Immunization of Mice with Urease Vaccine Affords Protection against Helicobacter pylori Infection in the Absence of Antibodies and Is Mediated by MHC Class II-restricted Responses

By Thomas H. Ermak,* Paul J. Giannasca,* Richard Nichols,* Gwendolyn A. Myers,* John Nedrud,‡ Richard Weltzin,* Cynthia K. Lee,* Harold Kleanthous,* and Thomas P. Monath*

From *OraVax, Inc, Cambridge, Massachusetts 02139; and ‡Case Western Reserve University, Cleveland, Ohio 44106

Summary

We examined the roles of cell- and antibody-mediated immunity in urease vaccine-induced protection against Helicobacter pylori infection. Normal and knockout mice deficient in major histocompatibility complex (MHC) class I, MHC class II, or B cell responses were mucosally immunized with urease plus Escherichia coli heat-labile enterotoxin (LT), or parenterally immunized with urease plus aluminum hydroxide or a glycolipid adjuvant, challenged with H. pylori strain X47-2AL, and H. pylori organisms and leukocyte infiltration in the gastric mucosa quantified. In an adjuvant/route study in normal mice, there was a direct correlation between the level of protection and the density of T cells recruited to the gastric mucosa. In knockout studies, oral immunization with urease plus LT protected MHC class I knockout mice [β2-microglobulin (β2/2)] but not MHC class II knockout mice [I-Aβ (β2/2)]. In B cell knockout mice [μMT (−/−)], vaccine-induced protection was equivalent to that observed in immunized wild-type (+/+ mice); no IgA+ cells were detected in the stomach, but levels of CD4+ cells equivalent to those in the wild-type strain (+/+) were seen. These studies indicate that protection of mice against H. pylori infection by immunization with the urease antigen is dependent on MHC class II-restricted, cell-mediated mechanisms, and antibody responses to urease are not required for protection.

Key words: Helicobacter pylori • knockout mice • adjuvants • gastric mucosa • T cells

Helicobacter pylori, a gram-negative, spiral bacterium that colonizes the gastric mucosa, is the principal cause of chronic gastritis, peptic ulcer disease, and gastric adenocarcinoma in humans (1, 2). The development of vaccines to prevent H. pylori–associated diseases will be facilitated by an understanding of the immune mechanisms responsible for protection. Mucosal immunization with recombinant H. pylori urease administered with the mucosal adjuvant Escherichia coli heat-labile enterotoxin (LT)1 protects mice against challenge with H. pylori, reducing bacterial density by ~100-fold (3). Efforts to identify specific mechanisms or cells responsible for protection using a number of Helicobacter antigens have failed to identify conclusively correlates of immunity (4–9). Immunized animals develop antigen-specific serum IgG and IgA, intestinal and salivary IgA, and, after challenge, a local (gastrectomy) cellular and antibody response (3, 5, 10). Protection is also associated with the presence of CD4+ cells and CD8+ cells in the gastric mucosa (3, 11), and reductions in bacterial load can be achieved in the absence of active immunization by adoptive transfer of T cells from immunized donor mice, suggesting that cell-mediated immune responses play a major role in protection in this species (12).

Other than mucosal immunization with bacterial antigens combined with LT or cholera toxin (3–13), few immunization regimens have been explored as a means to study the mechanisms of protection against H. pylori. Subcutaneous immunization with urease plus several different parenteral adjuvants generated high levels of serum IgG and showed various degrees of protection against Helicobacter fe-
lis or H. pylori (5, 14), whereas intranasal (IN) immunization with urease without adjuvant generated moderate levels of serum IgG, salivary IgA, and fecal IgA, but was not protective (15). These findings demonstrated that although appreciable antibody responses can be generated without a mucosal adjuvant, protective immunity mediated via urease immunization can only be achieved in the presence of a mucosal or parenteral adjuvant. The lack of protection in the absence of a suitable mucosal adjuvant suggested that antibody may not be an essential mediator of protection.

Recent developments in gene knockout technology have produced a variety of experimental mouse models to study mechanisms of immunity and their roles in infectious diseases. Mice in which the I-A gene has been disrupted lack MHC class II molecules, are deficient in CD4+ T cells, and have impaired cellular and antibody-mediated immunity (16, 17). Mice in which the β2-microglobulin (β2m) molecule is lacking are deficient in MHC class I molecules, fail to differentiate normal numbers of CD8+ T cells, and have deficient CTL responses (18). Antibody-deficient mice have been produced by disruption of the immunglobulin μ chain gene at the μMT exon (19). In these latter mice, peripheral B cells are absent, and no serum or mucosal antibody responses can be generated (20).

In this investigation, mechanisms of vaccine-induced protection against H. pylori were examined using mucosal and parenteral immunization regimens with recombinant urease in both wild-type and gene knockout mice. In wild-type mice, mucosal immunization with urease plus LT yielded higher levels of protection than did parenteral or combination parenteral/mucosal regimens. Protection best correlated with the density of T cells in the gastric mucosa after challenge with H. pylori. An essential role for MHC class II-dependent T cell responses in protection was determined using β2m and I-Aβ knockout mice. In B cell knockout mice, protection equivalent to that seen in immunized wild-type mice was demonstrated in the absence of specific antibodies against urease. These results suggest a central role of CD4+ T cell–dependent cell-mediated immunity in urease vaccine–induced protection of mice against H. pylori infection.

Materials and Methods

Animals. All procedures were conducted with approval of the OraVax Institutional Animal Care and Use Committee. Specific pathogen–free, 8-wk-old outbred female Swiss–Webster mice, inbred homozygous (−/−) and heterozygous (+/−) I-Aβ gene knockout mice, homozygous (−/−) and heterozygous (+/−) β2m gene knockout mice, and wild-type (+/+ ) C57BL/6 mice free from Helicobacter muridarum were obtained from Taconic Farms, Inc. (Germantown, NY). Specific pathogen–free, 8-wk-old μMT (Igh−/−) gene knockout mice back-crossed to the C57BL/6 background and wild-type (+/+) C57BL/6 mice free from Helicobacter spp. were obtained from The Jackson Lab. (Bar Harbor, ME). Experimental groups contained 10–12 mice each.

Urease Expression and Purification. Recombinant urease was used as the model antigen in all studies and was expressed and purified from E. coli strain O R V214 as described previously (5). Native H. pylori urease was used as the coating antigen in ELISA immunosassays and was purified from H. pylori strain ATCC 43504 (American Type Culture Collection, Rockville, Md) as described previously (5).

Adjuvants. LT was obtained from Berna Products Corp. (Coral Gables, Fl). Alum was obtained as an aluminum hydroxide gel (R hydrogel®) from R eehs, Inc. (Berkeley Heights, N J). The glucosylamidine Bay R 1005 [N-(2-deoxy-2-1-leucylaminob-d-glycophosphonyl)-N-octadecylododecanamide acetate] (14, 21) was provided by Bayer AG (Wuppertal, Germany).

Immunization Procedures. Mice were immunized at weekly or biweekly intervals with recombinant urease containing the appropriate dose of adjuvant (Table 1). For oral immunization, 25 μl of vaccine was delivered into the mouth (5). For IN immunization, 10 μl of vaccine was applied onto the external nares of unanesthetized mice (15). For parenteral immunization, 100 μl of solution was injected by the s.c. route along the midline of the lower back. 1 wk after the last immunization, blood samples were taken from the retro-orbital sinus to measure the antiusrease systemic immune responses before challenge.

Immunization Regimens. The immunization doses and schedules for each study are outlined in Table 1. T to determine the efficacy of different adjuvants and routes of urease vaccine administration, we compared mucosal immunization with parenteral immunization in an outbred mouse model. The roles of MHC I- and MHC II–restricted T cell responses in protection were examined in β2m and I-Aβ gene knockout mice, and the role of B cells and antibody in protection was examined in μMT knockout mice. In the adjuvant studies, urease plus LT was delivered by the IN route to give a direct comparison with IN delivery of urease without adjuvant. In the knockout studies, urease plus LT was administered by the more traditional oral route. Both IN and oral immunization with urease plus LT previously have been shown to give comparable levels of protection and recruitment of gastric T cells (3).

To determine the role of urease–specific serum IgG in protection, outbred Swiss–Webster mice were immunized by mucosal and parenteral routes as described in Table 1. 1 wk after immunization, donor mice were bled, the sera pooled, and 2 ml was injected by the i.p. route into seven recipient mice 2 d before H. pylori challenge. Blood from recipient mice was sampled the next day to confirm transfer of urease–specific antibodies.

H. pylori growth conditions and challenge. A streptomycin-resistant mutant of H. pylori strain X 47-2AL was used for all challenge experiments. H. pylori strain X 47-2AL (O R V 2001) was originally isolated from a domestic cat (Dr. J.G. Fox, Massachusetts Institute of Technology, Cambridge, M A) (22), and adapted to Swiss–Webster mice by sequential in vivo passages (3). To prepare the challenge inoculum, bacteria were grown on agar plates for 2 d followed by 1 d in suspension (3). 2 wk after the last immunization, mice were challenged intragastrically via a 20-gauge feeding needle with a 100-μl suspension of H. pylori strain X 47-2AL containing 107 CFU/ml. At ~2 wk after challenge, mice were killed and gastric tissue processed for urease activity, H. pylori culture, histology, or immunohistochemistry as described below.

Serum Antibodies. Serum samples collected 7 d after the last immunization were evaluated for urease-specific IgG, IgG1, or IgG2a by indirect ELISA as described previously (3, 15). Urease-specific IgM or IgA was detected by antibody-capture ELISA. Plates were coated with 1 μg/ml goat anti–mouse IgM or IgA (Southern Biotechnology Associates, Birmingham, Al); test sera were added, followed by 2.5 μg/ml purified native H. pylori
urease, rabbit anti-urease, and goat anti-rabbit IgG conjugated to alkaline phosphatase (Southern Biotechnology Associates). ELISA plates were developed with $p$-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) and absorbance read at OD 405 with a Vmax microplate reader (Molecular Devices Corp., Sunnyvale, CA). The concentrations of urease-specific serum IgG 1 and IgG2a were calculated using standard curves generated by titrating pooled high-titer sera (23). The results were expressed as U/ml, and the IgG1:IgG2a ratio was calculated for each mouse. Absorbance values were converted to specific antibody measurements using standard curves generated with SOFTmax 881 software (Molecular Devices Corp.).

Gastric Tissue Analyses. The stomach was dissected along the lesser curvature and divided into strips for urease activity, $H. pylori$ culture, histopathology, or immunohistochemical analyses. For histopathology, a longitudinal segment including the antrum and corpus plus a piece of attached intestine was fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 $\mu$m. For immunohistochemistry, gastric segments were mounted on a 3-mm slice of spleen by wrapping the mucosa around the outer capsule of the spleen with the gastric mucosa facing outwards. Each piece of tissue was then prepared and plated onto Helicobacter-selective agar, as described above. Bacterial counts were determined after 5 d of growth in a 7% CO2 incubator at 37°C.

Histopathology. Histological sections from adjuvant studies and MHC-II and MHC-I knockout studies were evaluated by a pathologist blinded to the sample code (randomized histological sections of gastric mucosa) as described previously (11). $H. pylori$ organisms in gastric sections were made visible with a modified Steiner silver stain (Sigma Chemical Co.). The density of $H. pylori$ per longitudinal section from the intestine through the entire antrum and corpus was scored as follows: 0, no bacteria; 1, 1–5 foci containing one or more bacteria; 2, 5–10 foci; 3, 10–20 foci; and 4, $>20$ foci.

Gastritis and epithelial alterations were evaluated on hematoxylin and eosin (H & E) stained sections as described previously (11). For gastritis, sections were scored from zero to four based upon the intensity of the infiltration of lymphocytes, plasma cells, and neutrophils. The degree of epithelial change was scored similarly based on the loss of parietal cells, hyperplasia of the surface epithelium, and the presence of microabscesses or cystic glands.

| Table 1. Vaccine Schedule for Protection Studies |
|---|---|
| **Group** | **N o. and route of immunizations** | **U rease dose** | **Adjuvant dose** |
| Adjuvant and route study in Swiss-Webster mice | | | |
| U rease/no adjuvant | 14 daily IN | 25 |  |
| Alum | 3 s.c. | 10 | 200 |
| Bay | 3 s.c. | 10 | 400 |
| LT/Alum | 1 IN/2 s.c. | 25/10 | 1/200 |
| LT/Bay | 1 IN/2 s.c. | 25/10 | 1/400 |
| LT | 3 IN | 25 | 1 |
| MHC-class I and class II study in C57BL/6 mice | | | |
| Alum | 3 s.c. | 10 | 200 |
| LT | 4 weekly oral | 25 | 1 |
| Passive transfer study in Swiss-Webster mice | | | |
| U rease/no adjuvant | 15 daily IN | 25 |  |
| Alum | 3 s.c. | 10 | 200 |
| LT | 3 IN | 25 | 1 |
| μMT study in C57BL/6j mice | | | |
| Alum | 3 s.c. | 10 | 200 |
| LT | 3 oral | 25 | 1 |

*Immunizations given biweekly unless otherwise noted; controls were untreated.
†Included 10 mice per group.
§Included 10 (+/+ and –/−) mice and 5 (+/+ −) mice per group.
5), CD8α (clone 53-6-7), CD103 (αE-integrin) (clone M290), β7-integrin (clone M293), CD45R (B220) (clone RA3 6B2), IgA (clone R 5-140), IgM (clone R 6-60.2), CD11b (Mac-1) (clone M 1/70), or Ly-6G (clone R6-8C5) (reactive with neutrophils) (PharMingen, San Diego, CA). Sections were then incubated with biotinylated rabbit anti-m IgG (Vector Laboratories Inc.), followed by horseradish peroxidase conjugated to an avidin–biotin complex (ABC-Elite KIT; Vector Laboratories Inc.), diamobenzidine, and methyl green. Control sections were incubated without primary mAb. Urease-specific antibody-containing cells (urease-ACC) were detected by an avidin–biotin glucose oxidase procedure, as described previously (3). The number of positive cells was counted in 0.5-mm segment lengths of gastric mucosa and expressed per mm² field as described previously (11).

Flow Cytometric Analysis of Lymphocyte Populations. Peripheral blood, spleen, and intestinal intraepithelial lymphocytes (IEL) were examined for depletion of T cells in knockout mice using rat mAb against mouse CD4 (clone RM4-5), CD8α (clone 53-6-7), CD103 (αE-integrin) (clone M290), CD90 (Thy-1.2) (clone 30-H12), CD45 (clone 30-F11), CD45R (B220) (clone RA3 6B2), IgM (clone R 6-60.2), I-A⁻ (A⁻α) (clone 25-9-17), or H-2Dᵇ (clone H K95) (PharMingen) as described previously (11, 24). The stained lymphocytes were analyzed (10,000 cells) using an Epics XL (Coulter Corp., Miami, FL).

Statistics. Statistical analyses were performed with either JMP™ or Graphpad Prism™ software using Wilcoxon/Kruskal-Wallis (rank sums) test for continuous or ordinal variables, or linear regression.

Results

Role of Adjuvant and Route of Immunization in Protection of Outstanding Mice

Protection against H. pylori infection. Levels of protection were determined by urease activity or quantitative culture of gastric tissue in a study using five different adjuvant regimens administered with urease (plus controls) to determine whether correlates of protection could be identified (Fig. 1). Unimmunized mice and mice receiving daily IN urease immunization without adjuvant were highly infected with geometric mean CFU s of ~5.0 × 10⁵ to 1.5 × 10⁷ per gastric segment at 14 d after challenge. The greatest level of protection was observed in mice given urease plus LT by the IN route; the geometric mean H. pylori density (3.6 × 10⁵) was 100-1,000-fold lower than in unimmunized mice or mice receiving daily IN urease without LT (P < 0.01 versus controls; Wilcoxon/Kruskal-Wallis rank sum test). Parenteral administration of the same dose and regimen of urease plus either alum or Bay adjuvants resulted in partial protection characterized by greatly reduced gastric urease activity (P < 0.01) but <10-fold decrease in H. pylori density by culture (significance achieved versus IN per day controls, P < 0.02; but not versus unimmunized controls). A combined regimen of mucosal priming with urease plus LT followed by parenteral boosting with urease plus alum or Bay gave intermediate levels of protection between urease plus LT and parenteral-only regimens (10–100-fold decrease in H. pylori versus controls, P < 0.01).

Effects on Gastric Mucosa. Histopathologic evaluations of gastritis and epithelial change were performed on H & E sections of gastric mucosa from mice that received urease plus LT, urease plus alum, or no immunization, and were then challenged with H. pylori. As reported elsewhere (14), immunization and protection was associated with increased recruitment of immune cells to the gastric mucosa in comparison with controls (each group, P < 0.01). The increased cellular infiltrate was accompanied by a slight increase in epithelial change in either the antrum or corpus (mean epithelial score in the corpus increased from 0 in controls to 1 in urease plus LT-immunized mice, P < 0.01).

Recruitment of T and B Cells to Gastric Tissue after Challenge. Previous studies demonstrated that significant recruitment of CD4⁺ and CD8⁺ cells occurs in the gastric mucosa of immunized mice after H. pylori challenge (3). In this study, we examined the regional localization of cells af-
ter challenge. The highest density of cells localized at the junction between the antrum and corpus (Fig. 2a). For example, immunized mice had three to four times as many CD4+ cells in the junction region as in the antrum or corpus. In histological sections of gastric tissue from unimmunized mice examined in preliminary studies, the junction region was also the area where most H. pylori were located (data not shown). Therefore, quantitative analyses of gastric lymphocytes focused on this region.

The density of gastric T cells, IgA+ plasma cells, and urease-ACC was determined for each group of mice. CD4+ cells were the most numerous of any lymphocyte subset, averaging a fivefold increase in density over unimmunized controls, and were found along the muscularis mucosa and in the lamina propria between gastric pits (Fig. 2b). The density of T cells varied as a function of immunization regimen (Fig. 3, A and B) and strongly correlated with the level of protection (CD4+ cells, r² = 0.956; CD8+ cells, r² = 0.947) (Fig. 3, D and E). Nonprotective daily IN urease immunization without adjuvant resulted in no gastric T cells, whereas immunization with urease plus adjuvant by any route produced a significant increase in gastric T cells (all groups, P < 0.01). IN immunization with urease plus LT induced the greatest number of gastric T cells.

IgA+ cells were also most numerous in the urease plus LT group, increased in the nonprotective regimen of IN urease without adjuvant (P < 0.04), and were somewhat variable in animals receiving the parenteral and combination regimens (Fig. 3C). IgA+ cells also showed a strong positive correlation with the level of protection (r² = 0.690) (Fig. 3F). Urease-ACC were detected in mice receiving urease plus LT (1–2 cells/mm² field in mice receiving urease plus Bay, LT/alum, LT/Bay, or LT alone), but no cells were detected in control, urease daily without adjuvant, or urease plus alum groups.

Urease-specific IgG1 and IgG2a levels as surrogate markers for Th2:Th1 responses. Urease-specific antibody levels were analyzed to determine if IgG1:IgG2a ratios correlated with protection (3, 12, 14). Daily IN administration of urease without adjuvant and parenteral immunization with alum or Bay produced higher IgG1 than IgG2a levels, whereas immunization with urease plus LT produced equivalent levels of IgG1 and IgG2a (Table 2). The ratio of urease-specific IgG1 to IgG2a showed a positive correlation with protection (r² = 0.703), with a ratio of 1 being associated with the greatest protection (urease plus LT). This correlation suggested that a balanced Th2:Th1 response might be important for the high level of protection observed after immunization with urease plus LT.
Protection in I-A\(^b\) (−/−) and \(\beta_2m\) (−/−) K knockout Mice

Effect of Immunization on H. pylori in Gastric Tissue after Challenge. Wild-type, \(\beta_2m\) (−/−), and I-A\(^b\) (−/−) C57BL/6 mice received urease administered either orally with LT, parenterally with alum, or were not immunized before challenge. The doses and regimens are outlined in Table 1. Analysis of gastric tissue revealed that wild-type (+/+) and \(\beta_2m\) (−/−) mice receiving urease plus LT had little detectable gastric urease activity and a >100-fold decrease in geometric mean CFUs as compared with unimmunized controls (P < 0.01) (Fig. 4). Subcutaneous immunization with urease plus alum resulted in a significant decrease in gastric urease activity (Fig. 4 A) and a fivefold decrease in the bacterial density of wild-type (+/+) mice (P = 0.02) (Fig. 4 B), but had no protective effect in \(\beta_2m\) (−/−) or heterozygous (+/−) strains (Fig. 4). Oral immunization of both wild-type and \(\beta_2m\) (−/−) mice with urease plus LT clearly gave superior protection in comparison to parenteral immunization with alum (P < 0.02).

Neither mucosal nor parenteral immunization regimens were effective in protecting I-A\(^b\) (−/−) knockout mice as determined by gastric urease activity and quantitative culture (Fig. 4). The geometric mean bacterial densities of immunized mice were no different from those of unimmunized controls. Furthermore, H. pylori infection was not enhanced in the absence of the host MHC II-restricted responses of control I-A\(^b\) (−/−) mice in comparison to wild-type mice (Fig. 4).

Antibody Responses to Urease Immunization. An analysis of urease-specific serum IgG, IgM, and IgA indicated systemic immune responses to urease in mice from all immunized groups except the I-A\(^b\) (−/−) knockout mice, which did not have detectable antibody responses (data not shown). \(\beta_2m\) (−/−) knockout mice had reduced serum IgG titers after immunization when compared with wild-type mice, as well as equivalent or lower levels of serum IgM and IgA.

Effects on Gastric Mucosa. As in outbred mice, immunization and challenge in wild-type and \(\beta_2m\) (−/−) mice but not I-A\(^b\) (−/−) mice was associated with increased lymphocyte recruitment to the gastric mucosa, as determined by gastritis scores obtained from H & E sections (data not shown). Wild-type (+/+) mice immunized with urease plus LT had minimal loss of parietal cells (mean corpus epithelial score of 1.1, range 0–2) relative to controls (mean of 0.2, range 0–2) (P < 0.04), whereas a more substantial loss of parietal cells was seen in \(\beta_2m\) (−/−) mice receiving the same regimen (mean score of 2.4, range 1–3) (P < 0.01 versus controls). Wild-type and \(\beta_2m\) (−/−) mice immunized with urease plus alum, and the unprotected I-A\(^b\) (−/−) mice immunized by either regimen, had no epithelial change relative to controls.

T and B cells in Gastric Tissue. The densities of CD4\(^+\), CD8\(^+\), and IgA\(^+\) cells in the gastric mucosa were evaluated as indicators of T and B cell activity in response to immunization and challenge. In addition, the densities of CD8\(^+\) and CD103\(^+\) (αE-integrin\(^+\)) cells were used as indicators of gastric IEL. Unimmunized controls had few T or B cells in the gastric mucosa 2 wk after H. pylori challenge. Signifi-

### Table 2. Urease-specific Ser um IgG\(_1\) and IgG\(_{2a}\) Levels by Immunization Regimen

| Group       | IgG\(_1\) (median) U/ml | IgG\(_{2a}\) (median) U/ml | IgG\(_1\):IgG\(_{2a}\) ratio* (median) |
|-------------|-------------------------|---------------------------|-------------------------------------|
| IN/day 2.61 | 5.71 \times 10^4        | 2.66 \times 10^7          | 41                                  |
| Alum        | 2.61 \times 10^4        | 2.66 \times 10^7          | 43                                  |
| Bay         | 3.20 \times 10^6        | 6.73 \times 10^6          | 10                                  |
| LT/Alum     | 9.61 \times 10^6        | 9.61 \times 10^6          | 1                                   |

*Median IgG\(_1\):IgG\(_{2a}\) ratio from individual mice; not the ratio of median IgG\(_1\) to median IgG\(_{2a}\).
Significant increases in CD4⁺, CD8⁺, CD103⁺, and IgA⁺ cells were seen in β₂m (−/−) and wild-type (+/+), but not I-A⁺ (−/−) mice immunized with urease plus LT (P < 0.05) (Fig. 5).

Smaller increases in T and B cells were seen after immunization with urease plus alum (all groups, P < 0.05, except CD4⁺ cells in β₂m/alum group and IgA⁺ cells in β₂m/alum group) (Fig. 5). β₂m (−/−) knockout mice had reduced CD8⁺ and CD103⁺ cells in the gastric mucosa after oral immunization with urease plus LT relative to wild-type mice (P < 0.05, except CD8⁺ cells did not achieve significance). Most CD8⁺ and CD103⁺ cells in the gastric mucosa of β₂m (−/−) mice were within the epithelium, suggesting that they were IEL.

The lack of protection in I-A⁺ (−/−) knockout mice was accompanied by an absence of T or B cells recruited to the gastric mucosa after challenge. Although some CD4⁺ T cells were present in peripheral blood, spleen, Peyer's patches, and intestinal lamina propria of I-A⁺ (−/−) mice, CD4⁺ cells and all other T and B cell subsets in the gastric mucosa were similar to those seen in unimmunized control mice (Fig. 5).

Role of Serum IgG in Protection

The ability of parenteral immunization to afford protection against H. pylori raised the possibility that serum antibodies could play a role in bacterial clearance. This conclusion was supported by studies in I-A⁺ (−/−) mice which had no antibody response to urease and were not protected against challenge. In an effort to clarify the contribution of circulating antiurease antibodies to protection, we tested whether immunity could be transferred to naive mice by serum, as described in Materials and Methods and Table 1.

Groups of donor mice were immunized with either a protective mucosal regimen (IN urease plus LT), a protective parenteral regimen (urease plus alum), a nonprotective mucosal regimen (intranasal urease without adjuvant), or were untreated. After passive transfer of sera, recipient mice had protective levels of urease-specific IgG similar to those of actively immunized mice before challenge (data not shown). Despite the presence of high levels of urease-specific IgG in recipient mice, including mice receiving serum from donor mice immunized according to the protective mucosal or parenteral regimens, no protection was observed in any mice after challenge.

Protection against H. pylori Infection in μMT Knockout Mice

Effect of Immunization on H. pylori Infection. The inability of immune sera to passively confer protection from H. pylori infection to naive mice demonstrated that circulating antibodies to urease alone played a minor, if any, role in protection. To elucidate the contribution of local antibody responses in the gastric mucosa, B cell-deficient mice were used. Wild-type (+/+), μMT (−/−), and C57BL/6J mice received either oral or parenteral immunization with urease, as did the MHC-I and MHC-II knockout mice (Table 1). Oral immunization with urease plus LT decreased the gastric urease activity to baseline levels and reduced the density of H. pylori by more than 100-fold in both wild-type and μMT (−/−) mice after challenge (both assays, all groups, P < 0.01 versus controls) (Fig. 6). Parenteral immunization with urease plus alum reduced the density of H. pylori 10-fold in wild-type mice (P < 0.03) and fivefold in μMT (−/−) mice (not significant) and decreased gastric urease activity to baseline levels in more than half of the mice in both groups (Fig. 6).

A nitroty R responses to U rease Immunization. No urease-specific serum IgG, IgM, or IgA responses to urease were detected in μMT (−/−) mice after oral immunization with urease plus LT or parenteral immunization with urease.
Infection in the Absence of Antibodies

Discussions

Protection against H. pylori is an important goal in the prevention and treatment of gastroduodenal diseases, including ulcers and stomach cancer. Despite the proven ability of immunization to prevent or reduce H. pylori infection in murine models, the precise mechanisms of protection have remained obscured by the numerous immunological pathways stimulated. Mucosal immunization with H. pylori extracts or purified recombinant proteins generates antigen-specific serum, salivary, and intestinal antibody responses as well as cellular immunity (3, 5, 12, 13, 29). Furthermore, after immunization and challenge, gastric IgA and T cell responses can be measured (3, 5, 6). Since H. pylori is largely an extracellular pathogen of the gastric mucosa, it was reasoned that secretory IgA might play an important role in protection through diminished bacterial-mucus or epithelial cell interactions (5, 30). In this study, we examined mechanisms of protection using different adjuvants and routes of administration and a single H. pylori candidate vaccine antigen in several murine knockout models with the goal of further defining aspects of cell- and antibody-mediated immunity essential for clearance of H. pylori.

By comparing various prophylactic immunization schemes, including mucosal, parenteral, or combination regimens, we consistently achieved the highest level of protection by mucosal immunization with urease plus LT, which decreased the gastric bacterial load 100-fold or more after challenge compared with controls. A combined protocol of mucosal and parenteral immunization conferred a level of protection closer to that of mucosal immunization. Protective efficacy was evaluated by quantitative culture of H. pylori, which has been shown (3) to be more sensitive than either measurement of gastric urease activity or histological enumeration of bacteria (3, 8, 31). The H. pylori
strain X47-2AL, originally isolated from a domestic cat (22), preferentially colonized the corpus-antrum junction region of the mouse stomach, a similar distribution to that of the human-derived *H. pylori* Sydney (SS1) strain (31). The requirement for LT with mucosal immunization was clear; no detectable protective activity could be measured in the absence of mucosal adjuvant when urease was administered by a daily IN regimen or in the form of weekly doses (15). Parenteral immunization with urease plus alum or Bay adjuvant showed less protection by comparison, demonstrating significant protection by gastric urease activity, but yielding at most a 10-fold reduction in bacterial burden after challenge.

Because a range of protective efficacy was observed with the immunization regimens tested, we examined several immunological readouts for correlation with protection. Previous analyses of the levels of urease-specific antibodies in sera and mucosal secretions after immunization with urease failed to identify a single isotype-restricted response which correlated with protection (3). However, the analysis of antigen-specific IgG1/IgG2a ratios as markers of Th activity in this investigation and a comparative study of several different parenteral adjuvants (14) determined that a more balanced (i.e., 1:1) profile was associated with greater protection, suggesting that the ability to stimulate both Th1 and Th2 responses was important for protection. These observations supported the emerging concept from vaccination studies that cell-mediated immunity is required for clearance of *H. pylori* from the murine gastric mucosa.

We focused our attention on the constituents of the cellular infiltrate that are present in the gastric mucosa after immunization and challenge. A common feature of the infiltrate was its preferential localization at the corpus-antrum junction, which coincided with the distribution of *H. pylori*. Interestingly, in humans, the junction region is also the site where intestinal metaplasia, gastric atrophy, and cancer are often found (32). When comparing the constituents of the infiltrate elicited by the various regimens, we found that certain immunization schemes stimulated recruitment of distinct cellular profiles in response to *H. pylori* challenge. For example, the nonprotective regimen of IN urease immunization without adjuvant (15) induced B cells but few T cells to the gastric mucosa. In contrast, IN immunization with urease plus LT was followed by extensive recruitment of both T and B cells to the gastric mucosa. Furthermore, the density of the local infiltrate after immunization and challenge correlated with enhanced bacterial clearance. Because the appearance of the cellular infiltrate was dependent on prior exposure to antigen, required *H. pylori* challenge, and could be modulated by adjuvants, an antigen-specific cellular response after local stimulation with *Helicobacter*-associated urease is the likely mechanism responsible for the extensive cellular trafficking to the gastric mucosa. Despite the recruitment of mononuclear cells to the gastric mucosa after protective vaccination and *H. pylori* challenge, extensive epithelial damage was not found in wild-type mice. At this time, it is not clear how these observations in the murine model will translate to humans, since it remains to be determined whether vaccination will limit both infection and pathology. In unimmunized humans, the severity of inflammation correlates with the level of infection (33–35), suggesting that controlling infection may also reduce inflammation.

Use of MHC-I and MHC-II knockout mice has been instrumental in elucidating mechanisms of immunity and vaccine-induced protection to a number of viruses, bacteria, and parasites (36–38). Using these research tools, we were able to demonstrate that protection against *H. pylori* can be achieved in MHC-I-deficient mice receiving oral urease plus LT. Mice that are lacking B2m are deficient in CD8αβ+ T cells and fail to generate cytotoxic T cell responses (39, 40). This finding strongly suggests that MHC-I-restricted CD8αβ cell responses do not play a major role in protection against murine *H. pylori* infection. Because CD8+ cells typically contribute to host defenses against viruses and intracellular bacteria (36), it is not surprising that these cells are not key players against this extracellular organism. The present study does not rule out a possible role of MHC-I in protection afforded by parenteral (alum) immunization, nor does it exclude a possible role of MHC-I-independent CD8+ cell responses such as that performed by gastric CD8αα+ IEL, which differentiate independently of MHC-I (39–41). However, considering the relatively low density of these cells in comparison to CD4+ cells or macrophages, it seems likely that they are not the principal cellular component involved in protection based upon urease vaccination.

In contrast to MHC-I-deficient mice, immunization and challenge of MHC-II knockout mice revealed a profound defect in the ability of immunization to afford protection. The lack of protection was associated with an absence of CD4+ and CD8αβ T cells and IgA+ B cells in the gastric mucosa. Although the MHC class II-deficient mice did not mount a protective immune response, the absence of detectable urease-specific antibodies in sera and IgA+ cells in the gastric mucosa of these mice did not clarify the relative roles of antibodies and CD4+ T cells as effectors of protection.

In an effort to determine the contribution of circulating antibodies to protection, we performed passive transfer studies using immune sera from mice vaccinated via mucosal or parenteral routes. Despite high antibody titers to urease in recipient mice, no detectable protection after *Helicobacter* challenge was observed, demonstrating that antigen-specific circulating antibodies play little or no role in protection in this model. However, these experiments could not definitively rule out the potentially protective role of locally synthesized antibodies. Our previous studies using mucosal immunization suggested that urease-specific IgA cells recruited to the lamina propria secreted antibody into the gastric pits (3, 5), and thus theoretically could play a role in immune clearance. It has also been proposed that local IgG antibodies in the gastric mucosa may play a role in protection (10). Conflicting data with these studies showing that mice deficient in IgA were protected against *H. felis* challenge (42) further contributed to the confusion as to the role of antibodies in protection against *Helicobacter*. 

2285 Ermak et al.
To define the role of humoral immunity in protection from H. pylori more precisely, we used the \( \mu \text{MT}^{2-/-} \) mouse model of B cell and antibody deficiency. This model has been instrumental in determining the role of humoral immunity in clearance of infectious diseases (43–45). Immunization followed by challenge of \( \mu \text{MT}^{2-/-} \) mice revealed, unexpectedly, that they were capable of mounting a protective response to H. pylori which was indistinguishable from that of wild-type mice. However, the results obtained with knockout mice do need to be interpreted with caution, as it is known that ablation of specific immune functions can result in compensation by alternative pathways (20, 43). Nonetheless, these results demonstrated that clearance of H. pylori from the gastric mucosa can occur by antibody-independent mechanisms and suggest that, in normal mice, neither gastric IgA (3) nor IgG antibodies (10) play an essential role in protection after immunization with urease.

In conclusion, this study demonstrated that, in a murine model, protection against H. pylori can be achieved by urease in the absence of B cells and antibodies but requires MHC class II CD4\(^+\) T cell responses. These findings reveal an previously unreported cell-mediated mechanism of immune clearance of a predominantly intraluminal pathogen of the gastrointestinal tract. We cannot predict that a similar mechanism is responsible for protection after vaccination with other Helicobacter antigens, either during long-term challenge or after therapeutic immunization. Future studies will determine whether this is a universal mechanism used in these various settings.

The authors thank Pierre Meuille, Marie-Jose Quentin-Millet, Bruno Guy, Ling Lissolo, Farrukh Rizvi (Pasteur Mérieux Connaught, France), and Steven C Zinn (Case Western Reserve University) for their scientific and experimental suggestions during the course of these studies, and Ru Ding, Kathleen M. Georgopoulos, Heather L. Gray, Jennifer W. Ingrasia, W. En-de Lei, Michel Pietropaulo, R. Schrader, and Timothy J. Tibbitts for excellent technical assistance. Bay R 1005 was kindly provided by B. Guy (Pasteur Mérieux Connaught).

This work was supported by a joint venture between Pasteur Mérieux Connaught and OraVax.

Address correspondence to Thomas Ermak, OraVax, Inc., 38 Sidney St., Cambridge, MA 02139. Phone: 617-494-1339; Fax: 617-494-0927; E-mail: termak@oravax.com

Received for publication 9 June 1998 and in revised form 13 October 1998.

## References

1. Blaser, M.J. 1990. Helicobacter pylori and the pathogenesis of gastroduodenal inflammation. J. Infect. Dis. 161:626–633.

2. Cover, T.L., and M.J. Blaser. 1992. Helicobacter pylori and gastroduodenal disease. Annu. Rev. Med. 43:135–145.

3. Kleanthous, H., G.A. Myers, K.M. Georgopoulos, T.J. Tibbitts, J.W. Ingrasias, H.L. Gray, R. Ding, Z.-Z. Zhang, W. Lei, R. Nichols et al. 1998. Recombinant and intranasal immunization with recombinant urease induce distinct local and systemic immune responses in mice and protect against Helicobacter pylori infection. Infect. Immun. 66:2879–2886.

4. Michetti, P., I. Corthesy-Theuilaz, C. Davin, R. Haas, A.-C. Vaney, H. Heitz, J. Bille, J.-P. Kraehenbuhl, E. Saraga, and A.L. Blum. 1994. Immunization of BALB/c mice against Helicobacter felis infection with Helicobacter pylori urease. Gastroenterology. 107:1002–1011.

5. Lee, C.K., R. Weltzin, W.D. Thomas, Jr., H. Kleanthous, T.H. Ermak, G. Soman, J.E. Hill, S.K. Ackerman, and T.P. Monath. 1995. Oral immunization with recombinant Helicobacter pylori urease induces secretory IgA antibodies and protects mice from challenge with Helicobacter felis. J. Infect. Dis. 172:161–172.

6. Ferrero, R.L., J.M. Thibeige, I. Kansau, N. Wuchser, M. Huerra, and A. Labigne. 1995. The GroES homologue of Helicobacter pylori confers protective immunity against mucosal infection in mice. Proc. Natl. Acad. Sci. USA. 92:6499–6503.

7. R. Adcliff, P.J., S.L. Hazzel, T. Kolesnikow, C. Doidge, and A. Lee. 1996. Catalase, a novel antigen for Helicobacter pylori vacccination. Infect. Immun. 65:4668–4674.

8. Marchetti, M., B. Arico, D. Burroni, N. Figure, R. Appuoli, and P. Ghiara. 1995. Development of a mouse model...
of Helicobacter pylori infection that mimics human disease. Science. 267:1655–1658.

9. Pappo, J., W.D. Thomas, Jr., Z. Kabok, N.S. Taylor, J.C. Murphy, and J.G. Fox. 1995. Effect of oral immunization with recombinant urease on murine Helicobacter fels gastritis. Infect. Immun. 63:1246–1252.

10. Ferrero, R.L., J.-M. Thibierge, and A. Labigne. 1997. Local immunoglobulin G antibodies in the stomach may contribute to immunity against Helicobacter in mice. Gastroenterology. 113:185–194.

11. Ermak, T.H., R. Ding, B. Ekstein, B. Hill, G.A. Myers, C.K. Lee, J. Pappo, H.K. Kleanthous, and T.P. Monath. 1997. Oral immunization with recombinant urease: gastritis after Helicobacter fels challenge may be due to residual bacteria. Gastroenterology. 113:1118–1128.

12. Mohammadi, M., J.N. Edrud, R. Redline, N. Lycke, and S.J. Czinn. 1997. Murine CD4 T-cell response to Helicobacter infection: TH1 cells enhance gastritis and TH2 cells reduce bacterial load. Gastroenterology. 113:1848–1857.

13. Czinn, S.J., A. Caï, and J.G. Edrud. 1993. Protection of germ-free mice from infection by Helicobacter fels after active oral or passive IgA immunization. Vaccine. 11:637–642.

14. Guy, B., C. Hessler, S. Fourage, J. Haensler, E. Vialon-Lafay, B. Roob, and M.J. Quentin-Millet. 1998. Systemic immunization with urease protects mice against Helicobacter pylori infection. Vaccine. 16:850–856.

15. Wietz, R., H. Kleanthous, F. Guirakhoo, T.P. Monath, and C.K. Lee. 1997. Novel intranasal immunization techniques for antibody induction and protection of mice against gastriHelicobacter pylori fels infection. Vaccine. 15:370–376.

16. Grusby, M.J., R.S. Johnson, V.E. Papaioannou, and L.H. Glimcher. 1991. Depletion of CD4+ T cells in major histocompatibility complex class II–deficient mice. Science. 253:1417–1420.

17. Koller, B.H., P. Marrack, J.W. Kappler, and O. Smithies. 1990. Normal development of mice deficient in beta-2m MHC class I proteins, and CD8+ T cells. Science. 248:1227–1229.

18. Spriggs, M.K., B. Koller, T. Sato, P.J. Morrissey, W.C. Fanslow, O. Smithies, R.F. Voice, M.B. Widmer, and C.R. Maliszewski. 1992. β2-microglobulin−/−, CD8+ T-cell-deficient mice survive inoculation with high doses of vaccinia virus and exhibit altered IgG responses Proc. Natl. Acad. Sci. U.S.A. 89:6070–6074.

19. Kitamura, D., J. Rouse, R. Künz, and K. Rajewsky. 1991. A B cell–deficient mouse by targeted disruption of the membrane exon of the immunoglobulin γ chain gene. Nature. 350:423–426.

20. Epstein, M.M., F. Di Rosa, D. Jankovic, A. Sher, and P. Mätzing. 1995. Successful T cell priming in B cell–deficient mice. J. Exp. Med. 182:915–922.

21. Lockhoff, O. 1991. Glycolipids as immunomodulators: syntheses and properties. Angew. Chem. Int. Ed. Engl. 30:1611–1620.

22. Handt, L.K., J.G. Fox, I.H. Stalis, R. Ruffo, G. Lee, J. Linn, X. Li, and H. Kleanthous. 1995. Characterization of feline Helicobacter pylori strains and associated gastritis in a colony of domestic cats. J. Clin. Microbiol. 33:2280–2289.

23. Kemény, D.M. 1992. Titration of antibodies. J. Immunol. Methods. 150:57–76.

24. Lefrançois, L., and N. Lycke. 1994. Isolation of mouse small intestinal intraepithelial lymphocytes, Peyer’s patch, and lamina propria cells. In Current Protocols in Immunology. J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Stobo, editors John Wiley & Sons, Inc., New York. 3.19.1–3.19.16.

25. Springer, T.A., G. Galfre, D.S. Secher, and C. Milstein. 1979. Mc-1: a macrophage differentiation antigen identified by a monoclonal antibody. Eur. J. Immunol. 9:301–306.

26. Kishimoto, T.K., M.A. Jutila, E.L. Berg, and E.C. Butcher. 1989. Neutrophilic Mac-1 and M E-L–14 adhesion proteins inversely regulated by chemotactic factors. Science. 245:1238–1241.

27. Fleming, T.J., M.L. Fleming, and T.R. Mek. 1993. Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. R B6-BC 5 mAb to granulocyte–differentiation antigen (GR-1) detects members of the Ly-6 family. J. Immunol. 151:2399–2409.

28. Lagasse, R.E., and I.L. Weissman. 1996. Flow cytometric identification of murine neutrophils and monocyes. J. Immunol. Methods. 197:139–150.

29. Mohammadi, M., S. Czinn, R. Redline, and J. Edrud. 1996. Helicobacter-specific cell–mediated immune responses display a predominant Th1 phenotype and promote a delayed-type hypersensitivity response in the stomachs of mice. J. Immunol. 156:4729–4738.

30. Blanchard, T.G., S.J. Czinn, R. Maurer, W.D. Thomas, G. Soman, and J.G. Edrud. 1995. Urease-specific monoclonal antibodies prevent Helicobacter fels infection in mice. Infect. Immun. 63:1394–1399.

31. Lee, A., J. O’Rourke, M.C. De Ungria, B. Roberson, G. Dakakopoulos, and M.F. Dixon. 1997. A standardized mouse model of Helicobacter pylori infection: introducing the Sydney strain. Gastroenterology. 112:1386–1397.

32. Veldhuyzen van Zanten, S.J.O., and A. Lee. 1997. The gastric transitional zones: the neglected link in Helicobacter pylori infection. Gut. 41(Suppl 1):A23–A24. (Abstr.)

33. Stolte, M., O. Stadlermann, B. Bethke, and G. Burkard. 1995. R relationships between the degree of Helicobacter pylori colonization and the degree of activity of gastritis, surface epithelial degeneration and mucus secretion. Z. Gastroenterol. 33:89–93.

34. Tokunaga, Y., H. Shirahase, E. Yamanoto, Y. Gouda, K. Kanaji, and K. Ohsumi. 1998. Semiquantitative evaluation for diagnosis of Helicobacter pylori infection in relation to histological changes. Amer. J. Gastroenterol. 93:26–29.

35. Atherton, J.C., K.T. Tham, R.M. Peek, Jr., T.L. Cover, and M.J. Blaser. 1996. Density of Helicobacter pylori infection in vivo as assessed by quantitative culture and histology. J. Infect. Dis. 174:552–556.

36. Ladell, C.H., I.E. Flesch, J. Arnoldi, and S.H. Kaufmann. 1994. Studies with MHC–deficient knock-out mice reveal impact of both MHC I– and MHC II–dependent T cell responses on Listeria monocytogenes infection. J. Immunol. 153:3116–3122.

37. Morrissey, R.P., K. Feilzer, and D.B. Tumas. 1995. Gene knockout mice establish a primary protective role for major histocompatibility complex class II–restricted responses in Chlamydia trachomatis genital tract infection. Infect. Immun. 63:4661–4668.

38. Hernandez, H.J., Y. Wang, N. Tzellas, and M.J. Stadecker. 1997. Expression of class II, but not class I, major histocompatibility complex molecules is required for granuloma formation in infection with Schistosoma mansoni. Eur. J. Immunol. 27:1170–1176.

39. Correa, I., M. Bix, N.S. Liao, M. Zijlstra, R. Jaenisch, and D.
Raulet. 1992. Most γδ T cells develop normally in β2-microglobulin-deficient mice. Proc. Natl. Acad. Sci. USA. 89:653-657.

40. von Boehmer, H., W. Swat, and P. Kisielow. 1993. Positive selection of immature αβ T cells. Immunol. Rev. 135:67-79.

41. Buzoni-Gatel, D., A.C. Lepage, I.H. Dimier-Poisson, D.T. Bout, and L.H. Kasper. 1997. Adoptive transfer of gut intraepithelial lymphocytes protects against murine infection with Toxoplasma gondii. J. Immunol. 158:5883-5889.

42. Nedrud, J., T. Blanchard, S. Czinn, and G. Harriman. 1996. Orally immunized IgA deficient mice are protected against H. felis infection. Gut. 39(Suppl 2):A45. (Abstr.)

43. Su, H., K. Feilzer, H.D. Caldwell, and K.P. Morrison. 1997. Chlamydia trachomatis genital tract infection of antibody-deficient gene knockout mice. Infect. Immun. 65:1993-1999.

44. Thomsen, A.R., J. Johansen, O. Marker, and J.P. Christensen. 1996. Exhaustion of CTL memory and recrudescence of viremia in lymphocytic choriomeningitis virus-infected MHC class II-deficient mice and B cell-deficient mice. J. Immunol. 157:3074-3080.

45. Langhorne, J., C. Cross, E. Seixas, C. Li, and T. von der Weid. 1998. A role for B cells in the development of T cell helper function in a malaria infection in mice. Proc. Natl. Acad. Sci. USA. 95:1730-1734.