Immunomodulation by non-absorbable antibiotics given by the intragastric route

Magdalena Leiva, Encarnacion Moreno, Alfonso Ruiz-Bravo, Maria Jimenez-Valera

Department of Microbiology, Faculty of Pharmacy, University of Granada, 18071 Granada, Spain

Received 29 July 2004; accepted 6 October 2004

Abstract

To test the role of bacterial fractions released from intestinal flora during immunomodulation by antimicrobial agents, BALB/c mice were treated with the non-absorbable antibiotics polymyxin B or teicoplanin by the intragastric route. The composition of faecal microbiota and the capacity of spleen cells to proliferate in response to B-cell and T-cell mitogens were assessed at several times during the treatment. Both antibiotics lowered the count of some bacteria of the intestinal flora and induced significant modifications in spleen cell ability to proliferate in response to mitogens. Thus, the active fractions released from intestinal bacteria during antibiotic treatments may be able to induce immunomodulating effects.

© 2004 Elsevier B.V. and the International Society of Chemotherapy. All rights reserved.

Keywords: Immunomodulation; Antibiotics; Indigenous bacteria; Intestinal microbiota

1. Introduction

Numerous antimicrobial agents are biological response modifiers; that is, they are able to modulate multiple inter- and intracellular molecular signals involved in the functioning of the immune system [1–4]. Antibiotics may act directly on cells of the immune system, which has been demonstrated by in vitro experiments with several antibiotic families, such as β-lactams [5,6], macrolides [7,8], or fluoroquinolones [9,10]. Additionally, it is conceivable that antibiotics, when given in vivo, may result in lysis of indigenous bacteria, thus releasing bacterial fractions having immunomodulatory activity. These are called modulins. Although there are some data suggesting a role for this indirect mechanism in the immunomodulation by specific antibiotics [11], conclusive experimental evidence has not been reported. The comparison of immunomodulation by antibiotics in both conventional and germ-free animals does not seem a suitable model, since the immaturity of the immune system of germ-free animals invalidates such comparisons. However, if non-absorbable antibiotics, when passing through the digestive tract, are able to exert immunomodulatory effects, then these effects should be attributed to modulins released by antibiotic-killed bacteria. In this paper, the effects of two non-absorbable antibiotics on the mouse intestinal microbiota and their capacity to modify the response of spleen cells to mitogenic stimuli were studied.

2. Methods

2.1. Mice

Six to eight-week 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. Antibiotic treatments

Polymyxin B was purchased from Sigma Chemical Co. (St. Louis, Mo). Teicoplanin was a gift from Aventis (Romainville, France). The drugs were dispersed at the desired concentration in saline solution and administered intragastrically in a total volume of 0.2 ml per animal on 3 consecutive days.
concentrations in distilled water and were administered by oesophageal gavage once a day. The drugs were given at the following dosages in milligrams per kilogram of body weight: polymyxin B, 10 and teicoplanin, 40.

2.3. Quantification of bacterial populations in faeces

Fresh faeces were weighed, homogenised in sterile saline solution, and suitable dilutions of the homogenates were plated onto culture media agar plates for viable bacteria counts. The following culture media (Difco Laboratories Inc., Detroit, MI) were used: Tryptic soy agar (TSA) for total aerobic bacteria, MacConkey agar for enterobacteria, MRS agar for lactobacilli and Brewer agar, under anoxic atmosphere (GasPak, BBL Microbiology Systems, Cockeysville, MD) in anaerobic jars, for anaerobic bacteria. Results were expressed as the number of colony-forming units (CFU) per g of fresh faeces.

2.4. Spleen cell proliferation assay

Spleens were removed aseptically and homogenised 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). Splenocytes were sedimented by centrifugation, resuspended in red blood cell lysing buffer (Sigma) for 10 min, washed, and resuspended in fresh medium. Cell suspensions were distributed (5 × 10⁵ viable cells per well) into 96-well tissue culture clusters with flat-bottom wells (Costar, Cambridge, Mass.). 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 [11]. After incubation at 37 °C in 5% CO₂ for 3 days, proliferation of spleen cells was measured by colorimetric reading of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction as described by Mosmann [12].

2.5. Statistical analysis

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

3. Results

3.1. Intestinal microbiota changes and immunomodulation following treatment with polymyxin B

The impact of polymyxin B on faecal bacterial counts is shown in Table 1. No relevant changes in the aerobic flora, anaerobic flora and lactobacilli were observed during the study period, but the number of enterobacteria dropped below the lowest detectable number of bacteria (<10³ CFU per g of faeces) after 24 h following the start of treatment and continued to be undetectable for the administration period.

We examined the effect of treatment with polymyxin on splenocyte proliferation in response to mitogens (Table 2). A 5-day treatment suppressed the Con A-driven response by 69% (P < 0.025), whereas the response to LPS was unaffected. A 12-day treatment enhanced the response to LPS by 66% (P < 0.005) and suppressed the response to Con A by 20% (P < 0.025). When the treatment was extended for 19 days, no significant changes were observed.

3.2. Intestinal microbiota changes and immunomodulation following treatment with teicoplanin

The effect of teicoplanin on the intestinal flora is shown in Table 3. Both aerobic and anaerobic bacterial counts were clearly increased. Dramatic increases in CFU counts on MacConkey plates were also observed. The macroscopic and mi-
10 ± 9.5 ± Mitogen-driven proliferation of spleen cells from mice treated with polymyxin B enhance the response to Con A by 61% (proliferation in response to mitogens. The 5-day treatment enable to growth on MacConkey agar. With a Gram-negative, facultatively anaerobic bacilli that was administered with changes in the intestinal microbiota. During the teicoplanin administration period, these Gram-positive bacteria were replaced. A microscopic study of colonies revealed the presence of several types of Gram-positive bacilli and cocci on Brewer plates. The 12-day treatment suppressed it by 27% (P < 0.05). The data presented here demonstrate that non-absorbable antibiotics are able to exert immunomodulatory effects when given by the intragastric route. These effects are associated with changes in the intestinal microbiota. Given the influence of intestinal bacteria on the immune functions, the immunomodulation reported here may be attributed to the disturbance of the ecological balance of intestinal flora caused by the administration of antimicrobial agents. However, immunomodulation disappeared when the treatment with polymyxin B was continued for 19 days, although the intestinal microbiota remained disturbed. This suggests that immunomodulation is due to modulins released from antibiotic-susceptible bacteria, since it is reasonable to accept that susceptible bacteria are suppressed when antibiotic treatment is long enough. A similar explanation has been proposed for the loss of immunosuppressive activity of ciprofloxacin in mice when the antibiotic treatment is extended for a week [11].

Besides the loss of effect with prolonged treatment, immunomodulation by both polymyxin B and teicoplanin was clearly influenced by the duration of the antibiotic administration period. This may be the consequence of a time-dependent pattern of release of helper and suppressive cytokines as described for some experimental models of immunomodulation by bacterial modulins [13,14]. Animal models and clinical studies have shown that the antibiotic treatment of bacteraemia may cause swift clinical deterioration, including septic shock, due to rapid bacterial lysis with release of biologically active cell wall components into the circulation [15]. The antibiotic-mediated release of cell wall components, such as endotoxin from Gram-negative bacteria promotes production of pro-inflammatory cytokines that trigger systemic inflammatory reactions [16]. However, there is no evidence that antibiotic-killed bacteria belonging to the intestinal flora can be a source of biologically active fractions inducing immunomodulation at the systemic level. To our knowledge, this is the first report to demonstrate that antimicrobial agents may exert immunomodulatory effects through the release of modulins from indigenous bacteria.

### Table 2

<table>
<thead>
<tr>
<th>Mitogen</th>
<th>Treatment of mice</th>
<th>Optical density (570–630 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 5</td>
</tr>
<tr>
<td>LPS</td>
<td>None</td>
<td>0.242 ± 0.0277</td>
</tr>
<tr>
<td></td>
<td>Polymyxin B</td>
<td>0.230 ± 0.0299</td>
</tr>
<tr>
<td>ConA</td>
<td>None</td>
<td>0.447 ± 0.1547</td>
</tr>
<tr>
<td></td>
<td>Polymyxin B</td>
<td>0.337 ± 0.0024</td>
</tr>
</tbody>
</table>

* Polymyxin B (10 mg/kg) was given by oesophageal gavage once a day. Results are mean ± standard deviation for four animals.

### Table 3

<table>
<thead>
<tr>
<th>Days from initiation of teicoplanin</th>
<th>TSA</th>
<th>MRS</th>
<th>Brewer</th>
<th>MacConkey</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.1 ± 0.22</td>
<td>8.4 ± 0.20</td>
<td>8.8 ± 0.32</td>
<td>5.8 ± 0.35</td>
</tr>
<tr>
<td>2</td>
<td>9.9 ± 0.24</td>
<td>10.1 ± 0.20</td>
<td>10.1 ± 0.21</td>
<td>9.7 ± 0.30</td>
</tr>
<tr>
<td>4</td>
<td>10 ± 0.19</td>
<td>10 ± 0.21</td>
<td>10 ± 0.25</td>
<td>9.6 ± 0.22</td>
</tr>
<tr>
<td>10</td>
<td>9.5 ± 0.20</td>
<td>9.3 ± 0.25</td>
<td>9.4 ± 0.26</td>
<td>9.4 ± 0.20</td>
</tr>
</tbody>
</table>

* Teicoplanin (40 mg/kg) was given by oesophageal gavage once a day. Data represent the mean log10 ± standard deviation of viable bacteria per gram of faeces from four animals.

### Table 4

<table>
<thead>
<tr>
<th>Mitogen</th>
<th>Treatment of mice</th>
<th>Optical density (570–630 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 5</td>
</tr>
<tr>
<td>LPS</td>
<td>None</td>
<td>0.278 ± 0.0330</td>
</tr>
<tr>
<td></td>
<td>Teicoplanin</td>
<td>0.324 ± 0.0244</td>
</tr>
<tr>
<td>ConA</td>
<td>None</td>
<td>0.308 ± 0.0246</td>
</tr>
<tr>
<td></td>
<td>Teicoplanin</td>
<td>0.495 ± 0.0504</td>
</tr>
</tbody>
</table>

* Teicoplanin (40 mg/kg) was given by oesophageal gavage once a day. Results are mean ± standard deviation for four animals.

### Discussion

The data presented here demonstrate that non-absorbable antibiotics are able to exert immunomodulatory effects when given by the intragastric route. These effects are associated with changes in the intestinal microbiota. Given the influence of intestinal bacteria on the immune functions, the immunomodulation reported here may be attributed to the disturbance of the ecological balance of intestinal flora caused by the administration of antimicrobial agents. However, immunomodulation disappeared when the treatment with polymyxin B was continued for 19 days, although the intestinal microbiota remained disturbed. This suggests that immunomodulation is due to modulins released from antibiotic-susceptible bacteria, since it is reasonable to accept that susceptible bacteria are suppressed when antibiotic treatment is long enough. A similar explanation has been proposed for the loss of immunosuppressive activity of ciprofloxacin in mice when the antibiotic treatment is extended for a week [11].

Besides the loss of effect with prolonged treatment, immunomodulation by both polymyxin B and teicoplanin was clearly influenced by the duration of the antibiotic administration period. This may be the consequence of a time-dependent pattern of release of helper and suppressive cytokines as described for some experimental models of immunomodulation by bacterial modulins [13,14]. Animal models and clinical studies have shown that the antibiotic treatment of bacteraemia may cause swift clinical deterioration, including septic shock, due to rapid bacterial lysis with release of biologically active cell wall components into the circulation [15]. The antibiotic-mediated release of cell wall components, such as endotoxin from Gram-negative bacteria promotes production of pro-inflammatory cytokines that trigger systemic inflammatory reactions [16]. However, there is no evidence that antibiotic-killed bacteria belonging to the intestinal flora can be a source of biologically active fractions inducing immunomodulation at the systemic level. To our knowledge, this is the first report to demonstrate that antimicrobial agents may exert immunomodulatory effects through the release of modulins from indigenous bacteria.
Polymyxin B is known to neutralize the biological activity of endotoxin [17,18], and a similar function has been found in teicoplanin [19]. Although we do not know the relative contribution that endotoxin made on the immunomodulation described here, it is reasonable to accept that the endotoxin role was diminished by the neutralizing ability of both drugs. The endotoxin contribution should be more effective in the case of treatment with antibiotics lacking in neutralizing activity. However, the immunomodulation due to fractions from indigenous bacteria is very difficult to demonstrate with absorbable antibiotics, which reach blood levels that are high enough to cause immunomodulation by direct action on the immunocompetent cells. The use of non-absorbable antibiotics administered to mice by the intragastric route provides an useful model to study the effect of bacterial modulins released from the intestinal flora.

Acknowledgments

This work was supported by Ministerio de Ciencia y Tecnología and the European Regional Development Fund (SAF 2002-01606), and by the Junta de Andalucía (Research Group CV1201). M. Leiva was supported by a grant from the Ministerio de Educacion, Cultura y Deporte (Beca-colaboracion).

References