INFECTION AND IMMUNITY, Sept. 1996, p. 3673–3681 Vol. 64, No. 9
0019-9567/96/$04.00+0

Persistent Hepatitis and Enterocolitis in Germfree Mice Infected with *Helicobacter hepaticus*

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Received 19 March 1996/Returned for modification 3 May 1996/Accepted 27 June 1996

Helicobacter hepaticus **has been associated with naturally occurring hepatitis in certain inbred strains of mice, and in A/JCr mice it has been linked to the development of hepatic adenomas and adenocarcinomas. *H. hepaticus* was orally inoculated into 30 axenic, outbred female mice, and the mice were studied longitudinally to fulfill Koch's postulates and to ascertain the pathogenic potential of the organism under defined germfree conditions. Ten cage contact mice were also housed in the same germfree isolator to study transmission patterns, and 10 germfree mice were maintained in separate isolators as controls. Mice serially euthanized from 3 weeks through 24 months postinoculation (p.i.) were surveyed by culture and PCR for *H. hepaticus* in liver and intestinal tissues. Tissues were analyzed for histopathological changes, and sera were assayed for the presence of immunoglobulin G antibody to *H. hepaticus* and changes in the liver enzyme alanine aminotransferase. Inoculated mice and cage contact mice were persistently infected with *H. hepaticus* as identified by culture and PCR, in both the intestine and, less frequently, the liver, for the duration of the 2-year study. Animals developed persistent chronic hepatitis, and in some animals enterocolitis was noted. Hepatocellular carcinoma was diagnosed in one *H. hepaticus*-infected mouse. The level of *H. hepaticus* serum antibody was highest in experimentally infected mice at 12 to 18 months p.i.; this corresponded in general to the time interval when the highest levels of alanine aminotransferase were recorded. Although cage contact mice became persistently infected with *H. hepaticus*, lesions were less severe and the levels of serological biomarkers utilized in the study were lower. The *H. hepaticus*-infected mouse will provide an ideal model to study putative bacterial virulence determinants and how they interact with the host to induce chronic inflammation and tumorigenesis.**

Helicobacter species isolated from the stomachs of humans and animals have been the focus of considerable research because of their association with gastric disease (33). *Helicobacter pylori* causes active, chronic gastritis and peptic ulcers in humans (24, 29, 33) and has also been recently linked to the development of gastric adenocarcinoma and gastric mucosa-associated lymphoma (17, 35, 36, 53). Several additional *Helicobacter* species isolated from the stomachs of various mammals (4, 7, 16, 19, 30) have been shown to cause various degrees of gastritis in their hosts (15, 16, 20, 28). *Helicobacter* species have also been isolated from the intestinal tracts of mammals (10, 42, 43, 48, 49) and birds (6). Two of them, *H. muridarum* and "*H. rappini*" colonize the ilea and ceca of rodents, but *H. muridarum* also can elicit gastritis after colonizing the gastric mucosae of older rodents (31, 37, 40). In addition, "*H. rappini*" has been associated with abortion in sheep and intestinal disease in animals and humans (2, 25, 39). Another bacterium has been isolated from diseased livers and intestines of aged inbred mice and has been characterized biochemically, by 16S rRNA sequence data, and named *H. bilis* (23). *H. pullorum* has been isolated from chickens with hepatitis and humans with gastroenteritis, and another intestinal helicobacter, *H. canis*, has been isolated from asymptomatic and diarrheic dogs as well as from humans with diarrhea (42, 43).

We recently isolated, characterized, and named *H. hepaticus*, a spiral-to-curved bacterium, observed with Steiner's silver stains in livers of barrier-maintained mice suffering from multifocal necrotic hepatitis (18, 52). *H. hepaticus* persistently colonizes the colon and cecum, is probably transmitted via the fecal-oral route, and is prevalent in many mouse colonies (41). The bacterium is associated with liver tumors in A/JCr mice as well as hepatitis in other susceptible inbred mouse strains (50, 52). Furthermore, a high incidence of inflammatory bowel disease in nude and SCID mice and several immunocompromised targeted gene mutant mice infected with *H. hepaticus* has been noted (12, 51). In a preliminary experiment, 10 A/JCr mice injected intraperitoneally with liver homogenate from a mouse with hepatitis developed liver lesions consistent with *H. hepaticus*-associated hepatitis (52). A 2-year longitudinal study in which the natural route of infection was mimicked by orally inoculating *H. hepaticus* into axenic mice was therefore designed to determine the organism's pathogenic potential and to fulfill Koch's postulates under defined germfree (GF) conditions.

MATERIALS AND METHODS

Animals. Fifty GF isolator-reared 4-week-old female Swiss Webster mice [Tac:(SW)f] were obtained from Taconic Farms (Germantown, N.Y.). The mice were maintained in GF isolators for the duration of the experiment. All materials for the GF units were sterilized with peracetic acid or Clidox. The mice were housed in polycarbonate cages on hardwood bedding and were fed an autoclaved pelleted diet and given sterile water ad libitum.

Bacteria. *H. hepaticus* type strain ATCC 51448 used for oral challenge was grown under microaerobic conditions at 37°C on Columbia blood agar for 3 days (Remel Laboratories, Lenexa, Kans.). The bacteria were harvested, resuspended in brucella broth, and visualized by phase microscopy for motility and morphology, and approximately 10^7 CFU/ml was used for inoculation. The inocula were assayed for evidence of aerobic and anaerobic bacterial contamination by culture on Colombia blood agar under aerobic and anaerobic conditions.

Experimental design. Thirty mice each received 0.2 ml of *H. hepaticus*-containing broth per os, and 10 mice served as cage contact controls. An additional 10 GF mice were housed in a separate GF isolator as controls. At 3, 10, 16, 28, 33, 44, and 58 weeks postinoculation (p.i.), as well as 16, 18.5, and 24.5 months p.i., one to five experimentally dosed and zero to three cage contact control mice from the GF isolator were euthanized and necropsied. Also, at similarly pre-

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scribed intervals, control mice from the separate GF isolator were euthanized and processed for evaluation.

***H. hepaticus* isolation.** At scheduled intervals (see above), mice were removed from the isolator, anesthetized with carbon dioxide for blood collection, and then euthanized with an overdose of carbon dioxide. Intestinal scrapings from the colon and ceca and a 2-mm cube of liver from each mouse were collected aseptically for culture (18). Spleens were cultured from mice analyzed at weeks 3 through 33 p.i., and uteri from mice at 80 and 106 weeks p.i. were cultured for *H. hepaticus*. Isolation of bacteria from infected mice was performed on Colombian blood agar (Remel Laboratories), with incubation at 37°C under microaerobic conditions for 5 days in vented jars containing N_2, H_2, and CO_2 (90:5:5). Characteristic colonies were Gram stained and examined by phase microscopy for motility. Oxidase, catalase, and urease reactions and sensitivities to nalidixic acid and cephalothin were also determined (18). Samples of feces, as well as caging material, were routinely incubated on sheep blood agar plates and incubated aerobically and anaerobically for 3 days at 37°C to assay for possible bacterial contamination of the GF mice and/or isolator.

Viral screening. Sera were collected from selected control and inoculated mice throughout the 2-year study. Sera were assayed for immunoglobulin G (IgG) antibody response by an enzyme-linked immunosorbent assay (ELISA) for the following murine viruses: minute virus of mice, sendai virus, GDVII, mouse hepatitis virus, ectromelia, pneumonia virus of mice, reovirus type 3, lymphocytic choriomeningitis virus, rotavirus, mouse adenovirus, polyomavirus, and K virus. The sera were also assayed for *Mycoplasma pulmonis* by ELISA and mouse orphan parvovirus by immunofluorescence by using standard procedures.

ELISA for anti-*H. hepaticus* antibody. (i) Antigen preparation. *H. hepaticus* sonicate for use in the ELISA was prepared in a manner similar to that for *H. felis* sonicate described elsewhere (15, 22). In brief, *H. hepaticus* was grown for 96 h in brucella broth (Difco Laboratories, Detroit, Mich.) containing 5% fetal calf serum. Cultures were incubated at 37°C in a microaerobic environment and were shaken at 10,000 rpm (Sorvall RC-5B centrifuge; Dupont, Newtown, Conn.) for 10 min, and the pellet was washed three times in phosphate-buffered saline (PBS). The pellet was then diluted in PBS to an optical density (at 600 nm) of ~1.0, and the bacterial cells were disrupted by sonication (sonicator from Artec K System Inc., Farmingdale, N.Y.). After centrifugation at 10,000 rpm for 10 min in a Sorvall RC-5B centrifuge, the protein content was determined by the Lowry technique as previously described (15, 22). Aliquots were frozen at −70°C until used in the assay.

(ii) ELISA. The ELISA was performed as previously described (15, 21). Wells of microtiter plates (Dynatech Laboratories, Chantilly, Va.) were incubated with 100 ng of *H. hepaticus* protein per ml in carbonate buffer. After the plates had been washed, serial twofold dilutions of sera from *H. hepaticus*-infected or control mice were incubated for 60 min at 37°C; the appropriate dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, St. Louis, Mo.) was then added to each well. Serum IgG antibody titers of <1:32 were considered negative.

Serum ALT. Serum alanine aminotransferase (ALT) was measured in *H. hepaticus*-infected cage contact and GF control mice on a longitudinal basis, by using sera from the same mice for which the *H. hepaticus* ELISA was performed. A 10-μl drop of serum was deposited on a multilayered film supported in plastic (Kodak Ektachem DT slide; Eastman Kodak Co., Rochester, N.Y.). The analysis was performed according to the manufacturer's direction and is based on an enzyme-coupled oxidation of NADH to NAD^+. The rate of oxidation was monitored by reflectance spectrophotometry and was used to measure ALT activity.

Histopathology. Gastrointestinal and liver tissues were fixed in neutral buffered 10% formalin, processed by standard methods, embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin and Warthin-Starry stains. The gastrointestinal and liver tissues were examined for histological changes and the presence of *H. hepaticus.*

Immunofluorescence staining of mouse liver. Livers and intestines from *H. hepaticus*-infected mice and control livers were processed for immunofluorescence staining by using polyclonal *H. hepaticus* rabbit antisera as previously described (22). Briefly, tissue sections were deparaffinized and rehydrated and slides were incubated with 0.05% pronase. The tissue sections were covered with either rabbit preimmune serum or postimmune serum raised against *H. hepaticus* whole-cell sonicate and incubated. Slides were then washed and incubated with anti-rabbit IgG fluorescein isothiocyanate conjugate. Slides were examined with a Zeiss fluorescence microscope.

PCR analysis. PCR evaluations of mucosal scrapings from ceca and colons and homogenates of liver tissue were performed to supplement culture techniques to identify infection with *H. hepaticus.*

DNA extraction. DNA was extracted from frozen cecal, colonic, and liver tissues isolated from control and *H. hepaticus*-infected mice. Approximately 15 mg of tissue was homogenized to uniformity by using a plastic, microcentrifuge-adapted pestle. Tissue was then processed by using the Rapid Prep Genomic DNA kit as outlined by the manufacturer (Pharmacia Biotech, Piscataway, N.J.).

PCR amplification. The primer sequences chosen for PCR amplification recognized a region of the 16S rRNA gene specific for *H. hepaticus* (41). The two oligonucleotides, 5′ GCA TTT GAA ACT GTT ACT CTG 3′ and 5′ CTG TTT TCA AGC TCC CC 3′, produced an amplified product of 417 bp. Between 12 and 18 μl of the DNA preparation was added to a 100-μl (final volume) reaction mixture containing 1× *Tth* polymerase buffer (supplied by the manufacturer but supplemented with 1 M $MgCl_2$ to a final concentration of 2.75 mM), 0.5 μM each the two primers, 200 μM each deoxynucleotide, and 200 μg of bovine serum albumin per ml. Samples were heated at 94°C for 4 min, briefly centrifuged, and cooled to 61°C. *Tth* polymerase (3.2 U) (Pharmacia) and 1.25 U of polymerase enhancer (Perfect Match; Stratagene, La Jolla, Calif.) were added, and these were followed by an overlay of 100 μl of mineral oil. The following conditions were used for amplification: denaturation at 94°C for 1 min, annealing at 61°C for 2 1/4 min, and elongation at 72°C for 2 1/2 min. A total of 35 cycles were performed, and these were followed by an elongation step of 7 min at 72°C. A 10- to 15-μl aliquot of the sample was then electrophoresed through a 6% Visigel separation matrix (Stratagene); this was followed by ethidium bromide staining and viewing by UV illumination.

Southern blots. For confirmation of the PCR-amplified DNA by Southern blot hybridization, selected PCR products were analyzed. A 15-μl volume of the PCR product was electrophoresed through a 1.0% NuSieve GTG agarose gel (FMC Bioproducts, Rockland, Maine) and transferred onto a Hybond N nylon membrane as outlined by the manufacturer (Amersham, Arlington Heights, Ill.); this was followed by UV cross-linking. The fixed DNA was then subjected to hybridization with a PCR-generated probe prepared by using *H. hepaticus* type strain DNA. This product was excised from a 1.0% low-melting-point agarose gel (FMC Bioproducts) and purified by using the Geneclean DNA purification kit (Bio 101, Vista, Calif.). The probe was labeled with horseradish peroxidase, hybridized overnight to the nylon membrane at 42°C, and exposed in the presence of luminol to Hyperfilm-ECL as outlined by the manufacturer (Amersham, Arlington Heights, Ill.).

RFLP. The primer sequences chosen for the PCR amplification recognized a region of the 16S rRNA common to all known *Helicobacter* species. The two oligonucleotides 5′ GCT ATG ACG GGT ATC C 3′ and 5′ ACT TCA CCC CAG TCG CT 3′ produced an amplified product of approximately 1,220 bp. PCRs were performed as indicated above under the following conditions: 94°C for 4 min, followed by 35 cycles composed of 1 min at 94°C, 2 1/2 min at 56°C, and 3 1/2 min at 72°C. A final elongation was performed at 72°C for 7 min. Fifteen-microliter aliquots were then removed and electrophoresed through a 6% Visigel as indicated previously. For the RFLP, 20-μl volumes from positive samples were restricted with *Alu*I and *Bfa*I in an excess of enzyme for 4 h at 37°C as outlined by the manufacturer. The digested PCR product was then electrophoresed through a 5.0% NuSieve GTG agarose gel (FMC Bioproducts) and stained in ethidium bromide, and the resultant RFLP patterns were photographed.

RESULTS

Colonization of *H. hepaticus*. The culture results of mice analyzed during the 2-year study are shown in Table 1. Compilation of culture results from different sites on individual mice showed *H. hepaticus* isolation in 16 of 26 (62%) mice. The lack of uniform *H. hepaticus* positivity by culture resulted from the failure to isolate *H. hepaticus* from mice sampled at weeks 10, 28, and 33 p.i. Sporadic recovery of *H. hepaticus* from livers and stomachs of *H. hepaticus*-infected mice was recorded, and *H. hepaticus* was isolated from the spleen of one mouse at 3 weeks p.i. Interestingly, 100% of the 12 experimentally infected mice whose tissue samples or samples of feces collected from the intestinal tract were cultured from week 44 through the completion of the study, 24.5 months p.i., were positive for *H. hepaticus*. A lower incidence of *H. hepaticus* recovery, from four of nine mice (44%), was noted for cage contact mice. Also, the uteri were negative for *H. hepaticus* by culture. *H. hepaticus* was not recovered from any GF mice housed in a separate GF isolator.

***H. hepaticus* detection by PCR.** The use of previously validated PCR primers with high levels of specificity and sensitivity increased the number of *H. hepaticus*-positive tissue samples, particularly from the liver (Fig. 1A) (41). *H. hepaticus*-specific PCR products were identified in 12 of 19 liver samples and in 20 of 20 (100%) intestinal samples from *H. hepaticus*-infected mice (Table 1). In cage contact mice the incidence was lower, with only two of six (33%) liver samples testing positive by PCR. However, all seven cage contact mouse intestine samples analyzed were positive by PCR. None of the germfree mice in the separate isolator were positive for *H. hepaticus* with these *H. hepaticus*-specific primers.

Southern blot. Selected intestinal and liver *H. hepaticus* PCR products from samples taken from *H. hepaticus*-infected and

FIG. 1. (A) Electrophoresis of DNA amplified by PCR on a 6% Visigel separation matrix with *H. hepaticus*-specific primers. Lane 1, DNA isolated from colonic tissue of an uninfected mouse; lane 2, DNA isolated from the liver of an *H. hepaticus*-infected mouse 10 weeks p.i.; lane 3, DNA isolated from the liver of an infected mouse 44 weeks p.i.; lane 4, DNA isolated from the liver of an infected mouse 24.5 months p.i.; lane 5, DNA isolated from colonic tissue of an infected mouse 18.5 months p.i.; lanes 6 and 7, DNA isolated from the livers of infected mice 24.5 months p.i.; in both cases, colonic and cecal tissue isolates tested positive by PCR; lane M, 100-bp molecular size markers (GIBCO/BRL). (B) Southern blot hybridization of PCR products after electrophoresis through a 1.0% agarose gel (FMC). Lanes are as described for panel A.

cage contact mice at different time points in the study hybridized specifically to the horseradish peroxidase-labeled PCR-generated probe prepared by using the *H. hepaticus* type strain as described previously (Fig. 1B) (41).

RFLP. By RFLP analysis, *H. hepaticus* isolated from mice 10 weeks and 24.5 months p.i. showed a pattern identical to that of the *H. hepaticus* strain used for inoculation (Fig. 2).

ELISA for viral antibody. All mice assayed from both GF

FIG. 2. Comparison of RFLP patterns obtained with restriction enzymes *Alu*I and *Bfa*I. Portions (20 μl) of the digested PCR products were electrophoresed through a 5.0% agarose gel. Lanes 1, tissue digest from a cecal sample taken at 10 weeks p.i.; lanes 2, tissue digest from a cecal sample taken at 24.5 months p.i.; lanes 3, purified DNA from the infecting strain of *H. hepaticus*; lanes 4, purified DNA from another *H. hepaticus* strain; lane M, 100-bp molecular size marker (GIBCO/BRL).

TABLE 1. *H. hepaticus* in orally inoculated and cage contact GF mice

Tissue	No. of mice with positive result/no. of mice with sample cultured[a] at wk p.i.:																			
	3		10		16		28		33		44 (I)	58 (I)[b]	69 (I)	80 (I)	106	
	I[b]	CC[c]	I	CC	I	CC	I	CC	I	CC					I	CC
Lower intestine	2/2 (2/2)	0/1 (1/1)	0/2 (2/2)	0/1 (1/1)	2/3 (3/3)	1/1 (1/1)	0/4 (3/3)	0/2	0/3 (3/3)	0/1 (1/1)	2/2 (1/1)	2/2 (2/2)	1/1 (1/1)	2/2	5/5 (5/5)	2/3 (3/3)
Liver	1/2 (1/2)	0/1 (0/1)	0/2 (1/2)	0/1 (1/1)	1/3 (3/3)	0/1 (1/1)	0/4	1/2	0/3 (1/1)	0/1	0/2	1/2 (1/2)	0/1 (1/1)	0/2 (2/2)	0/5 (2/5)	1/3 (0/3)
Stomach	2/2	0/1	0/2	0/1	0/3	0/1	NA[d]	NA	NA	NA	0/2	2/2	0/1	0/2	0/5	0/3
Spleen	1/2	0/1	0/2	0/2	0/3	0/1	0/4	0/2	0/3	0/1	NA	NA	NA	NA	NA	NA
Total	2/2 (2/2)	0/1 (1/1)	0/2 (2/2)	0/1 (1/1)	2/3 (3/3)	1/1 (1/1)	0/4 (3/3)	1/2	0/3 (3/3)	0/1 (1/1)	2/2 (1/1)	2/2 (2/2)	1/1 (1/1)	2/2 (2/2)	5/5 (5/5)	2/3 (3/3)

[a] Numbers in parentheses indicate PCR results.
[b] I, mice inoculated with *H. hepaticus*.
[c] CC, cage contact mice.
[d] NA, not applicable.

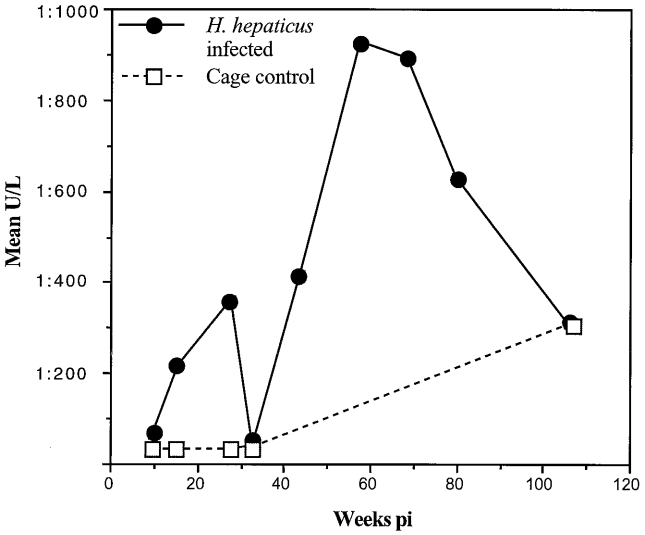

FIG. 3. *H. hepaticus* IgG levels in infected mice as determined by ELISA.

isolators had negative antibody titers to all viruses tested, as well as to *M. pulmonis.*

ELISA. Titers indicating *H. hepaticus* positivity in mice were not observed by ELISA until 16 weeks p.i. The mean *H. hepaticus* serum IgG levels by ELISA were highest at 12 to 18 months postinfection (Fig. 3). The peak *H. hepaticus* serum IgG titer of 1:928 noted by ELISA at 58 weeks p.i. corresponded to the time point when the highest mean ALT value was recorded for the same animals. The cage contact control mice had ELISA IgG titers of <1:64 throughout the study, until 24 months p.i. when a mean value of 1:305 was recorded.

ALT. Early in the study, i.e., 10 to 33 weeks p.i., the liver aminotransferase levels in the experimentally infected mice ranged from a low of 65 IU at 16 weeks to a high of 161 IU at 10 weeks p.i., but in general, measurements in the low 100's were recorded. However, at 44 weeks p.i., the ALT levels were elevated to 265 IU and at 58 weeks p.i. the ALT attained its highest level, 419 IU. Thereafter the levels dropped, with a mean of 231 IU observed for the last group of mice analyzed at 24.5 months p.i. In the cage contact animals, on the other hand, ALT levels were consistently lower, remaining in the low to mid 100's with the exception of a high of 204 IU and a low of 81 IU at 16 and 10 weeks p.i., respectively. The mean ALT levels measured in GF mice at different ages maintained in the separate isolator remained in the low to mid 100's.

Gross findings. During the 2-year study, six mice (four *H. hepaticus*-infected mice, one cage contact control mouse, and one GF mouse) died in the GF isolators and were unavailable for analysis. At 28 weeks p.i., inoculated mice had enlarged mesenteric lymph nodes and one mouse had multifocal 1- to 3-mm-diameter punctate white foci in two lobes of the liver. Focal liver lesions were also noted in several mice at 33 through 106 weeks p.i. One mouse at 33 weeks p.i. appeared lethargic with a distended abdomen. It had lymphosarcoma in the liver, mesenteric lymph nodes, and peripheral lymph nodes, as well as in the spleen, colon, and cecum. At 18 to 24 months p.i., several of the mice had fluid-filled, enlarged uteri.

Histopathology in gnotobiotic mice. Three weeks after inoculation with *H. hepaticus*, multifocal discrete coagulative necrosis of hepatocytes with minimal inflammatory cell infiltration at the periphery was noted. The necrotic foci mostly involved a few to many hepatocytes, and the inflammatory cells were mainly lymphocytes and neutrophils. The punctate lesions were often adjacent to or in close proximity to central veins and/or intralobular venules. The lesion was essentially limited to the parenchyma, and the portal triads were rarely affected.

At 10, 16, and 28 weeks after inoculation, hepatocyte necrosis became more severe, reaching a peak at 28 weeks (Fig. 4a). The size of the necrotic foci varied from many hepatocytes at 10 weeks to numerous hepatocytes at 28 weeks. The inflammatory cells, i.e., lymphocytes, macrophages, and neutrophils, infiltrated into the necrotic foci, the parenchyma around the central veins and intralobular venules, and the portal triads. Focal aggregates of macrophages containing granular yellow-brown pigment were also present, sometimes in association with foci of inflammation, but not consistently so. At 33 weeks to 16 months p.i., hepatocyte necrosis was still present and occasionally multinucleate giant cells were associated with the inflammation and necrosis. Inflammation became more prominent and was centered in the portal triads and the parenchyma around the intralobular venules (Fig. 4b). Hepatocyte necrosis was very mild at 18 months and essentially disappeared at 24 months. The inflammatory cells were centered in portal triads and intralobular venules and extended into the adjacent parenchyma, with occasional formation of lymphoid aggregates or follicles. Some of the portal and intralobular veins and bile ducts were also infiltrated by the inflammatory cells (Fig. 4c). A hepatocellular carcinoma was observed in one mouse at 24 months (Fig. 4d). The hepatocytes were markedly pleomorphic and arranged in anastomosing cords or lobules.

The cecum and colon were histologically normal in infected mice at 3 weeks p.i. Mixed cell infiltrates consisting of lymphocytes, plasmacytes, and occasional macrophages and neutrophils were observed in the ceca in all mice from 10 weeks to 24 months p.i., with frequent lymphoid aggregates or follicle formation in the mucosae and submucosae at 33 weeks and thereafter (Fig. 5a). Minimal to mild colitis was observed in one mouse at 28 weeks and in all mice at 33 weeks to 24 months p.i., with frequent lymphoid aggregates or follicles in the mucosae (Fig. 5b). Mucosal epithelial cell hyperplasia was observed in the ceca at 18 and 24 months (Fig. 5a). Peyer's patches became very prominent in the colons of these mice (Fig. 5c). A Warthin-Starry stain and immunofluorescence revealed *H. hepaticus* organisms in the mucosal crypts of the cecum and colon (Fig. 5d). Mucosal epithelial cell hyperplasia was also seen in the colons of five of eight mice (five *H. hepaticus*-infected and three cage contact mice) at 24 months. One mouse examined at 24 months also had marked segmental enteritis characterized by multifocal infiltration of mixed inflammatory cells in the mucosa, submucosa and muscle layers, with epithelial hyperplasia, erosions, and ulceration. Perivasculitis was also a prominent feature of the lesion (Figs. 5e and f). The changes in the livers, ceca, and colons of cage contact control mice appeared to be slightly less severe than those in the corresponding inoculated mice, particularly at early time points (3 to 16 weeks).

Mild to minimal or no changes in the livers of GF control mice were observed at all time points examined. An occasional older mouse had liver changes similar to but less severe than those in the *H. hepaticus*-inoculated mice at 3 to 10 weeks p.i. The focal, sporadic liver lesions in the GF mice, when present, were characterized by discrete areas of coagulation necrosis, with a minimal inflammatory component. The punctate lesions were often adjacent to, or in close proximity to, central veins and/or intralobular venules. A marginal thin zone of mononuclear inflammatory cells often provided a sharply demarcated ring to the lesion, separating affected parenchyma from normal

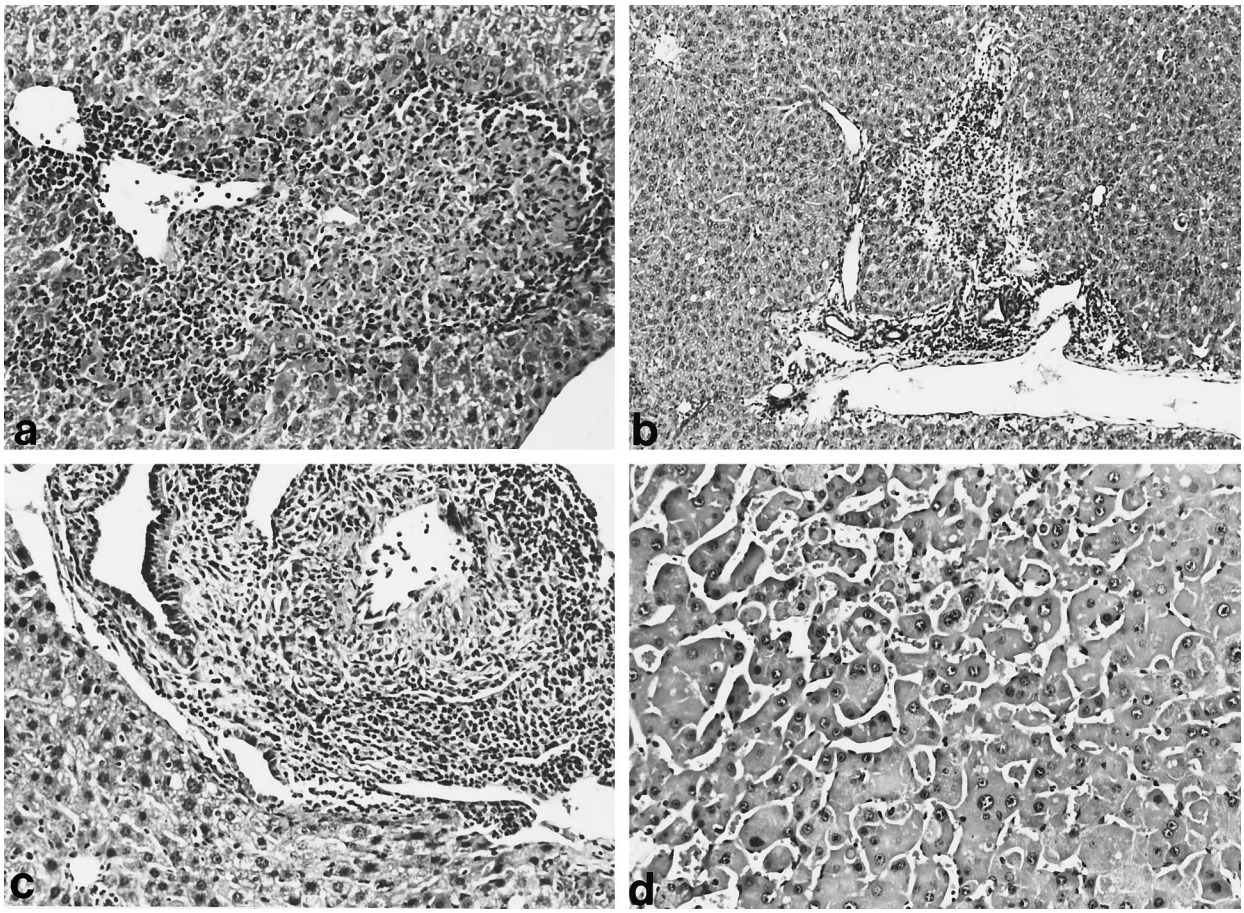

FIG. 4. Mouse liver tissue. (a) Focal hepatocyte necrosis with lymphocyte, macrophage, and neutrophil infiltration around the central vein; (b) marked inflammatory cell infiltration centered in the portal triad and extending into the adjacent parenchyma; (c) inflammatory cells in a portal triad infiltrating the vascular and bile duct walls; (d) hepatocellular carcinoma consisting of pleomorphic hepatocytes arranged in anastomosing cords or lobules in liver tissue of an *H. hepaticus*-infected mouse.

hepatic parenchyma. The lesion was classified as a parenchymal lesion, with the histopathological features of microvascular thrombosis and infarction. A few scattered mononuclear cells in the lamina propria were observed in the cecum at certain time points. No lesions in the colon were observed at any time points, and lymphoid aggregates or follicles, when seen, in the cecal and colonic mucosae of the GF mice were minimal.

Immunofluorescence assay. A fluorescent-antibody technique, described previously, utilizing rabbit polyclonal antibody to *H. hepaticus* was performed on livers and intestines of *H. hepaticus*-infected, cage contact *H. hepaticus*-infected, and GF control animals (22). *H. hepaticus* was stained with the fluorescence-labeled *H. hepaticus* polyclonal rabbit antibody in the ceca and colons of *H. hepaticus*-infected mice. The organisms were noted to be present on the mucus of surface epithelia as well as deep in the crypts (Fig. 4d). Fewer organisms were present in cage contact animals. However, careful examination of *H. hepaticus*-infected mice failed to discern *H. hepaticus* in their livers.

DISCUSSION

The results of this study showing that *H. hepaticus* can cause persistent, chronic hepatitis in mice fulfill Koch's postulates. The uniform identity of the inoculation strain of *H. hepaticus* throughout the study, as determined by RFLP, strengthens Koch's postulates on a molecular basis. Furthermore, the organism colonizes the lower intestine and in selected animals causes enterocolitis. In many respects, the results obtained by experimental oral inoculation of *H. hepaticus* into GF mice mirror our earlier observation of natural *H. hepaticus* infection in A/JCr mice (22, 50, 52). *H. hepaticus* colonized, on a persistent basis, the lower intestine, the organism being recovered by culture from 100% of the experimentally infected mice from week 44 through the completion of the study 2 years after the mice were orally dosed once with *H. hepaticus*. Although there were fewer positive *H. hepaticus* culture results for mice analyzed earlier in the study, the overall incidence of *H. hepaticus* infection as determined by both culture and PCR was 25 of 26 (96%) in the orally inoculated mice and 8 of 9 (89%) in the cage contact mice. Given that previously published studies indicate that other enteric organisms readily colonize the gastrointestinal tracts of GF mice, it was surprising that *H. hepaticus* did not colonize more efficiently and in higher numbers, particularly in the colon and cecum, its preferred ecological niches (44, 54). The 100% colonization of the colon and cecum in *H. hepaticus*-infected A/JCr mice may indicate that other enteric microflora are needed for more efficient colonization (22). *H. hepaticus* was also infrequently isolated by culture from the livers of mice in this study, as was the case for naturally *H. hepaticus*-infected A/JCr mice (22). Increasing the number of sample sites in the liver may have also increased the

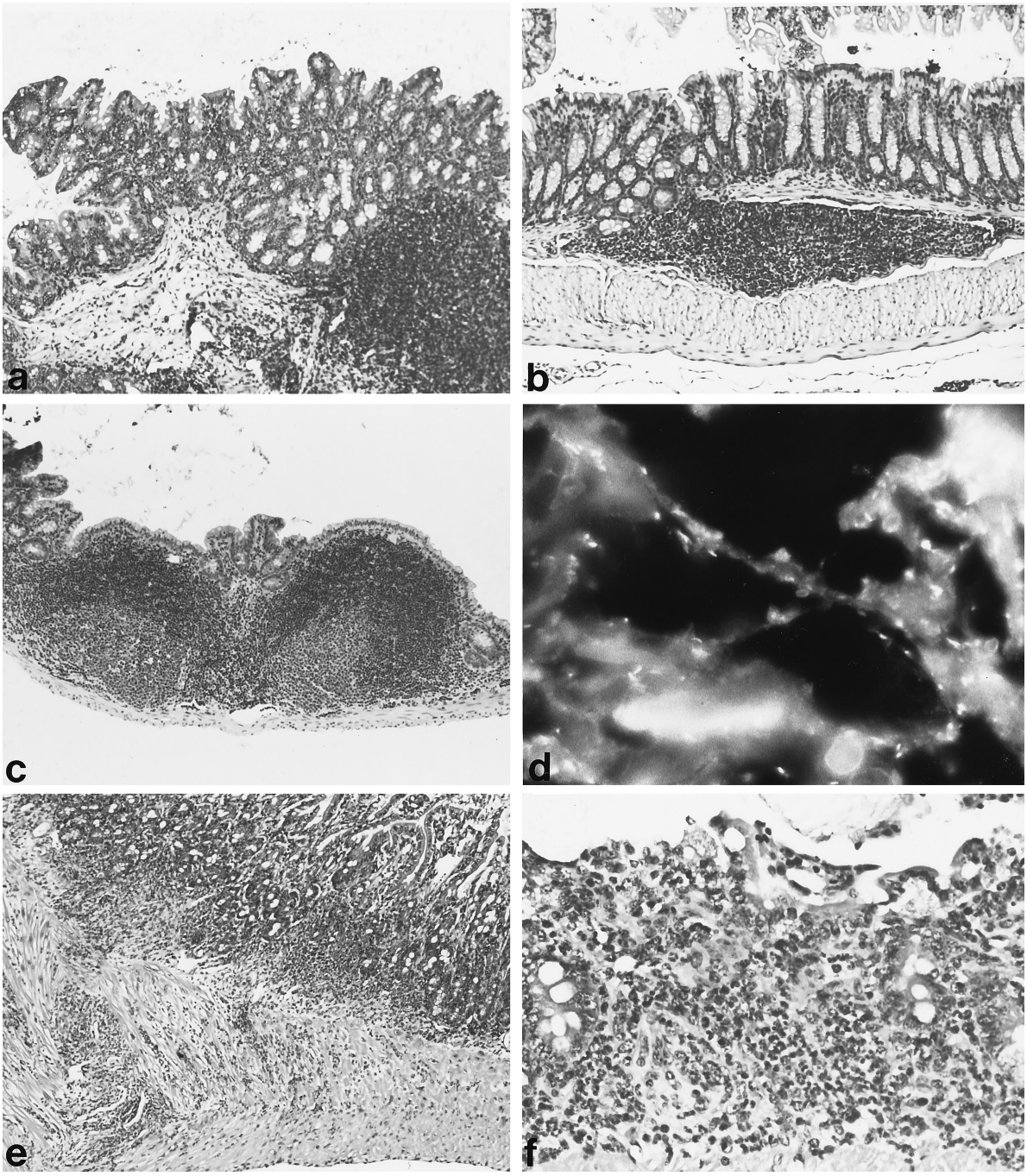

FIG. 5. Mouse tissues. (a) Cecum. Shown are mixed inflammatory cell infiltration and lymphoid aggregation in the lamina propria and submucosa with mild epithelial hyperplasia. (b) Colon. Mononuclear cell infiltration in the lamina propria and lymphoid aggregation in the submucosa can be seen. (c) Colon. Prominent Peyer's patches are visible. (d) Immunofluorescence revealed numerous *H. hepaticus* organisms in a crypt of cecal mucosa. (e) Ileum. Shown is interstitial to perivascular infiltration of mixed inflammatory cells in the mucosa and muscle layers. (f) Ileum. Intensive infiltration of neutrophils and mononuclear cells in the mucosa with deep erosion is visible.

number of positive *H. hepaticus* culture results. The organism, however, was identified to be present in the livers of the *H. hepaticus*-infected GF mice on a more consistent basis by PCR analysis.

The bipolar flagella on the organism probably allow *H. hepaticus* to actively migrate through the mucus layer of the lower intestinal crypts and persist in this milieu despite the peristaltic action of the intestine and active shedding of intestinal enterocytes. Gastric helicobacters also successfully establish persistent colonies in the mucus layer of the stomach (20). The importance of motility in colonization of gastric mucus is illustrated by studies using isogenic *Helicobacter* mutants; aflagellated mutants of *H. pylori* and *H. mustelae* were unable to colonize gnotobiotic piglets and ferrets, respectively, whereas the fully motile parent strains were efficient colonizers of the gastric mucosa (1, 9). Urease is also important for colonization

by *H. pylori* in gnotobiotic piglets and *H. mustelae* in ferrets, as demonstrated in vivo by the use of isogenic mutants lacking this enzyme (1a, 8). It will be interesting to ascertain whether these phenotypic features of motility and urease activity in *H. hepaticus* are also required for the organism to colonize and persist in both the intestines and livers of mice. Also, it is not known how *H. hepaticus* causes necrosis or inflammation in the liver and apparently, in certain mice, an inflammatory response in the intestine. Urease produces ammonia as a by-product of urea metabolism. Large amounts of ammonia generated by urease-positive *H. hepaticus* could cause tissue damage because of the compound's tissue irritant properties. In addition, urease can independently activate neutrophils and mediate inflammatory cytokines (5, 32, 38). Also, *H. hepaticus* produces a hepatocytotoxin in vitro, and this protein, like the cytotoxin of *H. pylori*, may play a role in eliciting tissue damage in vivo (47).

Because of the length of the study, concern about fighting among male mice, and the limited space in the GF isolator which dictated group housing of the animals, female mice were chosen for this study. As noted previously for A/JCr mice, the severity of the liver lesion is more pronounced in male mice than in female mice (22, 50, 52). Thus, the appearance of moderate hepatitis in the female mice in this study and the finding of only one mouse with a hepatocellular carcinoma are not surprising. It should also be stressed that oval-cell hyperplasia and karyomegaly were not as pronounced in these *H. hepaticus*-infected female mice as in male A/JCr mice infected with *H. hepaticus*. As noted earlier with specific-pathogen-free inbred strains of mice and confirmed in this study with axenic mice, focal idiopathic necrotic hepatitis without *H. hepaticus* infection does occur (45). The genesis of the lesion appears to be the result of microvascular thrombosis and infarction. However, unlike *H. hepaticus*-associated hepatitis, livers with this idiopathic vascular lesion do not have bile duct and oval cell hyperplasia and portal triad inflammation was minimal to absent. It is important however, to recognize that idiopathic hepatitis occurs in mice, as it did in this study, and may be confused with *H. hepaticus*-associated hepatitis. Careful cultural or PCR analysis for *H. hepaticus* and determining the susceptibility of given mouse strains to *H. hepaticus* hepatitis as well as idiopathic liver lesions are important considerations in arriving at a correct diagnosis.

In addition to gender, host genotype appears to be important in expression of hepatitis in *H. hepaticus*-infected mice. For example, *H. hepaticus* efficiently colonizes the lower bowels of C57B1 mice but this inbred strain appears to be resistant to development of chronic hepatitis (52). It may be that outbred mice are also more resistant to *H. hepaticus*-induced liver disease than the inbred A/JCr strain is. Host genotype is also important with regard to the degree of gastric inflammation and epithelial cell proliferation in mice experimentally infected with *H. felis* (21, 34).

Although the organism's natural ecological niche is the crypts of the cecum and colon, the mechanism whereby *H. hepaticus* colonizes and persistently infects the liver is unknown. Whether *H. hepaticus* subsequently colonizes the liver directly via the bile duct, by M-cell uptake, or by translocation across epithelia, its presence in bile canaliculi is undoubtedly facilitated by its resistance to high levels of bile (18, 22, 52). The isolation of *H. hepaticus* from the spleen of one mouse 3 weeks p.i. suggested hematogenous spread of *H. hepaticus* early in the course of disease, but *H. hepaticus* was not isolated from the spleen at later time points. A hematogenous route early in the course of infection, though suspected with certain strains of *Campylobacter jejuni* that cause persistent hepatitis in mice, was not shown experimentally (26, 27). Others have indicated, however, that the liver had a role in clearing *C. jejuni* from the blood of experimentally infected mice (3).

The cage contact mice were apparently infected by aerosolization of fecal matter in the GF isolator, since separate instruments were used to change bedding materials for cage contact mice and mice experimentally infected with *H. hepaticus*. The number of organisms infecting cage contact mice was smaller than the number infecting experimentally inoculated mice, on the basis of the lower rate of recovery of *H. hepaticus* by culture and the lack of appreciable elevation in ELISA and ALT values, and, importantly, the liver lesions in these animals, particularly early in the study, were less severe. These findings would argue that the level of *H. hepaticus* exposure (and hence the oral inoculating dose) in infected colonies may in part dictate the prevalence of *H. hepaticus* in a given colony as well as the level of severity of hepatic involvement in *H. hepaticus*-infected animals (41). In addition to use of sound sanitation protocols and perhaps the use of microisolator tops on mouse cages to minimize the spread of *H. hepaticus*, intensive antibiotic therapy can eradicate *H. hepaticus* from infected mice and prevent its spread in mouse colonies (13, 14). Unfortunately, the use of noninvasive biomarkers in lieu of culture and/or PCR analysis to presumptively detect the presence of *H. hepaticus* was disappointing. Even in GF mice experimentally infected with *H. hepaticus*, the ELISA and ALT values were not appreciably elevated until the animals had been infected for almost a year. These results are similar to our findings with A/JCr mice, where these biomarkers were more suggestive of *H. hepaticus* infection in male mice versus female mice but were not considered useful as a screening tool for early diagnosis of *H. hepaticus* infection (22).

The presence of enterocolitis in several of the older *H. hepaticus*-infected mice is consistent with earlier observations of a higher incidence of colitis and typhlitis in immunocompromised SCID and *nu*/*nu* mice and A/JCr mice infected with *H. hepaticus* than in those colonies free of the organism (22, 50, 51). We have also recorded a high incidence of *H. hepaticus* in several targeted-gene-mutant strains of mice with inflammatory bowel disease (12). The presence of enterocolitis in mice monocontaminated with *H. hepaticus* suggests that chronic colonization by *H. hepaticus* can induce persistent inflammation in intestinal tissue as well as the liver, but at a lower frequency. This contrasts with results of earlier studies in which GF mice monocontaminated with *Escherichia coli* showed no difference from control of mice in histology (46). Mechanisms operable in this process, as well as the influence of host genotype and immune status on disease expression, may be comparable to mechanisms operating in the pathogenesis of proctitis and colitis in immunocompromised men infected with *H. cinaedi* and *H. fennelliae* and immunocompetent macaques experimentally infected with these *Helicobacter* spp. (10, 11, 48).

In summary, the *H. hepaticus* mouse model offers the potential to study the pathogenesis of a chronic bacterial infection and its role in development of hepatic cancer and inflammatory bowel disease. As evidence that several *Helicobacter* spp. play a pathogenic role in gastroduodenal disease in humans and animals continues to accumulate, it is important to understand the pathogenesis of the disease, to identify important virulence determinants shared by these organisms, and to determine how these genes individually and collectively influence expression of disease induced by *Helicobacter* spp. Studies to define putative virulence factors of *H. hepaticus* and, once these have been determined, to construct *H. hepaticus* isogenic mutants lacking expression of these phenotypes are under way in our laboratory. Testing these isogenic mutants in mouse

models will allow us to specifically identify the role of these virulence factors in expression of *H. hepaticus*-associated gastrointestinal disease and will allow us to determine if they are homologous to the genetic microbial determinants responsible for *H. pylori*-associated gastritis, peptic ulcer disease, and gastric cancer.

ACKNOWLEDGMENTS

This study was supported by grant RO1 CA67529 and contract RFPS 94-69 from the National Cancer Institute.

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Editor: P. E. Orndorff