Helicobacter infection leads to chronic inflammation of the stomach. Although the infection persists in spite of an immune response, animal studies have shown that adjuvant-based oral vaccines can protect against infection and even eliminate established infection. These vaccines are thought to induce a Th2 immune response, counterbalancing the Th1 response seen with natural infections. As a prelude to using adenovirus vectors carrying cytokine genes to modulate the immune response to established Helicobacter felis infection, we first examined the effect of the replication-defective adenovirus (RDA) vector itself. C57BL/6 mice chronically infected with H. felis (8 to 10 weeks) received intramuscular injections of RDA. The effect of RDA on the severity of H. felis colonization and the degree of gastric inflammation was assessed 2 weeks later. RDA caused a significant decrease in H. felis colonization without significantly altering the associated inflammation. RDA did not alter the H. felis-specific immunoglobulin G1 (IgG1), IgG2a, and IgA responses in the serum but was associated with an increase in gamma interferon (IFN-γ)-producing CD8⁺ spleen cells. To determine if IFN-γ or Th1 cytokines were involved in the response to RDA, we examined RDA treatment of H. felis infection in mice lacking either IFN-γ or interleukin-12 (IL-12). RDA failed to alter H. felis colonization in either of these two mouse strains. Thus, viral infection of mice chronically infected with H. felis led to a significant decrease in H. felis colonization in an IFN-γ- and IL-12-dependent manner. These results demonstrate that Th1 responses associated with systemic viral infection can influence an established H. felis infection.

Helicobacter pylori causes gastritis, has a causal role in the development of peptic ulcers, and is considered a risk factor for the development of gastric cancer and mucosa-associated lymphoid tissue lymphomas (34). Once H. pylori infection is established, it persists in spite of the immune response that develops. Eradication of Helicobacter infection leads to cure of the ulcer and prevention of its recurrence (47). Studies of H. felis in mice suggest that oral vaccines given with a mucosal adjuvant such as cholera toxin can prevent Helicobacter infection and can even lead to elimination of established infection (6, 8, 12). Cholera toxin induces a shift in the helper T-cell response from a Th1- to a Th2-type cytokine response. Although it has been proposed that the shift from a Th1- to a Th2-type response is responsible for the induction of protection, cholera toxin can also induce mixed Th1 and Th2 responses (17, 43) and even Th1 responses (4). Therefore, the possibility remains that Th1 responses can influence Helicobacter infection.

The use of mucosal adjuvants like cholera toxin or the heat-labile enterotoxin of Escherichia coli in humans is potentially associated with toxicity, and preliminary data on the clinical effectiveness of these adjuvant-based H. pylori vaccines in humans is disappointing (36). Therefore, alternative means of modulating the immune response to Helicobacter infection need to be explored. We and others have shown that injection of replication-defective adenovirus (RDA) carrying cytokine genes can modulate the immune and inflammatory responses to a number of infectious agents and allergens (21, 33, 41, 44). The availability of recombinant RDA containing immunomodulatory molecules provides an alternate approach to the modulation of the immune response to established Helicobacter infections. The present study was designed to determine the effects of the RDA vector itself on H. felis colonization and on the inflammatory response to an established H. felis infection in the mouse model.

MATERIALS AND METHODS

Animals and bacteria. Specific-pathogen-free female C57BL/6 and gamma interferon (IFN-γ)−/− mice (6 to 8 weeks old) were purchased from The Jackson Laboratory, Bar Harbor, Maine. The generation of interleukin-12 (IL-12) p40−/− mice (C57BL/6 background) has previously been described (24). These mice were bred in our central animal facility. All mice were housed in microisolator cages with free access to autoclaved chow and water. H. felis ATCC 49179 (CS1) was obtained from the Laboratory Center for Disease Control of Canada and stored at −70°C in 80% brain heart infusion broth plus 10% horse serum and 10% glycerol. The bacteria were cultured on chocolate agar plates (PML Microbiologicals, Mississauga, Ontario, Canada) under microaerophilic conditions. Before harvesting in sterile saline, bacterial cultures were examined by Gram staining to exclude possible contamination and by phase-contrast microscopy to ensure viability and motility.

Establishment of gastric H. felis infection. Six- to 8-week-old mice were infected with three doses of 5 × 10⁸ H. felis bacteria (in 300 μl of sterile saline) by oral gavage at 2-day intervals. Sham-treated mice were gavaged with a solution of sterile saline. The date of the first infection was counted as day 1. This study was approved by the McMaster University Animal Care Committee and conforms to the guidelines of the Canadian Council on Animal Care.

Preparation of H. felis whole-cell sonicate. Whole-cell sonicate (WCS) was prepared from freshly harvested H. felis. Cell pellets of bacteria were resuspended in sterile distilled water and submitted to ultrasonication at 4°C in a...
Fisher sonic dissector (Artex Systems Corp., Farmingdale, N.Y.). The WCS was centrifuged for 10 min at 4°C to clear the cellular debris and then filtered through a 0.2-μm-pore-size Acrodics filter (Gelman Sciences, Ann Arbor, Mich.). The protein concentration of the filtrate was determined by the Lowry method (22), and aliquots were stored at −70°C until use. WCS was used to immunize mice to generate positive serum standards for an H. felis-specific antibody isotype enzyme-linked immunosorbent assay (ELISA).

**Grading of gastric inflammation and infection.** Stomachs of mice were removed and fixed in 10% neutral buffered formalin and then embedded in paraffin. Sagittal sections at three different levels were stained with hematoxylin and eosin. Histological evaluation of inflammation and infection was carried out in a blinded manner by an assessor designated by the Lowry method (28, 30). The inflammation was graded on the basis of the intensity of inflammation in the longitudinal axis of the mucosa and the vertical extent of inflammation within the gastric glands. The intensity of inflammation was measured in the areas showing the most significant changes under ×10 magnification and scored on a scale of 0 to 4 (grades: 0, no inflammatory cells; 1, rare inflammatory cells; 2, multiple clusters of inflammatory cells; 3, diffuse inflammation with variable intensity; 4, diffuse and uniformly severe inflammation). The longitudinal extent of inflammation was scored on the basis of the percentage of the mucosal surface involved in the inflammation as assessed for the entire sagittal section examined at ×10 magnification (grades: 0, none; 1, <25%; 2, 25 to 50%; 3, 50 to 75%; 4, >75%). The vertical extent of inflammation was scored on the basis of the degree to which the inflammation extended to the different mucosal layers in the area with the greatest involvement (grades: 0, none; 1, only basal area involved by inflammation [i.e., not extending to the surface of the mucosa]; 2, transmural [i.e., full-thickness involvement of the mucosa]; 3, deep [i.e., involvement of both the mucosa and the submucosa]).

The grading of gastric inflammation, intensity, longitudinal extent, and vertical extent were combined, and the sum was used to represent the degree of inflammation. The extent of infection was estimated by determining first the number of H. felis-positive glands per 20 glands and then the maximum number of H. felis organisms per gland. These two numbers were averaged. The average extent of infection in the antrum was then combined with the average extent of infection in the fundus. The degree of infection represents the combined extent of infection in the antrum and fundus (28).

**RDA.** The human type 5 RDA carries a deletion of the E1 gene and a partially crippled E3 gene in the adenoviral genome (adenovirus type 5 strain DL70-3) (46). RDA was harvested and purified by ultracentrifugation, and the titer was determined as previously described (2). The virus was diluted to a final concentration of 1 × 108 PFU/100 μl. Each mouse was infected twice with 6 × 106/ml of RDA in the hind legs over a 5-day period (46). The mice receiving the RDA injection had had an established H. felis infection for at least 8 weeks.

**Collection of serum and gut wash samples.** The serum used as a standard for the ELISA was obtained from C57BL/6 mice that had received four weekly infections in general and is also in keeping with the predominant Th1 responses favoring IgG2a over IgG1 isotype responses.

**Effect of RDA infection on established H. felis colonization of the mouse stomach.** C57BL/6 mice infected with H. felis for 8 weeks or more received RDA or PBS injections in a hind limb. H. felis infection is well established at 8 weeks and is associated with chronic gastritis and a significant antibody response (Fig. 1) (30). Two weeks after RDA injection, the mice were sacrificed and the stomachs were removed for histological examination. Mice receiving RDA injections showed a significant decrease in H. felis colonization compared to mice receiving PBS alone (P < 0.005). The degree of gastric inflammation did not change significantly after RDA injection (Table 1).

**Effect of RDA infection on the immune response to established H. felis colonization.** In C57BL/6 mice, an increase in serum IgG2a H. felis-specific antibody was detected by 7 weeks after H. felis infection (Fig. 1). In contrast, minimal serum IgG1 or IgA anti-H. felis antibody responses were measured, even up to 22 weeks after infection. Previous studies indicate that Th1 responses favor IgG2a over IgG1 isotype responses (31, 32). Therefore, the increase in IgG2a is in keeping with the tendency of C57BL/6 mice to develop Th1-type responses to infections in general and is also in keeping with the predominant Th1-type responses demonstrated in helicobacter infections specifically (14, 29). We compared the H. felis-specific antibody responses in H. felis-infected mice 2 weeks after RDA injection (10 to 12 weeks post H. felis infection) with that of H. felis-infected mice given PBS. No significant difference was found in any of the three isotype responses between PBS- and RDA-treated H. felis-infected mice (data not shown).

We also analyzed the effect of RDA infection on IFN-γ, IL-4, and IL-2 production by H. felis-stimulated spleen cells by using flow cytometry analysis of intracellular cytokine expression. In the absence of in vitro H. felis stimulation, there was no detectable cytokine (data not shown). After H. felis WCS stimulation of spleen cells from RDA-infected, H. felis-colonized mice, there was a significant increase in IFN-γ-producing CD8+ splenic T cells (6.5 to 25%) (Fig. 2). There was no

**RESULTS**

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significant change in the number of IL-4- or IL-2-producing spleen cells. RDA infection of naive mice not colonized with *H. felis* failed to increase IFN-γ-producing spleen cells after in vitro stimulation with *H. felis* antigen (data not shown). Therefore, RDA infection of *H. felis*-colonized mice increased IFN-γ-producing *H. felis* antigen-specific cells.

**Effect of RDA on *H. felis* infection in mice lacking the IFN-γ or IL-12 p40 gene.** IFN-γ and IL-12 are thought to contribute to the gastritis and mucosal damage that develop during *Helicobacter* infection in humans and mice (13, 29). In order to directly examine the role of these cytokines in the development of *H. felis*-associated gastritis and in regulating *H. felis* colonization, we infected mice lacking the IFN-γ or IL-12 p40 gene. Lack of the IFN-γ gene causes loss of IFN-γ production in vivo (9), and CD4+ T cells respond to antigens by differentiation to a Th2 response (42). IL-12-deficient mice are impaired in the ability to produce IFN-γ following endotoxin administration.

**TABLE 1. Effect of RDA on the severity of an established *H. felis* infection and the associated gastric inflammation in C57BL/6 mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of experiments</th>
<th>Mean degreea (±SEM) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Infection</td>
</tr>
<tr>
<td><em>H. felis</em></td>
<td>5</td>
<td>12.6 ± 1.3</td>
</tr>
<tr>
<td><em>H. felis</em> + RDA</td>
<td>4</td>
<td>3.2 ± 1.3b</td>
</tr>
</tbody>
</table>

*Degrees of infection and inflammation were graded as described in Materials and Methods. Two to six mice were used per experiment.

*Significantly different (P < 0.005) from the value for control *H. felis*-infected mice not treated with RDA as calculated by Student’s t test.*
lines decreased helicobacter infection in the mouse (29). On the other hand, there is data that suggests that oral vaccines do not require antibody since they are effective in mice deficient in antibody production (3).

Our data further showed that the effect of RDA on H. felis infection in the mouse was dependent on IFN-γ and IL-12, i.e., Th1 cytokines. This seems to contradict the prevailing notion that Th1 cytokines do not effect protective immunity as opposed to Th2 responses stimulated by cholera toxin-based vaccines. As mentioned, cholera toxin can induce a mixed Th1 and Th2 response and even Th1 responses. Furthermore, while some studies have shown that H. pylori infection in humans was associated with a Th1 cytokine response (1, 10, 16), others have shown that gastritis due to H. pylori was associated with fewer IFN-γ-producing cells in the gastric antrum than are seen in gastritis not due to H. pylori (18). It remains possible therefore, that Th1 immune responses are involved in controlling helicobacter infection. In a recent study by Blanchard et al. (3), systemic vaccination with complete Freund’s adjuvant, a strong inducer of Th1 responses, protected against H. felis infection in mice. This supports a role for Th1 responses in controlling helicobacter infection. The means by which Th1 cytokines effect protective immunity or decrease colonization by H. felis is not clear. Our data suggests that in the absence of IFN-γ and IL-12, there is a decrease in serum and secretory IgA levels. Previous work has shown that IL-12 administered intranasally can increase serum and secretory IgA levels in response to tetanus toxin (5). Therefore, it is tempting to speculate that these cytokines influence the infection by altering IgA responses. On the other hand, antibody responses do not seem to be required for the effectiveness of oral vaccines. The possibility that cellular responses influenced by IFN-γ and IL-12 are important in the control of helicobacter infection remains to be explored.

Viral infections influence T-cell responses to bacteria (48), but few studies have directly examined the relationship between viral infections and helicobacter. Studies of hepatitis

![Flow cytometry analysis of intracellular IL-4 and IFN-γ (top panels) or IL-4 and IL-2 (bottom panels) in CD4⁺ and CD8⁺ spleen cells isolated from mice at 10 weeks after infection with H. felis (left series of panels), 10 weeks after oral infection with 5 × 10⁶ H. felis bacteria, and 2 weeks after infection of 0.6 × 10⁶ PFU of RDA into each hind leg, twice over a 5-day period. The cell suspensions were incubated overnight with H. felis WCS antigen and then stimulated with phorbol myristate acetate (50 ng/ml; Sigma), ionomycin (5 ng/ml; Sigma), and 10 μg of brefeldin A (Sigma) per ml for 4 h. Three-color staining was performed as described in Materials and Methods. These results are representative of two experiments, each consisting of three mice. FITC, fluorescein isothiocyanate; PE, phycoerythrin.

![Viral infections influence T-cell responses to bacteria (48), but few studies have directly examined the relationship between viral infections and helicobacter. Studies of hepatitis.](image-url)

### Table 2. Effect of RDA on the severity of H. felis infection and gastric inflammation in IFN-γ- and IL-12-deficient mice

| Treatment        | Mouse strain | No. of mice | Mean degreea (±SEM) of:
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Infection</td>
</tr>
<tr>
<td>H. felis</td>
<td>IFN-γ⁻/⁻</td>
<td>9</td>
<td>9.1 ± 1.7</td>
</tr>
<tr>
<td>H. felis + RDA</td>
<td>IL-12 p40⁻/⁻</td>
<td>4</td>
<td>10.5 ± 1.0</td>
</tr>
<tr>
<td>H. felis + RDA</td>
<td>IFN-γ⁻/⁻</td>
<td>3</td>
<td>6.8 ± 3.1</td>
</tr>
<tr>
<td>H. felis + RDA</td>
<td>IL-12 p40⁻/⁻</td>
<td>3</td>
<td>7.2 ± 2.0</td>
</tr>
</tbody>
</table>

* Degrees of infection and inflammation were graded as described in Materials and Methods.
infection influencing helicobacter have focused primarily on hepatitis A as a surrogate marker of fecal-oral routes of transmission (23, 35). On the other hand, helicobacter infection was shown to influence viral infections in a study of vaccinia virus in the mouse. The decreased clearance of the vaccinia virus infection was mediated by a reduction in cytotoxic T-cell responses and Th1 cytokines associated with helicobacter infection (38). Helicobacter infection commonly occurs in childhood and remains chronic in spite of the development of an immune response (11, 15). Cohort and cross-sectional population studies indicate that the rate of spontaneous elimination of H. pylori infection is low (20, 27, 45); nonetheless, spontaneous eradication does occur. The mechanism leading to spontaneous clearance of helicobacter infection is not known. Furthermore, once helicobacter infection in humans is eradicated, the rate of reinfection is very low (27). One interpretation of this observation is that the immune response can become effective in protecting against helicobacter infection and may even participate in eliminating established infection. Our data suggest that the immune response can become effective after viral infection in the mouse.}

**ACKNOWLEDGMENTS**

This work was supported by grants from the Chedoke-McMaster Hospital Foundation and The Medical Research Council of Canada. K.C. gratefully acknowledges the award of an Ontario Ministry of Health Career Scientist Award.

We are grateful to Pam Lyn for skilled technical assistance.

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Editor: J. R. McGhee