Effects of *Corynebacterium parvum* on *Escherichia coli* Infection in Mice

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The contribution of activated macrophages to protection against *Escherichia coli* was studied in mice. Mice treated intraperitoneally with killed *Corynebacterium parvum* organisms 1 d prior to challenge showed an increased resistance to intraperitoneal infection with *E. coli*; the predominant leucocytes in the peritoneal cavity of these animals were polymorphonuclear cells. However, treatment with *C. parvum* 4 d prior to challenge induced mainly activated macrophages in the peritoneal cavity and host resistance to the infection was not increased. Activated macrophages from such mice showed both enhanced phagocytic activity *in vivo* and a high degree of intracellular killing of *E. coli* *in vitro*. At the same time these cells became more susceptible to the cytotoxic effect of endotoxin. After challenge with *E. coli* there was a marked decrease in the number of peritoneal macrophages in mice that were treated with *C. parvum* 4 d prior to challenge. Increased susceptibility of activated macrophages to the cytotoxic effect of endotoxin could explain the absence of enhanced resistance to *E. coli* infection in such animals.

### INTRODUCTION

Polymorphonuclear cells (PMN) and macrophages are known to play important roles in protection at the early stage of infection with a variety of bacteria. However, the relative contribution of PMN and macrophages to protection differs with individual micro-organisms. Bactericidal activity of PMN is important for host resistance against extracellular Gram-negative bacteria such as *Escherichia coli* or *Pseudomonas aeruginosa* (Tatsukawa *et al.*, 1979; Tsuru *et al.*, 1981). Macrophages are considered to be less effective in protection against these bacteria because they have less lysosomal enzyme activity and generate less toxic oxygen species during phagocytosis than PMN (Reiss & Roos, 1978; Roos *et al.*, 1980). However, monocytes from neutropenic patients were found to consume as much or even more oxygen than normal blood neutrophils (Baehner & Johnston, 1972) and it was suggested that macrophages might be able to substitute for PMN in protection against certain Gram-negative bacteria.

Macrophages activated by killed corynebacteria have been shown to contain high levels of lysosomal enzymes and to have an increased capacity to generate oxygen radicals, which equals or exceeds that of PMN (McBride *et al.*, 1974; Nathan *et al.*, 1978). Activated macrophages have been shown to contribute to enhanced resistance against subsequent infection with various intracellular bacteria such as *Brucella abortus* (Adlam, 1973), *Salmonella enteritidis* (Collins & Scott, 1974) and *Listeria monocytogenes* (Swartzberg *et al.*, 1975).

In this paper, the effects of pretreatment with *Corynebacterium parvum* on the resistance to *E. coli* infection were investigated with the object of determining whether or not activated macrophages could substitute for PMN in host resistance against *E. coli* infection in mice.

**Abbreviations:** HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline; PMN, polymorphonuclear cells.
METHODS

Animals. Female mice of an outbred CF, strain were obtained from the Breeding Unit of Kyushu University. Mice used for experiments were 10-12 weeks old.

Micro-organisms. Escherichia coli (E77156: O6: H1), Listeria monocytogenes strain EGD, prepared as described previously (Yoshikai et al., 1980; Tsuru et al., 1981) were used for experiments. A suspension of heat-killed and formalin-preserved Corynebacterium parvum (Propionibacterium avidum) was supplied by the Institute Merieux, Lyon, France. Mice were injected intraperitoneally with 0.25 ml of the suspension (equivalent to 0.5 mg dry weight of organism). Lyophilized lipopolysaccharide W (LPS) from E. coli 0111:B4 (Difco) was dissolved in phosphate-buffered saline (PBS) at a concentration of 200 µg ml⁻¹. The 50% lethal dose (LD50) was determined by using the method of Reed & Muench (1938).

Preparation of peritoneal cells. Peritoneal cells were recovered by lavage of the peritoneal cavity with Hanks’ balanced salt solution (HBSS) containing 3 unit heparin ml⁻¹; they were collected by centrifuging at 110 g for 10 min, washed once with HBSS and counted in a haemocytometer. Smear specimens for differential counts were stained with Giemsa solution.

Determination of bacterial growth. The challenge dose was administered intraperitoneally in untreated mice and in mice that had previously received C. parvum intraperitoneally 1 or 4 d previously; times of C. parvum treatments are designated −1 and −4 d, respectively. Each group consisted of five mice. At intervals after challenge animals were killed by cutting the femoral artery. To follow bacterial growth in the peritoneal cavity, its contents were washed with 5 ml PBS. Peritoneal leucocytes were lysed by freezing and thawing and the fluid was diluted 10-fold with PBS; 0.1 ml of each dilution was spread on nutrient agar plates containing 0.4% (v/v) glucose. Colonies were counted after incubation for 20 h at 37 °C. The number of colonies in 5 ml harvested fluid was expressed as log10. Bacterial growth in liver homogenates was determined as described previously (Mitsuyama et al., 1978).

Phagocytosis and intracellular killing of E. coli. Phagocytosis and intracellular killing by peritoneal cells were determined according to the methods of Van Furth & Van Zwet (1973). Briefly, a 1 ml suspension of E. coli (2 x 10⁶ ml⁻¹) in HBSS containing 0.1% (w/v) gelatin and 10% (v/v) foetal calf serum was injected intraperitoneally into untreated mice and into mice treated with C. parvum on day −1 and day −4. Animals were killed after exactly 3 min by cervical dislocation, and 1 min later 5 ml PBS with heparin was injected for collection of peritoneal leucocytes. Cell suspensions from five mice were pooled in a polypropylene tube (no. 2063, Falcon). To remove the extracellular bacteria, the suspensions were centrifuged at 110 g for 5 min and washed three times with HBSS. Cells containing ingested bacteria were then resuspended in gelatin-HBSS at a concentration of 2 x 10⁶ leucocytes ml⁻¹. After 0, 30 and 60 min of reincubation, the cells were centrifuged at 110 g for 5 min. Supernatants were removed and the cells were lysed by adding 1.0 ml distilled water and by freezing and thawing. The number of viable bacteria was then determined by culture on nutrient agar.

Assay of in vitro endotoxin-induced cytotoxicity for peritoneal cells. Peritoneal leucocytes were recovered by lavage of the peritoneal cavities of untreated mice and of mice injected intraperitoneally with C. parvum on day −1 or on day −4. Each group consisted of five mice. Peritoneal cells were suspended at 2 x 10⁶ cells ml⁻¹ in RPMI1640 medium (Difco) with 5% heat-inactivated foetal calf serum, 0.2% NaHCO₃, 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. Leucocytes were incubated in polystyrene tubes with either medium alone or LPS (200 µg ml⁻¹) at 37 °C in a water bath with shaking. In another experiment, peritoneal cells (1 x 10⁶ ml⁻¹) were added to plastic dishes (no. 3001, Falcon) containing cover slips. After incubation at 37 °C in an atmosphere of 5% (v/v) CO₂ and air for 1 h, non-adherent cells were removed by gentle pipetting. Macrophage monolayers were cultured in medium alone or medium containing LPS (200 µg ml⁻¹). After 3 h or 5 h incubation, viability was determined by the trypan blue dye exclusion test.

Statistics. The statistical significance of the data was determined by the Student's t-test. A P value of less than 0.05 was taken as significant.

RESULTS

Effects of an intraperitoneal injection of C. parvum on peritoneal leucocyte population and resistance to E. coli

The mean total number of peritoneal leucocytes obtained from untreated mice was 4.75 x 10⁶ ± 0.31 and 49% of these cells were macrophages as judged by morphological characteristics. At day −1 mice treated with C. parvum yielded 7.25 x 10⁶ ± 0.2 leucocytes, of which 72% were PMN. The number of peritoneal leucocytes increased at day −4 to reach 11.8 x 10⁶ ± 1.84, of which 62% were macrophages. The ability of peritoneal phagocytes to inhibit the growth of L. monocytogenes was significantly increased at day −4 (P < 0.001, Table 1) but not at day −1. On the other hand, the LD₅₀ of E. coli inoculated intraperitoneally increased from 4.5 x 10⁶ for untreated mice to 32.0 x 10⁶ in mice treated with C. parvum on day −1 but it did not increase in
Table 1. Effects of an intraperitoneal injection of C. parvum on cellular response in the peritoneal cavity and resistance to E. coli

<table>
<thead>
<tr>
<th>Days after injection of C. parvum (× 10⁶)</th>
<th>Percentage of each cell type</th>
<th>Log₁₀ listeria inoculated intraperitoneally</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PMN</td>
<td>Macrophages Others</td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>4.75 ± 0.31</td>
<td>48</td>
</tr>
<tr>
<td>1</td>
<td>7.20 ± 0.20</td>
<td>72</td>
</tr>
<tr>
<td>4</td>
<td>11.30 ± 1.84</td>
<td>14</td>
</tr>
</tbody>
</table>

* Mean total number of leucocytes obtained from five mice.
† Groups of five mice were infected intraperitoneally with 1 × 10⁶ L. monocytogenes. Total number of viable organisms in the peritoneal cavity was determined 20 h after infection (see Methods).
‡ Difference from normal control was significant at P < 0.001.

mice treated with C. parvum on day −4 (5.2 × 10⁶). These results suggested that C. parvum-activated macrophages contributed to the increased resistance to L. monocytogenes but not to E. coli.

Phagocytosis and intracellular killing of E. coli by C. parvum-induced peritoneal phagocytes

The ability of peritoneal leucocytes to phagocytose and kill E. coli was assessed. Preparations of peritoneal cells from untreated mice, PMN-rich populations obtained from C. parvum-treated mice on day −1, and macrophage-rich populations obtained from C. parvum-treated mice on day −4 were obtained. After intraperitoneal injection of 2 × 10⁶ E. coli, leucocytes were prepared as described in Methods, washed and then incubated at 37 °C in vitro for 0, 30 and 60 min. When incubated at 4 °C, the numbers of viable intracellular bacteria were constant during the incubation period of 60 min (data not shown). The numbers of viable intracellular bacteria at 0 min were regarded as indicating the phagocytic activity in vivo. The degrees of phagocytosis and intracellular killing were slightly higher in C. parvum-induced macrophage-rich populations than in C. parvum-induced PMN-rich populations (P < 0.05, Fig. 1).

Effects of pretreatment with C. parvum on the bacterial growth and the cellular response in the peritoneal cavity after intraperitoneal inoculation of E. coli

Untreated mice and mice injected intraperitoneally with C. parvum on day −1 or on day −4 were inoculated intraperitoneally with 1 × 10⁷ E. coli and the kinetics of bacterial growth and

![Fig. 1. Phagocytosis and intracellular killing of E. coli by C. parvum-induced peritoneal leucocytes. O, Leucocytes from normal mice; ●, leucocytes from mice treated with C. parvum on day −1; ■, leucocytes from mice treated with C. parvum on day −4. Each point and bar indicates the mean of triplicate tubes ± s.e., for a single representative experiment.](image-url)
cellular response in the peritoneal cavity were investigated. The bacterial count in the peritoneal cavity increased to around $1 \times 10^9$ at 20 h in untreated mice and in mice pretreated with C. parvum on day $-4$ and most of these animals died. In mice treated with C. parvum on day $-1$, however, the number of bacteria decreased progressively and all of these mice survived beyond 2 weeks (Fig. 2). The patterns of bacterial growth were similar in the liver.

The major changes in leucocyte population over the 10 h period following challenge are shown in Fig. 3. PMN levels increased, whereas macrophage levels decreased during this time; the greatest increase in PMN levels was in mice treated with C. parvum on day $-1$, whereas the greatest decrease in macrophages was in mice treated with C. parvum on day $-4$. The decrease in macrophages was not observed after an intraperitoneal infection with a lethal dose of L. monocytogenes (unpublished data).

Susceptibility of C. parvum-induced peritoneal leucocytes to the cytotoxic effect of LPS in vitro

It is generally accepted that endotoxin plays an important role in the pathogenesis of infection with Gram-negative bacteria. Bacille Calmette–Guérin (BCG) is known to increase the susceptibility of mice to the lethal effect of LPS (Suter, 1962). The lethality of LPS for mice has been shown to parallel the in vitro susceptibility of their macrophages to LPS (Peavy et al., 1979). In our preliminary experiments, the LD$_{50}$ of LPS administered intraperitoneally was 794 µg in untreated mice and decreased to 86 µg in mice treated with C. parvum on day $-4$. Therefore, any decrease in the number of peritoneal macrophages following infection with a lethal dose of E. coli in mice treated with C. parvum on day $-4$ could be the result of enhanced susceptibility to the cytotoxic effect of LPS. In order to confirm this, the in vitro cytotoxic effect of LPS on unseparated peritoneal cells and glass-adherent cells was studied. First, unseparated leucocytes

Fig. 2. Effects of pretreatment with C. parvum on bacterial growth in the peritoneal cavity (a) and liver (b) after intraperitoneal inoculation of $1 \times 10^7$ E. coli in mice. O, Normal mice; ●, mice treated with C. parvum on day $-1$; □, mice treated with C. parvum on day $-4$. Each point and bar indicates the mean of five mice ± s.e.
Effects of *C. parvum* on *E. coli* infection

Fig. 3. Changes in total count of (a) the whole leucocyte population, (b) peritoneal PMN and (c) peritoneal macrophages following intraperitoneal inoculation of $1 \times 10^7$ *E. coli* in mice. ○, Normal mice; ●, mice treated with *C. parvum* on day –1; □, mice treated with *C. parvum* on day –4. Each point and bar indicates the mean of five mice ± s.e.

were incubated with a high dose of LPS (200 µg ml$^{-1}$), a lethal dose in mice treated with *C. parvum* on day –4, but not in untreated mice. After 5 h incubation more than 90% of cells from untreated mice and from mice treated with *C. parvum* on day –1 remained viable. The number of viable peritoneal cells from mice treated with *C. parvum* on day –4 decreased after incubation with LPS ($P < 0.001$, Table 2). Second, LPS (200 µg ml$^{-1}$) was added to the adherent cell monolayers from normal mice and from mice treated with *C. parvum* on day –4. The percentage of trypan blue positive cells from mice treated with *C. parvum* on day –4 increased significantly as compared with that from untreated mice ($P < 0.001$, Table 2). These results indicated that *C. parvum*-activated macrophages had a greatly increased susceptibility to the cytotoxic effect of LPS.

**DISCUSSION**

Mice treated with *C. parvum* 1 d before intraperitoneal infection with *E. coli* showed an enhanced resistance against *E. coli* infection, but pretreatment with *C. parvum* 4 d before infection did not enhance the host resistance against the bacterial infection. The number and composition of peritoneal leucocytes changed after an intraperitoneal injection of *C. parvum*.
Table 2. Viability of C. parvum-induced peritoneal cells after incubation with or without LPS

(a) Whole leucocyte population

<table>
<thead>
<tr>
<th>Mouse treatment</th>
<th>LPS (200 µg ml⁻¹)</th>
<th>Leucocytes damaged* (%) at:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>Untreated</td>
<td>(&lt;)</td>
<td>3-4 ± 1-1†</td>
</tr>
<tr>
<td>C. parvum on day -1</td>
<td>(−)</td>
<td>3-4 ± 2-1</td>
</tr>
<tr>
<td>C. parvum on day -4</td>
<td>(+)</td>
<td>4-2 ± 2-2</td>
</tr>
</tbody>
</table>

(b) Macrophages

<table>
<thead>
<tr>
<th>Mouse treatment</th>
<th>LPS (200 µg ml⁻¹)</th>
<th>Leucocytes damaged (%) at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>Untreated</td>
<td>(&lt;)</td>
<td>4-2 ± 2-1</td>
</tr>
<tr>
<td>C. parvum on day -4</td>
<td>(+)</td>
<td>5-0 ± 1-6</td>
</tr>
</tbody>
</table>

* As measured by the trypan blue dye exclusion test.
† Mean of triplicate cultures ± standard errors.
‡ P < 0.001.

PMN accumulated mainly in the peritoneal cavity 1 d after the injection and the number of PMN increased progressively after intraperitoneal infection with a lethal dose of E. coli resulting in marked inhibition of the bacterial growth. There is a good correlation between the number of PMN and reduction of viable E. coli in the peritoneal cavity. These results were in agreement with our previous report that elimination of bacterial growth after E. coli infection depends mainly on the function of PMN (Tsuru et al., 1981).

Activated macrophages, as determined by listericidal activity, were mainly induced into the peritoneal cavity 4 d after treatment with C. parvum. These macrophages also showed enhanced phagocytic activity in vivo and a high degree of intracellular killing of E. coli in vitro. Nevertheless, limitation of bacterial growth in the peritoneal cavity of mice treated with C. parvum on day -4 was not observed after infection with a normally lethal dose of E. coli. In addition, a marked decrease in the number of peritoneal macrophages was observed in these mice after the infection. This decrease may be ascribed to cell death rather than migration of macrophages from the peritoneal cavity, because our data showed that macrophages obtained from mice treated with C. parvum on day -4 became more susceptible to the cytotoxic effect of LPS in vitro. It is suggested that C. parvum-activated macrophages are killed more readily by endotoxin from the killed bacteria and the rapid disappearance of these cells at the infection site perhaps allows the residual bacteria to grow in the peritoneal cavity, liver and other organs.

It is generally accepted that endotoxin plays an important role in the pathogenesis of infection with Gram-negative bacteria. At the cellular level, macrophages have been shown to be the main target of endotoxin in vivo (Nelson & Boyden, 1963) as well as in vitro (Shands et al., 1974; Peavy et al., 1978). Excess stimulation with LPS has been known to cause instability of lysosomal plasma membrane in newly created lysosomes (Weissmann & Thomas, 1962). Therefore, the mechanism of LPS-mediated cytotoxicity in our experiments may be explained as a result of autolysis by increased lysosomal enzyme and/or biologically active mediators released from C. parvum-activated macrophages. Peavy et al. (1979) reported that macrophages from BCG-infected mice were more susceptible to the cytotoxic effect of LPS in vitro and the release of toxic or vasoactive substances from LPS-sensitive macrophages might be in part responsible for a variety of biological activities of LPS. These include complement activation (Synderman et al., 1971), kinin generation (Nies & Melman, 1971), intravascular coagulation (Beller, 1969), increased capillary permeability (Wyler et al., 1969), shock and death (Suter, 1962). In our experiments, the possibility could not be ruled out that release of toxic or vasoactive substances
from *C. parvum*-activated macrophages inhibited the other defence mechanisms against *E. coli* infection. To obtain a definite explanation for the inability of activated macrophages to enhance the host resistance to *E. coli*, further investigation should be carried out.

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REFERENCES


