MICROBIAL PATHOGENICITY

The effect of colonisation by *Helicobacter pylori* in *Praomys* (Mastomys) *natalensis* on the incidence of carcinoids

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An animal model of experimental gastric *Helicobacter pylori* infection has been developed in the Z strain of *Praomys* (Mastomys) *natalensis*; this animal has been reported to develop gastric carcinoids and adenocarcinoma spontaneously. In the present study, male and female Mastomys were killed at 1, 2, 4, 8 and 16 weeks after *H. pylori* inoculation. Colonisation of *H. pylori* was maintained in the stomachs of all animals for up to 16 weeks. *H. pylori* were mainly found in the antrum. Lymphoid infiltration appeared in the antral lamina propria and submucosa in all male and female animals from 4 to 16 weeks after inoculation. On microscopic examination after immunostaining for *H. pylori*, the organisms were detected in the antral mucus layer of the gastric epithelium. Serum immunoglobulin G specific for *H. pylori* could be detected 2 weeks after inoculation in female and 4 weeks after inoculation in male Mastomys, and persisted throughout the 16-week study period. At 18 months after inoculation, *H. pylori* positive rates for male and female Mastomys were 15 of 21 and 7 of 27, respectively. Carcinoids developed in 27 of 100 inoculated and in 49 of 100 uninoculated male, and in 5 of 100 inoculated and in 21 of 100 uninoculated female animals at 18 months after inoculation. Adenocarcinoma developed in 1 of 100 male Mastomys in both the inoculated and uninoculated groups, but in none of the female animals in either the inoculated or uninoculated groups. These results indicate that antrum-predominant colonisation by *H. pylori* caused the decrease in incidence of carcinoid formation in Mastomys.

Introduction

Helicobacter pylori infection is strongly associated with gastroduodenal disease [1, 2]. The site of gastritis induced by *H. pylori* may play a role in determining clinical outcome. Gastritis involving the antral region is associated with excessive acid production and a high risk of duodenal ulcer disease [3]. In contrast, gastritis involving the acid-secreting corpus region leads to hypochlorhydria, progressive gastric atrophy and an increased risk of gastric cancer [4].

Praomys (Mastomys) *natalensis* is an African rodent with a high incidence of spontaneous stomach tumours [5]. A Z

strain of this species has been reported to develop carcinoids along the greater curvature of the body as well as adenocarcinoma along the lesser curvature of the antrum, the predilection site of human gastric adenocarcinoma [6]. In a study by Kumazawa *et al.*, carcinoids were found in 80.6% of male and in 64.7% of female animals, whereas adenocarcinoma was found in 22.6% of male and in 13.2% of female animals of this Z strain at the age of 18 months [7]. In Mastomys, as in man, gastric carcinoids are mainly composed of enterochromaffin-like (ECL) cells [8]. The adenocarcinomas in Mastomys appear to proceed according to a hyperplasia-dysplasia-adenocarcinoma sequence [7]. In man, gastric cancer consists of two distinct histological subtypes: intestinal and diffuse [9]. According to Correa's hypothesis, the intestinal type of gastric cancer develops from chronic inflammation leading to intestinal metaplasia, then dysplasia and finally cancer, while the diffuse type does not go through these precancerous conditions but moves

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straight from inflammation to cancer [10]. The type of adenocarcinoma seen in *Mastomys* resembles diffuse-type cancer.

ECL carcinoids in the human stomach are usually associated with chronic atrophic gastritis, Zollinger-Ellison syndrome with multiple endocrine neoplasia type 1, or pernicious anaemia [11], but are only rarely associated with *H. pylori* infection [12]. On the other hand, several studies have shown an epidemiological association between *H. pylori* and gastric cancer [13]. Thus, the World Health Organization's IARC designated *H. pylori* as a definite biological carcinogen in 1994 [14]. Recent studies have suggested that the association of *H. pylori* and intestinal-type gastric cancer is related to gastric mucosal atrophy and intestinal metaplasia [15], but there has been no consensus regarding the relationship of *H. pylori* to diffuse-type cancer [16]. This study examined the influence of *H. pylori* infection on the incidence of carcinoids and adenocarcinoma in *Mastomys*.

Materials and methods

Animals

Five-week-old specific-pathogen-free (SPF) male and female animals of the Z strain of *Praomys* (*Mastomys natalensis* (Japan SLC, Shizuoka, Japan) weighing c. 40 g were used in this study. Japan SLC established SPF *Mastomys* breeding colonies originating from the Z strain [17]. *Mastomys* were confirmed to be free of the following common pathogens: *Pseudomonas aeruginosa*, *Mycoplasma* spp., *Bordetella bronchiseptica*, *Streptococcus pneumoniae*, *Corynebacterium kutscheri*, *Pasteurella pneumotropica*, Tyzzer's organism, *Salmonella* spp., *Giardia* spp., *Spironucleus* spp., *Syphacia* spp., *Aspiculuris tetraptera*, Sendai virus and Pneumonia virus of mice. The presence or absence of gastric *Helicobacter* spp. was ascertained in the 10 male and female *Mastomys* before admission to the study by evaluating the animals' stomachs for urease activity, by examining tissue sections for the presence of *Helicobacter*-like organisms [18] and by culture for *Helicobacter* spp. [19]. All *Mastomys* were negative for *Helicobacter* spp. in all tests. *Mastomys* were housed in polycarbonate cages in isolators and fed a commercial pellet diet (F-2; Funabashi Farms, Chiba, Japan) with water *ad libitum*. All animal experiments were carried out according to the guidelines provided by the Institutional Animal Care and Use Committee of Sankyo Co. Ltd (Tokyo, Japan).

Bacterial inocula

H. pylori strain 9818 was isolated from gastric biopsy samples from a patient with gastric ulcers and was supplied by Dr T. Fujioka (Oita Medical University, Oita, Japan). This strain had the *cagA* gene and vacuolating cytotoxin, as confirmed by PCR amplifica-

tion with primers specific for *cagA* and cytotoxin assay, respectively [20, 21]. This *H. pylori* strain was passaged three times in *Mastomys*. Stock cultures were stored at -80°C in Brucella Broth (Becton Dickinson, Cockeysville, MD, USA) supplemented with fetal bovine serum (FBS; Dainippon Pharmaceutical, Osaka, Japan) 2%.

Experimental design

Bacteria were cultured in brucella broth supplemented with FBS 2% on a gyratory shaker at 110 rpm for 40 h at 37°C under micro-aerobic conditions. The culture was harvested and the cells were suspended in brucella broth supplemented with FBS 2% to 2×10^8 cfu/ml. The bacterial cell counts of all inocula were done with Brain Heart Infusion Agar (Difco Laboratories, Detroit, MI, USA) supplemented with horse blood 5%. Plates were incubated at 37°C in a GasPak jar (Becton Dickinson) for 5 days with Campy Paks (Becton Dickinson). *Mastomys* were inoculated orally with 2 ml of bacterial suspension after 1 day of fasting. Control groups were given brucella broth supplemented with FBS 2% alone. Six inoculated animals and six uninoculated controls were anaesthetised in a CO_2 chamber and killed by cervical dislocation at 1, 2, 4, 8 and 16 weeks after inoculation. Blood was obtained by heart puncture, and serum was stored at -20°C until testing for anti-*H. pylori* antibody by an enzyme-linked immunosorbent assay (ELISA) as described below. Three each of the six inoculated and uninoculated animals were used for culture of *H. pylori*, and the other three inoculated and uninoculated animals were used for microscopic examinations. Finally, 121 inoculated male, 127 inoculated female, 100 uninoculated male and 100 uninoculated female animals were examined for *H. pylori* by culture and for the incidence of gastric carcinoids and adenocarcinoma at 18 months after inoculation.

Microbiology

The stomachs were homogenised in 5 ml of brucella broth supplemented with FBS 2%, followed by dilution with the same broth; 100- μl samples of the dilutions were inoculated on to modified Skirrow's agar plates containing vancomycin (Sigma) 10 $\mu\text{g}/\text{ml}$, bacitracin (Sigma) 8 $\mu\text{g}/\text{ml}$, polymyxin B (Pfizer Pharmaceutical, Tokyo, Japan) 0.25 $\mu\text{g}/\text{ml}$, trimethoprim (Shionogi Pharmaceutical, Osaka, Japan) 2.5 $\mu\text{g}/\text{ml}$ and amphotericin B (Sigma) 3 $\mu\text{g}/\text{ml}$. Plates were incubated at 37°C under micro-aerobic conditions for 5 days and the colonies of *H. pylori* were counted. Bacterial counts are expressed as cfu/g of tissue.

Distribution of *H. pylori* in the stomach

One week after inoculation, three female *Mastomys* were used to study the distribution of *H. pylori* in the stomach. The body, antrum and duodenum of the

stomach were examined. These sections were cut separately and homogenised in 2 ml of brucella broth supplemented with FBS 2%. Viable cells of *H. pylori* were counted as described above.

Histopathological examination

Histopathological examination was performed by the method of Shimizu *et al.* [22]. Each stomach was fixed in cold Carnoy's solution (a mixture of ethanol:acetic acid:chloroform, 6:3:1, v:v:v) for 2 h at 4°C. After fixation, the stomachs were sliced longitudinally at 5-mm intervals. All tissue sections were then dehydrated in absolute alcohol, cleared in xylene and embedded in paraffin. Serial paraffin sections of 3- μ m thickness were prepared. From each block, one slide was stained with haematoxylin-eosin for histopathological observation and one was immunostained for *H. pylori* by an indirect immunoperoxidase method. Histopathological findings were evaluated for the presence of lymphocyte aggregates in each section. A lymphocyte aggregate was defined as >10 lymphocytes/ $\times 400$ field. After rehydration, sections fixed in Carnoy's solution were re-fixed with buffered formalin 20% for 30 min. Before applying anti-*H. pylori* polyclonal antibody (DAKO Japan, Kyoto, Japan), hydrated sections were treated with a trypsin solution (Sigma) (trypsin 0.2% and CaCl_2 0.1% in 0.05 M Tris buffer, pH 7.6) at 37°C for 10 min. After washing with 0.05 M Tris-HCl buffered saline (pH 7.6), diluted horseradish peroxidase-conjugated anti-rabbit IgG antibody (DAKO Japan) was added. Antibody binding sites were visualised with 3', 3'-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan), and the sections were counterstained with haematoxylin.

ELISA for *H. pylori* antibody

Serum IgG antibody was assayed by ELISA with a commercial kit (Helico G; International Reagents, Kobe, Japan). Serial two-fold dilutions of sera from *H. pylori*-infected Mastomys were incubated for 1 h at 37°C. The secondary antibody, peroxidase-conjugated rabbit anti-mouse IgG (Chemicon International, Temecula, CA, USA) diluted 1 in 1000 in commercial buffer, was applied to the wells for 30 min at 37°C, followed by addition of substrate for 10 min, and then addition of stopping solution. OD_{450} was recorded with an ELISA plate reader (MR 580; Dynatech Laboratories, Alexandria, VA, USA). The antibody titre of serum samples was prepared at a dilution with an OD_{450} of 3 SD above the mean OD_{450} of four negative control serum samples.

Incidence of carcinoids and adenocarcinoma 18 months after *H. pylori* inoculation

Inoculated ($n = 100$ of each sex) and uninoculated ($n = 100$ of each sex) Mastomys were used for the histopathological examinations, and 21 inoculated male

and 27 inoculated female animals were used for culture of *H. pylori*. Carcinoids were classified according to Bilchik *et al.* [23]. All sera were tested for anti-*H. pylori* antibody by the ELISA method.

PCR amplification and restriction fragment length polymorphism (RFLP)

H. pylori were recovered from both male and female Mastomys at 16 weeks and 18 months after inoculation. The DNA samples of the recovered *H. pylori* were used. Primer sequences chosen for amplification were specific for the *H. pylori flaA* gene [24]. PCR was performed as described previously [25], with some modifications. Following PCR amplification, 15 μ l of reaction mixture were removed and incubated at 37°C for 2 h, and the fragments were separated on an agarose 3% gel. The gels were stained with ethidium bromide.

Statistical analysis

All values are expressed as the means and SD. The incidence of gastric carcinoids and adenocarcinoma was compared by the χ^2 goodness of fit test. p values <0.05 were considered statistically significant.

Results

Colonisation of gastric tissue with *H. pylori*

As shown in Fig. 1, *H. pylori* were continuously detected in the stomachs of all male and female Mastomys. For 1–2 weeks, the count of *H. pylori* in the stomachs of male and female animals increased, and then the count of colonies decreased, reaching one-tenth of the peak level by 16 weeks after inoculation.

Fig. 1. Changes in viable counts in gastric mucosa of male (○) and female (●) Mastomys after inoculation with *H. pylori* 9818. Each value indicates the mean and SD of three animals.

No *H. pylori* were isolated from the uninoculated Mastomys.

Distribution of H. pylori in the stomach

The bacterial counts from the body and antrum were 4.94 SD 0.56 and 5.62 SD 0.09 log₁₀ cfu/g, respectively. The viable count of *H. pylori* colonising the antrum was five-fold higher than that in the body. No *H. pylori* were found in the duodenum.

Histopathology

A summary of the histopathological changes in the antrum of Mastomys inoculated or not inoculated with *H. pylori* is shown in Table 1. Inflammation was rarely present in the forestomach and body mucosa. At 1 week after inoculation in female animals or 2 weeks after inoculation in male animals, lymphoid infiltration was detected in the antral mucosa. From 4 weeks after inoculation in both male and female animals, multifocal lymphoid infiltration appeared in the lamina propria and submucosa in the pylorus (Fig. 2a, b and c). Pathological change in non-inoculated Mastomys was seen in only one male Mastomys. With immunostaining, *H. pylori* were detected in the antral mucus layer of the gastric epithelium in female Mastomys at 1 week after inoculation (Fig. 3).

Serum anti-H. pylori antibody

Anti-*H. pylori* IgG antibody levels increased at 2 weeks after inoculation in female or 4 weeks after inoculation in male Mastomys. The anti-*H. pylori* IgG levels persisted throughout the 16 weeks (Fig. 4). The uninoculated control Mastomys had no antibody response specific for *H. pylori*.

Incidence of carcinoids and adenocarcinoma 18 months after H. pylori inoculation

The pathological classification and the incidence of carcinoids 18 months after *H. pylori* inoculation are shown in Table 2. The incidence of carcinoids was significantly lower ($p < 0.05$) in animals inoculated with *H. pylori* than in uninoculated male and female animals. Microscopic examination revealed carcinoids

in the body of inoculated (Fig. 5a) and uninoculated (Fig. 5b) male animals. Adenocarcinoma developed in 1 of 100 male Mastomys in both the inoculated and uninoculated groups, but in none of the female Mastomys in either the inoculated or uninoculated groups. *H. pylori* positive rates for male and female animals were 15 of 21 and 7 of 27, respectively. No serum anti-*H. pylori* antibody was detected in any of the animals.

RFLP analysis of H. pylori

The identities of the isolates of *H. pylori* recovered from the experimentally inoculated Mastomys were compared by RFLP analysis of the 1.5-kb *flaA* gene segment with that of the infecting strain. The RFLP pattern of the *flaA* gene PCR product of the infecting strain was virtually identical to that of the isolates recovered from the inoculated animals (Fig. 6).

Discussion

These results demonstrate that Mastomys can be readily colonised by *H. pylori*. The *H. pylori*-inoculated female and male animals had mild antral gastritis within 1 and 2 weeks after inoculation, respectively, and they were persistently colonised for up to 16 weeks after inoculation. Raised antibody titres were detected within 2 weeks in female animals and within 4 weeks after inoculation in male animals. The reason for this sex difference is unknown. The most striking difference between the *H. pylori*-infected Mastomys model and man is the very low number of neutrophils and the predominant presence of lymphocytes in the inflamed mucosa of the former. A similar inflammatory response has been observed in several other models of animal infection [26–28], but the reason for this difference between animal models and man remains unclear. On the other hand, in man, lymphocyte infiltration and follicle infiltration are consistent with chronic *Helicobacter*-induced gastritis [29]. Therefore, the Mastomys model of *H. pylori* infection may resemble chronically infected humans. *H. pylori* colonised mainly in the antrum of the Mastomys and were detected by immunostaining. Furthermore, inflammatory cells were

Table 1. Histopathological findings in the antrum of Mastomys after inoculation or non-inoculation with *H. pylori*

Time after challenge (weeks)	Number of animals in which inflammatory change was detected			
	Male		Female	
	Inoculated	Non-inoculated	Inoculated	Non-inoculated
1	0	0	3	0
2	2	1	2	0
4	3	0	3	0
8	3	0	3	0
16	3	0	3	0

n = 3 in each test group at each time point.

Fig. 2. Experimental *H. pylori* infection caused antral gastritis at (a) 4, (b) 8 and (c) 16 weeks after inoculation. At 16 weeks after inoculation, lymphoid infiltration extended throughout the antrum. Bar, 100 μ m.

observed only in the antrum. A similar predominance of *H. pylori* colonisation in the antrum has also been reported in man [30]. The present study demonstrated that *Mastomys* is susceptible to gastric infection with *H. pylori*, as demonstrated by culture, immunohistochemical detection of *H. pylori*, antibody titres and

histopathological findings of local inflammatory response. Moreover, the RFLP pattern of the *flaA* gene PCR product of the infecting strain was virtually identical to those of the recovered isolates. This result confirmed the lack of co-infection with other *Helicobacter* spp. *Mastomys* could be an ideal animal model

Fig. 3. Immunostaining of *H. pylori* (arrow) in the antral mucosa of a female *Mastomys* 1 week after inoculation. Bar, 10 μ m.

Fig. 4. *H. pylori* IgG serum antibody titres as measured by ELISA in inoculated male (○) and female (●) *Mastomys*.

to study the association between *H. pylori* and carcinoids or adenocarcinoma.

Gastric carcinoids have been shown previously to develop in the oxyntic mucosa of aging male and female *Mastomys* [31]. In this study, the incidence of carcinoids in both male and female inoculated animals was significantly lower than that in uninoculated animals. It has also been shown that carcinoids develop very rapidly during H₂-receptor blockade in *Mastomys* [23]. It was suggested that the growth of these carcinoids may have been promoted by inhibition of acid secretion [23]. According to this assumption, high acid secretion would reduce the growth of carcinoids. El-Omar *et al.* reported that individuals with gastritis predominantly localised in the antrum by *H. pylori* infection retain high acid secretion [3]. As it has also been demonstrated that gastritis in the *Mastomys* infection model is localised in the antrum by *H. pylori*, the incidence of carcinoids may be decreased due to high acid secretion. Solcia *et al.* reported that the incidence of *H. pylori* infection was lower in the

Table 2. Pathological classification and incidence of carcinoids 18 months after inoculation or non-inoculation with *H. pylori*

Tumour type	Number of animals in which a tumour was detected			
	Male		Female	
	Inoculated (n = 100)	Non-inoculated (n = 100)	Inoculated (n = 100)	Non-inoculated (n = 100)
Invasive	15	29	2	10
Mucosal	10	13	2	7
Microcarcinoid	2	7	2	4
Total	27*	49	6*	21

*p < 0.05 compared with the uninoculated group.

Fig. 5. A carcinoid of *Mastomys* 18 months after (a) inoculation (bar, 500 μm) and (b) in uninoculated animals (bar, 1000 μm). D, duodenum.

presence of carcinoids than carcinomas and that carcinoid-associated gastritis was usually inactive or poorly active, even when human patients were *H. pylori* positive [32]. On the other hand, in a Mongolian gerbil model, *H. pylori* infection was shown to cause prominent suppression of acid secretion, which appears to be a major cause of hypergastrinaemia [33]. In consequence, ECL carcinoids developed following *H. pylori* infection in Mongolian gerbils [34].

The colonisation rate of male *Mastomys* tended to be higher than that of female animals at 18 months in the present study. The reasons for this sex difference remain unknown, although it was reported in another study that the rate of carcinoid formation of male *Mastomys* is higher than that of female animals for reasons which also remain unknown [7]. The difference in the rate of colonisation and that of carcinoid formation may be considered a sex difference. Further studies are needed to clarify the relationship between *H. pylori* infection and sex difference in *Mastomys*.

Adenocarcinomas of *Mastomys* have been shown to develop from pre-existing dysplastic glands that originate from several hyperplastic glands without gastric atrophy or metaplasia [7]. In the present study, there was no difference in the incidence of adenocarcinoma between male or female inoculated or uninoculated animals. In man, there have been discrepant results regarding the association between *H. pylori* infection and the diffuse types of gastric cancer [16, 35]. Miehke *et al.* compared the gastritis parameter in the antrum and corpus in *H. pylori*-positive gastric carcinoma patients with those in *H. pylori*-positive control patients who had only gastritis [4]. In the corpus only, the grade of *H. pylori* colonisation, grade of gastritis and activity of gastritis were statistically higher in both the intestinal and diffuse types of gastric carcinomas than in the control [4]. Based on the above findings that the *Mastomys* infection model shows antrum-predominant colonisation and gastritis, *H. pylori* infection in *Mastomys* would appear not to promote adenocarcinoma. Furthermore, whether *H.*

Fig. 6. RFLP analysis of the PCR-amplified 1.5-kb *flaA* segment of *H. pylori* with indicated restriction endonucleases. PCR products from: lane 1, infecting *H. pylori* 9818; 2, isolate recovered from a male *Mastomys* 16 weeks after inoculation; 3, isolate recovered from a female *Mastomys* 16 weeks after inoculation; 4, isolates recovered from a male *Mastomys* 18 months after inoculation; 5, isolates recovered from a female *Mastomys* 18 months after inoculation; M, 100-bp ladder markers (Amersham Pharmacia Biotech, Uppsala, Sweden).

pylori infection increases or decreases acid secretion appears to be an important question with respect to gastric cancer.

Eighteen months after inoculation, no serum antibody against *H. pylori* was detected in any of the animals, including those positive for *H. pylori*. The levels of *H. pylori* colonisation 18 months after inoculation were lower than those 16 weeks after inoculation (data not shown); however, the reasons for this finding remain unknown. Although no difficulties appeared in determining the antibody until 16 weeks after inoculation, further work might be needed to produce an anti-*Mastomys* IgG antibody.

The incidence of carcinoids and adenocarcinoma in this study was lower than those previously reported [7]. As the origin of the *Mastomys* in this study was the same as that in the previous study, this difference may be attributed to differences in gastric flora between conventional and specific-pathogen-free conditions or differences in environmental factors.

The clinical presentation of *H. pylori* infection is thought to be influenced by the strain diversity of *H. pylori*, factors involving the host or environment, and the duration of infection [36–38]. The present study attempted to inoculate *Mastomys* with two strains isolated from a patient with gastric cancer, two strains from patients with gastric ulcers and one strain from a patient with duodenal ulcers, but only one of the strains from a patient with gastric ulcers successfully colonised *Mastomys* (data not shown). It is possible that other strains capable of inducing corpus-predominant colonisation and gastritis might show different results.

In conclusion, this study demonstrated experimental *H. pylori* gastric infection in *Mastomys*. The findings suggest that the antrum-predominant colonisation of *H. pylori* caused a decrease in the incidence of carcinoid formation in *Mastomys*.

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