ANTILYMPHOCYTIC ACTIVITY OF ERYTHROMYCIN DISTINCT FROM THAT OF FK506 OR CYCLOSPORIN A

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(Received for publication January 26, 1993)

Erythromycin (EM), a macrolide antibiotic has been recently reported to depress the extent of inflammation irrespective of its antimicrobial action. Our study was initiated to examine the effect of EM on T cell proliferation *in vitro*, since other macrolide antibiotics FK506 and rapamycin (RAP) have been well known to possess strong immunosuppressive or anti-inflammatory potential. EM had a suppressive effect on the proliferative response of human lymphocytes stimulated with mitogens and antigens, while EM had no effect on concanavalin A (Con A)-induced interleukin-2 (IL-2) production or IL-2Rα (CD25) expression. Delayed addition of EM after the first 48 hours of mitogenic stimulation did suppress IL-2-dependent proliferation of Con A blasts, whereas pretreatment with EM for the first 48 hours of stimulation did not impede the subsequent IL-2-dependent proliferation of obtained blast cells. The results indicate that EM suppresses T cell proliferation at a late stage in the activation process by impairing their response to IL-2. This antilymphocytic action of EM was quite distinct from that of FK506 or cyclosporin A (CsA) but was similar to that of RAP. Unlike RAP, however, EM did not antagonize FK506-induced suppression but potentiated the action of FK506 and CsA. The addition of an enteric hormone motilin, a receptor of which was previously found to be occupied by EM, unaffected the lymphocyte proliferation and the subsequent EM-induced suppression. These data suggest that EM operates through an undefined mechanism probably distinct from that of FK506, CsA, RAP or motilin.

The usage of antibiotics is generally based on the concept of their selective toxicity to the target organisms and extensive studies have been directed towards specific effects of antimicrobial agents on pathogens. However, recent observations indicate that certain antibiotics alter the function of host cells *in vitro*[1,2].

Erythromycin (EM) is a macrolide antibiotic first isolated from cultures of *Streptomyces erythreus* in 1952 and has been widely used to treat a variety of infectious diseases[3]. In the past several years, it was reported that long-term treatment with EM appears to improve chronic inflammatory process including diffuse panbronchiolitis[4,5], bronchial asthma[6] and HTLV-1-associated myelopathy[7], irrespective of its antimicrobial property.

There is increasing evidence that EM has direct effects on host cells. To date, it has been reported that EM influences neutrophil functions[8,9] and reduces mucus secretion from secretory epithelial cells[10] and contracts gastrointestinal smooth muscles binding to a receptor of an enteric hormone, motilin[11].

Although previous work demonstrated that EM suppressed a mitogen-mediated lymphocyte proliferative response[12,13], the antilymphocytic activity of EM was never fully studied. On the other hand, the macrolide antibiotics FK506 and rapamycin (RAP) were isolated from other strains of *Streptomyces*

and are now well known to possess strong immunosuppressive or anti-inflammatory activity[14~19].

The aim of this study was to investigate the antilymphocytic action of EM and characterize the bioactivity, comparing with those of other immunosuppressants. We demonstrate that the antilymphocytic activity of EM is mild and that the property is quite distinct from that of FK506 or cyclosporin A (CsA) but similar to that of RAP.

Materials and Methods

Reagents

Erythromycin base was obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). It was dissolved in ethanol at 200 mg/ml and was diluted with culture medium in each experiment. Concanavalin A (Con A), indomethacin and methyl α-mannopyranoside were purchased from Sigma Chemical Co. (St. Louis, MO). Purified protein derivative (PPD) of *Mycobacterium tuberculosis* was kindly provided by Dr. T. KATAOKA (National Institute of Health, Tokyo, Japan). Recombinant human interleukin-1 (IL-1)α was donated from Dainippon Pharmaceutical Co., Ltd. and recombinant human interleukin-2 (IL-2) was supplied by Dr. M. HONDA (National Institute of Health, Tokyo). FK506 was obtained from Fujisawa Pharmaceutical Co. (Osaka, Japan) and CsA was from Sandoz Ltd. (Basel, Switzerland). These immunosuppressants were dissolved in ethanol at 10^{-2} M and were diluted with culture medium. Motilin (porcine) was purchased from Peptide Institute Inc. (Osaka, Japan) and dissolved in distilled water at 2×10^{-4} M and was diluted with culture medium.

Preparation of Human Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) were obtained from venous blood of normal healthy volunteers whom permission was granted from. PBMC were isolated by centrifugation on a Ficoll-Metrizoate density gradient (Lymphoprep; Nycomed, Oslo, Norway). The cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (Z. L. Bockneck Laboratories Inc., Ontario, Canada), 2 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin at 37°C in a humidified 5% CO_2 atmosphere.

Cell Lines

The human T-lymphoid (Molt-4, Jurkat and HPB-ALL), monocytic leukemia (THP-1) and melanoma (A375) cell lines were maintained in the medium described above.

Proliferation Assay

PBMC (5×10^5/ml) were cultured in 96-well flat-bottom tissue culture plates (Falcon No. 3072; Becton Dickinson Labware, Lincoln Park, NJ) and were stimulated with Con A (2 μg/ml) or PPD (50 U/ml) in the absence or presence of EM for 72 hours. EM with each stimulus was added at the initiation of the cultures. The percentage of viable cells was determined by trypan blue dye exclusion test. Mixed lymphocyte reactions (MLR) were established between PBMC (5×10^5/ml) from pairs of donors and these cultures were incubated in the absence or presence of EM for 5 days.

To examine the effects of exogenous IL-1, IL-2 and prostaglandin E_2 (PGE_2), recombinant human IL-1 (10~100 U/ml), IL-2 (100 U/ml) or indomethacin (5×10^{-6} M) together with EM (1~100 μg/ml) was added to the system of Con A-stimulated proliferation of PBMC.

Each culture was pulsed with 0.5 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA) per well for the final 18 hours of incubation before being harvesting onto glass fiber filters. The radioactivity collected was counted by a liquid scintillation counter[14]. Results are expressed as the mean ± SD.

Preparation and IL-2-Dependent Proliferation of Con A Blasts

PBMC were stimulated with Con A (2 μg/ml) for 48 hours and the non-adherent cells were collected and washed in phosphate buffered saline containing methyl α-mannopyranoside (25 mM) to eliminate further effect of Con A. Then obtained blast cells (Con A blasts) were incubated with exogenous IL-2 in the absence or presence of EM (1~100 μg/ml) for 96 hours.

Analysis of Lymphocyte Surface Markers

For the lymphocyte surface marker study, cells were cultured in tissue culture dishes (Falcon 3003, Becton Dickinson Labware) at 1×10^6/ml for 24~48 hours. Surface markers of lymphocytes were examined by using anti leu-4 (CD3), anti leu-3a (CD4), anti leu-2a (CD8) or anti-Tac (CD25) (Becton Dickinson Mountain View, CA) with fluorescein isothiocyanate-conjugated goat anti-mouse IgG $F(ab')_2$ fragment (Cooper Biomedical Inc., Malverm, PA). Flow cytometry was performed on a FACStar (Becton Dickinson)[14].

Human IL-2 ELISA Assay

The amount of IL-2 produced by cultured PBMC was determined using a ELISA system specific for IL-2 (E. I. Du Pont de Nemours & Co., Medical Products Department, Boston, MA). Cell-free culture supernatants of Con A-stimulated PBMC (5×10^5/ml) after 48 hours of incubation in the presence of EM or FK506 were assayed to the manufacturer's instructions[20]. Samples were assayed in triplicate and analyzed on an automated ELISA plate reader (SJeia Auto Reader, model ER-80000, Sanko Junyaku Co., Ltd., Tokyo, Japan). The detection limit of this ELISA kit was 0.5 IU/ml of IL-2.

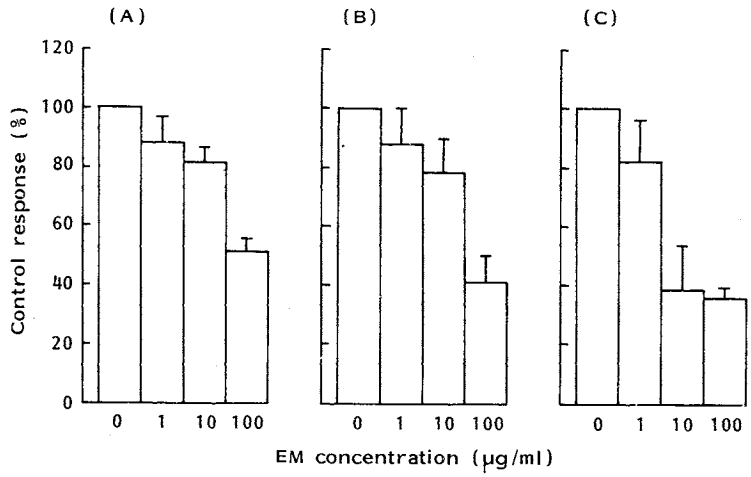
Statistical analysis was performed using STUDENT's *t*-test.

Results

EM Suppresses the Proliferative Response Induced by Con A, PPD and MLR in Human T Cells

To determine whether EM has antilymphocytic activity, we first evaluated the effects of EM on the proliferative response of human T cells stimulated with a mitogen (Con A), an exogenous antigen (PPD) and alloantigens (MLR). EM dose-dependently suppressed [^{3}H]thymidine incorporation by 10~20% at 1 μg/ml ($p < 0.01$) and by 40~60% at 100 μg/ml ($p < 0.001$) in each system (Fig. 1(A), (B) and (C)). Another lectin, phytohemagglutinin (PHA)-mediated proliferation was also suppressed by EM (data not shown). The percentage of viable cells did not change after 48~72 hours of incubation in the presence of EM and

Fig. 1. Suppressive effect of EM on the lymphocyte proliferative responses stimulated with A, Con A (2 μg/ml), B, PPD (50 U/ml) and C, MLR.



Vertical axis indicates the percentage of control response (cpm). The proliferative response was measured by [^{3}H]thymidine incorporation. Representative experiments (of at least three performed) are shown. Error bars are SD. The control responses (cpm $\times 10^{-3}$) of cells treated with the stimuli alone were: Con A = 22.6, PPD = 5.51, MLR = 3.71.

the final concentration of the diluent, 0.05% ethanol in the medium had no suppressive effect in this system (data not shown).

EM does not Suppress IL-2 Production or IL-2Rα (CD25) Expression

To explore the antilymphocytic action, we next examined a possibility that impairment of IL-1 or IL-2 production or enhancement of PGE_2 production causes EM-induced suppression. However, recombinant human IL-1, IL-2 or indomethacin did not reverse EM-induced suppression of Con A-stimulated lymphocyte proliferation (data not shown).

Another immunosuppressive macrolide FK506 is known to inhibit IL-2 production markedly[14,19]. Thus, we further evaluated whether EM directly influences IL-2 production. The amount of IL-2 in the culture supernatants of Con A-stimulated PBMC was measured by IL-2 ELISA. However, EM ($1 \sim 100\,\mu g/ml$) did not suppress IL-2 production by Con A-stimulated PBMC, while FK506 (1 nM) did suppress IL-2 production by 90% (data not shown).

The surface expression of CD25 on lymphocytes stimulated with Con A in the absence or presence of EM was also evaluated. Although EM ($100\,\mu g/ml$) suppressed Con A-stimulated lymphocyte proliferation by $40 \sim 60\%$ as shown in Fig. 1(A), it did not reduce fluorescence intensity of CD25 on Con A-stimulated lymphocytes (data not shown).

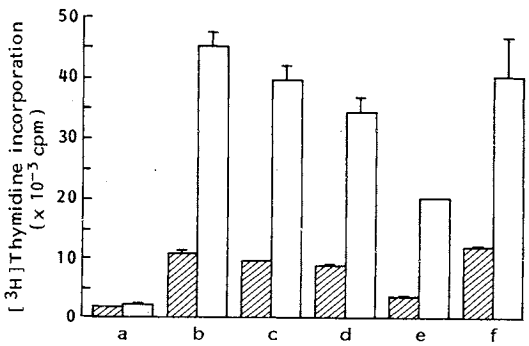
EM Suppresses the Lymphocyte Proliferative Response at the Late Stage of T Cell Activation, Impairing Their Response to IL-2

To elucidate the target phase of EM-mediated suppression, the time-course studies were carried out. Con A-stimulated human T cells began to incorporate [^{3}H]thymidine within $24 \sim 48$ hours to a peak rate of DNA synthesis at $72 \sim 96$ hours. Addition of EM did not cause any major alteration in these kinetics but the peak height was reduced ($53.7 \pm 7.4 \times 10^{-3}$ cpm; 57%; $p < 0.01$) as compared to control response ($93.7 \pm 10.7 \times 10^{-3}$ cpm). When EM was added to the culture after 24 hours from stimulation, the suppression was observed to the same degree (data not shown).

To further clarify the stage of T cell activation suppressed by EM, we examined whether IL-2-dependent growth of Con A-stimulated lymphocytes is directly suppressed by EM. Con A blasts were prepared as described before. As shown in Fig. 2, IL-2-dependent proliferation of Con A blasts was dose-dependently suppressed by EM. As compared with the suppressive effect of EM($100\,\mu g/ml$) on their IL-2-dependent growth, when PBMC were

Fig. 2. Suppressive effect of EM on IL-2-dependent growth of Con A blasts.

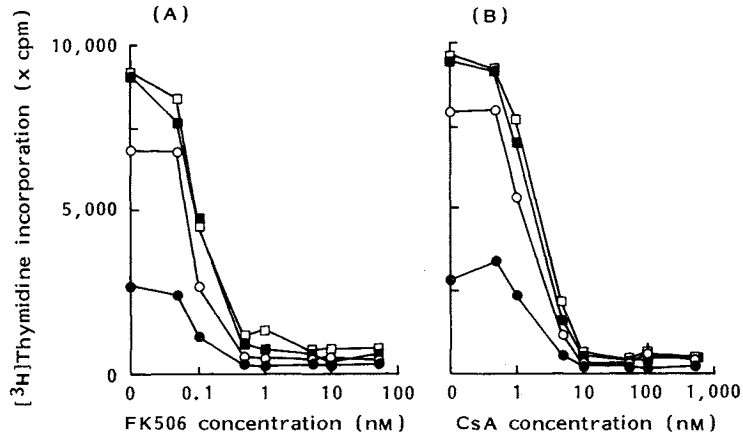
Closed: IL-2 (3 U/ml), open: IL-2 (9 U/ml).



Vertical axis indicates [^{3}H]thymidine incorporation ($\times 10^{-3}$ cpm). Con A blasts were prepared as described in Materials and Methods and obtained cells were cultured with exogenous IL-2 (3 or 9 U/ml). The first column [a] indicates that PBMC without Con A-stimulation were cultured with exogeneous IL-2 in the absence of EM. The next 4 columns [b, c, d, e] indicate that Con A blasts were cultured with exogenous IL-2 in the absence (b) or presence of EM [(c) $1\,\mu g/ml$, (d) $10\,\mu g/ml$, (e) $100\,\mu g/ml$]. The last column [f] indicates that PBMC were pretreated with Con A and EM ($100\,\mu g/ml$) for the first 48 hours and that obtained Con A blasts were cultured with exogenous IL-2 in the absence of EM. The proliferative response was measured by [^{3}H]thymidine incorporation after 96 hours of culture. Error bars are SD.

Fig. 3. Effect of combining fixed concentrations of EM with various doses of FK506 or CsA on the proliferation of Con A-stimulated PBMC.

A, various concentrations of FK506 were added either alone or together with EM at 1～100 μg/ml. B, various concentrations of CsA were added either alone or together with EM at 1～100 μg/ml. □ EM 0 (μg/ml), ■ EM 1, ○ EM 10, ● EM 100.



The proliferative response was measured by [^{3}H]thymidine incorporation after 48 hours of culture.

pretreated with EM(100 μg/ml) and rinsed, the subsequent IL-2-dependent proliferation was not hampered ($p<0.01$). Taken together, these results indicate that EM suppresses the stage of IL-2-dependent T cell proliferation.

To determine whether EM interferes with factors-independent cell proliferation, effects of EM on the autonomous proliferation of several human cell lines were examined. The proliferation of THP-1, A375, HPB-ALL and Molt-4 cells was unaffected by EM (data not shown). Only at high concentrations of EM (100 μg/ml), [^{3}H]thymidine incorporation to Jurkat cells was slightly suppressed (15%; $p<0.05$), although the suppression ratio was significantly lower than that in the proliferative response of normal lymphocytes induced by Con A (41%; $p<0.001$).

Additive Effect of EM on the Inhibition of Lymphocyte Proliferative Response by FK506 or CsA

The antilymphocytic action of EM was found to be similar to that of another immunosuppressive macrolide RAP, which was previously shown to impair IL-2-driven T cell proliferation with minimal effect on IL-2 production and IL-2R expression[18,19]. Interestingly, recent studies have revealed that RAP acts as an antagonist of FK506 suppression and interacts with a common receptor site(s) of FK506[21,22]. Thus, we examined whether EM also acts as an antagonist of FK506 or CsA. Unlike RAP-induced suppression, however, percent suppression is 26% at EM 10 μg/ml, 51% at FK506 0.1 nM and 71% at EM 10 μg/ml plus FK506 0.1 nM. Percent suppression is 18% at EM 10 μg/ml, 20% at CsA 1 nM and 45% at EM 10 μg/ml plus CsA 1 nM (Fig. 3). These indicate that the combination of EM and FK506 exerts additive inhibitory effect on the proliferative response, as well as the combination of EM and CsA.

Motilin, an Enteric Hormone does not Suppress Lymphocyte Proliferation and does not Reverse EM-Induced Suppression

EM was reported to bind to a receptor of an enteric hormone, motilin[11]. Although it is unlikely that

lymphocytes have motilin receptors on their surfaces, it could not be ruled out that EM binds to an unknown receptor structurally similar to the motilin receptor and suppresses the lymphocyte proliferation. However, motilin neither suppressed lymphocyte proliferation even at high concentrations ($0.01 \sim 10\,\mu$M) nor had a potential to reverse EM-induced suppression (data not shown).

Discussion

The present investigation provides the notion that EM possesses the potency to suppress lymphocyte proliferative response *in vitro*. EM suppressed the proliferation of human T cells induced by antigens (PPD or alloantigens) as well as mitogens (Con A or PHA) in a dose-dependent manner.

The antilymphocytic activity of two other macrolides, FK506 and RAP are quite different each other; FK506 strongly suppresses the early phase of T cell activation, like CsA, inhibiting IL-2 production and IL-2 receptor expression, while RAP primarily suppresses the stage of IL-2-dependent proliferation with minimal effect on IL-2 production or IL-2 receptor expression[14,18,19,22]. In our present studies, exogenous IL-2 could not overcome EM-induced suppression. IL-2 production and IL-2 receptor (CD25) expression induced by Con A were not suppressed by EM. The kinetic studies disclosed that EM did not alter the timing of the beginning of DNA synthetic phase but reduced the [^{3}H]thymidine incorporation. The experiment of Con A blasts demonstrated that EM affects the late phase of T cell activation suppressing IL-2-dependent proliferation. Collectively, these properties of EM are quite distinct from those of FK506 or CsA but similar to those of RAP.

The proliferation of several cell lines was unaffected by EM, although the proliferation of a T-lymphoid Jurkat cell line was slightly affected by EM. Similarly, RAP was shown to have little or no antiproliferative activity against several tumor lines[23], while RAP was reported to suppress the autonomous proliferation of a T cell hybridoma line[19]. A possibility that EM acts as a nonspecific cytotoxic agent seems unlikely, since even high concentrations of EM used did not reduce cell viability by trypan blue dye exclusion test and the pretreatment with EM unaffected the subsequent lymphocyte proliferation (the last column in Fig. 2). It has been reported that efflux of EM from mammalian cells after removal of the extracellular drug from tissue culture medium is rapid[3].

A possible effect of EM on contaminating monocytes seems negligible since EM similarly suppressed the proliferative response of non-adherent cells or Con A blasts without adherent cells. At least IL-1 and PGE_2 released from accessory cells appear to have little contribution to this suppression mechanism. In addition, a possibility that EM modifies membrane-associated events of T cell activation including interaction between T cells and accessory cells is unlikely because our preliminary study revealed that human lymphocyte proliferation induced by phorbol myristate acetate (PMA) and ionomycin which bypass the early membrane-associated step of T cell activation[24], was equally suppressed by EM (data not shown). Although a possibility that EM acts selectively on a certain T cell subset should be considered, at least the proportion of CD4 or CD8 positive lymphocytes was unaffected by EM (data not shown) and memory T cell proliferation by PPD and non-specific T cell proliferation by Con A were equally suppressed by EM as shown in Fig. 1.

Recent studies have shown that RAP competitively inhibits the activity of peptidylprolyl isomerase characterized as the major cytosolic FK506-binding protein (FKBP) and that RAP acts as an antagonist of FK506[21,25]. The analogy between RAP and EM prompted us to examine the combined effects of EM and other immunosuppressants. However, EM had an additive effect of FK506- or CsA-induced suppression. It is inferred that EM does not competitively bind to the cellular target(s) of FK506 or CsA. It is likely that RAP after binding to FKBP interacts another target molecule and suppresses a pathway of the late phase of T cell activation[25] and it was recently reported that the p70 S6 kinase activation cascade is blocked by the FKBP-RAP complex[26]. As compared with RAP, much higher concentrations of EM are needed to exert significant immunosuppressive effect, nevertheless, it should be further studied whether RAP and EM share the same target for their similar biological actions.

A putative target molecule of EM on lymphocytes dose not appear to be analogous to the motilin receptor which was reported to be occupied by EM[11], because the motilin at much higher concentrations

than those (>1 nM) to contract smooth muscles had no effect on the proliferation of T cells.

In our study, the antilymphocytic effect of EM at therapeutic concentrations (0.5~2 μg/ml) was much less potent than that of FK506 or RAP, but the suppression was dose-dependent. Since EM is well known to be accumulated into tissue cells[9], long-term treatment with EM might exert its effect on infiltrating lymphocytes at sites of chronic inflammation.

Acknowledgments

We thank Dr. SATOSHI ŌMURA for helpful discussion. This work was supported in part by Japan Health Science Foundation Grant No. 3-1-2 and by Research Committee of Diffuse Pulmonary Diseases, the Ministry of Health and Welfare of Japan.

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