Original article

Effect of macrolides on the expression of human leukocyte antigen-DR and costimulatory molecules on cultured human mononuclear cells

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ABSTRACT

Although the clinical effectiveness of long-term low-dose administration of macrolides for diffuse panbronchiolitis, sinobronchial syndrome, chronic sinusitis and otitis media with effusion has been well documented, the mechanism of action remains to be determined. To clarify the effect of macrolides on the initiation of immune responses, we investigated changes in the expression levels of human leukocyte antigen (HLA)-DR, a major histocompatibility complex class II antigen, and of costimulatory molecules, such as CD54, CD80 and CD86, on monocytes following administration of macrolides. The expression of HLA-DR, CD54, CD80 and CD86 on cultured human peripheral mononuclear cells following stimulation by interferon (IFN)-γ and lipopolysaccharides (LPS) was analyzed using flow cytometry in the presence and absence of macrolides. The macrolides tested inhibited the expression of CD54, CD80 and CD86 on cultured monocytes following stimulation by IFN-γ and LPS, while the expression of HLA-DR on monocytes was not affected. Suppression of CD80 expression was the most significant among the costimulatory molecules tested and occurred in a dose-dependent manner. These results suggest that macrolides may downregulate the expression levels of costimulatory molecules, such as CD80, and may normalize the hyperimmune responses that may be responsible for the chronic intractable inflammation.

Key words: CD54, CD80, CD86, costimulatory molecules, human leukocyte antigen-DR, macrolides, monocytes

INTRODUCTION

Macrolide therapy, that is, low-dose long-term administration of 14-membered ring macrolides, has recently been reported to be very effective in the treatment of chronic upper respiratory infections, including diffuse panbronchiolitis,1 sinobronchial syndrome,2 chronic sinusitis3,4 and otitis media with effusion.2 The mechanisms underlying the attenuation of chronic inflammation in the respiratory tract remain to be determined. However, the clinical efficacy of macrolide therapy has been suggested to be little related to the antimicrobial effect of the macrolide, because the treatment is also effective in patients infected with pathogens insensitive to macrolides. Kudoh et al.1 have suggested that 14-membered ring macrolides may have anti-inflammatory effects and regulate the hyperimmune responses that lead to chronic intractable inflammation. In accordance with this hypothesis, macrolides have been reported to exhibit many effects on immunocompetent cells, such as monocytes, lymphocytes and neutrophils. These include effects on the proliferation, chemotactic activities, cytokine/chemokine production, superoxide production and expression of adhesion molecules of these cells.

The mucosal surface of the respiratory tract is continuously exposed to foreign bodies and, hence, is an important site of immunologic activity. Immune responses against foreign bodies are initiated by the presentation of the antigen to T lymphocytes by antigen-presenting cells (APC). Recent studies have shown that the proliferation of T cells requires the recognition of antigen/major
histocompatibility complex (MHC) complexes by the T cell receptor (TCR) and, in addition, the delivery of other non-specific costimulatory signals via molecules expressed on the surface of the APC. The costimulatory signals are provided efficiently by costimulatory molecules, such as CD54 (intercellular adhesion molecule (ICAM)-1), CD80 (B7–1) and CD86 (B7–2). T cell activation by the main signal in the absence of costimulatory signals results in T cell unresponsiveness or anergy. In the present study, to clarify the effect of macrolides on the initiation of immune responses, we investigated the effect of macrolides on the expression levels of human leukocyte antigen (HLA)-DR, a MHC-II antigen, and costimulatory molecules, such as CD54, CD80 and CD86, on human monocytes in vitro, because a monocyte can act as an APC.

**METHODS**

**Materials**

Erythromycin lactobionate (EM) was obtained from Abbott Laboratories (North Chicago, IL, USA), clarithromycin (CAM) was obtained from Taisho Pharmaceutical (Tokyo, Japan), roxithromycin (RXM) was from Eisai Pharmaceutical (Tokyo, Japan) and dexamethasone (DX) was from Banyu Pharmaceutical (Tokyo, Japan). Both EM and DX were dissolved in distilled water and CAM and RXM were suspended in a minimal volume of dimethylsulfoxide (DMSO) and diluted with distilled water. *Escherichia coli* O111:B4 lipopolysaccharide (LPS; Difco Laboratories, Detroit, MI, USA) and interferon (IFN)-γ (Sigma Chemical Co., St Louis, MO, USA) were suspended in distilled water and sonicated. Phycoerythrin (PE)-antihuman HLA-DR, fluorescein isothiocyanate (FITC)-antihuman CD54, FITC-antihuman CD80 and monoclonal antibodies IgG1 FITC/IgG1 PE/IgG1 PE-Cy5 were purchased from Immunotek (Marseille, France). Fluorescein isothiocyanate-antihuman CD86 and Via-Probe were obtained from PharMingen (San Diego, CA, USA). Normal goat serum was purchased from Cedarlane Laboratories (Ontario, Canada).

**Human mononuclear cell cultures**

Heparinized peripheral blood was obtained from six healthy adults who did not smoke or have either acute/chronic infections or allergic diseases, including bronchial asthma, nasal allergy and atopic dermatitis. Blood samples were diluted twice with phosphate-buffered saline (PBS), layered onto Ficoll–Hypaque (Separate-L; Muto Pure Chemical, Tokyo, Japan) and then centrifuged to isolate mononuclear cells. The mononuclear cells thus obtained were washed three times with PBS and were suspended in RPMI 1640 medium (Nissui, Tokyo, Japan) containing 10% fetal calf serum (1 × 10⁶ cells/mL). The cell suspension was preincubated with EM, CAM, RXM (final concentrations 1 or 10 µg/mL) or DX (final concentration 10⁻⁶ mol/L) at 37°C for 36 h in culture wells under 5% carbon dioxide. As controls, RPMI medium with or without the same concentration of DMSO as that used for dissolving CAM and RXM was added to the culture medium. After preculture, a LPS suspension (final concentration 100 ng/mL) and IFN-γ (final concentration 500 IU/mL) were added to each well. Then, EM, CAM, RXM or DX at the same concentration was further added to the culture medium and incubated for 12 h under the same conditions.

**Cell viability**

Cell viability was measured at the end of the culture period by the trypan blue exclusion method. Cell viability was not altered by any concentration of the drugs tested.

**FACS analysis**

Cultured mononuclear cells were preincubated with normal goat serum for 30 min at room temperature and then with PE-labeled antibodies against HLA-DR or FITC-labeled antibodies against CD54, CD80 or CD86 for 30 min at 37°C. As a negative control, cells were also incubated with the monoclonal antibodies IgG1 FITC/IgG1 PE/IgG1 PE-Cy5. Cells were then washed twice and fixed with formalin. Data were analyzed by single histogram of 1 × 10⁴ cells on a FACScan (Becton Dickinson, Mountain View, CA, USA) gated on the monocyte position in scattered dots (forward scatter (FSC), side scatter (SSC)). Dead cells as detected by staining with PE-Cy5–Via-Probe were excluded from analysis.

**Statistical analysis**

Statistical analysis was performed using unpaired Student’s t-test. Two-tailed P values less than 0.05 were considered significant.
RESULTS

Effect of LPS and IFN-γ on the expression of HLA-DR and costimulatory molecules on monocytes

The expression of HLA-DR, CD54, CD80 and CD86 on cultured human monocytes was upregulated by the addition of LPS and IFN-γ compared with expression before in the culture medium alone (Fig. 1). Monocytes in the culture medium containing the same amount of DMSO as that used for dissolving CAM and RXM showed no changes in the expression levels of any of the surface antigens.

Expression of HLA-DR on cultured monocytes

Figure 2 shows the percentage of cells in the gated area M1 on the histogram of fluorescence intensity. None of the agents tested had any effect on the expression of HLA-DR on the monocytes.

Expression of CD54 on cultured monocytes

Following the addition of $10^{-4}$ mol/L DX to the culture medium, significant downregulation of CD54 expression on the monocytes was observed ($P < 0.001$). At concentrations of 1 and 10 µg/mL, both CAM and RXM inhibited the expression levels of CD54 on the monocytes. In contrast, EM at these concentrations had little effect on CD54 expression (Fig. 3).

Expression of CD80 on cultured monocytes

With the addition of DX, EM, CAM or RXM to the culture medium, the expression of CD80 on monocytes following stimulation by IFN-γ and LPS was significantly reduced compared with cells cultured without any drugs. The percentage inhibition of the expression of CD80 was the greatest with the addition of DX and the second greatest inhibition was observed following the addition of RXM (Fig. 4).
Fig. 2 Effects of dexamethasone (DX; 10^{-6} mol/L) and macrolides (1 and 10 µg/mL; EM, erythromycin lactobionate; CAM, clarithromycin; RXM, roxithromycin) on human leukocyte antigen (HLA)-DR expression on cultured mononuclear cells following stimulation by lipopolysaccharide (100 ng/mL) and interferon-γ (500 IU/mL). Data are the mean ± SD of the percentage of cells gated in the M1 area shown in Fig. 1a (n = 4).

![Fig. 2](image)

Fig. 3 Effects of dexamethasone (DX; 10^{-6} mol/L) and macrolides (1 and 10 µg/mL; EM, erythromycin lactobionate; CAM, clarithromycin; RXM, roxithromycin) on CD54 expression on cultured mononuclear cells following stimulation by lipopolysaccharide (100 ng/mL) and interferon-γ (500 IU/mL). Data are the mean ± SD of the percentage of cells gated in the M2 area shown in Fig. 1b (n = 4). *P < 0.05, **P < 0.01 and ***P < 0.001 compared with values in cultures without DX or macrolides (□).

![Fig. 3](image)

Fig. 4 Effects of dexamethasone (DX; 10^{-6} mol/L) and macrolides (1 and 10 µg/mL; EM, erythromycin lactobionate; CAM, clarithromycin; RXM, roxithromycin) on CD80 expression on cultured mononuclear cells following stimulation by lipopolysaccharide (100 ng/mL) and interferon-γ (500 IU/mL). Data are the mean ± SD of the percentage of cells gated in the M2 area shown in Fig. 1c (n = 4). *P < 0.05, **P < 0.01 and ***P < 0.001 compared with values in cultures without DX or macrolides (□).

![Fig. 4](image)
Expression of CD86 on cultured monocytes

Figure 5 shows the percentage of cells occupying the M2 area of the histogram in the fluorescence intensity. Although, DX failed to affect any significant reduction of the M2%, the peak of the histogram of fluorescence intensity was markedly shifted to the left, implying down-regulation of the expression of CD86 by DX. Both EM and CAM also showed an inhibitory effect on CD86 expression at high concentrations. At concentrations of both 1 and 10 µg/mL, RXM exhibited a significant down-regulatory effect on CD86 expression on monocytes.

DISCUSSION

Fourteen-membered ring macrolides, such as EM, RXM and CAM have been reported to be very effective in the treatment of chronic sinusitis and low-dose long-term administration of macrolides (macrolide therapy) is now the therapy of first choice for the treatment of chronic sinusitis in Japan. The effectiveness of macrolides in the treatment of chronic sinusitis may not be related to their antimicrobial actions but, rather, to their anti-inflammatory or immunoregulatory actions. Recent studies have demonstrated that macrolides exhibit various biologic and immunologic effects on immune-competent cells, fibroblasts, respiratory epithelial and secretory cells and endothelial cells. These include: (i) inhibition of proliferation and differentiation; (ii) stimulation or inhibition of migration, chemotactic activities and superoxide production; (iii) inhibition of cytokine or chemokine production; (iv) effects on ion-transport and Cl secretion; (v) suppression of mucus secretion; and (vi) acceleration of apoptosis. However, there have been few reports concerning the effects of macrolides on the expression of surface antigens on immunocompetent cells.

One of the most critical events in the initiation of the immune response is the activation of T helper (Th) cells by the APC. The activated Th cells produce cytokines and express receptors for these cytokines, thereby further promoting Th cell proliferation. In fact, various kinds of Th cell-derived cytokines have been detected in specimens of nasal and paranasal mucosa, nasal polyps and nasal secretion in rhinitis and sinusitis, indicating that T cells are activated in the inflammatory conditions in the respiratory tract. The expression of MHC class II molecules is primarily limited to professional APC, such as macrophages, dendritic cells and B cells. The presence of MHC class II antigen, in the absence of costimulatory signals, results, instead of T cell activation, in a T cell unresponsive state termed clonal anergy. Some examples of costimulatory molecules expressed on APC are CD54 (ICAM-1), CD80 (B7–1), CD86 (B7–2), vascular cell adhesion molecule (VCAM)-1 and CD58 (lymphocyte function-associated antigens-3; LFA-3).

We have recently demonstrated for the first time that macrophages, one of the APC in nasal polyps and paranasal mucosa, expressed both a MHC-II (HLA-DR) and costimulatory molecules (CD54 and CD80), using an immunohistochemical double-staining technique. We have also reported that following macrolide therapy in patients, the number of CD4+ cells in the nasal polyps
was decreased compared with the number in patients who had not received macrolide therapy. However, no difference in the number of HLA-DR-positive cells was observed between the two groups.24 These results suggest that macrolides may modulate the APC–T cell interaction. Therefore, we investigated the expression levels of HLA-DR and those of costimulatory molecules, such as CD54, CD80 and CD86, on monocytes, one of the APC, in vitro. Monocytes were stimulated by LPS and IFN-γ in the present study, because it has been shown that IFN-γ enhances MHC class II antigen expression on human monocytes in vitro25 and that IFN-γ induces the expression of costimulatory molecules, such as CD80 and CD86, on synovial dendritic cells in vitro.26 In addition, a significant level of IFN-γ can be detected in inflamed nasal mucosa and nasal polyps, and IFN-γ may play an important role in acute or persistent inflammation in the respiratory tract.22

The results of our current study indicate that the expression of HLA-DR on monocytes following stimulation by IFN-γ and LPS was not altered by the addition of either DX or macrolides to the culture medium. Because HLA-DR is one of the MHC class II antigens and is of importance in the induction of the T cell immune response against foreign bodies, it seems reasonable to assume for the human immune system that the expression of HLA-DR is not affected by drugs that are commonly used in medical field.

It is generally accepted that in inflammatory conditions the expression of adhesion molecules, such as CD54, VCAM-1 and E-selectin, on endothelial cells is increased to promote recruitment of leukocytes. In regard to the effect of macrolides on the expression of adhesion molecules, in vitro and in vivo studies have shown that the expression of ICAM-1 and E-selectin on endothelial cells or neutrophils is suppressed by the administration of macrolides.27,28 In APC, CD54 and VCAM-1 have been reported to act as costimulators that activate resting T cells in the early stages of the immune response.29,30 The present study also demonstrated that the expression of CD54 on monocytes, upregulated following stimulation by LPS and IFN-γ, was reduced not only with the addition of DX but also following the addition of CAM and RXM. However, the effect of macrolides on the expression of CD54 was very mild compared with that of DX.

CD80 and CD86 are costimulatory molecules that bind to a cell surface-differentiation antigen of T cells, namely CD28. CD80 and CD86 also bind to another T cell-differentiation antigen, cytotoxic T lymphocyte-associated receptor (CTLA)-4, which is expressed on activated, but not resting, T cells. Human leukocyte-DR and CD54 are always involved at the site of mucosal immune responses, but the subsequent immune reaction and immune characteristics may be controlled by CD80/CD86 expression. A recent study has demonstrated that both CD80 and CD86 transfectants exhibit efficient costimulation of T cell proliferation, cytokine production, such as interleukin (IL)-2 and IFN-γ, and generation of functional cytotoxic T lymphocytes (CTL). In addition, the magnitude and kinetics of these responses was similar between the CD80 and CD86 transfectants.31

In the present study, the macrolides tested significantly reduced the expression level of CD80 on monocytes in a dose-dependent manner following stimulation by IFN-γ and LPS. The expression of CD86 was also reduced by the addition of macrolides to the culture medium; however, the level of suppression of CD86 was smaller than that of CD80. CD80 is not present on monocytes until after their stimulation with IFN-γ, whereas CD86 is constitutively expressed on resting monocytes.33 In addition, CD80 restimulates activated T cells in the late stages of the immune response, whereas CD86 may be more important in the primary interaction between monocytes and T cells during early stages of the immune response. Therefore, in cultured monocytes in the present study, the difference in the pattern of suppression of costimulatory molecule expression by DX and macrolides is reasonable.

There seem to be two possibilities concerning the mechanism of the inhibitory effect of macrolides on the expression of costimulatory molecules on cultured monocytes. One is the direct effect on the costimulatory molecule protein production. The other is the indirect effect of suppression of cytokine production by mono-nuclear cells. Macrolides have been reported to inhibit the production of various cytokines, such as tumour necrosis factor (TNF)-α or IL-1, IL-4 and granulocyte–macrophage colony stimulating factor (GM-CSF).13–16 Because TNF-α and GM-CSF, similar to IFN-γ, have also been reported to stimulate the expression of costimulatory molecules on monocytes in vitro,27 the suppressive effect of macrolides on expression may be secondary to the suppression of the production of these cytokines by macrolides.

Antigen-specific T cell activation and differentiation sometimes induces the production of various cytokines and the consequent activation and infiltration of inflammatory cells. Excessive amounts of inflammatory mediators produced by activated cells induce tissue damage and
lead to intractable inflammation. In the absence of costimulatory molecules, T cells become anergic. In mucosal immune response, the expression of costimulatory molecules must be carefully regulated. The results of the present study suggest that macrolides could normalize the hyperimmune response by modulating the expression of costimulatory molecules, such as CD80 and CD86, on APC in chronic inflammatory conditions.

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