Role of murine macrophages and complement in experimental campylobacter infection

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Summary. The roles of macrophages and the complement system as potential host defence mechanisms in mice against campylobacter infection were studied in vivo, by depleting the murine serum-complement or the phagocytic cells. Macrophage-depletion was performed by intraperitoneal (i.p.) injection of silica dust, Liquoid or dextran sulphate. During 5 days after infection, such mice showed a significant increase in mortality, compared with controls. In contrast, mice that were previously decomplemented by i.p. injection of Cobra Venom Factor showed no significant increase in mortality. The results with combined macrophage depletion and decomplementation did not differ from those with macrophage depletion alone. These experiments suggest that macrophages seem to be more important than complement in the defence of mice against experimental campylobacter infection.

Introduction

Campylobacter jejuni is recognised as a major cause of human enteritis (Skirrow, 1977). In order to study virulence factors, many workers have tried to establish an in-vivo model for campylobacter infection; however, a suitable animal model has not been found (a list of investigated models was summarised by Fox, 1982).

Mice, like other animals, are fairly resistant to campylobacter infection (Blaser et al., 1983) and develop signs of disease, which resemble human campylobacteriosis, only when receiving very high doses (Humphrey et al., 1986; McCardell et al., 1986). Adult mice, infected orally with $10^8$ bacteria, became excreters; and, in histological preparations, acute inflammatory reactions were observed (Blaser et al., 1983). However, neonatal mice, infected with higher doses of bacteria, showed severe symptoms of infection such as diarrhoea and increased mortality (Madge, 1980; Kazmi et al., 1984). It was concluded that, although mice may be susceptible to campylobacter infection, a highly effective immune mechanism prevents adult mice from becoming seriously affected.

The observations that mice are quite resistant, even on first contact with Campylobacter spp., leads to the assumption that nonspecific defence mechanisms such as the complement system or mononuclear phagocytes might contribute to the host’s resistance. Several in-vitro studies support this view: C. jejuni and C. coli are reported to be serum-sensitive (Blaser et al., 1985), thus emphasising a possible role for complement as a potential defence mechanism in tissue and in the circulation. Furthermore, phagocytosis of C. jejuni by mononuclear phagocytes has been demonstrated (Kiehlbauch et al., 1985; Banfi et al., 1986), indicating a contribution of the macrophage (MØ) to the defence mechanism.

The purpose of this study was to examine in vivo the role of nonspecific humoral and cellular immune mechanisms. The protective effect was assessed by reducing the murine complement system or the phagocytic cells systemically, and then studying susceptibility to infection with C. jejuni. In-vivo decomplementation was performed by injecting Cobra Venom Factor (CVF), mainly affecting serum-C3 (Cochrane et al., 1970). Macrophage-depletion was achieved by injecting silica dust or polyanions—dextran sulphate or Liquoid (Blokema et al., 1980).

Materials and methods

Mice

Female NMRI mice (from Ivanovas Versuchstierzuchtanstalt, Kieslegg, FRG) were used at 4–6 weeks of age. They were kept in groups of 4–6 per cage, and were
given antibiotic-free food and water *ad libidum*. From each animal, a faecal specimen was obtained and cultured for *Campylobacter* spp. before the study, but no positive cultures resulted.

**Strain of Campylobacter**

The strain was a clinical isolate from a patient with diarrhoea. It was grown in a micro-aerophilic and capnophilic atmosphere at 37°C on Columbia Agar supplemented with laked horse blood 5%. The isolate was identified as *C. jejuni* by the following criteria: typical microscopic appearance, oxidase and catalase positive, sensitive to nalidixic acid, growth at 43°C, hippurate positive.

**Intraperitoneal and oral challenge**

Overnight cultures of bacteria were washed once with sterile saline, resuspended in phosphate-buffered saline (PBS; pH 7.2), and photometrically adjusted to $10^9$ bacteria/ml. The viable count was determined retrospectively by culture of serial tenfold dilutions on agar plates. Mice were infected either i.p., by injecting 0.5 ml of bacterial suspension into the abdominal cavity, or orally, by introducing 0.5 ml into the stomach with a plastic feeding tube (Boak Cannula 12"; Portex Ltd, Hythe). To preserve the virulence of the infecting strain, organisms were recovered from moribund or recently dead mice.

**Determination of serum-sensitivity**

Serum-sensitivity was tested as described by Blaser *et al.* (1985), except that 90% mouse serum was used in these experiments.

**Deaths of mice after challenge**

Deaths were recorded daily for 5 days after challenge. The data (figure) represent the mean values of three experiments. Statistical significance was assessed with the unilateral four field $\chi^2$-test.

**Table 1. Substances for decomplementation or MØ-depletion of female NMRI mice**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Mechanism of action</th>
<th>Biological consequences</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cobra Venom Factor</td>
<td>C3 activation via the alternative pathway</td>
<td>C3 depletion by generating C3 split products (C3a and C3b)</td>
<td>Cochrane <em>et al.</em> (1970)</td>
</tr>
<tr>
<td>Silica dust</td>
<td>Permanent induction of the &quot;oxidative burst&quot; in MØs after phagocytosis (release of lysozyme, $H_2O_2$, etc.)</td>
<td>Irreversible MØ-intoxication</td>
<td>Bitter-Suermann <em>et al.</em> (1972)</td>
</tr>
<tr>
<td>Polyanions (dextran sulphate and Liquoid)</td>
<td>Interaction with membranes of MØs and inhibition of Fc-receptors</td>
<td>Inhibition of phagocytosis</td>
<td>Allison <em>et al.</em> (1966)</td>
</tr>
<tr>
<td></td>
<td>Activation of complement (classical and alternative pathway)</td>
<td>Reduced activity of the classical and alternative complement pathways</td>
<td>du Buy (1975) Blomma <em>et al.</em> (1980)</td>
</tr>
</tbody>
</table>

**Decomplementation by Cobra Venom Factor (CVF)**

CVF (Miami Serpentarium Laboratories, FL, USA) was purified as described by Bitter-Suermann *et al.* (1972) and stored at $-70^\circ$C. Mice received 5 µg of CVF, dissolved in 0.5 ml of saline, by i.p. injection 12 h and 24 h before challenge (see also table I).

**Monitoring CVF-induced decomplementation.** CVF-induced decomplementation was shown by monitoring the serum C3 levels by rocket-immuno-electrophoresis (Laurell, 1972): Agarose gel 1% was prepared with Laurell-II buffer containing goat anti-guinea-pig C3 serum 5%; this cross-reacts with mouse C3. A row of wells was filled with 5 µl of two-fold dilutions of mouse serum; and electrophoresis was performed at 150 V for 8 h.

**MØ-depletion (see table I)**

- **Silica dust.** Mice were MØ-depleted by i.p. injection of 0.5 ml of a saline suspension of sonicated silica dust (40 mg/ml; Sigma, Taufkirchen, FRG; no. 5631—particle size 1–5 µm diam.) at different times before infection. The silica dust had been boiled initially in KOH 5% for 15 min, washed twice in distilled water, and heat-dried.

- **Dextran sulphate and Liquoid.** A fresh saline solution of dextran sulphate 500 (5 mg/ml; Serva, Heidelberg, FRG) or Liquoid (5 mg/ml; Sigma) was prepared for each experiment, the Liquoid being dissolved initially in hot saline. For MØ-depletion, mice were given i.p. injection (20 ml/kg of body weight) at different times before challenge.
Results

Virulence of C. jejuni in normal mice

The LD50 of a fresh isolate of C. jejuni was $5 \times 10^{10}$ cfu, in a group of 32 mice infected i.p. However, after five i.p. passages, it was lowered to $10^9$ cfu. Subsequent i.p. passages did not increase the virulence any more. In a control group of 20 mice given the equivalent of $10^9$ heat-inactivated bacteria, there were only three deaths (15%)—significantly less than in mice challenged with live bacteria.

The influence of the route of infection was studied by injection of $5 \times 10^8$ cfu of C. jejuni which had undergone several mouse passages. Of 51 mice inoculated i.p., 13 (26%) died, whereas only one (6%) died after oral inoculation of 18 mice; therefore the i.p. route was used subsequently.

Effect of CVF on serum C3 levels and infection

Decomplementation was monitored by immune-electrophoresis, because the murine complement system is not active in the haemolytic assay; 1 pg of CVF, given i.p. 12 h and 24 h before infection, reduced the C3 concentration to below 5% of normal. Exact determination of lower levels of C3 was not possible by electrophoresis, because of the insensitivity of this method. With the intention of achieving maximum decomplementation, all animals were given $2 \times 5 \mu g$ of CVF in subsequent experiments.

The in-vitro serum sensitivity of C. jejuni was assessed by inoculating 90% mouse-serum with bacteria to give a final concentration of $10^4$ cfu/ml. After 2 h, the recovery from normal serum was only 600 cfu/ml, indicating 94% killing. However, serum of CVF-treated mice exerted no bactericidal effect; after 2 h, the recovery from decomplemented serum was $1 \cdot 1 \times 10^4$ cfu/ml.

For 28 decomplemented mice that were challenged i.p. with $5 \times 10^8$ cfu, the mortality at day 5 was 21% (figure) and was similar to that after challenge of untreated controls. Moreover, CVF-treated controls, that were not challenged with C. jejuni, showed no overt signs of disease.

Effect of silica on infection

MØ-depletion was induced by i.p. injection of sonicated silica dust (20 mg/mouse). The influence of the interval between this treatment and subsequent challenge is shown in table II: the highest mortality was found when silica was given 2.5 h before challenge, so this interval was used in subsequent experiments. Silica had little effect when given 8 h before challenge.

Challenge of 28 silica-treated mice resulted in a mortality of 64%, compared with 25% of the untreated controls (figure). The difference was statistically significant from day 2 to day 5 ($p < 0.05$). Silica-treated controls, that were not challenged with C. jejuni, showed no signs of disease.

Table II. Influence of time-interval between MØ-depletion and bacterial challenge: mice were challenged i.p. with $5 \times 10^8$ cfu of C. jejuni

<table>
<thead>
<tr>
<th>MØ-depletion by prior i.p. injection (at 0 h) of</th>
<th>Number of mice dead within 5 days per total number challenged after a time-interval of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>Silica dust</td>
<td>4/10</td>
</tr>
<tr>
<td>Dextran sulphate</td>
<td>12/20</td>
</tr>
<tr>
<td>Liquoid</td>
<td>5/9</td>
</tr>
<tr>
<td>ND = not done.</td>
<td></td>
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</table>

Effect of polyanions on infection

MØ-inhibition was accomplished by i.p. injection of dextran sulphate or Liquoid (100 mg/kg of body weight). As with silica, these were most effective when injected 2.5 h before challenge (table II).

After previous treatment with dextran sulphate, the mortality in a group of 33 challenged mice was 58% (figure); and a similar mortality (52%) was observed in the same number of Liquoid-treated mice. With dextran sulphate, the mortality was raised significantly from day 2 to day 5 ($p < 0.05$), but only on day 5 in Liquoid-treated mice. After either form of treatment, controls that were not challenged with C. jejuni displayed no signs of disease.

Effect of combined CVF and silica on infection

Mice were decomplemented and MØ-depleted simultaneously by injecting CVF and silica before challenge. With 24 such mice, the mortality at day 5 was 54% (figure); and it was significantly greater than with untreated controls from day 3 to day 5 ($p < 0.05$). However, there was no significant difference between mice treated with silica alone and those treated with both CVF and silica.
were pre-treated with a venom from *Pseudomyrmex triplarus*, which impairs specifically the classical pathway (Schultz and Arnold, 1977). The infection after subsequent challenge was no different from that in controls or CVF-treated mice. In further experiments (data not shown), inbred AJ or DBA2 mice, which are known to have a genetic C5 deficiency, were challenged with the same strain. No significant increase in mortality was detected, indicating no major role for the cytolytic terminal complex of the complement system in this type of infection.

The decomplementation experiments suggest that normal levels of serum complement are not essential for the resistance of mice to *C. jejuni* infection, despite the susceptibility of the organism to the bactericidal activity of serum in vitro. However, it could be argued that residual C3 was sufficient in the decomplemented animals.

To study the role of the MØ in host defence, MØ activity was impaired by pre-treatment of mice with silica or with polyanions (dextran sulphate or Liquoid) to intoxicate MØs or to inhibit their phagocytic activity (table I). In each case, pre-treated mice showed a significant increase in mortality after challenge with *C. jejuni*, compared with untreated controls (figure). The effect of MØ-depletion was detectable only when the interval between treatment and challenge was no more than 5 h, presumably because of recruitment of more MØs. Moreover, the results showed no difference between silica treatment, which is directed mainly against MØs, and polyanion treatment, which affects the complement system also. Furthermore, the combined effect of silica and CVF resembled that of silica alone, not that of CVF (figure), suggesting that decomplementation does not add to the effect of silica treatment.

This study was not intended to determine which bacterial virulence factors were responsible for disease. Previous workers have shown an endotoxin-mediated effect, using a larger inoculum (>10⁹ cfu) and younger mice than in this study (Ng *et al.*, 1980). Because of the large inoculum, lipopolysaccharide (LPS) might be involved in the pathogenic process.

However, additional factors are probably involved too, because silica-treated mice that received heat-inactivated bacteria showed a significantly lower mortality than those that received the same number of live bacteria (data not shown). Furthermore, C3H/HeJ mice, which are genetically susceptible to the action of LPS, showed no increase in resistance to the experimental campylobacter infection (data not shown).

The present results indicated that MØs are an
important factor in the defence of mice against campylobacter infection, whereas non-specific humoral factors appear to play no important role. However, opsonisation might be mediated by cross-reacting immunoglobulins which are directed against other gram-negative bacilli (Perez-Perez et al., 1986). The involvement of mononuclear cells in mice is further supported by in-vitro studies, which showed phagocytosis of C. jejuni and intracellular survival in mononuclear phagocytes (Kiehlbauch et al., 1985; Banfi et al., 1986). Additional evidence for the role of MØs in campylobacter infection was provided by electronmicroscopy studies, which demonstrated the presence of phagocytic cells in the intestinal mucosa after experimental infection (Duffy et al., 1980).

REFERENCES


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