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Research Paper

Azithromycin suppresses proliferation, interleukin production and mitogen-activated protein kinases in human peripheral-blood mononuclear cells stimulated with bacterial superantigen

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Abstract

Objectives Macrolide antibiotics are used for the treatment of immunological disorders such as psoriasis. However, few studies have investigated the immunoregulatory efficacy of macrolides in bacterial superantigen-stimulated immune cells.

Methods The suppressive efficacies of azithromycin, clarithromycin, roxithromycin and prednisolone were evaluated *in vitro* against the concanavalin A- or toxic shock syndrome toxin 1 (TSST-1)-induced proliferation of peripheral-blood mononuclear cells (PBMCs) obtained from nine healthy subjects. The concentrations of six cytokines in a PBMC-culture medium were measured using bead-array procedures followed by flow cytometry. Cellular c-jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) activity were measured using cell-based ELISA procedures.

Key findings Azithromycin, clarithromycin and roxithromycin inhibited the proliferation of both the concanavalin A- and superantigen-stimulated PBMCs dose-dependently. The effect of azithromycin was the strongest, with IC50 values of less than 5 μg/ml. Furthermore, the suppressive efficacy of prednisolone against concanavalin A- or TSST-1-stimulated PBMCs was significantly promoted in combination with 5 μg/ml azithromycin ($P < 0.002$). The concentrations of TNF-α, interleukin (IL)-2, –4, –5 and –10 in the supernatant of concanavalin A- or TSST-1-stimulated PBMCs cultured for 72 h decreased by 65–98% in the presence of 5 μg/ml azithromycin. The stimulation of PBMCs with concanavalin A or TSST-1 increased cellular JNK and ERK activity, and 5 μg/ml azithromycin significantly attenuated the increased activity of JNK in the TSST-1-stimulated cells and ERK in the concanavalin A- and TSST-1-stimulated PBMCs, respectively ($P < 0.05$).

Conclusions Azithromycin suppresses mitogen- or superantigen-induced proliferation of PBMCs by possibly inhibiting both cellular JNK and ERK activity.

Keywords azithromycin; cytokines; ERK activity; JNK activity; peripheral blood mononuclear cells

Introduction

Bacterial infection may influence not only pathogenesis but also the clinical responses of patients to immunosuppressive drugs in treating autoimmune diseases.[1–3] The modification of the efficacy of treatment with immunosuppressive drugs by bacteria, including *Staphylococcus aureus* and hemolytic streptococci, can occur in autoimmune diseases. Moreover, microbial superantigens have been reported to induce glucocorticoid insensitivity in human PBMCs *in vitro*.[2–4]

Macrolide antibiotics are effective for the treatment of patients infected with several pathogenic microorganisms, but macrolides also possesses anti-inflammatory[5] and immunomodulatory[6,7] efficacies. Indeed, in psoriasis, the oral administration of roxithromycin has been reported to be effective in clinical practice.[8,9] A combination therapy of the antibiotic with topical administration of glucocorticoids appears to be especially beneficial

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when these agents exhibit differing mechanisms of action that provide additive or synergistic efficacy, often reducing the required doses of the individual agents in comparison to monotherapy and potentially limiting side effects.[10] However, few studies have investigated the immunoregulatory efficacy of macrolide antibiotics, either alone or in combination with glucocorticoids, in bacterial superantigen-stimulated immune cells.

We have previously raised the possibility that the macrolide antibiotic roxithromycin may suppress the proliferative response and cytokine production in human PBMCs stimulated with streptococcal pyrogenic enterotoxin A produced from hemolytic streptococci.[11] The roxithromycin effect appeared to result from the suppression of c-jun N-terminal kinase (JNK) activity in PBMCs stimulated with the superantigen.[11] From these observations, we suggested that the alternative or concomitant use of roxithromycin in combination with glucocorticoid may be an effective way to overcome decreased glucocorticoid response in patients having a risk of hemolytic streptococci infection.

The present study compared the suppressive efficacies of the macrolide antibiotics azithromycin, clarithromycin and roxithromycin and evaluated *in vitro* the efficacy of a combination of the drug and the synthetic glucocorticoid prednisolone against the proliferation of human PBMCs stimulated with a T cell mitogen, concanavalin A, or the bacterial superantigen, TSST-1. The effects of azithromycin on the expression of several cytokines and cellular mitogen-activated protein kinase activities in PBMCs were also examined to elucidate the mechanism(s) of the antiproliferative efficacy of the antibiotic. The results suggested that azithromycin suppresses mitogen- or superantigen-induced proliferation of PBMCs by possibly inhibiting cellular JNK and extracellular signal-regulated kinase (ERK) activities.

Materials and Methods

Subjects

The present study was approved by the Ethical Committee of Tokyo University of Pharmacy and Life Sciences and written informed consent was obtained from all healthy volunteers included in the study. The study included nine healthy subjects (four male and five female; 25.9 ± 8.5 years of age). These subjects had neither a history of immunological disorders nor a history of taking immunosuppressive drugs. All of the subjects were non-smokers.

Isolation of PBMCs and evaluation of drug effects *in vitro*

Twenty milliliters of venous blood were taken from the healthy subjects between 9:30 and 11:00 in the morning and the samples were heparinized. This 20 ml sample size was the smallest possible to carry out the drug sensitivity tests for the three to four immunomodulating agents, but occasionally not all the agents could be tested in each subject. The heparinized blood was loaded on 3 ml of Ficoll-Hypaque (Nakarai Co., Japan), centrifuged at 1300*g* for 20 min and the PBMCs were separated as described previously.[12–15] For the evaluation of PBMC sensitivity to immunosuppressive drugs, the cells were washed and suspended in RPMI 1640 medium containing 10% foetal bovine serum, 100 000 IU/l penicillin and 100 mg/l streptomycin to a final density of 1×10^6 cells/ml. Concanavalin A or TSST-1 was added to each well to a final concentration of 5.0 μg/ml or 100 ng/ml, respectively. Subsequently, 4 μl of an ethanol solution containing antibiotics or prednisolone was added to give final agent concentrations of 5–40 μg/ml for the antibiotics and 0.1–10 000 ng/ml for prednisolone, respectively. Four microliters of ethanol were added to the control wells. The plate was incubated for 96 h in 5% CO_2/air at 37°C. The cells were pulsed with 18.5 KBq/well of [^{3}H]-thymidine for the last 16 h of incubation, collected on a glass-fibre filter paper using a multiharvester device and then dried. The radioactivity retained on the filter was further processed for liquid scintillation counting. The mean of the counts for a triplicate of each sample was determined. The PBMC-stimulation index was calculated from the formula:

[^{3}H]-thymidine incorporated in presence of stimulant (dpm)/[^{3}H]-thymidine incorporated in absence of stimulant (dpm)

The IC50 values were determined from a linear regression of at least four points at different concentrations of the drugs.

Cytokine analysis

PBMCs were incubated for 24 and 72 h in the presence of concanavalin A or TSST-1 and the drugs as described above, and the culture supernatant was stored at −80°C until the cytokine concentrations were measured. The concentrations of TNF-α, interferon-γ, IL-2, −4, −5 and −10 in the supernatant of the culture medium were measured with a bead-array procedure followed by flow cytometry according to the instructions of BD Biosciences (San Jose, CA, USA).[11,16]

MAPK assays

PBMCs were seeded into 96-well plates precoated with 10 μg/ml poly-L-lysine to a cell density of 1×10^6 cells/ml and the cells were pre-incubated with or without concanavalin A or TSST-1 for 48 h. The phosphorylation of the mitogen-activated protein kinase (MAPK) families JNK and ERK was measured using a cell-based ELISA kit (SuperArray Inc., Bethesda, MD).[11,16] In brief, after stimulation of the cells, the cells were fixed with 37% formaldehyde in phosphate-buffered saline for 20 min at room temperature and washed with a washing buffer. Endogenous peroxidase was quenched with 30% H_2O_2 and 10% NaN_3 in a washing buffer for 20 min. After washing, the cells were blocked with a blocking buffer for 1 h at room temperature and then were incubated overnight with primary antibodies for phospho- and pan-protein-specific-JNK or ERK (diluted to 1 : 150 and 1 : 200, respectively) at 4°C. Next, the cells were washed and incubated with secondary antibodies diluted to 1 : 160 for 1 h at room temperature. The cells were then washed three times with a washing buffer and twice with phosphate-buffered saline. Subsequently, the cells were incubated with 100 μl of a developing solution for 20 min at room temperature in the dark. The reaction was stopped with 100 μl of a stop solution and the absorbance was measured at 450 nm using an optical

reference wavelength of 655 nm with an ELISA reader. The phospho-JNK or ERK level was corrected by absorbance at 595 nm from the cell staining.

Materials

The RPMI 1640 medium and foetal calf serum were purchased from Gibco Co., USA. Concanavalin A was obtained from Seikagaku Kogyo Co., Japan. Azithromycin, roxithromycin, prednisolone and TSST-1 were obtained from Sigma Chemical Co., USA. Clarithromycin was from Wako Chemicals, Co., Japan. [^{3}H]-thymidine (5.55×10^{11} Bq/mmol) was from New England Nuclear Corporation, USA. BD™ cytometric bead array human Th1/Th2 cytokine kits were obtained from BD Biosciences, USA. All other reagents were of the best available grade.

Statistics

For a comparison of the IC50 values between the PBMCs proliferated in the presence of prednisolone alone and those proliferated in the presence of prednisolone plus azithromycin, the data were first checked with a variance analysis. Subsequently, the data were analysed with Wilcoxon's tests. Comparison of the data in multiple groups was carried out by Kruskal–Wallis tests. These analyses were performed with Graph Pad Prism software v4.0. In each case, two-sided P values less than 0.05 were considered to be significant.

Results

Macrolide effects on concanavalin A- and TSST-1-stimulated PBMCs

The effects of azithromycin, clarithromycin and roxithromycin on the in-vitro proliferation of PBMCs obtained from the healthy subjects were examined (Figure 1). These macrolides suppressed PBMC-proliferations stimulated with either concanavalin A (5 μg/ml) or TSST-1 (100 ng/ml) dose-dependently as shown in Figure 1. The suppressive efficacy of azithromycin was the strongest, with IC50 values against the proliferation of both concanavalin A- and TSST-1-stimulated PBMCs of less than 5 μg/ml. These macrolides had little or no cytotoxic effect on the survival of PBMCs stimulated with concanavalin A or the cells stimulated with TSST-1, even at a high concentration (40 μg/ml), as examined by trypan blue exclusion test (data not shown).

Glucocorticoids are frequently applied topically in combination with oral macrolides for the treatment of psoriasis and therefore the effect of azithromycin on the IC50 values of prednisolone was examined against the proliferation of the PBMCs. Prednisolone inhibited the concanavalin A- or TSST-stimulated proliferation of PBMCs dose-dependently, but the effects on TSST-1 stimulated PBMC proliferation were apparently less than those for concanavalin A-stimulated proliferation (Figure 2). However, the suppressive efficacy of prednisolone as estimated by the IC50 values of the steroid against either the concanavalin A- or TSST-1-stimulated proliferation of PBMCs was significantly promoted in combination with 5 μg/ml azithromycin ($P < 0.002$; Table 1).

The proliferative response of the PBMCs as estimated by the stimulation indices, which calculate the ratio of cell proliferation in the presence of the stimulant and in the absence of the stimulant, was not significantly different between the concanavalin A- and TSST-1-stimulated cells (data not shown).

Effects of azithromycin on cytokine production from PBMCs

The concentrations of six cytokines, TNF-α, interferon-γ, IL-2, –4, –5 and –10 in the supernatant of concanavalin A- and TSST-1-stimulated PBMCs were measured with bead-array procedures 24 and 72 h after the cell culture (Tables 2 and 3). PBMCs stimulated with either concanavalin A or TSST-1 for 24 h produced most of these cytokines (Table 2), and the amounts produced increased at 72 h of culture (Table 3). Prednisolone at 100 ng/ml suppressed the production of most of these cytokines, except interferon-γ. Azithromycin also suppressed the production of these cytokines, except interferon-γ. The concentrations of TNF-α, IL-2, –4, –5 and –10 in the supernatant of the concanavalin A- or TSST-1-stimulated PBMCs cultured for 24 h decreased by 32–98% (Table 2), and those in the supernatant of the PBMCs

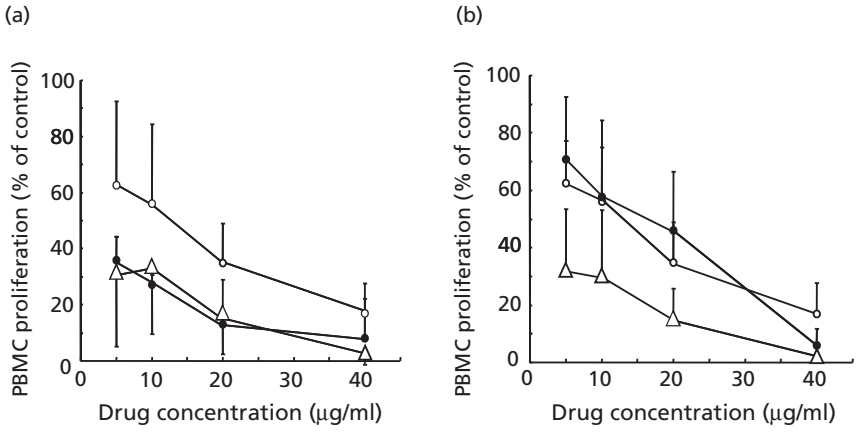

Figure 1 Dose–response curves of azithromycin (triangle), clarithromycin (closed circle) and roxithromycin (open circle) against the proliferation of PBMCs stimulated with concanavalin A (a) or TSST-1 (b). The data are the mean ± SD of nine healthy subjects.

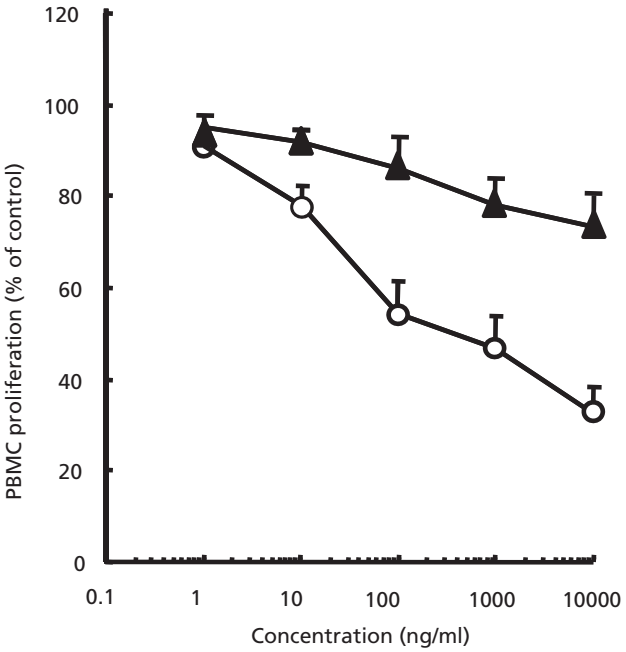

Figure 2 Dose–response curves of a synthetic glucocorticoid, prednisolone, against the proliferation of PBMCs stimulated with concanavalin A (open circle) or TSST-1 (closed triangle). The data are the mean ± SD of six healthy subjects.

cultured for 72 h decreased by 65–98% (Table 3) in the presence of 5 μg/ml azithromycin.

Effects of azithromycin on JNK and ERK activity of PBMCs

The effects of azithromycin on the activities of cellular JNK and ERK in PBMCs stimulated with concanavalin A or TSST-1 were examined (Figures 3 and 4). JNK and ERK activities in both concanavalin A- or TSST-1-stimulated PBMCs increased (Figures 3 and 4). Azithromycin at a concentration of 5 μg/ml did not suppress JNK activity significantly in the concanavalin A-stimulated PBMCs, but the antibiotic at 5 μg/ml significantly suppressed JNK activity in the TSST-1-stimulated PBMCs ($P < 0.05$; Figure 3). Azithromycin at a concentration of 5 μg/ml suppressed ERK activities significantly in both the concanavalin A- and TSST-1-stimulated PBMCs (Figure 4).

Discussion

The results above suggest that azithromycin suppresses the proliferation of PBMCs stimulated with the T cell mitogen concanavalin A or the bacterial superantigen TSST-1. These data also suggest that azithromycin suppresses the production of several cytokines, including TNF-α, IL-2, 4, 5 and 10, from the PBMCs by inhibiting the activity of the cellular MAPK families JNK and ERK. Activation of MAPK and production

Table 1 Comparison of the IC50 values of prednisolone against the proliferation of PBMCs stimulated with concanavalin A or TSST-1 between PBMCs treated in the absence and presence of 5 μg/ml azithromycin

Treatment	IC50 values of prednisolone (ng/ml) in PBMCs stimulation with:			
	Concanavalin A ($n = 9$)		TSST-1 ($n = 9$)	
	Median (range)	Mean (SD)	Median (range)	Mean (SD)
Prednisolone	53.8 (6.0–212.5)*	66.7 (62.7)	100.7 (19.4–10000)*	1244.3 (3286.0)
Prednisolone + Azithromycin	14.3 (0.1–82.3)*	14.0 (16.0)	1.0 (1.0–1763.0)*	202.7 (585.2)

*$P < 0.002$ between PBMCs treated in the absence and presence of azithromycin by Wilcoxon's tests.

Table 2 Effects of azithromycin on the production of TNF-α, interferon-γ, IL-2, –4, –5 and –10 in culture supernatant of PBMCs stimulated with concanavalin A or TSST-1 for 24 h

Treatment	Mean (SD) of cytokines produced (pg/ml)*					
	TNF-α	INF-γ	IL-2	IL-4	IL-5	IL-10
Concanavalin A						
Control	319.8 (113.5)	29.7 (5.5)	25.3 (17.7)	24.8 (5.0)	12.7 (16.0)	25.1 (11.2)
Prednisolone	12.3 (15.8)	6.0 (1.8)	4.4 (5.0)	7.3 (2.8)	1.2 (1.5)	11.6 (7.7)
Azithromycin	4.1 (12.1)	23.5 (10.3)	17.2 (23.4)	6.2 (1.4)	1.0 (1.2)	0.3 (6.1)
TSST-1						
Control	262.8 (90.0)	14.4 (4.8)	2312.5 (123.6)	20.6 (5.7)	4.3 (5.2)	13.8 (9.3)
Prednisolone	33.5 (28.4)	11.2 (6.6)	362.4 (182.0)	8.4 (2.1)	1.3 (1.5)	8.4 (10.1)
Azithromycin	14.2 (12.8)	76.7 (39.7)	194.3 (93.2)	6.4 (1.2)	1.1 (1.3)	2.0 (6.9)

*Values are expressed as the mean (SD) of the data obtained from four different healthy subjects.

Table 3 Effects of azithromycin on the production of TNF-α, interferon-γ, IL-2, –4, –5 and –10 in culture supernatant of PBMCs stimulated with concanavalin A or TSST-1 for 72 h

Treatment	Mean (SD) of cytokines produced (pg/ml)*					
	TNF-α	INF-γ	IL-2	IL-4	IL-5	IL-10
Concanavalin A						
Control	>5000	>5000	331.3	123.7	843.4	2599.4
	(0)	(0)	(105.1)	(2.4)	(851.8)	(3394.9)
Prednisolone	82.1	1909.2	95.7	20.1	20.1	158.9
	(35.5)	(1054.5)	(57.3)	(7.0)	(7.1)	(78.5)
Azithromycin	69.0	1209.4	92.2	21.1	20.7	72.4
	(49.3)	(383.1)	(59.4)	(1.9)	(32.6)	(33.1)
TSST-1						
Control	2147.7	>5000	2312.5	98.9	350.2	600.0
	(633.4)	(0)	(123.6)	(3.1)	(276.2)	(204.4)
Prednisolone	662.6	2656.9	362.4	60.2	45.8	685.9
	(511.1)	(2705.6)	(182.0)	(19.5)	(82.9)	(145.9)
Azithromycin	149.0	2829.6	194.3	35.0	10.0	188.5
	(42.5)	(990.2)	(93.2)	(11.2)	(17.2)	(80.3)

*Values are expressed as the mean (SD) of the data obtained from four different healthy subjects.

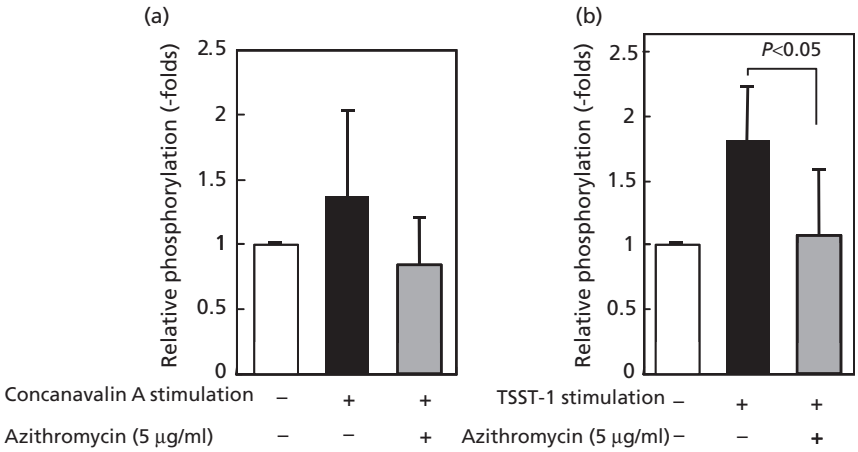

Figure 3 Effects of azithromycin on the JNK activity of PBMCs stimulated with concanavalin A (a) or TSST-1 (b). Treatments are indicated under each figure. Data are expressed as the mean ± SD of phosphorylated-protein amounts (multiples of control) in PBMCs obtained from four healthy subjects. In (b), a statistically significant difference between the two treatment groups was observed by the Kruskal–Wallis tests ($P < 0.05$).

of several cytokines by mitogen- or superantigen-stimulation might precede proliferation of PBMCs.[11,16] We analysed the cytokine levels and the MAPK activities at 24–72 h after culture, followed by measurement of PBMC-proliferation at 96 h after culture. Since the in-vitro effects of immunosuppressive drugs against PBMC-proliferation significantly correlate with the clinical efficacy of the drugs in immunological disorders,[12,14–16] the present observations suggest that azithromycin is effective in suppressing anomalistic immunity in patients having *Streptococcus aureus* infection.

The dye exclusion test suggested that azithromycin is not cytotoxic against human PBMCs in the present study. However, it has been reported that macrolide antibiotics induce apoptosis in human lymphocytes.[17,18] Mizunoe *et al*. concluded that the macrolide antibiotics clarithromycin and azithromycin induce apoptosis of activated lymphocytes via down-regulation of Bcl-xL.[19] Thus, apoptosis-inducing mechanism(s) have also been suggested as being implicated in the suppressive efficacies of macrolides against activated human lymphocytes.

In addition to their antibiotic properties, macrolide antibiotics are also known to have anti-inflammatory efficacy.[5] According to these observations, the immunomodulatory efficacy of macrolides has been clinically implicated in diffuse panbronchiolitis,[20] chronic bronchitis, bronchial asthma[5,6] and psoriasis.[8,21] This efficacy is suggested to result from the suppression of the production of several cytokines, inhibition of leukocyte migration, antioxidative effects and the suppression of cell adhesion.[5,7] A pharmacokinetic review of a macrolide antibiotic, roxithromycin, suggests that it has a high bioavailability and tissue penetration after oral administration.[22,23] The blood concentration of roxithromycin has been reported to reach as high as 10 μg/ml after multiple dosing.[23,24] The inhibitory effects of azithromycin against the

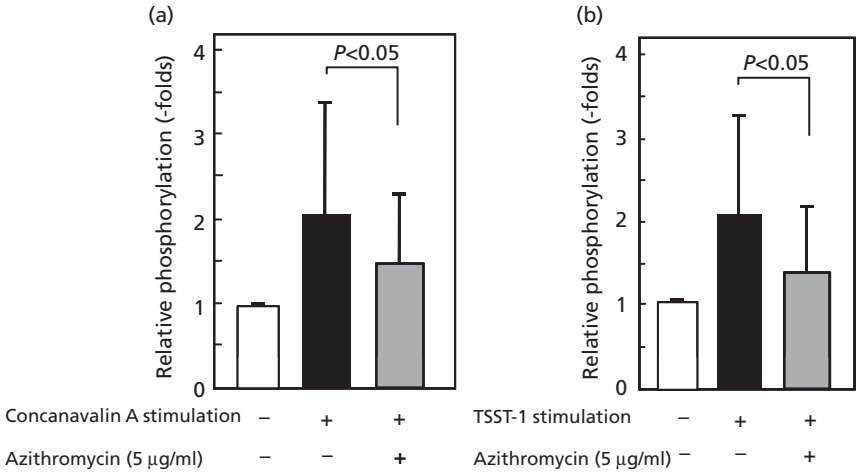

Figure 4 Effects of azithromycin on the ERK activity of PBMCs stimulated with concanavalin A (a) or TSST-1 (b). Treatments are indicated under each figure. Data are expressed as the mean ± SD of phosphorylated-protein amounts (multiples of control) in PBMCs obtained from four healthy subjects. Statistically significant differences between the two treatment groups were observed by the Kruskal–Wallis tests ($P < 0.05$).

proliferation of the TSST-1-stimulated PBMCs were observed at 5 μg/ml in the present study. The achievement of a concentration of 5 μg/ml or more would be quite possible in polymorphonuclear leukocytes or tonsils after oral administration of macrolide antibiotics, since the preferable entry and/or disposition of the antibiotic to these cells or tissues has been demonstrated.[25,26]

After binding to glucocorticoid receptor α in cytoplasm and entering the nucleus as a glucocorticoid–receptor complex, glucocorticoids suppress the function of nuclear transcription factors, such as activated protein-1 and nuclear factor (NF)-κB (transrepression), and subsequent transcription of several inflammatory cytokines.[13] In contrast, the glucocorticoid-mediating pathway(s) (transrepression), which appear to operate independently from the calcineurin/NFAT pathway, are thought to be interrupted by stimulation of PBMCs with superantigens.[27] TSST-1, a superantigen derived from *Streptococcus aureus*, is known to bind directly to MHC class II molecules on antigen-presenting cells without antigen processing and can stimulate T cells potently via selected T-cell receptor β variable region elements.[28] Superantigens are reported to induce glucocorticoid resistance of human T cells through the activation of the mitogen-activated kinase/ERK (MEK-ERK) pathway.[29] Superantigens are also suggested to activate NFAT and subsequent IL-2 production.[30] ERK activation is possibly associated with NFAT function and IL-2 production in a late phase of T cell activation.[31] According to these observations, therefore, at least any one of these signal-transduction pathway(s) appears to be activated by stimulation with TSST-1, which results in the attenuation of the suppressive efficacy of prednisolone. The present data show that the suppressive efficacy of prednisolone against the proliferation of the TSST-1-stimulated PBMCs was markedly improved in combination with 5 μg/ml azithromycin. Azithromycin is therefore suggested to suppress cellular JNK and ERK activities and enhance the *trans*-repression action of prednisolone, which might be interrupted by stimulation of PBMCs with the superantigen. Although we did not examine the effects of azithromycin alone without stimulation on the phosphorylation in the present study, the data for the effects of azithromycin on the MAPK activity in mitogen- or superantigen-stimulated PBMCs is the first report to the best of our knowledge.

In summary, the present study raises the possibility that azithromycin suppresses the proliferative response and cytokine production in PBMCs stimulated with TSST-1 induced from *Streptococcus aureus* or those stimulated with a T cell mitogen, concanavalin A. The azithromycin effect appears to result from the suppression of JNK and ERK activities in PBMCs stimulated with the mitogen or superantigen. The alternative or concomitant use of azithromycin in combination with glucocorticoid is thus considered to be an effective way to overcome decreased glucocorticoid response in patients having a risk of *Streptococcus aureus* infection.

Conclusion

The suppressive efficacies of azithromycin, clarithromycin and roxithromycin were evaluated *in vitro* against the concanavalin A- or TSST-1-induced proliferation of human PBMCs. Azithromycin, clarithromycin and roxithromycin inhibited the proliferation of both the concanavalin A- and superantigen-stimulated PBMCs dose-dependently. The effect of azithromycin was the strongest, with IC50 values of less than 5 μg/ml. The suppressive efficacy of prednisolone against the concanavalin A- or TSST-1-stimulated PBMCs was significantly promoted in combination with azithromycin. The concentrations of TNF-α, IL-2, –4, –5 and –10 in the supernatant of the concanavalin A- or TSST-1-stimulated PBMCs decreased by 65–98% in the presence of 5 μg/ml azithromycin. Azithromycin attenuated the increased activity of JNK in the TSST-1-stimulated cells and ERK in the concanavalin A- and TSST-1-stimulated PBMCs. These results suggest that azithromycin, either alone or in combination with glucocorticoid, suppresses mitogen- or superantigen-induced proliferation of T lymphocytes by inhibiting both cellular JNK and ERK activities.

Declarations

Conflict of interest

The Authors declare that there are no conflicts of interest.

Acknowledgements

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