MODELS OF INFECTION

A protective role for lymphocytes in cyclophosphamide-induced endogenous bacteraemia in mice

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Summary. Cyclophosphamide (CY) is used in many animal studies, including models of bacteraemia, to deplete peripheral neutrophils and induce a compromised state. Although CY also influences lymphocyte function, the protective role of lymphocytes in bacteraemia is unclear. Therefore, CY (200 mg/kg) was administered to ddY mice and its influence on the number, cellular composition, and function of lymphocytes in the spleen and Peyer’s patches was examined. A single dose of CY reduced the number of lymphocytes in a time-dependent fashion. Flow cytometry showed that B cells carrying B220 antigen decreased significantly. The production of IgA in Peyer’s patches, as measured by enzyme-linked immunosorbent assay, was also suppressed in a time-dependent fashion. Blastogenic responses of splenic lymphocytes to Concanavalin-A, lipopolysaccharide and heat-killed Pseudomonas aeruginosa were suppressed 48 h after CY administration. The results suggest that CY suppresses the number and function of lymphocytes, especially B cells. This may lead to bacterial overgrowth in the gut and result in bacteraemia. Intravenous transfusion of normal lymphocytes or oral inoculation of IgA to mice with P. aeruginosa endogenous bacteraemia significantly increased survival rates, indicating that lymphocytes and their products have a protective role in bacteraemia in mice.

Introduction

Septicaemia in immunocompromised patients frequently arises as a result of invasion by endogenous microflora, particularly from the gastrointestinal tract. Pseudomonas aeruginosa is a typical cause of endogenous bacteraemia originating in the intestinal flora and is associated with high mortality rates.

We reported previously that P. aeruginosa causes a high mortality rate in leucopenic mice with endogenous bacteraemia induced by cyclophosphamide (CY). We also established a model of endogenous bacteraemia induced by feeding specific P. aeruginosa strains to mice treated with CY. This model includes important steps involved in the infection process, such as bacterial colonisation, overgrowth in the intestinal tract, invasion into the bloodstream, production of virulence factors and death. Consequently, the pathological features of bacteraemia in this model are similar to those of septicaemia in human patients.

In many animal studies, CY is used to reduce and deplete peripheral neutrophils and induce a compromised state. CY also affects lymphocyte function, such as delayed-type hypersensitivity and is selectively toxic to B cells. However, the role of lymphocytes in protection against bacteraemia is not fully understood. CY was administered to mice and its influence on the number, cellular composition, and function of lymphocytes from the spleen and Peyer’s patches was examined. Subsequently, normal T or B splenic lymphocytes were introduced into mice with P. aeruginosa D4 endogenous bacteraemia and their survival was compared with that of control mice to establish the protective role of lymphocytes. Finally, the influence of oral administration of purified murine IgA on the mortality of mice was examined with the same model.

Materials and methods

Animals

Specific-pathogen-free (SPF) male ddY mice (Japan SLC Co., Shizuoka) weighing 20–24 g were used in the present experiments. BALB/c, BALB/c nu/nu and BALB/c nu/+ (Japan SLC) mice weighing 18–22 g were also used. Mice were fed a sterile diet and received...
sterile distilled water except during the period of oral administration of the bacteria.

**Spleen and Peyer's patch cells**

The numbers of spleen and Peyer's patch cells were examined at set time intervals after a single administration of CY (Endoxan; Shionogi Co. Ltd, Osaka, Japan) 200 mg/kg by intraperitoneal injection. Mice were anaesthetised by ether inhalation and the femoral vein was cut to exsanguinate the animals. The spleen and Peyer's patches were removed and teased with frosted glass in RPMI 1640. The suspension was passed through sterile gauze. Red blood cells in the spleen were lysed with 0.16 M ammonium chloride in Tris buffer. The resultant cells were washed twice with RPMI 1640, diluted in Turke's solution and counted by microscopy.

**FACS analysis**

Spleen and Peyer's patch cells were prepared as described above, adjusted to 1-0 x 10^6 cells/ml and stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD3 monoclonal antibody (MAb) (PharMingen, CA) or phycoerythrin (PE)-conjugated anti-B220 MAb (PharMingen) for 30 min at room temperature. After duplicate washing with Hanks's Balanced Salts Solution, cells were fixed with paraformaldehyde 1%. Labelled cells were washed again and analysed with a flow cytometer (FACStar; Becton Dickinson, CA, USA).

**Blastogenic response of spleen cells**

Spleen cells were obtained aseptically from ddY mice as described above. The cells were suspended at a density of 1-0 x 10^6 cells/ml in RPMI 1640 containing fetal calf serum 10% penicillin 100 U/ml, streptomycin 100 μg/ml, and 2-mercaptoethanol (1-0 x 10^-3 M); 50 μl of cell suspension were transferred (5000 cells/well) to flat-bottomed 96-well tissue culture plates. Spleen cells were incubated for 72 h in the presence or absence of Concanavalin-A (Con-A, Sigma) 5 μg/ml, lipopolysaccharide (LPS) from Escherichia coli (Difco) 25 μg/ml or heat killed P. aeruginosa D4 10^6 cells/ml. Cultures were pulsed with 0-5 μCi of [methyl-^3H] thymidine/well for the final 18 h of incubation. Cells were then collected with an automatic cell microharvester. [Methyl-^3H] thymidine incorporation was measured in a scintillation counter (Matrix 96; Packard Instrument Company, CT, USA). All specimens were run in triplicate and the mean result was used.

**Quantification of IgA**

Peyer's patch cells were incubated for 72 h in RPMI 1640 containing fetal calf serum 10%, penicillin 100 U/ml, streptomycin 100 μg/ml at a concentration of 100 cells/ml at 37°C in CO₂ 5%. The culture supernate was passed through a 0-22-μm microfilter. The level of IgA in the filtrate was measured with an enzyme-linked immunosorbent assay (ELISA) with goat anti-murine IgA antibody (E-Y Laboratories Inc., CA, USA) and peroxidase-conjugated rabbit anti-murine IgA antibody (Zymed Laboratories Inc., CA, USA). Purified rabbit IgA (Zymed) was used as a standard.

**Experimental design of endogenous bacteraemia**

Endogenous *P. aeruginosa* bacteraemia was produced as described previously. Briefly, *P. aeruginosa* D4 was isolated from cardiac blood of a mouse with systemic endogenous bacteraemia induced by CY. Bacteria were suspended in sterile saline 0-45% and adjusted to a suitable concentration for each experiment by optical density (UVIDEC-40 spectrophotometer, Tokyo, Japan). Faecal specimens were obtained before the study and examined to ensure the absence of *P. aeruginosa*. Mice were fed *P. aeruginosa* D4 in drinking water between days 1 and 4. They also received sodium ampicillin (Vicillin; Meiji Seika Kaisya Co. Ltd, Tokyo, Japan) 200 mg/kg during this period to disrupt the normal gastrointestinal flora. On days 5 and 8, CY 200 mg/kg/day was administered. Mice were observed four times daily and deaths were recorded up to day 15. Specimens of cardiac blood were obtained immediately from dead mice and mice were killed at the end of the experiment. All samples were cultured aerobically on Trypticase Soy Agar (BBL Microbiology Systems, Cockeysville, MD, USA).

**Table. Influence of CY on number of spleen and Peyer's patch cells**

<table>
<thead>
<tr>
<th>Time after CY administration (200 mg/kg)</th>
<th>Number (x 10^6/mouse) (%) of spleen cells</th>
<th>Number (x 10^6/mouse) (%) of Peyer's patch cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4-18 SEM 2-35 (100)</td>
<td>7-47 SEM 3-17 (100)</td>
</tr>
<tr>
<td>6 h</td>
<td>2-17 SEM 1-63 (51-9)</td>
<td>5-67 SEM 1-53 (75-9)</td>
</tr>
<tr>
<td>12 h</td>
<td>1-96 SEM 1-41 (46-9)</td>
<td>4-80 SEM 1-48 (64-3)</td>
</tr>
<tr>
<td>24 h</td>
<td>1-41 SEM 0-74* (33-7)</td>
<td>4-80 SEM 1-96 (64-3)</td>
</tr>
<tr>
<td>48 h</td>
<td>1-03 SEM 0-98* (24-6)</td>
<td>2-00 SEM 0-49* (26-8)</td>
</tr>
</tbody>
</table>

*Significantly lower than control value (p < 0.01).
Fig. 1. Two-colour FACS analysis of spleen cells from mice with or without administration of CY. CD3 and B220 antigens were stained with FITC- and PE-labelled MAbs, as markers of T and B cells, respectively. Note the time-dependent decrease in B cells (B220⁺ cells). a, Control; b, c and d, 12, 24 and 48 h after administration of CY.

USA) at 37°C in humidified air for 24 h and examined for the presence of *P. aeruginosa*.

**Effect of lymphocyte transfusion on survival rates of mice with endogenous *P. aeruginosa* bacteremia**

Spleen cells obtained from BALB/c nu/nu and BALB/c nu/+ were used as B and T cell sources, respectively. A single cell suspension was obtained as described above. Cells were adjusted to a concentration of 1 × 10⁴ cells/ml and incubated in plastic dishes at 37°C in CO₂ 6% for 1 h. Non-adherent cells were gathered and washed twice with RPMI 1640. Cells obtained from BALB/c nu/+ mice were passed through a T cell immunocolumn (Cellect Mouse T Cell Kit; Biotex Laboratories Inc., Alberta, Canada) to remove B cells. Over 95% of cells in preparations obtained from BALB/c nu/nu and nu/+ mice were positive for B220 and CD3, respectively. In the endogenous *P. aeruginosa* bacteremia model with neutropenic BALB/c mice, 1 × 10⁵ cells/mouse of T or B cell preparations were transfused into mice intravenously on days 6 and 9.
Fig. 2. Two-colour FACS analysis of Peyer's patch cells from mice with or without the administration of CY. Note the time-dependent reduction of B cells (B220⁺ cells). a. Control; b, c and d. 12, 24 and 48 h after administration of CY.

**Effect of oral inoculation of IgA on survival rate of mice with endogenous P. aeruginosa bacteraemia**

IgA 5 μg/ml in bovine serum albumin (BSA) 2% was mixed with drinking water and provided from day 5 until the end of experiment to ddY mice with endogenous *P. aeruginosa* bacteraemia. IgG 5 μg/ml in BSA 2% or BSA only were given as controls.

**Statistical methods**

The Student’s *t* test and χ² test were used to compare means and survival rates, respectively.

**Results**

**Number of spleen and Peyer’s patch cells**

As shown in the table, a single dose of CY reduced the number of spleen and Peyer’s patch cells in a time-dependent fashion. Spleen cell counts 24 and 48 h after CY administration were significantly lower than those of controls (*p* < 0.01). The number of Peyer’s patch cells 48 h after injection of CY was also significantly lower than that of controls (*p* < 0.01).
FACS analysis

A single dose of CY reduced B220+ cells in the spleen progressively with time (fig. 1). In control mice, B220+, CD3- cells comprised 48.9% of the total cell count. The values were 40.1, 29.2 and 16.2% at 12, 24 and 48 h, respectively, after administration of CY. In contrast, CD3+, B220+ spleen cells increased from 42.5% in control to 68.3% 48 h after CY injection. No change occurred in CD4+ cells and CD8+ cells (data not shown). Similar results were obtained in Peyer's patches as shown in fig. 2.

Blastogenic response

Spleen cells obtained from control mice showed a high blastogenic response against Con-A, E. coli LPS and heat-killed P. aeruginosa D4. The mean 3H-thymidine incorporation was 42088 SEM 26600 Matrix CPM when stimulated with Con-A 5 µg/ml. The values were 11523 SEM 1694 and 2695 SEM 309 CPM, when stimulated with LPS 25 µg/ml and heat-killed P. aeruginosa D4, 10^8 cells/ml, respectively. The count without stimulation was 1075 SEM 157 CPM. Spleen cells treated with CY showed almost no response against such stimulation. The counts were 34 SEM 9, 21 SEM 6 and 24 SEM 6 CPM when

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**Fig. 3.** IgA production by Peyer's patch cells from mice with or without the administration of CY; *p < 0.01.

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**Fig. 4.** Effect of lymphocyte transfusion on survival of mice with *P. aeruginosa* endogenous bacteraemia: ■, B cell transfusion, p < 0.01; ▲, ▲, T cell transfusion, p < 0.06; ●, ●, control.

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**Fig. 5.** Effect of oral administration of IgA or IgG on survival rates of mice with endogenous *P. aeruginosa* bacteraemia: ●, ●, control (BSA); ♦, ♦, IgA, p < 0.05; ■, ■, IgG (NS).
lymphocytes from mice treated with CY stimulated with Con-A, LPS and heat-killed *P. aeruginosa* D4, respectively.

**IgA production**

The concentration of IgA in the culture supernate of Peyer's patch cells obtained from CY-treated mice significantly decreased in a time-dependent fashion (p < 0.01 at any time interval after administration of CY). Control cells produced 407.9 ± 146 ng/ml of IgA compared with 1050 ± 8.3 ng/ml 24 h after the administration of CY (fig. 3).

**Effect of lymphocyte transfusion on survival rates of mice with endogenous *P. aeruginosa* bacteraemia**

As shown in fig. 4, transfusion of B cells obtained from the spleen of BALB/c nu/nu mice significantly protected BALB/c mice against a lethal endogenous *P. aeruginosa* septicaemia (70% survival rate, p < 0.01). Transfusion of T cells also increased survival rate from 0% to 50% (p < 0.06). Pure cultures of *P. aeruginosa* were obtained from cardiac blood samples of all mice that died of septicaemia. By contrast, blood cultures were sterile in all survivors at the end of the experiment.

**Effect of oral inoculation of IgA on survival rates of mice with endogenous *P. aeruginosa* bacteraemia**

Continuous oral inoculation of IgA significantly increased the survival rate of mice from 20% to 70% (p < 0.05) (fig. 5). IgG also increased survival rate of mice (60%), although this did not reach statistical significance.

**Discussion**

The results of this study showed that a single administration of CY 200 mg/kg dramatically reduced lymphocyte counts in the spleen and Peyer's patches in a time-dependent fashion. FACS analysis revealed that B cells were predominantly influenced by CY as compared with T cells. CY also significantly suppressed the blastogenic response of lymphocytes from the spleen to both T and B cell mitogens. CY also significantly decreased IgA production by lymphocytes from Peyer's patches in a time-dependent fashion. IgA is an important product of B cells and plays a primary role in protecting the host against exogenous and endogenous micro-organisms, including bacteria from the digestive tract. It is possible that by suppressing B cell function and reducing IgA production, CY suppresses the immunity of the intestinal tract. These results suggest that suppression of the number and function of not only neutrophils but also lymphocytes, particularly B cells, may contribute to the pathogenesis of endogenous bacteraemia arising from the gastrointestinal tract.

These results demonstrate that the transfusion of normal lymphocytes, especially B cells, protected mice from lethal endogenous *P. aeruginosa* septicaemia. Moreover, oral inoculation of IgA significantly increased the survival rate of mice. These results suggest that lymphocytes play a protective role in endogenous *P. aeruginosa* bacteraemia.

**References**

18. Okuyama H, Matsunaga T, Kobayashi S, Hashimoto Y,


