Neutralizing antibodies for *Helicobacter pylori* urease inhibit bacterial colonization in the murine stomach *in vivo*

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**ABSTRACT**

*Helicobacter pylori* (*H. pylori*) urease is a key protein for persistent infection of the bacteria in the stomach. Although *H. pylori* generally induce anti-*H. pylori*-specific antibodies (Abs), these Abs do not usually work for eradication or prevention of the *H. pylori* infection. In our previous study, we identified a linear epitope composed of 19-mer peptides termed UB-33, CHHLDKSIKEDVQFADSRI, within the large subunit of *H. pylori* urease. Anti-UB-33-specific Abs neutralized the enzymatic activity of *H. pylori* urease *in vitro*. In the present study, we evaluated the effect of immunization of BALB/c mice with *H. pylori* UB-33 peptide. After confirming the production of anti-UB-33-specific Abs, mice were challenged orally with *H. pylori* Sydney Strain-1 (SS-1). Mice producing anti-UB-33-specific Abs were not infected with SS-1, and the amount of SS-1 isolate in their stomach was significantly reduced. Also, the urease-negative mutant of *H. pylori*, HPP1801, did not colonize in the stomach, indicating that *H. pylori* urease was a critical element for infection of *H. pylori* in the gastric mucosa. Moreover, mice producing UB-33-specific Abs apparently suppressed *H. pylori* infection in the stomach where anti-UB-33 Abs were secreted in the gastric juice, indicating that *H. pylori* colonization was inhibited in the presence of anti-UB-33 Abs. In addition, the neutralization activity of sera from mice immunized with purified urease was less potent than that in the sera from mice immunized with UB-33. Furthermore, the recognition of epitope UB-33 was mediated through Toll-like receptor 2 (TLR2) on the B-1 cells using TLR2-knockout BALB/c mice *in vivo*. These results indicate that linear peptide UB-33 should be used for immunization to induce neutralizing Abs instead of purified *H. pylori* urease to prevent *H. pylori* infection and their colonization in the stomach.

*Helicobacter pylori* (*H. pylori*) is a gram-negative spiral bacterium that colonizes the gastric mucosa and initiates various gastro-duodenal diseases such as peptic ulcers, chronic gastritis, gastric cancers, and mucosa-associated lymphoid tissue lymphomas (21). In addition, *H. pylori* infection may be correlated with induction of various autoimmune disorders such as rheumatoid arthritis (9), idiopathic thrombocytopenic purpura (also called immune thrombocytopenic purpura) (4), and Sjögren’s syndrome (3). We proposed possible mechanisms by which initiation of these various autoimmune diseases may be associated with innate immune responses such as B-1 cells that are stimulated by *H. pylori*-specific urease during the production of autoantibodies (22). Autoantibody secretion by B-1 cells is inhibited when bacterium-coated plates are pre-treated with anti-*H. pylori* urease-specific antibodies (Abs). In particular, pretreatment with Abs specific to UB-33, an epitope of *H. pylori* urease, showed strong inhibitory activity (11).

Two types of *H. pylori* urease-specific Abs, in-
cluding conformational structure-specific Abs and linear epitope (UB-33; composed of 19 amino acid residues: CHHLDKSIKEDVQFADSRI)-specific Abs, have been reported. The former *H. pylori* urease-specific Abs induced with purified *H. pylori* urease do not have any positive effect on *H. pylori* urease activity (8), whereas the latter anti-linear epitope (UB-33)-specific Abs show strong inhibitory activity against the enzymatic activity of *H. pylori* urease (7). Inhibitors of *H. pylori* urease abrogated *H. pylori* persistence in the gastric mucosa and prevented infection by *H. pylori* (12, 17). However, whole *H. pylori* urease seems to generate insufficient immunity that may not abolish the enzymatic activity. Thus, we recommend use of the epitope UB-33 to induce Abs that prevent *H. pylori* infection as well as persistence.

Moreover, we have examined whether *H. pylori* urease is expressed on/in the bacteria with immunohistochemical staining using anti-*H. pylori* urease-specific Abs (11). Compared with *H. pylori* isolate (HPP1801) as a urease deficient control (20), *H. pylori* urease is predominantly expressed on the surface of bacteria rather than inside. Thus, *H. pylori* can directly attach to the gastric mucosa via urease. Also, as shown previously, the crystal structure of the active site in *H. pylori* urease, residues 327 to 334 of the UreB subunit forming a helix-turn-helix motif, completely overlaps with the *H. pylori* UB-33 portion where anti-UB-33-specific Abs interact (6, 7). Therefore, anti-UB-33-specific Abs may recognize the portion of the molecule responsible for the enzymatic activity of *H. pylori* urease and terminate *H. pylori* enzymatic activity to block infection and persistence.

In humans, although a number of individuals infected with *H. pylori* have usually some *H. pylori* urease-specific Abs, *H. pylori* will still persist and the *H. pylori* urease-specific Abs cannot prevent *H. pylori* infection and persistence (10). Moreover, after eradication of *H. pylori* with a combination of anti-bacterial drugs, *H. pylori*-specific Abs can still be detected. Thus, more effective *H. pylori*-specific Abs that can inactivate the enzymatic activity of *H. pylori* urease must be developed to eradicate the bacteria throughout the body.

In the present study, we established BALB/c mice that produce either anti-*H. pylori* urease-specific or anti-*H. pylori* UB-33-specific Abs. These mice were orally challenged with the infectious Sydney Strain-1 (SS-1) isolate to compare their infectivity and persistence in vivo and to confirm which types of Abs are more effective. Based on the experimental findings obtained from the murine data, we need to reconsider a new therapy that combines the current therapy using antibiotics and inhibitory drugs for anti-gastric juice secretion with immunotherapy using *H. pylori* UB-33 peptide immunization for continuous eradication of *H. pylori* from the body.

**MATERIALS AND METHODS**

**Animals.** Six-week-old female BALB/c mice were purchased from Nisseizai (Tokyo, Japan), and 8-week-old female Toll-like receptor 2 (TLR2) knockout BALB/c mice (TLR2 (−/−)) (19) were purchased from Oriental Bio Service (Kyoto, Japan). These animals were housed in micro-isolator cages under pathogen-free conditions and fed autoclaved laboratory chow and water. All animal experiments were carried out according to the guidelines of the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the Review Board of Nippon Medical School.

**Bacterial strains and growth conditions.** The bacterium used in the present study was the wild-type *H. pylori* strain, SS-1 (13). To obtain a large amount of bacterial cells, we used the following methods. SS-1 was cultured on either Brucella agar (BD Biosciences, San Diego, CA) containing 5% horse serum (Sigma-Aldrich, St. Louis, MO) and 1% cyclodextrin (Wako Junyaku, Osaka, Japan) or Columbia blood agar (OXOID, Hants, UK) containing *H. pylori* Selective Supplement (OXOID), 0.001% tetrazolium blue (MP Bio, Solon, OH), and 10% horse serum (Sigma-Aldrich, St. Louis, MO) at 37°C under micro-aerophilic conditions (5% O₂, 15% CO₂, and 80% N₂) with an Anaero-Pack (MicroAero; Mitsubishi Gas Chemical, Tokyo, Japan). After 2 days of culture, the colonies were harvested by scraping with a sterile metal spatula, transferred to 50 mL Brucella Broth (BD Biosciences, San Jose, CA) containing 5% horse serum and 1% cyclodextrin, and further cultured for 24 h at 37°C. Then, 500 μL cell-containing medium was plated on Brucella agar for an additional 3 days at 37°C, and the grown bacterial cells were harvested and washed with cold phosphate-buffered saline (PBS) at pH 7.0. The cells were pelleted by centrifugation (10,000 × g) for 10 min at 4°C, and the cell pellet was stored at −80°C. The urease-negative mutant of *H. pylori*, HPP1801 (20), was a kind gift from Dr. Asako Mizote at Yamaguchi Prefectural University, and was cultured on Brucella Broth containing 5% horse serum, 1% cyclodextrin, and 10 μg/mL kanamycin.
Preparation of anti-\textit{H. pylori} urease-specific Abs. Anti-\textit{H. pylori} urease-specific Abs were obtained from rabbits immunized with either purified urease (11) or synthetic peptides purchased from Greiner Bio-One (Kremsmünster, Austria). To induce production of neutralizing Abs that abrogate the enzymatic activity of \textit{H. pylori} urease, a 19-mer peptide, UB-33 from the large subunit of \textit{H. pylori} urease, \textit{UreB} (7), was administered intramuscularly to rabbits in the same volume of complete Freund adjuvant (CFA) (Becton, Dickinson and Company (BD), Franklin Lakes, NJ). Two and 8 weeks after the immunization, rabbits were boosted with UB-33 peptide mixed with the same volume of incomplete Freund adjuvant (IFA) (BD). Serum containing Abs was collected 3 weeks after the last immunization and purified using a protein A column (GE Healthcare, Uppala, Sweden).

Immunization and Infection. The protocol for immunization is shown in Fig. 1. Vaccines contained 0.5 mg/mL peptide antigen mixed with CFA or IFA. One month after the first intraperitoneal (i.p.) injection, immunized mice were infected with \textit{H. pylori} SS-1 (10\(^7\)–8 colony-forming units (CFU)) by intubation into their stomach. SS-1 isolate was ingested three times every 48 h. One month after the infection, mice were sacrificed to measure the amounts of \textit{H. pylori} in their stomach.

Western blotting. \textit{H. pylori} lysates were loaded onto a sodium dodecyl sulfate-polyacrylamide gel for electrophoresis, and separated proteins were transferred to a nitrocellulose-polyvinylidene difluoride membrane (Atto, Tokyo, Japan) with blotting buffer, which contained 2 M Tris (pH 8.0), 1.43% glycine, and 5% methanol. The blot was blocked overnight at 4°C with Block Ace (Dainihon Seiyaku, Osaka, Japan) and then incubated with samples (serum from immunized mice or anti-UB-33 Abs containing rabbit serum) for 90 min. The blots were washed three times with Tris-buffered saline (TBS; 0.8% NaCl, 0.02% KCl, and 0.3% Tris) containing 0.05% Tween 20 and incubated with biotin-labeled anti-rabbit or anti-mouse immunoglobulin (1:200) in TBS for 1 h. After washing three times, the blots were incubated with streptavidin-alkaline phosphatase (Nichirei, Tokyo, Japan) diluted 1:4 in TBS for 30 min. The signals were detected with the ProteiBlot nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate color development system (Promega Corporation, Madison, WI).

Detection of anti-UB-33 Abs and anti-urease Abs. A 50-μL aliquot of 1 μg/mL UB-33 or purified urease was added on a 96-wells plate and incubated. After an overnight incubation, the antigen-coated plates were blocked with 1% bovine serum albumin in PBS. A 50-μL aliquot of diluted (1:4000) serum was incubated for 2 h at room temperature. After washing with PBS containing 0.05% Tween 20 (T-PBS), a 50-μL aliquot of diluted biotinylated rabbit anti-mouse Igs (1:2000; Dako, Santa Clara, CA) was added for 60 min, followed by horseradish peroxidase-avidin D (1:1000; Vector Laboratories, Burlingame, CA). After washing with T-PBS, a 3, 3’, 5’, 5’-tetramethylbenzidine (TMB) staining solution was added, plates were incubated for 20 min, and TMB stop solution was added. The optical density was measured by absorbance at 450 nm with a microplate reader. The concentration of serum IgG was determined based on the mouse reference serum for standard IgG (Bethyl Laboratories, Montgomery, TX).

Effect of \textit{H. pylori} urease-specific Abs on \textit{H. pylori} urease activity. To investigate the inhibitory effect of \textit{H. pylori} urease-specific Abs on the enzymatic activity of \textit{H. pylori} urease, \textit{H. pylori} lysates were mixed with the immune sera in 96-well plates and incubated for 120 min. After incubation, 50 μL reacted solution was mixed with 50 μL urea medium (FUJIFILM Wako Pure Chemical Corporation). Using a microplate reader, the time-course of the color development was monitored by its absorbance at 540 nm at 15-min intervals for 90 min.

Measuring the amounts of bacteria in the murine stomach. One month after the infection, mice were sacrificed to measure the amounts of \textit{H. pylori} in their stomach. The stomachs removed from mice were cut in half, and one fragment was homogenized in 2 mL Brucella Broth with a tissue homogenizer. A 10-fold dilution series of homogenate was prepared and spread with a bacteria spreader on Columbia blood agar containing \textit{H. pylori} Selective Supplement. Dishes with spread homogenate were cultured for 4 days under microaerobic conditions at 37°C, and then the numbers of colonies of \textit{H. pylori} were counted.

Statistical analyses. All data were analyzed with GraphPad Prism 8 software and expressed as the mean ± standard error of the mean (SEM). Statisti-
Abs in BALB/c mice. We examined whether these Abs had neutralizing activity against *H. pylori* urease. The immunization protocol is shown in Fig. 1. In the sera of immunized mice, specific Abs against either UB-33 or whole *H. pylori* urease were detected with an enzyme-linked immunosorbent assay (ELISA) using UB-33-KLH-coated plates (Fig. 3A-a) or purified *H. pylori* urease-coated plates (Fig. 3A-b). In addition, specific IgA against either UB-33 or whole *H. pylori* urease was present in the sera of immunized mice (Fig. 3B), while specific IgM Abs were not detected in the sera (data not shown). Almost all specific Abs induced with this systemic immunization protocol were IgGs. Anti-UB-33-specific Abs detected in the sera of UB-33-immunized mice were identified as 75 kDa by western blotting analysis (Fig. 3C). Moreover, sera from mice immunized with UB-33 significantly suppressed the enzymatic activity of *H. pylori* urease, suggesting that UB-33-specific Abs had neutralizing activity against urease (Fig. 3D). In contrast, sera from mice immunized with purified *H. pylori* urease did not neutralize the enzymatic activity of *H. pylori* urease completely.

Therefore, immunization with UB-33-KLH together with CFA in BALB/c mice induced anti-UB-33-specific Abs with neutralizing activity against urease.

Analysis of *H. pylori* colonization in the stomach of BALB/c mice immunized with linear epitope peptide UB-33

To examine the effects of immunization of BALB/c mice on *H. pylori* colonization in the stomach, we used the *H. pylori* SS-1 strain, which can be colonized in the stomach of BALB/c mice. After oral administration of the SS-1 isolate, BALB/c mice immunized with UB-33-KLH together with CFA in BALB/c mice showed significantly lower amounts of bacterial colonies in the stomach compared with un-immunized control mice (Fig. 4A).

**RESULTS**

**Effects of anti-UB-33-specific Abs on *H. pylori* growth and their enzymatic activities**

To confirm the toxicity and effect of anti-UB-33-specific Abs on *H. pylori* growth, the antibody-treated *H. pylori* was stained with Rhodamine (Sigma-Aldrich, St. Louis, MO), which binds live *H. pylori*. We incubated *H. pylori* with or without rabbit anti-UB-33-specific Abs and stained them with Rhodamine. Rabbit anti-UB-33-specific Abs were not toxic to live bacteria at all (Fig. 2A). Thus, we could measure the urease activity against *H. pylori* after incubating them with rabbit anti-UB-33-specific Abs in a dose-dependent manner (Fig. 2B). Because *H. pylori* SS-1 isolate was not eradicated by rabbit anti-UB-33-specific Abs, we administered *H. pylori* pre-incubated with or without rabbit anti-UB-33-specific Abs into BALB/c mice. The SS-1 isolate of *H. pylori* did not colonize in the stomach in the presence of rabbit anti-UB-33-specific Abs when compared them in the absence of the Abs (Fig. 2C). It should be noted that the mutant strain of *H. pylori* defective in urease, termed UreB-negative *H. pylori* SS-1 isolate (HPP1801), did not infect the stomach of BALB/c mice (Fig. 2C). These data suggest that *H. pylori* urease is important for bacterial colonization in the stomach.

**Induction of anti-UB-33-specific Abs in BALB/c mice by immunization with linear epitope peptide UB-33**

Then we tried to confirm that the i.p. administration of UB-33-keyhole limpet hemocyanin (UB-33-KLH) with CFA efficiently induced anti-UB-33-specific Abs in BALB/c mice. We examined whether these Abs had neutralizing activity against *H. pylori* urease. The immunization protocol is shown in Fig. 1. In the sera of immunized mice, specific Abs against either UB-33 or whole *H. pylori* urease were detected with an enzyme-linked immunosorbent assay (ELISA) using UB-33-KLH-coated plates (Fig. 3A-a) or purified *H. pylori* urease-coated plates (Fig. 3A-b). In addition, specific IgA against either UB-33 or *H. pylori* urease was present in the sera of immunized mice (Fig. 3B), while specific IgM Abs were not detected in the sera (data not shown). Almost all specific Abs induced with this systemic immunization protocol were IgGs. Anti-UB-33-specific Abs detected in the sera of UB-33-immunized mice were identified as 75 kDa by western blotting analysis (Fig. 3C). Moreover, sera from mice immunized with UB-33 significantly suppressed the enzymatic activity of *H. pylori* urease, suggesting that UB-33-specific Abs had neutralizing activity against urease (Fig. 3D). In contrast, sera from mice immunized with purified *H. pylori* urease did not neutralize the enzymatic activity of *H. pylori* urease completely. Therefore, immunization with UB-33-KLH together with CFA in BALB/c mice induced anti-UB-33-specific Abs with neutralizing activity against urease.

**Analysis of *H. pylori* colonization in the stomach of BALB/c mice immunized with linear epitope peptide UB-33**

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Fig. 2  Effect of anti-UB-33 Abs on H. pylori growing. (A) H. pylori treated with 5% hypochlorous acid were used as the negative control. H. pylori were incubated with control rabbit serum or rabbit anti-UB-33-specific Abs for 2 h and stained with Rhodamine and analyzed by flow cytometry. (B) To examine the urease enzymatic activity to hydrolyze urea, H. pylori were treated with a variety concentration of rabbit anti-UB-33 Abs (0–50 mg/mL) and their decomposition ability of urea into ammonia was tested, and the optical density at 540 nm was measured every 5 min. Data are the mean ± SD (n = 6). (C) Colonization in the stomach of BALB/c mice by H. pylori incubated with or without rabbit anti-UB-33 Abs. ND means not detected. Data are the mean ± SEM (n = 5). Statistical significance was tested using a Mann-Whitney U test (**P < 0.01).
Fig. 3  Specific Abs following immunization against urease and UB-33 in BALB/c mice. (A) Specific-IgG Abs were measured in serum from immunized mice with ELISA using UB-33-KLH coated onto the plate (a). ELISA with purified urease coated onto the plate (b). Data are the mean ± SEM (n = 5). Statistical significance was tested using a one-way ANOVA (****P < 0.0001). (B) Specific-IgA Abs were measured with ELISA using UB-33-KLH coated onto the plate (a) or with purified urease (b). Data are the mean ± SEM (n = 5). (C) Western blot of sera from immunized mice (UB-33, urease) and control mice. The sera from non-immunized mice as the negative control (Lane 1). The sera from mice immunized with purified urease (Lane 2) and with UB-33 (Lane 3). Rabbit anti-UB-33 Abs were the positive control (Lane 4). The arrow indicates a band of antibodies combined with urease. (D) To examine the neutralization ability of sera from immunized mice, the reaction solution that included *H. pylori* lysates and sera from mice immunized with antigens were mixed in urea medium. The optical density at 540 nm was measured every 15 min. Data are the mean ± SD (n = 3).
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... cific and the other is linear epitope (UB-33)-specific Abs. In the present study, we found that the former structure-specific Abs induced with purified urease showed no effect on the enzymatic activity of H. pylori. BALB/c mice immunized with purified H. pylori urease also showed variable suppression of SS-1 colonies in the stomach to some extent. These data suggest that anti-UB-33-specific Abs have a far more stable suppressive effect on colonization of SS-1 than anti-H. pylori urease-specific Abs. Thus, systemic immunization of BALB/c mice with the UB-33 linear peptide provides effective protection against H. pylori infection. Anti-UB-33-specific Abs were also detected in the gastric juice of the stomach (Fig. 4B). These Abs probably react with urease on the surface of H. pylori in the stomach, and this reaction leads to inhibition of H. pylori colonization.

UB-33 was recognized through TLR2

Our previous reports showed that TLR2 recognizes urease in vitro (11). We thus examined whether immunization with UB-33-KLH induced UB-33-specific Abs in TLR2 knockout BALB/c (TLR2 (−/−)) mice. As shown in Fig. 5, TLR2 (−/−) mice immunized with UB-33-KLH together with CFA did not develop anti-UB-33-specific Abs in the sera at all (Fig. 5). These data indicate that H. pylori UB-33 is recognized by antigen-presenting cells through TLR2.

DISCUSSION

As has been demonstrated previously (7), there are two distinct types of IgG Abs against H. pylori urease. One is conformational structure of urease-spe-
activity of \textit{H. pylori} urease represented strong inhibition against the enzymatic activity of \textit{H. pylori} urease to hydrolyze urea. Because \textit{H. pylori} urease is dominantly expressed on the surface of the bacteria, functional UB-33-specific Abs that inactivate their enzymatic activities may inhibit attachment of the bacteria on the gastric mucosa. Thus, we investigated here whether UB-33-specific Abs will inhibit the \textit{H. pylori} colonization in the murine stomach.

According to the protocol shown in Fig. 1, BALB/c mice were i.p. immunized with either KLH conjugated UB-33 peptide or whole \textit{H. pylori} urease together with adjuvant CFA. After confirming the antibody production in the immunized BALB/c mice, the immune mice were orally challenged with infectious SS-1. As expected, the mice producing anti-UB-33 Abs were not infected by SS-1, while the mice making anti-urease-specific Abs were apparently infected, indicating that we can prevent \textit{H. pylori} infection by internal production of functional anti-UB-33 Abs that may neutralize enzymatic activity of \textit{H. pylori} urease. Although there have been developed a number of immunization protocols for preventing \textit{H. pylori} infection, such as multivalent epitope-based vaccines (5, 18), recombinant \textit{H. pylori} urease (14), DNA vaccines (15), and live vector vaccines (1), most of the protocol induced a mixture of \textit{H. pylori}-specific Abs that may interact with conformational structure of the \textit{H. pylori} and hardly inhibit the infection by SS-1 isolate. Therefore, Abs that neutralize the enzymatic activity of \textit{H. pylori} urease should be critical for the inhibition of the \textit{H. pylori} colonization in the murine stomach.

People suffered from \textit{H. pylori} infection usually obtained high titers of specific IgG, IgA, and IgM Abs against the bacteria (2), nonetheless, these Abs do not inhibit colonization of the bacteria in the body, and thus \textit{H. pylori} can still persistently exist in the gastric mucosa of infected individuals, suggesting that these anti-\textit{H. pylori}-specific Abs do not have neutralizing capacities (10), a critical factor for inhibiting their colonization. In contrast, as demonstrated here, mice with stronger neutralization capacities against \textit{H. pylori} urease showed apparent protective abilities against \textit{H. pylori} infection, while mice with weaker neutralization activity developed an \textit{H. pylori} infection. In the present study, anti-UB-33-specific Abs induced with linear UB-33 peptide showed much potent neutralizing capacity against enzymatic activities of \textit{H. pylori} urease. Taken together, these findings suggest that linear epitope of UB-33 will elicit Abs having strong neutralizing activity to \textit{pylori} urease and thus UB-33 should be involved to make effective vaccines for both prophylactic or therapeutic vaccines. As for \textit{H. pylori} eradication, current therapy using a combination of antibiotics appeared successful, but the therapy still showed many serious problems such as antibiotic-resistance (16). We would like to propose here an additional strategy for inhibiting \textit{H. pylori} infection and their persistency through neutralizing the enzymatic activity of urease by immunization with UB-33 linear epitope.

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DISCLOSURES

The authors have no financial conflicts of interest.

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