Identification of an Antigenic Epitope in *Helicobacter pylori* Urease That Induces Neutralizing Antibody Production

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We previously reported a mouse monoclonal antibody (MAb), termed L2, specific for *Helicobacter pylori* urease strongly inhibited its enzymatic activity. Here, to gain insight into how this antibody affects urease activity, the epitope that was recognized by the antibody was determined. By screening a panel of overlapping synthetic peptides covering the entire sequence of the two subunits (UreA and UreB), we identified a stretch of ureB-derived 19 amino acid (aa) residues (UB-33; aa 321 to 339, CHHLDKSIKEDVQFADSRI) that was specifically recognized by the L2 antibody. Further sequential amino acid deletion of the 19-mer peptide from either end allowed us to determine the minimal epitope as 8 amino acid residues (F8; SIKEDVQF) for L2 reactivity. This epitope appears to lie exactly on a short sequence which formed a flap over the active site of urease, suggesting that binding of the L2 antibody sterically inhibits access of urea, the substrate of urease. Finally, immunization of rabbits with either the 19-mer peptide or the 8-mer minimal epitope resulted in generation of antiurease antibodies that were capable of inhibiting the enzymatic activity. Since urease is critical for virulence of *H. pylori*, antigenic peptides that induce production of antibodies to inhibit its enzymatic activity may potentially be a useful tool as a vaccine for prevention and treatment of *H. pylori* infection.
preventing bacterial growth and attachment to the gastric mucosa. In this study, using the *H. pylori* urease-specific murine MAb L2 having a strong capacity to neutralize the urease activity, we tried to identify the neutralizing epitope with a series of overlapping peptides covering the entire sequence of *H. pylori* urease to gain insight into how this antibody affects urease activity.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *H. pylori* NCTC 11637 was cultured on modified Belo-Horizonte medium (pylori agar medium) (Nikken Bio Medical Lab., Kyoto, Japan) for 3 days at 37°C under a microaerophilic atmosphere (5% O₂, 15% CO₂, and 80% N₂) by AnaeroPack Campylo (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). A colony was inoculated into 20 ml of Brucella broth (Becton Dickinson, Cockeysville, Md.) containing 0.1% β-cyclodextrin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 5% (vol/vol) horse serum in a 250-ml culture bottle, and the cultures under the same conditions. After incubation for 24 h with shaking (100 rpm) at 37°C, 2 ml of culture was transferred to a 250-ml culture bottle containing 40 ml of fresh medium, and the cells were reincubated twice under the same conditions. One milliliter of the incubating medium containing the cells mostly spiral and coccoidi, was plated on Brucella agar (Becton Dickinson) containing 7% (vol/vol) defibrinated horse blood and cultured for an additional 3 days at 37°C in a microaerophilic atmosphere. Bacterial cells were harvested and washed twice with cold phosphate-buffered saline (PBS) at pH 7.0. Then the cells were sedi-

**Peptide synthesis.** As shown in Fig. 1, 23 sets (UA-1 through UA-23) of 18 truncated peptides originating from the large subunit of *H. pylori* urease, as indicated in Table 1, a series of 18 truncated peptides originating from the large subunit of *H. pylori* urease, were also synthesized to determine the minimum epitope in UB-3 by the same procedure. In brief, the tert-butylcarbonyl (t-Boc)-protected amino acids were obtained with a model 430 multipptide synthesizer (Applied Biosystems, Foster City, Calif.). Each peptide was partially deblocked and cleaved from the resin in liquid anhydrous HF. All synthetic peptides were purified and isolated by reverse-phase high-pressure liquid chromatography (RP-HPLC) performed with a liquid chromatograph (Waters, Milford, Mass.). The purity and molar concen-

**Measurement of *H. pylori* urease enzymatic activity.** The urease activity was prepared from various fractions of bacteria: the urease fraction, purified by column chromato-

**Urease inhibition (neutralizing) test using specific antibodies.** The *H. pylori* urease inhibition test was carried out similar to the urease assay described above. In brief, 25 μl of *H. pylori* urease was incubated with either 25 μl of urease-specific MAb or IgG purified from immune rabbit serum (equivalent to 0 to 25 μg of IgG protein) in 96-well microtiter plates overnight at 4°C, and then 50 μl of a 50 mM phosphate buffer (pH 6.8) containing 500 mM urea, 0.02% phenol red, and 0.1 mM DTT was added to each well. The color development was monitored at 550 nm with a microplate reader (model 3550; Bio-Rad) over a 3-h incubation period at 23°C, and the rates of development were calculated from the linear portions of the curves. Urease activity was estimated with jack bean urease as a standard, the specific activity of which was 33,600 U/g, dry weight. One unit is equivalent to 1 μmol of ammonia liberated per min.

**RESULTS**

Mapping of neutralization epitope identified with *H. pylori* urease-specific MAb L2. We have previously reported that we happened to obtain a murine hybridoma specific for *H. pylori* urease, termed L2, that could significantly inhibit the urease activity (27), which we have confirmed again in the present study (Fig. 2, indicated as open circles). Using a series of overlapping peptides covering the entire sequence of *H. pylori* urease, we tried to identify the neutralizing epitope by ELISA. The L2 reactivity to each peptide fragment derived from UreA and UreB is shown in Fig. 1. Among 79 overlapping peptides tested, only L showed a significant response to L2. The epitopic peptide was identified in the large subunit of *H. pylori* urease, UreB, as a UB-33 (aa 321 to 339) C-terminal phosphatase-conjugated goat anti-rabbit immunoglobulin (Sigma Chemical Co.) to the yellow product; p-nitrophenylphosphate (Sigma Chemical Co.) to the yellow product; p-nitrophenylphosphate (Sigma Chemical Co.) to the yellow product; p-nitrophenylphosphate (Sigma Chemical Co.) to the yellow product; p-nitrophenylphosphate (Sigma Chemical Co.) to the yellow product; p-nitrophenylphosphate (Sigma Chemical Co.) to the yellow product; p-nitrophenylphosphate (Sigma Chemical Co.) to the yellow product; p-nitrophenylphosphate (Sigma Chemical Co.) to the yellow product; p-nitrophenylphosphate (Sigma Chemical Co.) to the yellow product; p-nitrophenylphosphate (Sigma Chemical Co.) to the yellow product; p-nitrophenylphosphate (Sigma Chemical Co.) to the yellow product; p-nitrophenylphosphate (Sigma Chemical Co.) to the yellow product; p-nitrophenylphosphatase-conjugated goat anti-rabbit immunoglobulin (Cappel, Durham, N.C.) or alka-

**Rabbit immunization protocols.** A synthetic epitope peptide, UB-33 (CCHLDSKISEDVQFADRSI), from *H. pylori* urease was synthesized by solid-phase peptide synthesis as described above. Then, keyhole limpet hemocyanin (KLH) was conjugated to the N-terminal cystein of UB-33 by the Maleimido method (16) using *meru*-maleimidobenzyl N-hydroxyssuccinimide ester (MBS) as a coupling reagent. Preimmune sera (negative control) were obtained from three rabbits, which were then immunized subcutaneously (s.c.) with 1 mg of UB-33-KLH dissolved in PBS emulsified 1:1 in complete Freund’s adjuvant (CFA) in a total volume of 1 ml per animal. The rabbits were bled once (prebooster), and then they were given boosters in the same manner, except that CFA was changed to incomplete Freund’s adjuvant (IFA), at 2-week intervals. The immune rabbits were bled every 7 days after the booster immunization, and each serum sample was tested for *H. pylori* urease-specific antibody activity by ELISA. Similarly, another three sets of rabbits (each set composed of three rabbits) were immu-

All animal experiments were performed according to the guideline of the NIH Guide for the Care and Use of Laboratory Animals and approved by the Review Board of Nippon Medical School.
SRI [28a]) that showed a predominant response, and the titer was almost equal to that against the whole purified H. pylori urease (positive control).

Induction of linear epitope (UB-33)-specific antibody by immunization with UB-33-KLH. Then we tried to confirm whether the UB-33 (linear epitope)-specific antibody could actually inhibit (neutralize) H. pylori urease activity. We made a carrier protein (KLH)-conjugated UB-33 (UB-33-KLH) and immunized three rabbits with that UB-33-KLH together with CFA to obtain UB-33-specific antibody. The mixture of sera from three rabbits was purified into IgG with an affinity column, and its specific response was measured by ELISA. The obtained rabbit IgG antibody was specific for the linear peptide UB-33 and does not cross-react with the neighboring peptides UB-28 to UB-35 (data not shown), suggesting that the UB-33-derived antibody may respond to its central portion.

Capacity of UB-33-specific rabbit IgG antibody to inhibit (neutralize) H. pylori urease activity. Based on the inhibition assay described in the Materials and Methods section, we examined whether the obtained UB-33-specific rabbit IgG antibody had the capacity to inhibit H. pylori urease activity. As demonstrated in Fig. 2, in comparison with purified IgG from the mixed sera of three rabbits immunized with purified H. pylori urease, the rabbit UB-33-specific IgG antibody showed a significant inhibition of H. pylori urease activity. As also shown in Fig. 2, less than 10 μg of IgG/well of L2 MAb induced complete inhibition of the H. pylori urease activity, whereas around 25 μg of IgG/well of UB-33-specific rabbit antibody showed 60% inhibition, a value obtained with approximately 3 μg of IgG/well of L2. Thus, the inhibitory capacity of L2 appears to be 8 to 9 times greater than that of UB-33-specific rabbit IgG antibody.

Comparison of minimum antigenic epitope between L2 and UB-33-specific rabbit IgG antibody. We tried to compare the minimum antigenic epitope(s) for both L2 and UB-33-specific rabbit IgG antibody using a series of truncated peptides (Table 1). As shown in Fig. 3 as well as in Table 1, both UB-33(12R) (IKEDVQFADSRI) and UB-33(13L) (CHHLDKSIKEDVQ) significantly reduced the L2 response, the minimum antigenic epitope for L2 was identified as an 8-mer peptide, SIKEDVQF (UB-33-F8) within UB-33 (CHHLDK-SIKEDVQFADSRI, shown in italics). In contrast, because the UB-33-specific rabbit polyclonal IgG did respond to both UB-33(12R) and UB-33(11R) (KEDVQFADSRI) but weakly to UB-33(10R) (EDVQFADSRI), the minimum antigenic epitope among the rabbit polyclonal antibodies was a 5- or 6-mer peptide, (K)EDVQF. However, these polyclonal antibodies also seem to contain UB-33-F8 specific MAb to some extent that might elicit L2-like inhibition shown in Fig. 2. To examine whether the 8-mer peptide can induce neutralizing
Induction of minimal epitope-specific antibody by F8-MAP peptide. To induce antibodies specific for minimal epitope (UB-33(F8)), we then tried to immunize rabbits with F8-based MAP composed of 8 residues of F8 (F8-MAP) in a unit using lysine (K) as binders (see Materials and Methods). Because the generated rabbit IgG showed clear UB-33 specificity, we tried to examine the capacity to inhibit H. pylori urease activity. As also shown in Fig. 2, rabbit IgG fraction purified from F8-MAP-immunized rabbits inhibited the urease activity better than UB-33-KLH-derived IgG. Thus, we tried to identify the minimal epitope of that antibody using similar sets of truncated peptides. As demonstrated in Table 1, the minimal epitope of F8-MAP-elicited antibodies was determined to be SIKEDVQF. However, Table 1 also indicated that the F8-MAP-induced polyclonal rabbit IgG antibodies also seem to contain F8 (SIKEDVQF)-specific MAb that might elicit L2-like inhibition shown in Fig. 2.

Cross-reactivity to ureases of other species and peptide corresponding to UB-33 of each urease. We further studied whether UB-33-specific antibody might cross-react with the ureases of other species using L2. As shown in Fig. 4, although L2 did cross-react with purified ureases of the Helicobacter species H. pylori, H. felis, and H. nemastrinae, the MAbs did not cross-react with ureases of Proteus mirabilis or jack bean. Thus, we synthesized peptides from the ureases corresponding to the UB-33 portion to examine the cross-reactivity using three distinct antibodies, such as L2, UB-33-specific IgG, and F8-MAP IgG. As indicated in Table 1, although L2 and F8-MAP IgG did not cross-react, the urease-derived peptides from jack bean and P. mirabilis, UB-33-specific IgG did respond. Therefore, we synthesized a series of peptides with a single amino acid substitution in the epitope of UB-33 corresponding to the jack bean urease (327E, 329P, 332L, 333A, and 336H) to determine the critical amino acid(s) for the response. The results clearly demonstrated that both L2- and UB-33-specific antibodies cross-reacted with 327E, 332L, 333A, and 336H, while L2 and F8-MAP antibodies did not respond to 329P at all, though the UB-33-specific antibody did cross-react with that substituted peptide. These results strongly indicated the importance of 329K for F8-specific recognition, and 329K is not a critical amino acid for the recognition of UB-33-KLH-induced antibodies.

Response of purified H. pylori urease immune sera to epitope UB-33. Finally, we tried to confirm whether purified H. pylori urease immune sera could respond to the neutralization epitope UB-33. The result indicates that the mixed IgG antibodies from the sera of three rabbits immunized with purified H. pylori urease did not respond to UB-33 at all, though it
showed the strong response to purified *H. pylori* urease at almost the same level as UB-33-KLH- or F8-MAP-immunized rabbit IgG antibodies (Fig. 5). In addition, as far as we have investigated, we could not find any specific linear epitope recognized by that immune serum (data not shown). Thus, when we use purified urease as an immunogen, we might obtain antibodies mostly specific for conformational structure of the *H. pylori* urease.

**DISCUSSION**

In this paper, we demonstrated that there are two types of IgG antibodies against *H. pylori* urease. One is conformational structure specific and the other is linear epitope (UB-33) specific. Our present observation indicates that dominant production of the former antibodies that have no effect on *H. pylori* urease activity when the whole purified urease was used as the immunogen. In contrast, when the linear epitope was used as the immunogen, we could obtain a good amount of the latter epitope-specific antibodies, which inhibit the enzymatic activity of *H. pylori* urease. Indeed, Mooney and colleagues demonstrated that inhibitors for the urease abrogated *H. pylori* persistence in the gastric mucosa and prevent infection (25). To date, there are several reports on *H. pylori* vaccination using *H. pylori* urease (24) or its B subunit as immunogens (5). However, the present study in an animal model suggests that the whole *H. pylori* urease seems to generate insufficient immunity that might not abolish the enzymatic activity. If *H. pylori* urease is an actual target for making an effective vaccine, it is better to elicit antibodies that abrogate urease activity. Therefore, we would like to recommend that the epitope UB-33, in particular the minimal 8-mer peptide SIKEDVQF, be included to make a vaccine that prevents *H. pylori* infection as well as persistence.

In the present study, we synthesized a series of overlapping peptides covering the entire sequence of *H. pylori* urease and...
Antigen

None

F8MAP

UB33-KLH

HP urease

Anti f8MAPS rabbit IgG

Anti UB33-KLH rabbit IgG

Anti HPurease rabbit IgG

O.D. at 405nm

FIG. 5. Antibodies from purified \( H. pylori \) urease immune sera did not respond to linear UB-33 and F8 peptides. Diluted antigens (F8-MAP, UB-33, or \( H. pylori [HP]\)urease) were coated onto 96-well ELISA plates overnight with blocking, and then 50 \( \mu \)l of each sample (diluted 1:20) (anti-F8-MAP rabbit IgG, anti-UB33-KLH rabbit IgG, anti-H. pylori urease rabbit IgG) was added. After washing, either alkaline phosphatase-conjugated goat anti-mouse immunoglobulin or goat anti-rabbit immunoglobulin was added, and optical density (O.D.) was measured at 405 nm by ELISA.

tried to identify the epitope of a neutralizing antibody specific for the urease and found the linear epitope UB-33 (residues 321 to 339; CHHLDKSIKEDVQFADSRI). As reported by Jabri et al., in general most MAbs for the intact protein tend to be directed against the conformational discontinuous epitope of the protein recognized by anti-\( H. pylori \) antibodies, high titers corresponding to the linear epitope seem to have greater ability to neutralize the urease. Thus, L2 may fit to the flap region and consequently inhibit enzymatic activity. The amino acid residues corresponding to the flap were highly conserved among ureases from \( H. pylori \), Klebsiella aerogenes, and Bacillus pasteurii except for a few amino acids in the turn-helix (14a). It will be interesting to examine the inhibitory activity of L2 against the ureases of \( K. aerogenes \) and \( B. pasteurii \).

The findings shown here offer important information for making a vaccine that neutralizes \( H. pylori \) urease in preventing both \( H. pylori \) infection as well as its persistence in the stomach.

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