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Influence of *Helicobacter pylori* infection on development of stress-induced gastric mucosal injury

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Abstract: Immediately after the Great Hanshin Earthquake in Kobe in 1995, the recurrence rate of peptic ulcer in patients infected with *Helicobacter pylori* was higher than that in patients in whom *H. pylori* had been eradicated. We evaluated the influence of *H. pylori* infection on stress-induced gastric mucosal injury in Mongolian gerbils and C57BL/6 mice. These animals were immersed in water for 30, 120, and 720 min 12 weeks after inoculation with *H. pylori*, and then killed to assess gastric mucosal damage, and to measure cytokine production (interleukin [IL]-1β, IL-4, IL-6, and IL-10; interferon [IFN]-γ; and tumor necrosis factor [TNF]-α) in the gastric tissue of the mice. The stress treatment for 30 min resulted in a significantly higher bleeding rate and bleeding index among infected gerbils and mice compared with results in uninfected animals. Conversely, the bleeding and ulcer indexes were significantly higher in uninfected gerbils after 720 min of the stress treatment than in infected gerbils. Prior to the stress treatment, gastric IL-1β and IFN-γ production was significantly higher in the infected group than in the uninfected group. After 120 min of the stress treatment, TNF-α production was increased in the infected group, and IL-1β and IL-10 production was increased in the uninfected group. However, the production of these cytokines showed no change at 30 min of the stress treatment. These results suggest that *H. pylori* infection influences the development of gastric mucosal injury in the early phase of stress exposure; cytokines do not play a major role in this process.

Key words: *Helicobacter pylori*, gastric ulcer, water-immersion stress, cytokine, animal model

Introduction

Helicobacter pylori infection is closely correlated not only with gastritis and peptic ulcers but also with gastric carcinoma and lymphoma.[1–3] The finding that recurrence of peptic ulcer is prevented by eradication of *H. pylori* strongly supports the causal role of *H. pylori* in ulcer formation.[4] Although the precise mechanism of peptic ulcer formation has not yet been clarified, pathogenic bacterial strains, host susceptibility, the use of non-steroid anti-inflammatory drugs (NSAIDs), smoking, and stress have been implicated as possible contributory factors.[5–7] Regarding the role of stress, we can recall the experience in the 1995 Great Hanshin Earthquake in Kobe, which placed tremendous stress on its victims. After the earthquake, an increase in the number of patients with peptic ulcer was observed clinically. Upper digestive tract endoscopy in earthquake victims with epigastralgia revealed that gastric ulcers had recurred in all patients infected with *H. pylori*, but not in patients in whom *H. pylori* had been eradicated. The recurrence rate of duodenal ulcers was 93% in patients infected with *H. pylori*, but was zero in *H. pylori*-eradicated patients.[8] It has been suggested that psychological stress probably functions as a co-factor in *H. pylori*-related ulcer diseases.[7] These findings suggest the involvement of *H. pylori* infection in stress-induced peptic ulcers. In addition, a recent study showed that *H. felis* infection augmented stress-induced gastric lesion.[9] To investigate the effects of *H. pylori* infection on stress-induced gastric mucosal injuries, we assessed gastric mucosal injuries induced by water-immersion in Mongolian gerbils and C56BL/6 mice, as these animals can be infected with *H. pylori*, and the infection leads to the development of gastric ulcers and/or gastritis.[10–12] We also measured the levels of cytokines (interleukin [IL]-1β, IL-4, IL-6, and IL-10; interferon (IFN)-γ; and tumor necrosis factor [TNF]-α) in the gastric tissue in the C56BL/6 mice, because *H. pylori* infection alters the

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production of various cytokines, some of which are induced by psychological stress and contribute to the development of gastric mucosal injuries and inflammatory reactions.[13–15]

Materials and methods

Animals and bacteria

Six-week-old male specific pathogen-free (SPF) Mongolian gerbils (MGS/Sea) (purchased from Seac Yoshintomi, Fukuoka, Japan) and 6-week-old female SPF C57BL/6 mice (purchased from Charles River Japan, Kanagawa, Japan) were used, following the guideline for use of laboratory animals of Hyogo College of Medicine. The animals were kept in our animal house at housing conditions of: five per cage; room temperature, 23°C; humidity, 50%; and 12-h alternating light and dark periods. They were fed on CE-2 (Clea Japan, Tokyo, Japan). After a 2-week equilibrium period, these animals were used in the following experiments.

Sydney strain (SS1) of *H. pylori* (University of New South Wales, Sydney, Australia) was incubated overnight by the shake-culture method under a microaerobic environment at 37°C (Anaero Pack Helico, Mitsubishi Gas Chemical, Tokyo, Japan), using a Brucella Broth culture medium containing 5% inactivated horse serum and 1% β-cyclodextrin. Bacterial suspension was prepared at a concentration of 10^8 colony-forming units (CFU)/ml, using Brucella Broth culture medium, and used for inoculation challenge to animals.

Experimental schedule

When animals were 8 weeks old, 41 Mongolian gerbils and 48 C57BL/6 mice were administered 0.5 ml of *H. pylori* culture solution once a day through a gastric tube for 3 consecutive days. The remaining animals were not infected with the bacterium. The animals infected with *H. pylori* and those not infected were caged separately for 12 weeks under the above-described conditions. The animals were subjected to water-immersion 12 weeks after *H. pylori* infection, when chronic gastritis, but not ulcers, was known to be present.[16]

According to the method of Takagi and Okabe,[17] each animal was restrained and immersed in water at 21°C in an upright position, up to its xiphoid process, for 30, 120, or 720 min. Immediately after the immersion, the stomach was removed, and an incision was made along the greater curvature. Bleeding and mucosal lesions were macroscopically assessed, and the excised stomach was then cut in half at the lesser curvature. One half was immediately frozen in liquid nitrogen. Part of the other half was cut out for the rapid urease test,[18] and the remaining stomach was fixed in 10% formalin. The number of animals in each group was 8–19, and immersion times in the control groups for both the *H. pylori*-infected and uninfected animal were designated as zero.

Assessment of gastric lesions and colonization

As the stomachs of the Mongolian gerbils and the mice were small, we used the ulcer index, bleeding rate, and bleeding index for analyses. The bleeding rate and the bleeding index were determined from macroscopic assessments. The bleeding index was determined as follows: 0 points for no bleeding, 1 point for mild bleeding (a small amount of coagula in the stomach), 2 points for moderate bleeding (intermediate between 1 point and 3 points), and 3 points for severe bleeding (contents of the stomach were filled with blood). A bleeding index of 1 point or more was considered a "positive" bleeding rate. The ulcer index (sum of the length of ulcers, including erosions) of gastric mucosal lesions was assessed according to Takagi and Okabe.[17] These assessments were conducted in a blind fashion by one of the authors who did not administer the water-immersion treatment. The specimens fixed in formalin were embedded in paraffin, sliced finely, and stained with hematoxylin-eosin (H&E) and Giemsa to assess the mucosal inflammation microscopically and to examine for the presence of *H. pylori*, respectively. To confirm the infection, we used the rapid urease test, employing a CLO test kit (Delta West, Bentley, Australia), and the results were assessed 24 h later. The animals were judged to be infected by *H. pylori* when the result of either the urease test or the Giemsa staining was positive. The results of the CLO test were always positive in Mongolian gerbils and C57BL/6 mice infected with *H. pylori*. In contrast, none of the uninfected animals tested positive.

Cytokine measurement

The concentrations of IL-1β, IL-4, IL-6, IL-10, IFN-γ, and TNF-α in the gastric tissue of C57BL/6 mice were measured by enzyme-linked immunosorbent assay (ELISA). First, 2 ml of phosphate-buffered saline (PBS) containing 0.1% sodium dodecylsulfate (SDS) and 0.1% Tween 20 was added to each frozen specimen. The specimen was then homogenized for 90 s in cold water. After overnight storage at −20°C, it was centrifuged at 400 *g* for 15 min at 4°C. The supernatant was analyzed with a Biotrak mouse cytokine ELISA system (Amersham International, Buckinghamshire, England). Results were expressed in terms of concentration per mg protein. SPECTRAmax 250 (Molecular Devices, Sunnyvale, CA, USA) was used as the ELISA reader,

and protein was measured using Bicinchoninic acid (BCA) Protein Assay Reagents (Pierce Chemical, Rockford, IL, USA).

Statistical analysis

Values for results were expressed as means ± SD. Fisher's exact probability test was used to analyze bleeding rate, and the Wilcoxon rank-sum test (an unpaired test) was used to analyze the other parameters. Two-way analysis of variance (ANOVA) was carried out with these data, including an evaluation of the effect of the interaction between *H. pylori* infection and duration of stress on cytokine production. All P values were calculated by a two-sided test, and a P value of less than 0.05 was considered significant.

Results

Macroscopic findings

Mongolian gerbils. When the Mongolian gerbils were not subjected to the water-immersion treatment, bleeding and gastric mucosal lesions were not seen macroscopically in either the infected or uninfected group. When the Mongolian gerbils were subjected to the stress treatment (except at 30-min stress), bleeding and the formation of ulcers were observed regardless of whether or not the animals were infected with *H. pylori* (Table 1).

After 30 min of the stress treatment, the bleeding rate and bleeding index in the infected group were signifi cantly higher than those in the uninfected group (P = 0.036 and P = 0.038, respectively). The ulcer index in the infected group was higher than that in the uninfected group. After 120 min of the stress treatment, the bleeding rate, bleeding index, and ulcer index in the infected group were, conversely, lower than those in the infected group. After 720 min of the stress treatment, the bleeding index and ulcer index in the infected group were significantly lower than those in the uninfected group ($P < 0.01$ and P = 0.043, respectively).

C57BL/6 mice. When C57BL/6 mice were not subjected to the water-immersion treatment, bleeding and gastric mucosal lesions were not observed macroscopically in either the infected or uninfected group. When the mice were subjected to the stress treatment (except at 30-min stress), bleeding and the formation of ulcers were observed regardless of whether or not the animals were infected with *H. pylori* (Table 2).

Table 1. Comparisons of bleeding rates, bleeding indexes, and ulcer indexes in uninfected and infected groups of Mongolian gerbils with water-immersion stress

Rate or index of mucosal lesion	*Helicobater pylori*	Immersion period (min)			
		0	30	120	720
Bleeding rate (%)[a]	Uninfected	0/13 (0)	0/8 (0) **	6/8 (75.0)	9/9 (100)
	Infected	0/15 (0)	5/8 (62.5)	2/8 (25.0)	9/10 (90.0)
Bleeding index[a]	Uninfected	0	0 **	1.25 ± 1.16	3.00 ± 0 ****
	Infected	0	0.63 ± 0.52	0.25 ± 0.46	1.40 ± 0.84
Ulcer index[a]	Uninfected	0	0	1.25 ± 1.75	9.67 ± 3.20 **
	Infected	0	0.56 ± 0.62	0.25 ± 0.46	5.95 ± 4.19

Differences in bleeding rates were analyzed by Fisher's exact probability test, and other differences were analyzed by Wilcoxon rank-sum test (* $P < 0.05$; ** $P < 0.01$)

[a] See text for explanation

Tabke 2. Comarisons of bleeding rates, bleeding indexes, and ulcer indexes in uninfected and infected groups of C57BL/6 mice with water-immersion stress

Rate or index of mucosal lesion	*H. pylori*	Immersion period (min)			
		0	30	120	720
Bleeding rate (%)	Uninfected	0/19 (0)	2/18 (11.1) ****	7/9 (77.8)	8/9 (88.9)
	Infected	0/14 (0)	10/17 (58.8)	7/9 (77.8)	7/8 (87.5)
Bleeding index	Uninfected	0	0.17 ± 0.51 **	1.44 ± 1.01	1.22 ± 0.67
	Infected	0	0.71 ± 0.77	0.89 ± 0.60	1.25 ± 0.71
Ulcer index	Uninfected	0	0	0.67 ± 1.09	2.39 ± 1.96
	Infected	0	0.06 ± 0.18	0.56 ± 0.73	2.31 ± 1.49

Differences in bleeding rates were analyzed by Fisher's exact probability text, and other differences were analyzed by Wilcoxon rank-sum test (* $P < 0.05$; ** $P < 0.01$)

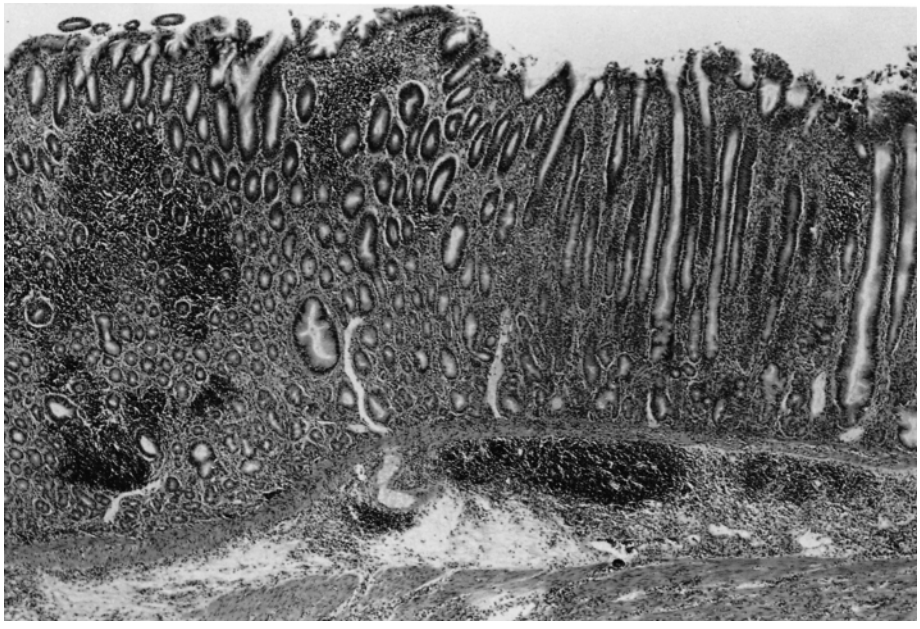

Fig. 1. Histopathological findings of *Helicobacter pylori*-infected Mongolian gerbil 12 weeks after inoculation (non-stress treatment group). Infiltration of inflammatory cells into the lamina propria is observed. H&E, ×34

After 30 min of the stress treatment, the bleeding rate in the infected group was significantly higher than that in the uninfected group ($P < 0.01$). Although the bleeding index in the infected group was significantly higher than that in the uninfected group ($P = 0.035$), there was no significant difference in the ulcer index between the two groups. After 120 and 720 min of the stress treatment, there were no significant differences in the bleeding rates, bleeding indexes, or ulcer indexes between the two groups.

Microscopic findings

When Mongolian gerbils and C57BL/6 mice were infected with *H. pylori*, the infiltration of inflammatory cells, consisting primarily of mononuclear cells, into the lamina propria was observed histologically (Figs. 1, 2). Hyperplastic regeneration of epithelium, pit abscess, and formation of lymphoid follicles were also observed. In *H. pylori*-intected and *H. pylori*-uninfected animals, focal necrosis of the epithelium with hemorrhaging (UL-I) was detected in those subjected to water-immersion, but there was no infiltration of inflammatory cells within the lesions (Figs. 3, 4). Thus, stress treatment did not induce histological inflammation, nor did it influence the histological degree of inflammation associated with *H. pylori*.

Profiles of cytokine production in gastric tissue of C57BL/6 mice

IL-1β. Prior to the stress treatment, the production of IL-1β was significantly higher in the infected group (92.82 ± 25.39 pg/mg protein), than in the uninfected group (26.88 ± 15.10 pg/mg protein) ($P < 0.01$). In the uninfected groups, the production was significantly higher in the 120-min stress treatment group (45.58 ± 12.17 pg/mg protein) than in the non-stress treatment group ($P < 0.01$). In contrast, the production was significantly lower in the 720-min stress treatment group (16.82 ± 6.21 pg/mg protein) than in the 30-min (37.56 ± 17.62 pg/mg protein), and 120-min stress treatment groups ($P < 0.01$). Similarly, in the infected groups, the production was significantly lower in the 720-min stress treatment group (36.60 ± 34.46 pg/mg protein) than in the non-stress treatment, the 30-min (81.75 ± 48.07 pg/mg protein) and the 120-min (95.00 ± 59.21 pg/mg protein) stress treatment groups ($P < 0.01$) (Fig. 5a). Results of two-way ANOVA included F values of 6.55 ($P = 0.0004$), 47.37 ($P < 0.0001$), and 2.09 ($P = 0.11$) for water-immersion, *H. pylori* infection, and the combination of these two factors, respectively.

IL-4. Prior to the stress treatment, there was no significant difference in the production of IL-4 between the infected and uninfected groups. In both the infected and the uninfected groups, the production was higher in the 120-min stress treatment group than in the non-stress treatment group, whereas the production was slightly lower in the 720-min stress treatment group than in the 120-min stress treatment group; however, these differences were not significant (Fig. 5b). Results of two-way ANOVA included F values of 2.20 ($P = 0.097$), 0.74 ($P = 0.39$), and 0.71 ($P = 0.55$) for water-immersion, *H. pylori* infection, and the combination of these two factors, respectively.

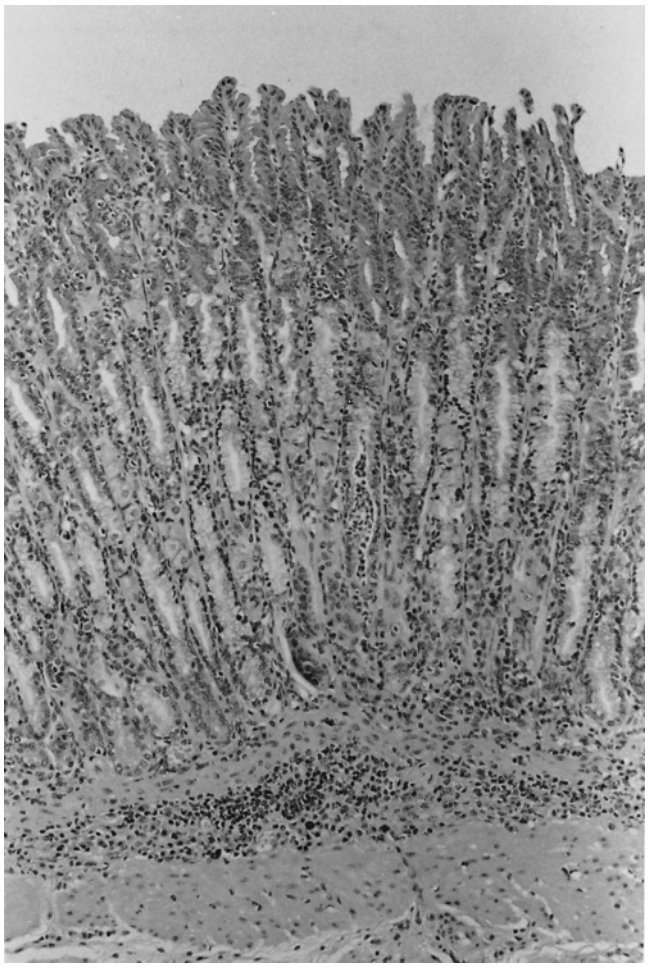

Fig. 2. Histopathological findings of *H. pylori*-infected C56BL/6 mouse 12 weeks after inoculation (non-stress treatment group). Infiltration of inflammatory cells into the lamina propria is observed. H&E, ×89

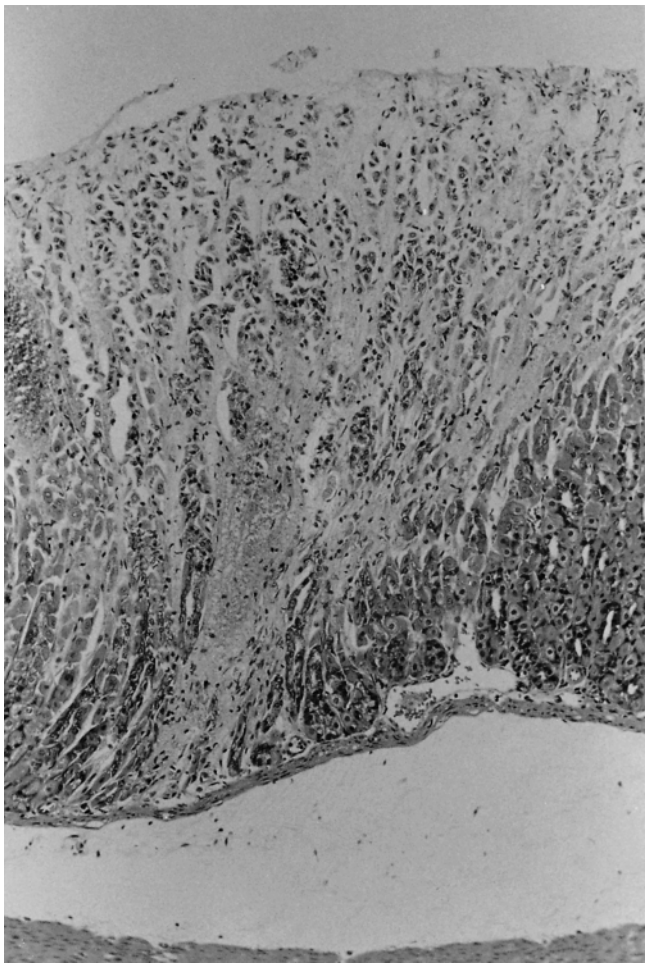

Fig. 3. Gastric mucosal lesion induced by water-immersion stress in *H. pylori*-uninfected Mongolian gerbil (720-min stress treatment group). Focal necrosis of the epithelium is observed. H&E, ×89

IL-6. Prior to the stress treatment, there was no significant difference in the production of IL-6 between the infected and uninfected groups. In the uninfected groups, the production was higher in the 720-min stress treatment group than in the non-stress treatment and 120-min stress treatment groups but the difference was not significant. Similar tendencies were observed in the infected groups, but, again, these differences were not significant (Fig. 5c). Results of two-way ANOVA included F values of 0.91 ($P = 0.44$), 0.36 ($P = 0.55$), and 0.30 ($P = 0.83$) for water-immersion, *H. pylori* infection, and the combination of these two factors, respectively.

IL-10. Prior to the stress treatment, there was no significant difference in the production of IL-10 between the infected and uninfected groups. In the uninfected groups, the production was significantly higher in the 120-min stress treatment group (3712 ± 1226 pg/mg protein) than in the non-stress treatment (1593 ± 1037 pg/mg protein) and 30-min stress treatment (1857 ± 1481 pg/mg protein) groups ($P < 0.01$ and $P = 0.020$, respectively), whereas the production was significantly lower in the 720-min stress treatment group (1950 ± 1069 pg/mg protein) than in the 120-min stress treatment group ($P < 0.01$). Similar tendencies were detected in the infected groups, but the differences were not significant (Fig. 5d). Results of two-way ANOVA included F values of 6.60 ($P = 0.0006$), 0.025 ($P = 0.88$), and 0.27 ($P = 0.85$) for water-immersion, *H. pylori* infection and the combination of these two factors, respectively.

IFN-γ. Prior to the stress treatment, the production of IFN-γ was significantly higher in the infected group (58.38 ± 36.34 pg/mg protein) than in the uninfected group (7.08 ± 10.10 pg/mg protein) ($P < 0.01$). In the uninfected groups, the production was higher in the

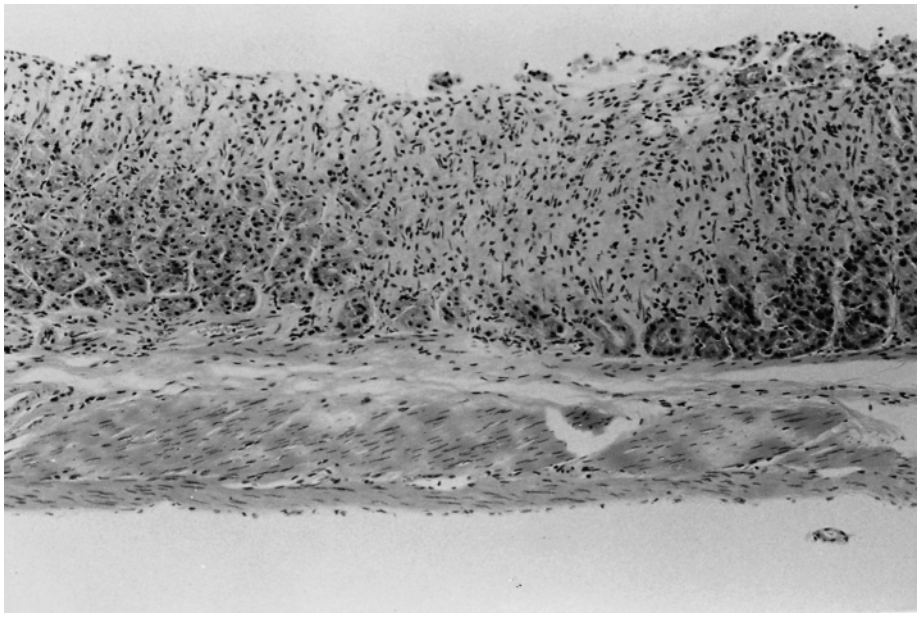

Fig. 4. Gastric mucosal lesion induced by water-immersion stress in *H. pylori*-uninfected C56BL/6 mouse (720-min stress treatment group). Focal necrosis of the epithelium is observed. H&E, ×86

120-min stress treatment group than in the non-stress treatment group, whereas the production was slightly lower in the 720-min stress treatment group than in the 120-min stress treatment group. In the infected groups, the production was lower in the 720-min stress treatment group than in the non-stress treatment and 30-min stress treatment groups. These differences were not significant (Fig. 5e). Results of two-way ANOVA included F values of 2.23 ($P = 0.090$), 27.81 ($P < 0.0001$), and 2.14 ($P = 0.10$) for water-immersion, *H. pylori* infection, and the combination of these two factors, respectively.

TNF-α. Prior to the stress treatment, there was no significant difference in the production of TNF-α between the infected and uninfected groups. In the uninfected groups, the production was higher in the 120-min stress treatment group than in the non-stress treatment group, whereas the production was lower in the 720-min stress treatment group than in the 120-min stress treatment group; however, these differences were not significant. In the infected groups, the production was significantly higher in the 120-min stress treatment group (83.53 ± 21.57 pg/mg protein) than in the non-stress treatment (45.24 ± 32.58 pg/mg protein) and 30-min stress treatment (50.54 ± 30.40 pg/mg protein) groups ($P < 0.01$). Further, the production was lower in the 720-min stress treatment group than in the 120-min stress treatment group; however, this difference was not significant (Fig. 5f). Results of two-way ANOVA included F values of 3.57 ($P = 0.017$), 0.41 ($P = 0.52$), and 1.06 ($P = 0.37$) for water-immersion, *H. pylori* infection, and the combination of these two factors, respectively.

Discussion

Our clinical experience in the Great Hanshin Earthquake suggested that *H. pylori* infection was involved in the recurrence of stress-induced peptic ulcers. In this study, we attempted to gain more insight into the interaction between stress and *H. pylori* infection in ulceration, using Mongolian gerbils and C57BL/6 mice as animal models. When Mongolian gerbils were subjected to water-immersion treatment, the incidence of mucosal lesions was higher in the early phase and lower in the later phase of the stress exposure in animals infected with *H. pylori*, compared with findings in uninfected gerbils. Similar tendencies were observed in C57BL/6 mice, but the severity of lesions was milder than that in the gerbils (only two mice were judged to have had severe bleeding). In other words, *H. pylori* infection lowers the threshold for gastric mucosal injuries in the early phase of stress exposure, whereas *H. pylori* infection suppresses the formation of mucosal lesions in the later phase of stress exposure.

The production of IL-1β and IFN-γ was significantly higher in the infected than in the uninfected group of mice, which supports the findings of previous studies.[19–21] However, there were no significant differences in the production of IL-4, IL-6, IL-10, and TNF-α between these two groups. Previously, *H. pylori* infection was shown to induce the production of TNF-α,[19,21–23] but the result of an in-vitro experiment with peripheral blood monocyte stimulation showed that lipid A in the lipopolysaccharides of *H. pylori* did not induce TNF-α production as much as *Escherichia coli.*-type lipid A did.[24] *H. pylori* infection has been shown to

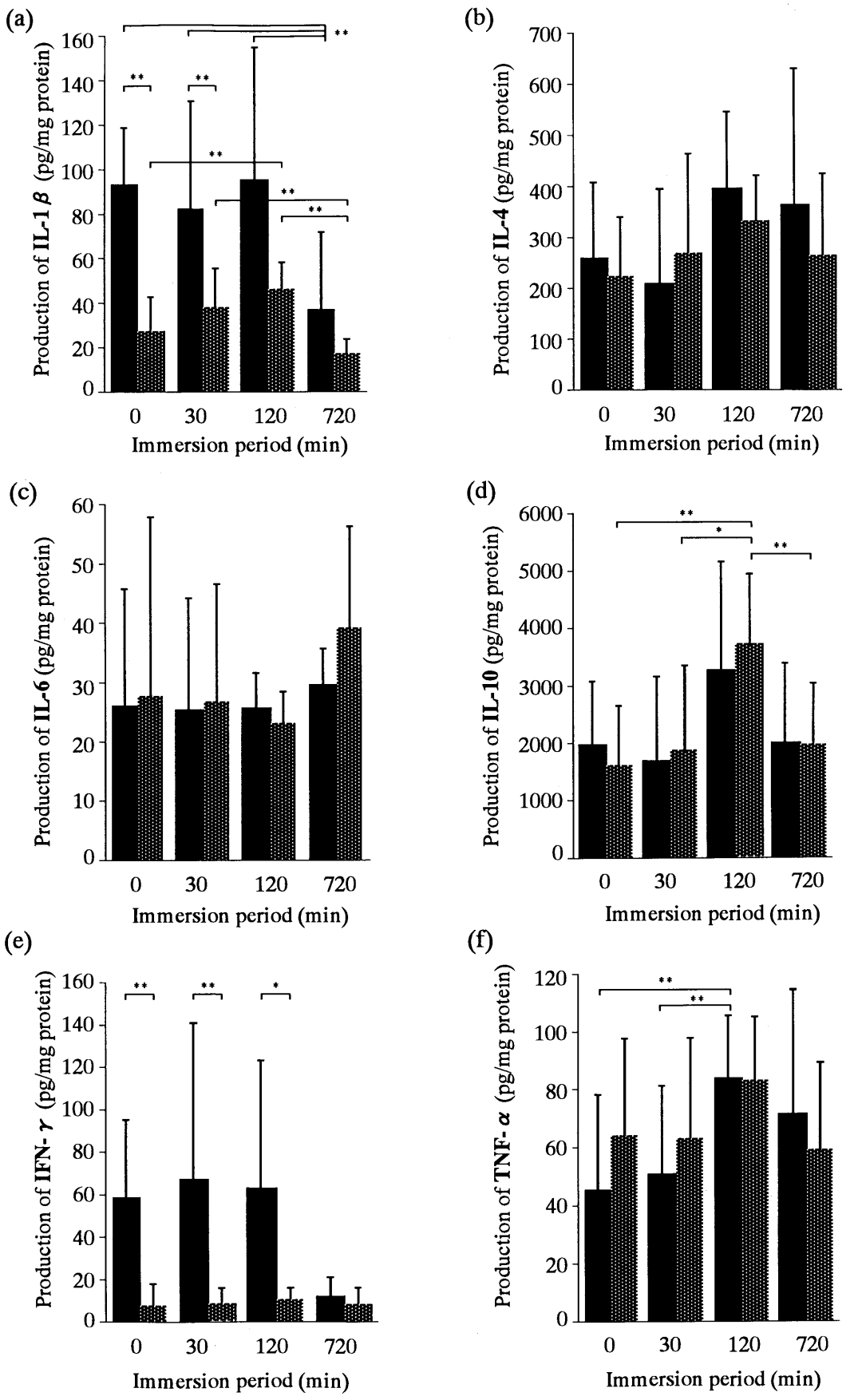

Fig. 5a–f. Production of **a** interleukin (*IL*)-1β, **b** IL-4, **c** IL-6, **d** IL-10, **e** interferon (IFN)-γ, and **f** tumor necrosis factor (TNF)-α in the gastric tissue of *H. pylori*-infected (*black columns*) and uninfected (*gray columns*) C57BL/6 mice with water-immersion stress. *Vertical bars*, Means ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$

induce gastritis in TNF-α receptor knockout mice.[25] Thus, the induction of TNF-α may not be a prerequisite for inflammation in *H. pylori* infection. Regarding the effect of stress, the production of cytokines tended to increase after the stress treatment in both the *H. pylori*-uninfected and *H. pylori*-infected groups of mice. Thus, the presence of chronic inflammation with *H. pylori* infection did not have a superimposing effect toward the production of cytokines induced by stress. The changes in IL-1β and IFN-γ induced by *H. pylori* infection and stress treatment indicate that these cytokines may be candidates for the cause of acute mucosal injury of the stomach. Indeed, enhanced IL-1β gene expression was observed in the gastric mucosa of stress-treated *H. felis*-infected mice.[9] On the other hand, the time lag between the occurrence of mucosal injuries and the alterations in cytokine production, and the absence of inflammatory cell reaction suggest that additional mechanisms, other than cytokines, may be involved in the formation of gastric mucosal injuries in the early phase of stress treatment. One of these candidates may be heat shock proteins (HSPs), which are induced by exposure to stress such as water-immersion.[26,27] Although the relationship between *H. pylori* infection and host-cell HSPs has not been fully clarified, a recent study has shown that *H. pylori* infection reduces HSP70 in gastric mucosa induced by the repeated administration of aspirin.[28]

Our present finding that *H. pylori* infection suppressed the formation of lesions after 720 min of the stress treatment cannot be explained by cytokine production, because the decreased production of IL-1β and IFN-γ was observed with longer stress treatment in the *H. pylori*-infected group. It has been shown that *H. felis* infection augmented stress-induced gastric lesions, with a concomitant increase in IL-1β gene expression when mice were immersed for 24 h.[9] It is notable that the inflammatory reaction was weak and mucosal atrophy was not induced in BALB/c mice after 8 weeks of *H. felis* infection.[11] Thus, the phenomena observed in the later phase of stress exposure may be explained by several mechanisms, such as a reduction in acid secretion, caused by atrophy of the gastric mucosa.[29] These differences in the responses of gastric mucosal injuries and cytokines depending on the duration of stress treatment emphasize that it is necessary to clarify those phenomena which occur in an early phase of stress exposure when the cytokine network is activated, and those which are induced in a later phase of stress exposure when the production of cytokines is suppressed. Moreover, it is important to keep in mind that the formation of the hemorrhagic gastric mucosal lesions that were seen in the present study represents only the initial stages of gastric mucosal injuries. Ischemic mucosal lesions that are seen in restraint water-immersion stress models[30] are thought to be modified by re-perfusion after ischemia, and inflammatory reactions are responsible for tissue repair. *H. pylori* infection may affect this process, as we previously reported that *H. pylori* infection delayed the healing of gastric mucosal injuries caused by ammonia.[31] *H. pylori* is suggested to be involved in the relapse of peptic ulcers by affecting the production of IL-1β.[32]

In conclusion, the present study demonstrated that *H. pylori* infection exacerbated gastric mucosal injury in the early phase of stress treatment and suppressed the formation of gastric mucosal injury in the later phase of stress treatment. Cytokine production also depended on the duration of stress treatment. Furthermore, these effects of *H. pylori* and stress seemed to be dependent on the condition of the background mucosa. Further research is necessary to clarify the reason for the differences between our clinical observations and the experimental results in relation to *H. pylori* infection and stress.

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