

Safety and Immunogenicity of Oral Inactivated Whole-Cell *Helicobacter pylori* Vaccine with Adjuvant among Volunteers with or without Subclinical Infection

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Helicobacter pylori infection of the gastric mucosa can be found in approximately 50% of the world's population and is associated with a range of pathology, including peptic ulcer, atrophic gastritis, and gastric cancer. To explore immunization as a strategy for preventing and treating *H. pylori*-associated disease, we assessed the safety and immunogenicity in healthy adults of a formalin-inactivated, oral *H. pylori* whole-cell (HWC) vaccine, administered with or without mutant *Escherichia coli* heat-labile toxin (LT_{R192G}) as a mucosal adjuvant. In a dose-response study, 23 subjects with or without *H. pylori* infection were vaccinated with either 2.5×10^6 HWC, 2.5×10^8 HWC, or 2.5×10^{10} HWC, plus 25 μ g of LT_{R192G}. Thereafter, a randomized study was conducted in which 18 *H. pylori*-infected subjects were assigned, in a double-blind fashion, to receive either 2.5×10^{10} HWC plus placebo-adjuvant, placebo-vaccine plus 25 μ g of LT_{R192G}, placebo-vaccine plus placebo-adjuvant, or 2.5×10^{10} HWC plus 25 μ g of LT_{R192G}. Diarrhea (six subjects), low-grade fever (five subjects), and vomiting (two subjects) were observed, usually after the first dose. Significant rises in geometric mean mucosal (fecal and salivary) anti-HWC immunoglobulin A antibodies occurred among *H. pylori*-infected and uninfected subjects following inoculation with 2.5×10^{10} HWC plus 25 μ g of LT_{R192G}. Moreover, among *H. pylori*-negative volunteers, this regimen induced significant lymphoproliferative responses in 5 of 10 subjects and gamma interferon production responses to *H. pylori* sonicate in 7 of 10 subjects. There was no evidence that vaccination eradicated *H. pylori* in infected volunteers. These results suggest that it is possible to stimulate mucosal and systemic immune responses in humans to *H. pylori* antigens by using an HWC vaccine.

Helicobacter pylori infects nearly half of the world's population, resulting in chronic active gastritis, which persists throughout life unless the organism is eradicated (16, 22). Although most individuals experience no symptoms, 10 to 20% develop peptic ulcer disease (25, 55). Furthermore, chronic *H. pylori* infection confers a 3- to 12-fold increased risk of developing gastric cancers such as adenocarcinoma and low-grade B-cell lymphoma (6, 23, 28, 41, 51, 52).

Randomized, placebo-controlled trials have demonstrated that eradication of *H. pylori* infection from the stomach with antimicrobial therapy heals chronic gastritis and peptic ulcers, prevents ulcers from recurring (25, 31, 47, 55) and may lead to regression of gastric lymphoma (50, 65). However, there are impediments to identifying a simple, inexpensive, safe, and effective treatment, including the high cost and side effects associated with standard multidrug regimens (57), the appearance of antibiotic-resistant *H. pylori* strains (3), and the measurable risk of reinfection following antibiotic-induced

eradication (36, 56). For these reasons, the use of vaccines for treatment and prevention of *H. pylori* infection has been explored.

Preclinical studies have identified a number of promising *Helicobacter* antigens, including urease (20, 44, 48), VacA (45), CagA (46), heat shock protein (64), neutrophil-activating protein (59), and outer membrane lipoprotein (34). Mucosal administration of inactivated *Helicobacter* whole-cell (HWC) preparations is another approach that has been extensively explored. A series of independent experiments in animal models has demonstrated that mucosal vaccination with whole-cell preparations of *H. pylori* confers protection against challenge with wild-type *H. pylori* or *H. felis* organisms (11, 21, 44–46; M. Chen, A. Lee, and S. Hazell, Letter, *Lancet* **339**:1120–1121, 1992). Coadministration of a mucosal adjuvant, such as cholera toxin (CT) (11; Chen et al., Letter), CT B subunit (42), the heat-labile enterotoxin (LT) of *Escherichia coli* (45), and mutant LT K63 (46), has been essential to elicit these protective responses. HWC vaccination has also been explored as a therapeutic strategy (24, 32). For example, administration of either *H. felis* or *H. pylori* sonicate plus CT eradicated *H. felis* infection in mice; 94% of the animals remained cured of their infection for 3 months after vaccination, as detected by histology and local urease activity (15).

Despite the growing body of preclinical data, there have been few clinical trials to determine whether *Helicobacter* vac-

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TABLE 1. Open-label, dose-response study design^a

| HWC vaccine inoculum | LT _{R192G} dose (μg) | <i>H. pylori</i> status | No. of subjects |
|------------------------|-------------------------------|-------------------------|-----------------|
| 2.5 × 10 ⁶ | 25 | Uninfected | 3 |
| 2.5 × 10 ⁶ | 25 | Infected | 3 |
| 2.5 × 10 ⁸ | 25 | Uninfected | 4 |
| 2.5 × 10 ⁸ | 25 | Infected | 3 |
| 2.5 × 10 ¹⁰ | 25 | Uninfected | 10 |

^a For the purpose of characterizing the dose response, the eight *H. pylori*-infected subjects who received 2.5 × 10¹⁰ inactivated HWC vaccine plus 25 μg of LT_{R192G} adjuvant as part of the randomized safety and immunogenicity study (Table 2) are also included in the analysis of this portion of the study.

cines can achieve similar success in humans, and these have thus far involved either recombinant urease (rUrease) or urease expressed by *Salmonella* spp. (1, 14, 49; T. Buclin, M. Cosma, I. Corthesy-Theulaz, and P. Michetti, *Lancet* 347:1630–1631, 1996). We report here the clinical acceptability and immunogenicity of formalin-inactivated HWC vaccine administered to healthy adults with or without natural subclinical *H. pylori* infection and the effect of coadministered mucosal adjuvant on these responses.

MATERIALS AND METHODS

Vaccine. The formalin-inactivated HWC vaccine used in this study (lot 0290, under commercial development by a subsidiary of Antex Biologics, Inc.) was derived from a frozen stock of a clinical strain (ATCC 55713) that was originally isolated from a human duodenal ulcer biopsy. The parent strain, designated G1-4, is highly motile and expresses CagA, VacA, urease, and catalase. In addition, G1-4 binds to asialo-GM₁ (39) but not to other gangliosides (GB₄, GD₁-B, and GM₃) (40). The vaccine was prepared at the Walter Reed Army Institute of Research (WRAIR) Forest Glen Annex Facility using Good Manufacturing Practice. In brief, G1-4 was grown to a concentration of 5 × 10⁸ bacterial cells per ml in 320 liters of brain heart infusion broth supplemented with bovine calf serum. At the time of harvest, the culture medium was centrifuged, and the bacteria were resuspended in phosphate-buffered saline (PBS), to which formalin was added to a concentration of 0.025 M for 18 h at room temperature. Inactivated cells were then separated by centrifugation and suspended in sterile PBS to achieve a final optical density at 625 nm (OD₆₂₅) of 30 ± 2. Vaccine was packaged in 20-dose (20 ml) vials each containing 2.5 × 10¹⁰ to 5.0 × 10¹⁰ bacterial cells and 0.1 mg of sodium thimerisol per ml of PBS (formaldehyde content, <0.01 M) and stored at 4°C. When it was necessary to administer an inoculum of either 2.5 × 10⁸ or 2.5 × 10⁶, the vaccine was diluted with PBS immediately prior to use.

The formalin-inactivated cells contain lipopolysaccharide (as shown by *Limulus* lysate assay), do not produce urease, but nonetheless induce antibodies to urease, catalase, and flagellin in mice as shown by enzyme-linked immunosorbent assay (ELISA) and retain the overlying binding characteristics of live cells. The vaccine strain also induces mouse antibodies by Western blot to immunodominant proteins in the 30 to 60-kDa range from homologous and heterologous *H. pylori* strains.

Adjuvant. The adjuvant is a modified form of the heat-labile enterotoxin of *E. coli*, designated LT_{R192G}, having a glycine residue substituted for the arginine at position 192 from the amino terminus of the A1 subunit of the molecule (12). Removal of this arginine residue renders the molecule trypsin-insensitive, thereby interfering with its activation to an enterotoxic form. LT_{R192G} was produced to specifications by the Swiss Serum and Vaccine Institute, Berne, Switzerland. Each 1-mg portion was lyophilized in 3.29 mg of Tris, 0.146 mg of EDTA, and 5.84 mg of NaCl per ml and 5% (wt/vol) lactose and then stored at 4°C until use. The adjuvant (lot mHT419) was rehydrated with 1 ml of sterile water. The dose administered was 25 μl (25 μg).

Placebo for vaccine and adjuvant. The placebo for the vaccine (designated placebo-vaccine) and for the adjuvant (designated placebo-adjuvant) consisted of sterile buffer mixed with powdered skimmed milk to match the turbidity of the vaccine and adjuvant formulations.

Subjects. Healthy volunteers 18 to 55 years of age were recruited from the Baltimore-Washington metropolitan area. They were determined to be in good health on the basis of medical history, physical examination, and a battery of

clinical laboratory tests. Prospective volunteers were excluded if they gave a history of major gastrointestinal surgery or illness, current gastrointestinal symptoms such as dyspepsia requiring daily therapy, regular use of aspirin or nonsteroidal anti-inflammatory drugs, allergy to a study medication, or receipt of a vaccine or investigational drug during the 30 days prior to enrollment. Women received a serum pregnancy test before each vaccination to ensure that pregnant women were not vaccinated. Volunteers completed a written examination to test their comprehension of the purpose, procedures, and risks of the trial and were required to answer at least 70% of the questions correctly in order to participate. All enrolled subjects provided informed, written consent according to the guidelines of the University of Maryland, Baltimore, Institutional Review Board.

Screening for *H. pylori* infection. A two-stage process was used to determine whether prospective volunteers were infected with *H. pylori*. First, subjects were screened for the presence of serum antibody to *H. pylori* using a commercial ELISA manufactured by BioWhittaker, Inc. (Walkersville, Md.) in the dose-response study and by Wampole Laboratories, Dist., Carter-Wallace, Inc. [Cranbury, N.J.] in the randomized safety and immunogenicity study. Next, a ¹³C urea breath test (¹³C UBT; Meretek, Inc., Houston, Tex.) was used to confirm the presence or absence of active infection. Volunteers who were positive by both the ELISA and breath test were considered *H. pylori*-infected and those negative by both assays were considered uninfected. Seropositive subjects who had negative breath tests were excluded from participation.

Study design. (i) Dose-response study among *H. pylori*-infected and uninfected subjects. An initial dose-response study was conducted among 23 volunteers to determine whether increasing inocula of HWC, coadministered with 25 μg of LT_{R192G}, were well tolerated and to evaluate whether increasing HWC inocula enhanced the immune response. It was anticipated that the optimal dose would contain 2.5 × 10¹⁰ HWC plus 25 μg of LT_{R192G}. Groups of 3 to 10 *H. pylori*-infected or *H. pylori*-uninfected subjects were assigned in an unblinded fashion to receive three oral doses of vaccine on days 0, 14, and 28 at an inoculum of either 2.5 × 10⁶, 2.5 × 10⁸, or 2.5 × 10¹⁰ HWC plus 25 μg of LT_{R192G} (Table 1). Safety was established at each dose level before a new group of volunteers received a higher inoculum of vaccine. For the purpose of characterizing the dose response, the eight *H. pylori*-infected subjects who received 2.5 × 10¹⁰ HWC plus 25 μg of LT_{R192G} as part of the randomized safety and immunogenicity study, described below (Table 2), are also included in this analysis.

(ii) Randomized safety and immunogenicity study among *H. pylori*-infected subjects. After determining that subjects tolerated the target inoculum of 2.5 × 10¹⁰ HWC plus 25 μg of LT_{R192G}, we conducted a randomized study among *H. pylori*-infected subjects to investigate in a preliminary fashion the safety and immunogenicity of the oral HWC vaccine administered with or without mucosal adjuvant. Twenty *H. pylori*-infected subjects were randomly assigned, in a double-blind, placebo-controlled fashion, to receive, on days 0, 14, and 28, either 2.5 × 10¹⁰ HWC plus placebo-adjuvant, placebo-vaccine plus 25 μg of LT_{R192G}, placebo-vaccine plus placebo-adjuvant, or 2.5 × 10¹⁰ HWC plus 25 μg of LT_{R192G} (Table 2). Two subjects were withdrawn after a single inoculation because of scheduling conflicts (one subject had received HWC with placebo-adjuvant, and the other subject had received placebo-vaccine plus LT_{R192G}), leaving 18 analyzable subjects.

(iii) Inoculation. Volunteers fasted for 90 min before and after inoculation. Immediately before inoculation a buffer solution was prepared by dissolving 2 g of NaHCO₃ in 150 ml of sterile water. Volunteers drank 120 ml of buffer solution followed 1 min later by the test inoculum suspended in the remaining 30 ml of buffer solution.

(iv) Clinical evaluation. Volunteers were observed at the study site for at least 1 h before and after inoculation to ensure that fasting was maintained and to monitor for immediate reactions. For 7 days following each inoculation, volunteers completed a standardized diary form to assess their clinical response. They recorded their evening oral temperature, the presence of symptoms (epigastric

TABLE 2. Randomized, double-blind, placebo-controlled safety and immunogenicity study design among subjects with subclinical *H. pylori* infection

| No. of subjects | Randomized assignment | |
|-----------------|----------------------------|---------------------|
| | Vaccine | Adjuvant |
| 2 | 2.5 × 10 ¹⁰ HWC | Placebo-adjuvant |
| 3 | Placebo-vaccine | LT _{R192G} |
| 5 | Placebo-vaccine | Placebo-adjuvant |
| 8 | 2.5 × 10 ¹⁰ HWC | LT _{R192G} |

pain, heartburn, malaise, nausea, or bloating), vomiting, and the consistency (loose or formed) and presence of gross blood in each stool passed. Symptoms were graded as follows: 0, absent; 1, mild (hardly noticed); 2, moderate (bothersome, but continued the same activities); and 3, severe (interrupted activities or sleep). Fever was defined as an oral temperature of 100°F or higher, and diarrhea was defined as three or more loose stools within a 24-h period.

H. pylori-uninfected subjects underwent a repeat ¹³C UBT on day 56 to exclude the possibility of interim *H. pylori* infection. Infected subjects in the dose-response study completed follow-up breath tests on day 56, and those in the randomized study completed follow-up breath tests on days 56, 180, and 210 to determine whether *H. pylori* had been eradicated following vaccination.

Immunology. (i) Serum antibody. Blood was collected on days 0, 14, 28, 35, 56, and 180 to measure immunoglobulin A (IgA) and IgG responses to LT_{R129G} and HWC by ELISA; published assays were used for LT_{R129G} and adapted for use for the HWC antigen (62). Briefly, microtiter plates were coated in rows alternating with specific antigen (LT_{R129G} at 1 µg/ml or HWC at 1:1,000 in PBS [pH 7.2]) or with PBS alone, as the negative control. A single 1:100 dilution of each test serum sample was used for the IgG LT assay; twofold dilutions of serum were used for all other antigen and isotype assays starting at 1:100 for the IgA LT and IgG HWC assays and 1:25 for IgA HWC assay. Endpoints for the titers were determined using an *H. pylori*-seronegative population in standardization studies; 0.1 for IgA LT and HWC and 0.3 for IgG HWC.

(ii) Mucosal antibody. Samples of stool (prepared as a 10% stool supernatant) and saliva (after a 1-h fast) were collected for measurement of total and antigen-specific IgA antibody production on days 0, 7, 14, 21, 28, 56, and 180. Protease inhibitors were added, and the samples were stored at -70°C until testing.

Total IgA was measured using a quantitative ELISA (limit of detection, 6 ng/ml) according to previously described methods (62). Specific IgA antibody responses to HWC and LT_{R129G} were determined, as described above, on samples which contained at least 2 mg of total IgA per ml (using twofold dilutions of the test sample); IgA levels were determined by extrapolating to the linear part of the quantitative ELISA curve, which was run in parallel with each ELISA and then standardizing the value to 20 mg of total IgA per ml.

(iii) ASCs. As a measure of intestinal priming induced by inoculation, circulating specific antibody-secreting cells (ASCs) were quantified on days 0, 7, 10, 14, 21, 28, and 35 using ELISPOT, as previously described (64). Microdilution plates were coated with either 1 µg of LT_{R129G} per ml or 1:000 dilution of HWC (WRAIR, BPR 144-01).

(iv) CMI. Cell-mediated immunity (CMI) responses were assessed in subjects who received 2.5×10^{10} HWC plus 25 µg of LT_{R129G} using peripheral blood mononuclear cells (PBMC) isolated before and 56 days after the first inoculation and frozen in liquid nitrogen until use, as previously described (61).

(a) Lymphoproliferative responses. Lymphoproliferative responses to *H. pylori* sonicate and purified recombinant catalase (a putative immunoprotective antigen (54) expressed by the vaccine) were measured. *H. pylori* sonicate was prepared from an overnight broth culture, which was pelleted by centrifugation, washed, resuspended in deionized water, disrupted by sonication, and then stored at -20°C. To derive recombinant catalase, the Fe²⁺ catalase gene from *H. pylori* ATCC 49503 was PCR cloned into plasmid pKK223-3 (Pharmacia Biotech, Inc., Piscataway, N.J.) as a -1.1-kbp *Eco*RI fragment and transformed into *E. coli* JM105. Derivatives of JM105 expressing high levels of catalase were identified by examining crude whole-cell lysates on sodium dodecyl sulfate (SDS)-gels for the presence of a novel ~55-kDa IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible protein. Soluble catalase was purified by DEAE-Sepharose chromatography to ~90% homogeneity as previously described (53). Purity was assessed on SDS-gels, and the material was tested for activity using H₂O₂.

Phytohemagglutinin (HA-17; Wellcome Diagnostics, Beckenham, United Kingdom) and tetanus toxoid (Wyeth, Marietta, Pa.) were used to confirm the ability of PBMC to proliferate to mitogenic stimulation and antigenic stimulation, respectively (61). Antigens were added in the following final concentrations: *H. pylori* sonicate, 0.2, 2, and 20 µg/ml; recombinant catalase, 0.2, 2, and 20 µg/ml; bovine serum albumin (BSA), 20 µg/ml; tetanus toxoid, 2 µg/ml; and phytohemagglutinin, 2 µg/ml. Net counts per minute (cpm) represented the difference in ³H-labeled thymidine incorporation by antigen-exposed versus unstimulated cells, for that same day and subject. A positive response was defined as a difference ($P < 0.05$, two-tailed *t* test) in mean net cpm between triplicate pre- and postvaccination samples stimulated with each individual antigen (i.e., *H. pylori* sonicate, recombinant catalase or BSA).

(b) IFN-γ and IL-5 production. Cytokines were measured by chemiluminescence ELISA (the limits of detection were 4 pg/ml for gamma interferon [IFN-γ] and 2 pg/ml for interleukin-5 [IL-5]) as previously described (53, 66). Net cytokine production levels (in picograms/milliliter) were calculated by subtracting cytokine levels in negative control wells (no antigen exposure) from the levels in

test sample wells for that same day and subject. A positive IFN-γ or IL-5 response was defined as a difference ($P < 0.05$, two-tailed *t* test) in mean chemiluminescence units between duplicate pre- and postvaccination samples stimulated with each individual antigen (i.e., *H. pylori* sonicate, recombinant catalase, or BSA).

Statistical analysis. Serum and local immunoglobulin titers were converted to natural logarithms prior to analysis. ASC counts were converted to logarithms after coding (one was added to remove values of zero). Standard errors presented in this report represent the back-transformed standard error of the log-transformed observations.

In the dose-response study, pre-versus peak postvaccination geometric means were compared using Wilcoxon's signed-rank test. Significant differences in mean lymphoproliferative and cytokine responses pre-versus postvaccination were detected with the paired Student's *t* test.

In the randomized safety and immunogenicity study, two immunologic comparisons were of interest: (i) responses to HWC antigen following administration of HWC plus placebo-adjuvant versus placebo-vaccine plus placebo-adjuvant to assess the immunogenicity of the vaccine when given without mucosal adjuvant and (ii) responses to HWC antigen following administration of HWC plus LT_{R129G} versus HWC plus placebo-adjuvant to determine whether addition of mucosal adjuvant enhances the immune response to the HWC vaccine. Geometric mean values (peak postvaccination) were compared between the randomized vaccine groups using analysis of covariance, adjusting for prevaccination values.

Two-tailed hypotheses were evaluated throughout, with statistical significance determined at the 5% level. Corrections for multiple comparisons were not made.

RESULTS

Clinical tolerance. The clinical response to vaccination among the 41 subjects who participated in the trial is shown in Table 3. Six subjects experienced diarrhea (three of whom had baseline *H. pylori* infection); one subject received placebo-vaccine plus LT_{R129G}, and the remaining five received 2.5×10^{10} HWC plus LT_{R129G} (Table 3). Thus, diarrhea was seen only among subjects who received LT_{R129G} (with or without vaccine) and only following the highest (2.5×10^{10}) HWC dose. Diarrhea followed the first inoculation in all but one subject. The episodes lasted for 1 to 3 days, during which time these subjects passed a total of 3 to 17 loose stools. Five subjects met the definition of fever (including one who received only placebo) but experienced only a single temperature elevation of 100 to 101°F 2 to 5 days after the first inoculation (Table 3). Two recipients of 2.5×10^{10} HWC plus LT_{R129G} vomited once after the first inoculation; one also had diarrhea, and the other also had a fever.

One or more gastrointestinal complaints (nausea, anorexia, malaise, heartburn, stomachache, and abdominal pain) was reported by subjects in all study groups (Table 3). There was no apparent effect of *H. pylori* infection status on the occurrence of these symptoms following vaccination. Affected subjects rated their symptoms as mild or moderate in severity with the exception of two recipients of 2.5×10^{10} HWC vaccine plus LT_{R129G} who experienced abdominal pain that made them sufficiently uncomfortable to alter their normal activity. Two *H. pylori*-infected subjects observed blood streaks in a formed stool; one subject had received 2.5×10^{10} HWC plus LT_{R129G}, and the other had received placebo-adjuvant plus placebo-vaccine.

Effect of vaccination on *H. pylori* infection, as measured by ¹³C UBT. *H. pylori*-infected subjects had repeat ¹³C UBT after vaccination. In the dose-response study, all six subjects remained positive when the test was repeated 2 months after vaccination. In the randomized study, the ¹³C UBT was repeated 2, 6, and 7.5 months after vaccination, and 17 of 18 remained

TABLE 3. Clinical tolerance of oral inactivated whole-cell vaccine plus adjuvant by study group

| Symptom | No. of subjects | | | | | | | | |
|-----------------------|--|------------------------------------|--------------------------------------|------------------------------------|------------------------------------|---------------------------------|---------------------|-----------------|-------------------------------------|
| | <i>H. pylori</i> uninfected (unblinded dose response at:) | | | <i>H. pylori</i> infected | | | | | |
| | | | | Unblinded dose response at: | | Randomized assignment at: | | | |
| | 10 ⁶ , 25 μg (n = 3) ^a | 10 ⁸ , 25 μg (n = 4) | 10 ¹⁰ , 25 μg (n = 10) | 10 ⁶ , 25 μg (n = 3) | 10 ⁸ , 25 μg (n = 3) | 10 ¹⁰ , 0 (n = 2) | 0, 25 μg (n = 3) | 0, 0 (n = 5) | 10 ¹⁰ , 25 μg (n = 8) |
| Nausea | 1 | 2 | 2 | 1 | 0 | 1 | 1 | 2 | 3 |
| Vomiting | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| Anorexia | 1 | 2 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| Malaise | 2 | 0 | 3 | 1 | 0 | 1 | 1 | 2 | 3 |
| Heartburn | 1 | 2 | 1 | 2 | 1 | 0 | 1 | 1 | 2 |
| Stomachache | 1 | 1 | 3 | 2 | 0 | 0 | 1 | 3 | 3 |
| Abdominal pain | 0 | 1 | 4 | 3 | 0 | 0 | 1 | 2 | 2 |
| Fever of >100°F | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 |
| Diarrhea ^b | 0 | 0 | 3 | 0 | 0 | 0 | 1 | 0 | 2 |
| Blood in stool | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |

^a Each study design is presented as follows: HWC dose, LT_{R192G} dose (n = number of subjects). The actual doses of HWC were 2.5 × 10⁶, 2.5 × 10⁸, or 2.5 × 10¹⁰.

^b Defined as three or more loose or watery stools within a 24-h period.

positive. One recipient of placebo-vaccine plus LT_{R192G} reverted to negative at 6 months; she had received a 1-week course of metronidazole to treat an upper respiratory infection approximately 1 month earlier. All 17 *H. pylori*-uninfected subjects had negative ¹³C UBT results when the test was repeated 2 months after vaccination.

Dose-dependent immune responses to HWC vaccine plus LT_{R192G} among *H. pylori*-infected and uninfected subjects. (i) **Anti-HWC responses.** Immunization elicited rises in the geometric mean serum and mucosal anti-HWC antibodies only among subjects who received the highest (2.5 × 10¹⁰ HWC) vaccine dose (Fig. 1). Whereas postvaccination increases in geometric mean peak serum IgA and IgG titers were marginal ($P = 0.06$) and were seen only among *H. pylori*-infected subjects, the fecal and salivary IgA responses were statistically significant and occurred in both *H. pylori*-infected and uninfected volunteers. Anti-HWC ASC responses were meager (none exceeded 10 cells per 10⁶ PBMC) and so were not subjected to statistical analysis (Fig. 1).

(ii) **Anti-LT_{R192G} responses.** As shown in Fig. 2, rises in serum and mucosal anti-LT_{R192G} antibodies were observed following vaccination. Interestingly, postvaccination anti-LT_{R192G} levels appeared to rise as the dose of HWC vaccine increased, despite a constant dose of adjuvant. Statistically significant anti-LT_{R192G} antibody increases occurred only in the groups (both *H. pylori*-infected and uninfected) receiving the highest (2.5 × 10¹⁰ HWC) vaccine dose, for serum IgG (but not IgA), fecal IgA, and salivary IgA. In contrast to the minimal ASC responses to HWC, nearly half of the anti-LT_{R192G} ASC responses exceeded 100 cells per 10⁶ PBMC. Significant increases in the geometric mean number of LT_{R192G}-specific IgA- and IgG-producing ASCs were observed following vaccination among *H. pylori*-infected and uninfected subjects who received 2.5 × 10¹⁰ HWC (Fig. 2).

CMI responses among *H. pylori*-infected subjects to immunization with 2.5 × 10¹⁰ HWC plus LT_{R192G}. (i) **Lymphoproliferative responses.** Immunization with 2.5 × 10¹⁰ HWC plus LT_{R192G} resulted in increased, albeit statistically insignificant ($P = 0.22$) mean group proliferative responses to the *H. pylori* sonicate among *H. pylori* uninfected volunteers (Fig. 3A). Sig-

nificant rises in proliferative responses to 2 μg of *H. pylori* sonicate per ml were observed in 5 of the 10 volunteers evaluated, while no significant increases in proliferative responses were observed when PBMC were incubated with either recombinant catalase or BSA (Fig. 3A). In contrast, no significant increases in mean lymphoproliferative responses to the *H. pylori* sonicate were observed following immunization of *H. pylori*-infected volunteers (Fig. 3B).

(ii) **Production of IFN-γ and IL-5.** Immunization with 2.5 × 10¹⁰ HWC plus LT_{R192G} resulted in significant ($P < 0.05$) increases among *H. pylori* uninfected volunteers in mean group IFN-γ production to the *H. pylori* sonicate at 2 μg/ml (Fig. 4A); significant rises were observed in 7 of the 10 volunteers studied. Postvaccination responses, albeit not statistically significant, were also observed when cultures contained 0.2 and 20 μg of the *H. pylori* sonicate per ml (data not shown). No significant increases in mean IFN-γ production were observed when PBMC were incubated with either recombinant catalase or BSA (Fig. 4A). In contrast, significant increases in mean IFN-γ production to the *H. pylori* sonicate were not observed following the immunization of *H. pylori*-infected volunteers (Fig. 4B). Undetectable or minimal levels of IL-5 were observed in culture supernatants from PBMC obtained before and after immunization of *H. pylori*-infected and uninfected volunteers (data not shown).

Immune response of *H. pylori*-infected subjects to HWC vaccine with or without coadministered LT_{R192G}. In the first analysis, the anti-HWC responses among recipients of 2.5 × 10¹⁰ HWC plus placebo-adjuvant were compared with the responses among recipients of placebo-vaccine plus placebo-adjuvant to assess the immunogenicity of the vaccine alone in *H. pylori*-infected subjects. It was observed that HWC recipients achieved significantly higher geometric mean fecal IgA titers than those receiving placebo (301 versus 88, $P < 0.001$; Fig. 5).

In the second analysis, comparisons were performed to determine whether coadministered adjuvant increased the immunogenicity of the high dose of HWC. The only comparison of anti-HWC responses that approached statistical significance was the geometric mean serum IgA titer among subjects who

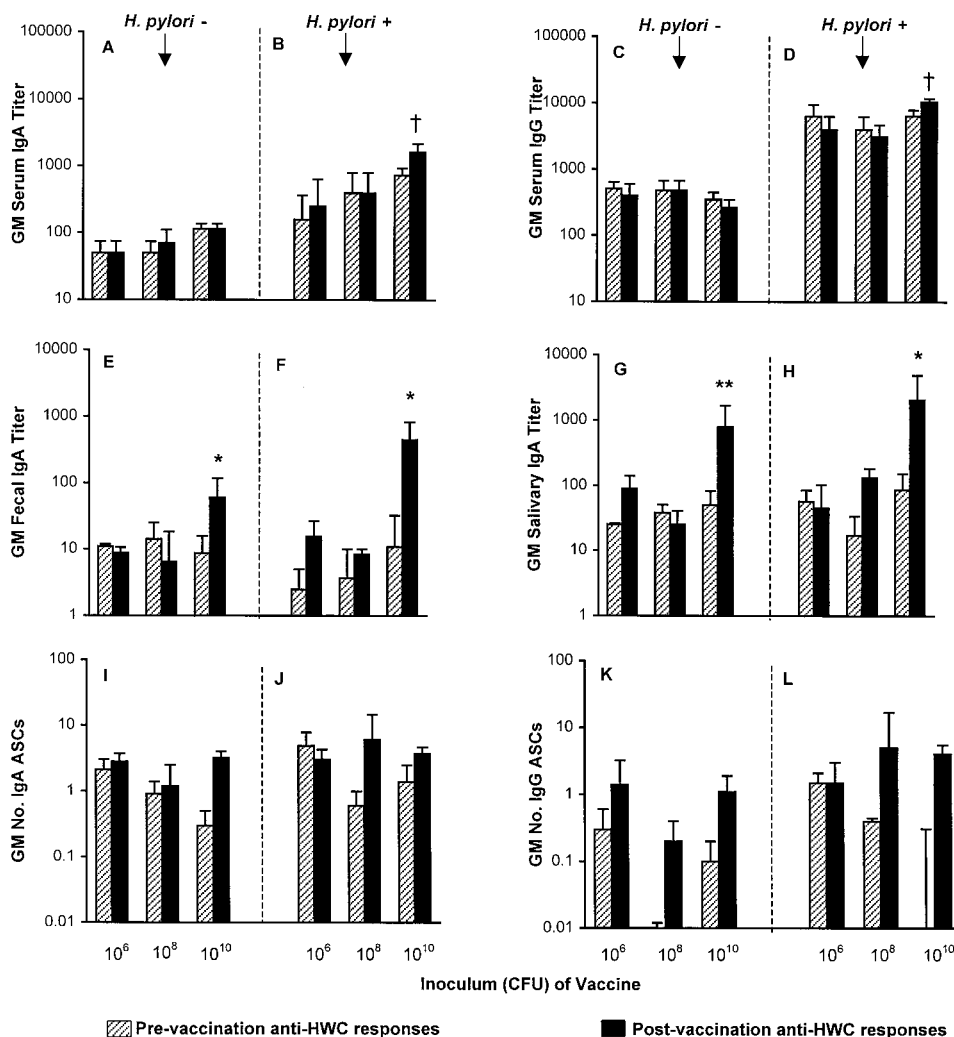


FIG. 1. Immune responses to HWC antigen according to *H. pylori* infection status and vaccine dose. Volunteers received an oral dose of either 2.5×10^6 , 2.5×10^8 , or 2.5×10^{10} inactivated HWC vaccine plus 25 μ g of LT_{R192G} adjuvant on days 0, 14, and 28. Responses are expressed as the geometric mean (GM) titer or geometric mean number of ASCs per 10^6 PBMC \pm the back-transformed standard error measured prevaccination and postvaccination. Panels A, C, E, G, I, and K represent volunteers with no evidence of *H. pylori* infection (there were 3, 4, and 10 recipients of the 10^6 , 10^8 , and 10^{10} doses of HWC, respectively). Panels B, D, F, H, J, and L represent volunteers with subclinical *H. pylori* infection at baseline (there were 3, 3, and 8 recipients of the 10^6 , 10^8 , and 10^{10} doses of HWC, respectively). Comparisons of pre- and postvaccination titers: *, $P < 0.05$; **, $P < 0.01$; †, $P = 0.06$.

received HWC with adjuvant versus those who received HWC alone (1,646 versus 400, $P = 0.06$; Fig. 5).

DISCUSSION

These results demonstrate that vaccination with inactivated HWC vaccine is immunogenic when given to volunteers with or without subclinical *H. pylori* infection. Furthermore, it provides the first indication in humans that an orally administered vaccine against *H. pylori* can induce mucosal IgA responses, as measured in stool and saliva, and elicit both IFN- γ and the appearance of circulating sensitized lymphocytes that proliferate.

Despite extensive investigation in animals demonstrating that mucosal adjuvants are essential to produce protective immunity to *Helicobacter*, comparable human experience is lacking. Although the sample sizes were small in our study and the

results must be considered preliminary, one response (serum anti-HWC IgA) approached statistical significance when subjects who received HWC with adjuvant were compared to those who received HWC alone (1,646 versus 400, $P = 0.06$). A previous series of clinical trials suggested that native LT adjuvanted the immune responses to rUrease, although direct comparisons of rUrease with or without adjuvant were not made (49). In these trials, native LT coadministered with rUrease vaccine induced serum and ASC IgA responses but not local (salivary and gastric) responses to the vaccine antigen (49), whereas in a previous trial, rUrease vaccine alone failed to induce an immune response (Kreiss et al., Letter). Although no recipients of LT plus rUrease were cured of their *H. pylori* infection, a significant decrease in gastric *H. pylori* bacterial density (but not inflammation) was observed in biopsy tissue. We were unable to determine whether vaccination similarly

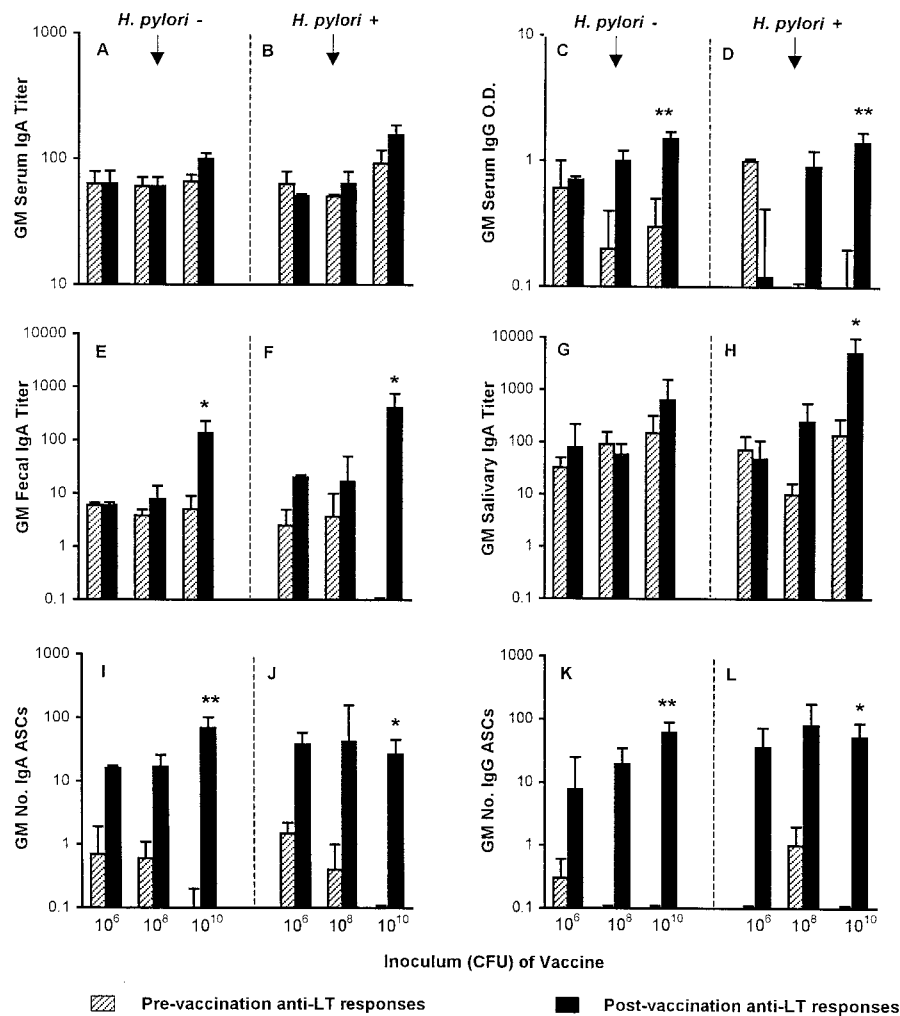


FIG. 2. Immune responses to the mucosal adjuvant LT_{R192G} according to *H. pylori* infection status and vaccine dose. Volunteers received an oral dose of either 2.5×10^6 , 2.5×10^8 , or 2.5×10^{10} inactivated HWC vaccine plus 25 μ g of LT_{R192G} adjuvant on days 0, 14, and 28. Responses are expressed as the geometric mean (GM) titer, the geometric mean OD, or the geometric mean number of ASCs per 10^6 PBMC \pm the back-transformed standard error measured prevaccination and postvaccination. Panels A, C, E, G, I, and K represent volunteers with no evidence of *H. pylori* infection (there were 3, 4, and 10 recipients of the 10^6 , 10^8 , and 10^{10} doses of HWC, respectively). Panels B, D, F, H, J, and L represent volunteers with subclinical *H. pylori* infection at baseline (there were 3, 3, and 8 recipients of the 10^6 , 10^8 , and 10^{10} doses of HWC, respectively). Comparisons of pre- and postvaccination titers: *, $P < 0.05$; **, $P < 0.01$.

reduced the bacterial burden in our trial because biopsies were not taken. Growing evidence in animal models suggests that both prophylactic and therapeutic *Helicobacter* vaccines do not achieve sterilizing immunity but rather reduce levels of bacterial colonization (13, 17, 35, 43). It remains to be determined whether sterilizing immunity can be achieved and, if not, whether suppression alone can prevent the pathological consequences of *H. pylori* infection.

It has been hypothesized that a balance of Th1 and Th2 responses is necessary to invoke protective immunity against *H. pylori*. Initial *H. pylori* vaccines were designed to target Th2-type responses, reasoning that activation of antigen-specific IgA at the mucosal surface would facilitate the clearance of bacteria from the stomach (10, 24). In mice, the enhanced efficacy of vaccine antigens conferred by coadministered native or nontoxic mutants of LT and CT in preventing and eradicating *H. pylori* infection has been attributed to the ability of these

mucosal adjuvants to drive preferential activation of Th2-type CD4⁺ responses (7, 15, 24, 58, 63, 68). This view is supported by observations that mice given monoclonal anti-*H. felis* (11) or anti-urease (4) IgA at the time of wild-type challenge were significantly protected against infection. Furthermore, the presence of antigen-specific secretory IgA in mucosal secretions (44) and not serum IgG (21) has been associated with protection in mice against acquisition of *H. felis* infection following challenge. In contrast, natural infection induces a more proinflammatory Th1-type response in the mouse *H. felis* model (21) and also in humans with *H. pylori*-associated peptic disease (30). However, the optimal type of immune response to be induced by vaccination requires further investigation. Recent observations showed that protection induced by mucosal immunization with rUrease plus LT in B-cell knockout mice was equivalent to that observed in the wild-type mouse strain, suggesting that antibody responses to urease are not required

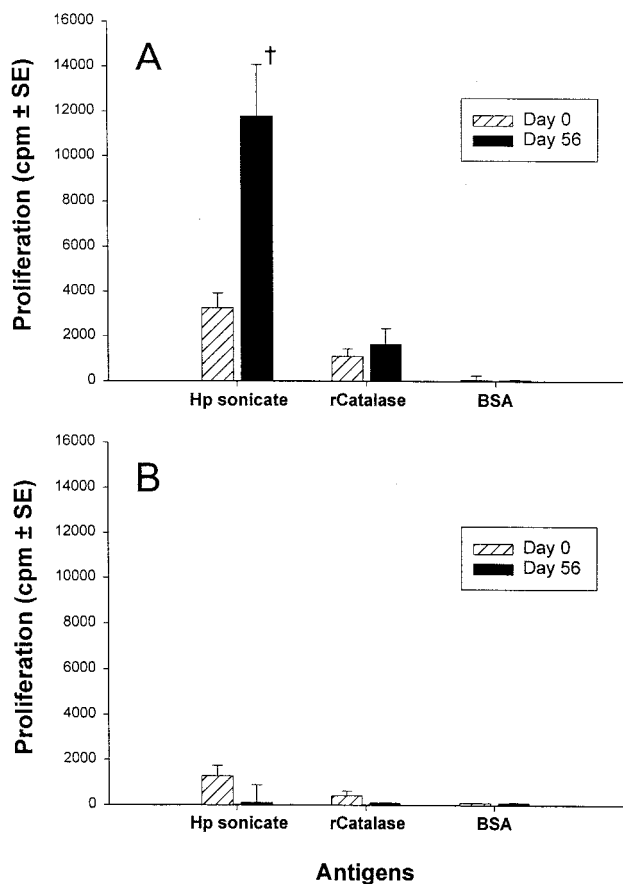


FIG. 3. Proliferative responses by PBMC from eight *H. pylori* uninfected (A) and 10 *H. pylori*-infected (B) volunteers following ingestion of 2.5×10^{10} inactivated HWC vaccine plus 25 μg of LT_{R192G} adjuvant. PBMC obtained from volunteers before or 56 days after immunization were evaluated for lymphoproliferative responses to an *H. pylori* sonicate or purified recombinant catalase. The results are expressed as the mean net cpm \pm the standard error for all volunteers in each group †, $P = 0.22$ (paired t test) of mean postimmunization versus preimmunization values.

for protection (18). Furthermore, rUrease vaccine injected with adjuvants that induce strong Th1- and Th2-type responses (e.g., saponin and glycol-lipopeptide) elicits better protection of mice against *H. pylori* challenge than rUrease mixed with adjuvants that induced a predominant Th2-type response (e.g., LT) (29).

Interestingly, vaccination with 2.5×10^{10} HWC plus 25 μg of LT_{R192G} elicited significant increases in sensitized lymphocytes that proliferated and produced IFN- γ , but not IL-5, in response to an *H. pylori* antigenic preparation. However, these responses were observed only in volunteers who were *H. pylori* negative. These results suggest that as an immunoprophylactic agent in *H. pylori*-negative individuals, the HWC vaccine can induce both type 1 and type 2 responses. Recent data suggest a marked predominance of a type 1 pattern of cytokine production, characterized by a prevalence of IFN- γ over IL-4 and IL-5 production by cells isolated from gastric biopsies of *H. pylori*-infected volunteers (2, 33). Our observations that immunization of *H. pylori*-uninfected volunteers with a whole-cell *H. pylori* vaccine elicits the appearance in peripheral blood of

sensitized cells that proliferate and produce predominantly type 1 cytokines (i.e., IFN- γ but not IL-5 production) to *H. pylori* antigens suggest that immunization with whole-cell vaccine may mimic to some extent the responses observed during natural infection. However, the fact that immunization of volunteers also produces increases in anti-*H. pylori* fecal and salivary IgA suggests that type 2 cytokine responses are also elicited. In contrast, the responses observed following immunization in *H. pylori*-infected volunteers, characterized by increases in serum, fecal, and salivary IgA in the absence of proliferation or IFN- γ production, suggest a predominance of type 2 responses. Alternatively, our inability to detect IFN- γ and proliferative responses in *H. pylori*-infected volunteers might be related to the previously observed phenomenon that exposure of PBMC and lamina propria lymphocytes from *H. pylori*-infected volunteers to *H. pylori* antigens resulted in lower proliferative responses and IFN- γ production than that observed

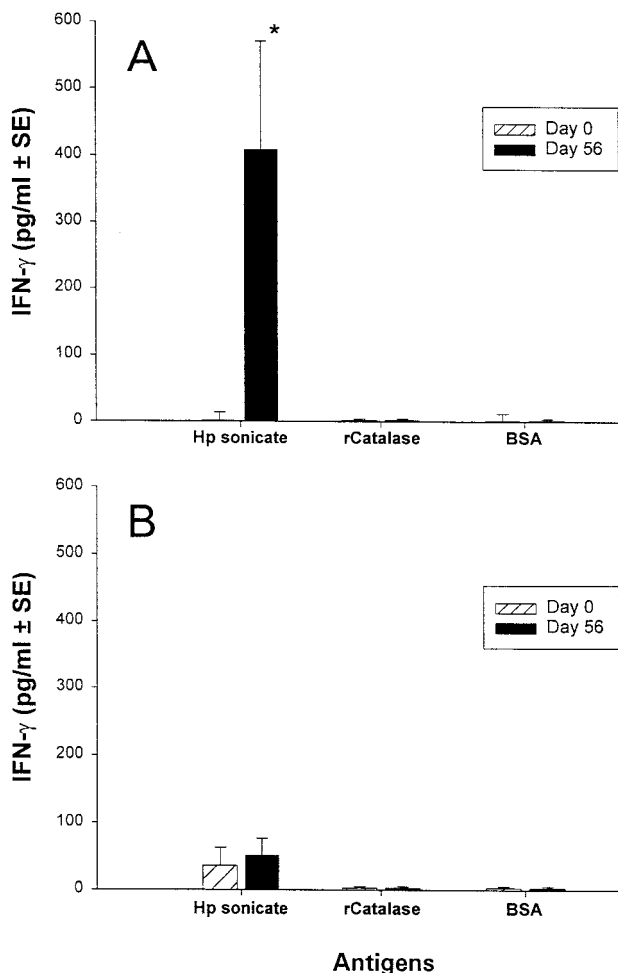


FIG. 4. IFN- γ production by PBMC from eight *H. pylori*-uninfected (A) and 10 *H. pylori*-infected (B) volunteers following ingestion of 2.5×10^{10} inactivated HWC vaccine plus 25 μg of LT_{R192G} adjuvant. PBMC obtained from the volunteers before or 56 days after immunization were evaluated for IFN- γ production to an *H. pylori* sonicate or purified recombinant catalase. The results are expressed as the mean net pg/ml \pm the standard error for all volunteers in each group. *, $P < 0.05$ (paired t test) of mean postimmunization versus preimmunization measurements.

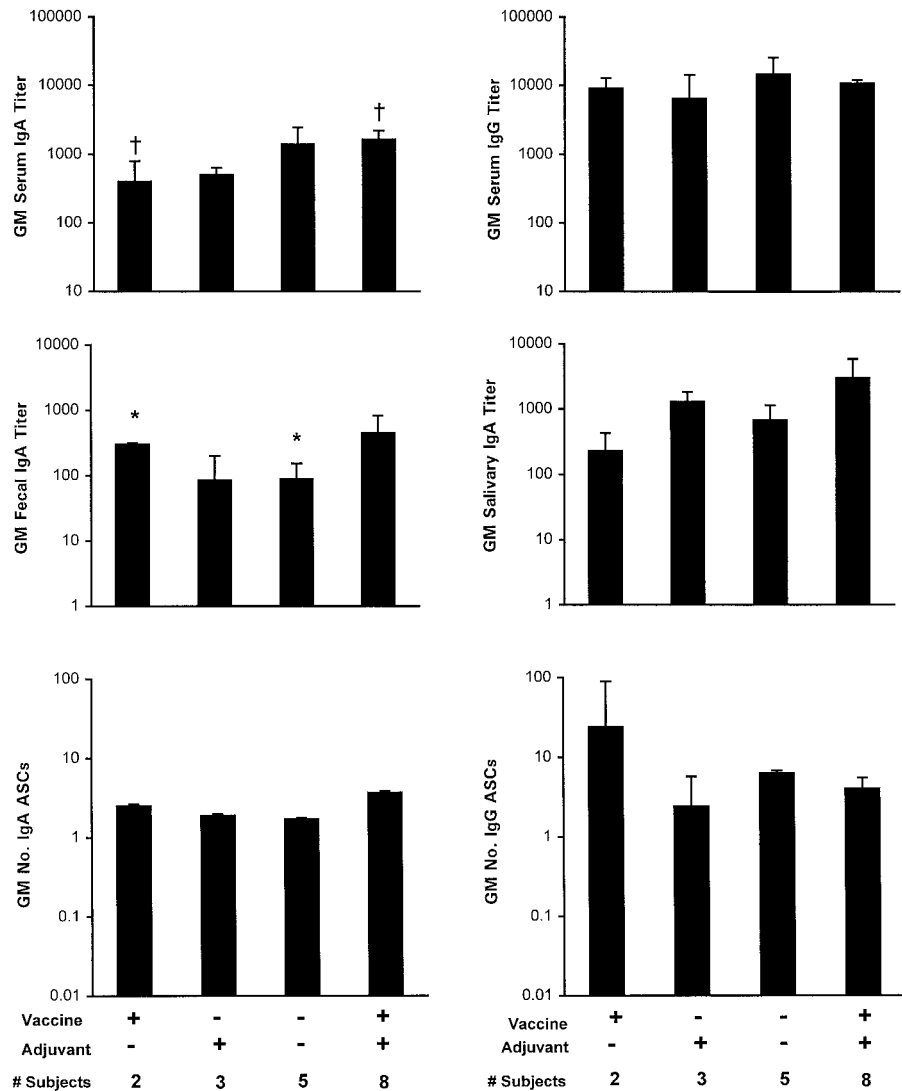


FIG. 5. Postvaccination peak immune responses of *H. pylori*-infected subjects to HWC antigen according to a randomized immunizing regimen. Volunteers received an oral dose of either 2.5×10^{10} HWC vaccine (vaccine+) or placebo-vaccine (vaccine-), plus either 25 μg of LT_{R192G} (adjuvant+) or placebo-adjuvant (adjuvant-). The responses are expressed as the geometric mean (GM) titer or the geometric mean number of ASCs per 10^6 PBMC \pm the back-transformed standard error. *, $P < 0.001$, comparing recipients vaccine+ plus adjuvant- with recipients of vaccine- plus adjuvant-; †, $P = 0.06$, comparing recipients of vaccine+ plus adjuvant- with recipients of vaccine+ plus adjuvant+. Note that the responses in the group that received vaccine+ and adjuvant+ are also shown in Fig. 1.

with cells isolated from noninfected volunteers, suggesting that *H. pylori* antigens might suppress specific immune responses (19).

Vaccination was generally well tolerated, although self-limited diarrhea occurred (generally only after the first dose) in 28% of subjects who received 2.5×10^{10} HWC plus LT_{R192G} and in one additional subject who received LT_{R192G} alone. Some subjects also experienced vomiting and low-grade fever. In comparison, diarrhea occurred in 66% of subjects participating in another study who received native LT (49). The self-limited diarrheal illnesses in our study may have resulted from residual enterotoxigenicity of LT_{R192G} , which retains some activity in the mouse Y-1 adrenal tumor cell assay (37). The substituted arginine residue at position 192 on the LT molecule is a trypsin cleavage site of A subunit to A1 and A2

components. LT has been reported to be activated by proteolytic cleavage at this site (5); however, cleavage at this site is not essential for the expression of enzymatic activity (26). Given the relationship between diarrhea and increasing doses of vaccine, it is also possible that the enterotoxic activity of *Helicobacter's* vacuolating toxin (VacA) was not completely eliminated with formalin processing (27).

In sum, the encouraging results of this study suggest that it is possible to stimulate an immune response to *H. pylori* antigens using an inactivated whole-cell vaccine. However, there is controversy regarding which immune responses are necessary to prevent or cure infection, particularly in light of the fact that chronic *H. pylori* infection occurs in the face of measurable systemic and local (gastric and salivary) antibody responses (8, 9, 38) and individuals who have been cured of their *H. pylori*

infection are occasionally reinfected (67), even with a homologous strain (60). The success of any *H. pylori* vaccine to prevent and/or cure infection in humans hinges on the ability to identify antigens and delivery systems which stimulate active immunity without inducing undesirable inflammatory processes and to target these responses to the stomach.

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