In vitro and ex vivo effects of erythromycin A, azithromycin and josamycin on the splenic response to specific antigens and mitogens

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Received 18 October 2001; returned 15 January 2002; revised 8 February 2002; accepted 11 March 2002

The immunomodulatory properties of antimicrobial agents and their clinical impact have been the focus of worldwide interest in recent years. In this study, the effects of different treatments with 14-, 15- and 16-membered ring macrolides on the mitogen-induced proliferative response of lymphocytes and the splenic response to immunization with sheep erythrocytes have been tested by in vitro and ex vivo assays in a murine experimental model. We observed that the in vivo administration of these antibiotics to mice induces a compensatory mechanism that abrogates the suppression observed by in vitro assays. Thus, physiological parameters may be important when testing the immunopharmacological effects of antibiotics.

Introduction

Macrolide antibiotics are polyoxygenated fungal and bacterial secondary metabolites that possess large ring structures, hence the name macrocyclic or macrolide. These antibiotics exhibit anti-inflammatory activity, and are especially effective in the therapy of infections caused by facultative intracellular bacteria. The possibility that intracellular accumulation also alters host cell function has triggered new interest in the therapeutic potential of macrolides in clinical settings other than infectious diseases.1 The interaction between macrolides and the bactericidal mechanisms of phagocytes is an area of active research.2 However, few studies have been devoted to the interference of macrolides with the immune responses of lymphocytes.

In this study we examine the effect of different treatments with erythromycin A (14-membered ring), azithromycin (15-membered ring) and josamycin (16-membered ring) on the cellular and humoral responses of lymphocytes in a murine experimental model.

Materials and methods

Animals

Male BALB/c mice were obtained from the breeding colony of the University of Jaén. They were maintained under pathogen-free conditions, with free access to food and water. The experiments were approved by the ethics committee for animal experiments at the University of Jaén.

Antibiotics

Erythromycin A (Estedi, Barcelona, Spain), azithromycin (Pfizer, Madrid, Spain) and josamycin (Yamanouchi Pharma, Madrid, Spain) were dissolved in dimethyl sulphoxide (DMSO; Sigma Chemical Co., St Louis, MO, USA) at 10 g/L and further diluted in sterile phosphate-buffered saline (PBS; Sigma). The drugs were given to mice by ip injection at 24 h intervals for 7 consecutive days. The dosages were calculated on a per kg body weight basis, in accordance with doses given to humans (57, 28, 14 and 7 mg/kg/day).3 Control mice received equal volumes of PBS for 7 days.

The antibiotic concentrations for in vitro assays were similar to serum levels attained with the treatment schedules used in human therapy (8, 4, 2 and 1 mg/L for erythromycin; 3.2, 1.6, 0.8 and 0.4 mg/L for azithromycin; 12, 6, 3 and 1.5 mg/L for josamycin).

Mitogen-induced proliferation of lymphocytes

Antibiotic-treated and control mice were killed, and their spleens were aseptically removed and homogenized in sterile Hanks balanced salt solution (HBSS; Sigma). Splenocytes
were washed twice with HBSS and finally resuspended in complete medium RPMI 1640 (Sigma) supplemented with 1% penicillin G/streptomycin solution (Sigma).

Cell suspensions were adjusted to $7 \times 10^6$ viable cells/mL and distributed (100 µL/well) into 96-well flat-bottomed microtitre plates (Costar, Cambridge, MA, USA). Lipopolysaccharide (LPS; Sigma) at 25 mg/L was used as a B-cell mitogen and concanavalin A (ConA; Sigma) at 10 mg/L was used as a T-cell mitogen. After incubation at 37°C in 5% CO$_2$ for 48 h, cellular proliferation was measured by colorimetric reading of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) reduction as described by Mosmann.4

In vitro assays

Antibiotics were added at different time intervals before and after the incubation of lymphocytes in order to determine when the effect of the macrolides occurs.

Lymphocyte viability. In order to detect a possible effect of macrolides on lymphocyte viability we cultured the cells in the presence or absence of antibiotics for 48 h.

Pre-incubation of lymphocytes with the macrolides. Lymphocytes were pre-incubated in the presence or absence of the drugs for 24 h. Cells were then thoroughly washed, and further cultured for an additional 48 h in the presence of T- or B-cell-specific mitogens.

Kinetic study of the activity of the macrolides. Antibiotics were added together with the mitogens at the initiation of the culture period, or for the last 24 h of the 2 day culture period.

Splenic response to sheep erythrocytes

Mice were immunized by ip administration of 0.5 mL of 2% (v/v) sheep erythrocytes (SRBCs; bioMérieux, Marcy l’Étoile, France). Five days after immunization, the mice were killed and the spleens were aseptically removed and homogenized in HBSS. The number of cells secreting anti-SRBC antibodies was determined in haemolytic plaque assays, as described by Henry.5

Statistics

All results are shown as means ± S.D. for seven mice, tested in duplicate for each sample. Statistical analysis was carried out using two-way factorial analysis of variance. Treated and control groups were compared with the least significant difference test. A $P$ value of $<0.05$ was considered significant.

Results and discussion

In vitro assays

All results are summarized in Table 1.

Lymphocyte viability. Macrolides seem not to have a direct cytotoxic effect on splenocytes, as cells cultured for 48 h in the presence of the macrolides showed similar viability to those cells cultured in the absence of the drugs.

Kinetic study of the activity of the macrolides. All macrolide antibiotics showed a potent inhibitory effect on the LPS- or ConA-induced proliferation of splenocytes when the drug was added at the initiation of the culture period, but when erythromycin A or azithromycin was added for the last 24 h of the culture period, using ConA as specific mitogen, this inhibitory effect was significantly reduced. In contrast, the 16-membered ring macrolide josamycin maintained its inhibitory effect on splenocyte proliferation when the drug was added later to the cell cultures.

These results about lymphocyte viability and proliferation are in agreement with those described by Morikawa et al.6 for the macrolides midecamycin acetate, josamycin and clarithromycin; these drugs do not modify lymphocyte viability but are able to inhibit the mitogen-induced proliferative response of these cells.

Pre-incubation of lymphocytes with the macrolides. The pre-incubation of splenocytes with macrolides did not interfere with the subsequent proliferation of mitogen-stimulated cells, so the coexistence of these drugs at the initiation of splenocyte stimulation might be necessary to inhibit cell proliferation. These results confirm that macrolides may work on an early event in cell activation, as Morikawa et al.6 have described previously.

Ex vivo assays

In ex vivo assays (Table 2) both azithromycin and josamycin, in the lowest dosages, impaired the LPS- or ConA-induced proliferation of lymphocytes. In contrast, higher concentrations of the drugs injected into mice induced a compensatory mechanism, leading to an increase in the proliferative response of these cells. This could be because of the antibiotic-mediated killing of indigenous bacteria in the intestinal tract, which may result in the release of immunomodulating bacterial fractions, as previously described for the quinolone ciprofloxacin.7 On the other hand, erythromycin A was able to abrogate the inhibitory effect observed in vitro even when the lowest dosage was injected into mice. Thus, the bactericidal activity of these macrolides might be responsible for some compensatory mechanism that abrogates the suppression observed by in vitro assays.
Table 1. Kinetic study of the effect of macrolides on LPS- and ConA-induced proliferation of lymphocytes

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (mg/L)</th>
<th>48 h Lymphocyte viability</th>
<th>LPS</th>
<th>ConA</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>48 h</td>
<td>Pre-incubation</td>
<td>48 h</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>71.12 ± 7.47</td>
<td>88.95 ± 5.02</td>
<td>132.55 ± 13.95</td>
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<tr>
<td>EM</td>
<td>8.0</td>
<td>77.00 ± 4.75</td>
<td>79.80 ± 11.03</td>
<td>89.14 ± 12.68 a</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>70.75 ± 7.36</td>
<td>91.35 ± 8.13</td>
<td>113.14 ± 37.33 a</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>73.12 ± 5.19</td>
<td>88.10 ± 6.15</td>
<td>97.00 ± 13.35 a</td>
</tr>
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<td></td>
<td>1.0</td>
<td>71.38 ± 6.16</td>
<td>73.00 ± 5.80</td>
<td>105.75 ± 25.56 a</td>
</tr>
<tr>
<td>AM</td>
<td>3.2</td>
<td>70.75 ± 11.74</td>
<td>98.25 ± 0.50</td>
<td>104.86 ± 20.19 a</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>67.12 ± 7.14</td>
<td>87.35 ± 6.15</td>
<td>101.85 ± 19.07 a</td>
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<tr>
<td></td>
<td>0.8</td>
<td>65.38 ± 2.62</td>
<td>80.90 ± 2.83</td>
<td>115.28 ± 18.46 a</td>
</tr>
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<td></td>
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<td>83.77 ± 8.87</td>
<td>121.43 ± 15.94 a</td>
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<td>JM</td>
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<td>83.90 ± 8.34</td>
<td>102.00 ± 14.54 a</td>
</tr>
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<td>107.57 ± 20.51 a</td>
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<tr>
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<td>87.00 ± 13.94 a</td>
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<tr>
<td></td>
<td>1.5</td>
<td>76.00 ± 6.72</td>
<td>65.00 ± 14.57</td>
<td>114.28 ± 29.92 a</td>
</tr>
</tbody>
</table>

EM, erythromycin A; AM, azithromycin; JM, josamycin. aP < 0.05.
Mice injected with azithromycin or josamycin also showed a dose-dependent decrease in the number of cells secreting anti-SRBC antibodies compared with the control group. On the other hand, when the lowest dosages of erythromycin A were injected into mice a significant decrease in this immune function was detected, whereas higher dosages induced a radical change, so no effect (in the case of IgM-secreting cells) or even a significant increase in the production of IgG by lymphocytes was induced. Thus, we have demonstrated that physiological parameters (not detected by in vitro assays) may be important when testing the immunopharmacological effects of antibiotics.

The immunosuppressive effect of macrolides has been proposed to have clinical relevance for modulation of the immune response in transplant patients, immunocompromised patients and patients with inflammatory diseases. However, the in vivo effects of these antibiotics on human lymphocyte functions should be evaluated further in clinical studies, as physiological parameters can alter the effect of these drugs on the immune response.

### Acknowledgements

This work was supported by the Plan Andaluz de Investigación (No. CTS 0105).

### References


