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Ashoka Dey* Kenji Yokota† Keita Kobayashi‡

Keiji Oguma** Yoshikazu Hirai†† Tadaatsu Akagi‡‡

*Okayama University,

†Okayama University,

‡Okayama Univeristy,

**Okayama University,

††Okayama University,

‡‡Okayama University,

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Ashoka Dey, Kenji Yokota, Keita Kobayashi, Keiji Oguma, Yoshikazu Hirai, and Tadaatsu Akagi

Abstract

Helicobacter pylori (H. pylori) infection in the stomach is etiologically closely associated with chronic active gastritis, peptic ulcer, gastric cancer and gastric mucosa-associated lymphoid tissue lymphoma. In this study, we examined the antibody responses and cytokine profiles of three strains of mice (BALB/c, C3H/He, and C57BL/6) infected with H. pylori. Following this, correlations between host-immune reactions and intensity of inflammation were analyzed. H. pylori (ATCC43504) was intragastrically administered once a week to the mice from 4 weeks of age, and they were sacrificed at the ages of 4 and 7 months. In these mice, we examined the histology of the stomach, antibody titers against H. pylori, and serum levels of cytokines (IL-4, IL-10, TNF-alpha, IL-2 and Interferon-gamma). In BALB/c mice, inflammation of the stomach was minimal. Inflammation was observed in 63.6% of C57BL/6 mice and 33.3% of C3h/He mice. In C57BL/6 and C3H/He mice, all the cytokines tended to increase. In contrast, BALB/c mice were inactive in cytokine production except for IL-2. Two C3H/He mice developed severe inflammation with lymph follicles; one showed a response largely typical of Th-1, and the other showed a response largely typical of Th-2. Although a definite correlation was not shown between Th-1/Th-2 response evaluated by cytokine production and intensity of inflammation, it appears that in H. pylori-induced inflammation both cell-mediated (Th-1) and humoral (Th-2) immunity play a role in pathogenesis.

KEYWORDS: Helicobacter pylori, cytokine, humoral immunity, cell-mediated immunity, gastritis

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Antibody and Cytokine Responses in *Helicobacter pylori*-Infected Various Mouse Strains

Ashoka Dey[a], Kenji Yokota[b], Keita Kobayashi[a], Keiji Oguma[b], Yoshikazu Hirai[c] and Tadaatsu Akagi[a]*

[a]Second Department of Pathology, [b]Department of Bacteriology, Okayama University Medical School, Okayama 700-8558, Japan and [c]Department of Microbiology, Jichi Medical School, Tochigi 329-0431, Japan

Helicobacter pylori (H. pylori) infection in the stomach is etiologically closely associated with chronic active gastritis, peptic ulcer, gastric cancer and gastric mucosa-associated lymphoid tissue lymphoma. In this study, we examined the antibody responses and cytokine profiles of three strains of mice (BALB/c, C3H/He, and C57BL/6) infected with *H. pylori*. Following this, correlations between host-immune reactions and intensity of inflammation were analyzed. *H. pylori* (ATCC43504) was intragastrically administered once a week to the mice from 4 weeks of age, and they were sacrificed at the ages of 4 and 7 months. In these mice, we examined the histology of the stomach, antibody titers against *H. pylori*, and serum levels of cytokines (IL-4, IL-10, TNF-α, IL-2 and Interferon-γ). In BALB/c mice, inflammation of the stomach was minimal. Inflammation was observed in 63.6% of C57BL/6 mice and 33.3% of C3H/He mice. In C57BL/6 and C3H/He mice, all the cytokines tended to increase. In contrast, BALB/c mice were inactive in cytokine production except for IL-2. Two C3H/He mice developed severe inflammation with lymph follicles; one showed a response largely typical of Th-1, and the other showed a response largely typical of Th-2. Although a definite correlation was not shown between Th-1/Th-2 response evaluated by cytokine production and intensity of inflammation, it appears that in *H. pylori*-induced inflammation both cell-mediated (Th-1) and humoral (Th-2) immunity play a role in pathogenesis.

Key words: *Helicobacter pylori*, cytokine, humoral immunity, cell-mediated immunity, gastritis

It is generally accepted that *Helicobacter pylori (H. pylori)* infection of the stomach has a close causal association with chronic active gastritis, duodenal and gastric ulcers, and/or the predisposition to gastric cancer and mucosa-associated lymphoid tissue lymphoma (1-5). There have been many reports concerning the pathogenic factors of this organism. Urease, flagella, and various adhesins are considered to be factors necessary for the organism to survive and colonize (6). A cytotoxin named VacA and immunodominant antigens named CagA, which are encoded by *vacA* gene and *cagA* to *cagI* genes, respectively, are thought to be important in *H. pylori* pathogenesis (7-10). Oral inoculation of the VacA or *H. pylori* itself induced gastric and duodenal injuries in mice (11). Ghiara *et al.* (12) reported, however, that VacA plays an important role in the induction of gastric epithelial cell lesions, but not in producing inflammation. Urease activity was not important in producing the observed gastric damage, and other components which are present in *H. pylori* strains carrying the *cagA* gene but distinct from CagA itself, were involved in eliciting the inflammatory response (12).

Recently, some reports have suggested that host factors are also important for producing inflammation in *H. pylori* infection. Occurrence of atrophic gastric changes and inflammation has been reported to depend on mouse strains (13), and *H. pylori*-induced inflammation was associated with the major histocompatibility complex (MHC) (14). In this study, antibody response against organisms and cytokine production were examined in three different inbred mouse strains infected with *H. pylori*: BALB/c, C3H/He, and C57BL/6. The correlation between host immune reaction and severity of inflammation was also evaluated.

* To whom correspondence should be addressed.

Materials and Methods

Animals and bacteria. Four weeks old specific pathogen-free female mice (BALB/c, C3H/He, and C57BL/6) were purchased from Clea Japan (Tokyo). They were maintained in clean isolators and fed with sterilized commercial pellets (Oriental Yeast Co. Ltd., Chiba, Japan). *H. pylori* (ATCC43504) was obtained from American Type Culture Collection and cultured on Brucella agar (Difco, MI, USA) plates containing 7% horse blood in a microaerobic condition (N_2: 85%, O_2: 5%, CO_2: 10%, in aerobic globe box) at 37°C for 5 days.

Experimental design. *H. pylori* (10^7 CFU) was intragastrically administered to each mouse through a feeding needle once a week. The mice were sacrificed at the ages of 4 and 7 months, one week after their final inoculation with *H. pylori*. Following this, samples were taken from their blood and stomachs.

Histopathology. The stomachs were cut into strips, fixed in 10% buffered-formalin solution and embedded in paraffin. The sampling techniques were standardized so that two longitudinal samples contained the forestomach, fundic mucosa, antrum and duodenum. The 4 μm-thick tissue sections were processed with hematoxylin and eosin stain and Giemsa stain. Then, they were immunohistochemically analyzed using rabbit anti-*H. pylori* polyclonal antibody.

Inflammation score. Intensity of gastric inflammation was scored as follows (Fig. 1): Grade 0, no inflammatory changes in the gastric mucosa; Grade 1, mild infiltration of inflammatory cells which were confined to the junction of the mucosa and submucosa; Grade 2a, marked diffuse infiltration of inflammatory cells without lymph follicle formation, involving both the mucosa and submucosa; Grade 2b, localized inflammation with lymph follicle formation involving the mucosa and/or submucosa. Inflammatory cells were polymorphonuclear leucocytes with few lymphocytes, plasma cells and monocytes in Grade 1. Whereas, in Grade 2 (a, b), lymphocytes, plasma cells and monocytes were mainly observed.

Serum antibody assay. IgG antibody against *H. pylori* in the serum was measured using an enzyme-linked immunosorbent assay (ELISA). Each well of the microtiter plates was coated with 4 μg of whole cell sonicates of *H. pylori*. Sample sera were diluted 100 times with 10% skimmed milk in PBS, and reacted with *H. pylori* antigens for 2 h at room temperature. Specific antibodies against *H. pylori* were detected using peroxidase-conjugated anti-mouse IgG as the second antibody and O-phenylendiamine as the substrate. The reaction was stopped with 4N H_2SO_4. The optical density was measured by reading absorbances at 490 nm using a microplate reader (Bio-Rad Novopath, CA, USA).

Cytokine Assay. Sera contents of mouse interleukin (IL)-4, IL-10, IL-2, tumour necrosis factor (TNF)-α and interferon (IFN)-γ were estimated by sandwich enzyme immunoassay according to the manufacturer's instructions (Mouse Titer Screen II or Mouse Titer Screen III, Enzyme Immunoassay Kit, Perseptive Diagnostics Inc., MA, USA). A polystyrene 96-well plate was precoated with monoclonal antibodies to these cytokines. Serum samples were added to the precoated wells, and the cytokines in the sera were allowed to bind to the antibody. A paired-detecting rabbit antibody to each mouse cytokine was added, which binds to the respective captured mouse cytokines. After each step, excess components were removed by rinsing with a washing buffer solution included in the kit. Horseradish peroxidase-conjugated goat anti-rabbit IgG/streptavidine was then added. The unreacted enzyme conjugate was removed by washing. Then, TMB (3, 3′, 5, 5′,-tetramethyl benzidine) as a substrate and hydrogen peroxide were added to the wells. The optical density of the colored reaction products was determined at 450 nm with a microplate reader. Sample concentration of mouse IL-4, IL-10, IL-2, TNF-α and IFN-γ was determined from a standard curve obtained by assaying serial dilutions of respective cytokines. The whole procedure was done at room temperature. The detection limit of mouse IL-4, IL-10, IL-2, TNF-α, and IFN-γ was 11.6, 78.4, 4.92, 13.8, and 1.74 pg/ml, respectively.

Results

Histopathological evaluation. The extent of inflammation varied among the mouse strains. In BALB/c mice, only one mouse showed inflammation of Grade 1 at 6 months after inoculation. In contrast, 6 of 18 (33.3%) C3H/He mice developed gastritis, as did 7 of 11 (63.6%) of C57BL/6 mice (Table 1). Severe gastritis with lymph follicles (Grade 2b) was observed in two C3H/He mice (C-301 and J-302) at 3 months after inoculation (Fig. 2). The colonization of *H. pylori* was detected in all inoculated mice by Giemsa staining and

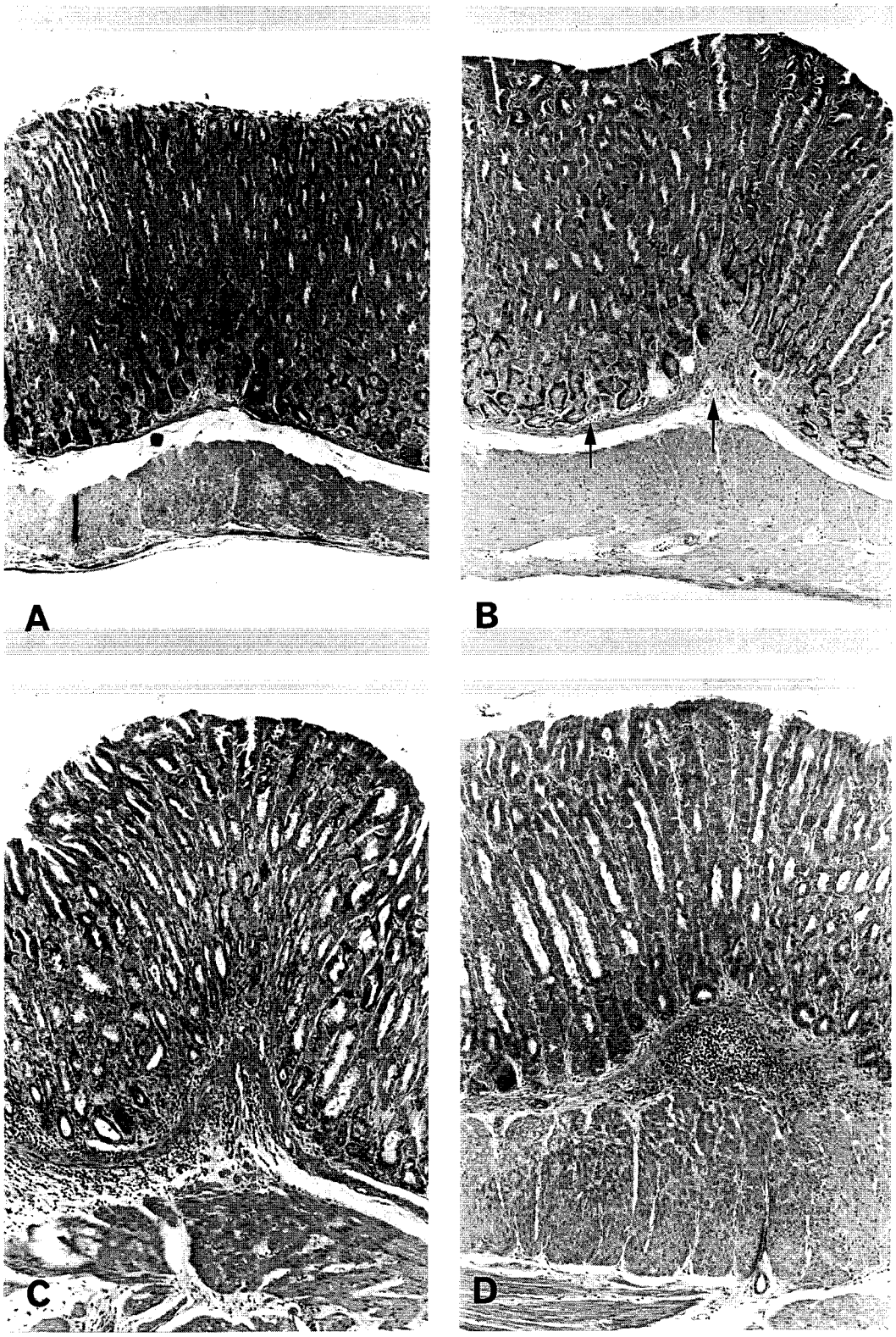

Fig. 1 Grading of inflammation. Arrows indicate the minimal accumulation of inflammatory cells. **A**: Grade 0; **B**: Grade 1; **C**: Grade 2a; **D**: Grade 2b.

Table 1 Histological findings of the stomach evaluated by histological grade and colonization of *Helicobacter pylori*

Mouse strain	Period after *H. pylori* inoculation (months)	Total number of mice examined	Total inoculations	Number of mice with:				
				Histological grade				Colonization of *H. pylori*
				0	I	2a	2b	
BALB/c	0 (Negative control)	5	0	5	0	0	0	0
C3H/He	0	3	0	3	0	0	0	0
C57BL/6	0	5	0	5	0	0	0	0
BALB/c	3	4	12	4	0	0	0	4
C3H/He	3	11	12	7	2	0	2	11
C57BL/6	3	6	12	2	3	1	0	6
BALB/c	6	11	24	10	1	0	0	11
C3H/He	6	7	24	5	1	1	0	7
C57BL/6	6	5	24	2	1	2	0	5

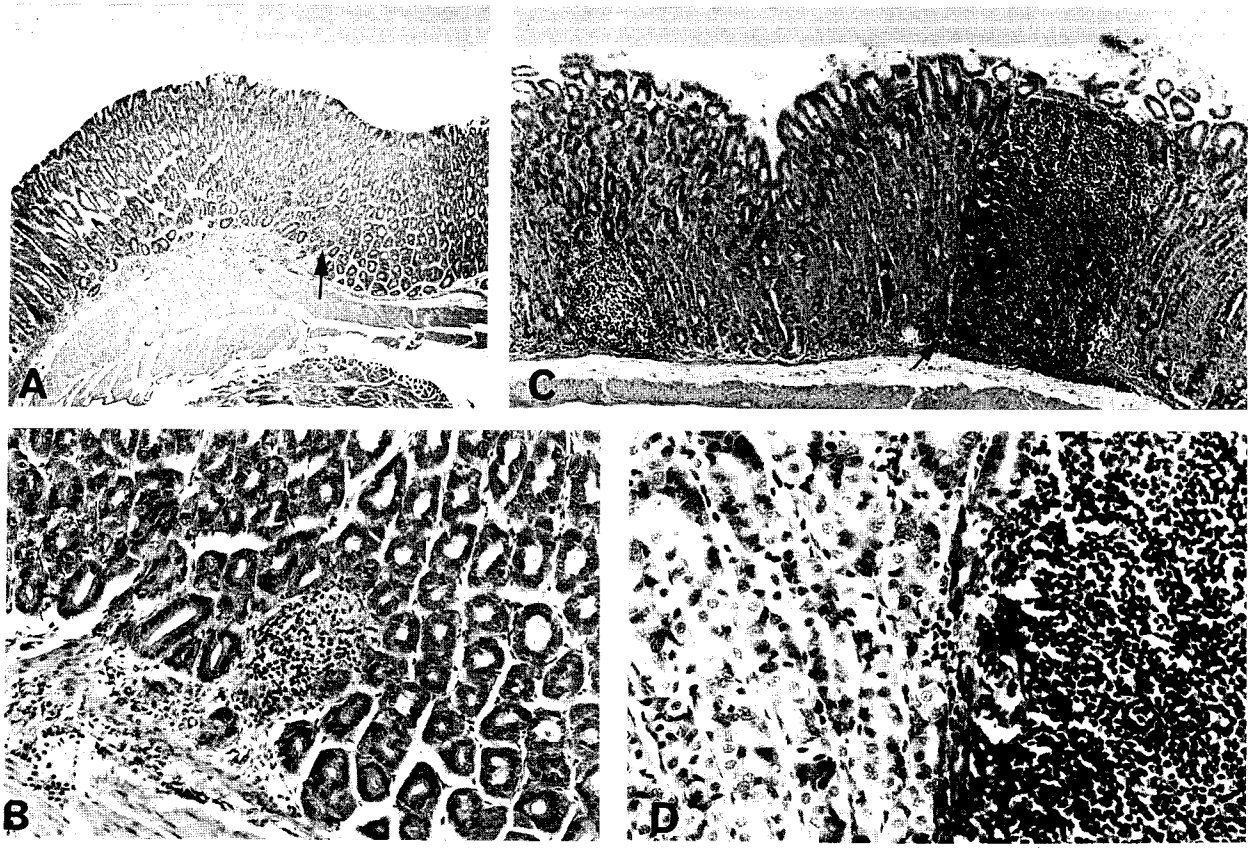

Fig. 2 Gastric histology of two C3H/He mice which developed severe gastritis with lymph follicles. **A** and **B**: J302; **C** and **D**: C301. Arrows indicate lymph follicles in the mucosa.

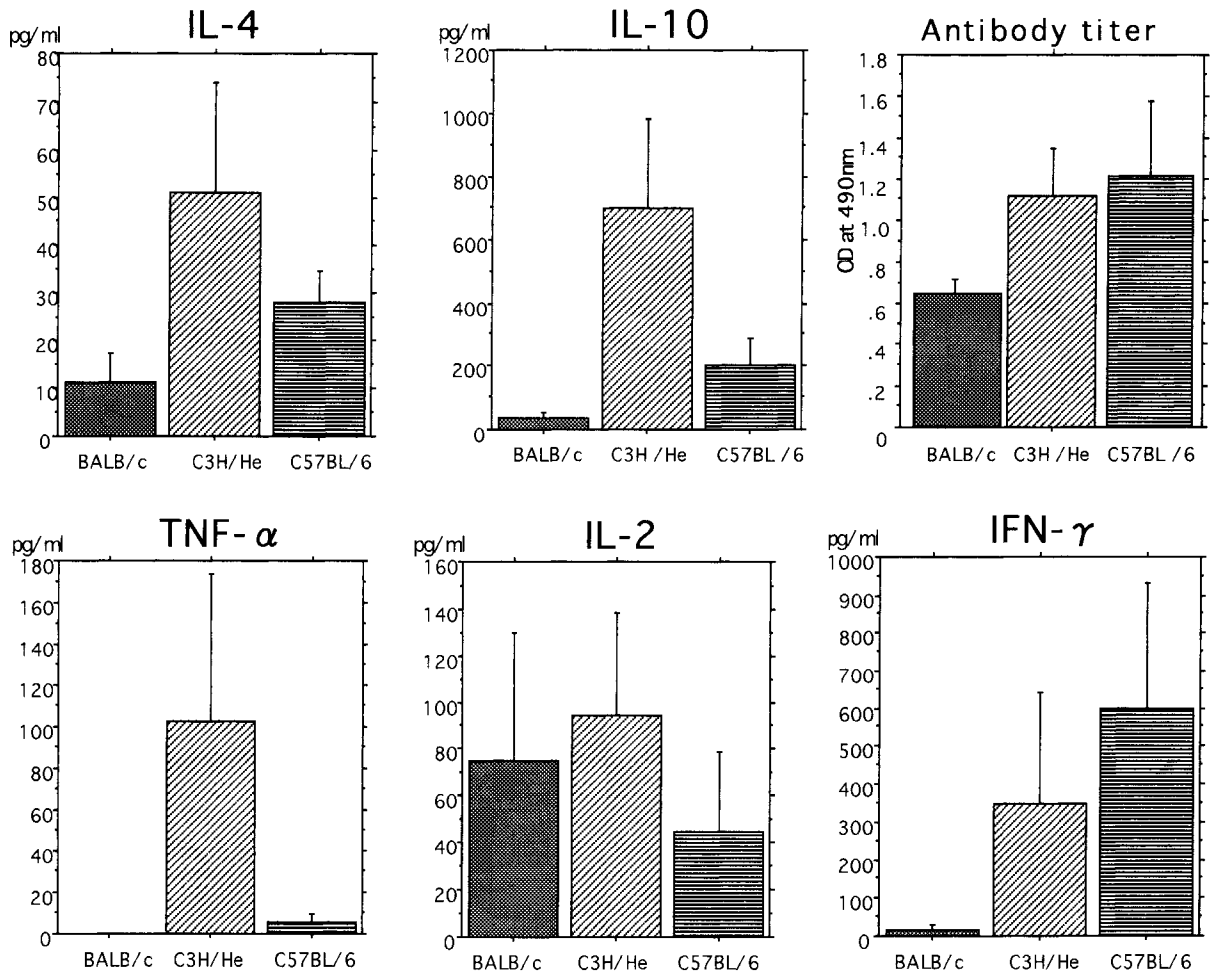

Fig. 3 Levels of the various cytokines and anti-*H. pylori* antibody titers in three different strains of mice after oral inoculation with *H. pylori*, which were estimated at the age of 4 or 7 months. (BALB/c, n = 15; C3H/He, n = 18; C57BL/6, n = 11). The values are expressed as the mean ± S.E.

immunohistochemistry.

Cytokine levels and anti-H. pylori antibody titers in different strains of mice. Antibody titers and cytokine levels in different mouse strains estimated at the age of 4 or 7 months are shown in Fig. 3. Serum IL-4 levels of BALB/c, C3H/He and C57BL/6 mice were 11.2 ± 6.3, 50 ± 22.9, and 28 ± 6.6 pg/ml, respectively, and serum IL-10 levels of these mice were 34 ± 20.3, 698 ± 287.4, and 203 ± 84.8 pg/ml, respectively. C3H/He mice showed the highest serum IL-4 and IL-10 levels. TNF-α in sera was highest in C3H/He mice and undetectable in BALB/c mice. Serum IL-2 levels were not markedly different among these three strains: BALB/c, 75.2 ± 52; C3H/He, 94.5 ± 43.5; C57BL/6, 44.7 ± 34.0 pg/ml. Serum IFN-γ levels were high in C3H/He mice (346 ± 295.7 pg/ml) and C57BL/6 mice (600 ± 330.6 pg/ml), but low in BALB/c mice (16 ± 10.4 pg/ml). The average antibody titers against *H. pylori* antigens of BALB/c, C3H/He and C57BL/6 mice were 0.65 ± 0.08, 1.12 ± 0.97, and 1.2 ± 0.37 (OD at 490 nm), respectively. Serum levels of these cytokines and antibody titers against *H. pylori* were under detection limits in the uninfected control mice.

Cytokine profile of two C3H/He mice which developed severe gastric inflammation with lymph follicle formation. Cytokine profiles in sera and *anti-H. pylori* antibody titres of two C3H/He mice (mouse numbers C-301 and J-302), which developed severe inflammation with lymph follicles, are

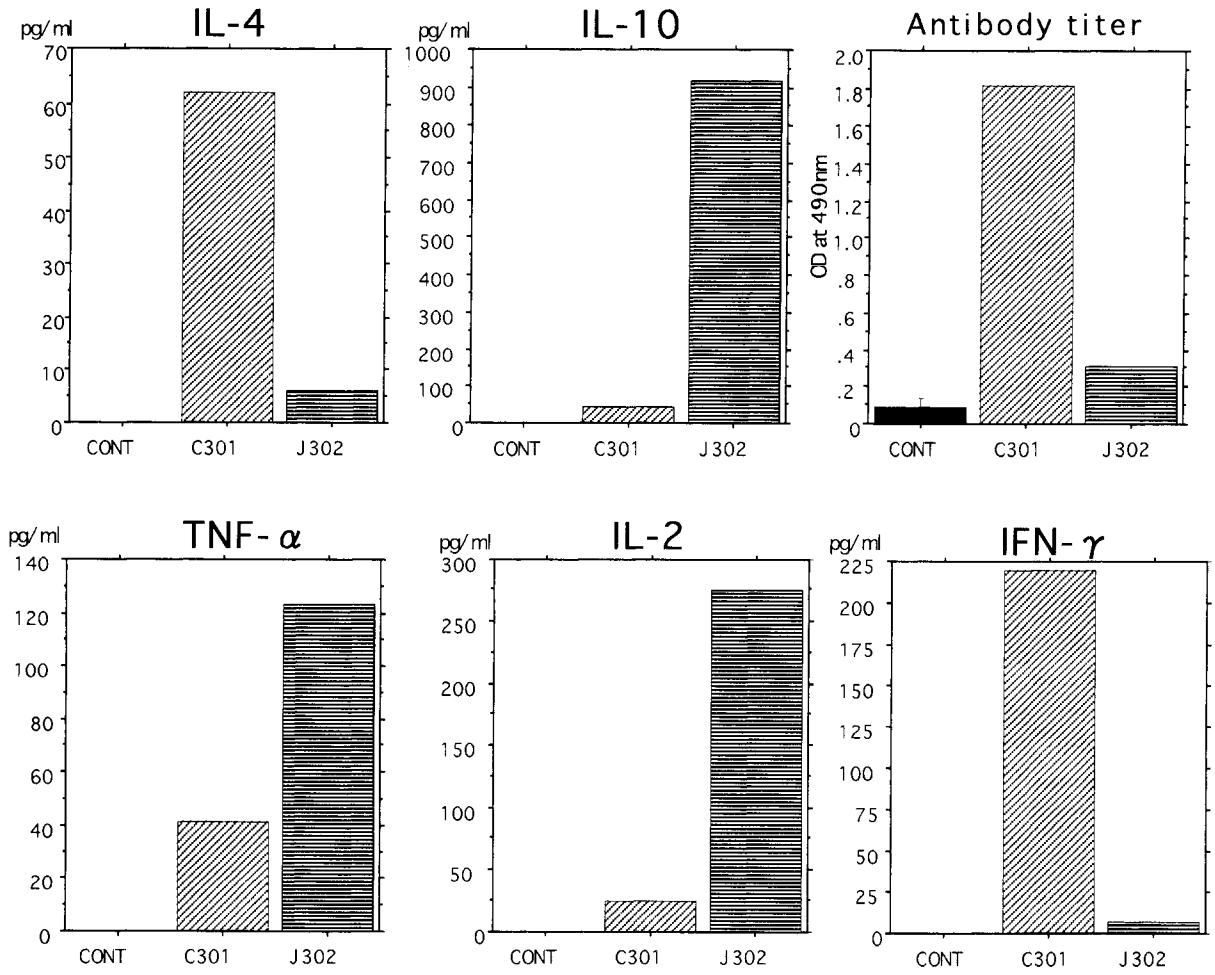

Fig. 4 Cytokine levels and anti-*H. pylori* antibody titers in two C3H/He mice which developed severe gastritis with lymph follicles after oral inoculation with *H. pylori*. The values were estimated at the age of 4 months.

shown in Fig. 4. In the C-301 mouse, IL-4 and IFN-γ levels and antibody titers were elevated. In contrast, J-302 mouse showed high TNF-α, IL-2, and IL-10 levels.

Discussion

The prevalence of *H. pylori* infection in Japanese adults has been estimated at between 50% to 90%. However, only a relatively small minority of these infected individuals progress to gastroduodenal diseases. One hypothesis to explain the discrepancy between the prevalence of *H. pylori* infection and the limited occurrence of ulcers is the possible existence of ulcerogenic strains of *H. pylori*. However, it remains unclear as to whether patients with peptic ulcer are infected with these ulcerogenic strains. Alternatively, host immunity might also play a role in *H. pylori* infection and its pathogenesis. T helper (Th) cells are responsible for both humoral and cell-mediated immunity (CMI) in response to different infectious agents. The response of Th cells can be divided into two groups according to their patterns of cytokine production. Th-1 cells produce IFN-γ, IL-2 and TNF-α, which mediate macrophage activation and delayed-type hypersensitivity reactions, whereas Th-2 cells produce IL-4, IL-5, IL-6 and IL-10, which act as growth and/or differentiation factors for B cells, thus providing a simple explanation for the observed dichotomy of immune responses (15).

In the present study, we examined the inflammatory response of the stomach and serum cytokine profiles in different inbred strains of mice after oral administration of

a single *H. pylori* strain. The least inflammation was observed in the BALB/c mice, which were inactive in cytokine production except for IL-2, compared with the other two mouse strains. It is known that the immune response of BALB/c mice tends to be of the humoral (Th-2) variety, whereas that of C57BL/6 mice tends to be of the cell-mediated (Th-1) variety (16, 17). In this study, however, cytokine profiles of the three strains of mice infected with *H. pylori* infection were complex, and simple classification of these mouse strains into Th-1 or Th-2 immune responses was difficult. C57BL/6 mice showed elevation of all cytokine levels, although they tended to show a relatively higher IFN-γ response. Therefore, C57BL/6 mice showed Th-2 response in addition to Th-1 immune response. Most of C57BL/6 had a tendancy to develop gastric inflammation. C3H/He mice also showed elevation of all cytokine levels but the degree of elevation varied markedly among individuals. Inflammatory changes were also various among different individuals. Moreover, all three strains showed high antibody titers; however, there was no correlation between the antibody titers and the severity of inflammation. Likewise, cytokine levels were individually various in all mouse strains.

Interestingly, two mice of the C3H/He strain developed severe inflammation with lymph follicle formation (Grade 2b). One mouse (J-302) had high TNF-α and IL-2 levels and a low antibody titer in the serum, indicating that this mouse showed a predominantly Th-1 reaction. The other mouse (C-301) had high antibody and IL-4 levels, which corresponds to a Th-2 response. Large variations in the severity of disease were observed after infecting different inbred and congenic mouse strains with a single isolate of *H. feris* or *H. pylori* (13, 14). Our data indicated that the association between inflammation and the nature of the immune response, *i.e.*, Th-1 or Th-2 immune response was not definite. The individual J-302 and C-301 mice tended to show Th-1-predominant and Th-2-predominant reactions, respectively, but both developed severe gastritis. It appears that Th-1 and Th-2 responses co-regulate and mutually influence each other, and this self regulation prevents inappropriate immune reactions against host (15). The J-302 mouse showed a predominantly Th-1 response, but its IL-10 level was also high. IL-10 is known as an inhibitory cytokine of Th-1 cells and macrophage activation. In this case, the IL-10 might not be enough to downregulate Th-1 reactions. While the C-301 mouse showed a predominantly Th-2 response, its IFN-γ was also high. Usually, IFN-γ inhibits the Th-2 reaction, but it may not have been present in sufficient quantities in the C-301 mouse to exert this influence. Thus, the immune reactions in the *H. pylori*-infected mice may not be controlled only by cytokine itself. The reactions in the two C3H mice suggest that the Th-1/Th-2 balance may be important. However, further studies are needed to draw a definite conclusion.

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