Mononuclear cell response in the liver of mice infected with hepatotoxigenic Campylobacter jejuni

E. KITA, F. NISHIKAWA, N. KAMIKAIDOU, A. NAKANO, N. KATSUI and S. KASHIBA

Department of Bacteriology, Nara Medical University, 840 Shiyoyocho, Kashihara, Nara 634, Japan

Summary. Intragastric inoculation with hepatotoxigenic strains of Campylobacter jejuni led to the death of mice during the late phase of infection. Histological study disclosed a massive infiltration of mononuclear cells in the liver, mimicking intrahepatic hypersensitivity. Neither enterotoxigenic nor enteroinvasive Escherichia coli induced such a lesion. However, the same histopathological change was induced by injecting the hepatotoxic factor of hepatotoxigenic C. jejuni intravenously on two occasions separated by 14 days. Neither a single injection of an increased dose of the hepatotoxic factor nor two injections, the second of which was heat-inactivated, induced this change. Pre-treatment with rabbit antibody to the hepatotoxic factor inhibited the development of the hepatic lesion. These results suggest that C. jejuni-induced hepatic lesions in mice may be caused, at least in part, by the active moiety of the hepatotoxic factor. The possible mechanisms by which the toxic factor induces hepatitis as a consequence of hypersensitivity are discussed in relation to Guillain-Barré syndrome and Reiter's syndrome associated with C. jejuni enteritis.

Introduction

Campylobacter jejuni is a major cause of diarrhoea in man throughout the world. Both extracellular enterotoxin1–3 and invasiveness4–8 are considered to be important virulence factors. C. jejuni causes both intestinal and hepatic lesions in man.7–9 Previous investigations10 showed that C. jejuni caused hepatitis in mice after intragastric inoculation. More recently, a toxic factor for mouse hepatocytes was isolated from several clinical strains of C. jejuni.11

In a previous investigation, C. jejuni-infected mice died after 12 months, with massive infiltration of mononuclear cells in the liver, mimicking sustained intrahepatic hypersensitivity. The present study was made to ascertain whether a hepatotoxic factor of C. jejuni could induce such a lesion in the liver of mice.

Materials and methods

Micro-organisms

C. jejuni strains GIFU 8734 and Nara-24 were used as hepatotoxigenic strains, and strains Nara-69 and KZ-12 as non-toxigenic strains, as described previously.11 Enterotoxigenic Escherichia coli 271–6, a gift from M. Ehara, Institute for Tropical Medicine, Nagasaki, Japan, and enteroinvasive E. coli N-15 (O167: H4), isolated from a patient with acute enteritis clinically resembling salmonellosis, were used as control strains.

Campylobacter culture

All C. jejuni strains were stored at −20°C in gelatin disks12 of the same batches as those used in previous studies.10,11 Bacteria were grown at 42°C for 24 h on plates of Brucella Agar (Difco) supplemented with defibrinated horse blood 10% v/v in an anaerobic jar with a Gas-Pak (Baltimore Biological Laboratory, Cockeysville, MD, USA) but without catalyst. The bacteria grown on these plates were used to inoculate a liquid medium10 composed of Tryptose (Difco) 2% w/v, K₂HPO₄ 0.2% w/v, NaCl 0.6% w/v, soluble starch 1% w/v, l-cysteine 0.02% w/v, yeast extract (Difco) 1%, and CO₂-saturated sodium bicarbonate 0.1% w/v, and incubated at 42°C for 48 h in a micro-aerobic atmosphere. For inoculation, the bacteria were centrifuged, washed with pre-warmed Brucella Broth (Difco) diluted 10-fold with a sodium bicarbonate 0.2% w/v solution containing l-cysteine 0.02% w/v, and then suspended (10⁸ cfu/ml) in this wash solution. The number of bacteria was quantitated spectrophotometrically and confirmed by plate counts. E. coli was cultured in tryptic soy broth and the inoculum was prepared in the same way as that of C. jejuni.

Animals

Female specific pathogen-free ddY mice were purchased from Japan SLC (Hamamatsu, Shizuoka) and...
maintained on sterile pelleted food and water ad libitum. Mice were 5 weeks old when they were infected with *C. jejuni* or control bacteria.

**Preparation of hepatotoxic factor**

The crude hepatotoxic factor, designated P65, was isolated from whole-cell lysates of hepatotoxicogenic strains of *C. jejuni*. It was purified by two chromatographic separation series (gel filtration by Sepharose 4B and ion-exchange chromatography by DEAE-Sepharose 4B) as described previously. The P65 fractions of non-hepatotoxigenic *C. jejuni* and *E. coli* were also used.

**Infection of mice**

In each of three experiments, groups of 25 mice were infected orally with the four *C. jejuni* strains by introducing 10⁷ cfu in a volume of 0.1 ml directly and atraumatically into the stomach through a thin vinyl tube (Intramedic, Clay Adams, Becton, Dickinson and Company, NJ, USA) connected to a blunted 23-gauge needle. The mice were starved for 24 h before inoculation. To confirm colonisation by *C. jejuni*, five randomly selected mice from each group were bled and killed 30 days after inoculation, and their intestinal contents and liver homogenates were cultured microaerobically on Skirrow's medium at 42°C for 48 h. Serum samples were tested for indices of liver function to confirm the presence of hepatic disorder. Ten of the remaining 20 mice in each group were bled and killed 10 months after inoculation. The mice that survived were killed 14 months after inoculation. During the period from 10 to 14 months, mice that died were dissected to examine histopathological changes in the liver.

The three experiments included groups of 20 mice given 107 cfu of each of two *E. coli* strains. In each group 10 mice were killed for examination 10 months after inoculation and the remainder were kept for 14 months. Nine uninfected control mice were included in each experiment, three being killed after 1, 10 and 14 months.

**Injection of hepatotoxic factor**

Groups of 10 mice were given two intravenous injections (at a 14-day interval) of graded doses of the purified hepatotoxic factor from *C. jejuni* strain GIFU 8734 or Nara-24; or of 100 μg of P65 from strain Nara-69 or KZ-12; or of preparations from strain 271-6 or N-15 of *E. coli*. Mice were bled and killed 30 days after the second injection, and examined histopathologically and by liver function tests.

**Preparation of rabbit hepatotoxic factor antibody**

One ml of the heat-inactivated (60°C, 30 min) hepatotoxic factor (300 μg) prepared from strain Nara-24, or P65 of strain Nara-69, was mixed with an equal volume of Freund's complete adjuvant. Female rabbits were inoculated intradermally with the mixture at five sites (400 μl/site) on the back. Each rabbit was immunised three times at 14-day intervals. Immunoglobulin fractions, prepared from the sera obtained 7 days after the last immunising dose, were precipitated with ammonium sulphate and used after extensive dialysis. The immunoglobulin fraction from a normal rabbit was used as a control.

**Neutralisation test**

Groups of 10 mice were inoculated intraperitoneally with 100 μg of the hepatotoxic factor antibody, of strain Nara-69 P65 antibody, or of normal rabbit immunoglobulin 2 h before an intravenous injection of the toxic factor of strain Nara-24 (30 μg/mouse). Mice were given the same dose of each preparation 2 h before a second injection of the toxic factor 14 days later. Histopathological examination and liver function tests were done 30 days after the second injection.

**Histology**

Livers were removed from infected mice 10 and 14 months after inoculation, and also when they died between these two time points. Tissues were fixed with formaldehyde 3.5% v/v, embedded in paraffin wax, sectioned, and stained with haematoxylin and eosin (HE). The sections from each mouse were examined "blind".

**Liver function tests**

Liver function of infected mice was determined by measuring the activity of serum glutamic-oxaloacetic transaminase (EC 2.6.1.1; GOT), serum glutamic-pyruvic transaminase (EC 2.6.1.2; GPT) and lactic dehydrogenase (EC 1.1.1.27; LDH) and also the albumin content as described previously.

**Results**

**Histopathological changes and liver function tests in *C. jejuni*-infected mice**

Ten months after an intragastric inoculation of strain Nara-24, there were multiple hepatic lesions consisting of marked infiltration of mononuclear cells (fig. 1a). Most of the lesions were situated around the portal triad, a site at which the inflammatory cell response has been shown to become prominent 1–2 months after inoculation. At 10 months after inoculation, however, most of the remaining parenchymal cells remained intact. Mononuclear cell infiltration increased strikingly with very few intact hepatocytes 12 months after inoculation (fig. 1b), as
Fig. 1. Histopathological changes in the livers of ddY mice infected intragastrically with *C. jejuni* Nara-24. HE. Bar = 100 μm. (a) Multiple lesions of mononuclear cell infiltration 10 months after infection. (b) Increased mononuclear cell infiltration 12 months after infection. (c) Massive infiltration of mononuclear cells spreading through the parenchyma 14 months after infection.

shown by the mice that died 10–14 months after inoculation. Similar pathological changes were seen in the livers of mice infected with *C. jejuni* GIFU 8734. In contrast, neither the non-hepatotoxic strains of *C. jejuni* nor the enterotoxigenic or enteroinvasive strains of *E. coli* evoked such cellular responses 10 months after infection; the histopathology of the liver was indistinguishable from that of untreated control mice. Mononuclear cell infiltration in the livers of mice infected with hepatotoxic strains of *C. jejuni* had increased in intensity 14 months after inoculation (fig. 1c).

Liver function tests disclosed marked hypoalbuminæmia with modestly elevated concentrations of LDH and decreased concentrations of GOT and GPT 10 months after infection with *C. jejuni* Nara-24 (table I). Similar results were obtained with *C. jejuni* GIFU 8734. LDH concentrations and hypoalbuminæmia increased further 14 months after infection.

Table II shows that of six bacterial strains, only two (the hepatotoxigenic strains of *C. jejuni*) induced hepatic lesions and resulted in death, the mortality rate being > 70% 14 months after inoculation. In contrast, neither non-hepatotoxigenic strains of *C. jejuni* nor strains of *E. coli* induced liver lesions or caused death. Throughout the experiments, none of the nine uninoculated control mice used in each experiment exhibited any hepatic disorder as judged by histological examination and liver function tests.

**In-vivo effect of the hepatotoxic factor**

The intravenous LD50 of the hepatotoxic factor was determined with two-fold dilutions (10 mice/dilution) by the method of Reed and Muench. The LD50 of GIFU 8734 hepatotoxic factor was c. 210 μg, and for strain Nara-24 c. 180 μg. Graded doses of each toxic factor, from 10 μg to 100 μg/mouse, were injected intravenously in two doses separated by a 14-day interval. The minimal doses required to induce the same liver lesion as that seen in mice infected intragastrically with living organisms were 55 μg/mouse (strain GIFU 8734) and 30 μg/mouse (strain Nara-24). These doses produced massive mononuclear cell infiltration in the liver within 30 days of the second injection (fig. 2).

However, at least two injections of the toxic factor, even at higher doses, were required to induce the pathological changes produced by intragastric inoculation with living organisms. Furthermore, two injections of P65 fraction from the non-hepatotoxigenic strains of *C. jejuni* did not induce marked histopathological changes in the liver.

Groups of 10 mice were next inoculated intravenously with 30 μg of the hepatotoxic factor of strain Nara-24, and 14 days later received intravenously the same dose of the heat-inactivated (60°C, 30 min) factor. However, this procedure induced only the necrotic infiltrative lesions seen in mice that received a single dose (100 μg) of the hepatotoxic factor. Control groups of mice receiving two injections (at a 14-day interval) of the heat-inactivated factor, showed neither necrotic infiltrative lesions nor a mononuclear cell response in the liver.

Pre-treatment of mice with 100 μg of the hepatotoxic factor antibody 2 h before each intravenous injection of the toxic factor (30 μg) of strain Nara-24 protected mice against the development of the hepatic lesions as determined by the liver function tests and histological examination (data not shown). In contrast, neither control rabbit immunoglobulin fraction nor an antibody to P65 of strain Nara-69 (non-hepatotoxigenic strain) protected mice.
Table I. Liver function tests in mice infected intra-gastrically with *C. jejuni* Nara-24

<table>
<thead>
<tr>
<th>Test</th>
<th>Mean (SD) values at intervals after infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected 1 month</td>
</tr>
<tr>
<td>GOT (IU/L)</td>
<td>128 (15)</td>
</tr>
<tr>
<td>GPT (IU/L)</td>
<td>54 (6)</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>452 (18)</td>
</tr>
<tr>
<td>Albumin (mg/L)</td>
<td>4.6 (0.4)</td>
</tr>
</tbody>
</table>

Data were obtained from three separate experiments. The number of test samples from infected mice at each time point was: 15 at 1 month, 30 at 10 months and seven at 14 months; there were 15 samples from control mice at each time.

Table II. Hepatic lesion-inducing activity in mice of *C. jejuni* 10 months after infection

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Mice in groups of 10 showing hepatic lesions 10 months after infection in</th>
<th>Mice in groups of 10 surviving 14 months after infection in</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt 1</td>
<td>Expt 2</td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>GIFU 8734</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nara-24</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nara-69</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>271-6 (enterotoxigenic)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N-15 (enteroinvasive)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

ND, not done.

Fig. 2. Histopathological change in mice given two intravenous injections of 30 μg of the hepatotoxic factor prepared from *C. jejuni* Nara-24; focal infiltration of mononuclear cells as seen 30 days after the second injection. HE. Bar = 100 μm.

Discussion

The present study has shown that mice infected intragastrically with hepatotoxigenic strains of *C. jejuni* exhibited a chronic hepatic disorder, the characteristic lesion being mononuclear cell infiltration. It showed also that intravenous injections of hepatotoxic factor from these strains produced similar lesions. Rabbit antibodies to the hepatotoxic factor protected mice against the development of the hepatic lesions induced by intravenous injections of the hepatotoxic factor. In contrast, neither enterotoxigenic nor enteroinvasive *E. coli* produced such a lesion in the livers of infected mice. These results strongly suggest that an active moiety of the hepatotoxic factor contributes to the development of a chronic mononuclear cell infiltration in the liver of *C. jejuni*-infected mice.

Several pathogens are capable of evoking hepatic lesions in mice, e.g. *Salmonella typhimurium* and *Listeria monocytogenes*, and the characteristic histological change induced by these bacteria is a chronic proliferative response. The capacity of these pathogens to stimulate the host defence system to elicit a proliferative response seems to be related to their ability to resist the intracellular killing system in mononuclear phagocytes. Kiehlbauch et al. reported that *C. jejuni* is readily phagocytosed by human mononuclear phagocytes in the presence of specific opsonins but continues to survive intracellularly for a long period. In *C. jejuni*-infected mice, infiltration of mononuclear cells was first prominent at the portal triad and soon spread to the adjoining area of the liver, whereas proliferative or granulomatous changes became prominent in the parenchymal area of livers of *S. typhimurium*-infected mice. The latter phenomenon was found to be associated with the development of salmonella-specific cell-mediated immunity, and the hepatic lesion remained unchanged over a long period unless the animals were re-infected with *S. typhi*.
In contrast, the infiltration of mononuclear cells in the liver of *C. jejuni*-infected mice increased in intensity with time, thereby resulting in the impairment of liver function. Nevertheless, the number of viable organisms recovered from the liver decreased with time, and in the advanced stage *C. jejuni* was recovered only from the gall bladder. In liver function tests, *C. jejuni* infection resulted in hypalbuminaemia but not in elevation of GOT and GPT after 14 months. This is probably explained by a substantial decrease in the number of hepatocytes, resulting from the massive infiltration of mononuclear cells. The striking increase in mononuclear cells in the liver may account for the relatively high level of LDH in *C. jejuni*-infected mice.

The formation of liver lesions was not accelerated by increasing the doses of the hepatotoxic factor unless the factor was injected twice; a single injection with a high dose (100 µg/mouse) resulted in acute death with massive necrotic changes in the liver, probably due to acute toxemia. This may be explained by our previous finding that higher concentrations of the hepatotoxic factor (> 20 mg/L) induced cytolysis of hepatocytes in vitro, whereas low doses (< 5 mg/L) caused only a “rounding-up” of hepatocytes. Furthermore, heat-inactivated toxic factor was no substitute for the native toxic factor at the second injection. This suggests that the sustained and expanding mononuclear cell infiltration in the liver is due to a persistent host response to the active moiety of this factor. Such speculation is supported by the histopathological features in the liver of *C. jejuni*-infected mice. The persistent mononuclear cell infiltration of the portal and periportal area of the liver indicates that hepatitis is a consequence of an immunological reaction. The formation of this lesion in the liver was not dependent on the dose of hepatotoxic factor. The hepatic lesion associated with *C. jejuni* infection may therefore be a type of hypersensitivity reaction. Studies are in progress to determine the nature of the mononuclear cells histochemically and immunohistochemically.

So far, it has not been ascertained whether *C. jejuni* can evoke pathological changes in man such as those observed in the liver of *C. jejuni*-infected mice. However, some forms of bacterial enteritis, including those caused by *Shigella*, *Salmonella* and *Yersinia*, are associated with Reiter’s syndrome, which consists of arthritis, urethritis and conjunctivitis. In this syndrome, either a special property of these pathogens or a peculiar host response may play an important role in pathogenesis. In particular, a specific histocompatibility antigen, HLA-B27, is closely linked to the incidence of the disease.* C. jejuni* has been implicated as one aetiological agent in Reiter’s syndrome and there is more recent evidence of its role in Guillain-Barré syndrome, an immunopathological disease. Although reactivity with *C. jejuni* and human sciatic nerve proteins has not been demonstrated in sera from patients with Guillain-Barré syndrome, sera from patients with this syndrome following *C. jejuni* enteritis were shown to contain high titres of IgG antibody against GM-1 ganglioside. From these reports and the data obtained in the present study, the mechanism by which the hepatotoxic factor induces an immunopathological response may be suggested as follows. The toxic factor may act as a hapten and combine with a membrane component of the hepatocytes which are damaged by the factor, or it may denature a self-antigen. This will result in a response to native or denatured hepatocytes. The heat-inactivated toxin is unable to substitute for the native one, since it lacks the ability to affect the membrane of hepatocytes.

That *C. jejuni* infection does not always initiate Reiter’s syndrome or Guillain-Barré syndrome may be explained by the fact that only a limited proportion of *C. jejuni* strains are capable of producing the hepatotoxin or that reactive arthritis associated with *C. jejuni* enteritis has been reported in an HLA-B27-positive patient. Histopathological and immunohistochemical studies of other organs including the spleen, lymph nodes and joints might provide further valuable information on the nature of the host responses to *C. jejuni* infection.

We greatly appreciate the assistance of I. Tanikawa in the preparation of this manuscript.

### References

15. Kiehlbauch JA, Albach RA, Baum LL, Chang K-P.


