

# Catalytic Features and Eradication Ability of Antibody Light-chain UA15-L against *Helicobacter pylori*\*<sup>§</sup>

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We have successfully developed a catalytic antibody capable of degrading the active site of the urease of *Helicobacter pylori* and eradicating the bacterial infection in a mouse stomach. This monoclonal antibody UA15 was generated using a designed recombinant protein UreB, which contained the crucial region of the *H. pylori* urease  $\beta$ -subunit active site, for immunization. The light chain of this antibody (UA15-L) by itself showed a proteolytic activity to substantially degrade both UreB and the intact urease. Oral administration of UA15-L also significantly reduced the number of *H. pylori* in a mouse stomach. This is the first example of a monoclonal catalytic antibody capable of functioning *in vivo*, and such an antibody may have a therapeutic utility in the future.

One of the potential utilities of catalytic antibodies is to use them for therapeutics, especially against the infectious agents, through the specific destruction of the essential proteins in a virus or bacteria. Many catalytic antibodies degrading antigens such as VIP<sup>2</sup> (1), DNA (2), HIV gp41 (3), HIV gp120 (4), and factor VIII (5) etc. have been reported in the past decade. However, none of these catalytic antibodies have been developed into a therapeutic agent. *Helicobacter pylori*, a Gram-negative spiral bacteria (6) infecting ~50% of the world's populations, is an etiologic agent in a variety of gastroduodenal diseases and is the only microorganism known to regularly inhabit the human stomach (7). *H. pylori* produces a large amount of urease, which is essential for the colonization of the stomach and pathogenesis. Although *H. pylori* infection can currently be treated with

antibiotics (8, 9), its complete eradication from the stomach is often difficult, due to the adverse effect of the antibiotics and the presence of a resistant bacterium.

*H. pylori* urease enables the bacterium to colonize the human stomach by neutralizing the acidic condition, through the conversion of urea into ammonium (bicarbonate is also produced as a by-product). Therefore, a monoclonal catalytic antibody capable of destroying urease has a potential to be an effective therapeutic strategy to protect the stomach from the infection of *H. pylori*.

We have already prepared HpU-9-L catalytic antibody light chain (10), which was obtained by the immunization of whole molecules of the urease. One of the most important subjects in the study on catalytic antibody is whether or not we are able to prepare the catalytic antibody for the designated portion, which possesses the crucial function of the protein. Such catalytic antibody can erase the crucial function of targeting protein. In this study, a designed antigen (UreB), whose sequence is essential for the active site of the urease, was prepared and a catalytic antibody, UA15-L, was derived.

## EXPERIMENTAL PROCEDURES

The urease of *H. pylori* is a multimeric enzyme, which consists of an  $\alpha$ -subunit and a  $\beta$ -subunit. The active site resides in the latter subunit. The crucial region to exhibit the enzymatic activity of the urease is considered to locate from aa 201–338 in the sequence of  $\beta$ -subunit of the urease (see the *underlining* in Fig. 1a). The polypeptide, referred as UreB, having the 138 amino acids was prepared in the following method.

### Expression of Immunized Antigen (UreB)

Genomic DNA extracted from *H. pylori* (ATCC 43504) was used as a template for PCR with oligonucleotide primers, in which the following primers were employed: forward primer (5'-AAGGATCCGCTTCTAACGATGCGAGC-3'), which contains a BamHI site (*underlined*), and the reverse (5'-GGG-AATTCCTTGAATCAGCGAACTG-3'), which contains an EcoRI site (*underlined*). An aliquot of the PCR mixture was analyzed by agarose gel electrophoresis.

To construct a plasmid for the expression of recombinant UreB fused with the GST protein, the PCR-amplified DNA fragment was ligated to the expression vector, pGEX-6P-1 (Amersham Biosciences). *Escherichia coli* BL21 was transformed and induced the recombinant UreB protein by an

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<sup>2</sup> The abbreviations used are: VIP, vasoactive intestinal peptide; aa, amino acid; CFU, colony-forming unit; GST, glutathione S-transferase; mAb, monoclonal antibody; pAb, polyclonal antibody; POD, peroxidase; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline; HIV, human immunodeficiency virus; PBS, phosphate-buffered saline; VH, heavy-chain variable region; VL, light-chain variable region.

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addition of a final concentration of 1 mM isopropyl 1-thio- $\beta$ -D-galactopyranoside.

### Production and Characterization of mAb

BALB/c mice were primed subcutaneously using 100  $\mu$ g/mouse of the recombinant UreB-GST emulsified with Freund's complete adjuvant (Difco Laboratories, Detroit, MI). Two weeks after the first immunization, a booster dose composed of an equal amount of the recombinant UreB-GST in Freund's incomplete adjuvant was given. A final booster dose without adjuvant was administered 2 weeks after the second immunization. Three days after the final booster, splenocytes were fused with NS1/1.Ag4.1 or X63-Ag8.635 myeloma cells using polyethylene glycol 1500 (Boehringer GmbH, Mannheim, Germany), followed by hypoxanthine-aminopterin-thymidine selection and screening. Hybridomas were screened using the culture supernatant by enzyme-linked immunosorbent assay using UreB-GST and GST as the coated antigens. The mAbs reacting with UreB-GST but not with GST were picked up. Positive hybridomas were subcloned more than thrice by the limited-dilution method. The class and subclass of each mAb were determined with a mouse mAb isotyping kit (Amersham Biosciences).

### Production of pAb against UreB

For obtaining the polyclonal antibodies, rabbits were immunized subcutaneously as follows. One milligram of the UreB was dissolved in 1 ml of PBS, which was emulsified with 1 ml of Freund's complete adjuvant. The emulsified mixture (totally 2 ml) was immunized into one rabbit. The rabbits were given boosters at 2-week intervals in the same manner except Freund's incomplete adjuvant instead of Freund's complete adjuvant. The immunized rabbit was bled after the fourth booster immunization. The resultant antiserum was purified using affinity chromatography (Sephacrose 4B coupled with *H. pylori* urease) for the experiment of immunohistochemical staining.

### Sequencing and Molecular Modeling

Messenger RNA was isolated from the hybridoma secreting UA15 mAb using an mRNA purification kit (Amersham Biosciences). The cDNA of the light and heavy chain were synthesized by a first-strand cDNA Synthesis Kit (Life Science Inc.). The VH and VL fragments were amplified directly by adding them to a mixture containing PCR components and Mouse Ig primers specific for IgG (Mouse Ig primer kit, Novagen, Darmstadt, Germany). The amplified DNA was visualized on 2.0% agarose gel containing 0.5  $\mu$ g/ml ethidium bromide. A band of  $\sim$ 450 bp was observed, which corresponds to the size of the variable fragment of the antibody gene with little or no extraneous product. The PCR product was cloned into a pGEM-T easy vector (Promega, Madison, WI). Sequencing was conducted using the Auto Read Sequencing Kit (Amersham Biosciences) and an automated DNA sequencer (OpenGene System, Long-Read Tower, Amersham Biosciences).

Computational analysis of the antibody structures was performed using the deduced VL and VH amino acid sequences by a workstation (Octane 2, Silicon Graphics Inc., PA) running AbM software (Oxford Molecular Ltd., Oxford, UK), which is

used for building models of three-dimensional molecules. The resulting Protein Data Bank data were applied to minimize the total energy by using DS-Modeling (Accelrys Software Inc., San Diego, CA). This software uses the CHARMM algorithm for minimizing the energy of a molecule (11). Protein Adviser v. 3.5 (FQS Ltd., Fukuoka, Japan) was employed to visualize, analyze, and draw the structures.

### Immunohistochemical Staining of Gastric Biopsy Specimens

Formalin-fixed/paraffin-embedded human gastric tissues infected with *H. pylori* were used. The sections (2  $\mu$ m thick) were deparaffinized in xylene and dehydrated in graded ethanol, and then endogenous peroxidase activity was blocked by 3%  $H_2O_2$ . After rinsing in PBS, the sections were incubated with the antiserum (which was purified by affinity chromatography using Sepharose 4B coupled with *H. pylori* urease) at a concentration of 10  $\mu$ g/ml in 1% bovine serum albumin/PBS at room temperature for 60 min, and then washed three times with PBS for 5 min each. The slides were incubated with EnVision+ (horseradish peroxidase rabbit) reagent (Dako Japan Co. Ltd., Kyoto, Japan) for 60 min at room temperature and washed three times with PBS. Staining was performed using a 3,3'-diaminobenzidine substrate kit (Nichirei Co., Tokyo, Japan).

Gimenez staining was performed as follows. The gastric biopsy specimens were deparaffinized and hydrated in distilled water. The sections were filtered in a carbol fuchsin solution for 1 min. After washing in water, the slides were stained with 1% malachite green for 5 s, then washed again in water and dried in air.

### Purification of the Antibody and the Isolation of the Light Chain

UA15 mAb (IgG<sub>1</sub>( $\kappa$ )) was purified according to the purification protocol from Bio-Rad Protein A MAPS-II kit (Nippon Bio-Rad, Tokyo, Japan) according to the purification protocol recommended in the Bio-Rad Protein A MAPS-II kit (Nippon Bio-Rad). Detailed procedures were described in the reference (10, 12–14).

### Cleavage Assays

To avoid contamination, most glassware, plastic ware, and buffer solutions used in this experiment were sterilized by heating (180 °C, 2 h), autoclaving (121 °C, 20 min), or filtration through a 0.20- $\mu$ m sterilized filter as far as possible. The experiments were mostly performed in a biological safety cabinet to avoid air-borne contamination.

In the cleavage assay for UreB, the expressed UreB-GST protein was digested by trypsin for the purpose of recovery of UreB. Then the UreB was purified using UA-15 mAb-fixed affinity chromatography. The purity was over 99% in SDS-PAGE analysis. The whole antibody of UA15 does not have a catalytic activity. Hence, the purification could be performed without trouble (see under "Results").

Prior to the cleavage test for UreB protein and the purified urease of *H. pylori*, a peptide, TPRGPDRPEGIEEGGERDRD (TP41-1), which has mostly been used for monitoring the catalytic activity of the antibody and/or its subunits (3, 10, 12–14), was completely digested by the catalytic reaction of UA15-L. By

this reaction, the catalytic activity of UA15-L was held constant, showing no induction time (3, 12–15). In the cleavage assay of UreB and the urease, the UA15-L mentioned above was used. Cleavages of recombinant UreB (10.7  $\mu$ M) and purified *H. pylori* urease (57 nM) were carried out using UA15-L in a 15 mM phosphate buffer (pH 6.5) at 25 °C. The reactions were measured with Coomassie Brilliant Blue and silver-stained SDS-PAGE for UreB and urease, respectively, under non-reduced conditions. For the cleavage assay using living *H. pylori* cells, the surviving number of *H. pylori* cells was first counted after 0 to 48 h in phosphate buffer. The number of the cells ( $10^8$  cells were prepared) remained almost constant during the incubation without the catalytic antibody. The cleavage reaction was monitored by Western blot analysis using HpU-17 (16) labeled with POD.

### Analysis of the N-terminal Sequence

For UreB and the urease, the reaction solution at 7–8 h of incubation was recovered and concentrated up to 15- and 10-fold, respectively, using an ultrafiltration membrane (Amicon Ultra-4 5000MWCO, Millipore Corp., Bedford, MA). Then the samples were submitted to a 12% gel SDS-PAGE (reduced condition for UreB and non-reduced for the urease). The bands were transferred for 1 h at 112 mA onto an Immobilon-PQS PVDF membrane (Millipore Corp.) in 0.1 M Tris-HCl, 0.19 M glycine, 5% methanol at pH 8.7. After being stained with Coomassie Brilliant Blue, visible bands were cut and subjected to the N-terminal sequence analysis (Automated Protein Sequencer, Prosizer 494 HT, Applied Biosystems, Foster City, CA) with the amount of protein used ranging from 2 to 40 pmol. For 0.5–2 pmol of the fragment, an automatic protein microsequencer Prosizer 494 cLC (Applied Biosystems) was used.

### Immunoblot Analysis

After SDS-PAGE was carried out without staining, the proteins were transferred from the gel onto an Immobilon-P PVDF membrane. The PVDF membrane was blocked with TBS containing 3% skim milk and 0.05% Tween 20. After washing with TBS containing 0.05% Tween 20 (TBS-T), it was incubated with mAb HpU-17 (16), conjugated with peroxidase for 1 h at room temperature. After washing with TBS-T, the color development was performed by using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Kirkegaard & Perry Laboratories). For *H. pylori* cells, 7% SDS-PAGE at 20 mA in a non-reducing condition was employed for detection of the fragments of *H. pylori* urease.

### Preparation of *H. pylori* Urease

*H. pylori* of the Sydney strain (SS1) was cultured on a *Brucella* broth (BBL, Cockeysville, MD) agar medium containing 10% fetal bovine serum at 37 °C for 4 days under a microaerophilic environment (5% O<sub>2</sub>, 10% CO<sub>2</sub>, 85% N<sub>2</sub>). Detailed purification methods of the *H. pylori* urease from the harvested pellet were described in the literatures (16).

### In Vivo Assay

**Animals**—Specific pathogen-free, 6-week-old female C57BL/6 mice were purchased from Shimizu Laboratory Supplies Co.,

Ltd. (Kyoto, Japan). Mice were housed under specific pathogen-free conditions and were allowed free access to food and water. Experiments were performed according to the guideline of the Ethical Committee for Animal Experiments at the Faculty of Medicine of Oita University (Oita, Japan) and Prefectural University of Hiroshima (Hiroshima, Japan).

**Infection**—Mice were orally challenged two times at 1-day intervals with 0.5 ml of *H. pylori* SS1 ( $1 \times 10^8$  colony-forming units (CFU)/ml). Seventeen days after the last inoculation, 2 of 29 mice were sacrificed to confirm the colonization of *H. pylori* in the stomach.

**Oral Administration of UA15-L**—Each mouse ( $n = 9$ ) was orally administered with a 0.5 ml of UA15-L (20  $\mu$ g/ml) containing solution in which Meyron (7% sodium bicarbonate, 833.2 mM, Otsuka Pharmaceuticals, Osaka, Japan) was added with one-tenth of the volume in order to neutralize the gastric acidity. In this study, nine mice were used for the administration. The nine mice were used for the control experiment in which the same solution containing Meyron without UA15-L was orally delivered. The administered UA15-L was prepared as described in the section of cleavage assay under “Experimental Procedures.”

**Assessment of Eradication of *H. pylori***—All mice were sacrificed, and the stomach of each mouse was isolated a day after the oral administration for bacterial and histological examination. The stomach was washed in sterile 0.8% NaCl and cut longitudinally into two pieces. One half was homogenized in 500  $\mu$ l of *Brucella* broth by using a glass homogenizer (Iwaki Glass Co. Ltd., Tokyo, Japan). Fifty microliters of gastric homogenate was serially diluted with *Brucella* broth and inoculated onto *Helicobacter*-selective agar plates (NISSUI pharmaceutical Co., Ltd., Tokyo, Japan) at 37 °C for 4 days under microaerobic conditions. Colonies were counted, and expressed as CFU/gram stomach tissue. The numbers of bacterial colony were analyzed and compared by the *t* test. *p* values of <0.05 were considered to indicate a significant difference.

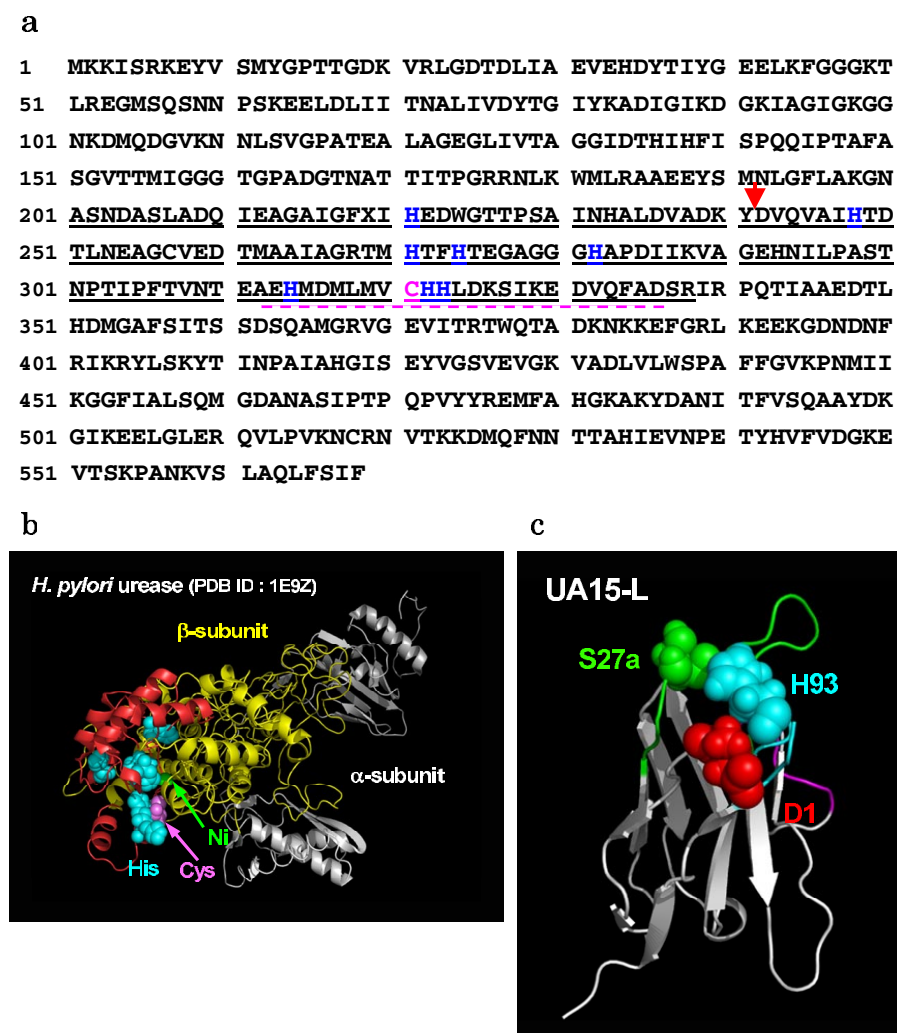
The other half of the stomach was used for the histological examination. Histological examination of gastric mucosa included longitudinal sections of the stomach, from the esophageal-cardiac junction through the duodenum, which were fixed in neutral-buffered 10% formalin and embedded in paraffin. Sections 5  $\mu$ m in length were stained with hematoxylin & eosin (gastric sections were scored and evaluated by the presence of inflammation characterized by the intensity of neutrophilic and/or lymphocytic infiltration in blind fashion by two independent examiners.)

## RESULTS

For the accuracy, some data were confirmed by repeated experiments.

**Design of the Immunizing Antigen UreB**—*H. pylori* urease is a hexamer consisting of  $\alpha$ - and  $\beta$ -subunits, with molecular sizes of 26.4 and 61.6 kDa, respectively (17). In the  $\beta$ -subunit, the amino acid residues number (aa) from aa 201 to 338 are essential for its activity. Cys<sup>321</sup> (violet colored character in Fig. 1a) strongly interacts with two nickel ions (bi-nickel center) and His<sup>221</sup>, His<sup>248</sup>, and His<sup>274</sup> also coordinate the ions (18, 19). In addition to these, other amino acid residues including His<sup>271</sup>,





**FIGURE 1. Sequences and structure of *H. pylori* urease.** *a*, amino acid sequence of  $\beta$ -subunit of *H. pylori* urease. The aa sequence of UreB is underlined, whose sequence involves many important histidine residues (which are conserved in many bacteria: blue letters) and one cysteine (which is also conserved: violet) residue. His<sup>248</sup> and His<sup>274</sup> strongly interact with the nickel ions. His<sup>221</sup> and His<sup>323</sup> locate very close to the ions. Cys<sup>321</sup> is present in a close position to the ions. UreB involves these important histidine and cysteine residues. The flanking region is indicated with dotted violet underline (see "Discussion"). The red arrow indicates the cleaved bond of UreB by UA15-L. *b*, the structure of *H. pylori* urease.  $\beta$ -subunit: yellow ribbon,  $\alpha$ -subunit: white ribbon, UreB: red ribbon (included in  $\beta$ -subunit); His: blue space fill, Cys: violet space fill, nickel ion: green ball. Eight conserved histidine residues (histidines 221, 248, 271, 274, 282, 314, 322, and 323) present in UreB sequence are indicated in blue space fill (PDB number: 1E9Z). *c*, structure of UA15-L by molecular modeling. The structure of the variable region of UA15-L was created by molecular modeling. Three amino acid residues (Asp<sup>1</sup>, Ser<sup>27a</sup>, and His<sup>93</sup>) locate in close position each other, which may function as a catalytic triad in a similar manner as other cases (14, 15).

His<sup>282</sup>, His<sup>314</sup>, His<sup>322</sup>, and His<sup>323</sup> are conserved among many bacteria such as *Helicobacter felis*, *Bacillus* sp., Jack bean, *Klebsiella aerogenes*, *Proteus mirabilis*, and *Ureaplasma urealiticum*. Cys<sup>321</sup> is conserved in all of these bacteria. Thus we decided to target this region (UreB) for the catalytic antibody in this study. The sequence of the UreB region is shown in Fig. 1*a* (solid underline), and the location of UreB in the structure of urease is indicated with a red ribbon in Fig. 1*b*.

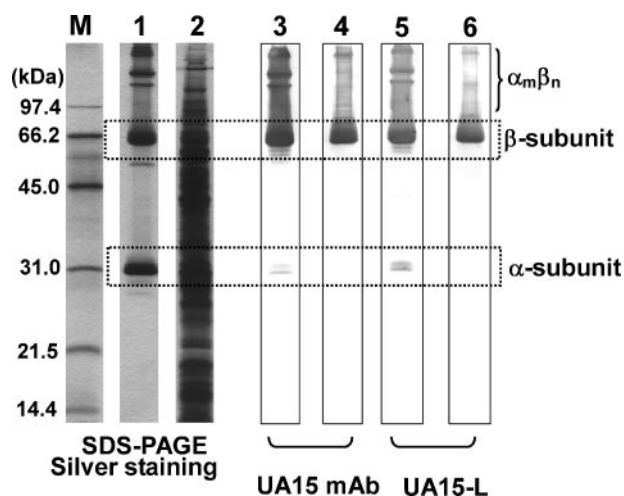
**Expression of UreB and the Production of the mAb**—UreB was expressed in *E. coli* (BL21) as a fusion protein with GST (UreB-GST). After the immunization of UreB-GST into BALB/c mice, a monoclonal antibody (mAb: UA15) was established using the conventional hybridoma generation technique (16). The mAb obtained specifically reacted with UreB and the intact *H. pylori*

urease but not with GST, bovine serum albumin, human serum albumin, and human  $\gamma$ -globulin. However, it slightly cross-reacted with Jack bean urease.

**Molecular Modeling**—Sequencing of cDNAs of variable region of the light (UA15-L) and heavy chain of UA15 mAb was conducted, and the amino acid sequences were deduced. Five aa residues at the N terminus of UA15-L were determined, and the sequence (Asp<sup>1</sup>-Val<sup>2</sup>-Val<sup>3</sup>-Met<sup>4</sup>-Thr<sup>5</sup>) was agreed with those deduced by the cDNA sequencing. The germ line gene of UA15-L was identified as *bd2*. The three-dimensional structure of the variable region of UA15-L was created by molecular modeling according to the method as described in Refs. 14 and 15 (Fig. 1*c*). Three aa residues (Asp<sup>1</sup>, Ser<sup>27a</sup>, and His<sup>93</sup>) locate in a close position, which are assumed to form a catalytic triad in such catalytic antibody light chains as VIPase (vaso-intestinal peptide) (20) and i41SL1-2 (15) (these belong to the same germ line gene *bd2*).

**Features of the Immunological Binding of UA-15 mAb and Its Light Chain (UA15-L)**—Lane 1 and 2 in Fig. 2 show the results of SDS-PAGE of *H. pylori* urease purified from the Sydney strain (SS1) and the lysate of the cells, respectively. In lane 1, the monomers of both the  $\beta$ - and the  $\alpha$ -subunits were clearly observed at 66.0 ( $\pm 2.8$  kDa) and 31.0 ( $\pm 0.8$  kDa), respectively. Some partially dissociated forms ( $\alpha_m\beta_n$ ) at high molecular sizes (over 96.4 kDa) were also observed. (We confirmed them to be derived from the urease

by a Western blotting analysis using the mAbs (HpU-2 and -17) against the  $\alpha$ - and the  $\beta$ -subunits, respectively (16). The natural form of this enzyme is  $\alpha_6\beta_6$ .) In lane 2, neither the  $\beta$ -subunit nor the  $\alpha$ -subunit of urease was detected, due to the presence of other bacterial proteins. The Western blots using UA15 mAb demonstrated that this mAb specifically reacted with the  $\beta$ -subunit as shown in lane 3 with the purified urease and lane 4 with the lysate of the bacteria. Interestingly, the light chain (UA15-L) by itself showed a similar reactivity as the native UA15 mAb (in lane 5 for purified urease and lane 6 for the bacteria). The mAb and the light chain slightly bound to the  $\alpha$ -subunit, which were hardly detected in lanes 4 and 6, because of relatively low content of the subunit in the lysate compared with the purified urease.

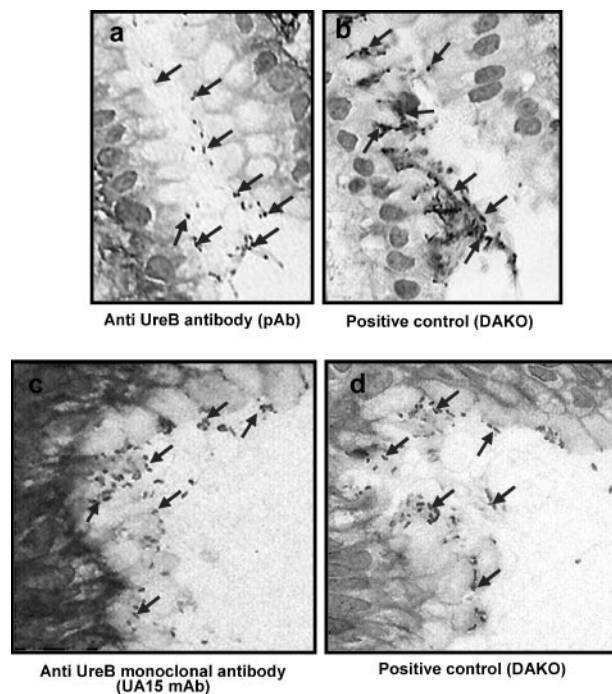


**FIGURE 2. Immunological binding features.** Immunological binding features of UA15 mAb and its light chain (UA15-L). Lanes 1 and 2, SDS-PAGE for purified urease and lysate of the bacterium, respectively; lane 3 and 4, Western blots by UA15 mAb for purified urease and lysate of the bacterium, respectively; lanes 5 and 6, Western blots by UA15-L for purified urease and lysate of the bacterium, respectively; M, marker. SDS-PAGE: 12% gel under non-reduced. Urease: 0.2  $\mu\text{g/ml}$  (57 nm). *H. pylori*:  $2 \times 10^8$  cells/ml. Western blots: immunoreaction: 1 h at room temperature. UA15 mAb: 1  $\mu\text{g/ml}$ . UA15-L: 77  $\mu\text{g/ml}$ . Both antibodies, UA-15 mAb and its light chain (UA15-L), reacted with the  $\beta$ -subunit of the urease of *H. pylori* (SS1 strain). This result was confirmed by repeated experiments.

Fig. 3 shows the results of the immunohistochemical staining of the formalin-fixed section of the human gastric tissues infected with *H. pylori*. As previously reported (19), an anti-UreB polyclonal antibody (pAb) produced by the immunization of UreB into rabbit specifically reacted with *H. pylori*, and the infected sites were clearly observed on the mucosal surface of human stomach (Fig. 3a), Fig. 3b shows the result using a commercially available pAb (DAKO Japan Co. Ltd., Kyoto, Japan) as a positive control. It is considered that the antibodies mainly reacted to the surface urease of *H. pylori*. Interestingly, the UA-15 mAb reacted similarly with *H. pylori* (a different specimen from the experiment using pAb) as shown in Fig. 3 (c and d), although it had been known that monoclonal antibodies tend not to react with the formalin fixed specimens.

**Cleavage Activity against UreB**—Because the epitope of UA15 mAb is not determined, a peptidase activity of UA15-L was examined by using a TP41-1 peptide (TPRGPDRPEGIEE-GGERDRD), which has been used to monitor the peptidase activity of the catalytic antibody or its subunits (3, 12, 13, 15, 16). The degradation of TP41-1 peptide by UA15-L followed the Michaelis-Menten equation, and its kinetic parameters were obtained as  $k_{\text{cat}} = 0.24 \text{ min}^{-1}$  and  $K_m = 6.2 \times 10^{-5} \text{ M}$  (see supplemental Appendix S1). These values were similar to those of other catalytic antibodies reported so far (3, 10, 14, 16). The heavy chain by itself or the whole antibody (UA15 mAb) did not show any catalytic activity in this assay. In addition, the light and heavy chains of UA11 mAb, which specifically binds to UreB, hardly showed the catalytic activity when they were monitored up to 200-h incubation.

According to the experimental protocol under “Experimental Procedures,” highly purified UreB (>99%; 10.7  $\mu\text{M}$ ) was mixed with UA15-L (0.6  $\mu\text{M}$ ) in phosphate buffer (pH 6.5) at 25 °C, and the degradation of UreB was monitored by SDS-



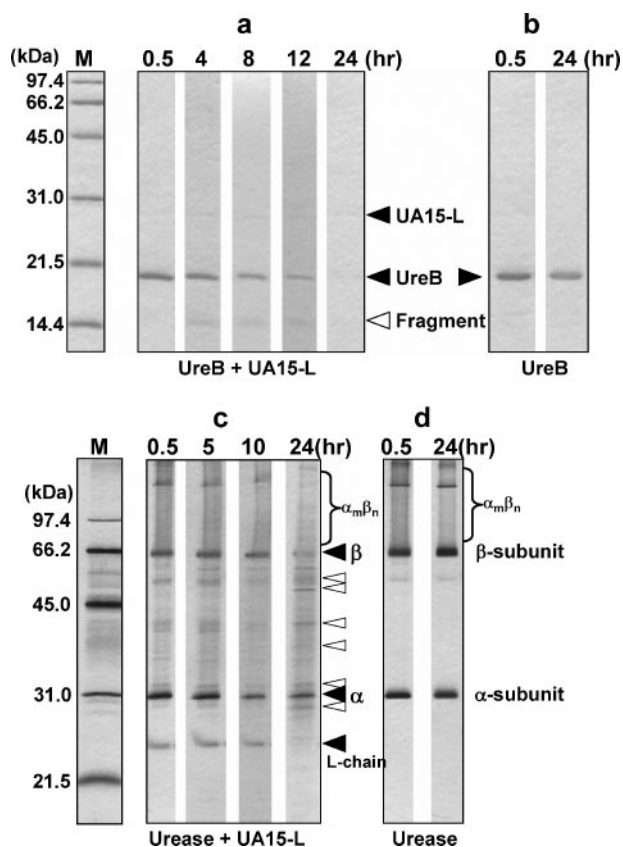
**FIGURE 3. Immunohistochemical staining.** *H. pylori*-infected gastric biopsy specimens (formalin-fixed section) were used in this experiment. a, UA15 polyclonal antibody (pAb); b, positive control (for polyclonal antibody); c, UA-15 mAb; d, positive control (using the same polyclonal antibody as in b). pAb against UreB was obtained by immunizing UreB into rabbits, while the mAb was prepared by the cell fusion using BALB/c mice. The small sites indicated with red arrows are the stained *H. pylori* in a–d. The polyclonal antibody induced by UreB antigen could bind to the *H. pylori* infecting the gastric mucosa (a). The UA-15 mAb could also stain the *H. pylori*-infected specimen (c). *H. pylori* was also stained using the anti-*H. pylori* urease polyclonal antibody from DAKO (b and d).

PAGE under reduced condition with Coomassie Brilliant Blue staining, as shown in Fig. 4a. The band of UreB ( $19 \pm 0.4 \text{ kDa}$ ) became gradually faint with an increased incubation time, with its complete disappearance after 24 h. At 4- and 8-h time points, a novel fragment with a size of  $14.4 (\pm 0.2) \text{ kDa}$  was observed, which became faint with further incubation. At 24-h incubation, this 14.4-kDa band disappeared as well as that of UreB, suggesting that the consecutive degradations took place. No other fragment was visible. UreB was not degraded without UA15-L (Fig. 4b).

We characterized the cleavage sites of UreB by N-terminal amino acid sequencing. From the 14.4-kDa fragment, a sequence of DVQVA was detected (the detection intensity = 33 pmol), indicating that the bond between Tyr<sup>46</sup> and Asp<sup>47</sup> was cleaved. The sequence of this scissile bond is indicated with a red arrow in Fig. 1a.

**Cleavage of Purified Urease**—The cleavage of *H. pylori* urease (57 nm) by UA15-L (0.4  $\mu\text{M}$ ) was performed under the same reaction condition. The reaction was monitored by SDS-PAGE under a non-reduced condition with silver staining at 0.5, 5, 10, and 24 h of incubation (Fig. 4c). *H. pylori* urease is a hetero-hexamers of the  $\alpha$ - and the  $\beta$ -subunits with a size of 528 kDa ( $(26.4 \text{ kDa} \times 6) + (61.6 \text{ kDa} \times 6)$ ). In a control experiment with the SDS-PAGE under non-reduced condition, we detected the monomers and the multimers of the urease subunits (Fig. 4d). The bands at  $31.0 (\pm 0.5) \text{ kDa}$  and at  $66.0 (\pm 2.8) \text{ kDa}$  appeared





**FIGURE 4. Cleavage assays for UreB and purified urease by UA15-L.** *a*, cleavage for UreB with UA15-L (UreB, 10.7  $\mu$ M; UA15-L, 0.6  $\mu$ M); *b*, control (without UA15-L). The SDS-PAGE (12% gel) was performed under reduced condition with Coomassie Brilliant Blue staining. The catalytic reaction was carried out in 15 mM phosphate buffer (pH = 6.5) at 25 °C. The band of UreB became faint with an increase of the reaction time. At 4-h incubation, a new band at 14.4 kDa appeared, and it completely disappeared at 24 h of incubation, suggesting that the consecutive degradation took place. In the control experiment, UreB was unchanged. *c*, cleavage of purified urease by UA15-L (purified urease: 57 nM; UA15-L: 0.4  $\mu$ M); *d*, control experiment without UA15-L. SDS-PAGE (12% gel) was performed with silver staining under non-reduced condition. The reaction at 25 °C was carried out under similar conditions as employed in *a* and *b*. There were many fragmented bands observed (indicate with open triangle arrows). UA15-L could completely decompose the  $\beta$ -subunit of the urease in a time-dependent manner. In the control experiment, no degradation of the  $\beta$ -subunit (and  $\alpha$ -subunit) was observed. The cleavage assay was performed six times. All experiments showed similar results.

to be the monomers of the  $\alpha$ - and the  $\beta$ -subunit, respectively. (The expected sizes of these subunits were 26.4 and 61.6 kDa based on their amino acid sequences.) When the purified urease was mixed with UA15-L (Fig. 4c), the  $\beta$ -subunit band became faint at 0.5 h, and it became fainter with further incubation. After 24 h of incubation, the band was barely visible. During the degradation of the  $\beta$ -subunit, many fragmented bands, as indicated with open arrows, were observed (Fig. 4c). The bands corresponding to the heteromultimers of the  $\alpha$ - and  $\beta$ -subunits ( $\alpha_m\beta_n$ ) were identified by Western blotting using the mAbs HpU-2 and -17, which were specific to the  $\alpha$ - and  $\beta$ -subunits, respectively (16). These heteromultimers as well as the monomeric  $\beta$ -subunit were also degraded with the increased incubation time. On the other hand, the  $\alpha$ -subunit band was still clearly visible even after 24 h of incubation. The UA15-L band became faint presumably due to the self-digestion. In the control without UA15-L (Fig. 4d), no change of the  $\beta$ -subunit band

intensity was detected up to 24 h. Thus we concluded UA15-L could specifically cleave the  $\beta$ -subunit.

The N-terminal amino acid sequences of the urease-derived fragments were determined by a procedure similar to the one used for the UreB-derived fragments. After concentrating the samples by up to 15-fold through ultrafiltration, we were able to analyze six bands as summarized in Table 1. *Band 1* was derived from the cleavage of the bond at Glu<sup>124</sup>-Gly<sup>125</sup> of the  $\beta$ -subunit (presumably, Gly<sup>125</sup> to the C terminus of the  $\beta$ -subunit). From *band 2*, the cleavages at Tyr<sup>241</sup>-Asp<sup>242</sup> and Met<sup>262</sup>-Ala<sup>263</sup> were identified. *Band 3* was identified as the monomeric form of the  $\alpha$ -subunit. *Band 4* included three fragments. The strongest intensity was QAMGR. Two other were SQAMG and DSQAM corresponding to the cleavages at Asp<sup>362</sup>-Ser<sup>363</sup> and Ser<sup>361</sup>-Asp<sup>362</sup>, respectively. *Band 5* had a sequence of MKKIS matching with the  $\beta$ -subunit N terminus. Judging from its size, this fragment was likely to be a fragmented portion of the  $\beta$ -subunit, Met<sup>1</sup> to Glu<sup>124</sup> generated by a cleavage at Glu<sup>124</sup>-Gly<sup>125</sup>. A minor sequence GLTVT was also detected in *band 5*, which was likely to be generated through the successive cleavage of *band 1*. From *band 6*, three N-terminal sequences, GLIVT, MKKIS, and MKLTP, were identified. The GLIVT and MKKIS were present in the  $\beta$ -subunit sequence and likely were the products of successive cleavages of *bands 1* and *3*. The MKLTP sequence matched the N terminus of the  $\alpha$ -subunit. Thus UA15-L appeared to have cleaved the  $\alpha$ -subunit slightly too. All the detected bands were derived from the urease or its fragments. Thus UA15-L was capable of consecutive cleavages of urease. The enzymatic activity of urease decreased to ~3% of the original activity with the advancement of the degradation.

**No Bovine Serum Albumin Cleavage by UA15-L**—To examine the substrate specificity, UA15-L (0.4  $\mu$ M) was incubated with bovine serum albumin (0.58  $\mu$ M), under the conditions identical to those employed for *H. pylori* urease. No cleavage of bovine serum albumin was detected after 24 h of incubation (data not shown).

**Reaction with Intact *H. pylori* Cells**—UA15-L (0.8  $\mu$ M) similarly prepared as the case of previous experiments was mixed with living *H. pylori* cells ( $5 \times 10^7$  cells/ml) in 15 mM phosphate buffer (pH 6.5) followed by an incubation at 25 °C with shaking. In this experiment, the reaction was monitored by a Western blot analysis (7% gel was used in SDS-PAGE) using POD-labeled HpU-17 mAb (16), which specifically recognizes the  $\beta$ -subunit. As shown in Fig. 5a, when UA15-L and the bacterium were mixed, the urease  $\beta$ -subunit was gradually degraded with incubation. The degradation time course of the  $\beta$ -subunit is presented in Fig. 5c. It was also observed that the  $\alpha_m\beta_n$  bands gradually became faint with a prolonged incubation. At 0.3 h, a new band appeared at <50 kDa, whose molecular size was correctly estimated to be 30 kDa using 14% gel in SDS-PAGE. The band strength increased with the incubation time. This band is considered to be a fragment of the  $\beta$ -subunit. In contrast, the  $\alpha$ -subunit of the urease was barely degraded (data not shown). In a control experiment (Fig. 5b), the  $\beta$ -subunit was clearly visible at 66 kDa, and  $\alpha_m\beta_n$  also remained. These bands were not affected by an incubation up to 24 h without UA15-L.

**In Vivo Assay**—The most appropriate schedule, which was found by many preliminary experiments, for *H. pylori* infection

TABLE 1

Results of N-terminal amino acid sequence analysis for fragmented polypeptides from the urease of *H. pylori*

Fragmented band	Size	aa residues of N-terminal (5 residues)	Cleavage site
	<i>kDa</i>	<i>pmol</i>	
Band 1	51.4	Gly (0.73), Leu (0.72), Ile (0.73), Val (0.55), and Thr (0.37)	Glu <sup>124</sup> -Gly <sup>125</sup> of $\beta$ -subunit
Band 2	41.4	Asp (0.21), Val (0.30), Gln (0.24), and Val (0.34)	Tyr <sup>241</sup> -Asp <sup>242</sup> of $\beta$ -subunit
		Ala (1.17), Ala (1.34), Ile (0.89), Ala (1.02), and Gly (0.52)	Met <sup>262</sup> -Ala <sup>263</sup> of $\beta$ -subunit
Band 3	31.1	Met (11.3), Lys (10.2), Leu (10.0), Thr (8.4), and Pro (4.8)	N-terminal of $\alpha$ -subunit
Band 4	28.8	Gln (6.3), Ala (7.8), Met (4.8), Gly (5.0), and Arg (1.7)	Ser <sup>363</sup> -Gln <sup>364</sup> of $\beta$ -subunit
		Ser (4.6), Gln (4.6), Ala (6.2), Met (3.5), and Gly (4.3)	Asp <sup>362</sup> -Ser <sup>363</sup> of $\beta$ -subunit
		Asp (3.7), Ser (2.9), Gln (3.5), Ala (4.2), and Met (2.2)	Ser <sup>361</sup> -Asp <sup>362</sup> of $\beta$ -subunit
Band 5	17.5	Met (31.4), Lys (29.3), Lys (32.3), Ile (22.3), and Ser (14.3)	N-terminal of $\beta$ -subunit
		Gly (7.4), Leu (4.8), Ile (5.2), Val (4.3), and Thr (3.5)	Fragment of band 1
Band 6	15.3	Gly (7.9), Leu (8.6), Val (7.8), Ile (8.0), and Thr (7.5)	Glu <sup>124</sup> -Gly <sup>125</sup> of $\beta$ -subunit
		Met (18.1*), Lys (19.2*), Lys (12.5), Ile (9.9), and Ser (6.6)	Fragment of band 5
		Met (18.1*), Lys (19.2*), Leu (9.5), Thr (7.6), and Pro (4.8)	Fragment of band 3

\* These values were not highly accurate.

through oral administration is shown in Fig. 6a. First, C57BL/6J mice were orally inoculated two times at 1-day intervals with *H. pylori* (SS1:  $50 \times 10^6$  CFU for each mouse). To confirm the infection of the bacteria in the mice stomach, 2 of 29 mice were sacrificed and were examined for the number of colonized *H. pylori*. At 17 days after the last inoculation,  $15.9 \times 10^6$  CFU of bacteria per 1.0 g of stomach tissue were recovered. At 24 days after the confirmation of infection, 0.5 ml of UA15-L (0.8  $\mu$ M), UA15 mAb (0.4  $\mu$ M), or phosphate buffer (control) were orally administered to each C57BL/6J mouse. All the administered solution contained 10% Meyron (7% sodium bicarbonate, 833.2 mM) for the neutralization of the gastric acidity in the stomach. For each group, nine or eight mice were used. No mucosal adjuvant such as cholera toxin, which is mostly used for the oral vaccination, was employed in this experiment.

After the next day of the administration, all mice were sacrificed, and the stomach was isolated for the assessment of eradication of *H. pylori* and the histological examination of the gastric mucosa. One half of the specimen was homogenized for the bacterial examination to assess the number of infected *H. pylori*. The other was used for the histological examination. The results of the bacterial examination are shown in Fig. 6b. The mean bacteria number calculated for the UA15-L administered mice with was  $1.71 \times 10^6$  CFU/g of stomach tissue. The value for whole antibody UA15 mAb was  $2.82 \times 10^6$  CFU/g of stomach tissue. On the other hand, the control mice showed the mean value of  $4.93 \times 10^6$  CFU/g of stomach tissue. Thus the number of bacteria colonizing the stomach was reduced to one-third less with the administration of UA15-L, compared with the control group ( $p < 0.05$ ). The decrease of  $15.9 \times 10^6$  CFU of the sacrificed mice to  $4.93 \times 10^6$  CFU of the control mice may be due to the interval of 24 days by the administration of UA15-L, because the number of colonies has a tendency to decline along with the time after infection. Whole antibody, UA15 mAb, did not show a statistically significant difference from the control group.

Histological analysis was performed by hematoxylin & eosin staining using the *H. pylori*-infected stomach. Although *H. pylori* was partially eradicated by the administration of UA15-L, the gastritis scores of the stomach among UA15-L-, UA15 mAb-, and PB-administered groups were not different. These results might be due to the fact that the histological analysis was carried out the next day of the administration of

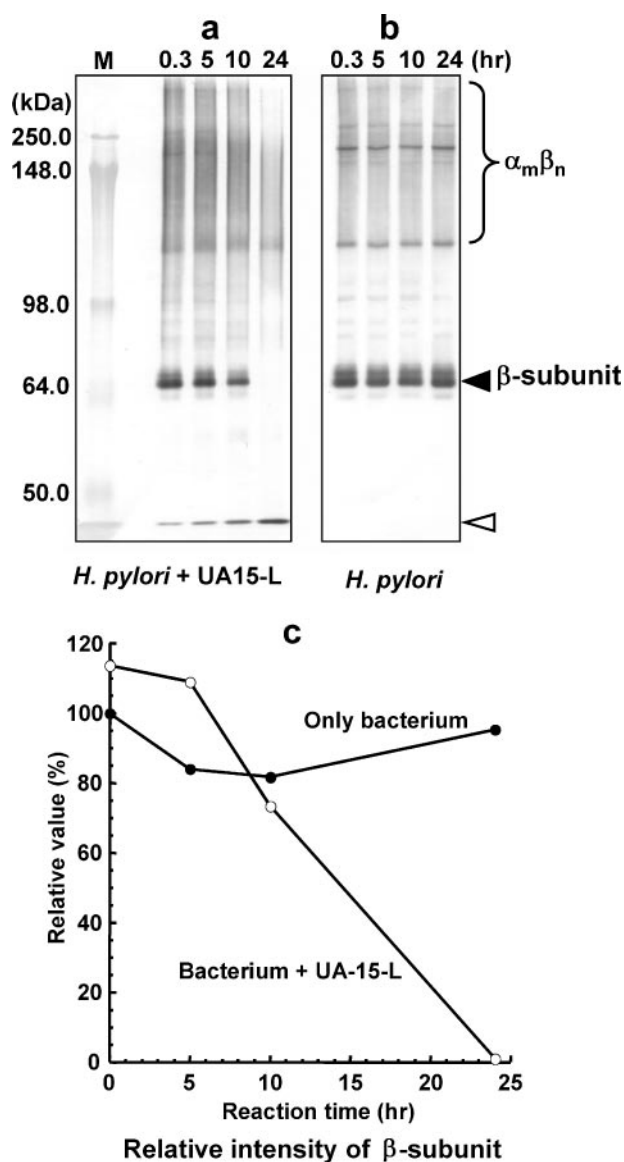
UA15-L. The effect on gastric scores might have needed a longer time to take place.

## DISCUSSION

A monoclonal antibody UA15 was produced by immunization using a recombinant protein UreB, which contained the crucial part of the *H. pylori* urease (19) active site. UA15 mAb could bind to UreB as well as the urease  $\beta$ -subunit. Interestingly, its light chain, UA15-L by itself showed the same binding feature as that of UA-15.

Erhan *et al.* (22) suggested that the light chain of the antibody could function as a peptidase/protease by itself based on the homology between antibody light chains and serine proteases. It has been reported that the active site composed of catalytic dyad or triad of the catalytic antibody can function to hydrolyze the antigens. Kolesnikov *et al.* (23) reported that the antibody possessing the catalytic dyad (His<sup>35</sup> and Ser<sup>99</sup>) in the heavy chain is the active site that hydrolyzes the acetylthiocholine molecule. In addition, Paul *et al.* (20) also revealed that the catalytic triad composed of Asp<sup>1</sup>, Ser<sup>27a</sup>, and His<sup>93</sup> in the light chain (whose germ line belongs to *bd2*) could catalytically hydrolyze the antigen VIP. In our case, UA15-L belonged to *bd2* germ line (DDBJ; accession No. AB286872) and possess the identical aa residues to the catalytic triad of VIPase (20), ECL2B-L (13, 21), and i41SL2-1-L (15, 21). Thus, it was predicted that UA15-L could hydrolyze the antigen UreB. In contrast, we could not see any possible catalytic triad structure in the heavy chain because of the lack of His in the variable region.

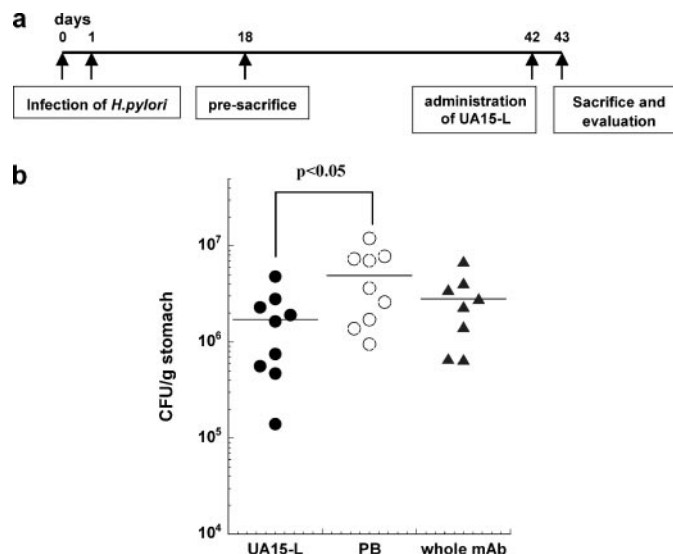
As the result of the cleavage assay, UA15-L decomposed the peptide bond at Tyr<sup>46</sup>-Asp<sup>47</sup> of UreB. In addition, the light chain could cleave the same peptide bond in the urease  $\beta$ -subunit. Hence, it appeared that UA15-L first bound to the UreB region and cleaved the peptide bonds at Tyr<sup>241</sup>-Asp<sup>242</sup> or Met<sup>262</sup>-Ala<sup>263</sup>. Successively, peptide bonds at Ser<sup>361</sup>-Asp<sup>362</sup>, Asp<sup>362</sup>-Ser<sup>363</sup>, and Ser<sup>363</sup>-Gln<sup>364</sup> were cleaved. The position Glu<sup>124</sup>-Gly<sup>125</sup> was also cleaved, generating two bands corresponding to Met<sup>1</sup>-Glu<sup>125</sup> and Gly<sup>125</sup>-Asn<sup>520</sup>. There have been reports of natural antibodies capable of cleaving several peptide bonds. Kaveri *et al.* reported that several peptide bonds were cleaved in their natural catalytic antibody for factor VIII (5). Paul *et al.* also reported multiple cleavage sites in HIVgp120 by a catalytic IgM monoclonal antibody (4). The UA15-L also was capable of peptide bond cleavage at multiple sites. For the cleav-



**FIGURE 5. Effect of UA15-L on *H. pylori* bacterium.** *a*, incubation of *H. pylori* ( $5 \times 10^7$  cells/ml) and UA15-L ( $0.8 \mu\text{M}$ ). *b*, control experiment without UA15-L. *H. pylori* ( $5 \times 10^7$  cells/ml) were incubated with UA15-L ( $0.8 \mu\text{M}$ ) in phosphate buffer. After SDS-PAGE (7% gel) of the reaction solution, the bands were transferred to Immobilon-P PVDF membrane. By using POD-HpU17 mAb, the bands were detected. By using UA15-L, the  $\beta$ -subunit and  $\alpha_m\beta_n$  were degraded with the increase of the incubation time. During the degradation, a strong band below 50 kDa (whose molecular size was correctly estimated at 30 kDa size using 14% gel, because it situated at the bottom end) was observed, suggesting that the degradation of urease of living *H. pylori* cells occurred. *c*, time course of the  $\beta$ -subunit.  $\circ$ ,  $\beta$ -subunit;  $\bullet$ , control (only bacterium). For quantitative analysis, the bands of  $\beta$ -subunit in *a* were analyzed using the computer program Image (National Institutes of Health). The  $\beta$ -subunit was clearly degraded with incubation. This cleavage assay was performed three of times. All experiments showed similar results.

ages, there is a small possibility that UreB or urease can acquire the proteolytic activity by its conformational change by mixing with UA15-L. The possibility for auto-proteolytic activity induced by UA15-L is not excluded.

Regarding the possession of catalytic activity of the light chain, we consider that the location of catalytic triad (Ser, His, and Asp) in the antibody structure may be crucial. If the triad is situated close to the surface of the antibody, both light chain and the whole antibody may have a catalytic activity. However,



**FIGURE 6. In vivo assay.** *a*, schedule for *H. pylori* infection and administration of UA15-L. Mice were infected orally with *H. pylori* inoculum ( $50 \times 10^6$  CFU) two times at 1-day intervals. Six weeks after infection, mice were treated with 0.5 ml of the catalytic antibody light-chain UA15-L ( $20 \mu\text{g}/\text{ml}$ ,  $0.8 \mu\text{M}$ ). The levels of *H. pylori*-specific antibodies, densities of colonizing bacteria, and histological analysis were evaluated. *b*, bacterial examination. After the administration with UA15-L, the number of the bacteria colonizing in the stomach was apparently reduced to  $\sim 30\%$  of the control group ( $p < 0.05$ ). Here, *H. pylori* colony counts were analyzed, and a *t* test was performed for the results. *p* values of  $< 0.05$  were considered as indicating a significant difference. (The statistical *t* test was performed using the equation presented in supplemental Appendix S2.)

when the triad locates at the interface between the light and the heavy chain, the whole antibody cannot exhibit the catalytic activity. Once the light chain is separated from the heavy chain, we are able to observe the catalytic activity. One more possibility is considered. The structure of whole antibody is rigid. In contrast, that of light chain is flexible, so that it can easily change the conformation. As a result, three amino acids (Ser, His, and Asp) can come into a close position so as to make a catalytic triad showing the catalytic activity. We have already reported that the conformational change of the light chain takes place in the induction period of the cleavage reaction (12). During the cleavage reaction, the enzymatic activity of urease declined to 3%, indicating that UA15-L destroyed not only the  $\beta$ -subunit but also the enzymatic function of the urease. As pointed out by Ha *et al.* (18), the sequence from Glu<sup>313</sup> to Asp<sup>336</sup> is the flap (helix-turn-helix) and flanking region (*magenta dotted underline* in Fig. 1a) of *H. pylori* urease. The region is highly conserved among the bacteria such as *K. aerogenes* and *P. mirabilis*, and the flap region is essential for these bacteria. UA15-L cleaved the peptide bonds of Tyr<sup>241</sup>-Asp<sup>242</sup> and Met<sup>262</sup>-Ala<sup>263</sup>, located upstream of the flap region. It also digested the peptide bonds of Ser<sup>361</sup>-Asp<sup>362</sup>-Ser<sup>363</sup>-Gln<sup>364</sup> locating downstream of the flap region. The cleavages of these peptide bonds by UA15-L released this region from the  $\beta$ -subunit leading to the activity loss. Note that the urease was also degraded when the intact bacteria were treated with UA15-L. The degraded band at  $\sim 30$  kDa corresponds to *band 4* in Table 1. Moreover, the antibody light chain could suppress the number of colonizing *H. pylori* in the stomach ( $p < 0.05$ ) *in vivo*.

Recently, triple therapy, which typically consists of two antibiotics with anti-acid drugs, has become "the gold standard" for



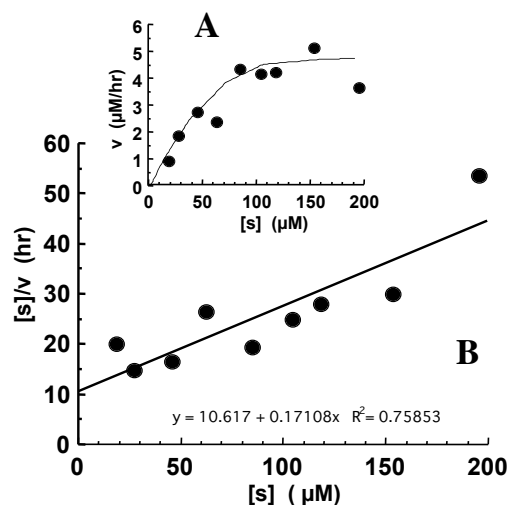
treatment to eradicate *H. pylori*. However, adverse reactions, including allergy, liver dysfunction, and diarrhea, remain to be overcome. In addition, the emergence of antibiotic-resistant bacteria has complicated therapeutic strategies. A single dose administration of antibiotics exhibited only a ~37% eradication rate (24). Therefore, the antibiotics are usually administered every 7 days. Although in our case because of the difficulty of the production of enough quantity of the catalytic antibody for *in vivo* assay, one dose was given. Nonetheless, colonizing bacteria in mouse stomach was reduced up to 70% compared with control. This should be a significant reduction as well as the administration of antibiotics. On the other hand, prophylactic and therapeutic vaccinations have been extensively studied as the alternatives to antimicrobial treatment. In these studies, repeated vaccinations (three to five repeats) using antigens such as a bacterial lysate of *H. pylori*, a recombinant urease protein, inactivated bacterial protein, and others were orally performed using mucosal adjuvant (e.g. cholera toxin) (25–28). It was reported that the number of bacteria reduced to 10% of control even in the most effective case.

Taking together these data and results, the effect of the administration of the catalytic antibody might be comparable or superior to the antibiotics and the oral vaccinations. More detailed experiments should be conducted in the near future. Although the mechanism of this eradication of *H. pylori* living in the stomach is not clear at the present time, it is likely that *H. pylori* was killed by the strong acid in the stomach, because of the reduced urease activity due to this catalytic antibody.

Conclusively, this type of antibody could have utility as a therapeutic medicine after more rigorous testing of cleavage specificity. Moreover, it is desirable that the antigen-decomposing rate and the affinity could be improved by other methods such as affinity maturation and/or genetic engineering.

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### Hanes-Woolf plots

B: Hanes-Woolf plots by UA15-L.

[S]: concentration of the TP41-1;

[V]: initial rate of the cleavage reaction.

The plot demonstrates that the cleavage reaction by UA15-L obeys the Michaelis-Menten kinetics equation, indicating that the reactions are enzymatic.

The obtained kinetic values are  $K_m = 6.2 \times 10^{-5} \text{ M}^{-1}$ ,  $k_{cat} = 2.4 \times 10^{-1} \text{ min}^{-1}$ ,  $k_{cat}/K_m = 3.9 \times 10^3 \text{ M}^{-1} \text{ min}^{-1}$ .

### APPENDIX-2

The statistical t-test was performed using the following equation.

$$t = \frac{|\bar{X}_A - \bar{X}_B|}{\sqrt{\left[ \frac{(N_A - 1)s_A^2 + (N_B - 1)s_B^2}{N_A + N_B - 2} \right] \left[ \frac{1}{N_A} + \frac{1}{N_B} \right]}}$$

Here, A = group one, B = group two,  $\bar{X}_\alpha$  = average of the participants for either group,  $N_\alpha$  = the number of participants,  $s_\alpha$  = standard deviation for either group.

### APPENDIX-1

#### Kinetic analysis:

The concentration of UA15-L was fixed at 0.8 μM and that of the TP41-1 peptide was varied at 25°C in phosphate buffer (pH 6.5).

A: Plot of concentration of TP41-1 peptide [S] vs. the initial rate [V]

**Catalytic Features and Eradication Ability of Antibody Light-chain UA15-L  
against *Helicobacter pylori***

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