Mucosal Immunization with *Helicobacter*, CpG DNA, and Cholera Toxin Is Protective

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The mucosal delivery of antigens requires an effective adjuvant to induce mucosal immunity. Current mucosal adjuvants include cholera toxin (CT) and *Escherichia coli* heat-labile toxin. Unmethylated CpG immunostimulatory oligodeoxynucleotides (ODNs) have been proposed as novel mucosal adjuvants. In this study, mice were immunized with sonicated *Helicobacter felis* with CT and/or CpG ODN adjuvants. All groups receiving either adjuvant singly or in combination developed increased serum anti-*H. felis* immunoglobulin G (IgG). The addition of either CpG or CT, or both, produced a specific fecal anti-*H. felis* IgA response, with the highest IgA levels occurring in animals immunized intranasally with sonicated *H. felis* with CT and CpG. Following *H. felis* challenge, addition of the adjuvant CpG ODN provided no significant protection, while groups given CT showed a high degree of protection, although not complete. When CpG ODN was combined with CT and the vaccine combination was delivered intranasally, no bacterial colonization was detected by quantitative PCR, providing “sterile immunity” and demonstrating synergy between CpG ODN and CT.

Helicobacter pylori, first isolated in 1982, is a gram-negative, spiral-shaped organism that requires a microaerophilic environment to grow in vitro (37). The organisms usually colonize the mucus layer of the gastric epithelium in humans. *H. pylori* infects >50% of the world's population. Among infected individuals, <10% develop clinical signs of disease. *H. pylori* infection has now been linked to type B gastritis (4), peptic ulcer (4), gastric adenocarcinoma (45), and mucosa-associated lymphoid tissue lymphoma (46). *H. pylori* infection is characterized by mucosal infiltration of polymorphonuclear cells, monocytes, and lymphocytes. All infected individuals mount a vigorous but ineffective immune response regardless of the outcome of the infection. The immune response to natural *Helicobacter* infection is predominantly a T helper 1 (Th1) response in both humans and experimentally infected animals (1, 13, 40). The pathogenesis of *H. pylori* infection is not well elucidated; however, several pathogenic factors, such as cytotoxin-associated gene A (CagA), urease, vacuolating cytotoxin A (VacA), lipopolysaccharide, and host genetic background, have been suggested to be associated with pathogenicity (16, 41). Other *Helicobacter* spp. have been isolated from animals ranging from birds to nonhuman primates. There are currently 17 formally named *Helicobacter* spp. and several related organisms that have not been named (17). Animal-to-human transmission has been proposed but is not yet well defined. However, several human clinical cases associated with animal contact have been reported (30, 50). Natural *H. pylori* colonization of commercially raised cats has also indicated a possible animal reservoir for the organism (19). Other *Helicobacter* spp. that do not colonize humans naturally were isolated from healthy (16) and

immunocompromised (16, 25, 48) patients. The *Helicobacter felis* strain (ATCC 49179) used in this study was first isolated from a cat (31). Due to its ability to induce gastritis similar to human disease in conventionally housed mice, *H. felis* challenge in mice has been employed as one of the standard animal models for *H. pylori* vaccine development (17). Recently, the establishment of experimental *H. pylori* colonization in the mouse stomach allowed direct efficacy assessment of *H. pylori* antigen-targeted vaccines (17). However, we should note that there are no models of *Helicobacter* infection that exactly mimic the human disease.

Conventional chemotherapeutic regimens provide successful suppression of the bacteria; however, relapses do occur in some patients, and the threat of antibiotic resistance is a growing concern. Several recombinant vaccines have been tested in animal models and provided protection when the animals were challenged with *Helicobacter* (12, 26, 32). These experimental vaccines all use either cholera toxin (CT) or *Escherichia coli* heat-labile toxin as a mucosal adjuvant.

The immunostimulatory properties of bacterial DNA were first reported by Tokunaga et al. in 1984 (51). In 1995, Krieg et al. demonstrated that this phenomenon was due to unmethylated CpG dinucleotides in the bacterial genome (28). Prokaryotes lack a cytosine methylase; thus, their CpG dinucleotides are not methylated at the 5 position of the cytosine. However, the majority of CpG dinucleotides are methylated in vertebrate genomic DNA (3). This difference in methylation between vertebrate DNA and prokaryote DNA may very well serve as a “danger signal” upon microbial invasion. Unmethylated CpG oligodeoxynucleotides (ODNs) have the ability to stimulate B-cell proliferation (29). They also stimulate macrophages, dendritic cells, and natural killer cells (9, 23, 47). CpG ODNs can also induce the production of cytokines, such as interleukin 6 (IL-6), IL-12, gamma interferon (IFN- γ), and tumor necrosis factor alpha (9, 46, 47, 55). One of the mechanisms of CpG ODN action on cells of the immune system was recently determined to depend on Toll-like receptor 9 (TLR-

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9), which binds CpG-containing oligonucleotides and activates a cellular signaling pathway (2). TLR-9 activates MyD88, IRAK, and TRAF6, which in turn activate NF- κ B. TLR-9 is similar to many members of the TLR family which resemble the IL-1 receptor. These molecules recognize a variety of pathogen-associated molecular patterns triggering the production of proinflammatory cytokines, resulting in both intrinsic and adaptive immune responses (49).

In two murine models, CpG ODNs administered without a protein antigen provided protection against *Leishmania major* (14) and *Listeria monocytogenes* (29). CpG ODNs given with antigens, such as hepatitis B surface antigen and influenza nucleoprotein, also induced specific immune responses (33). When given with antigen systemically, CpG ODNs induce a Th1 antigen-specific immune response (11, 20). CpG ODN treatment can also redirect a Th2 allergic immune response to a Th1 response, thus alleviating allergic responses (27, 41). CpG ODNs have been tested as mucosal adjuvants with viral antigens in a few studies (18, 38, 39, 43). Both systemic and mucosal immune responses were induced, and one study showed protection against influenza virus challenge (43).

The aim of this study was to determine whether CpG ODNs can induce a mucosal immune response when given with *Helicobacter* antigen and to determine if that response would protect against infection in the *H. felis*-mouse model.

MATERIALS AND METHODS

Mice. *Helicobacter* spp.-free, 6-week-old female BALB/c mice (Harlan, Indianapolis, Ind.) were housed in microisolation cages. Autoclaved food and water were provided ad libitum. Experiments using animals were approved by the Auburn University Animal Care and Use Committee.

Bacterial strains and antigen preparation. *H. felis* (ATCC 49179) was grown in brucella broth containing 5% heat-inactivated fetal bovine serum and 1% V-C-N (vancomycin-colistin-nystatin) inhibitor (BD Pharmingen, San Diego, Calif.) under microaerophilic conditions at 37°C for 3 days. The organisms were harvested by centrifugation at $5,000 \times g$. The typical morphology, mobility, and urease activity of each culture were determined. Master stocks were stored in 50% brucella broth–25% glycerol–25% heat-inactivated fetal bovine serum at -80°C for future culture.

Antigens were prepared by sonicating whole bacterial organisms. Briefly, harvested *H. felis* cells were diluted in phosphate-buffered saline (PBS) and sonicated five times for 30 s each time with a 30-s pause on ice, using a sonicator (Torbeo ultrasonic processor; Cole-Parmer, Vernon Hills, Ill.). The sonicated antigens were then centrifuged at $5,000 \times g$ for 10 min at 4°C to clear cell debris and intact organisms. The supernatant was collected and filtered (0.2- μm pore size), and the protein concentration was determined by the protein assay of Lowry et al. (34). The supernatant was aliquoted and stored at -80°C until it was used for immunization, in vitro stimulation, or antibody assay by enzyme-linked immunosorbent assay. The number of CFU of cultured *H. felis* was determined by reading the optical density at 550 nm (OD_{550}). At this wavelength, 0.1 OD unit equaled approximately 10^7 CFU of *H. felis* (32).

Immunization and infection. Seventy BALB/c mice were assigned to 14 groups as follows: sonicated *H. felis*, subcutaneous (s.c.); sonicated *H. felis*, intranasal (i.n.); sonicated *H. felis*, intragastric (i.g.); sonicated *H. felis* plus CpG, s.c.; sonicated *H. felis* plus CpG, i.n.; sonicated *H. felis* plus CpG, i.g.; sonicated *H. felis* plus CT (Calbiochem, San Diego, Calif.), i.n.; sonicated *H. felis* plus CT, i.g.; sonicated *H. felis* plus CT plus CpG, i.n.; sonicated *H. felis* plus CT plus CpG, i.g.; CpG, i.n.; CpG, i.g.; and unvaccinated (not challenged) and unimmunized (challenged) controls.

Vaccines consisted of 10 μg of whole-cell sonicate mixed with either 10 μg of CT or 10 μg of CpG ODN. The optimal immunostimulatory mouse CpG ODN sequence (5'-TCCATGACGTTCTGACGT-3') was provided by Arthur Krieg. The CpG ODN was manufactured by Operon Technologies, Inc. (Alameda, Calif.), with a phosphorothioate backbone.

All of the mice were sedated lightly by isoflurane inhalation before immunization. The antigens and adjuvants were diluted in 20 μl of PBS for i.n. delivery,

with about half of the preparation delivered to each nostril with an Eppendorf pipette. The oral-immunization groups received 200 μl of vaccine through i.g. delivery with an 18-gauge feeding needle attached to a tuberculin syringe. S.c. immunization was in the right flank with a 27-gauge needle. BALB/c mice were immunized five times at intervals of 2 weeks. Two weeks after the last immunization, all animals except the negative controls were challenged with 10^7 CFU of *H. felis* three times over a 5-day period. The challenge dose was given by gastric intubation while the animals were lightly sedated with isoflurane. All of the animals were sacrificed 2 weeks after the challenge by cervical dislocation.

Serology. Serum samples were collected by tail bleeding at week 10 to assay for serum immunoglobulin G (IgG), IgG1, and IgG2a against sonicated-*H. felis* antigen. Fecal samples were collected biweekly on the day of bleeding. Fecal pellets were dissolved in PBS (5% [wt/vol]) by vortexing them vigorously followed by centrifugation at $5,000 \times g$ for 10 min. The supernatant was collected to assay it for IgA against sonicated *H. felis*. Ninety-six-well plates (Nalgene, Rochester, N.Y.) were coated with 8 ng of sonicated-*H. felis* protein/ml in 100 μl of PBS, pH 7.4, overnight at 4°C. The plates were washed three times with PBS and blocked with blocking buffer (BD Pharmingen) for 1 h at room temperature. All samples, secondary antibodies, and avidin-horseradish peroxidase (HRP) conjugates were diluted in blocking buffer. All incubations were performed at room temperature for 1 h. The plates were washed three times with PBS between steps. After the blocking step, 100- μl duplicate diluted samples (1:200) were added followed by goat anti-mouse IgG, IgG1, or IgG2a conjugates (Southern Biotechnology Associates, Birmingham, Ala.) diluted 1:5,000 and avidin-HRP conjugates diluted 1:4,000. For fecal-IgA detection, the samples were diluted 1:5 in blocking buffer and added to the antigen-coated plates in duplicate followed by goat anti-mouse IgA conjugated with HRP (1:1,000), and the avidin-HRP step was omitted. The plates were then incubated with substrate TMB (BD Pharmingen) for 20 min at room temperature, and the reactions were stopped by the addition of 1 N H_2SO_4 . The results were read at 450 nm with a microplate reader.

Cytokine assays. Spleens were removed aseptically and disrupted to prepare single-cell suspensions. The suspensions were adjusted to 10^6 cells/well in 24-well plates containing 1 ml of RPMI 1640 medium (Gibco BRL Life Technologies, Rockville, Md.) with or without the optimal concentration of sonicated *H. felis* (20 $\mu\text{g}/\text{ml}$) or concanavalin A (10 $\mu\text{g}/\text{ml}$). The cells were incubated for 48 h at 37°C with 5% CO_2 , and the supernatant was harvested for cytokine assays of IFN- γ and IL-4. The assay kits were purchased from R&D systems (Minneapolis, Minn.). The assay conditions were in accordance with the manufacturer's recommendations. The reactions were read at 450 nm with a microplate reader.

Urease broth test assessment of *H. felis* infection. At necropsy, the stomachs from the BALB/c mice were cut along the greater curvature. A quarter of the longitudinal section of the stomach was cut out and placed in a tube containing 1 ml of urease broth (60 g of urea, 2 g of KH_2PO_4 , 5 g of NaCl, 10 g of glucose, 1 g of peptone, and 0.012 g of phenol red in 1 liter of distilled water), which was incubated at 37°C for 4 h. A change of color from yellow to pink was considered positive for urease and an indication of *Helicobacter* colonization.

Quantitative-PCR assessment of *H. felis* colonization. Half of the stomach was frozen in liquid nitrogen until it was processed. Genomic DNA was isolated using the High Pure PCR Template Preparation kit (Roche Molecular Biochemicals, Indianapolis, Ind.). The stomach tissue was homogenized and incubated overnight at 55°C with 20 mg of proteinase K/ml. The homogenate was then treated with binding buffers that allowed specific binding of nucleic acids to a glass fiber column. The lysate and other residual impurities were removed by washing and centrifugation, and subsequently nucleic acids were eluted in elution buffer provided by the manufacturer. The DNA concentration was determined by the OD_{260} . Quantitative PCR was carried out using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). SYBR Green PCR reagents were obtained from Perkin-Elmer Applied Biosystems. PCRs were performed as follows: 2 min at 50°C and 10 min at 95°C for 1 cycle followed by 15 s at 95°C and 60 s at 60°C for 40 cycles. Forward (5'-CTGAGAGGGTGAACGACACA-3') and reverse (5'-CTTCTCTGACAAAAGGAGTTACAATCC-3') primers were designed based on *H. felis* 16S rRNA and synthesized at the Nucleic Acid Facility, Scott-Ritchey Research Center, Auburn University, Auburn, Ala. Each reaction mixture contained 2 μg of template DNA. Standard *H. felis* DNA was made by extracting 10^7 CFU of *H. felis* using Roche's High Pure PCR Template Preparation kit and diluted to the desired concentration. The SYBR Green fluorescence activity was detected by the ABI 7700, and data was processed by Perkin-Elmer Applied Biosystems Sequence Detector version 1.6.3 using a Power Macintosh 7200. The computer software converts the fluorescence activity into CFU counts based on a standard curve.

Statistical analysis. All serum and fecal-antibody results were tested by one-way analysis of variance (ANOVA). Urease broth test results were analyzed by

FIG. 1. Serum IgG levels against *H. felis*. Serum samples were collected at week 10 and diluted 1:200. The samples were tested in duplicate. The bars represent the means of OD₄₅₀ plus standard deviations. *, significant compared to either sonicated *H. felis* (sHf) alone or the unimmunized negative control (Negative) ($P < 0.05$). S.c. and i.g. administration of sonicated *H. felis* alone produced background OD₄₅₀ readings comparable to those of the unimmunized control. CpG ODN treatment also did not yield increased IgG levels above background.

the Fisher exact test. Quantitative-PCR results were analyzed using Student's *t* test on log-transformed data.

RESULTS

Immune responses against *H. felis*. Both the i.g. and i.n. routes were studied, since previous research (6) indicated that immunization at different local induction sites might provide different responses at different effector sites. Ten weeks after the initial immunization and 2 weeks after the fifth immunization, all groups immunized with antigen and any adjuvant combination had increased serum IgG levels (Fig. 1). Animals immunized i.n. had higher IgG levels than those immunized i.g. The group that received sonicated *H. felis* plus CpG s.c. also had a high IgG level, comparable to that of the groups that received sonicated *H. felis* with CpG and/or CT adjuvants i.n.. Sonicated *H. felis* given alone showed a slight increase in IgG levels, while the unimmunized negative control group and CpG-alone group did not show increased serum IgG levels.

When serum IgG subtypes were examined, all the groups which received i.g. immunization with sonicated *H. felis* with CpG and/or CT adjuvants showed a predominantly Th2 response with IgG1/IgG2a ratios ranging from 2.27 to 2.81. All of the i.n.- and s.c.-immunized groups showed a balanced Th1-Th2 response with IgG1/IgG2a ratios around 1.00 (Fig. 2).

Fecal samples were collected and analyzed for mucosal IgA. All of the groups immunized with sonicated *H. felis* plus CpG and/or CT either i.n. or i.g. showed increased fecal-IgA levels at week 10, 2 weeks after the last immunization (Fig. 3). The groups which received i.n. immunization with sonicated *H. felis* plus CT with or without CpG ODN showed higher fecal IgA levels than other groups ($P < 0.05$). All negative control, protein-alone, and CpG-alone groups showed either no or minimal increases of fecal IgA.

Protection against *H. felis* infection. To examine the protective role of the induced immune responses in this study, all BALB/c mice were challenged with 10^7 CFU of *H. felis* three

FIG. 2. Serum IgG subtype levels against *H. felis*. Serum samples were collected at week 10 and diluted 1:200. The samples were tested in duplicate. IgG1 (solid bars) and IgG2a (open bars) OD₄₅₀ levels are presented as means plus standard deviations. The number above each set of bars indicates the IgG1/IgG2a ratio. sHf, sonicated *H. felis*.

times over a 5-day period. Two weeks after the challenge, all of the animals were sacrificed, and stomach samples were taken for the urease broth test and quantitative PCR to determine bacterial colonization.

A rapid urease broth test was employed as a method for qualitative assessment of the infection status in mouse stomach

FIG. 3. Fecal-IgA levels against *H. felis*. Fecal samples were collected at week 10 and dissolved in PBS (5% [wt/vol]) by vigorous vortexing followed by centrifugation at $5,000 \times g$ for 10 min, and the supernatants were collected to assay for IgA. Samples were tested in duplicate. Fecal-IgA OD₄₅₀ levels are expressed as means plus standard deviations. *, significant compared to the unimmunized and unchallenged control (Negative) ($P < 0.05$); \$, significant compared to sonicated *H. felis* (sHf) alone ($P < 0.05$); +, significant compared to sonicated *H. felis* plus CpG by the same route ($P < 0.01$); &, significant compared to sonicated *H. felis* plus CT by the same route ($P < 0.05$). S.c. and i.g. administration of sonicated *H. felis* alone did not induce fecal-IgA levels above the background level, nor did CpG ODN treatment. Statistical analysis was done by one-way ANOVA.

TABLE 1. Stomach sample urease broth test

Group	Urease test results ^a (1/4 stomach)
Negative control	0/5 ^b
Positive control	5/5
Sonicated <i>H. felis</i> , i.g.	5/5
Sonicated <i>H. felis</i> , s.c.	5/5
Sonicated <i>H. felis</i> , i.n.	3/5
Sonicated <i>H. felis</i> , + CpG, i.n.	2/5
Sonicated <i>H. felis</i> + CpG, s.c.	4/5
Sonicated <i>H. felis</i> + CpG, i.g.	5/5
Sonicated <i>H. felis</i> + CT, i.n.	0/3 ^b
Sonicated <i>H. felis</i> + CT, i.g.	1/5 ^b
Sonicated <i>H. felis</i> + CT + CpG, i.n.	0/3 ^b
Sonicated <i>H. felis</i> + CT + CpG, i.g.	0/5 ^b
CpG, i.g.	5/5
CpG, i.n.	5/5

^a Number positive/number tested.^b Significantly lower ($P < 0.05$) than positive control group by the Fisher exact test.

samples. Based on the urease test, the groups which received sonicated *H. felis* plus CT with or without CpG, either i.n. or i.g., had fewer positive results, indicating protection or limited infection. All the positive controls (unimmunized and challenged) had a color change from yellow to pink, indicating no protection or abundant colonization (Table 1). The unvaccinated, unchallenged controls had no indication of *H. felis* col-

onization. All other groups had color changes, which indicated no significant protection compared to the positive controls.

Since the urease broth test is only a qualitative method for measuring bacterial colonization, a robust quantitative-PCR assay was used. According to *H. felis*-specific SYBR Green PCR, the positive control animals were colonized with $\sim 10^5$ to 10^6 CFU of *H. felis* per stomach, while the negative control group showed only background readings (Fig. 4). All of the groups which received only protein antigen showed no significant decrease in *H. felis* colony numbers regardless of the immunization route. The groups receiving only CpG ODN as well as systemic immunization with sonicated *H. felis* plus CpG also showed between zero and one-half-log-unit decrease in colonization, which was not statistically significant. The group receiving i.n. immunization with sonicated *H. felis* plus CT showed a significant decrease in *H. felis* colonization ($P < 0.05$) compared to the unimmunized, sonicated-*H. felis*, CpG, and sonicated-*H. felis* plus CpG groups. The combination of CpG and CT given i.n. with antigen further decreased *H. felis* colonization to background levels compared to the negative controls. This decrease was significant ($P < 0.05$) compared to i.n. immunization with sonicated *H. felis* and CT. Forty percent of the animals in the group vaccinated i.g. with sonicated *H. felis* plus CT had high CFU counts that were comparable to those of positive controls, while the remaining 60% had at least a 2-log-unit decrease in CFU. All of the animals in the group

FIG. 4. Quantitative-PCR determination of *H. felis* colonization. Genomic DNA was isolated from mouse stomach samples, and each sample was tested in quadruplicate. The results from PCR were converted to CFU per stomach based on a standard curve derived from *H. felis* genomic DNA. Each data point represents a single mouse, and the solid lines indicate the CFU means. The dashed line indicates the *H. felis* CFU cutoff value (~ 40), and readings under this line should be considered zero. sHf, sonicated *H. felis*.

FIG. 5. Splenocyte IFN- γ levels after ex vivo stimulation with sonicated *H. felis* (sHf). Splenocytes (10^6) were cocultured with 20 μ g of sonicated *H. felis*/ml for 48 h, and the supernatant was analyzed for IFN- γ levels. Each bar represents the mean IFN- γ concentration plus the standard deviation. *, significant difference compared to the negative control group (Negative) ($P < 0.01$); +, significant difference compared to sonicated *H. felis* with CpG adjuvant administered by the same route ($P < 0.01$). Statistical analysis was done by one-way ANOVA.

vaccinated i.g. with sonicated *H. felis* with CT and CpG ODN adjuvants had at least a 1-log-unit decrease in bacterial colonization.

Cytokine profile. Splenocyte cytokine profiles were examined to determine the specific T-helper responses that might contribute to protection. On the day of euthanasia, splenocytes were obtained and stimulated with sonicated-*H. felis* protein. All animals that received sonicated *H. felis* plus CpG and/or CT had increased IFN- γ levels upon stimulation (Fig. 5), with those groups receiving protein plus CT with or without CpG ODN i.n. having higher IFN- γ levels than the other groups. The unvaccinated controls and the groups that received CpG alone and sonicated *H. felis* alone had either no or minimal increases in IFN- γ levels. All of the groups had no detectable IL-4 production (data not shown).

DISCUSSION

In both mice and humans, *Helicobacter* infection induces chronic inflammation, a strong humoral immune response, and a predominantly Th1 response in the gastric lamina propria (1, 13, 40). The host Th1 immune response is unable to clear the infection and may actually contribute to disease. Treatment of *Helicobacter*-infected mice with neutralizing antibodies against IFN- γ resulted in decreased gastric inflammation (40). *Helicobacter* organisms are generally considered to be extracellular parasites, and a specific Th2 response is thought to be a protective response against extracellular pathogens. In *Helicobacter*-vaccinated-animal models, challenged immunized animals also developed a predominantly Th1 response (15). However, the organisms are cleared over time and gastric inflammation is diminished. This difference may be due to a masked Th2 response, as treatment of immunized and challenged animals with monoclonal antibodies against IFN- γ revealed a masked Th2 response (40). However, recombinant urease vaccination in major histocompatibility complex (MHC) and B-cell knock-

out mice revealed that class I molecules were involved in protection while MHC class II molecules played a more critical role. Other evidence suggests that the CD4⁺ response generated by immunization was effective in preventing infection (42). In this study, the effects of CT, a Th2 adjuvant, and CpG ODN, an adjuvant usually described as Th1 biased, were studied.

Mucosal administration of CpG ODNs with sonicated *Helicobacter* bacterial antigen induced both a mucosal immune response and a systemic immune response. Circulating IgG levels were significantly increased in mice receiving sonicated *H. felis* s.c. with CpG or i.n. with CpG, CT, or the combination of CpG and CT. I.g. administration of antigen resulted in much weaker IgG responses, regardless of the adjuvant.

Serum IgG subtyping in i.n.-CpG adjuvant groups revealed more of a balanced T-helper response. The combination of CpG ODNs and CT did not direct the immune response to a predominantly Th1 response in either of the vaccinated groups, while i.g. administration showed a Th2 response regardless of the adjuvant. McCluskie and Davis used CpG ODN as an adjuvant with hepatitis B surface antigen and showed a balanced Th1-Th2 response (38). However, when administered with a well-known Th2 mucosal adjuvant, CT, CpG ODNs were able to convert the response to a Th1 response. The differences in antigen, vaccine volume, vaccination schedule, and mouse strain may explain these findings. The antigen used in this study was a whole-cell lysate. Type 1 *Helicobacter* lipopolysaccharide has been demonstrated to be a potent activator of the TLR-4 receptor, providing an alternate pathway for activation of the innate immune response (24). In addition, mucosal administration of CT with protein does not always lead to a pure Th2 response (1, 22, 36, 40, 54). Balanced Th1-Th2 responses (1, 21, 40, 53) or even a Th1 response can result (1, 5, 40).

Immunization through the i.n. route induced higher fecal-IgA levels than i.g. immunization. The combination of CpG ODNs and CT with sonicated-*H. felis* antigen, given i.n., induced a statistically significant increase in secretory IgA levels over those seen with sonicated *H. felis* with CT adjuvant alone, indicating a synergistic effect between the two adjuvants.

Toxicity associated with i.n. administration of CT was higher than that associated with i.n. administration of CpG ODN or either adjuvant when given orally, as two of five mice died in each of the i.n.-CT groups.

Based on the urease broth assay, animals receiving sonicated *H. felis* with CT adjuvant were protected, whether or not they received CpG ODN. However, this assay is neither quantitative nor specific, and PCR is considered the standard for diagnosis (17). Several studies have used competitive quantitative PCR in the detection of *Helicobacter* (44) and other infectious agents (8, 35). In this study, quantitative PCR was used to determine stomach colonization. A single time point was examined; thus, it should be remembered that animals with detectable colonization may completely clear their infection at a later time. Unimmunized and unchallenged negative control mice showed only background CFU counts based on fluorescence activity. Animals that received mucosal immunization with sonicated *H. felis* and CpG showed increased antibody responses but no protection against challenge. This suggests that CpG adjuvant cannot by itself provide protection

against *Helicobacter* infection. However, CpG ODNs may play a role in "sterile immunization." When CpG ODNs were combined with CT, bacterial colonization dramatically decreased in both i.g.- and i.n.-immunization groups. I.g. immunization with CT, with or without CpG ODN, showed less protection than the i.n. route. However, longer-term observation after challenge is needed to determine the outcome of i.g. immunization. The different outcomes with CpG ODNs and CT suggest that these two adjuvants probably target different cell types or act through different but complementary pathways. CpG ODNs have been shown to down-regulate macrophage MHC class II synthesis in vitro (10). On the other hand, CT can stimulate IL-1 production and enhance macrophage antigen presentation by the MHC in vitro (7). However, better protection was observed in this study when both adjuvants were used, suggesting a synergistic protective effect between the two adjuvants. Complete protection may require the induction of multiple immune components, as the combination of CT and CpG with protein given i.n. showed virtually no bacterial colonization.

In animals immunized with sonicated *H. felis* plus CT and/or CpG, splenocytes secrete more IFN- γ than those of control groups when stimulated ex vivo. Interestingly, the groups that received i.n. protein plus CT with or without CpG, which had the lowest level of colonization by *H. felis*, showed the highest IFN- γ levels compared to other groups. Although splenocyte cytokine production does not necessarily reflect local cytokine profiles, previous studies showed that the cytokine profiles in spleen and gastric lamina propria lymphocytes were similar after infection and immunization (40). IL-4 levels were not detectable in any group, which was similar to other studies (15). Other Th2 cytokines, such as IL-5 and IL-10, need to be included in future studies to determine the role of cytokines in vaccinated animals.

This study demonstrated that both CpG ODN and CT are potent mucosal adjuvants. CpG ODNs and CT have synergistic effects when given mucosally with *Helicobacter* antigen. CpG ODN appears to be safer than CT, and CpG ODN is also safer than other adjuvants (52). Whole *Helicobacter* sonicate with CT adjuvant provided better protection against *Helicobacter* challenge than CpG ODN adjuvants alone. The combination of both adjuvants with antigen provided sterile immunity when given i.n. In. immunization generated a better immune response and better protection than oral immunization; however, it was associated with higher toxicity, probably due to CT. Longer-term and larger studies need to be performed to further determine the effects of CpG ODNs in immune responses to *Helicobacter* pathogens. The efficacies of these adjuvants with recombinant proteins against *Helicobacter* infection also need to be determined in the *H. pylori*-mouse model.

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