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

N. S. TAYLOR, J. G. FOX and L. YAN

Division of Comparative Medicine, Massachusetts Institute of Technology, 37 Vassar Street, Cambridge, MA 02139, USA

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. 100000 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 as well as being linked to the development of gastric adenocarcinoma and gastric mucosal-associated lymphoma. Other species of Helicobacter cause varying degrees of gastritis in other mammals. H. muridarum primarily colonises the caecum and ileum of rodents, but can cause gastritis when it colonises the gastric mucosa of older rodents. "Helicobacter (Flexispira) rappini" has been isolated from the colon and caecum of mice. Recently, a new species of Helicobacter, H. hepaticus, was isolated from the liver, caecum and colon of mice with multifocal necrotic hepatitis.

H. pylori is known to produce a cytotoxin that causes vacuolisation of tissue-culture cells. Patients infected with H. pylori cytotoxin-producing strains develop an antibody response to this cytotoxin, indicating that the cytotoxin is produced in vivo. The mol. wt of purified, denatured cytotoxin is reported to be 87000 ± 320000. The mol. wt of the native toxin is ≥ 972000. Another protein with a mol. wt of 120000 is produced by strains of H. pylori from patients with peptic ulcer disease. While this protein has been shown to differ from the cytotoxin that causes vacuoles in cells, its function remains unknown.

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, Kansas).
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 CO₂ 8%. For cytotoxin assays, a suspension of cells was adjusted to a concentration of 1 × 10⁵ cells/ml and 100 μl plated in
the wells of 96-well microtitration plates. The plates were incubated overnight and used within 3–4 days.

Filter-sterilised supernatants were diluted two-fold serially in complete medium and 100 µl 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 µg to 0·4 ng. Cells were observed as above for morphological changes.

The physical properties of the *H. hepaticus* cytototoxin 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 supernatant 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 × 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 UAS-2 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 vacuulating 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>
<thead>
<tr>
<th>Strain</th>
<th>Titre of</th>
<th>Granulating activity in mouse liver cells</th>
<th>Vacuulating activity in HeLa cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. hepaticus</em> 3B1</td>
<td>1000</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>H. hepaticus</em> †</td>
<td>1000</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>H. hepaticus</em> 93-1875</td>
<td>512</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>H. hepaticus</em></td>
<td>256</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>H. pylori</em> †</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>H. alcohyla</em></td>
<td>64</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>H. mustelae</em> (mink)</td>
<td>64</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>H. pylori</em> 1027-1</td>
<td>8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>H. pylori</em> 1037-22</td>
<td>32</td>
<td>Undilute</td>
<td></td>
</tr>
<tr>
<td><em>H. pylori</em> 2015-1</td>
<td>&gt; 8</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>H. pylori</em> 2013-2</td>
<td>&gt; 8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>H. pylori</em> 1016-2</td>
<td>&gt; 16</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td><em>H. pylori</em> 1014-1</td>
<td>8</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td><em>H. pylori</em> 2006-2</td>
<td>&gt; 8</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td><em>H. pylori</em> 2006-1</td>
<td>&gt; 8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td><em>H. pylori</em> 1050-2</td>
<td>&gt; 8</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>H. pylori</em> 1009-1</td>
<td>&gt; 8</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>H. pylori</em> 1009-3</td>
<td>32</td>
<td>64</td>
<td></td>
</tr>
</tbody>
</table>

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.
HEPATOTOXIN PRODUCTION BY HELICOBACTER SPP.

Fig. 2. Sephadex G200 elution profile of H. hepaticus supernate; OD$_{280}$; —— cytotoxic activity.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mouse liver cell tissue culture titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernate</td>
<td>256</td>
</tr>
<tr>
<td>YM10 retentate</td>
<td>1000</td>
</tr>
<tr>
<td>YM10 filtrate</td>
<td>0</td>
</tr>
<tr>
<td>G200 fraction 24</td>
<td>32</td>
</tr>
<tr>
<td>heated at 56°C, 30 min</td>
<td>32</td>
</tr>
<tr>
<td>boiled 15 min</td>
<td>0</td>
</tr>
<tr>
<td>trypsin 0.25% at 37°C, 15 min</td>
<td>8</td>
</tr>
</tbody>
</table>

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 cytotoxic 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. The hepatotoxic factor was associated with subcellular components of the organism and was produced by some strains of C. jejuni. Hepatotoxic C. jejuni must colonise the liver for lesions to occur; 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 cytotoxic is a protein. Chromatography of the native protein indicated that the mol. wt of the partially purified toxin was calculated to be c. 100000.

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.

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
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.

References