

Immunization with Recombinant *Helicobacter pylori* Urease in Specific-Pathogen-Free Rhesus Monkeys (*Macaca mulatta*)

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Immunization with urease can protect mice from challenge with *Helicobacter pylori*, though results vary depending on the particular vaccine, challenge strain, and method of evaluation. Unlike mice, rhesus monkeys are naturally colonized with *H. pylori* and so may provide a better estimate of vaccine efficacy in humans. The purpose of this study was to examine the effectiveness of *H. pylori* urease as a vaccine in specific-pathogen (*H. pylori*)-free rhesus monkeys. Monkeys raised from birth and documented to be free of *H. pylori* were vaccinated with orogastric ($n = 4$) or intramuscular ($n = 5$) urease. Two control monkeys were sham vaccinated. All monkeys were challenged with a rhesus monkey-derived strain of *H. pylori*, and the effects of vaccination were evaluated by use of quantitative cultures of gastric tissue, histology, and measurement of serum immunoglobulin G (IgG) and salivary IgA. Despite a humoral immune response, all monkeys were infected after *H. pylori* challenge, and there were no differences in the density of colonization. Immunization with urease therefore does not fully protect against challenge with *H. pylori*. An effective vaccine to prevent *H. pylori* infection will require different or more likely additional antigens, as well as improvements in the stimulation of the host immune response.

Helicobacter pylori infects the gastric epithelium of approximately half of the world's population and causes a histologic gastritis (9). An estimated 20% of those infected will go on to develop either peptic ulcer or gastric adenocarcinoma, which is the second-most-common cause of cancer mortality worldwide. Therapy of *H. pylori* requires the prolonged administration of several agents, and even under the best of circumstances it fails in 10 to 15% of cases. The growing development of antibiotic resistance further complicates the future of *H. pylori* management (16).

Immunization is an alternative strategy that may be useful as primary prevention and perhaps also as therapy for *H. pylori* infection. The early encouraging results obtained in the *H. felis* mouse model (4, 12, 13, 22, 27, 29) were confirmed by subsequent studies which showed that mice can be protected from *H. pylori* challenge by immunization with a variety of whole-cell or recombinant antigens (3, 14, 15, 26, 31). Most studies have utilized oral administration of recombinant urease, which has been shown to be effective as therapy and as primary prevention. Protection typically ranges from ca. 60 to 100%, depending on the particular adjuvant and challenge strain. More recently, parenteral administration of urease, together with a synthetic glycolipid adjuvant, has also been shown to be effective for primary prevention of *H. pylori* (18).

These promising results obtained in the mouse model have been only partially supported by recent studies in the rhesus monkey. Captive rhesus monkeys are naturally colonized with *H. pylori* that is indistinguishable from that found in humans (5–7, 33) and therefore may represent a more appropriate test of vaccine efficacy than the mouse, which is not a natural host for *H. felis* or *H. pylori*. Three studies have examined the

efficacy of urease vaccine in rhesus monkeys. Lee et al. showed that immunization of naturally infected rhesus monkeys with *H. pylori* urease plus *Escherichia coli* enterotoxin (LT) was not effective therapy for *H. pylori* and prevented infection in only a small percentage of naturally colonized animals that were challenged following antibiotic treatment (24). However, a reduced level of colonization was seen in vaccinated monkeys compared to sham controls. A subsequent study with antibiotic-treated monkeys found that all vaccinated animals as well as controls were infected following challenge, but again the colonization level was reduced in monkeys that received oral vaccine followed by a parenteral boost (23). These studies, however, differ from the anticipated use of a vaccine in humans because the animals were not immunologically naive to *H. pylori*, and the effects of prior infection on vaccination are unknown. In a study of natural and presumed primary acquisition of *H. pylori* in very young animals, 69% of monkeys immunized with oral urease and LT developed infection, compared with 93% of controls (8). However, the results are difficult to interpret because the initial absence of infection was documented only by serology, which is not sensitive for the detection of newly acquired infection. Unlike in the challenge experiments, the vaccine produced no reduction in bacterial colonization among the animals that became infected.

We have developed a protocol for derivation of specific-pathogen (*H. pylori*-free (SPF) rhesus monkeys (33). These monkeys may provide an attractive model for evaluation of vaccine efficacy because they are well characterized and have no prior immune response to *H. pylori*. The purpose of this experiment was to examine the effect of oral and parenteral urease vaccination on challenge with *H. pylori* in SPF rhesus monkeys.

MATERIALS AND METHODS

Animals. Eleven newborn rhesus monkeys were taken from their dam within 24 h of birth and pair housed in temperature- and humidity-controlled incubators in the nursery. At approximately 1.5 years of age monkeys were examined for *H. pylori* by serology, [¹⁴C]urea breath test, and histology and culture of gastric

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biopsies by using methods previously described (33). All 11 animals were free of *H. pylori* and "*H. heilmannii*," the uncultivated gastric spiral organism that commonly colonizes monkeys and other animals (6, 28, 33, 34). The [¹⁴C]urea breath test and endoscopy were repeated at approximately 2 years of age, and all 11 animals remained uninfected.

Animals were individually housed during their participation in these experiments. Ketamine (10 mg/kg of body weight) was administered intramuscularly as sedation for all procedures, which were performed following an overnight fast. Euthanasia was performed on ketamine-sedated monkeys by intravenous administration of pentobarbital (50 mg/kg of body weight). Experiments were conducted according to the *Guide for the Care and Use of Laboratory Animals* and were approved by the Research Advisory Committee of the California Regional Primate Research Center.

Vaccine and adjuvant preparation. Vaccines and adjuvants were provided by OraVax (Cambridge, Mass.). Catalytically inactive but fully assembled urease apoenzyme was purified from *E. coli* containing either plasmid pORV214 (oral vaccine) or pORV273 (parenteral vaccine) by methods described previously (25). pORV273 is a proprietary construct identical to pORV214 except that the urease binding sites are substituted, and it was used to prepare parenteral vaccine for production rather than scientific reasons. There are no detectable antigenic differences between urease made from these two constructs. Urease for parenteral administration was purified by passing it through a Sartobind matrix (Sartorius Corp., Edgewood, N.Y.) in order to reduce contamination by *E. coli* endotoxin to 1.5 ng of urease or less per mg. Urease was lyophilized in 2% sucrose and stored at -20°C prior to use.

For oral immunization, 4 mg of recombinant *H. pylori* urease and 100 µg of *E. coli* LT (Berna Products Corp., Coral Gables, Fla.) were mixed and diluted to a final volume of 5 ml of lactated Ringer's solution. For parenteral immunization, 100 µg of low-endotoxin urease was mixed immediately prior to injection with 600 µg of glycolipid adjuvant (BAY R 1005; Bayer AG, Leverkusen, Germany) in lactated Ringer's solution to a final volume of 0.5 ml. The dose of oral vaccine was based on a body weight-adjusted dose found to be effective in mice (25), while the parenteral dose was chosen as the maximum likely to be used in humans.

Immunization. Monkeys were given oral urease plus LT adjuvant ($n = 4$), parenteral low-endotoxin urease plus Bay adjuvant ($n = 5$), or sham immunization with oral lactated Ringer's solution ($n = 2$). Animals to receive oral vaccine were first given 2.5 ml of 8% NaHCO₃ per os to neutralize gastric acidity immediately prior to immunization. Vaccine was then administered by gradually dripping the solution into the oral cavity to ensure that mucosal surfaces were exposed prior to swallowing, followed by 3 ml of phosphate-buffered saline to rinse the mouth. Parenteral vaccine was administered by intramuscular injection of half the volume (0.25 ml) into the deltoid muscle and the remaining half (0.25 ml) into the lateral thigh muscle. Each animal received four doses of vaccine or sham immunization, which were given at weeks 0, 3, 6, and 12.

Experimental challenge with *H. pylori*. Two weeks after completion of vaccination (week 14), the immunized and sham control animals were orogastrically inoculated with *H. pylori* N246C₃, a rhesus monkey-derived strain previously shown to effectively colonize rhesus monkeys (24). Low-passage stocks were subcultured once on brucella agar with 5% newborn calf serum (Gibco-BRL, Gaithersburg, Md.) supplemented with TVPA (trimethoprim, 5 mg/liter; vancomycin, 10 mg/liter; polymixin B, 2.5 IU/liter; amphoterin B, 4 mg/liter; all were from Sigma) and incubated at 37°C in 5% CO₂. The subculture was then used to inoculate brucella broth (Difco Laboratories, Detroit, Mich.) with 5% newborn calf serum and TVPA, which was incubated without shaking at 37°C in 5% CO₂ until the optical density at 600 nm (OD₆₀₀) was approximately 0.2 (ca. 15 h). The culture was centrifuged and resuspended in brucella broth at 2×10^8 CFU/3 ml. Prior to each inoculation, the culture was examined by Gram stain, wet mount, and rapid urease assay with urea-indole medium (21). The inoculum was quantitated by plating serial dilutions. All animals were pretreated with cimetidine (10 mg/kg of body weight) 1 h prior to challenge to neutralize the gastric acid and then given 2×10^8 CFU in 3 ml delivered by orogastric tube. The challenge was repeated every other day for a total of three doses.

Endoscopy and necropsy. Four weeks after *H. pylori* challenge (week 18) all monkeys underwent endoscopy. Mucosal biopsies were obtained from the gastric antrum and body for quantitative culture (three from each site) and for histology (one from each site). Eleven weeks after *H. pylori* challenge (week 25), vaccinated and sham control monkeys were sacrificed and the stomach was opened aseptically along the greater and lesser curvature. One half of the stomach was examined by quantitative culture using 10 0.5-cm-diameter punch biopsies distributed over the gastric cardia ($n = 1$), body ($n = 2$), transition zone ($n = 3$), antrum ($n = 3$), and pylorus ($n = 1$). The remaining mucosa was collected by gently scraping it with a sterile microscope slide. The other half of the stomach was examined histologically using 10 1.0-cm² tissue samples distributed over the regions of the stomach as described above.

Quantitative culture of gastric tissue. Gastric tissue was placed in preweighed vials containing 0.9% sterile saline, weighed again, and transported immediately to the laboratory. The tissue was homogenized with a sterile ground-glass pestle and 100 µl of neat, 1:10, 1:100 and 1:1,000 dilutions were inoculated onto brucella agar containing 5% bovine calf serum (Gibco-BRL) and TVPA. The plates were incubated in an atmosphere of 5% CO₂ for up to 10 days. *H. pylori* was identified in the conventional manner by colony morphology (pinhead-sized

translucent colonies), microscopy (gram-negative curved organisms), and biochemistry (oxidase, catalase, and urease positive). The CFU per gram of gastric mucosa was calculated by enumerating colonies, adjusting for the dilution, and dividing by the tissue weight.

Histology. Tissues were formalin fixed, paraffin embedded, and processed with hematoxylin and eosin and Warthin-Starry stains. Gastritis was determined on blinded samples by assessment of acute (polymorphonuclear leukocytes) and chronic (mononuclear cells) inflammation on a scale of 0 (none), 1 (mild), 2 (moderate), or 3 (severe) as described in the Sydney system (30).

Urease-specific *H. pylori* antibodies. Blood was collected by femoral venipuncture at week 0 (prior to vaccination), week 14 (challenge), and at weeks 18 (endoscopy) and 25 (necropsy). Sera were separated by centrifugation and stored at -70°C. Saliva was collected at weeks 0, 14, and 18 by placing two cellulose wicks (Polyfiltron, Rockland, Maine) between the lower lip and gum for 5 min or more. Wicks saturated with saliva were stored at -70°C. At the time of assay, 0.5 ml of phosphate-buffered saline containing protease inhibitors (0.2 µM amin-oethyl-benzene sulfonyl fluoride, 1 µg of aprotinin per ml, and 10 µM leupeptin) was added, and saliva was recovered by centrifugation.

Urease-specific antibodies were detected by enzyme-linked immunosorbent assay with native *H. pylori* recombinant urease as the antigen. Serum (100 µl) was added in serial dilutions (beginning at 1:100) to flat-bottom 96-well plates coated with 0.5 µg of purified *H. pylori* urease blocked with nonfat milk. Saliva (100 µl) was treated similarly but at a 1:5 dilution only. Goat anti-rhesus immunoglobulin G-horse radish peroxidase (IgG-HRP) conjugate or anti-rhesus IgA-HRP conjugate (Nordic Immunological Laboratories, Capistrano Beach, Calif.) were used as secondary antibodies. After incubation of plates with substrate (ABTS [*N*-(2-acetamido)-2-aminomethanesulfonic acid] plus H₂O₂ in citrate phosphate buffer) for 20 min at room temperature, the absorbance at 550 nm was determined. Results for salivary IgA were expressed as OD₅₅₀; results for serum IgG were expressed as the reciprocal of the lowest titer to exceed an arbitrary threshold of 0.10 absorbance unit. All samples were run in duplicate.

Molecular fingerprinting of *H. pylori*. Restriction fragment length polymorphism (RFLP) analysis was used to confirm that *H. pylori* recovered from vaccinated monkeys was identical to the inoculated strain and different from strains that are enzootic at the primate center. Chromosomal *H. pylori* DNA was prepared from bacteria grown on brucella agar with 5% bovine serum using a method previously described (2). DNA (1 µg) was digested with an excess of *Hae*III (New England BioLabs, Beverly, Mass.) for 3 h at 37°C and electrophoresed in a 0.7% agarose gel (Gibco-BRL) with 1× Tris-borate buffer at 55 V for 16 h. The gel was stained with ethidium bromide and photographed under UV light.

RESULTS

Antibody response to immunization. Immunization with oral and especially parenteral urease produced a marked rise in urease-specific serum IgG, which was apparent at week 14 when monkeys were challenged with *H. pylori* (Fig. 1). Serum IgG remained elevated at weeks 18 and 25, when monkeys underwent endoscopy and necropsy, respectively, though titers declined somewhat in monkeys that received parenteral vaccine. Immunization produced only modest elevations in urease-specific salivary IgA. As expected, control monkeys showed no immune response to sham immunization.

Protection from *H. pylori* challenge. *H. pylori* was cultivated from gastric tissue in all control and vaccinated monkeys, both at endoscopy 4 weeks after challenge (week 18) and at necropsy 11 weeks after challenge (week 25). In order to determine if there were quantitative differences in *H. pylori* colonization among the three groups, the mean log₁₀ CFU per gram of gastric tissue was calculated for each monkey. Data were averaged over the six endoscopic biopsies obtained 4 weeks after challenge (Fig. 2A) and over the 11 samples (10 punch biopsies and the gastric scraping) obtained at necropsy 11 weeks after challenge (Fig. 2B). There was considerable variability in bacterial density, which in most animals ranged between 10² and 10⁶ CFU/g of tissue. No quantitative differences in *H. pylori* colony counts were apparent between the control and either vaccination group.

In order to confirm that the recovered *H. pylori* was strain N246C₃ and not a strain enzootic at the primate center, we examined by RFLP a strain isolated from each of the 11 experimental animals and three representative strains isolated from naturally infected monkeys at the primate center. The fingerprint of *H. pylori* recovered from each monkey matched

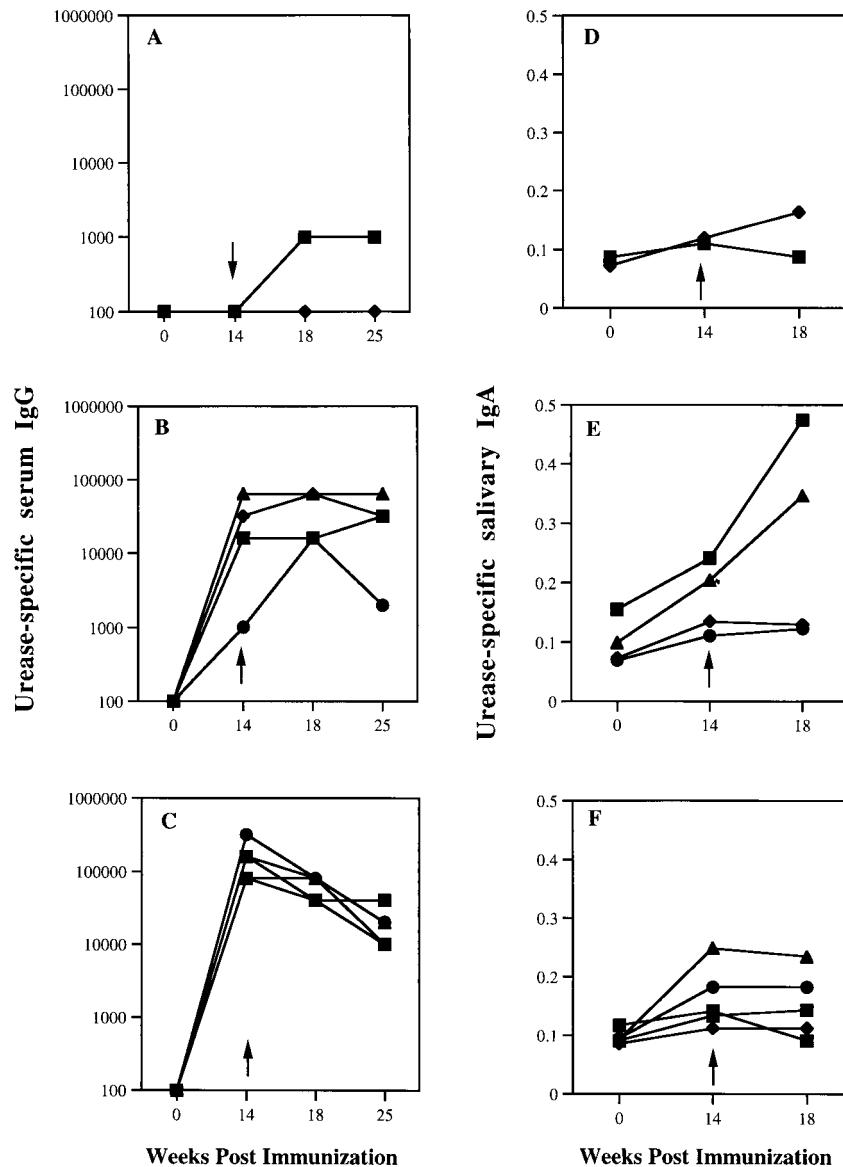


FIG. 1. Urease-specific serum IgG (A, B, and C) and salivary IgA (D, E, and F) for monkeys which received sham immunization (A and D), oral immunization with urease plus *E. coli* LT (B and E), or intramuscular urease plus Bay (C and F). Results are shown for samples obtained before immunization (0 week), 2 weeks following immunization (14 weeks), just prior to challenge (arrow), and at 4 and 11 weeks (IgG only) after challenge (18 and 25 weeks, respectively). Serum IgG is expressed as the reciprocal of the lowest titer to exceed a threshold of 0.10 absorbance unit. Salivary IgA is expressed as A_{550} .

that of strain N246C₃ and was easily distinguished from representative strains found in naturally infected monkeys (Fig. 3).

Topography of *H. pylori* infection. In order to examine topographical differences in *H. pylori* density, we calculated the log₁₀ CFU per gram of tissue obtained at necropsy from each region of the stomach (Fig. 4). Data for the cardia and pylorus were based on a single sample, while the means of multiple samples were calculated for the body (2), transitional zone or junction (3), and antrum (3). Since animals in all groups were infected and there were no apparent topographical differences among groups, the means were computed for all 11 monkeys combined. *H. pylori* density was consistently lowest in the cardia and pylorus and was most often greatest in the junction (transitional zone).

Histopathology. Gastritis was evaluated before immunization and at 4 and 11 weeks after *H. pylori* challenge. At 4 weeks after *H. pylori* challenge (week 18), all monkeys developed an

increase in polymorphonuclear leukocytes that tended to increase at 11 weeks (week 25) after challenge (Fig. 5). Similar results were seen in other regions of the stomach and for chronic (mononuclear) inflammation (results not shown). "*H. heilmannii*" was not seen in any monkeys prior to vaccination but was found in two animals at 4 weeks after challenge (one oral and one parenteral immunization) and in six animals at 11 weeks after challenge (one control, two oral, and three parenteral immunizations).

DISCUSSION

Development of the rhesus monkey model of *H. pylori* provides an opportunity to study vaccine efficacy in a naturally infected host that is closely related to humans. Using procedures we developed to derive SPF rhesus monkeys, we examined for the first time the effectiveness of an *H. pylori* vaccine

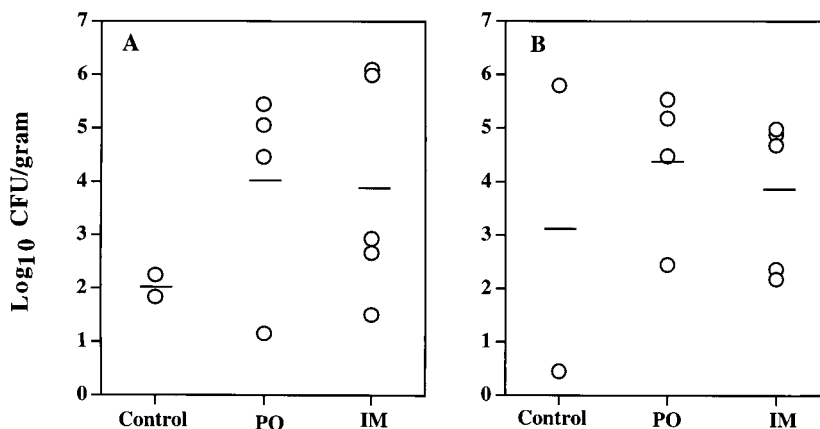


FIG. 2. Mean \log_{10} CFU/gram of tissue obtained at endoscopy 4 weeks after challenge (A) and at necropsy 11 weeks after challenge (B) with *H. pylori*. Results for each animal are averaged for six samples at endoscopy and for 11 samples at necropsy. Means for animals that received sham vaccination (control), oral urease (PO), and parenteral urease vaccination (IM) are shown as bars.

in monkeys that were documented to have no prior *H. pylori* infection. The results showed that immunization with recombinant urease, formulated with *E. coli* LT for oral delivery or a synthetic glycolipid for intramuscular delivery, does not prevent infection after experimental challenge with rhesus monkey-derived *H. pylori*. These results are consistent with two previous studies in rhesus monkeys which showed that immunization with four different urease and adjuvant preparations, including oral urease plus LT (24) and intramuscular urease plus Bay (23), prevented infection in only 1 of 19 animals challenged with *H. pylori*.

Although previous studies in rhesus monkeys did not demonstrate complete protection, they suggested that oral urease-LT alone or followed by a urease parenteral boost could

produce 1- to 2-log reductions in *H. pylori* colony counts compared with sham-vaccinated controls (23, 24). Recent studies in gnotobiotic piglets (10) and in mice (20) have also found that when careful quantitative cultures are performed, the effect of vaccination may be quantitative rather than complete protection. We failed to find quantitative differences in *H. pylori* colonization between vaccinated and control animals, perhaps because of small sample size and variability in quantitative cultures. However, in unpublished studies we found that in five treated monkeys experimentally infected with *H. pylori* N246C₃, the mean (range) \log_{10} CFU/g at 2 and 10 weeks postinoculation (similar time intervals to those in the present study) were 4.3 (0 to 6.1) and 3.6 (0 to 7.5), respectively (one of five animals was uninfected). These results are similar to those we found in vaccinated animals (Fig. 2). Others have also failed to demonstrate quantitative differences in natural *H. pylori* colonization of rhesus monkeys after urease vaccination (8). Future studies will be required to determine whether immunization can consistently reduce *H. pylori* density and whether such a reduction is clinically important in the reduc-

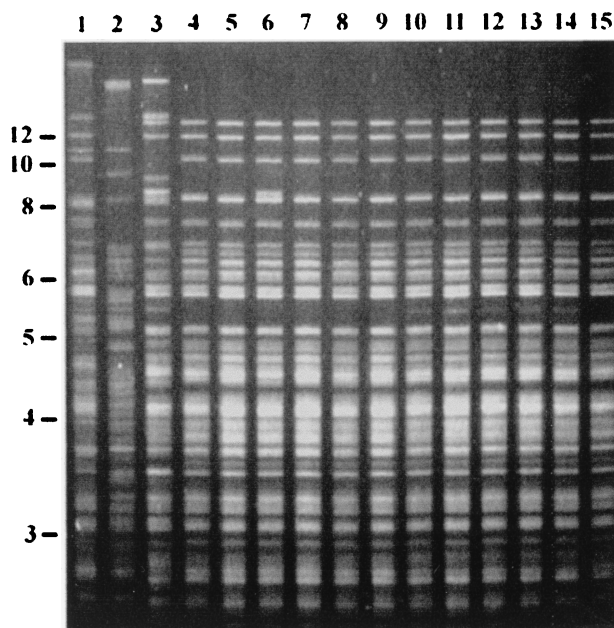


FIG. 3. RFLP analysis of *H. pylori* DNA digested with *Hae*III. Samples are from strains isolated from naturally infected monkeys at the primate center (lanes 1 to 3), strain N246C₃ used as the inoculum in the present experiment (lane 4), and strains isolated from each of the 11 experimental animals (lanes 5 to 15). A size ladder in kilobases is shown at left.

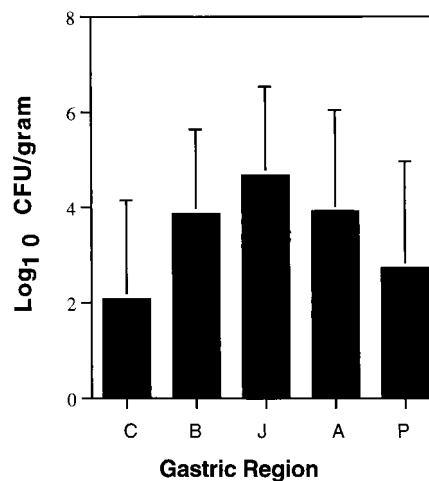


FIG. 4. Mean *H. pylori* \log_{10} CFU/gram of gastric tissue in the cardia (C), body (B), junction (J), antrum (A), and pylorus (P) averaged over all 11 monkeys. The standard deviation is indicated above each bar.

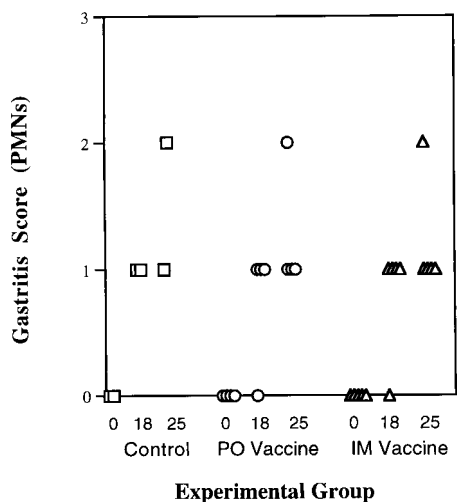


FIG. 5. Gastritis score for acute (polymorphonuclear leukocytes) inflammation on a scale of 0 (none), 1 (mild), 2 (moderate), or 3 (severe) as described in the Sydney System (30). Data are shown for each animal in the control, oral vaccine (PO), and parenteral vaccine (IM) groups before immunization (0 week), 4 weeks after challenge (18 weeks), and 11 weeks after challenge (25 weeks).

tion of clinical endpoints such as peptic ulcer disease and gastric cancer. Studies on the latter point have produced mixed results (1, 17, 19). However, since the clinical sequelae of *H. pylori* infection are presumed to result from chronic inflammation, a quantitative reduction in *H. pylori* density might be expected to be clinically significant.

A recent study examined the effects of oral vaccination with urease plus LT on naturally acquired *H. pylori* infection in 9-month-old rhesus monkeys (8). The results showed that 69% of vaccinated animals were *H. pylori* infected 10 months after vaccination compared to 93% of controls. Strengths of this study were that the follow-up was prolonged and that natural infection may provide a more realistic estimate of vaccine efficacy than experimental challenge with a large inoculum. But the absence of *H. pylori* prior to vaccination was determined only by serology, which may be falsely negative during the period of rapid seroconversion which occurs in young rhesus monkeys during the first few years of life (7, 33). Therefore, the authors used seroprevalence data to estimate the actual prevalence of infection among vaccinated and control animals at the time of vaccination and then used these data to calculate that the vaccine was 69% effective. However, since the animals did not undergo endoscopy prior to immunization, the actual effect of the vaccine could not be determined.

Infection with *H. pylori* developed in all vaccinated animals despite a significant induction of humoral immunity. Immunization induced a marked increase in urease-specific serum IgG, particularly when administered parenterally, but only a modest increase in salivary IgA (Fig. 1). Similar results have been reported previously in rhesus monkeys vaccinated with *H. pylori* urease (8, 24). These results indicate that although the vaccine preparations were immunogenic, they were not sufficient to elicit protection. Recent results in B-cell knockout mice suggest that in the mouse model, protection from *H. pylori* challenge can occur in the absence of an antibody response (11).

Histologic examination showed that colonization with *H. pylori* in all monkeys was often greatest at the junction or the transitional zone between the gastric antrum and body and least at the cardia and pylorus (Fig. 4). Similar results have

been obtained in rhesus monkeys (24) and in mice (11). This finding emphasizes the recent proposal that the transitional zone is a unique environment in the stomach where local acid production may determine whether *H. pylori* is restricted distally to the antrum, where it is more likely to cause duodenal ulcer, or moves proximally into the body where it may lead to atrophy, metaplasia, and adenocarcinoma (32). It was surprising that "*H. heilmannii*" was observed in some monkeys following *H. pylori* challenge, despite the fact that two prior endoscopic examinations failed to show it. Since "*H. heilmannii*" was seen much more frequently at necropsy than at endoscopy, this may mean that low-grade infections were present in some animals despite the fact that they were isolated within 24 h of birth.

In conclusion, we have examined the effect of immunization with oral and parenteral urease against challenge with *H. pylori* in SPF rhesus monkeys. The results show that although the vaccine is immunogenic, it does not prevent infection. While we did not observe a reduction in colonization density in vaccinated animals compared to controls, the possibility that vaccination produces a reduction in the level of colonization cannot be excluded due to the small sample size. An effective vaccine to prevent *H. pylori* infection will probably require different or more likely additional antigens, as well as improvements in stimulation of the host immune response.

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