NOTE *Laboratory Animal Science*

Histopathological Studies on Cases of Chronic Mouse Hepatitis by Natural *Helicobacter* Infections

Chunja NAM[1)], Yasushi OHMACHI[1)]*, Toshiaki KOKUBO[2)], Tetsu NISHIKAWA[2, 3)], Kazuyuki UCHIDA[4)] and Hiroyuki NAKAYAMA[4)]

[1)]*Animal Pathology Section, Department of Advanced Technologies for Radiation Protection Research, Research Center for Radiation Protection, National Institute of Radiological Sciences, 4–9–1 Anagawa, Inage-ku, Chiba, 263–8555, Japan*
[2)]*Laboratory Animal Section, Department of Technical Support and Development, Fundamental Technology Center, National Institute of Radiological Sciences, 4–9–1 Anagawa, Inage-ku, Chiba, 263–8555, Japan*
[3)]*Animal Research Center, Kyoto Pharmaceutical University, 5 Misasagi-Nakauchicho, Yamashina-ku, Kyoto 607–8414, Japan*
[4)]*Department of Veterinary Pathology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo, 113–8657, Japan*

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ABSTRACT. It is known that *Helicobacter hepaticus* or *Helicobacter bilis* infection causes chronic inflammation of the colon and liver. Chronic active hepatitis was found in radiation exposure experiments using male C3H/HeNrs mice at our institute. Histopathologically, 103 cases among 978 mice (64–91 weeks of age at autopsy) had hepatic lesions regardless of irradiation exposure. Mild lesions showed only focal necrosis and focal inflammation in the liver. Severe cases were accompanied by hepatocytomegaly, bile duct hyperplasia, hypertrophy and activation of Kupffer cells, cholangitis, pleomorphic hepatocytes and/or tumor. Helical-shaped bacteria were detected between hepatocytes by Warthin-Starry silver stain and immunohistochemistry (IHC) with an antibody against *Helicobacter pylori*. It was suggested that these cases of chronic hepatitis were caused by *Helicobacter* spp. Although chronic hepatitis occurred frequently in mice exposed high-dose irradiation compared with nonirradiated mice in one lot, it was not concluded that radiation might influence the incidence or degree of hepatitis. Our report suggested that natural *Helicobacter* spp. infection in mice can occur in an experimental animal facility. Therefore, it is suggested that monitoring of *Helicobacter* infection is very important for quality control of animal experiments.
KEY WORDS: C3H/HeNrs mouse, chronic hepatitis, *Helicobacter*, natural infection.

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Helicobacter spp. are often isolated from humans and animals and cause inflammation and tumors in the gastrointestinal tract and liver [9, 20]. Some enterohepatic *Helicobacter* spp. in mice normally colonize the lower intestinal tract and liver and cause chronic hepatitis or inflammatory bowel diseases [1, 17–20]. *H. hepaticus* is associated with bowel and liver disease in susceptible mouse strains, such as A/J, Balb/c, SJL, B6C3F1, C3H/He and SCID mice [1, 3, 7, 8, 11, 18, 20]. *H. bilis* is also associated with hepatitis or inflammatory bowel disease in C3H /He, C57BL6 and Swiss Webster mice [6, 20]. *H. bilis* often colonizes the cecum/colon, and it colonizes the liver less often [6]. *H. hepaticus* and *H. bilis* are also capable of colonizing in the bile canaliculi of susceptible mouse strains [6, 13, 17, 20]. It has been well known that strain, age and gender of mice can influence *Helicobacter* hepatic infections [10, 12, 17]. Of the susceptible strains, aged and male mice develop severe hepatitis inflammatory lesions early, and these may progress to hepatic carcinoma [5, 14, 15, 19]. *H. hepaticus* can be visualized as small helical bacteria between hepatocytes or in bile canaliculi by Warthin-Starry silver staining or Steiner staining [10, 13, 17]. On the other hand, it has been rarely reported that *H. bilis* is less often observed in silver-stained liver sections [6]. Female mice infected with *Helicobacter* spp. often develop an inflammatory bowel disease characterized by mucosal hyperplasia with erosions/ulcers and diverse inflammatory cells infiltration [12, 16, 18]. Either gender of immunodeficient SCID mice can develop both liver and intestinal disease [7, 11, 18].

Chronic hepatitis induced by natural *Helicobacter* infection in mice has rarely been reported in Japan. In radiation carcinogenesis experiments at our institute, chronic active hepatitis and its related hepatic tumor, which were caused by natural *Helicobacte*r infection, occurred in 64–91-week-old C3H/HeNrs mice regardless of irradiation exposure. Lesions of hepatic chronic inflammation and the tumor were observed through histopathological examination, such as hematoxylin and eosin (HE) staining. In this report, we also described histopathological and immunohistological features of chronic hepatitis and hepatic tumor caused by natural by *Helicobacter* infection in C3H/HeNrs mice under the barrier systems.

Male C3H/HeNrs mice were bred at our institute. They were housed in a barrier system (SPF laboratory animal system) under conditions of 23 ± 1°C, relative humidity of

*CORRESPONDENCE TO: Dr. OHMACHI, Y., Animal Pathology Section, Department of Advanced Technologies for Radiation Protection Research, Research Center for Radiation Protection, National Institute of Radiological Sciences, 4–9–1 Anagawa, Inage-ku, Chiba, 263-8555, Japan.
e-mail: y-omachi@nirs.go.jp

Table 1. Incidence of hepatitis and proliferative lesions in male C3H/HeNrs mice with or without exposure to irradiation

Group/Lot	Age at exposure	γ-ray (Gy)	Animal No.	Incidence (%)		
				Chronic hepatitis	Hyperplastic foci	Hepatocellular adenoma or carcinoma
A	1 w	0	40	12.5	5.0	2.5
		0.2	30	13.3	6.7	0.0
		0.5	40	5.0	0.0	0.0
		1	49	20.4	4.1	6.1
		2	40	17.5	12.5	10.0
		3	43	25.6	18.6	14.0
B	3 w	0	35	10.0	0.0	0.0
		0.2	45	6.7	0.0	0.0
		0.5	35	7.7	2.6	2.6
		1	35	10.3	7.7	0.0
		2	35	5.0	2.5	0.0
		3	45	11.4	5.7	2.9
C	8 w	0	30	11.4	5.7	5.7
		0.2	45	8.9	2.2	4.4
		0.5	39	12.8	0.0	0.0
		1	40	15.0	5.0	0.0
		2	40	12.5	7.5	5.0
		3	35	0.0	0.0	0.0
D	15 w	0	39	7.7	7.7	2.6
		3	44	0.0	0.0	0.0
E	35 w	0	34	8.8	5.9	2.9
		0.2	50	8.0	8.0	4.0
		0.5	25	8.0	4.0	0.0
		1	20	15.0	10.0	15.0
		2	45	6.7	0.0	2.2
		3	20	10.0	5.0	5.0

55 ± 5% and an alternating 12-hr light/dark schedule. Mice were fed a sterilized laboratory diet (Funabashi Farm Co., Ltd., Tokyo, Japan) and given chlorinated water *ad libitum*. Exposure to Cs-137 gamma rays was conducted with a Gammacell irradiator (Nordion Inc., Ottawa, ON, Canada). All experimental procedures were approved by the Animal Use and Care Committee of the National Institute of Radiological Sciences.

During the experiment, a few mice died due to lymphoma. The 64- to 91- week-old mice were euthanized by exsanguination under ether anesthesia. The liver, kidneys, Harderian glands, thymus and bone marrow were weighed, fixed with neutral formalin and subjected to histological examination. Four-micrometer paraffin sections were subjected to HE and Warthin-Starry (WS) silver staining and IHC.

WS silver staining (Kerr's modification) was conducted as follows. The deparaffinized and rehydrated sections were incubated in a 1% silver nitrate solution dissolved in 1% citric acid buffer (pH 4.0) for 1 hr at 43°C. The sections were then incubated with developing solution at 54°C until they became pale brown; the developing solution was made separately and preheated at 54°C, and the following components were added before use: 15 m*l* of 2% silver nitrate solution, 37.5 m*l* of 5% gelatin solution and 2 m*l* of 0.15% hydroquinone dissolved in 1% citric acid buffer. The sections were then washed twice with distilled water at 54°C and tap water, dehydrated and mounted for examination.

IHC was carried out by the streptavidin (LSAB) method against anti-*Helicobacter pylori* (Dako, Carpinteria, CA, U.S.A.), anti-CD3-ε (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-CD45R (BD Biosciences, Franklin Lakes, NH, U.S.A.) and anti-F4/80 (Abcam, Cambridge, U.K.) antibodies. Paraffin sections were deparaffinized and immersed in 10 mM citrate buffer, pH 6.0, and heated for 20 min at 105°C. After washing in Tris-buffered saline (TBS), the sections were placed in 3% H_2O_2 for 10 min to inactivate endogenous peroxidase. After incubation in 10% goat or mouse serum at room temperature for 10 min to reduce nonspecific staining, the sections were reacted with primary antibodies at 4°C overnight with secondary antibodies at room temperature for 30 min and then with peroxidase-labeled streptavidin (Dako) at room temperature for 30 min, respectively. The sections were visualized by peroxidase-diaminobenzidine (DAB) reaction and then counterstained with hematoxylin.

The livers of affected mice showed variable degrees of hepatitis, and in some case, hyperplastic nodules and neoplasia were observed. Hepatitis or hepatic tumors occurred

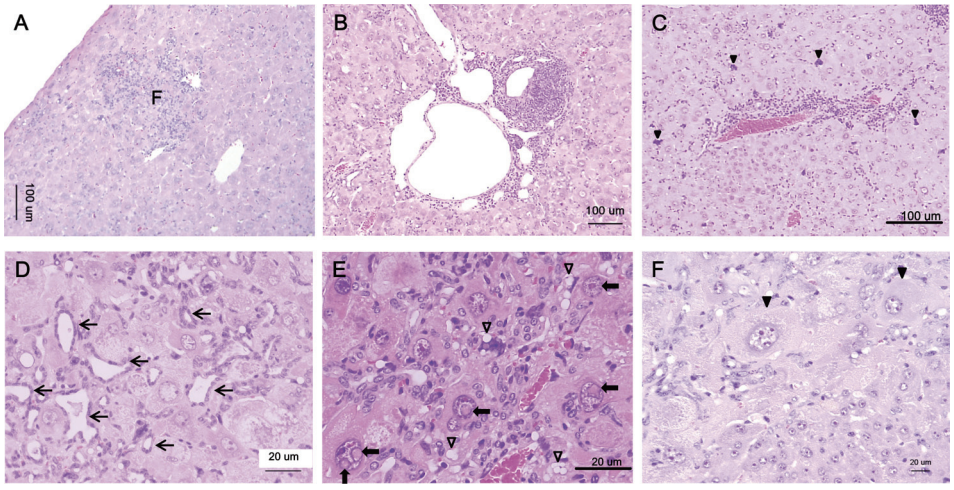

Fig. 1. Variable degrees of hepatitis lesions after *Helicobacter* spp. infection. (A) A necrotic focus (F) with nonsuppurative inflammation. (B) Moderate inflammation in hepatic portal area and severe cholangitis with mononuclear cell infiltration. (C) Hyperplasia of bile ductular epithelial cells and oval cells (←). (D) Hypertrophied macrophages (▼). (E) Compared with normal-sized hepatocytes, some hepatocytes showed hepatocytomegaly (▼). (F) Intranuclear pseudoinclusion bodies (arrows) and Ito cell proliferation (∇) .

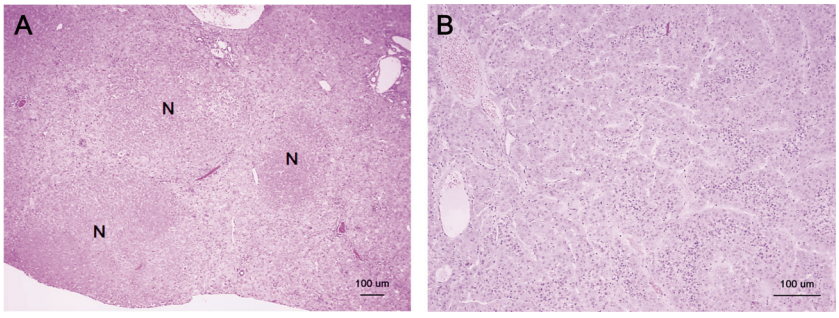

Fig. 2. Preneoplastic and neoplastic lesions in the liver. (A) Hyperplastic nodule (N). (B) Hepatic cellular carcinoma with trabecular proliferation.

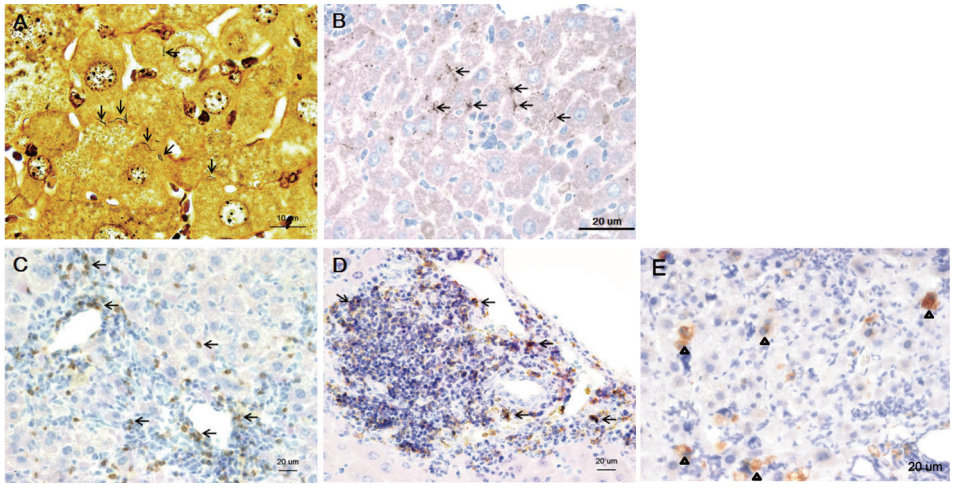

Fig. 3. Helical-shaped bacteria (arrow) are visible between hepatocytes in the liver. (A) Warthin-Starry silver stain. (B) IHC with an antibody against *Helicobacter pylori*. (C–E) IHC with an antibody against immune cell: IHC for anti-CD3-ε (C), CD45R (D) and F4/80 (E). CD3-ε- and CD45R-positive cells are T lymphocytes and B lymphocytes, respectively. F4/80-positive cells are mature tissue macrophages, Kupffer cells (Δ).

in either nonirradiated or irradiated mice (Table 1). In this study, liver lesions of the mice showed the identical lesions to *H. hepaticus*-induced chronic hepatitis [8, 11], such as focal necrosis, cholangitis, bile ductular hyperplasia, Kupffer cell proliferation, Ito cell proliferation, pseudoinclusion bodies in hepatic nuclei and/or hepatocytomegaly.

Some mice showed mild lesions accompanied by nonsuppurative inflammatory foci (Fig. 1A). Some mice showed moderate or severe lesions with mononuclear inflammatory cell infiltration in portal areas around bile ducts or ductules (Fig. 1B). Small aggregates of inflammatory cells were occasionally seen in the affected livers. Inflammatory cells of chronic hepatitis consisted of mononuclear cells including most lymphocytes and some macrophages (Fig. 1B). Some macrophages were hypertrophied in sinusoids in the severely affected liver (Fig. 1C). As the lesion became more extensive and severe, chronic hepatitis was accompanied by hyperplasia of bile ductules or oval cells, bile duct proliferation formation, Ito cell proliferation, pseudoinclusions in hepatic nuclei and hepatocytomegaly with megakaryocytes (Fig. 1D–F). In the most severe cases, some cases of chronic hepatitis developed preneoplastic and neoplastic lesions in the liver, such as hyperplastic nodules or hepatic tumors (Fig.2A–B).

In infected mice with hepatitis, bacteria were detected by WS silver staining and IHC for *Helicobacter pylori* near hepatitis lesions (Fig. 3A–B). Helical bacteria were observed between hepatocytes in the affected mouse liver. In IHC for *H. pylori*, bacteria organisms stained in spots or in a linear pattern. Furthermore, a few bacteria in the livers of mice with hepatic carcinoma showed positive reactions in IHC for *H. pylori*.

The inflammatory cells in the livers of mice with hepatitis were positively stained against CD3-ε, CD45R and/or F4/80 (Fig. 3C–E). CD45R-positive B lymphocytes and CD3-ε-positive T lymphocytes mostly infiltrated into portal inflammation areas and also dispersed in the liver (Fig. 3C–D). Some hypertrophied and activated Kupffer cells in hepatic sinusoids of infected mice were positively stained against F4/80 (Fig. 3E). Otherwise, there was no positive reaction of resident Kupffer cells in nonirradiated mice. Because F4/80 has been known as an antigen marker sensitive to fixation time, a long period of fixation with formalin might weaken the antigenicity of F4/80. Actually, antigen retrieval of F4/80 was tried using several methods, but all attempts failed to recover the F4/80 antigenicity of resident Kupffer cells.

Regarding organs other than the liver, the incidence of enteritis was unknown, because the intestines were not collected in these cases. Because a fecal examination was not carried out, *Helicobacter* colonization in the mouse intestine was also not clear. Detection of *Helicobacter hepaticus*-specific 16S rRNA in liver sections (formalin-fixed and paraffin embedded sample) [2, 4] by PCR was attempted for differential identification from other *Helicobacter spp*., but it failed. One of the possible explanations for this was that long-term storage of the paraffin blocks (e.g., over 3 years) might degrade bacterial nucleotides.

It has been well reported that male A/J mice develop active chronic hepatitis and hepatic tumors by natural and experimental infections with *H. hepaticus* [5, 17, 21]. *H. hepaticus*-induced chronic active hepatitis is characterized by portal inflammation and/or cholangitis with mononuclear inflammatory cells, bile ductular hyperplasia, pseudoinclusion bodies in hepatocyte nuclei, hepatocytomegaly and hepatocytic pleomorphism [5, 13, 15, 17, 21]. In our study, the histopathological lesions were closed to identical lesions of *H. hepaticus*-induced chronic active hepatitis in A/J mice, including intranuclear pseudoinclusion bodies and hepatocytomegaly. Because this study was not an intentional infectious experiment and the mouse colony had been already closed, the exact *Helicobacter* species and infectious route of the causative microorganism were unclear. Infiltrated inflammatory cells in chronic hepatitis consisted of mostly B lymphocytes, T lymphocytes and/or activated and hypertrophied Kupffer cells.

In this study, hepatic inflammation and tumors were also detected in some nonirradiated and irradiated mice. Despite the irradiation effects on mice being unclear, hepatic inflammation and tumors induced by natural *Helicobacter* infection could influence on experimental results. It was suggested that these chronic hepatitis and hepatic tumors might be related to *Helicobacter* infection. Helical-shaped bacteria were observed and confirmed between hepatocytes by WS silver staining and IHC for *H. pylori*.

In lot A mice, the incidence of chronic active hepatitis and hepatic neoplasm after γ-ray irradiation showed a dose-dependent pattern relative to irradiation dose. It was presumed that immuosuppression caused by irradiation might aggravate inflammation and tumorigenesis in the mouse liver. However, the results for the other 4 lots did not indicate that radiation influenced the incidence. Also, the incidence of the lesions showed no remarkable age dependency. In any case, natural *Helicobacter* spp. infection in mice can occur in experimental animal facilities. Because *Helicobacter* infections in mice are capable of interfering with experimental results in a long-term animal study, it is suggested that monitoring and control of the infection should be performed in laboratory animal facilities.

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