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Evaluation of Factors that Can Affect Protective Immune Responses Following Oral Immunization of Recombinant *Helicobacter pylori* Urease Apoenzyme

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Abstract *Helicobacter pylori* is the major cause of gastritis, peptic ulcer, and a principal risk factor for gastric cancer. As the first step towards a vaccine against *H. pylori* infection, *H. pylori* urease was expressed and purified as a recombinant apoenzyme (rUrease) in *E. coli*. In order to develop an effective immunization protocol using rUrease, the host immune responses were evaluated after the oral immunization of mice with rUrease preparations plus cholera toxin relative to various conditions, such as the physical nature of the antigen, the frequency of the booster immunization, the dose of the antigen, and the route of administration. The protective efficacy was assessed using a quantitative culture following an *H. pylori* SS1 challenge. It was demonstrated that rUrease, due to its particulate nature, was more superior than the UreB subunit as a vaccine antigen. The oral immunization of rUrease elicited significant systemic and secretory antibody responses, and activated predominantly Th2-type cellular responses. The bacterial colonization was significantly reduced (~100-fold) in those mice immunized with three or four weekly oral doses of rUrease plus cholera toxin ($p<0.05$), when compared to the non-immunized/challenged controls. The protection correlated well with the elicited secretory IgA level against rUrease, and these secretory antibody responses were highly dependent on the frequency of the booster immunization, yet unaffected by the dose of the antigen (25- 200 μg). These results demonstrate the remarkable potential of rUrease as a vaccine antigen, thereby strengthening the possibility of developing an *H. pylori* vaccine for humans.

Key words: *Helicobacter pylori* SS1-mouse model, *H. pylori* urease, *H. pylori* vaccine, oral immunization

Helicobacter pylori is a Gram-negative, spiral bacterium that has been isolated and cultured from the stomach biopsies of patients with chronic gastritis [35]. *H. pylori* colonizes the gastric mucosa of humans and is known to be an etiological agent for chronic gastritis and peptic ulcers. Moreover, *H. pylori* infection is associated with gastric adenocarcinoma and gastric lymphoma, and the bacterium was classified as a class I carcinogen by the World Health Organization [14]. Although current antibiotic treatment regimens are effective, problems such as the development of antibiotic-resistant strains, poor patient compliance, and the high cost of therapy are major drawbacks that limit the efficacy of chemotherapeutic intervention on a large scale [21, 22]. Furthermore, infections cured with antimicrobial agents in adults do not induce immunity against reinfection [31]. As such, the prevention of *H. pylori*-associated gastroduodenal diseases by an effective vaccine against *H. pylori* is thus of a high priority.

Once infected with *H. pylori*, the systemic and local antibody levels are significantly elevated [36], and, despite of strong immune responses, *H. pylori* remains firmly entrenched in the gastric mucosa. Consequently, vaccination has not been considered as a method of prophylaxis against *H. pylori* infection. However, the prevention of gastric *Helicobacter* infection has been demonstrated in animal models following the mucosal immunization of whole-cell lysates of *Helicobacter* or recombinant subunit vaccines, together with a mucosal adjuvant such as cholera toxin (CT) or the heat-labile enterotoxin of enterotoxigenic *Escherichia coli* (LT). At present, urease and its UreB subunit [6, 17, 19, 25, 28, 37], heat shock protein A (HspA) [7], vacuolating cytotoxin (VacA) [23], and catalase [30] have all been identified as protective antigens.

H. pylori urease is a hexameric enzyme composed of two structural subunits, UreA (29.5 kDa) and UreB (66 kDa) [13]. There is a strong rationale for selecting *H. pylori* urease as a vaccine antigen. Urease is required for colonization as it neutralizes gastric acid and its importance in colonization has been previously revealed by studies using the genotobiotic piglet model of infection [4]. The urease enzyme is

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constitutively expressed, reflecting the critical role of this enzyme, and is thus present as a target for immunity during the entire course of infection. *H. pylori* urease is a major component (up to 6%) of the total soluble protein of this bacterium [13] and is localized in both the cytoplasm and on the bacterial cell surface, where it is accessible to host immune effector molecules [11].

H. pylori urease is a potential vaccine antigen, however, the use of the native enzyme can involve some problems. The ammonia produced by the urease-mediated degradation of urea is toxic to human gastric epithelial cells [33]. The heat-shock protein of *H. pylori* often co-purified with the urease enzyme and immunization with native urease preparations can subsequently induce autoimmunity through the generation of antibodies directed against the heat-shock proteins, which in turn may cross-react with cellular stress proteins [25]. These problems can be eliminated by producing *H. pylori* urease as an enzymatically-inactive, recombinant apoenzyme.

Mice were considered as outside the host range of *H. pylori* until Karita *et al.* [15] first established a transient *H. pylori* infection in immunodeficient Balb/c mice. Since then, several *H. pylori* strains have been reported to colonize the gastric mucosa of mice [17, 18, 23]. Among them, the Sydney strain (SS1), a mouse-adapted strain, has been shown to colonize mice with high infection levels, a specific adhesion to gastric epithelial cells, and a pathology similar to that seen in humans [18]. Accordingly, *H. pylori* SS1 has been suggested as a standard strain for animal studies including *H. pylori* vaccine research.

In this study, the *H. pylori* SS1-mouse model was used to test the protective efficacy of the *H. pylori* vaccine preparations. A recombinant urease apoenzyme produced in *E. coli* was used as the vaccine antigen and CT as the mucosal adjuvant. In an effort to develop an effective mucosal immunization protocol using a recombinant urease apoenzyme, the effect of factors such as the physical nature of the antigen, the frequency of antigen exposure, the dose of antigen, and the route of administration on the host immune response and protection were evaluated.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The *H. pylori* SS1, provided by Dr. A. Lee (University of New South Wales, Sydney, Australia), was grown on blood agar plates containing 20 g/l of a tryptic soy agar (Difco, Detroit, U.S.A.), 22 g/l of a Columbia blood agar base (Difco, Detroit, U.S.A.), 7% (v/v) sheep blood with Glaxo selective supplement A [10 mg/ml vancomycin, 3.3 μg/ml polymyxin B, 20 mg/ml bacitracin, 10.7 μg/ml nalidixic acid, and 5 mg/ml amphotericin B (all from Sigma Chemical Co., St. Louis, U.S.A.)] [16]. The plates were incubated at 37°C under 10% CO_2. The *H. pylori* SS1 was adapted to colonize the gastric mucosa of the mouse stomach by repeated *in vivo* passages consisting of several isolation and reinoculation cycles. After the *in vivo* passages, the *H. pylori* SS1, subcultured less than five times *in vitro*, was then used for the bacterial challenge. The *H. pylori* SS1 culture was harvested in a brain heart infusion (BHI) broth containing 25% glycerol and stored at -70°C until use.

Experimental Animals

Specific-pathogen-free, six-week-old, female Balb/c mice were purchased from Charles River Laboratories (Tokyo, Japan). All mice were fed a commercial diet and given water *ad libitum*.

Expression and Purification of Recombinant *H. pylori* Urease Apoenzyme

H. pylori urease genes encoding UreA and UreB were amplified by a PCR with primers UreA/*Nde*I (5'-GGAAT-TCATATGAAACTCACCCCAAAAG-3') and UreB/*Eco*RI (5'-GGAATTCCTAGAAAATGCTAAAGAGTTG-3'), using the genomic DNA of *H. pylori* KCTC0217BP as the template. The amplified 2.4-kb DNA fragment was then digested with *Nde*I and *Eco*RI, and cloned into the expression vector ΔpMA to construct the plasmid ΔpMA/UreAB.

Escherichia coli MC1061 was transformed with ΔpMA/UreAB and the transformant was cultured at 37°C in a Luria-Bertani broth containing ampicillin (50 μg/ml) with shaking. When the OD_{600} of the culture reached 0.4, L-arabinose was added to a final concentration of 1% (w/v) and incubated overnight. The bacterial cells were harvested and pulse sonicated with an ultrasonifier (model 450, Branson Ultrasonics, Danbury, U.S.A.) for 5 min at 50% capacity, while being kept in an ice bath. After centrifugation at 12,000 rpm for 20 min (SS-34 rotor, Sorvall RC5-B), the supernatant was collected and used for the purification of the recombinant urease apoenzyme (rUrease) according to the method previously described by Hu *et al.* [12] with some modification. Briefly, the lysates were passed through a DEAE-Sepharose column. Thereafter, the rUrease in the flow-through was concentrated and purified further by phenyl-Sepharose and Sephacryl S-300 HR column chromatography.

Protective Immunization, *H. pylori* SS1 Challenge, and Quantitative Culture

The control mice were only given sterile PBS. For oral immunization, the mice were immunized orally with 25 μg, 100 μg, and 200 μg of the purified recombinant urease apoenzyme, or 100 μg of the UreB subunit plus 10 μg of CT as a mucosal adjuvant. For the parenteral immunization, 10 μg of rUrease was immunized intramuscularly. Unless otherwise stated, the immunization was performed four times at weekly intervals.

Two weeks after the fourth immunization, the mice were challenged with two orogastric doses of live *H. pylori* SS1 cells (10^7 cells/dose) over a 2-day period. The mice were then sacrificed 10 days after the last *H. pylori* challenge, and the stomach was collected for a protection analysis using a quantitative bacterial culture. The stomach was cut along the lesser curvature and rinsed with sterile saline to remove the contents. After weighing, the stomach tissue was placed in 0.5 ml of BHI broth and homogenized using a pellet pestle (Kontes Scientific Glassware, Vineland, U.S.A.). The suspension was then plated onto blood agar plates. The protection level was assessed by a viability count of the recovered bacteria and expressed as the colony-forming units (CFUs) per gram of gastric tissue.

Collection of Samples

Blood and fecal pellets were obtained at each immunization step, before the challenge, and at the termination of the experiment. Sera were prepared from the blood and used for determining the serum IgG and IgA levels. Secretory IgA was extracted from the fecal pellets by incubation in PBS containing 5% nonfat dry milk and 1% phenylmethylsulfonylfluoride (Sigma Chemical Co., St. Louis, U.S.A.). After extensive vortexing, the fecal material was centrifuged (16,000 ×g for 20 min), and the supernatants used for determining the antibody level [10].

Enzyme-Linked Immunosorbent Assay (ELISA)

Microtiter plates (Immulon-1, Dynex Technologies Inc., Chantilly, U.S.A.) were coated with 1 μg of purified rUrease per well for 12 h at 4°C and blocked with PBS containing 1% BSA for 2 h at room temperature. This blocking solution was then used as the diluent for the sera, fecal extracts, and conjugates for all subsequent steps. After blocking, the plates were incubated for 1 h at 37°C with appropriate dilutions of the sera and fecal extracts. The dilutions for the detection of serum IgG, serum IgA, and fecal IgA were 1:1,000, 1:100, and 1:20, respectively. After washing 5 times with a wash buffer (PBS containing 0.01% Tween-20), horseradish peroxidase-conjugated goat anti-mouse IgG(γ) (1:5,000) or goat anti-mouse IgA(α) (1:3,000) (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, U.S.A.) was added and the plates incubated for 1 h at 37°C. The plates were then washed 5 times and incubated with a substrate (3, 3', 5, 5'-tetramethylbenzidine) for 30 min at room temperature. A stop solution (1.6 N H_2SO_4) was added to each well and A_{450} was determined using a microplate reader.

Statistical Analysis

The differences in the colonization level by *H. pylori* SS1 in immunized and non-immunized mice were evaluated using the Mann-Whitney U test. The differences were considered significant for $P \leq 0.05$.

RESULTS AND DISCUSSION

Expression and Purification of Recombinant Urease Apoenzyme

Previously, Lee *et al.* [20] have reported that they expressed genes for two structural subunits for *H. pylori* urease (*ureA* and *ureB*) as inclusion bodies and reconstituted them as a native enzyme after solubilization and renaturation of the inclusion bodies. In this study, *H. pylori* urease was highly expressed as a soluble protein in *E. coli*, and purified to homogeneity by DEAE-Sepharose, phenyl-Sepharose, and Sephacryl S-300 HR column chromatographies, in the order stated (Fig. 1). The purified molecules did not show any urease activities as assessed by a rapid urease assay [32] and had a molecular mass of ~550 kDa (data not shown). From the result of the SDS-PAGE analysis, the purified urease protein was proven to consist of two structural subunits whose molecular weights were 66 and 30 kDa (Fig. 1). The stoichiometry of these two subunits appeared to be approximately 1:1, thereby indicating that the purified molecule was consisted of 6 molecules of each subunit to form a ~550 kDa protein. All these results indicate that the purified molecule is a hexameric apoenzyme of *H. pylori* urease (rUrease).

Since the antisera against rUrease reacted with native urease on a similar level and *vice versa* (data not shown), rUrease appeared to retain an antigenicity and immunogenicity identical to those of the native urease holoenzyme.

Factors That May Affect Mucosal Immune Responses

There have been many previous reports on successful vaccination against *H. pylori* infection using *H. pylori*

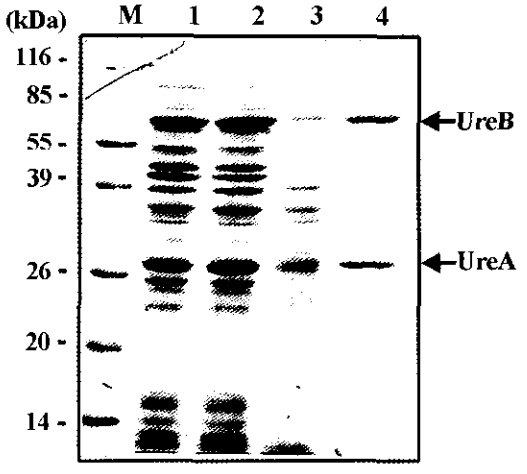

Fig. 1. Purification of recombinant *H. pylori* urease apoenzyme. The protein from each purification step was electrophoresed on a 15% polyacrylamide gel and stained with Coomassie blue. Lane M, protein standards; lane 1, whole-cell lysates of *E. coli* MC1061 containing the plasmid ΔpMA/UreAB; lane 2, DEAE Sepharose; lane 3, phenyl Sepharose; lane 4, Sephacryl S-300. The UreA and UreB proteins are indicated by arrows.

Table 1. Protection from gastric *H. pylori* infection.

Antigen	Dose (μg)	Route	Frequency	Log [*H. pylori* CFUs/g gastric tissue (±SD)][a]	No. of mice[b]	Statistics[c]
rUrease	25	Oral	1 or 2	3.38±3.26	6	$p>0.05$
			3 or 4	1.71±1.92	6(2)	$p=0.012$
	100		4	2.09±2.12	6(2)	$p=0.012$
	200		4	2.10±2.07	4(1)	$p=0.028$
rUrease	10	IM[d]	4	3.59±3.47	3	$p>0.05$
rUreB	100	Oral	4	3.53±3.40	3	$p>0.05$
Control				4.00±4.03	3	

[a]Bacterial colonization was assessed by quantitative *H. pylori* culture.
[b]Number of mice showing complete protection is shown in parenthesis.
[c]Mann-Whitney U test.
[d]Intramuscular immunization.

proteins and appropriate mucosal adjuvants, however, the immunization protocols were not fully optimized yet. Accordingly, in this study, we attempted to improve the currently available immunization conditions by evaluating the following factors involved in the host immune responses and protective efficacies.

(1) Physical nature of antigen. A particulate antigen is known to efficiently stimulate the induction of a mucosal immune response by facilitating the uptake and transport by the M cells overlying lymphoid follicles in the gastrointestinal tract [3]. Both the native and recombinant *H. pylori* urease molecules, but not its UreB subunit, have been reported to form a particle with a 12 nm diameter [19]. To study the effect of the physical nature of these two antigens on the antibody responses, mice were immunized orally four times at weekly intervals with equal amounts (100 μg/dose) of rUrease or rUreB, together with 10 μg of CT as an adjuvant. The rUrease-immunized mice consistently exhibited strong secretory and serum antibody responses and were significantly protected against gastric *H. pylori* SS1 infection (Table 1). However, the rUreB-immunized mice exhibited much lower and highly variable antibody responses (Fig. 2), and no protection was achieved (Table 1). These results indicated that a particulated antigen such as rUrease was superior over a soluble one like rUreB as a vaccine antigen, and for this reason, the following immunization studies were carried out with rUrease.

(2) Frequency of booster immunization. In an effort to optimize the immunization conditions with rUrease, the effect of the immunization frequency on the immune responses was analyzed. Groups of mice were immunized orally one through four times at weekly intervals with 25 μg of rUrease together with 10 μg of CT. Sera and fecal pellets were collected after each immunization step and used for measuring the rUrease-specific antibody levels.

Additional booster immunizations were found to be associated with a concomitant elevation of the rUrease-specific secretory and serum antibody levels, however, the dependency and profiles of the antibody increments were different depending on the antibody type. A significant elevation of the serum IgG level was observed even in those mice that were only given the primary immunization, and the antibody level rapidly increased towards a peak level with time (Fig. 3A). The serum IgA level was moderately

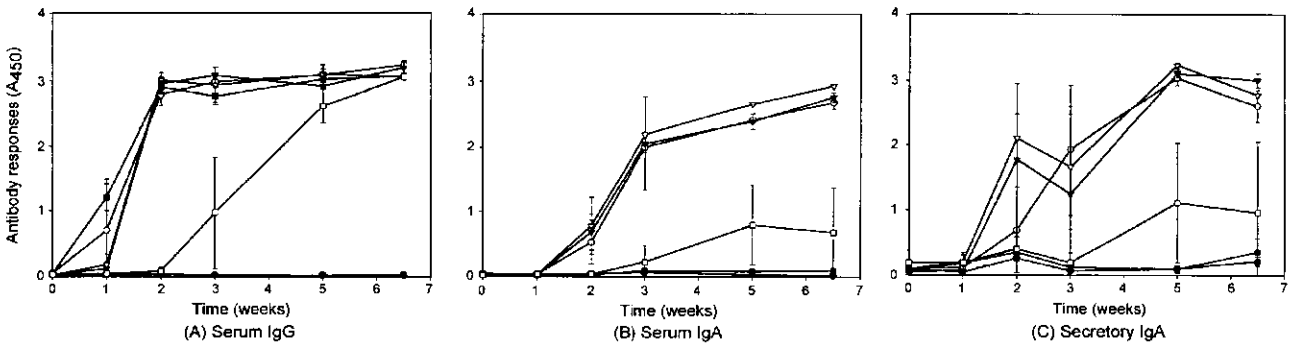

Fig. 2. Antibody responses according to dose of antigen, route of administration, and nature of antigen.
Mice were immunized four times at weekly intervals with the following variables. For oral immunization, 4 groups of mice were immunized with 25 (open circle), 100 (filled triangle), and 200 μg/dose (open triangle) of rUrease, respectively, or 100 μg/dose of rUreB (open rectangle). For the parenteral immunization, the mice were immunized intramuscularly with 10 μg/dose of rUrease (filled rectangle). The control mice were only given PBS (filled circle). Sera and fecal pellets were collected at each immunization step and used to measure (A) the serum IgG, (B) serum IgA, and (C) secretory IgA levels as described in Materials and Methods. The mean ELISA absorbance units of each group of mice are shown with standard deviations.

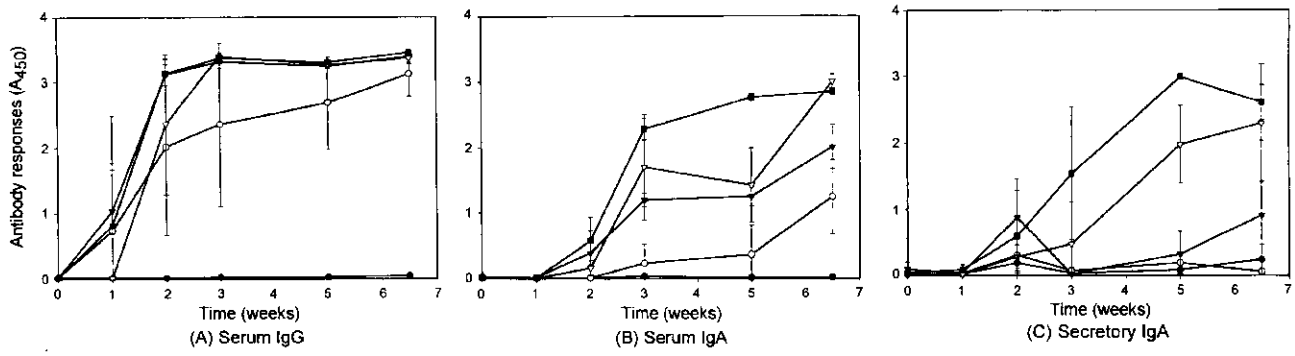

Fig. 3. Antibody responses according to different immunization frequencies.
After oral immunization with rUrease (25 μg/dose) plus CT under variable immunization frequencies, sera and fecal pellets were collected from each group of mice and used to measure (A) the serum IgG, (B) serum IgA, and (C) secretory IgA levels against rUrease. The oral immunization was performed once (open circle), twice (filled triangle), three- (open triangle), and four-times (filled rectangle) at weekly intervals, respectively. The control mice (filled circle) were only given PBS. The mean ELISA absorbance units of each group of mice are shown with standard deviations.

increased and highly proportional to the number of additional immunizations (Fig. 3B). In contrast, the mucosal antibody responses were only slowly induced and highly dependent on the immunization frequency when compared with the systematic antibody responses. Under the experimental conditions used in this study, three or more oral immunizations appeared to be required to induce a detectable level of mucosal antibody responses, while the mice given only one or two weekly oral administrations did not show any sero-conversion (Fig. 3C). It is of interest to note that protection from *H. pylori* infection was only achieved in those mice with detectable secretory IgA responses, thereby indicating the important role of secretory IgA in protection. Oral vaccines are advantageous over parenteral vaccines in that they have the potential for an almost unlimited frequency of boosting [27]. When considering this trait of oral immunization, the frequency-dependent nature of the secretory antibody responses, and resulting protective efficacies following oral immunization, it would seem possible to maximize the protective mucosal immune responses by increasing the frequency of boosting.

(3) Dose of antigen. Groups of mice were immunized with different doses of rUrease (25, 100, and 200 μg of proteins, respectively) together with 10 μg of CT in weeks 0, 1, 2, and 3. Sera and fecal pellets were then collected and used for determining the antibody levels. Significant yet similar levels of secretory IgA, serum IgG, and serum IgA responses have been induced in all three different dosing groups (Fig. 2). All these immunized mice exhibited significant protection against the *H. pylori* challenge. When compared to the non-immunized/challenged control mice, all the immunized/challenged mice showed significant decreases (~100-fold) in their bacterial densities. However, complete protection was only achieved in 31% of the immunized animals with four weekly oral doses of rUrease plus CT (Table 1). Kleanthous *et al.* [17] also reported similar results: They suggested that the residual infection may have been due to (1) the high challenge inoculum (1,000-fold 90% infective doses in that study) resulted in a breakthrough of the level of immunity achieved by the vaccination, (2) the urease shed from the bacterial surface acted as a decoy for the antibody, and (3) the residual *H. pylori* organisms occupied an immunologically privileged site that is inaccessible to the antibody or the effector function of the T cells. In addition, the current authors believe that this was partially due to the fact that the experiment was terminated too early for the host immune system to respond fully against the *H. pylori* challenge. When considering that the secretory IgA level increased continuously over time and that the secretory immune responses may also play an important role in protection, a further increase in the complete protection level seemed to be achieved with the secretory immune responses, although the level was not determined quantitatively, i.e. whether or not they were fully activated. This argument is supported by Michetti *et al.* [25] who showed that a low-level infection in mice early after the challenge was eliminated over time. The time required for a host to develop full protection is uncertain; however, once developed, the protective immunity has been reported to last over a 40-week period [24]. The fact that similar immune responses and protection were achieved after the immunization with an eight-fold lower amount of rUrease (25 vs. 200 μg protein) clearly reflects the remarkable antigenic potential of rUrease. Although a lower limit for an effective dose was not determined in this study, it is expected that an effective dose of rUrease could be less than 25 μg/dose.

(4) Route of administration. A group of mice was immunized intramuscularly four times at weekly intervals with 10 μg of rUrease. Thereafter, the protective efficacies and antibody responses induced in this group of mice were compared to those groups of mice immunized orally.

Although serum IgG was induced, no secretory IgA or serum IgA antibodies were detected in those mice given rUrease parenterally (Fig. 2). Moreover, the parenteral

immunization of rUrease did not confer any protection against the *H. pylori* challenge (Table 1). These results indicate an important role for the induction of mucosal immune responses and consequent production of secretory IgA in the prevention of *H. pylori* infection. This is one reason why a vaccine against *H. pylori* infection should be immunized by a mucosal route. Previous findings that an orally administered *H. felis* urease-specific monoclonal antibody protected mice against *H. felis* infection [2] and that a delayed *H. pylori* infection was observed in infants receiving breast milk with the anti-*H. pylori* IgA antibody [34] also support the role of secretory IgA in protection. A process termed 'immune exclusion' has been suggested to be involved in the protection by secretory IgA against *H. pylori* infection [19]. Briefly, secretory IgA antibodies secreted into the gastric mucus layer bind to the urease on the bacterial surface. This has the effect of cross-linking and agglutinating the bacteria and trapping them in the mucus, thereby enhancing their removal by peristalsis.

In several reports including these studies [19, 28], protection against *Helicobacter* infection has been shown to be strongly correlated with the induced secretory IgA level. However, the protective role of secretory IgA is still controversial. Weltzin *et al.* [37] reported that after repeated intranasal immunizations of rUrease without a mucosal adjuvant, although significant secretory IgA responses were elicited, no protection was conferred. In addition, protection has also been achieved without a detectable secretory IgA response [25]. Accordingly, these results reflect that secretory IgA may not be an actual immune effector molecule in the protection against *H. pylori* infection. This was also supported by a recent immunization study with knock-out mice, showing that the protection of mice against *H. pylori* infection through immunization with a urease antigen is basically dependent on major histocompatibility complex class II-restricted cell-mediated mechanisms, such that antibody responses to urease would not seem to be required for protection [5].

Induction of IgG Isotype Antibodies after Oral Immunization

To investigate the cellular immune responses induced in immunized/protected mice, the level of IgG isotype antibodies in mice after four weekly oral immunizations with 25 μg of rUrease plus 10 μg of CT was determined.

The immunization of animals with protein antigens plus CT has been shown to activate Th2 cellular responses [38] against the administered antigen. However, in this study, although significant levels of both serum IgG1 and IgG2a antibodies were detected in the immunized mice, IgG1 was the prevailing IgG isotype against both urease and CT. These results indicate that antigen-specific cellular immune responses, predominantly Th2-type, were activated (Fig. 4).

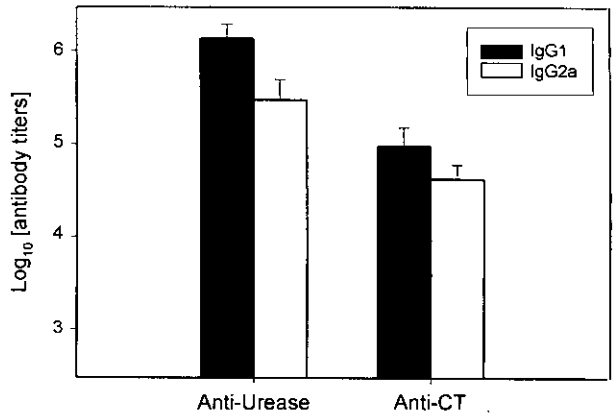

Fig. 4. IgG isotype antibody responses.
Mice were given four weekly oral doses of rUrease (25 μg/dose) plus CT, then the IgG1 and IgG2a titers against rUrease and CT were measured from the sera collected a week after the fourth immunization. The mean antibody titers are shown with standard deviations.

A *Helicobacter* infection has been shown to induce antigen-specific cellular immune responses, predominantly Th1 phenotype, and these Th1 responses are associated with the pathogenesis of disease [26]. In contrast, in immunized mice, Th2 cellular responses are activated and these are associated with the protection and control of infection [26]. Consequently, the switch of the T helper cell response from a Th1 to a Th2 response has been thought to be required for the prevention and elimination of a *Helicobacter* infection. Yet, a comparative study of several different parenteral adjuvants suggested that an appropriate balance between Th1 and Th2 type responses is required to achieve complete protection [9]. Therefore, to design novel strategies of immune modulation and to develop vaccines for the prevention of *H. pylori*, a clear understanding of the mechanisms by which the immune responses control bacterial infection is required.

The present study successfully expressed and purified the recombinant apoenzyme (rUrease) of *H. pylori* in *E. coli*. rUrease has shown significant potential as a vaccine antigen. The mucosal immune responses following the oral immunization of rUrease plus CT were found to be highly dependent on the frequency of the boosting and much less dependent on the dose of the antigen. The oral immunization of rUrease plus CT elicited significant systemic and mucosal antibody responses, and activated Th2 cellular immune responses. Furthermore, after the bacterial challenge, *H. pylori* colonization was significantly reduced in the immunized mice. When considering the association of the bacterial density with the clinical symptoms [1], these results indicate that the oral immunization of rUrease may be able to regress the gastropathy caused by chronic *H. pylori* infection. However, due to their toxicities, CT and LT can not be used in an *H. pylori* vaccine for humans. Consequently, there is an urgent need for a nontoxic and

safe mucosal adjuvant, despite that several nontoxic derivatives of LT have been developed [8, 29]. In conclusion, it is anticipated that further improvements in the immunization protocol, in combination with the development of an effective and safe mucosal adjuvant, will soon result in the development of an *H. pylori* vaccine for humans.

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