

Long-Term Infection of Mongolian Gerbils with *Helicobacter pylori*: Microbiological, Histopathological, and Serological Analyses

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The effects of long-term infection with *Helicobacter pylori* on the gastric mucosa of Mongolian gerbils were examined. Colonization by *H. pylori* was evaluated by both microaerobic cultivation and real-time reverse transcriptase PCR (RT-PCR). Persistent infection with *H. pylori* in gastric mucosa was detected by real-time RT-PCR during 6 months after infection, but no *H. pylori* was isolated 4 months after infection by cultivation. Infiltration with neutrophils and mononuclear cells was observed from 2 months after infection. Both intestinal metaplasia and gastric atrophy were also detected from 2 months after infection. The results by enzyme-linked immunosorbent assay indicated that antibody titers against whole *H. pylori* antigens, *H. pylori* heat shock protein 60 (HSP60), and *Escherichia coli* GroEL were significantly higher in the infected gerbils than in noninfected gerbils. After long-term infection with *H. pylori* for 18 months, marked atrophy of gastric mucosa and multiple cysts in the submucosa were observed in the glandular stomach of the infected gerbils. In addition, squamous cell papilloma with hyperkeratosis was observed in cardia of all the infected gerbils. These results indicate that evaluation of bacterial colonization during long-term infection can be done by real-time RT-PCR and that mucosal damage might be induced by host immune response against whole *H. pylori* antigen.

Warren and Marshall first isolated *Helicobacter pylori* in 1983 from the gastric mucosa of patients with chronic active gastritis (39), and since then the relation between this microorganism and gastric diseases has been widely studied (27, 28, 33, 37). A working group of the World Health Organization International Agency for Research on Cancer concluded in 1994 that *H. pylori* is a group I definite carcinogen in humans (13). However, the prevalence of *H. pylori* in patients with gastric carcinoma still remains considerably variable among studies, and there has been no direct demonstration that *H. pylori* is actually associated with gastric carcinogenesis.

Since Hirayama et al. (10) reported the establishment of persistent *H. pylori* infection in Mongolian gerbils as an animal model in 1996, many investigations on the association between *H. pylori* and gastric diseases have been carried out (12, 17, 21, 29, 30) and, recently, some reported that *H. pylori* was directly related to gastric carcinogenesis (9, 11, 32, 40). The purposes of almost all of the studies which have ever been carried out were predominantly focused on the measurement of the amount of gastric colonization, the serum antibody titer against *H. pylori*, and histopathological observation of the stomach. In the meantime, many investigators have reported that heat shock protein 60 (HSP60) or Lewis antigens of *H. pylori* are associated with the immune response (16, 20, 34, 35, 43). In this study, by using Mongolian gerbils infected with *H.*

pylori we examined how *H. pylori* contributes to the autoimmune response by checking the relation between the histopathological changes in the stomach and the antibody titers against the various antigens, including HSP60 and Lewis antigens. In addition, we performed a basic examination on the difference in the detectable sensitivity of the number of *H. pylori* organisms between the routine culture method and the real-time reverse transcriptase PCR (RT-PCR) method.

MATERIALS AND METHODS

Animals. Male 4-week-old Mongolian gerbils (MGS/Sea; specific pathogen free; body weight, 20 to 30 g) were purchased from Seac Yoshitomi (Fukuoka, Japan) and maintained in plastic cages under standard laboratory conditions (room temperature, 23 ± 2°C; relative humidity, 40 to 60%; 12-h light-dark cycle). A standard diet (CE-2; Clea Japan, Tokyo, Japan) and sterilized tap water were provided ad libitum.

Bacterial strain. The bacterial strain used was *H. pylori* TK1402, isolated from gastric biopsy specimens of a patient with gastric ulcer and duodenal ulcer. It was determined by the PCR method that the strain has the vacuolating cytotoxin gene (*vacA*) and the *cagA* gene. That is, the primers used for the detection of *cagA* were F1 (5'-GATAACAGGCAAGCTTTTGGAGG) and B1 (5'-CTGCAAAAGATTGTTTGGCAGA) (2). For the detection of the *vacA* gene, the primers used were VA3-F (5'-GGTCAAAATGCGGTCATGG) and VA3-R (5'-CCATTGGTACCTGTAGAAAC) and VA4-F (5'-GGAGCCCCAGGAAACATTG) and VA4-R (5'-CATAACTAGCGCCTTGCA) (2). Genomic DNA was extracted using MagExtractor (Toyobo). One microliter of extracted genomic DNA was mixed with each primer solution (5 pmol) and 0.5 U of *Taq* polymerase in a final volume of 20 µl. Reactions were performed for 30 cycles of 94°C for 1 min, 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min with a thermal cycler (Gene Amp PCR system 9600-R; Perkin-Elmer).

Furthermore, it was shown that the strain had the Lewis^y antigen (Le^y) on the cell surface. The bacteria were cultured in brain heart infusion agar (Difco, Detroit, Mich.) with 7% defibrinated horse blood (Wako Pure Chemical Industries Ltd., Osaka, Japan) under microaerobic conditions using GasPak jars (Mit-

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subishi Gas Chemical Company, Inc.) consisting of 85% N₂, 10% CO₂, and 5% O₂ at 37°C for 2 days.

Experimental design. Twenty-three Mongolian gerbils were inoculated with 1 ml of inoculum of 10⁹ CFU of *H. pylori* TK1402/ml intragastrically for two consecutive days by using a feeding needle. The gerbils were fasted from 16 h before the first inoculation to completion of the second inoculation. Two to four gerbils were sacrificed under anesthesia with pentobarbital (50 mg/kg of body weight, intraperitoneally; Dainippon Pharmaceutical Co., Ltd.) at 0.5, 1, 2, 3, 4, 5, and 6 months after inoculation with *H. pylori*. After sacrifice, determination of the number of the microorganisms in the stomach, determination of serum antibody titers against various antigens, and both macroscopic and histopathological examinations of the stomach were carried out as described below. As a control group, the noninfected group was set in order to compare with the *H. pylori*-infected group, and one to two gerbils were sacrificed at the same measurable points as from the *H. pylori*-infected group.

Measurement of the number of microorganisms in the stomach by culture methods. After inoculation with *H. pylori*, the stomachs were extirpated from the sacrificed gerbils at each point. The stomach was dissected along the greater curvature and washed with the 0.01 M phosphate-buffered saline (PBS, pH 7.4). Then, it was divided longitudinally into two halves, including the forestomach-to-pylorus part. The right half was used for the determination of the number of microorganisms. The left half was used for the histopathological examinations. The mucus layer was collected from the right half-stomach with Spertel in 1 ml of Hanks balanced salt solution and homogenized. Then, the suspended solution was inoculated onto M-BHM selective medium (Nikken Seibutsu, Kyoto, Japan) and incubated at 37°C for 7 days. After incubation, the number of gold colonies was counted and the number of viable organisms in the gastric mucosa was calculated.

Real-time RT-PCR assay. A real-time RT-PCR assay was performed using the method reported by Lesley et al. (19) with some modifications. Tissue samples were lysed with lysozyme (400 µg/ml) in 100 µl of TE buffer (10 mM Tris-Cl, 1 mM EDTA; pH 8) by vortexing, followed by addition of guanidine isothiocyanate-containing lysis buffer (QIAGEN, Valencia, Calif.). Total RNA was isolated from the lysed sample according to the instructions using an RNeasy Mini kit. Quantification of RNA was done by measuring the A_{260}/A_{280} ratio. Contaminating chromosomal DNA was digested with DNase I (DNA free; 1 U/µg of RNA; Ambion, Austin, Tex.) for 20 min at 37°C. One microgram of DNase I-treated total RNA was incubated with avian myeloblastosis virus reverse transcriptase with random primers in a commercial reaction mixture (20 µl; AMV reverse transcription system; Promega, Madison, Wis.). First-strand cDNA synthesis was performed.

cDNA was amplified using PCR primers for 16S rRNA of *H. pylori*, 16SB-F (5'-GCTAAGATCAGCCTATGTC-3') and 16SB-R (5'-TGGCAATCAGCGTCAGGTAATG-3') (4). Amplification of the *G3PDH* gene on cDNA derived from Mongolian gerbils by using the primers G3PDH-F (5'-ACCACAGTCCATGCCATCAC-3') and G3PDH-R (5'-TCCACCACCTGTGTGCTGTA-3') was used for a noninfected control of total RNA extraction and for standardization of results of target gene transcriptional activity.

Quantitative analysis was performed using SYBR Green methods. The generation of quantitative data was based on the different PCR kinetics of samples with different levels of target gene expression. We used a relative quantification in which the expression levels of *H. pylori* target genes were compared to the data from a standard curve, which was generated by amplifying serial dilutions of a known quantity of amplicons. For each primer set, PCR was performed in parallel reactions using different amount of *H. pylori* strain TK1402 chromosomal DNA. Quantification data were analyzed using 5700 quantification software (Applied Biosystems). In this analysis, the background fluorescence was removed by manually setting a noise band. The long-linear portion of the standard's amplification curve was identified, and the crossing point was the intersection of the best-fit line through the long-linear region and the noise band. The standard curve was a plot of the crossing points versus the log bacterial number (in CFU per milliliter). The quantification software determined the unknown concentration by interpolating the noise band intercept of an unknown sample against the standard curve of known concentrations. The quantitative data were calculated from the standard curve of the PCR. For this approach, the identity and specificity of the PCR product were confirmed by dissociation curve analysis, which is part of the 5700 quantification program.

Histopathological examination. After macroscopic observation of the stomach at necropsy, the remaining left halves of stomachs were fixed at 4°C for 2 h in cold Carnoy's solution (ethanol-chloroform-acetic acid, 6:3:1). After fixation, the stomachs were sliced longitudinally to include the forestomach to the glandular stomachs at intervals of 5-mm width. All specimens were then dehydrated in absolute alcohol and embedded in paraffin wax. Longitudinal sections with 5-µm

TABLE 1. Isolation of *H. pylori* from stomachs of infected Mongolian gerbils

Time after infection (mos)	Infection status ^a	Mean no. of <i>H. pylori</i> isolated ^b (CFU/g of tissue)
0.5	5/6	2.60 ± 1.42
1	5/8	1.90 ± 1.70
2	3/8	1.41 ± 1.96
3	6/8	4.45 ± 2.75
4	0/4	— ^c
5	0/4	—
6	0/4	—

^a Number of *H. pylori*-positive gerbils/number of gerbils examined.

^b Mean ± standard deviation (log 10).

^c No *H. pylori* was isolated.

thickness were processed from these paraffin blocks. The sections were stained with hematoxylin and eosin (H-E) and periodic acid-Schiff. The histopathological examinations were carried out, and the classification and grading of gastritis were based on the updated Sydney system (3).

ELISA. Each antigen for ELISA was prepared by the following procedures. After *H. pylori* TK1402 was cultured on the blood agar medium at 37°C for 3 days, the harvested whole organisms were suspended in the 0.01 M PBS (pH 7.4) and sonicated by using an ultrasonic Sonifier 250 (Branson Ultrasonics Co., Danbury, Conn.) for 5 min at 20 kHz. Microtitration plates (Greiner Labortechnik Japan, Tokyo, Japan) were coated at 4°C for 18 h with the whole-sonicate antigens (3 µg/well) of *H. pylori*. The fixed *H. pylori* (whole) cells were used as the surface antigens of the organism. After this, *H. pylori* TK1402 (10⁷ CFU) was poured in each well and fixed with 2% paraformaldehyde at 4°C for 18 h. Lipopolysaccharide (LPS) derived from *H. pylori* strain TK1313, which was extracted by the hot phenol-water technique (41), was used. *Klebsiella pneumoniae* LPS preparation (Sigma), Lewis Y tetrasaccharide (Le^Y; Sigma), human-derived recombinant HSP60 (Sigma), and *E. coli*-derived recombinant GroEL (Sigma) were also applied to the microtitration plates at 1 µg/well. In addition to them, *H. pylori*-derived HSP60 was prepared using the method of Yamaguchi et al. (43) and applied on the plates in the same way. Mucosal cells of the gastric body and pylorus, as antigens of 6-month-old Mongolian gerbils, were prepared by ultrasonication and applied to the plates at 2 µg/well. In addition, as human antigens, the AGS cell line derived from a gastric cancer and HEP2 cell line derived from a laryngeal cancer were sonicated by the method described above and applied on the plates at 2 µg/well. After coating the plates with each antigen at 4°C overnight, they were washed with PBS three times. After this, each antigen was blocked with PBS containing 1% skim milk (PBS-S; Yukijirushi Nyugyo Co., Tokyo, Japan) at 37°C for 1 h. After washing with PBS, each serum sample taken from the infected or noninfected gerbils with *H. pylori* was used after dilution with PBS by 20- or 300-fold. One hundred microliters of each diluted serum was added to the plates and reacted with each antigen at 37°C for 2 h. After washing with PBS three times, 100 µl of horseradish peroxidase-protein G (Sigma) at 25 µg/ml in PBS-S was added to the plates and reacted with the antigen-antibody complex at 37°C for 1 h. The plates were treated with 0.1% *o*-phenylenediamine in the developing buffer (0.1 M citric acid, 0.07 M sodium phosphate dibasic, 0.035% H₂O₂). After incubation at room temperature for 5 min, the reaction was stopped by adding 50 µl of 2 N H₂SO₄. The optical density was measured at 490 nm by using a microplate reader (model 550; Bio-Rad).

Statistic analysis. The correlation between the number of microorganisms and serum antibody titer and the correlation between serum antibody titer and the inflammation scores based on the updated Sydney system were examined by the statistic analysis software StatView (HuLinks Co. Ltd.). Also, statistically significant differences in serum antibody titers between the infected and the noninfected gerbils with *H. pylori* were examined by using Welch's *t* test.

RESULTS

Measurement of the number of microorganisms in the stomach. A change in the number of *H. pylori* organisms in the stomach by the culture method is shown in Table 1. The numbers of microorganisms were 10^{2.60} ± 10^{1.42}, 10^{1.90} ± 10^{1.70}, 10^{1.41} ± 10^{1.96}, and 10^{4.45} ± 10^{2.75} CFU/g of tissue at 0.5, 1, 2, and 3 months after inoculation with *H. pylori*, respectively, but

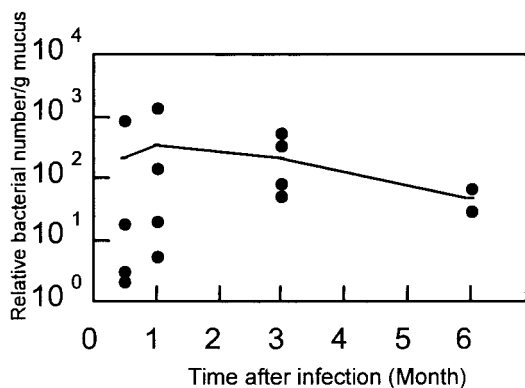


FIG. 1. Number of *H. pylori* in stomachs of Mongolian gerbils infected with *H. pylori* strain TK1402, calculated by the real-time RT-PCR method. ●, individual relative bacterial number per gram of mucus from *H. pylori*-infected gerbils; —, average relative bacterial number per gram of mucus.

less than the detectable limit (20 CFU/g of tissue) later at 4 months.

The change in the relative number of *H. pylori* strain TK1402 organisms in the stomach (estimated microorganism number per PCR mixture) by the real-time RT-PCR method is shown in Fig. 1. The melting point in the real-time RT-PCR method was 89.3°C. The number of microorganisms was found to be 10 to 10³ at 1 month after infection with *H. pylori* and did not change by 3 months after infection.

Histopathological findings of the stomach. As shown in Table 2, two to four gerbils infected with *H. pylori* were sacrificed per time point for pathology. There were no differences in the histopathological findings between the infected gerbils (Fig. 2B) and the noninfected gerbils (Fig. 2A) 1 month after inoculation with *H. pylori*. However, 2 months after infection with *H. pylori*, inflammatory cell infiltration by neutrophils and lymphocytes, hyperplasia of regenerative epithelium in the lamina propria mucosa, and atrophic changes were observed (Fig. 2C). Some prominent lymphoid follicles were also observed in both the lamina propria mucosa and submucosa. In severe cases, the inflammatory cell infiltration expanded to the lamina muscularis mucosa or more deeply, reaching the serosa. In the histopathological examination at 3 and 6 months after infection, intestinal metaplasia was also observed in one out of three gerbils and one out of four gerbils, respectively (Fig. 2D). Judging from the severity of the inflammation, there were no histopathological differences between 2 and 6 months after infection.

The histopathological findings observed in each Mongolian gerbil were scored based on the updated Sydney system (neutrophil infiltration [activity], mononuclear cell infiltration, intestinal metaplasia, and atrophy) and are summarized in Table 2. The severity of the findings was classified as mild (+ or 1), moderate (++ or 2), and marked (+++ or 3). The score for neutrophil infiltration or mononuclear cell infiltration was 2.5 to 3.0 at later than 2 months after infection with *H. pylori*. The score for intestinal metaplasia was 0.3 at 3 months and 6 months after infection with *H. pylori*. The score for atrophy was 1.0 to 1.5. Neutrophil infiltration and mononuclear cell infiltration were predominant, rather than intestinal metaplasia or atrophy.

ELISA results. The results of the ELISA are shown in Table 3. Antibody titer against TK1402 whole sonicate of *H. pylori*-infected gerbils tended to increase at later than 2 months after infection with *H. pylori*, and it was significantly higher than that of the noninfected gerbils ($P < 0.01$) (Fig. 3A). Antibody titers against *H. pylori* HSP60 and GroEL in the infected gerbils at 3 to 6 months after infection also increased and were significantly higher than those of the noninfected gerbils ($P < 0.01$ and 0.05, respectively). The antibody titers against human HSP60 and gerbil tissues (pylorus and gastric body) in *H. pylori*-infected gerbils were higher than those in noninfected gerbils, but there were no statistically significant differences between groups because of their deviations. These antigens were considered to have no cross-reaction with anti-*H. pylori* antibody in the infected Mongolian gerbils.

There was no statistically significant difference in antibody titer against *K. pneumoniae* LPS, Le^Y, pylorus and gastric body of the Mongolian gerbils, AGS cell line, and HEP2 cell line between *H. pylori*-infected gerbils and the noninfected gerbils. A correlation between antibody titer against *H. pylori* TK1402 sonicates and neutrophil infiltration based on the updated Sydney system was seen ($r = 0.7778, P < 0.05$) (Fig. 3B). Antibody titer against TK1402 whole sonicate increased 2 months after *H. pylori* infection, and a positive correlation between its antibody titer and neutrophil infiltration was shown.

Effect of long-term infection with *H. pylori* on stomachs of Mongolian gerbils. The relative number of *H. pylori* organisms estimated by real-time RT-PCR was below the detectable level 18 months after infection with *H. pylori*. At autopsy, macroscopically polyps in the glandular stomachs and thickening of the cardia in all three infected gerbils were detected. Histopathologically, marked atrophy of mucosa and multiple cysts in the submucosa were observed in the glandular stomachs of the three gerbils (Fig. 4A). In part of one glandular stomach,

TABLE 2. Scoring of histopathological findings based on the updated Sydney system in stomachs of Mongolian gerbils infected with *H. pylori*

Finding	Time after infection with <i>H. pylori</i> (mo) ^a													
	0.5		1		2		3		4		5		6	
	N (0)	H (2)	N (1)	H (3)	N (1)	H (3)	N (2)	H (3)	N (2)	H (4)	N (2)	H (4)	N (2)	H (4)
Neutrophil infiltration (activity)	—	0	0	0	0	3.0	0	3.0	0	2.8	0	2.5	0	3.0
Mononuclear cell infiltration	—	0	0	0	0	3.0	0	3.0	0	2.8	0	2.5	0	3.0
Intestinal metaplasia	—	0	0	0	0	0	0	0.3	0	0	0	0	0	0.3
Atrophy	—	0	0	0	0	1.3	0	1.3	0	1.0	0	1.0	0	1.5

^a N, noninfected gerbils; H, *H. pylori*-infected gerbils; —, not examined. Values in parentheses are the number of gerbils in each group.

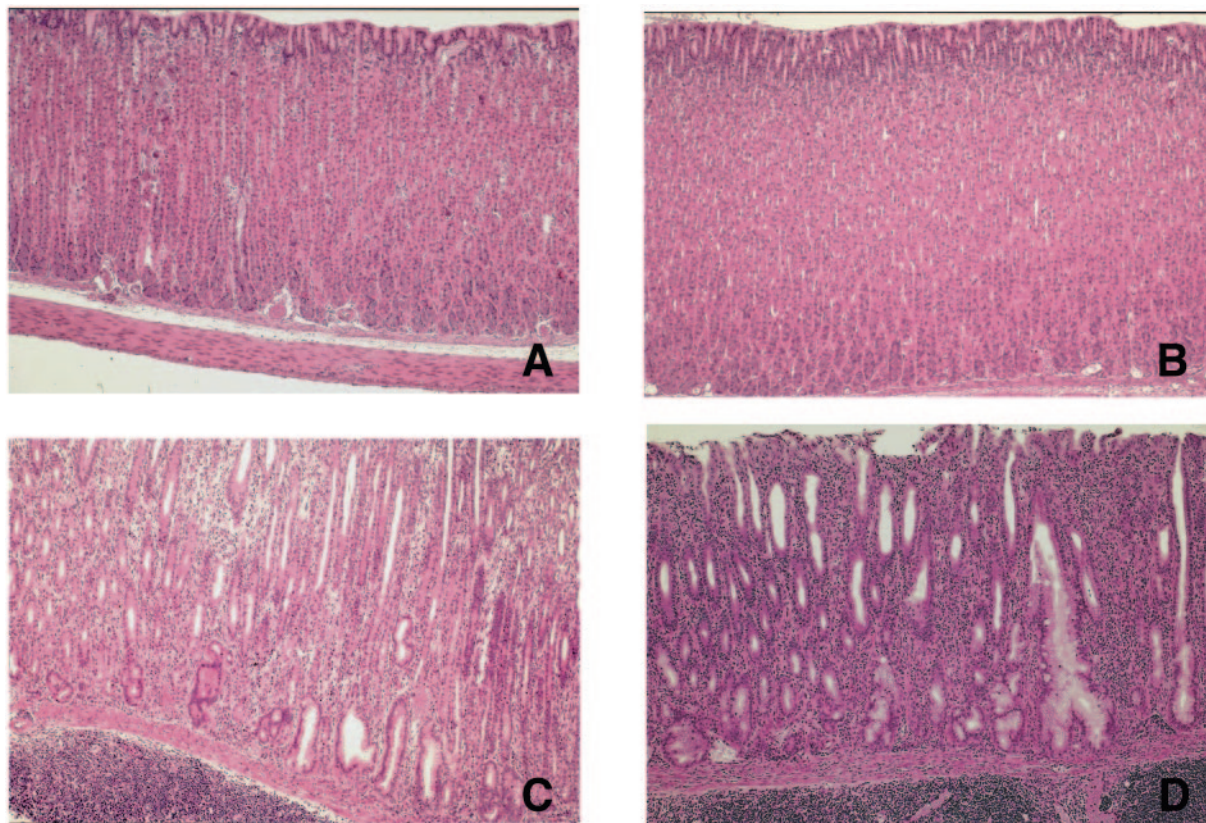


FIG. 2. Histopathological findings in gastric mucosa of Mongolian gerbils at 1, 2, and 6 months after *H. pylori* infection (H-E stain). (A) Noninfected; (B) 1 month after infection; (C) 2 months after infection; (D) 6 months after infection. Magnification, $\times 90$.

the disappearance of almost all gastric glands and fibrosis were observed in the lamina propria mucosa. This lesion was considered to be well-differentiated adenocarcinoma; however, the glandular epithelium was strongly atypical, and the nucle-

us/cytoplasm ratio was high (Fig. 4B). At the thick part of the cardia of three gerbils, squamous cell papillomas with hyperkeratosis were observed (Fig. 4C). In two noninfected gerbils sacrificed at the same time as the above-mentioned three infected gerbils, neither macroscopic nor histopathological changes were observed in either glandular stomach or cardia (Fig. 4D and E). The antibody titers against *H. pylori* TK1402 (sonicate) and *H. pylori* HSP60 were 1.868 ± 0.959 ($n = 3$) and 0.287 ± 0.120 ($n = 3$), respectively, 18 months after infection, and these were significantly higher than those of noninfected gerbils. There was no increase of antibody titers in the noninfected gerbils sacrificed at the same time.

TABLE 3. Serum antibody titers to the various antigens in Mongolian gerbils with or without *H. pylori* infection

Antigen tested	Antibody titer (mean \pm SD)	
	Noninfected gerbils ^a	<i>H. pylori</i> -infected gerbils ^a
<i>H. pylori</i> TK1402 (sonicate)	0.011 \pm 0.052	1.355 \pm 0.726**
<i>H. pylori</i> TK1402 (whole)	0.000 \pm 0.010	0.109 \pm 0.091**
HEp2	0.013 \pm 0.023	0.019 \pm 0.042
AGS	0.037 \pm 0.080	0.000 \pm 0.050
<i>H. pylori</i> HSP60	0.035 \pm 0.018	0.386 \pm 0.200**
Human HSP60	0.000 \pm 0.047	0.187 \pm 0.339
<i>E. coli</i> GroEL	0.021 \pm 0.068	0.139 \pm 0.167*
Le ^Y	0.003 \pm 0.009	0.012 \pm 0.030
<i>H. pylori</i> TK1313 LPS	0.547 \pm 0.428	0.521 \pm 0.399
<i>K. pneumoniae</i> LPS	0.010 \pm 0.007	0.020 \pm 0.054
Tissue (pylorus)	0.415 \pm 0.700	0.811 \pm 1.031
Tissue (gastric body)	0.205 \pm 0.501	0.813 \pm 0.943

^a For *H. pylori* TK1402 antigens (sonicate or whole), HEp2, and AGS, $n = 10$ noninfected gerbils or 23 *H. pylori*-infected gerbils and the ELISA was conducted 2 to 6 months after infection (serum was diluted 300-fold in PBS). For all other antigens, $n = 6$ noninfected gerbils or 17 *H. pylori* infected gerbils and the ELISA was conducted 3 to 6 months after infection (serum was diluted 20-fold in PBS). *, significantly different from noninfected gerbils ($P < 0.05$); **, significantly different from noninfected gerbils ($P < 0.01$).

DISCUSSION

The infection model for *H. pylori* using Mongolian gerbils is considered to be a useful model, since gastric mucosal changes in stomachs of Mongolian gerbils were similar to those in human stomachs following *H. pylori* infection. Gastric colonization by *H. pylori* in Mongolian gerbils was reported to be related to several factors of the organism, such as urease productivity, motility, etc. Iwao et al. reported that the motility of *H. pylori* was an important factor for gastric colonization in Mongolian gerbils, and there were differences in its gastric colonization due to differences in phenotypic strains of the gerbils (14). Ogura et al. reported that there were differences in gastric colonization, suppression of body weight gain, and

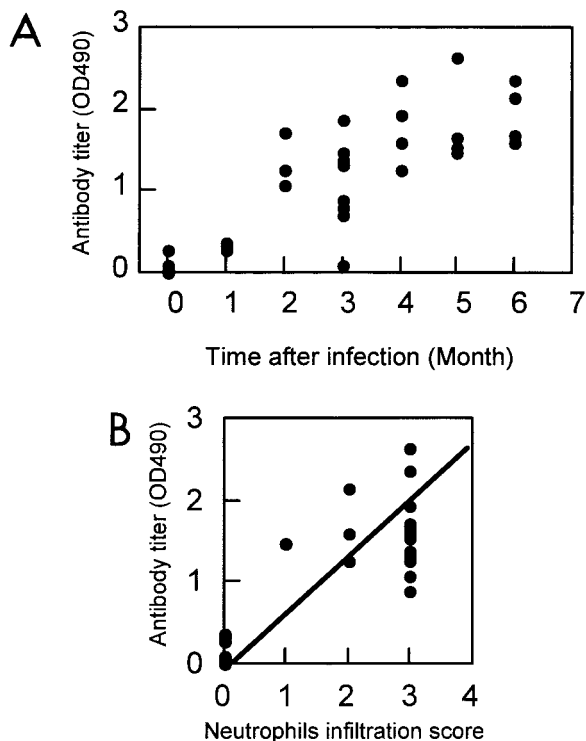


FIG. 3. Correlation between antibodies against *H. pylori* TK1402 sonicates and cell infiltration based on the updated Sydney system. (A) Antibody titer against *H. pylori* TK1402 sonicates after infection. (B) Correlation between antibody titer and score of neutrophil infiltration (activity).

histopathology following *H. pylori* infection, depending on the *H. pylori* strains (24). Kurita et al. (18) and Ohkusa et al. (25) also recognized that different strains induced different severities of infection in Mongolian gerbils. Strain TK1402, isolated from a patient with duodenal ulcer and gastric ulcer, has both *cagA* and *vacA*. There are two reports on the virulence of this strain in gnotobiotic mice (26) and C57BL mice (42). In this study, we found that histopathologically severe inflammatory cell infiltration with neutrophils and mononuclear cells, lymphoid follicles, and intestinal metaplasia were induced after infection with *H. pylori*. Judging from the onset time and severity of the histopathological changes, these were almost the same changes as with the standard strain, ATCC 43504, which has been used in many infection experiments with Mongolian gerbils (10–12, 14, 25, 30). The pathogenicity of strain TK1402 is considered to be almost equal to that of strain ATCC 43504.

Causality between the presence of *H. pylori* and its carcinogenicity in the stomach has not been made clear yet. Recently, however, there were several papers suggesting the causality between *H. pylori* and its carcinogenicity, as adenocarcinoma or carcinoid were observed in a long-term infection experiment with *H. pylori* in Mongolian gerbil (9, 40) and *H. pylori*, as promoter, enhanced the adenocarcinoma induced by *N*-methyl-*N*-nitrosourea (22, 31). In addition, Touati et al. (38) examined the role of *H. pylori* infections in the induction of mutations in gastric epithelial cells by using Big Blue transgenic mice, and they reported that the gastric mutant frequency was fourfold higher in *H. pylori*-infected mice than in noninfected

mice 6 months after inoculation and that this genotoxicity could be attributable to oxidative DNA damage involving the host inflammatory response. We did not find gastric cancer in the 6-month study. But in the long-term study for 18 months under the same conditions, well-differentiated adenocarcinoma was observed in one glandular stomach of three animals and squamous cell papillomas with hyperkeratosis were observed in the cardia of all three animals infected with *H. pylori*. No studies with squamous cell papillomas with hyperkeratosis have ever been reported for Mongolian gerbils infected with *H. pylori*.

In this study, we tried to quantify the number of colonized organisms in the stomach by the real-time RT-PCR method in addition to the routine culture method. With the culture method, many investigators have reported that they could detect the 10^4 to 10^5 CFU of *H. pylori* in the gastric mucosa when 10^7 to 10^9 CFU of *H. pylori* were inoculated (21, 29, 40). On the other hand, there was a report in which *H. pylori* was not detected by the culture method in spite of a high titer of anti-*H. pylori* antibody in the serum (9). The number of *H. pylori* organisms isolated was not always accordant among the reports using the culture method. In our case, we could detect viable *H. pylori* in the stomach by the routine culture method at 1 to 3 months after inoculation, but not 4 months later. On the other hand, we could detect *H. pylori* even 6 months after inoculation and obtained better sensitivity with the real-time RT-PCR method than the culture method. As we could calculate the number of *H. pylori* in all the infected Mongolian gerbils by using the real-time RT-PCR method at 6 months after inoculation, this PCR-based method is a useful tool for quantification of *H. pylori*.

Antibody titer against *H. pylori* whole-sonicate antigen or its surface antigen significantly increased from 2 months after inoculation. Many studies suggest that gastritis or gastric atrophy in humans is related to the production of autoantibodies (5, 15, 23, 35, 36). HSP60 is considered to be related to the induction of gastritis mediated by autoimmunity (16), and we have reported that the anti-HSP60 antibody titer increased in sera taken from patients with various gastric diseases (43). Gobert et al. (7) reported that *H. pylori* HSP60 mediated production of interleukin-6 and played a patho-physiological role in the autoimmune response of chronic gastritis. In addition, as Le^x and Le^y antigens, which are the components of the *H. pylori* LPS, are the same antigens as expressed on blood cells and parietal cells, the possibility that antibodies against these molecules react as an autoantibody has been pointed out (1, 8). We examined whether autoantibodies against these antigens were produced, and we confirmed the elevation of antibody titer against the HSP60 family. That is, autoantibody against GroEL derived from *E. coli*, which showed molecular homogeneity with *H. pylori* HSP60, also increased. Ferrero et al. (6) assessed *H. pylori* HSPs as potential protective antigens in mice with gastric *H. pylori* infection, and they reported that a protein belonging to the GroES class of HSPs induced protective immunity. Yamaguchi et al. (43) also pointed out the possibility of protection against *H. pylori* infection by an HSP60 homologue. The results of our present study may be related to theirs. On the contrary, there was no increase in antibody titers against Le^y or LPS derived from *H. pylori*.

It is possible that the raised antibodies to these antigens

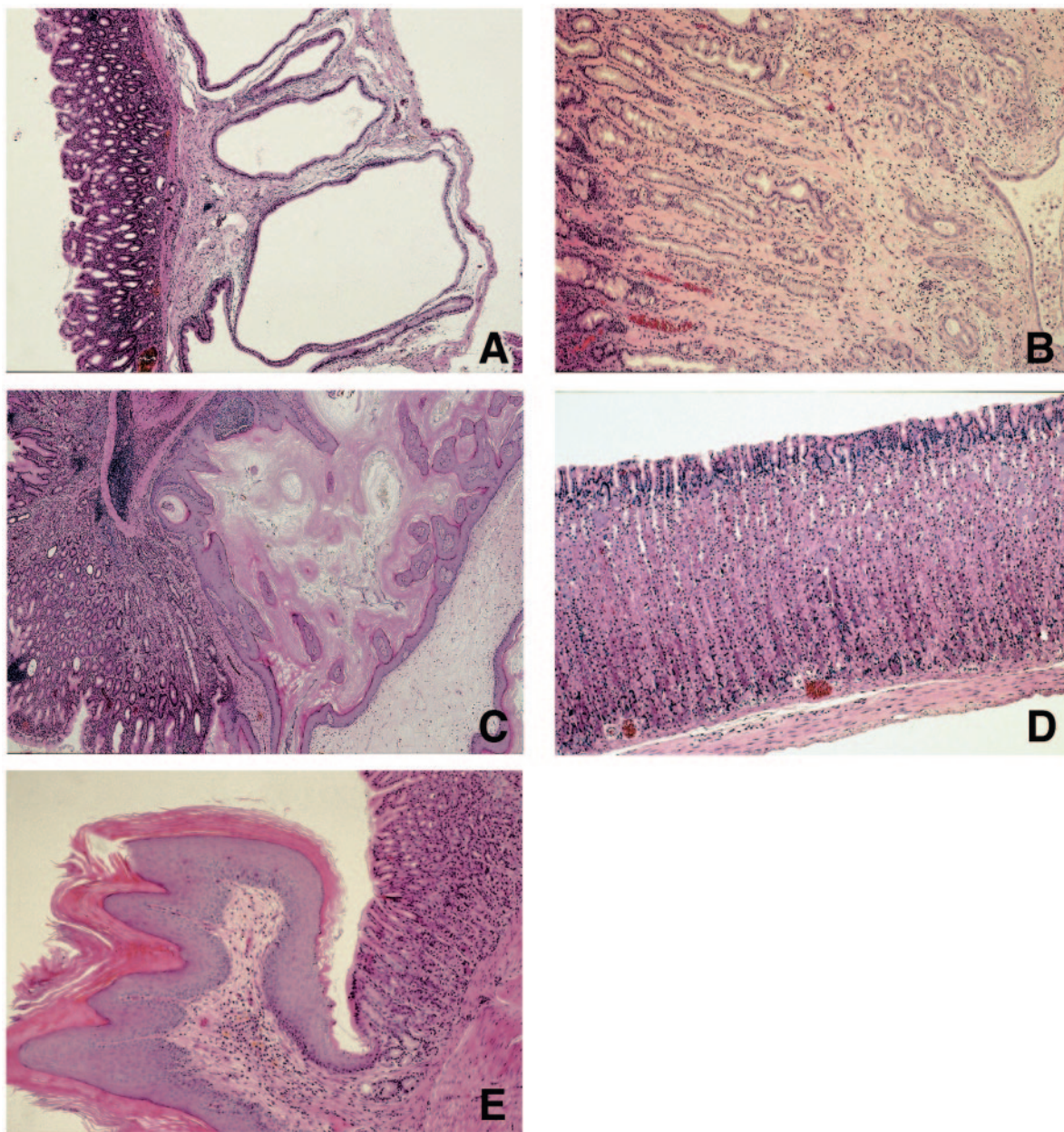


FIG. 4. Histopathological findings in gastric mucosa of Mongolian gerbils with (A, B, and C) or without (D and E) long-term *H. pylori* infection for 18 months (H-E stain). (A) Glandular stomach. Multiple cysts were seen in the submucosa. Magnification, $\times 50$. (B) Glandular stomach. The submucosa was fibrous, and a well-differentiated adenocarcinoma was seen. Magnification, $\times 90$. (C) Cardia. A squamous cell papilloma with hyperkeratosis was seen in the forestomach. Magnification, $\times 35$. (D) Glandular stomach of a noninfected gerbil. No significant change was seen. Magnification, $\times 90$. (E) Cardia of a noninfected gerbil. No significant change was seen. Magnification, $\times 90$.

following long-term infection with *H. pylori* might play an important role for induction of the mucosal damage in the stomach of the infected gerbils. However, the correlation between the production of these antibodies and the induction of gastric tumors, such as adenocarcinoma and squamous cell papilloma, remains to be determined.

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