PATHOGENICITY

In-vitro hepatotoxic factor in *Helicobacter hepaticus*, *H. pylori* and other *Helicobacter* species

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Summary. Several inbred strains of mice in closed breeding colonies were found to have spiral-shaped bacteria associated with active, chronic hepatitis. A new species of *Helicobacter*, *H. hepaticus*, was isolated from the infected livers of some strains of mice. Other strains of mice were colonised with *H. hepaticus* in the caecum and colon, but not the liver. Filter-sterilised supernatant fluid from five strains of *H. hepaticus* was tested in a mouse liver cell line (ATCC no. CCL 9.1) for cytotoxic activity. All strains produced a toxic factor causing morphological changes in the cells at dilutions up to 1 in 1000. Toxicity was observed after exposure to the supernatant fluid for 48–72 h. Other *Helicobacter* spp. that also produced the cytopathic effect (CPE) in the liver cell line were *H. felis*, *H. acinonyx*, *H. pylori* and one strain of *H. mustelae*. “*Helicobacter rappini*” and *H. muridarum* did not cause CPE in the liver cells. The soluble factor was stable at 4°C for up to 3 months. It was also stable at 56°C for 30 min, but was inactivated by boiling for 15 min. It was inactivated by incubation with trypsin. A partially purified preparation of the cytotoxin had a mol. wt of *c.* 100 000 and did not have urease activity. The cytotoxin produced by *H. hepaticus* did not cause vacuole formation in HeLa cells.

Introduction

Organisms belonging to the genus *Helicobacter* have been the focus of considerable research because of their role in gastric disease. *H. pylori* causes active, chronic gastritis and peptic ulcer disease in man^{1–3} as well as being linked to the development of gastric adenocarcinoma and gastric mucosal-associated lymphoma.^{4–7} Other species of *Helicobacter* cause varying degrees of gastritis in other mammals.^{8–10} *H. muridarum* primarily colonises the caecum and ileum of rodents, but can cause gastritis when it colonises the gastric mucosa of older rodents.^{11,12} “*Helicobacter (Flexispira) rappini*” has been isolated from the colon and caecum of mice.¹³ Recently, a new species of *Helicobacter*, *H. hepaticus*, was isolated from the liver, caecum and colon of mice with multifocal necrotic hepatitis.^{14–16}

H. pylori is known to produce a cytotoxin that causes vacuolisation of tissue-culture cells.^{17,18} Patients infected with *H. pylori* cytotoxin-producing strains develop an antibody response to this cytotoxin, indicating that the cytotoxin is produced *in vivo*.^{17–20} The mol. wt of purified, denatured cytotoxin is

reported to be $87\,000 \pm 320\,000$. The mol. wt of the native toxin is $\geq 972\,000$.¹⁹ Another protein with a mol. wt of 120 000 is produced by strains of *H. pylori* from patients with peptic ulcer disease.²¹ While this protein has been shown to differ from the cytotoxin that causes vacuoles in cells, its function remains unknown.²²

The finding that *H. hepaticus* could be isolated from the colons and caeca but not the livers of some mice with liver pathology suggested that a soluble toxic factor might be responsible for the lesions. *H. hepaticus* and other *Helicobacter* spp. were screened for cytotoxic effects on a mouse liver cell line and on HeLa cells.

Materials and methods

Bacterial cultures

Source. *H. hepaticus* was isolated from liver and mucosal scrapings of caecum and colon which were obtained at post-mortem examination. Tissue was homogenised in a tissue grinder in phosphate-buffered saline (PBS) and plated on to Sheep Blood 5% Agar and TVP Blood Agar (Remel Laboratories, Lenexa,

Fig. 1. Cytotoxic effect of *H. hepaticus* supernatant fluid on mouse liver cell line ATCC no. CCL 9.1. **A**, normal mouse liver cells; **B**, mouse liver cells after exposure to supernate of *H. hepaticus* broth culture for 72 h. Insert shows dense granules.

KA, USA). Mucosal scrapings were plated on TVP medium. After incubation for 7 days in micro-aerophilic conditions, the plates were examined for green, metallic, swarming growth. All suspect growth was gram-stained and examined under phase-contrast microscopy. Colonies were screened for urease, catalase, and oxidase reactions and characterised further as described previously.¹⁶

H. felis and *H. muridarum* were obtained from Dr A. Lee, University of New South Wales, Sydney, Australia; *H. acinonyx* from Dr K. Eaton, Ohio State University, Columbus, OH, USA; "*H. (Flexispira) rappini*" from Dr D. Schauer, Division of Comparative Medicine, MIT, Cambridge, MA, USA; *H. mustelae* isolated from a mink was donated by Drs Marshall and LaCross, University of Virginia, Charlottesville, VA, USA.

Cytotoxin production. Isolates were inoculated from blood agar plates into 10 ml of Brucella Broth (Difco) containing fetal calf serum (Hyclone Laboratories,

Logan, UT, USA) 20% in petri dishes. The petri dishes were stacked in Gas Pak jars (BBL Laboratories, Cockeysville, MD, USA) and the jars were filled with anaerobic gas mixture ($N_2:H_2:CO_2$, 90:5:5). Flasks were incubated on a rotary shaker (New Brunswick Scientific, Edison, NJ, USA) at 60 rpm at 37°C for 4–5 days. When the cultures appeared turbid, the supernatant fluid was harvested by centrifugation at 10000 rpm for 20 min (Sorvall Model RC4B, Dupont Instruments, Boston, MA, USA). The supernate was passed through a sterile 0.22- μ m filter and kept frozen at –70°C until used.

Cytotoxicity assay. A mouse liver cell line (ATCC no. CCL 9.1) and HeLa S3 cells were obtained from ATCC, Rockville, MD, USA. The cells were cultured in Dulbecco's Modified Eagle's Medium with glucose (Sigma) 4.5 g/L and fetal calf serum (Hyclone Laboratories) 10% at 37°C with CO_2 8%. For cytotoxin assays, a suspension of cells was adjusted to a concentration of 1×10^5 cells/ml and 100 μ l plated in

the wells of 96-well microtitration plates. The plates were incubated overnight and used within 3–4 days.

Filter-sterilised supernates were diluted two-fold serially in complete medium and 100 μ l were added to wells containing cells. Cells were observed daily for 5 days for morphological changes. Controls of sterile brucella broth containing serum 20% were also tested.

Tissue-culture cells were exposed to concentrations of *Escherichia coli* LPS (Sigma) from 4 μ g to 0.4 ng. Cells were observed as above for morphological changes.

The physical properties of the *H. hepaticus* cytotoxin were characterised with crude and partially purified cytotoxin preparations as described elsewhere for the toxins of *Clostridium difficile*.²³

Partial purification

Fifty ml of culture supernate were concentrated over a YM10 membrane (Amicon Corporation, Danvers, MA, USA) with an ultrafiltration cell (Amicon Corporation). Preparations were washed over the membrane with at least 2 volumes of column buffer (0.01 M Tris-HCl, 0.05 M NaCl, pH 8.0) and adjusted to a volume of 2–5 ml.

Sephadex G200 was prepared according to the manufacturer's instructions (Pharmacia Fine Chemicals, Piscataway, NJ, USA). A 1.5 $\times$ 30-cm column was poured and equilibrated at 4°C by reverse flow. A concentrated sample (0.5 ml) was applied to the column and 1-ml fractions were collected. UV absorbance was monitored with an ISCO UA5 monitor (ISCO Instruments, Lincoln, NB, USA). Alternative fractions were filter sterilised and assayed in cell culture for cytotoxic activity. Fractions with cytotoxic activity were serially diluted two-fold and assayed to determine peak cytotoxic activity.

LPS determination. Fractions obtained from the Sephadex G200 column were assayed for LPS with the E-Toxate Assay Kit (Sigma) following the protocol recommended by the manufacturer.

Mol. wt determination

The mol. wt of the toxic factor was determined by Sephadex G200 column chromatography with mol. wt standards (Sigma), as recommended by the manufacturer and as described previously.²⁴ The K_{av} value of toxin was compared to the K_{av} values of proteins of known mol. wt; i.e., cytochrome C, albumin, carbonic anhydrase and alcohol dehydrogenase.

Urease activity

A spectrophotometric assay was performed according to Dunn *et al.* with no modifications.²⁵

Results

Cytotoxic activity

Fig. 1A shows normal mouse liver cells in culture.

While it is normal for this cell line to release some cells into the medium, the majority of cells remain adherent to the plastic surface. Fig. 1B shows the cytotoxic effect on the cells after exposure to *H. hepaticus* supernatant fluid for 72 h. Cells exposed to the cytotoxin developed a dense, granular appearance (insert in fig. 1B). Cells exposed to high concentrations of the cytotoxin became rounded, but remained adherent to the plastic. Most (> 90%) of these cells were able to exclude trypan blue. The CPE observed in culture supernatant fluids from other *Helicobacter* species was identical to that seen with *H. hepaticus*. The results from screening several organisms for the cytotoxic effect in mouse liver cells, and for any vacuolating cytotoxin response of HeLa cells to the supernatant fluids, is shown in table I.

Partial purification of the cytotoxin

The tissue culture assay in mouse liver cells was used to determine whether the toxic factor in *H. hepaticus* and *H. pylori* supernatant fluid was retained by a YM10 membrane. The elution profile of *H. hepaticus* YM10 membrane-concentrated supernatant fluid by Sephadex G200 column chromatography is shown in fig. 2. A similar profile was observed for concentrated *H. pylori* supernatant fluid. Tissue-culture assay of fractions followed by serial dilutions of all fractions with toxic activity was performed to determine the elution profile of the toxic factor. A plot of the peak

Table I. Granulating cytotoxic activity in mouse liver cells and vacuolating cytotoxic activity in HeLa cells caused by supernatant fluids from five *H. hepaticus* strains* and related species

Strain	Titre of	
	Granulating activity in mouse liver cells	Vacuolating activity in HeLa cells
<i>H. hepaticus</i> 3B1	1000	0
<i>H. hepaticus</i> T†	1000	0
<i>H. hepaticus</i>	1000	0
<i>H. hepaticus</i> 93-1875	512	0
<i>H. hepaticus</i>	256	0
<i>H. felis</i> T†	4	0
<i>H. acinonyx</i>	64	0
<i>H. mustelae</i> (mink)	64	0
<i>H. pylori</i> 1027-1	8	0
<i>H. pylori</i> 1037-22	32	Undilute
<i>H. pylori</i> 2015-1	> 8	4
<i>H. pylori</i> 2013-2	> 8	0
<i>H. pylori</i> 1016-2	> 16	8
<i>H. pylori</i> 1014-1	8	16
<i>H. pylori</i> 2006-2	> 8	16
<i>H. pylori</i> 2006-1	> 8	8
<i>H. pylori</i> 1050-2	> 8	4
<i>H. pylori</i> 1009-1	> 8	2
<i>H. pylori</i> 1009-3	32	64

The ATCC type strains of *H. mustelae* and *H. muridarum*, and "*H. rappini*" did not cause CPE on either the mouse liver cell line or the HeLa cells.

*Five strains were tested from different sources.

†T designates ATCC type strain.

Fig. 2. Sephadex G200 elution profile of *H. hepaticus* supernate; — OD₂₈₀; ●---● cytotoxic activity.

Table II. Partial characterisation of the Sephadex G200 purified toxin from *H. hepaticus*

Treatment	Mouse liver cell tissue culture titre
Supernate	256
YM10 retentate	1000
YM10 filtrate	0
G200 fraction 24	32
heated at 56°C, 30 min	32
boiled 15 min	0
trypsin 0.25% at 37°C, 15 min	8

activity is shown in fig. 2. Cytotoxic activity was eluted in the same fractions when *H. pylori* chromatographed fractions were assayed. YM10 concentrated brucella broth containing serum 20% was also chromatographed. Assay of these fractions showed no effect on the mouse liver cell line, indicating that the CPE observed was not due to media components. A standard curve was constructed with known standards, and the mol. wt of the partially purified toxin was calculated to be *c.* 100 000.

Partial characterisation of the cytotoxin

The results of partial characterisation of the toxin are shown in table II.

Toxic activity of the partially purified cytotoxin was resistant to heating at 56°C for 30 min, but was inactivated by boiling for 15 min. Toxic activity of supernatant fluids and partially purified preparations was stable at 4°C and -70°C for at least 3 months. The cytotoxin was partially inactivated by trypsin 0.25% after 15 min at 37°C. Partially purified preparations had no urease activity. *H. hepaticus* supernatant fluids and partially purified preparations had no effect on HeLa cell culture. LPS concentrations up to 4 µg had no effect on the tissue culture cells. The concentration of LPS in the peak fractions was 1.9 ng/ml.

Discussion

A soluble factor that caused cytotoxic effects in a mouse liver cell line was demonstrated in the supernatant fluids of cultures of five strains of *H. hepaticus*. Other *Helicobacter* species that caused similar CPE were *H. felis*, *H. acinonyx*, *H. pylori* and one strain of *H. mustelae*. Organisms lacking the cytotoxin were "*H. rappini*", one strain of *H. mustelae* and *H. muridarum*. Cytotoxic activity in a mouse liver cell line which was related to hepatotoxic activity *in vivo* has been reported previously in strains of *Campylobacter jejuni*.²⁶⁻²⁸ The hepatotoxic factor was associated with subcellular components of the organism and was produced by some strains of *C. jejuni*.²⁷ Hepatotoxic *C. jejuni* must colonise the liver for lesions to occur;²⁷ thus it appears that hepatotoxicity occurs when there is direct contact of the organism with liver cells. We have been unable to culture *H. hepaticus* from the livers of most A/JCr mice, although these animals develop severe liver lesions. This suggests that *H. hepaticus* may produce a soluble factor that causes or contributes to the lesions observed in the livers of infected animals.

Inactivation by boiling and exposure to trypsin suggest that the cytotoxin is a protein. Chromatography of the native protein indicated that the mol. wt of the cytotoxin is *c.* 100 000.

The cytotoxin of *H. hepaticus* differs from the vacuolating cytotoxin of *H. pylori* in several ways. The latter is reported to have a native mol. wt of ≥ 972000 ,¹⁹ in contrast to the native mol. wt of *c.* 100 000 for *H. hepaticus* cytotoxin. Unlike the vacuolating cytotoxin, the *H. hepaticus* cytotoxin is stable at 4°C for up to 3 months. Furthermore, the crude supernatant fluids from *H. hepaticus* cultures and the partially purified cytotoxin had no effect on HeLa cells.

We conclude that *H. hepaticus* produces a soluble cytotoxic factor that is a protein and causes CPE in a mouse liver cell line. This cytotoxic factor is different

from the vacuolating cytotoxin produced by *H. pylori*. We are further purifying the cytotoxic factor to determine its role in the production of liver lesions *in*

vivo in mice and investigating the immune response of mice infected with *H. hepaticus* to determine if antibody to this cytotoxin is produced *in vivo*.

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