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Immunomodulatory Effects of Three Macrolides, Midecamycin Acetate, Josamycin, and Clarithromycin, on Human T-Lymphocyte Function In Vitro

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The effect of three macrolide antibiotics, midecamycin acetate, josamycin, and clarithromycin, on human T-cell function was investigated in vitro. Midecamycin acetate and josamycin suppressed the proliferative response of peripheral blood mononuclear cells stimulated by polyclonal T-cell mitogens at concentrations between 1.6 and 8 μg/ml. At higher concentrations (40 to 200 μg/ml), all these drugs showed a marked inhibitory effect. At concentrations of 1.6 to 40 μg/ml, these drugs suppressed interleukin-2 (IL-2) production induced by mitogen-stimulated T cells, but not the expression of IL-2 receptor (CD25), in a dose-dependent manner. Therefore, the suppressive action on T-lymphocyte proliferation seems to be based on the ability of these drugs to inhibit IL-2 production by T cells. The drugs also inhibited mixed lymphocyte reaction at the same concentrations. Combined treatment with these macrolides and the known immunosuppressants such as FK506 and cyclosporin A resulted in an increased inhibition of T-cell proliferation. The immunomodulatory properties of the antibiotics may have clinical relevance for modulation of the immune response in transplant patients and in patients with inflammatory diseases.

During the last decades, macrolide antibiotics have been widely used for the treatment of various types of bacterial infections in clinical settings. They have recently been reported to be very effective against chronic lower respiratory tract infections (1, 17, 18). However, the mechanism of action of macrolides in chronic inflammation of the respiratory tract has not been elucidated. On the other hand, it has been suggested that the clinical efficacy of low-dose, long-term treatment with erythromycin (EM) is unrelated to the antimicrobial effect of EM on the pathogens in the respiratory tract, because the treatment is also effective in patients infected with pathogens resistant to EM (18). It has been known that macrolides can penetrate and accumulate in bacterial and eukaryotic cells (7, 15). In addition, it has been reported that macrolide antibiotics influence the cellular functions of immunocompetent cells. EM has an inhibitory effect on the incorporation of [^{3}H]thymidine by human lymphocytes stimulated by phytohemagglutinin or *Staphylococcus aureus* Cowan I (4). EM can suppress production of inflammatory cytokines such as tumor necrosis factor alpha or interleukin-1 (IL-1) by monocytes or macrophages (9, 22). Clarithromycin (CAM) is a macrolide with a 14-atom lactone ring and has a suppressive effect on IL-1 production (22) by macrophages. Josamycin (JM) and midecamycin acetate (MOM) have a 16-atom lactone ring. MOM has been found to stimulate phagocytosis (2, 5, 13) and enhance natural killer (NK) activity (1, 5). The currently available immunosuppressants, such as FK506 and rapamycin, belong to the macrolide family (10, 19, 20). They are powerful suppressors of the immune system, especially on T cells. In the present study, we examined whether three macrolides, MOM, JM, and CAM, have an immunomodulatory property for human T lymphocytes in vitro.

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MATERIALS AND METHODS

Cell preparation. Heparinized peripheral blood was obtained from healthy donors, and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density centrifugation. PBMC were separated into rosetted (E^+) and unrosetted (E^-) cells after rosetting with sheep erythrocytes (E) treated with 2-aminoethylisothiouronium bromide (Sigma, St. Louis, Mo.). T cells were obtained by treating E^+ cells with lysing buffer. E^- cells were utilized as B-cell sources (12).

Reagents. MOM was provided by Meiji (Tokyo, Japan), JM was provided by Yamanouchi (Tokyo, Japan), and CAM was provided by Dainabot (Tokyo, Japan). FK506 and cyclosporin A (Cs-A) were donated by Fujisawa Pharmaceutical Company (Osaka, Japan) and Sandoz (Basel, Switzerland), respectively. The drugs were dissolved in ethanol, further diluted in medium at appropriate concentrations, and added to the cultures. Phytohemagglutinin P (PHA), concanavalin A (ConA), and pokeweed mitogen (PWM) were obtained from Difco Laboratories (Detroit, Mich.) and Gibco Laboratories (Grand Island, N.Y.), respectively. Fluorescein isothiocyanate-conjugated anti-CD25 monoclonal antibody (IL-2R1) was purchased from Coulter Immunology (Hialeah, Fla.).

Measurement of lymphocyte proliferation. PBMC were cultured in triplicate in 96-well flat-bottomed microplates at 37°C in 5% CO_2. The culture medium consisted of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, streptomycin (100 μg/ml), penicillin (100 U/ml), and 2-mercaptoethanol (0.005 mM). PBMC (10^5 cells per 200 μl) were cultured in the presence or absence of stimulators for 3 days. The cells were pulsed with 1 μCi of [^{3}H]thymidine per well during the last 18 h of the cultures and were harvested with a multiple cell harvester.

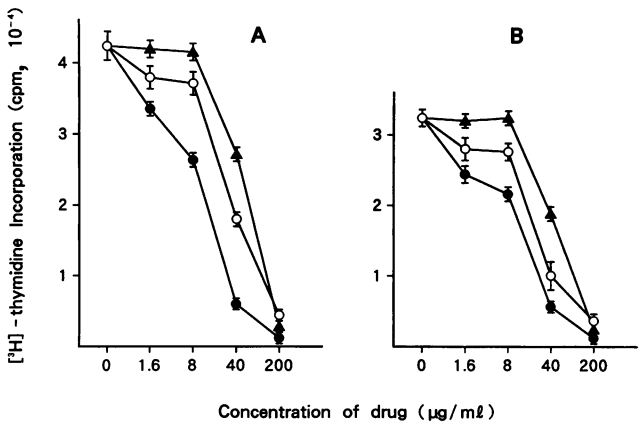

FIG. 1. PHA- and ConA-stimulated lymphocyte proliferation in the presence of macrolides. PBMC were cultured for 3 days with PHA (1:100 [vol/vol] [A]) or ConA (10 μg/ml [B]) and in the presence of various concentrations of MOM (○), JM (●), or CAM (▲). The proliferative response to the mitogens was measured by the incorporation of [^{3}H]thymidine over the last 18 h of cultures. The values represent the mean ± standard error (SE) of triplicate cultures.

Immunofluorescence. To detect cell surface antigen, cells cultured with or without PHA for 3 days were washed twice with medium and examined by direct immunofluorescence after being stained with fluorescein isothiocyanate-conjugated monoclonal antibody. Cell surface immunofluorescence was analyzed by flow cytometry.

IL-2 production. PBMC were seeded at a cell density of 10^6 cells per ml (1 ml per well) and cultured in 24-well plates in the presence of PWM (1:50 [vol/vol]) with or without macrolides for 48 h at 37°C in a 5% CO_2 incubator. The supernatants were recovered by centrifugation and stored at −20°C until assayed.

Assessment of IL-2 activity in culture supernatants. IL-2 concentration in culture supernatant was determined with commercial enzyme-linked immunosorbent assay (ELISA) kits (Serotec, Oxford, England). IL-2 content was calculated with a standard curve derived by linear dilution of the cytokine standards supplied with the respective kits. The minimal sensitivity of IL-2 determination in this assay is 67 pg/ml.

MLR. Allogeneic mixed lymphocyte reaction (MLR) was performed as described elsewhere (10). Briefly, responder T lymphocytes (10^5 E^+ cells per well) were cocultured with an equal number of mitomycin C-treated (50 μg/ml at 37°C for 30 min) stimulator B lymphocytes (10^5 E^- cells per well) in 200 μl of culture medium. The cells were incubated at 37°C in a 5% CO_2 incubator for 6 days. The cultures were pulsed with 1 μCi of [^{3}H]thymidine per well during the last 18 h and harvested with a multiple cell harvester.

Cell viability. PBMC (10^6/ml) were cultured in the presence or absence of the macrolides, and the cell viability after 3 days of culture was examined by trypan blue dye exclusion test.

RESULTS

Effect of macrolides on the proliferation of PBMC stimulated with PHA or ConA. At concentrations of 1.6 to 8 μg/ml, MOM and JM, but not CAM, inhibited cell proliferation induced by the polyclonal T-cell mitogens PHA and ConA (Fig. 1). At concentrations of 40 to 200 μg/ml, the three drugs inhibited cell proliferation markedly and in a concentration-dependent manner. JM, followed by MOM and CAM in that order, showed the most potent inhibitory effect.

TABLE 1. Kinetic studies of the effect of macrolides on PHA-induced DNA synthesis in PBMC[a]

Drug	Concn (μg/ml)	[^{3}H]thymidine incorporation (% inhibition) after time of treatment (h):		
		72	48	24
MOM	8	14.1 ± 0.5 (48)	19.1 ± 1.2 (30)	27.4 ± 2.0 (0)
	40	5.6 ± 0.3 (79)	14.9 ± 0.3 (45)	27.3 ± 1.8 (0)
JM	8	10.8 ± 0.4 (60)	18.5 ± 1.9 (32)	25.4 ± 0.8 (0)
	40	2.7 ± 0.2 (90)	12.2 ± 0.3 (55)	24.4 ± 0.9 (10)
CAM	8	25.1 ± 0.2 (8)	27.9 ± 0.9 (0)	27.8 ± 1.8 (0)
	40	16.5 ± 0.1 (39)	20.0 ± 0.8 (27)	27.3 ± 0.9 (0)

[a] PBMC were incubated in the presence of PHA (1:100 [vol/vol]). MOM, JM, and CAM were added at 0, 24 and 48 h, and all cultures were harvested at 72 h. The cultures were pulsed with [^{3}H]thymidine 18 h before harvesting. The control response in the absence or presence of PHA was 304 ± 21 cpm and 27,251 ± 274 cpm, respectively. The values represent mean (10^3 counts per minute) ± SE of triplicate cultures.

Kinetic study of the inhibitory activity of the macrolides. MOM, JM, and CAM (8 and 40 μg/ml) were added at different time intervals after the incubation of PBMC with PHA in order to examine when the effect of the macrolides occurs during the 3-day culture period. At both concentrations of 8 and 40 μg/ml, JM and MOM exerted a strong inhibitory effect when added at the initiation of 3-day culture, although the same concentrations of CAM showed weak inhibitory activity. The inhibitory effect was completely abrogated when the drug was added at the last 24 h of the 3-day culture period (Table 1). These results indicate that these drugs were most effective when added at the initiation of the 3-day culture interval. These data also imply that the inhibitory effect on T-cell proliferation of CAM (a 14-atom lactone ring) was less powerful than that of MOM and JM (a 16-atom ring).

Effect of preincubation of PBMC with the macrolides. We examined the effect of preincubation of PBMC with the drugs on the proliferative response to PHA stimulation. We incubated PBMC for 24 h in the presence or absence of the drugs (200 μg/ml). PBMC were then thoroughly washed, cell density was adjusted to 10^6/ml, and the PBMC were further cultured for 3 days in the presence or absence of PHA. Preincubation of PBMC with the drugs did not affect subsequent stimulation with PHA (data not shown).

Effect of macrolides on IL-2 production. Grading doses of the macrolides were added from the beginning together with PWM and were then cultured for 2 days. At the end of culture, the supernatants were collected and tested for IL-2 activity as described in Materials and Methods. As shown in Table 2, at concentrations of 1.6 to 40 μg/ml, the IL-2 contents in the culture supernatants were reduced concentration dependently. The reducing effect was most potent in JM, followed by MOM and CAM in that order. Thus, the potency of the inhibitory effect on IL-2 production was identical to that of the effect on T-cell proliferative responses. These results suggest that macrolides suppress the proliferation of human T cells by inhibiting their IL-2 productive capacity.

Effect of macrolides on IL-2 receptor (CD25) expression. We examined the effect of the drugs on IL-2 receptor (CD25) expression in PBMC stimulated with PHA for 3 days. As shown in Table 3, PHA-stimulated PBMC showed a marked increase in CD25-positive cells. The drugs, JM, MOM, and CAM, did not decrease the CD25 antigen-positive cells, although they slightly reduced the fluorescence intensity of these cells.

Suppression of MLR by macrolides. As shown in Table 4, allogeneic MLR was inhibited in the presence of MOM, JM,

TABLE 2. Effect of macrolides on PWM-induced IL-2 production by PBMC[a]

Drug	Concn (μg/ml)	IL-2 content (pg/ml)	Inhibition (%)
None		791	
MOM	1.6	610	23
	8	428	46
	40	195	75
JM	1.6	545	31
	8	141	82
	40	ND	100
CAM	1.6	693	12
	8	576	27
	40	348	56

[a] Human PBMC were cultured at a cell density of 10^6 cells per ml in 24-well microplates with PWM (1:50 [vol/vol]) in the presence or absence of MOM, JM, or CAM for 48 h. The supernatant was recovered after centrifugation and assayed for IL-2 content. The IL-2 content was detected by using commercial ELISA kits; concentrations were calculated with a standard curve derived by linear dilution of the cytokine standards supplied with the respective kits. ND, less than minimal sensitivity (67 pg/ml) of the IL-2 contents. Representative data from two separate experiments are shown.

and CAM in a concentration-dependent manner at concentrations of 1.6 to 40 μg/ml. The inhibition ranged from about 30% when 1.6 μg of the drugs per ml was added to 90% when the concentration of the antibiotics was 40 μg/ml.

Effect of macrolides on human lymphocyte viability. It was examined whether these macrolides have cytotoxic activities for PBMC. Human PBMC were cultured for 3 days in the presence or absence of macrolides at concentrations of 1.6 to 200 μg/ml. The viability of PBMC determined by trypan blue dye exclusion was (91 ± 1.2)% in the absence of macrolides and (93 ± 1.2), (93 ± 4.0), (96 ± 1.6), and (89 ± 3.2)% in the presence of 1.6, 8, 40, and 200 μg of each macrolide per ml, respectively. Thus, the inhibition of the proliferative response by these drugs was not induced by the cytotoxic effect.

Effect of macrolides in combination with FK506 or Cs-A on T-cell proliferation. PBMC were cultured with PHA for 3 days in the presence of FK506 (0.0001 and 0.001 μg/ml) or Cs-A (0.01 and 0.1 μg/ml) to examine the effect of macrolides in combination with the immunosuppressants. Each macrolide by itself suppressed the proliferative response of PHA-stimulated PBMC in such a manner as shown in Fig. 1. Although low doses of FK506 (0.0001 μg/ml; Fig. 2, b) or Cs-A (0.01 μg/ml; Fig. 2, a) alone evidently suppressed the proliferation of PBMC, coexistence of the immunosuppressants and the macrolides enhanced the suppressive effect in a concentration-dependent fashion (Fig. 2). The combinations of the lower concentration of JM or MOM (1.6 μg/ml) or of CAM (8 μg/ml) with FK506 (0.0001 μg/ml) or Cs-A (0.01 μg/ml) demonstrated a marked suppression of T-cell proliferation (Fig. 2). None of the drugs either alone or in combination affected the viability of PBMC under these experimental conditions. These results indicate that macrolides enhance the effect of two known immunosuppressants on human T-cell proliferation.

TABLE 3. Effect of macrolides on IL-2 receptor (CD25) expression in PHA-stimulated PBMC[a]

Concn (μg/ml)	PHA alone	% Positive (mean channel) for PHA plus:		
		MOM	JM	CAM
0	65 (395)			
1.6		64 (391)	74 (422)	71 (462)
8		63 (337)	69 (375)	60 (351)
40		68 (319)	70 (371)	60 (307)

[a] PBMC were stimulated with PHA (1:100) in the presence or absence of various concentrations of macrolides for 3 days. Thereafter, the cells were washed with medium and were stained with fluorescein isothiocyanate-conjugated anti-CD25 monoclonal antibody. PBMC cultured in the absence of PHA for 3 days showed 12% CD25-positive cells (mean channel, 162). Representative data from two separate experiments are shown.

TABLE 4. Effect of macrolides in MLR[a]

Drug	Concn (μg/ml)	[^{3}H]thymidine incorporation (10^3 cpm ± SE)	Inhibition (%)
None		15.3 ± 0.4	
MOM	1.6	11.3 ± 0.9	26
	8	9.4 ± 0.4	39
	40	1.7 ± 0.1	89
JM	1.6	9.7 ± 0.2	37
	8	6.0 ± 0.2	61
	40	1.7 ± 0.1	89
CAM	1.6	9.8 ± 0.8	36
	8	5.8 ± 0.2	62
	40	1.9 ± 0.7	88

[a] Mitomycin C-treated E^- cells (10^5 cells) were used as stimulator cells. They were cultured with 10^5 responding T cells in 96-well microtest plates for 6 days (allogeneic MLR). Graded doses of drugs were added at the initiation of the cultures. Proliferation was measured with a pulse of 1 μCi of [^{3}H]thymidine at 20 h before harvesting. The results are expressed as mean ± SE of triplicate cultures.

DISCUSSION

The data obtained from the present experiments showed that three macrolide antibiotics, MOM, JM, and CAM, clearly inhibited the proliferation of human lymphocytes stimulated with polyclonal T-cell mitogens. The inhibitory effect on T-cell proliferation of the drugs with a 16-atom lactone ring (JM, MOM) appeared to be more potent than that of the drug with a 14-atom lactone ring (CAM). However, the statistical evaluation of the difference in the inhibitory effect between 14- and 16-membered-ring macrolides is impossible, because of the limited number of macrolides tested in the present study. Kinetic study of their effects on T-cell proliferation indicates that the macrolides act within 24 h of the activation stimulus. If the drugs are given more than 48 h after the stimulation,

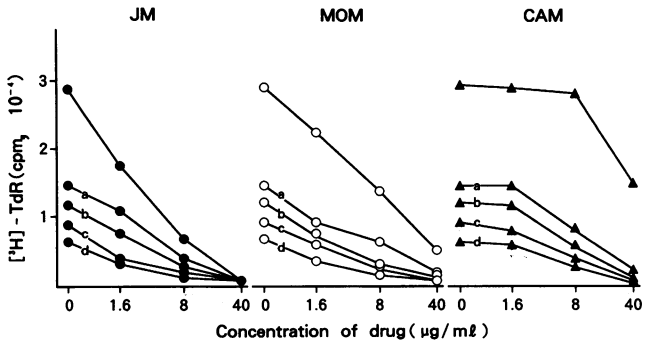

FIG. 2. Effect of the macrolides with the immunosuppressive agents on [^{3}H]thymidine incorporation in PHA-stimulated T cells. PBMC were cultured for 3 days with PHA, PHA and FK506 (0.0001 μg/ml, b; 0.001 μg/ml, c), or PHA and Cs-A (0.01 μg/ml, a; 0.1 μg/ml, d) and in the presence of MOM, JM, or CAM.

there is little effect on T-cell proliferation. Thus, these drugs might be working on an early event in T-cell activation. As pretreatment of lymphocytes with the drugs did not interfere with the subsequent proliferation of mitogen-stimulated T cells, the coexistence of the drugs at the initiation of T-cell stimulation seems to be necessary to inhibit the T-cell proliferation.

A series of events, the production of IL-2 and the induction of IL-2 receptor and then subsequent binding of IL-2 to IL-2 receptor, is pivotal in T-cell activation. FK506 and Cs-A inhibit the transcription of early T-cell activation genes such as the lymphokine gene of IL-2 and result in the suppression of IL-2 production (19). Whereas rapamycin has no effect on the production of IL-2, it potently inhibits the response of the T cell to IL-2 (19). We examined the effect of the drugs on the production of IL-2 and the expression of IL-2 receptor (CD25). The results demonstrated that the macrolides inhibited the production of IL-2 by mitogen-stimulated T lymphocytes. The order of the inhibitory potency in IL-2 production is identical to that observed in T-cell proliferation. These data indicate that the immunosuppressive activity of the macrolides may be based, at least in part, on their ability to inhibit production of IL-2 by T lymphocytes. Interestingly, these drugs scarcely interfered with the expression of IL-2 receptor (CD25). Thus, the mode of action of the macrolides may be similar to those of FK506 and Cs-A.

The macrolides also suppressed the MLR in a dose-dependent manner. The suppressive activity for MLR correlated with that for the cell proliferation in JM and MOM, but CAM demonstrated more powerful action for MLR than for the cell proliferative response (Table 4). The reason why CAM has more potent action in MLR is not known at present, but CAM might act on T cells in a way different from that of MOM or JM in MLR. CAM has been reported to suppress IL-1 production (22). As MLR used here is generally regarded as an in vitro indicator of allograft rejection (8), this finding also indicates the immunosuppressive property of these macrolide antibiotics.

Combined treatment with these macrolides and known immunosuppressants such as FK506 and Cs-A enhanced the suppressive effect of either drug on T-cell proliferation. As the macrolides potentiate the inhibitory activity of the immunosuppressants when used in combination, it is suggested that the macrolides and immunosuppressants such as FK506 and Cs-A do not share a receptor site on mediating the action. Studies on the mechanism by which Cs-A and FK506 block IL-2 gene activation in T cells suggest that these immunosuppressive agents bind to cytoplasmic receptors termed immunophilin (19, 20). The immunophilin-drug complex, and not the drug alone, is the agent responsible for the immunosuppressive action of the drugs. Although the direct biological target for the action of this complex remains unclear, recent study suggests that protein phosphatase or calcineurin is a possible candidate (20). However, the mechanisms of action of many recently discovered immunosuppressive agents remain to be defined.

Is the immunosuppressive effect of these macrolides of clinical relevance? According to the pharmacokinetic study, concentrations of CAM, MOM, and JM in serum peak at 2.42, 2.38, and 2.86 μg/ml 1 h after oral administration of 400 mg of CAM per ml (21), 600 mg of MOM per ml (6), and 1,000 mg of JM per ml (14), respectively. On the basis of those data, CAM is the highest and is followed by MOM and JM in that order in the level per milligram of drug in plasma. Levels of the macrolides in tissue are higher than those achieved in the plasma in experimental animals (6, 14, 21), indicating a good diffusion of the macrolides from plasma to tissue. Thus, the macrolides can penetrate and accumulate at high concentrations in tissues and cells. As a matter of fact, T lymphocytes may very well find themselves exposed to a relatively high concentration of drug at a specific location.

Though the mechanism of action of macrolides in chronic inflammatory respiratory tract infections has not been elucidated, the immunomodulatory effect of macrolides presented here may combine with their efficacy in affecting infectious status. Because the immunological response can lead to the injury of host cells and tissues during persistent infections, besides protecting the host against microbial infections (3), the immunomodulatory therapy may aid in the improvement of the status.

The immunosuppressive effect of some antibiotics may have clinical relevance for modulation of the immune response in transplant patients, immunocompromised patients, and patients with inflammatory diseases. Therefore, the in vivo effects of antibiotics on human lymphocyte function should be further evaluated in animal experiments and clinical studies.

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