Replication-Defective Adenovirus Infection Reduces Helicobacter felis Colonization in the Mouse in a Gamma Interferon- and Interleukin-12-Dependent Manner

BO JIANG,† MANEL JORDANA, ZHOU XING, FIONNA SMAILL, DENIS P. SNIDER, RAJKA BOROJEVIC, DARLENE STEELE-NORWOOD, RICHARD H. HUNT, AND KENNETH CROITORU*

Intestinal Diseases Research and Immunology and Infection Programs, Departments of Medicine and Pathology, McMaster University, Hamilton, Ontario, Canada

Received 24 February 1999/Returned for modification 9 April 1999/Accepted 29 June 1999

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 associated with systemic viral infection can influence an established H. felis infection.

The use of mucosal adjuvants like cholera toxin or the heat-labile enterotoxin of Escherichia coli in humans is potentially 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 associated with systemic viral infection can influence an established H. felis 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 (34). 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 × 10^8 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 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...
given an arbitrary value of 10,000 U/ml. Standard sera were diluted from 1:100 to
measure the activity in each of the isotype-specific assays. Each standard serum was
diluted, biotin-labeled secondary antibodies were added, and incubated for 60 min at 37°C. Wells were subsequently washed, and 100 l of substrate solution (containing 1 mg of
phospho-phenyl phosphate per ml) was added. The mixture was incubated for 10 min at room temperature. After washing with PBS, surface staining was carried out by incubating cells in 100 l of fluorescein isothiocyanate-conjugated anti-IFN-γ (0.1 µg) or anti-IL-2 (0.1 µg) and phycoerythrin-conjugated anti-IL-4 (0.2 µg) (Pharmingen) diluted in permeabilization buffer (PBS containing 1% saponin, 1% fetal calf serum, and 0.1% NaN₃) for 30 min. Finally, the cells were washed and fixed in 500 l of 1% paraformaldehyde. Data on a minimum of 10,000 events was collected for each sample on a Becton Dickinson FACScan using CellQuest software. Mononuclear cells were identified by their forward-by-side-scatter properties and were gated for analysis using PC Lysys software.

Statistical analysis. Data is presented as the mean value plus the standard error of the mean (SEM). Statistical significance was determined by the Student's t test or, if the data was not normally distributed, the nonparametric Mann-Whitney U test. Differences between groups were considered statistically significant at P < 0.05.

RESULTS

Effect of RDA infection on established H. felis colonization of the mouse stomach. CS75/6/6 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 infection, 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 CS75/6/6 mice, an increase in serum IgG2a H. felis-specific antibody was detected by 7 weeks after H. felis infection (Fig. 1). In contrast, minimal 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 CS75/6/6 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

Fisher sonic dismembrator (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 Acrodisc filter (Gelman Sciences, Ann Arbor, Mich.). The protein percentage was assayed as 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 re-
moved and fixed in 10% neutral buffered formalin and then embedded in par-
affin. 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 as previously described (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 inflamma-
tory 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, <5%; 2, 5 to 30%; 3, 30 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 the intensity, longitudinal extent, and vertical extent of inflammation was 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 murine human type 5 RDA carries a deletion of the E1 gene and a partially crippled E3 gene in the adenovirus 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 concen-
tration of 10⁶ PFU/100 µl. Each mouse was infected twice with 6 × 10⁶ PFU 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 was used as a standard for the ELISA was obtained from CS75/6/6 mice that had received four weekly immunizations with H. felis WCS antigen plus Freund's incomplete adjuvant (Gibco, Grand Island, N.Y.). Negative control serum was collected from naive CS75/6/6 mice. Serum was collected and stored at -20°C until use. To measure H. felis-specific antibody levels were deter-
mined by an isotype-specific ELISA. Heat-killed H. felis C57BL/6 mice. Serum was collected and stored at -20°C until use. To measure

H. felis colonization in the mouse stomach. CS75/6/6 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 CS75/6/6 mice, an increase in serum IgG2a H. felis-specific antibody was detected by 7 weeks after H. felis infection (Fig. 1). In contrast, minimal 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 CS75/6/6 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
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 their ability to produce IFN-γ following endotoxin administration.

**DISCUSSION**

RDA lacking functional E1 and E3 genes was constructed to serve as a vector capable of incorporating cytokine genes for in vivo delivery (2, 46). We have shown previously that RDA can influence the induction of the immune response to a protein allergen (41). In an effort to explore the utility of such vectors in modulating mucosal infections and inflammation, we examined the effect of RDA alone on chronic *H. felis* infection in the mouse. The results show that infection of mice with RDA led to a significant reduction of *H. felis* colonization in the stomachs of C57BL/6 mice but not in those of mice deficient in the IFN-γ or IL-12 cytokine gene. The effect of RDA on *H. felis* was independent of changes in the profile of antibody isotype responses but was associated with an increase in IFN-γ-producing splenic CD8+ T cells.

Previous work has documented that virus infection stimulates IFN-γ and IL-12 cytokine responses (7). Natural helicobacter infection is also associated with predominately Th1 responses characterized by increases in local IFN-γ (28). However, in the face of a vigorous immune response to helicobacter infection, this immune response is ineffective in eliminating the helicobacter infection. The reasons for this are not clear. Oral vaccines incorporating helicobacter antigens require mucosal adjuvants such as cholera toxin to effectively prevent or eliminate *H. felis* and *H. pylori* infections in the mouse (6, 25, 26, 28, 29, 37). These observations have led to the argument that cholera toxin shifts the immune response toward a Th2 response, leading to a more effective antibody response (37, 39). This argument is further supported by studies in which adoptive transfer of helicobacter-specific Th2 cell
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 op-
posed to Th2 responses stimulated by cholera toxin-based vac-
cines. 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 helico-
bacter 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 ef-
fact 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 toxoid (5). Therefore, it is tempting to speculate that
these cytokines influence the infection by altering IgA re-
sponses. On the other hand, antibody responses do not seem to
be required for the effectiveness of oral vaccines. The possi-
bility 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 be-
tween viral infections and helicobacter. Studies of hepatitis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mouse strain</th>
<th>No. of mice</th>
<th>Mean degree ± SEM of:</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. felis</em></td>
<td>IFN-γ−/−</td>
<td>9</td>
<td>9.1 ± 1.7</td>
</tr>
<tr>
<td><em>H. felis</em></td>
<td>IL-12 p40−/−</td>
<td>4</td>
<td>10.5 ± 1.0</td>
</tr>
<tr>
<td><em>H. felis</em> + RDA</td>
<td>IFN-γ−/−</td>
<td>3</td>
<td>6.8 ± 3.1</td>
</tr>
<tr>
<td><em>H. felis</em> + 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 cytokote 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 Helicobacter pylori infection is low (20, 27, 45); nonetheless, spontaneous eradication does occur. The mechanism leading to spontaneous elimination 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 and that the immune response can become effective and that the immune response can become effective and that the immune response can become effective.

**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.

**REFERENCES**


![FIG. 3. Mean (±SEM) anti-H. felis antibody isotype levels in serum and gut wash of: C57BL/6 mice (n = 6); IFN-γ knockout (KO) mice (n = 6); and IL-12 p40 knockout mice (n = 6). Samples were taken 8 to 10 weeks after oral infection with 5 × 10⁸ H. felis bacteria. Serum antibody was measured as described in Materials and Methods.](http://iai.asm.org/article/113/1/345/27)
in pathogenesis of Helicobacter-associated gastriﬁt: H. felis infection of in-
33. Papp, Z., D. M. Middleton, S. K. Mittal, L. A. Babiuk, and M. E. Baca-
Estrada. 1997. Mucosal immunization with recombinant adenoviruses: in-
duction of immunity and protection of cotton rats against respiratory bovine
S6–S9.
35. Pretolani, S., T. Stroffolini, M. Rapicetta, F. Bonvicini, L. Baldini, F. Me-
graud, G. C. Ghironzi, F. Sampogna, U. Villano, F. Cecchetti, G. Giulianelli,
M. L. Stefanelli, A. Armuzzi, F. Miglio, and G. Gasbarrini. 1997. Seropreva-
lence of hepatitis A virus and Helicobacter pylori infections in the general
population of a developed European country (the San Marino study): evi-
dence for similar pattern of spread. Eur. J. Gastroenterol. Hepatol. 9:
1081–1084.
37. Saldinger, P. F., N. Porta, P. Launois, J. A. Louis, G. A. Waanders, H.
Immunization of BALB/c mice with Helicobacter urease B induces a T helper
infection by Helicobacter pylori down-modulates virus-specific CD8+ cyto-
40. Snider, D. P., and B. J. Underdown. 1986. Quantitative and temporal anal-
yses of murine antibody response in serum and gut secretions to infection
Croitoru, and M. Jordon. 1998. Adenoviral infection inhibits allergic air-
CD4+ effector cells default to the Th2 pathway in interferon gamma-deﬁ-
production of cytokines by mucosal lymphocytes immunized by oral admin-
istration of keyhole limpet hemocyanin using cholera toxin as an adjuvant.
44. Wilson, M. E., B. M. Young, B. L. Davidson, K. A. Mente, and S. E.
McGowan. 1998. The importance of TGF-beta in murine visceral leishman-
elimination of Helicobacter pylori infection: clinical implications. Am. J.
Gastroenterol. 92:1780–1787.
Adenoviral vector-mediated interleukin-10 expression in vivo: intramuscular
gene transfer inhibits cytokine responses in endotoxemia. Gene Ther. 4:
140–149.
Woodland, and M. A. Blackman. 1996. Lethal synergism between inﬂuenza