice and challenge of *H. pylori*.** Specific-pathogen-free 5-week-old female C57BL/6 mice were purchased from Shimizu Laboratory Supplies. The mice were housed in a specific-pathogen-free environment and were provided with free access to food and water.

The mice were divided into three groups: group 1, immunized with 100 μg per dose of ureB138 (n=13); group 2, immunized with 100 μg per dose of BSA (n=13); group 3, not immunized (n=8). Each antigen (2 mg ml⁻¹) was emulsified with Freund’s complete adjuvant (Wako Pure Chemical). The emulsion was subcutaneously injected into each mouse at a total volume of 100 μl per animal. The mice
were given boosters in the same manner, except that Freund's complete adjuvant was changed to Freund's incomplete adjuvant (Wako Pure Chemical). The immunization (vaccination) was repeated at weekly intervals for 3 weeks. One week after the last vaccination, a blood sample was collected from each animal to monitor the immune response and stored at −80°C until use. One week after the last vaccination, all mice were challenged two times with 0.5 ml live *H. pylori* (1 × 10^8 c.f.u. ml⁻¹).

**Titre of the antibody in serum.** Blood was obtained from mice at 1 week after the final vaccination, and whole IgG and IgA titres were determined by ELISA. Briefly, microtitre plates (Nunc) were coated with 50 μl antigen [purified urease (>95%) from *H. pylori*, 250 ng per well] in PBS overnight at 4°C. Plates were blocked for 30 min at room temperature with 5% skimmed milk in PBS, and washed with PBS containing 0.05% Tween 20. The sera were diluted to 100-fold with the addition of PBS containing 1% BSA to the wells and incubated for 1 h at room temperature. After a wash step, the wells were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Vector Laboratories) or IgA (Kirkegaard & Perry Laboratories) antibodies for 1 h. After further washing, a solution containing o-phenylenediamine was added as a substrate for colour development. After 15 min incubation, absorbance was measured at 490 nm by a microplate reader (Molecular Devices). Each sample was tested in duplicate.

**H. pylori colony count.** At 6 weeks after the second challenge, all mice were sacrificed and the stomachs isolated. Each stomach was cut longitudinally into two pieces. One half was homogenized in 500 ml *Brucella* broth and inoculated onto *Brucella* broth using a glass homogenizer (Iwaki Glass). Fifty microlitres of gastric homogenate was serially diluted with *Brucella* broth and inoculated onto *Helicobacter*-selective agar plates (Nissui Pharmaceutical) at 37°C for 4 days under microaerobic conditions. Colonies were counted and expressed as c.f.u. (g stomach tissue)⁻¹.

**Histological evaluation.** The remaining half of each stomach was fixed with 10% formalin and embedded in paraffin. Sections (2 μm thick) were stained with haematoxylin-eosin (HE).

**Immunohistochemical staining with IgA antibody.** The stomach sections were deparaffinized in xylene, dehydrated in graded ethanol and then endogenous peroxidase activity was blocked by 3% H₂O₂. They were preincubated with normal goat serum for 10 min at room temperature and stained with rabbit anti-mouse IgA antibody (Zymed Laboratories) (1 μg ml⁻¹) in PBS for 60 min. After being washed three times with PBS for 3 min each, the slides were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) (Zymed Laboratories) in 1% BSA/PBS for 60 min at room temperature and washed with PBS. Staining was performed using a DAB (3,3′-diaminobenzidine tetrahydrochloride; Dijindo Laboratories) substrate kit (Nichirei) and the nuclei were counterstained with haematoxylin.

**Statistical analysis.** *H. pylori* colony counts were analysed and compared by t-test. P values of <0.05 were considered as a significant difference.

### RESULTS

#### Characterization of antibody induced by the ureB138 antigen

A pAb was produced by immunizing rabbits with the purified ureB138 antigen. The immunoreactivity of the pAb obtained was investigated by ELISA. The pAb strongly reacted with *H. pylori* urease, as well as jack bean urease (Fig. 1a), as it possesses the conserved sequence of the urease, but did not react with BSA. Immunohistochemical staining by the pAb using the specimen of human gastric tissues infected with *H. pylori* was also performed. The results demonstrated that the pAb could specifically bind with *H. pylori* urease (Fig. 1b, c).

#### Suppression of *H. pylori* urease activity by pAb induced with ureB138 protein in rabbit

The pAb was purified by *H. pylori* urease-fixed affinity chromatography. As shown in Fig. 2, the ureB138 specific pAb inhibited *H. pylori* urease activity in a concentration-dependent manner. Concentrations of 25 and 5 μg

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**Fig. 1.** Characterization of ureB138 pAb. (a) Immunoreactivity of ureB138 pAb to proteins examined by ELISA: ●, *H. pylori* urease; ▲, recombinant ureB138 protein; ■, Jack bean urease; ○, BSA. The pAb purified by affinity chromatography reacted with ureB138 protein, *H. pylori* urease and Jack bean urease but not with BSA. (b, c) Immunohistological staining with pAb. *H. pylori*-infected gastric biopsy specimens were used in this experiment: (b) Gimenez staining and (c) pAb staining. The pAb could bind to the *H. pylori* infecting human gastric tissues. The arrows show the stained *H. pylori* cells.
ureB138 pAb could suppress the activity by about 49% and 38%, respectively. In contrast, the pAb for *H. pylori* urease did not inhibit the urease activity at all, which was similar to the results reported up to now (Fujii et al., 2004).

**Titres of IgA and IgG**

IgA and IgG antibodies induced by the immunization of the ureB138 antigen were investigated using ELISA. As a control, BSA was immunized in the same manner as in the case of ureB138. The results are shown in Fig. 3, in which ELISAs were performed with *H. pylori* urease. In the case of IgG, when mice were immunized with ureB138, the titre became much higher than for BSA. In the case of IgA induced by immunization with ureB138, the values of the titre scattered to some extent. In this case, the titres were low compared with IgG.

**Effects of vaccination against *H. pylori* infection**

Vaccination effects were investigated using C57BL/6J mice by the protocol described in Methods. As shown in Fig. 4, a mean bacterial value of 16 \((\pm 9.6) \times 10^6\) c.f.u. \((\text{g stomach tissue})^{-1}\) was recovered for the non-vaccinated mice. For the BSA-immunized mice, the value was 9.9 \((\pm 4.2) \times 10^6\) c.f.u. \((\text{g stomach tissue})^{-1}\). In contrast, the value for the ureB138-vaccinated mice was 4.7 \((\pm 4.0) \times 10^6\) c.f.u. \((\text{g stomach tissue})^{-1}\), demonstrating a significant reduction in the number of colonies compared to the non-vaccinated mice \((P<0.05)\). In the case of ureB138- and BSA-vaccinated mice, there was a partial overlap, but a difference was still apparent.

**Effects of vaccination against local inflammation in the stomach**

In order to analyse the effect of ureB138 vaccination in mice, histological changes were determined by HE staining. The results are shown in Fig. 5. For ureB138-vaccinated mice, a significant reduction in the number of colonizing bacteria was observed in ureB138 mice compared to those not vaccinated. Horizontal bars indicate mean value of the colony counts ± SD of the results for each mouse.

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**Fig. 2.** Inhibition test of *H. pylori* urease activity by pAb. The anti-ureB138 pAb demonstrated concentration-dependent inhibition against urease activity. The strongest inhibitory effect (49%) was displayed by 25 μg anti-ureB138 pAb. With 5 μg, the effect was 38%. In contrast, the anti-urease pAb did not exhibit any inhibitory effect. HpU-2 mAb interacts with an epitope existing in the α-subunit of the urease of *H. pylori* and reduces the enzymic activity down to about 80% (Ikeda et al., 1998).

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**Fig. 3.** Detection of antibody responses in the sera of vaccinated mice. Blood samples were taken from mice that were treated with ureB138 and BSA at 1 week after the final vaccination, and the antibody titres against *H. pylori* urease were determined by ELISA: (a) IgG and (b) IgA. Significant increases in IgG levels were detected in the ureB138-vaccinated mice compared to the BSA-mice. On the other hand, in (b), the IgA titres of ureB138 were scattered to some extent compared with those of IgG, and the difference between ureB138 and BSA was less than the case of IgG. Horizontal bars indicate mean value of the titres ± SD of the results for each mouse.

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**Fig. 4.** Quantitative *H. pylori* culture of gastric tissue. Colony counts are presented as log c.f.u. \((\text{g stomach tissue})^{-1}\). A significant reduction in the number of colonizing bacteria was observed in ureB138 mice compared to those not vaccinated. Horizontal bars indicate mean value of the colony counts ± SD of the results for each mouse.
mice, the grade of gastritis was more severe than that for BSA. In particular, the gastritis score in protected mice was significantly higher than that in unprotected mice (Fig. 6). Infiltration of numerous neutrophils, plasma cells and mononuclear cells was observed in the stomach of the protected (ureB138-vaccinated) mice. Fig. 7 shows that the degree of gastritis had a tendency to increase in the stomach of the ureB138-vaccinated mice, which demonstrated a marked reduction of bacterial colonization. (The data points became fewer, especially for non-vaccinated samples, because some samples failed to make formalin-fixed tissues.)

**Immunohistological evaluation of gastric tissue**

To evaluate local IgA expression caused by the vaccination, immunohistological staining for IgA antibody in gastric mucosa was also performed 6 weeks after the challenge of *H. pylori*. In the case of ureB138-vaccinated mice, a high level of IgA was detected in the glandular lumen (Fig. 8).

**DISCUSSION**

*H. pylori* is a cause of infectious disease in the stomach. Because its route of infection has not been completely elucidated and an effective vaccine has not yet been developed, the prevention of *H. pylori* infection is difficult. Antibiotics cannot be used to eliminate *H. pylori* from all infected individuals because of the presence of resistant bacteria and side effects. Although *H. pylori* vaccine therapy is expected, it is still at the development stage.

The 138 amino acids extracted in this study, termed as ureB138, were expressed as a fused protein with GST in *E. coli*. As previously reported, we produced mAbs against ureB138 (Fujii et al. 2004). In the experiment, 11 out of 17 mAbs obtained suppressed the urease activity of *H. pylori*, indicating that the proportion of the obtained mAbs
capable of inhibiting the activity was approaching 70%. Furthermore, the pAb generated by immunization with recombinant ureB138 protein could inhibit the enzymatic activity of *H. pylori* urease. On the other hand, pAb obtained by immunization with urease did not show the inhibitory effect at all. Thus the ureB138 antigen has a characteristic feature able to induce unique antibodies capable of suppressing the urease activity.

*H. pylori* produces highly active urease, which enzymically catalyses the hydrolysis of urea to carbon dioxide and ammonia. *H. pylori* urease is found on the surface of the organism during spontaneous lyses of some of the bacteria (Phadnis *et al.*, 1996). Up to 30% of total urease is present on the surface of *H. pylori in vivo* (Dunn *et al.*, 1997). However, as urease-negative mutants fail to colonize gastric tissue of nude mice (Tsuda *et al.*, 1994), urease may represent an important target for the prevention of disease. The important region (138 aa residues of the β-subunit of the *H. pylori* urease) was identified by comparison of urease sequences among several bacteria and by the analysis of the structure of *H. pylori* urease. Based on these analyses, the sequence of ureB138 was determined, and ureB138 was expressed as a new antigen for a vaccine candidate in *E. coli* in the form of a GST-fusion protein.

The inhibitory effect of the ureB138 pAb on the enzymatic activity of *H. pylori* urease was investigated. An amount of 25 μg (125 μg ml⁻¹) of purified ureB138 pAb could reduce the enzymatic activity of *H. pylori* urease by 49%. However, anti-*H. pylori* urease pAb did not neutralize the urease activity. Similar evidence has been reported by Nagata *et al.* (1992), in which the urease-specific pAb generated by immunization with purified whole *H. pylori* urease protein did not exhibit an inhibitory effect. It is believed that the pAb induced by the whole protein mainly recognizes parts of the antigen other than the active site. ureB138 corresponds to the region of the active site. Thus the anti-ureB138 pAb can recognize the active site of urease, and hence inhibit the enzymic activity of the urease.

Nolan *et al.* (2002) reported that the level of urease activity is an important factor in colonization. Hence, the inhibition of urease activity might be a key factor in determining the fitness of *H. pylori* for colonization. The designed antigen, ureB138, can induce the antibodies inhibiting urease activity and recognizing *H. pylori* infecting human gastric mucosa. The concentration of immunoglobulin in human serum is about 20–30 mg ml⁻¹; 125 μg ml⁻¹ of ureB138 specific pAb corresponds to 1% of the immunoglobulin. It is considered that a sufficient amount of the antibody capable of strongly suppressing urease activity can be induced by ureB138 immunization.

Various studies have proved that *H. pylori* urease can serve as a protective antigen in a vaccine (Corthesy-Theulaz *et al.*, 1995; Goto *et al.*, 1999; Raghavan *et al.*, 2002). Hence, to investigate the effect of ureB138 vaccination against *H. pylori* infection using a mouse model, we subcutaneously treated mice with the antigens. At one week after the final vaccination, the ureB138 group produced serum IgG and IgA antibodies. We also observed an acute inflammatory cell infiltration in the corpus region 6 weeks after the *H. pylori* challenge. Goto *et al.* (1999) demonstrated that the development of a more severe inflammation might lead to protection. A number of investigators have reported that gastric inflammation occurs in immunized mice after an *H. pylori* challenge. This effect has been referred to as post-immunization gastritis, which might correlate with protection against *H. pylori* infection (Mohammadi *et al.*, 1996). In our case, the degree of the gastritis showed a tendency to increase in the stomach of the ureB138-vaccinated mice, which demonstrated a marked reduction of bacteria colonization (Fig. 7). It is considered that post-immunization gastritis occurs for protection other than that provided by the inhibitory effect of the antibody.

Goto *et al.* (1999) clarified the important role of IgA in the stomach against *H. pylori* colonization using a prophylactic vaccine. In the present study, we observed protection against *H. pylori* infection in ureB138-vaccinated mice, in
which high levels of IgA were detected in gastric mucosa in 8 out of 13 mice. This IgA is not necessarily specific for ureB138. However, it was not detected when BSA was immunized, though both mice (ureB138- and BSA-vaccination) were challenged with H. pylori. Therefore, the IgA was certainly generated by the immunization with ureB138. Furthermore, the IgA in the serum (Fig. 3) increased in the case of the immunization with ureB138 but not BSA. From these facts, we conclude that the IgA may be specific for ureB138.

At present, it is not clear how the antibody causes the inhibitory effect for urease activity against infection by H. pylori. However, it may contribute to a reduction of H. pylori survival to some extent. This mechanism must be clarified in the future. In conclusion, the current observations suggest that immunization of the recombinant protein ureB138 interfered with the establishment of infection with H. pylori.

ACKNOWLEDGEMENTS

This study was supported by Japan Science and Technology Agency (research area: Creation of Bio-devices and Bio-systems with Chemical and Biological Molecules for Medicinal Use).

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