Modification of mitogen-driven lymphoproliferation by ceftriaxone in normal and immunocompromised mice

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Abstract

Cyclophosphamide-treated mice are proposed as a model to assay immunomodulation by antimicrobial agents in immunocompromised animals. Cyclophosphamide-treated BALB/c mice developed temporary leukopenia, myelopenia and spleen atrophy that was followed by splenomegaly. Cells from both atrophic and hypertrophic spleens exhibited impaired responses to mitogens and suppressed the mitogen-driven proliferation of normal splenocytes. This experimental model was applied to the study of immunomodulation by ceftriaxone. Ceftriaxone did not worsen the cyclophosphamide-damaged immunity mechanisms. On the contrary, cyclophosphamide-induced suppression of lymphoproliferation in response to concanavalin A was attenuated by ceftriaxone.

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1. Introduction

Numerous antimicrobial agents have been shown to possess immunomodulatory effects. They are able to enhance or suppress the host immunological mechanisms, such as phagocytosis, cytokine production, lymphocyte proliferation, antibody response, and cellular immunity [1–3]. Immunomodulatory antibiotics may act as biological response modifiers by two ways: (i) by direct interaction with immunity cells; and (ii) by induction of lysis of indigenous microorganisms, resulting in release of microbial fractions with immunomodulatory activity [2]. A commonly approach used to study drug-induced immunomodulation is the use of animal models, in which animals are immunologically intact. However, antimicrobial agents are often used not only for therapy but also for the prevention of infections in neutropenic and immunocompromised patients [4,5]. To improve the clinical application of these drugs, it is very important to know whether they are able to promote restoration of damaged immune functions or, on the contrary, to increase the severity of immunodeficiency. In this report, a cyclophosphamide-treated mouse model is proposed as an approximation for immunomodulation studies in immunocompromised individuals. In addition, ceftriaxone, a widely prescribed cephalosporin used in the treatment of infections in patients with febrile neutropenia [6], was tested in this model.

2. Materials and methods

2.1. Animals

Six- to eight-week-old female BALB/c mice were provided by the Technical Services of the University of Granada (Granada, Spain). They were maintained under pathogen-free conditions. The experiments were approved and supervised by the local ethical committee at the University of Granada.
2.2. Treatment with cyclophosphamide

Cyclophosphamide (Asta Medica, Prasfarma S.A.) was administered by intraperitoneal (i.p.) injections (150 mg/kg on day 0 and 100 mg/kg on day 3).

2.3. Treatment with ceftriaxone

Mice were treated with ceftriaxone (Laboratorios Normon S.A.) administered by i.p. injection every day at a dose of 50 mg/kg. Treatment was initiated on the day following the last cyclophosphamide injection and continued for a total of 3 consecutive days (days 4–6).

2.4. Cell counts

For peripheral blood leukocyte (PBL) counts, blood samples were obtained by retro-orbital puncture with heparinized capillary tubes in ether-anesthetized mice. Blood was immediately diluted 1:5 with Türk solution (0.1% crystal violet in 1% acetic acid), and nucleated cells were counted in an haemocytometer. For bone marrow nucleated cell (BMNC) counts, cells were obtained by flushing the femurs with HBSS (1 ml per femur), diluted 1:2 with Türk solution, and counted. Splenocytes were counted as described for lymphoproliferation assay.

2.5. Mitogen-induced proliferation of splenocytes

Spleens were removed aseptically and homogenized in Hanks’ balanced salt solution (Sigma Chemical Co., St. Louis, MO). Splenocytes were sedimented by centrifugation, resuspended in red blood cell lysing buffer (Sigma) for 10 min, washed, and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum, 50 μM 2-mercaptoethanol, penicillin G (100 U/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml), 1 mM sodium pyruvate, and 2 mM L-glutamine (Sigma). Cell suspensions were distributed (5 × 10^5 viable cells per well) into 96-well tissue culture clusters with flat-bottom wells (Costar, Cambridge, MA). Salmonella typhi lipopolysaccharide (LPS; Sigma) was used at 2.5 μg/ml as B-cell mitogen, and concanavalin A (Con A; Sigma) was used at 1 μg/ml as T-cell mitogen; these mitogen concentrations have been shown to induce optimum splenocyte proliferation in our assay conditions [7]. After incubation at 37 °C in 5% CO₂ for 3 days, proliferation of spleen cells was measured by colorimetric reading of 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction as described by Mosmann [8].

2.6. Statistical analyses

The differences between infected and control groups were analyzed by using Student’s t-test. A P value of less than 0.05 was considered significant.

3. Results

3.1. Mouse immunosuppression by cyclophosphamide

We first evaluated the effects of cyclophosphamide treatment on the immunity functions of mice. Fig. 1 shows the time course of cyclophosphamide effects on PBL, BMNC, and spleen cells. Significant leukopenia was seen from 24 to 96 h after the end of treatment (days 4–7, with P < 0.03, 0.004, 0.03, and 0.05, respectively) (Fig. 1A). Leukopenia was associated with significant decrease in BMNC number on days 4–6 (P values < 0.04, 0.03, and 0.05, respectively) (Fig. 1B). The spleen cell count exhibited a biphasic time course curve (Fig. 1C): cyclophosphamide-treated mice had significantly reduced counts of splenocytes compared with untreated controls on days 4–7 (P values < 0.003, 0.04, 0.02, and 0.001), but these counts increased in a second step, with significant splenomegaly on days 9, 14, and 17 (P values < 0.05, 0.04, and 0.02, respectively).

Since mitogen-driven lymphoproliferation was used as a test to study immunomodulation by biological response modifier agents [7,9], we examined the ability of splenocytes from cyclophosphamide-treated mice to proliferate in response to standard mitogens LPS and Con A. Results are shown in Fig. 2. The proliferation in response to LPS was significantly suppressed on days 5, 7, 11, and 14 (P values < 0.03, 0.02, and 0.05, respectively) (Fig. 2A). Significant suppression of the Con A-induced lymphoproliferation was also observed on days 5, 7 (P < 0.01) and 11 (P < 0.05) (Fig. 2B).

In certain conditions, the injection of cyclophosphamide is known to induce suppressor cells [10–12]. To investigate whether decreased proliferation of splenocytes from cyclophosphamide-treated mice correlated with the presence of suppressor cells, spleen cells from untreated controls and from treated mice were placed in coculture with spleen cells from control mice at a ratio of 1:5. Splenocytes from treated mice were obtained on days 5 (within the initial phase of spleen atrophy) and 11 (within the phase of splenomegaly). In both cases, the addition of spleen cells from cyclophosphamide-treated mice to cultures of splenocytes from untreated controls exerted significant suppressive effects on responses to LPS and Con A (Table 1).
3.2. Effects of ceftriaxone on normal and immunosuppressed mice

To study the immunomodulation by ceftriaxone on intact and damaged immune system, experiments were designed with four experimental groups: untreated mice, ceftriaxone-treated mice, cyclophosphamide-treated mice, and mice sequentially treated with cyclophosphamide and ceftriaxone. As shown in Fig. 3, splenocyte counts were significantly reduced in ceftriaxone-treated
mice ($P < 0.04$); neither PBL nor BMNC counts were significantly modified in this group. Considering that cyclophosphamide-treated mice were sacrificed on day 7, data from this group were as expected: PBL and BMNC counts were significantly reduced ($P$ values $< 0.005$ and $0.03$, respectively). When cyclophosphamide-treated mice received ceftriaxone, PBL and BMNC remained significantly decreased with regard to untreated controls ($P$ values $< 0.002$ and $0.03$, respectively). There were no significant differences in cell counts between cyclophosphamide-treated mice and mice that received cyclophosphamide plus ceftriaxone.

Finally, we investigated the influence of ceftriaxone on mitogen-driven proliferation of normal and cyclophosphamide-treated mice. Results are presented in Fig. 4. Ceftriaxone had no effect on the capacity of spleen cells from normal mice to proliferate in response to LPS or Con A. As reported above, the proliferation of splenocytes from cyclophosphamide-treated mice in response to both mitogens was significantly suppressed compared with that of splenocytes from normal mice ($P$ values $< 0.05$ and $0.003$, respectively). However, when ceftriaxone was given to cyclophosphamide-treated mice, the ability of spleen cells to proliferate in response to Con A was partially restored (the difference with the response of Con A-stimulated splenocytes from untreated controls was not significant), whereas the response to LPS remained significantly suppressed ($P < 0.04$).

### 4. Discussion

Agents with immunomodulatory activity may induce contrasting effects in normal and immunocompromised individuals. Some antibiotics with immunosuppressor properties are able to impair the already damaged immune system of immunocompromised patients, whereas some others may contribute to restoration of previously depressed immune functions [3,13]. Thus, a comparison of immunomodulatory effects in normal and immunosuppressed animals should be considered in studies on immunomodulation by antimicrobial agents. In our present work, cyclophosphamide, a classical myelotoxic agent, was used to establish an experimental model applicable to the assay of immunomodulation by antibiotics in normal and immunocompromised mice. Cyclophosphamide has been frequently used to induce neutropenia in laboratory animals and so to turn them into susceptible hosts for experimental infection with opportunistic pathogens [14–17]. The cyclophosphamide regimen used in the present work was reported by other workers as able to produce neutropenia in mice for 5 days [18]. Our data showed that leukopenia was associated with myelopenia and with a remarkable

<table>
<thead>
<tr>
<th>Origin of cells added to normal splenocytes$^a$</th>
<th>Optical density (570–630 nm) in cultures stimulated with$^b$</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>LPS</td>
<td>Con A</td>
</tr>
<tr>
<td>Normal mice</td>
<td>$0.148 \pm 0.206$</td>
<td>$0.328 \pm 0.0129$</td>
</tr>
<tr>
<td>Treated mice (day 5)</td>
<td>$0.038 \pm 0.0113$</td>
<td>$0.091 \pm 0.0224$</td>
</tr>
<tr>
<td>Treated mice (day 11)</td>
<td>$-0.002 \pm 0.0243$</td>
<td>$0.131 \pm 0.0184$</td>
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Fig. 3. Levels of PBL (A), BMNC (B), and spleen cell (C) in the following experimental groups: untreated mice (□), ceftriaxone-treated mice (■), cyclophosphamide-treated mice (□), and mice sequentially treated with cyclophosphamide and ceftriaxone (■). Results are means for three mice. Error bars represent S.D. Differences with untreated mice were significant ($P$ values are indicated in the text).
reduction in the number of splenocytes. The initial step of spleen atrophy was followed by splenomegaly, showing a biphasic time course curve that is in agreement with earlier studies on splenic modifications induced by cyclophosphamide in other mouse strains [10,11,19]. We also found that splenocytes from cyclophosphamide-treated mice exhibited impaired responses to mitogens and significantly suppressed the ability of normal splenocytes to respond to mitogens, suggesting the presence of cyclophosphamide-induced suppressor cells both in atrophic and in hypertrophic spleens. The effect of cyclophosphamide on suppressor cells is complex and it depends on the cyclophosphamide doses and the nature of investigated cells. In certain conditions, cyclophosphamide is able to enhance some T cell-mediated responses by inhibition of suppressor cells [20–22], but cells with suppressor properties revealed by both in vitro [10–12] and in vivo assays [23], have also been described in cyclophosphamide-treated mice.

The cyclophosphamide regimen used in the present work provides a mouse model with well-defined conditions of leukopenia, myelopenia and immunosuppression, that offers a tool for the simultaneous evaluation of modulatory effects of antimicrobial agents on normal and damaged immune system. We have applied this model to examine the immunomodulation by ceftriaxone. Although some cephalosporins possess immunomodulatory properties [3,24–26], previous studies on ceftriaxone have not found significant effects on serum immunoglobulin concentrations, phagocytic activity, cytokine production and lymphocyte subsets in humans [27–29]. We found that ceftriaxone, when given to normal mice, did not modify PBL counts, BMNC counts, or spleen cell capacity to proliferate in response to mitogens. Only a reduction of splenocyte number was detected in ceftriaxone-treated mice, but this effect did not increase the splenocyte depletion due to cyclophosphamide in mice treated with both drugs. In addition, the suppressive effect of cyclophosphamide on the Con A-driven lymphoproliferation was attenuated by ceftriaxone. Thus, our results suggest that ceftriaxone do not worsen the cyclophosphamide-damaged immunity mechanisms, but may improve some lymphocyte responses in immunocompromised hosts.

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**References**


