Local Secretory Immunoglobulin A and Postimmunization Gastritis Correlate with Protection against *Helicobacter pylori* Infection after Oral Vaccination of Mice

TAKAYUKI GOTO, AKIRA NISHIZONO, TOSHIO FUJIYOKA, JUNKO IKEWAKI, KUMATO MIFUNE, AND MASARU NASU

Second Department of Internal Medicine, Department of Infectious Disease Control, and Department of Microbiology, Oita Medical University, Hasama-machi, Oita, Japan

Received 19 August 1998/Returned for modification 10 November 1998/Accepted 20 January 1999

C57BL/6 mice were orally immunized with five weekly doses of 2 mg, 200 μg, or 2 μg of *Helicobacter pylori* (Sydney strain) whole-cell sonicate combined with cholera toxin. One week after the last vaccination, mice were challenged with 5 × 10⁷ CFU of live *H. pylori* three times at 2-day intervals. At 6 or 18 weeks after the challenge, mice were sacrificed and bacterial cultures and histological studies of the stomach were performed. Vaccination with 2 μg/session or 200 μg/session inhibited *H. pylori* colonization by 90 and 100%, respectively. These mice were considered protected. Lower levels of *H. pylori*-specific immunoglobulin A (IgA) were detected in fecal and saliva samples before challenge. However, a significant increase in IgA secretion in mucosal tissue and a higher labeling index for IgA-positive lumina of pyloric glands were noted in these mice in response to challenge and in a vaccine dose-dependent manner. In protected mice, however, severe gastritis characterized by marked infiltration of inflammation mononuclear cells was noted at 6 weeks after challenge, compared with the gastritis seen in unprotected mice or nonvaccinated, ordinarily infected mice. Marked expression of gamma interferon mRNA was detected in the stomach of all protected mice, and 50% of these mice expressed interleukin 4 (IL-4) or IL-5 mRNA. Our findings suggest that local secretory IgA antibody and severe postimmunization gastritis correlate well with protection of mice against *H. pylori* infection.

**MATERIALS AND METHODS**

**Animals.** Specific-pathogen-free 6-week-old female C57BL/6 mice were obtained from Seac Yoshitomi (Fukuoka, Japan). Mice were housed in a specific-pathogen-free environment and were provided with free access to food and water. Experiments were performed according to the guidelines of the Ethical Committee for Animal Experiments at Oita Medical University, Oita, Japan.

**Bacterial strain and preparation of *H. pylori* antigen.** The Sydney strain of *H. pylori*, which was kindly provided by A. Lee (School of Microbiology and Immunology, University of New South Wales, Sydney, New South Wales, Australia), (9), was grown in brucella broth containing 10% horse serum under microaerobic conditions (5% O₂, 10% CO₂, 85% N₂) at 37°C. *H. pylori* sonicated antigen was prepared as described previously (16). Briefly, cultures were centrifuged at 1,000 × g for 10 min. The pellet was washed in phosphate-buffered saline (PBS), and cells were disrupted by sonication. After centrifugation at 1,000 × g for 10 min, the supernatant was collected, the protein concentration was determined, and the supernatant was frozen at −80°C until use.

**Vaccination and challenge of mice.** Mice were divided into four groups: groups 1 to 3, oral vaccination with cholera toxin (5 μg) as an adjuvant and *H. pylori* whole-cell sonicate at a dose of 2 mg (group 1, n = 20), 200 μg (group 2, n = 20), or 2 μg (group 3, n = 20), respectively (vaccinated/challenged groups); and group 4 (n = 20), ordinary infection without vaccination (nonvaccinated/infect group). Vaccination was repeated at weekly intervals for 5 weeks with the same doses of *H. pylori* as those listed above. One week after the last vaccination, blood, fecal, and saliva samples were collected to monitor the immune response and were stored at −80°C until use. One week after the last vaccination, all mice were challenged with 0.5 ml of live *H. pylori* (5 × 10⁷ CFU/ml) three times at 2-day intervals.

**Assessment of *H. pylori* in gastric tissue.** At 6 or 18 weeks after the last challenge, all mice were sacrificed and the stomach was isolated for examination for *H. pylori*, histological examination, and determination of cytokine expression by reverse transcription (RT)-PCR. The stomach was washed in sterile 0.8% NaCl and cut longitudinally into two pieces. One half was used for bacterial examination, while the other was used for histological examination and determination of cytokine expression.
Examination for *H. pylori.* Immediately after homogenization of the stomach specimens, they were smeared on 7% sheep blood agar (basic medium, Mueller-Hinton agar; BBL Microbiology Systems, Cockeysville, Md.) and Belo-Horizonte medium and incubated at 37°C for 4 days under microaerobic conditions. The presence of *H. pylori* in gastric tissue sections was also examined after Gram staining.

**Histological examination of gastric mucosa.** Longitudinal sections of gastric tissues from the esophageal-cardiac junction to the duodenum were fixed with neutral buffered 10% formalin and embedded in paraffin. Five-micrometer sections were stained with hematoxylin-eosin (HE) and Giemsa stains. Gastric sections were examined in a blinded fashion by two independent examiners, who provided an assessment of the overall grade of inflammation (on a scale of 0 to 7), which was expressed as a sum of the overall intensity and extent of inflammation. The intensity of inflammation was scored on a scale of 0 to 3 based on criteria modified slightly from those described by Mohammadi et al. (13): grade 0, rare inflammatory cells; grade 1, mild; grade 2, moderate; and grade 3, severe. The extent of inflammation was scored on a scale of 0 to 4 based on the percentage of inflammatory cell infiltration of the mucosal surface (13): grade 0, none; grade 1, <25%; grade 2, 25 to 50%; grade 3, 50 to 75%; and grade 4, >75%.

**Immunohistochemical staining with secretory IgA antibody in gastric tissue.** For immunohistochemical studies, 5-μm-thick paraffin sections were preincubated with normal goat serum diluted 1:9 for 10 min at room temperature. Sections were then stained with rabbit anti-mouse immunoglobulin A (IgA) antibody (Zymed Laboratories Inc., South San Francisco, Calif.) for 20 min at room temperature, followed by the avidin-biotin complex staining method (5). Sections were reacted with 0.05 mol of Tris-HCl buffer per liter containing 0.02% 3,3′-diaminobenzidine tetrahydrochloride (Wako Pure Chemicals, Osaka, Japan) and 0.005% H2O2, and the nuclei were counterstained with hematoxylin. Control sections incubated with normal rabbit IgG instead of the primary antibody did not show nonspecific staining. The labeling index (LI) for IgA-positive lumina of pyloric glands was determined by examination of 500 glands in sections of pyloric mucosa, and the percentage of lumina labeled with anti-mouse IgA antibody was used for analysis.

**Determination of antibody levels against *H. pylori* antigens in serum, feces, and saliva.** Blood samples were obtained from the tail vein at 7 days after the last immunization or by cardiac puncture at 6 weeks after the last challenge. Secretory IgA antibody in stool specimens was extracted from fecal pellets by incubation of the samples with PBS containing 0.02% 3,3′-diaminobenzidine tetrahydrochloride (Wako Pure Chemicals, Osaka, Japan) and 0.005% H2O2, and the nuclei were counterstained with hematoxylin. Control sections incubated with normal rabbit IgG instead of the primary antibody did not show nonspecific staining. The labeling index (LI) for IgA-positive lumina of pyloric glands was determined by examination of 500 glands in sections of pyloric mucosa, and the percentage of lumina labeled with anti-mouse IgA antibody was used for analysis.

**Determination of antibody levels against *H. pylori* antigens in serum, feces, and saliva.** Blood samples were obtained from the tail vein at 7 days after the last immunization or by cardiac puncture at 6 weeks after the last challenge. Secretory IgA antibody in stool specimens was extracted from fecal pellets by incubation of the samples with PBS containing 5% nonfat dry milk to yield approximately a 7% emulsion, 1 μg of aprotinin per ml, and 10 μM leupeptin (Wako) (16). After extensive vortexing, the fecal material was centrifuged at 13,000 × g for 10 min, and the supernatants were used for the determination of IgA antibody. Saliva samples were collected with a micropipette after intraperitoneal injection of 200 μg of pilocarpine (Sigma) in sterile PBS.

**Determination of IgG and IgA antibodies in blood, feces, and saliva samples** was performed by an enzyme-linked immunosorbent assay (ELISA). Sera were tested for IgG at a dilution of 1:100 and for IgG1 and IgG2a at a dilution of 1:25. Saliva and fecal samples were tested for IgA at a dilution of 1:4 and undiluted.
For semiquantitative determination of the expression of cytokine mRNA, we respectively. Microtiter plates (Nunc, Roskilde, Denmark) were first coated with 100 µl of antigen (H. pylori whole recombinated antigen 1 µg/well) in carbonate buffer (pH 9.6) for 1 h at 37°C. They were then incubated with PBS containing 5% bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo.) for 1 h at 37°C to block nonspecific binding and washed with PBS-Tween 20. Wells containing 100 µl of each test sample were incubated for 1 h at 37°C. After being washed, the wells were incubated with 100 µl of peroxidase-conjugated goat anti-mouse IgG, goat-anti mouse IgG2a, or goat-anti mouse IgA antibodies (Cappel, Malvern, Pa.) for 1 h at 37°C. After the wells were washed, conjugated peroxidase was detected with 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) and hydrogen peroxide. The absorbance at 414 nm was measured after 30 min of incubation at room temperature. Each sample was tested in duplicate.

FIG. 2. Effect of oral vaccination with 5 µg of cholera toxin and 2 mg (group 1), 200 µg (group 2), or 2 µg (group 3) of H. pylori whole-cell sonicate on H. pylori infection. Protection against H. pylori infection was based on the absence of H. pylori, determined by both bacterial culturing and light microscopic examination of stomach sections (Giemsa stain) at 6 weeks after the last challenge (n, 10 mice). An asterisk indicates that the P value was <0.001 compared with the value for the corresponding nonvaccinated/injected mice (Fisher's exact test).

There were no significant differences among the samples with regard to the expression of β-actin mRNA.

Statistical analysis. Differences in H. pylori-specific IgG and IgA antibody levels, grade of gastric inflammation, and LI for IgA-positive lumina of pyloric glands among experimental groups were examined for statistical significance by analysis of variance or Student's t test. Differences in the rate of protection against H. pylori infection were analyzed by Fisher's exact probability test. A P value of <0.05 was considered statistically significant.

RESULTS

H. pylori-specific antibody levels in feces, saliva, and sera obtained from orally vaccinated mice. Blood, fecal, and saliva samples were collected, and the antibody responses against H. pylori antigens were examined by an ELISA 1 week after the last vaccination. Although the antibody levels were low and the biologic significance was undefined, increases in fecal and saliva IgA antibody levels were observed in vaccinated (group 1 and 2) (Fig. 1A) mice and in group 1 mice (Fig. 1B), respectively, compared with the nonvaccinated controls (P <0.05).

In the vaccinated groups, the levels of serum H. pylori-specific IgA and IgG antibodies were elevated in proportion to the doses of H. pylori whole-cell sonicate (Fig. 1C and D).

Effect of vaccination against H. pylori infection. The effect of vaccination was based upon protection against live H. pylori challenge infection. Protection was judged by the absence of H. pylori, determined by both culturing for H. pylori and light microscopic examination of gastric sections stained with Giemsa stain at 6 and 18 weeks after challenge. In nonvaccinated/injected mice, H. pylori had colonized well at 6 and 18 weeks after the challenge. The percentages of protection were 90 and 100% in vaccinated/challenged groups 1 and 2, respectively, at 6 weeks after the challenge. In contrast, in 8 of 10 mice in group 3, H. pylori had colonized in the stomach, indicating that these mice were not protected. The difference in the levels of H. pylori colonization between vaccinated/challenged mice (groups 1 and 2) and nonvaccinated/injected mice was statistically significant (P <0.001) (Fig. 2).

Michetti et al. (12) reported that oral vaccination occasionally induces gastritis without H. pylori challenge. Therefore, we examined whether our oral vaccination alone induced gastric inflammation. Inflammatory cell infiltration was not observed in the stomachs of all vaccinated mice at 6 weeks after the last vaccination (data not shown). In nonvaccinated/injected mice, however, mild inflammation was present in the antral region at 6 weeks after infection (Fig. 3A). In gastric tissue sections of these mice, H. pylori was found in the antral region (Fig. 3B). The cell infiltrates consisted of diffuse aggregates of numerous neutrophils, plasma cells, and a few mononuclear cells. These histopathological inflammatory features were compatible with active chronic gastritis.

In vaccinated/challenged mice (groups 1 and 2) the grade of gastritis was more severe than that in nonvaccinated/injected mice; marked infiltration of mononuclear cells was noted, mainly in the corpus of the stomach (Fig. 3C). In unprotected mice the gastritis score at 6 weeks after the challenge was significantly lower than that at 18 weeks after the challenge in nonvaccinated/injected mice (Fig. 4). On the other hand, the gastritis score at 6 weeks after the challenge in protected mice was significantly higher than that in unprotected mice (P <0.0005). In addition, gastritis observed in protected mice lasted for 18 weeks, and the severity at 6 weeks after the challenge had reached levels almost equal to those observed at 18 weeks for unprotected or nonvaccinated/injected mice.

Immunohistopathological examination of gastric tissue. To examine whether secretory IgA is induced and actively secreted in gastric mucosa following oral vaccination, immuno-
The different natures of oral immunogens with regard to immunogenicity or cross-reactivity against host proteins, differences in mouse strains used, and the strain diversity of Helicobacter species might be the factors responsible for the differences in the extent of postimmunization gastritis. The same can be said of the discrepant finding that gastric inflammation was not observed in all vaccinated mice without H. pylori challenge in the present study, in contrast to the occasional induction of gastritis by vaccination only demonstrated by Michetti et al. (12).

Measurement of mRNAs for a group of cytokines in the stomach in the present study showed that IFN-γ mRNA was significantly higher in protected than in unprotected mice. In nonvaccinated/infected mice the level of IFN-γ mRNA was lower than 10^2 copies and was lower than that in vaccinated/challenged mice (Fig. 7). The level of IFN-γ mRNA in vaccinated mice without challenge was undetectable at 6 weeks after the last vaccination (data not shown). On the other hand, IL-4 mRNA was detected in 3 of 10 protected mice and IL-5 mRNA was detected in another 2 of 10 protected mice. In other words, the levels were lower than 10^5 copies. The same mRNAs were not detected in stomach homogenates of unprotected mice and nonvaccinated/infected mice (Fig. 7).

**DISCUSSION**

Several investigators have recently suggested that oral vaccination with either *H. pylori* whole-cell antigen or purified antigen and appropriate adjuvants can prevent infection by *Helicobacter* species (1, 8, 10, 12, 13, 16). In the present study, we showed that oral vaccination with a dose of greater than 200 µg of *H. pylori* whole-cell sonicate per session for 5 weeks was effective in protecting C57BL/6 mice against *H. pylori* infection.

We first noted a severe inflammatory cell infiltration in the corpus at 6 weeks after the challenge, especially in protected mice. We also demonstrated that the development of more severe inflammation at this stage might lead to protection. A number of investigators (1, 13, 16) have reported that gastric inflammation occurs in immunized mice after challenge; this effect has been referred to as postimmunization gastritis. However, the details of postimmunization gastritis differ among studies. For example, Pappo et al. (16) showed that oral immunization with recombinant urease and cholera toxin resulted in protection against *H. felis* infection and that the gastritis in recombinant urease-immunized mice subsequently challenged with *H. felis* exhibited Thy1.2^+^ T cells in mucosal and submucosal layers. On the other hand, Ferrero et al. (1) demonstrated that orogastric immunization of mice with recombinant *H. pylori* GroES-like and urease B-subunit proteins protected against *H. felis* challenge and that mild gastric inflammation may be a necessary prerequisite for successful immunization. In that study, the severity of postimmunization gastritis was milder than that of ordinary *H. felis* infection. Mohammadi et al. (13) showed that the grade of postimmunization gastritis was more severe than that of ordinary *H. felis* infection when *H. felis* whole-cell sonicate was used as the oral immunogen, similar to our findings. These results suggest that postimmunization gastritis might correlate with protection against *Helicobacter* challenge. However, the extent of postimmunization gastritis is different in each experimental design. The different natures of oral immunogens with regard to immunogenicity or cross-reactivity against host proteins, differences in mouse strains used, and the strain diversity of *Helicobacter* species might be the factors responsible for the differences in the extent of postimmunization gastritis.

**FIG. 4.** Gastritis scores in vaccinated/challenged and nonvaccinated/infected mice at 6 (open bars) and 18 (filled bars) weeks (W) after the challenge. HE-stained gastric sections were scored based on the overall grade of inflammation (on a scale of 0 to 7), which included the intensity and extent of inflammation (see Materials and Methods). Data represent the mean ± standard deviation (n = 5 to 10 mice). The asterisk indicates that the P value was <0.0005 compared with the value for the corresponding nonvaccinated/infected mice at 6 weeks after the challenge (Student's t-test).
strongly expressed in protected mice. In contrast, the expression was weaker (less than $10^3$ copies) in unprotected mice as well as in nonvaccinated/infected mice. We also showed that the expression of the mRNA for either IL-4 or IL-5 was detected in 50% of protected mice. These findings suggest that postimmunization gastritis represents a reactive inflammation of the gastric mucosa induced primarily by Th1 immune responses together with secondary Th2 responses induced by the challenge. In support of the involvement of Th1 immune responses or IFN-γ in postimmunization gastritis, Mohammadi and coworkers (13) showed that treatment of nonimmunized/infected and immunized/challenged mice with anti-IFN-γ antibody led to a significant reduction in the severity of gastric inflammation.

With regard to mucosal immunity, it is clear that local IgA antibody plays a primary role in protection against foreign
organisms. However, there are no reports directly indicating that gastric tissue functions as an effector organ of mucosal immunity, similar to gut-associated lymphoid tissue. In the present study, we observed protection against *H. pylori* in vaccinated mice (mainly groups 1 and 2), in which salivary and fecal *H. pylori*-specific IgA was detected. In these mice, high levels of IgA were also secreted in the pyloric mucosa, and these levels were vaccine dose dependent (Fig. 5). However, this IgA is not necessarily specific for *H. pylori*, since it has been demonstrated that IFN-γ and IL-4 up-regulate the expression of the polymeric immunoglobulin receptor, which transports IgA in human intestinal epithelium (18). In support of the involvement of Th2 cell-mediated immune responses in the stomach, we demonstrated the expression of mRNAs for IL-4 and IL-5 in the stomachs of mice protected against challenge infection (Fig. 7). To our knowledge, this is the first report of mRNAs for these cytokines in situ, although a previous study demonstrated the secretion of these cytokines in the supernatants of lymphocyte cultures from the stomach after antigenic stimulation (13). Taken together, these findings suggest that local secretory IgA antibody in gastric mucosal tissue correlates well with protection against *H. pylori* infection. However, whether the stomach functions as a primary priming site for inducing the protective IgA response remains to be elucidated in future studies.

Cellular immune responses to *H. pylori* have been studied for humans, but the nature of such responses, including the possible involvement of protective immunity, is not well defined. In the present study, we demonstrated that Th1 cell-mediated immune responses occur in the stomach, as indicated by the marked expression of IFN-γ mRNA. However, the mechanism(s) through which a challenge infection of vacci-
nated mice induces the strong expression of IFN-γ is still not clear, particularly in protected mice. The expression of IFN-γ might be associated with the appearance of postimmunization gastritis (13). Furthermore, the development of severe postimmunization gastritis during the early postchallenge infection period is probably an essential prerequisite for protection, as demonstrated in the present study and previous reports (1, 13). However, the mechanism of protection after the appearance of postimmunization gastritis remains unidentified.

Further studies are necessary to clarify these issues and identify the antigen(s) that induces protective immunity following oral vaccination, with the hope of preventing postimmunization gastritis.

ACKNOWLEDGMENTS

We thank Adrian Lee for donating the Sydney strain and for careful reading of the manuscript and Kiyomi Ohno and Mami Kimoto for technical assistance. We also thank F. G. Issa (Word-Medex, Sydney, Australia) for careful reading and editing of the manuscript.

REFERENCES

15. Otsuka, T., D. Villaret, and T. Yokota. 1987. Structural analysis of the mouse chromosomal gene encoding interleukin 4 which expresses B cell, T cell and...
mast cell stimulating activities. Nucleic Acids Res. 15:333–344.