Inflamm. res. 48 (1999) 461–468
1023-3830/99/080461-08 $ 1.50+0.20/0

Inflammation Research

JTE-607, a novel inflammatory cytokine synthesis inhibitor without immunosuppression, protects from endotoxin shock in mice

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Received 15 February 1999; returned for revision 17 March 1999; accepted by M. J. Parnham 14 May 1999

Abstract. *Objective and Design:* We investigated the effect of a novel N-benzoyl-L-phenylalanine derivative compound (JTE-607) on production of various cytokines and other immune responses in vitro and on endotoxin shock in vivo. *Materials and Methods:* Human, monkey, rabbit, mouse and rat peripheral blood mononuclear cells (PBMCs), and human fibroblasts, umbilical vein endothelial cells (HUVEC), mesangial cells and T cells were used in vitro. Endotoxin shock was induced by lipopolysaccharide (LPS) in *Corynebacterium parvum (C. parvum)* sensitized male C57BL/6 mice in vivo. *Results:* JTE-607 inhibited inflammatory cytokine production, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8 and IL-10, from LPS-stimulated human PBMCs, with IC_{50} values of 11, 5.9, 8.8, 7.3 and 9.1 nM, respectively. The inhibitory effects of JTE-607 were also seen in mRNA expression of those cytokines. The potency of JTE-607 on cytokine production from PBMCs of other species, and from other human cells were much lower than that on human PBMCs. JTE-607 did not affect either LPS-stimulated microbead phagocytosis or reactive oxygen species production at 1 μM in human PBMCs but slightly suppressed expression of major histocompatibility complex class II antigen at 1 μM, although it was 100-fold less active than it was as a cytokine inhibitor. JTE-607 (0.3–10 mg/kg, i.v.) showed dose dependent inhibition of mortality after LPS challenge in *C. parvum* sensitized mice in accordance with a decrease of plasma TNF-α. *Conclusions:* These results suggest that JTE-607 is a multiple cytokine inhibitor specific for human PBMCs. This compound may be useful for the treatment of various cytokine mediated diseases such as septic shock without causing immunosuppression.

Key words: Cytokine inhibitor – JTE-607 – Anti-inflammatory drug – Immunosuppression

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Introduction

The cytokines, which include tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, IL-10 and others, constitute complicated networks with one another. These cytokines are referred to as inflammatory cytokines since individually they have several functions and control inflammatory reactions.

The inflammatory cytokines are classified into two groups, one of which is the pro-inflammatory cytokine group and includes TNF-α, IL-1β, IL-6 and IL-8. These pro-inflammatory cytokines are known to be important mediators in the pathogenesis of lethal septic shock, systemic inflammatory response syndrome, tissue injury, cachexia, hemorrhagic shock, vascular leakage syndrome, transplant rejection and various inflammatory diseases [1–5]. The other group comprises the anti-inflammatory cytokines, such as IL-10, IL-1 receptor antagonist (IL-1RA), transforming growth factor-beta (TGF-β) and soluble TNF receptor (sTNFR). Treatment with such anti-inflammatory cytokines inhibits pro-inflammatory cytokine production and suppresses their inflammatory reactions [6–9]. However, blocking only one cytokine by anti-TNF-α antibody [10] or IL-1RA [11] fails to improve survival in septic patients. In addition, anti-inflammatory cytokines downregulate the major histocompatibility complex (MHC) class II antigen and suppress the other functions of monocytes [12, 13]. They also diminish T-lymphocyte activity including antigen specific proliferation [14]. It is therefore suggested, that if anti-inflammatory cytokines predominant over pro-inflammatory cytokines, this will manifest clinically as anergy, an increased susceptibility to infection [15].

Glucocorticoids are broadly used in clinical practice as anti-inflammatory drugs and are well known to inhibit both pro- and anti-inflammatory cytokine synthesis in monocytes. However, they also inhibit T-lymphocyte derived cytokine production such as IL-2 and interferon (IFN)-γ [16, 17], bacteria phagocytosis and the secretion of reactive oxygen species (ROS) from macrophages [18, 19], which are important for self defense. Glucocorticoids also induce T-lymphocyte apoptosis [20]. Treatment with glucocorticoids, therefore, may increase the risk of infection [21, 22].

Here, we describe the pharmacological features of JTE-607, (-)-ethyl N-{3,5-dichloro-2-hydroxy-4-[2-(4-methyl-piperazin-1-yl) ethoxy] benzoyl}-L-phenylalaninate dihydrochloride, as a novel cytokine synthesis inhibitor which inhibits both pro- and anti- inflammatory cytokines derived from peripheral blood mononuclear cells (PBMCs), but does not influence either cytokine production in T-lymphocytes or phagocytosis, ROS production and MHC class II antigen expression in human PBMCs in vitro, and has protective effect on mouse endotoxin shock in vivo.

Materials and Methods

Test compounds

JTE-607 was synthesized by Japan Tobacco Inc. (Osaka, Japan) JTE-607 and prednisolone (Sigma Chemical Co., St. Louis, MO, USA) were dissolved in dimethylsulfoxide (DMSO; Kanto chemicals, Tokyo, Japan) at a concentration of 20 mM and diluted with medium in vitro. Five-percent mannitol was used to dissolve JTE-607 and prednisolone (Shionogi & Co., LTD., Osaka, Japan) at concentrations of 1 mg/ml and 0.1 mg/ml, respectively and diluted with 5% mannitol in vivo.

Blood samples for cell preparation

Rats (Donryu, male, 7 to 8 weeks old) and mice (ICR, male, 7 to 9 weeks old) were purchased from Charles River (Yokohama, Japan). Rabbits (New Zealand White, male, 18 weeks old) were purchased from KBL (Kitayama Rabesu, Nagano, Japan). Rats and mice were anesthetized with ether and heparinized blood was collected from the inferior vena cava. Rabbit heparinized blood was collected from the auricular vein. Monkey (Crab eating, male, 4 years old) heparinized blood was purchased from Keari (Wakayama, Japan). Human heparinized blood was collected from healthy donors. These blood was used within 3 h after collection.

Cell preparation

PBMCs were isolated from heparinized blood by density gradient centrifugation on Ficoll-Hypaque® (Pharmacia, Uppsala, Sweden). The PBMC layer was harvested and washed 2 times with Eagle-minimum essential medium (E-MEM: Nissui, Tokyo, Japan) and resuspended in a culture medium consisting of Roswell Park Memorial Institute (RPMI)-1640 medium (Nissui, Tokyo, Japan) supplemented with 5% fetal calf serum (FCS), 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin (5% FCS RPMI-1640).

T-lymphocytes were purified with Pan T cell isolation kit (Miltenyi Biotec GmbH, Glad Bach, Germany) from human PBMCs. PBMCs (1.5×10^8 cells) suspended in 1.2 ml E-MEM were treated with 300 µl of hapten-antibody cocktail for 10 min at 4 °C. After 2 washes with phosphate-buffered saline (PBS, Ca^{2+}/Mg^{2+}-free; Nissui, Tokyo, Japan), the cells were treated with anti-hapten microbeads. After repeating the wash, the cells were applied to magnetic cell separator I (Miltenyi Biotec GmbH, Glad Bach, Germany). The fraction which passed the separator column was collected and analyzed by flow cytometry (FACsort, Becton Dickinson, Tokyo, Japan).

Human umbilical vein endothelial cells (HUVEC) were harvested from human umbilical cord veins with 0.015% collagenase and grown to confluence in 2% gelatin coated culture dishes containing M199 medium (Gibco, Tokyo, Japan) supplemented with 10% FCS, 3 U/ml Hepacarin® (Eisai, Tokyo, Japan), 10 ml/l antibiotic-antimycotic (Gibco, Tokyo, Japan), 10 ng/ml basic fibroblast growth factor (Genzyme, Cambridge, MA, USA) and 0.015 mg/ml endothelial cell growth supplement (Sigma).

Human lung fibroblasts and mesangial cells were purchased from Clonetics (San Diego, CA, USA). Human lung fibroblasts were cultured with 10% FCS RPMI 1640, mesangial cells were cultured with 5% FCS RPMI-1640.

Cytokine production assay

Cells were resuspended (HUVEC and human mesangial cells; 1×10^5 cells/ml, human lung fibroblasts; 1.5×10^5 cell/ ml, PBMCs; 5×10^5 cells/ml, T-lymphocytes; 1×10^6 cells/ml) and incubated for 1 h with various concentrations of JTE-607, prednisolone or vehicle (DMSO, 0.5% final concentration) at 37 °C in 5% CO_2 atmosphere. Various stimuli were then added at optimal concentration (lipopolysaccharide (LPS, *E. coli* 055:B5, Difco, Detroit, MI, USA); 10 µg/ml, TNF-α (Hayashibara Biochemical Laboratories, Okayama, Japan); 100 JRU/ml, and anti-CD28 antibody (Pharmingen, San Diego, CA, USA); 10 µg/ml on anti-CD3 antibody (Coulter, Miami, FL, U.S.A.) coated plates (5 µg/10 µl/well). After 20 h (T-lymphocytes; 16 h) incubation, each supernatant was collected and stored at −80 °C until specific ELISA measurement (for human TNF-α, IL-6, IL-10, mouse TNF-α and rat TNF-α (Amersham, Tokyo, Japan), and for human IL-1β, IL-8, IL-2 and IFN-γ (R&D, Minneapolis, MN, USA)).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Human PBMCs (5×10^5 cells/ml) were incubated for 20 h with 10 µg/ml LPS in the presence or absence of inhibitors (100 nM JTE-607, 100 nM prednisolone or vehicle (DMSO, 0.5% final concentration)) at 37 °C in 5% CO_2 atmosphere. Total RNA (1 µg) was extracted with Isogen (Wako, Osaka, Japan) and reverse transcribed into cDNA according to the manufacturer's protocol (Clontech Labs, Palo Alto, CA, USA) with 2.5 µM oligo-dT, 5 mM dNTP, 20 U RNase inhibitor and 50 U MMLV reverse transcriptase. The mixture was heated at 42 °C for 60 min and cooled to room temperature. PCR was performed in a final volume of 50 µl containing all reverse transcriptase reaction products, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 0.5 µM of each primer and 2.5 U of AmpliTaq DNA polymerase. Amplification for 30 cycles was performed for 30 s at 94 °C, 1 min at 55C and 2 min at 72 °C. PCR products were run on 2% agarose gel and visualized by ethidium bromide staining.

Phagocytosis

Human PBMCs were suspended at a concentration of 5×10^6 cells/ml in 5% FCS RPMI 1640 and incubated with JTE-607 (1 µM) or vehicle (DMSO, 0.5% final concentration) at 37 °C for 10 min prior to addition of finally 1000-fold diluted fluorescence microbeads (Funakoshi, Tokyo, Japan) and LPS (10 µg/ml). After 1 h, cells were immediately placed on ice to stop phagocytosis and washed 2 times with PBS followed by analysis with flow cytometry.

ROS production

Human PBMCs were suspended at a concentration of 5×10^7 cells/ml in HBSS (Nissui, Tokyo, Japan) and incubated with luminol solution (1×10^{-6} mol/ml) and JTE-607 (1 µM) or vehicle (DMSO, 0.5% final concentration) at 37 °C for 10 min. ROS production stimulated by LPS (10 µg/ml) was measured with luminometer instrument (LB905C; Berthold, Osaka, Japan) at 37 °C for 60 min.

Expression of MHC class II antigen

Human PBMCs were suspended at a concentration of 5×10^5 cells/ml in 5% FCS RPMI 1640, incubated for 1 h with JTE-607 (0.1 to 1 µM)

or vehicle (DMSO, 0.5% final concentration), and then stimulated with 100 JRU/ml of IFN-γ (Hayashibara Biochemical Laboratories, Okayama, Japan) for 20 h at 37C in 5% CO_2 atmosphere. PBMCs were dispersed by treatment with cold 0.2% EDTA in PBS. Cells were washed 2 times with PBS and suspended in PBS containing 1% BSA and 0.1% sodium azide. Cell suspensions were treated with FITC-conjugated anti-HLA-DR (Funakoshi, Tokyo, Japan) antibody for 30 min on ice. HLA-DR positive and negative cells were analyzed by flow cytometry.

C. parvum – LPS induced endotoxin shock in mice

Male C57BL/6 mice (Charles River, 5 to 6 weeks old) were sensitized by injecting *Corynebacterium parvum (C. parvum,* Immunochem. Research. Inc., Hamilton, MT, USA; 10 mg/kg, i.v.) at day 0. At day 6, LPS was injected (50 µg/kg, i.p.) to induce endotoxin shock and JTE-607 (0.3, 1, 3, 10 mg/kg) or prednisolone (1 mg/kg) was injected intravenously (10 ml/kg) 10 min before the LPS challenge. Heparinized blood was obtained from the orbital sinus 1 h after the LPS challenge, and the blood was centrifuged at 12000 rpm for 10 min at 4 °C to collect supernatant for the measurement of TNF-α by mouse ELISA kit (Genzyme, Cambridge, MA, USA). Mortality was observed at 24 h and 48 h after the LPS challenge to the mice.

Statistical analysis

Mortality data were analyzed using Log-Rank test and Wilcoxon test with Bonferroni corrections for multiple comparisons of JTE-607 treatment and for prednisolone, respectively. Other data were analyzed using a one-way ANOVA with Dunnett's test or Student's t-test. Probability p-value of $p < 0.05$ was considered to be significant.

Results

Effects of JTE-607 and prednisolone on inflammatory cytokine production from LPS-stimulated human PBMCs

LPS (10 µg/ml, for 20 h)-stimulated PBMCs produced a large quantity of various inflammatory cytokines. The quantities of TNF-α, IL-1β, IL-6, IL-8 and IL-10 in the LPS-stimulated control were 3928 ± 337, 4007 ± 665, 15235 ± 2609, 63641 ± 8851 and 297 ± 52 pg/ml, respectively (n = 3). When relevant concentrations of JTE-607 (0.3 to 100 nM) were added to PBMCs 1 h before LPS-stimulation, production of these cytokines was suppressed dose dependently (Fig. 1). The IC_{50}

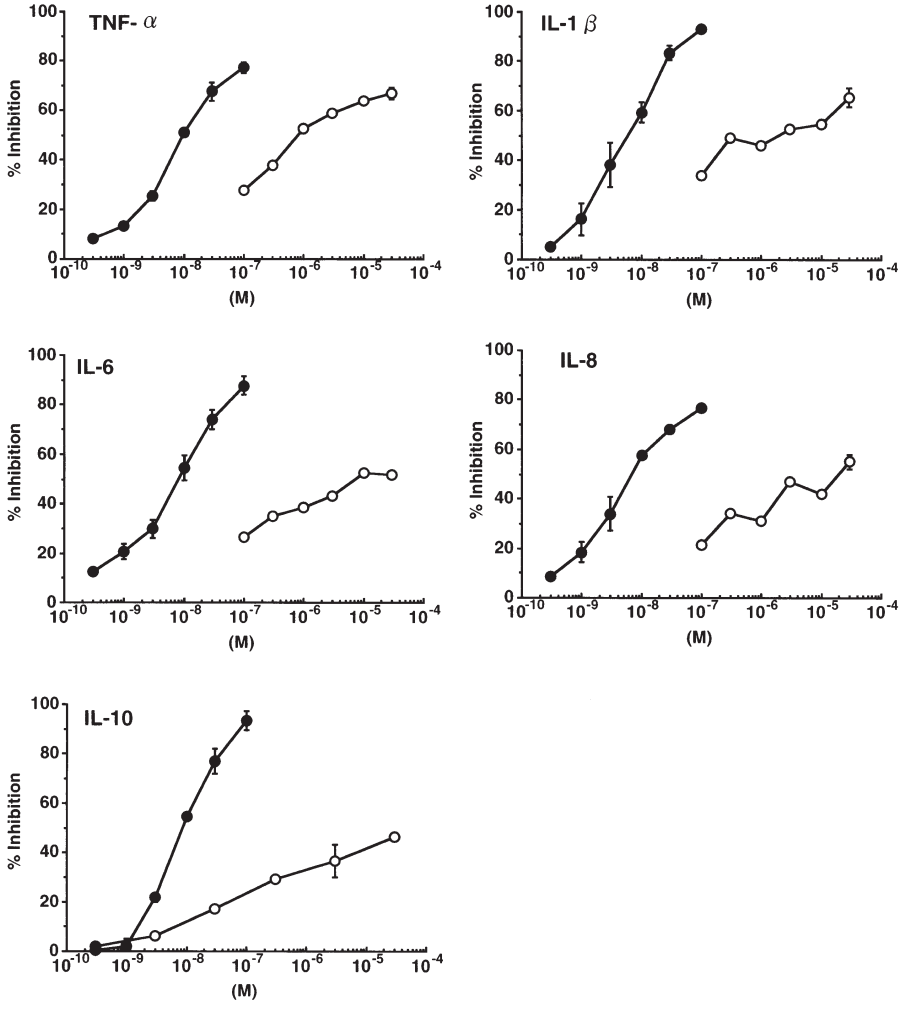

Fig. 1. Effects of JTE-607 (●) and prednisolone (○) on inflammatory cytokine production from LPS-stimulated human PBMCs. Cells were incubated for 1 h with JTE-607 (0.3 to 100 nM), prednisolone (0.3 nM to 30 µM) or vehicle (DMSO, 0.5% final concentration) prior to LPS-stimulation (10 µg/ ml). Cells were cultured for 20 h, and then cell-free supernatants were collected and stored at –80 °C prior to cytokine level determination by specific ELISA. Results are expressed as the mean of percent inhibition (% inhibition) ± SEM (n = 3, duplicate), compared to the vehicle.

values for JTE-607 to these cytokines were 11.0 ± 1.9, 5.9 ± 1.7, 8.8 ± 1.7, 7.3 ± 1.6 and 9.1 ± 1.0 nM, respectively (n = 3). The other cytokines, granulocyte-macrophage colony stimulating factor and IL-1RA, were also suppressed, with IC_{50} values of 2.4 ± 0.8 and 5.4 ± 0.4 nM, respectively (n = 3). On the other hand, the inhibitory activity of prednisolone on LPS-induced cytokine production was much less than that of JTE-607 (Fig. 1). No cytotoxicity was observed at 1 µM of JTE-607 by trypan blue exclusion method (data not shown).

Effect of JTE-607 on mRNA levels of inflammatory cytokines

To further elucidate the molecular mechanism of cytokine inhibition, we tested the effect of JTE-607 on several inflammatory cytokine mRNA levels in LPS stimulated human PBMCs. JTE-607 at 100 nM reduced the increase in the level of mRNAs of TNF-α, IL-1β, IL-6 and IL-8 (Fig. 2). Prednisolone at 100 nM also reduced mRNA level of these cytokines, but the inhibitory activity was less than that of JTE-607. The level of mRNA for β-actin was not affected by these inhibitors.

Distinction between inhibition of cytokine production by PBMCs from various species

We also examined the cytokine inhibitory activity of JTE-607 on PBMCs from various species. The experimental procedure in each animal was similar to that in human. Monkey and rabbit showed cross reactivity in the human IL-8 ELISA used, we therefore evaluated the level of IL-8 in monkey and rabbit by human IL-8 ELISA, while the level of TNF-α was measured in rat and mouse by rat and mouse TNF-α ELISA, respectively. Due to absence of IL-8 production in rat and mouse, and no cross-reactivity in available TNF-α ELISA kit for monkey and rabbit, the cytokines in those species were not measured. JTE-607 inhibited cytokine production in monkey, rabbit, mouse and rat with IC_{50} values of 59 ± 26, 780 ± 120, 1600 ± 650 and 19000 ± 3200 nM, respectively (n = 3, Table 1). The cytokine concentrations of the controls were 7428 ± 1099, 3212 ± 447, 872 ± 126 and 2997 ± 405 pg/ml, respectively (n = 3).

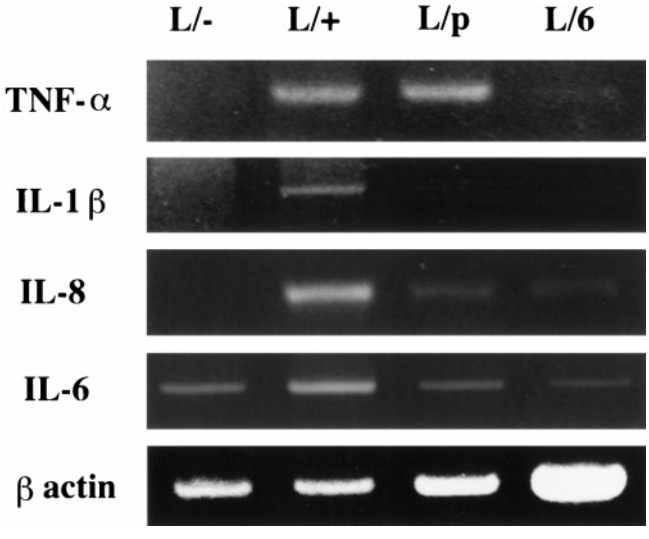

Fig. 2. Effects of JTE-607 and prednisolone on LPS-induced mRNA levels of TNF-α, IL-1β, IL-8 and IL-6 in human PBMCs. Cells were stimulated with 10 µg/ml LPS in the presence or absence of inhibitors for 20 h (L/–; non-stimulated, L/+; stimulated with LPS, L/p; stimulated with LPS in the presence of 100 nM prednisolone, L/6; stimulated with LPS in the presence of 100 nM JTE-607). Total RNA (1 µg/ml) was reverse transcribed into cDNA followed by RT-PCR.

Table 1. IC_{50} values for JTE-607 in inhibiting cytokine production from LPS-stimulated PBMCs of various species.

	IC_{50} (nM)	
	IL-8	TNF-α
Human	7.3 ± 1.6	11 ± 1.9
Monkey	59 ± 26	–
Rabbit	780 ± 120	–
Mouse	–	1600 ± 650
Rat	–	19000 ± 3200

Cells were incubated for 1 h with JTE-607 (0.3 nM to 30 µM) or vehicle (DMSO, 0.5% final concentration) prior to LPS-stimulation (10 µg/ml). Cells were cultured for 20 h, and then cell-free supernatants were collected and stored at –80 °C prior to cytokine level determination by specific ELISA. IC_{50} values are calculated from sigmoidal inhibition curves. Each data represents mean ± SEM (n = 3, duplicate).

Effect of JTE-607 on IL-8, IL-2 and IFN-γ production in various human cell types

We further investigated whether JTE-607 could inhibit pro-inflammatory cytokine production in various human cell types. IC_{50} values for JTE-607 in IL-8 production from fibloblasts, HUVEC and mesangial cells stimulated with LPS were >30000, 6000 and >30000 nM (IL-8 concentrations of the controls were 521, 15931 and 1652 pg/ml), respectively. The IC_{50} values in the same experiment with TNF-α were 6700, 2900 and 2600 nM (IL-8 concentrations of the controls were 29200, 16305 and 5910 pg/ml), respectively (Table 2). These values were much higher than those in PBMCs. In TNF-α stimulated PBMCs, JTE-607 inhibited IL-8 production at the same level as with LPS stimulation

Table 2. Effect of JTE-607 on IL-8 production from various human cells stimulated by LPS or TNF-α.

	IC_{50} (nM)			
	PBMC	Fibroblast	HUVEC	Mesangial cell
LPS	3.9	>30000	6000	>30000
TNF-α	4.5	6700	2900	2600

Cells were incubated for 1 h with JTE-607 (0.3 nM to 30 µM), or vehicle (DMSO, 0.5% final concentration) prior to LPS (10 µg/ml) or TNF-α (100 JRU/ml) stimulation. Cells were cultured for 20 h, and then cell free supernatants were collected and stored at –80 °C prior to IL-8 level determination by specific ELISA. Each data represents the IC_{50} value obtained from sigmoidal inhibition curves (triplicate).

(IC_{50} values in LPS-stimulation and TNF-α stimulation were 3.9 and 4.5 nM, respectively, Table 2).

We next investigated the effect of JTE-607 on IL-2 and IFN-γ production from purified human T-lymphocytes. After purification of T-lymphocytes, CD3 positive cells constituted 89–96% while CD14 positive cells were 0.2–1.6% (data not shown). In concanavalin A or only anti-CD3 antibody stimulation, IL-2 and IFN-γ production were negligible (data not shown). This demonstrated that monocytes and B-lymphocytes with co-stimulatory antigens rarely existed. When the purified T-lymphocytes were stimulated with a combination of anti-CD3 and anti-CD28 antibodies, IL-2 and IFN-γ were produced at concentrations of 3295 ± 756 and 111 ± 32 pg/ml. The IC_{50} values for JTE-607 in IL-2 and IFN-γ production were 1.3 ± 0.3 and >10 μM, respectively (n = 3, Table 3). Prednisolone inhibited these cytokines to the same extent as the monocyte derived cytokines (Fig. 1, Table 3).

Table 3. Effects of JTE-607 and prednisolone on IL-2 and IFN-γ production from anti-CD3 and anti-CD28 antibody-stimulated human T-lymphocytes.

	IC_{50} (μM)	
	IL-2	IFN-γ
JTE-607	1.3 ± 0.3	>10
Prednisolone	0.70 ± 0.36	3.3 ± 2.1

T-lymphocytes (5×10^5 cells/well) were incubated for 1 h with JTE-607 (0.3 to 10 μM), prednisolone (0.3 to 10 μM) or vehicle (DMSO, 0.5% final concentration) prior to anti-CD3 and anti-CD28 antibody-stimulation. Cells were cultured for 16 h, and then cell-free supernatants were collected and stored at −80 °C prior to cytokine level determination by specific ELISA. IC_{50} values are calculated from sigmoidal inhibition curves. Each data represents mean ± SEM (n = 3, duplicate).

Effect of JTE-607 on other functions of monocytes

JTE-607 did not inhibit either microbead phagocytosis or ROS production at 1 μM in LPS-stimulated (10 μg/ml) human PBMCs (Table 4 and 5). In addition, we tested JTE-607 in IFN-γ-stimulated MHC class II antigen expression on monocytes. JTE-607 at 1 μM showed slight suppression of IFN-γ mediated upregulation of the antigen (Fig. 3), but it was 100-fold higher than the IC_{50} values of cytokine production from PBMCs.

Table 4. Effect of JTE-607 on phagocytosis.

	LPS (–)		LPS (10 μg/ml)	
	control	JTE-607	control	JTE-607
Fluorescence intensity	148.5±11.1	150.7±14.9	228.3±15.8	238.9±12.2

Human PBMCs were incubated for 10 min with JTE-607 (1 μM), or vehicle (DMSO, 0.5% final concentration) prior to FITC-labeled microbeads and LPS (10 μg/ml) addition. After 1 h, cells were immediately placed on ice to stop phagocytosis and analyzed by flow cytometry. Each data represents mean ± SEM (n = 3).

Effect of JTE-607 on endotoxin shock in mice

Injection of LPS into *C. parvum* sensitized mice induced endotoxin shock and mortality of 87.5% and 100% at 24 h and 48 h after the LPS challenge, respectively. JTE-607 showed dose dependent inhibition of the mortality at 0.3 to 10 mg/kg and significant effect at 3 and 10 mg/kg ($p < 0.01$, Fig. 4). This effect was in accordance with a dose dependent inhibition of plasma level of TNF-α (Fig. 5). Prednisolone significantly inhibited both the mortality and the plasma TNF-α at 1 mg/kg ($p < 0.01$, Fig. 4 and 5).

Discussion

Various cytokines including TNF-α, IL-1 β, IL-6, IL-8 and IL-10 have several important roles individually and together

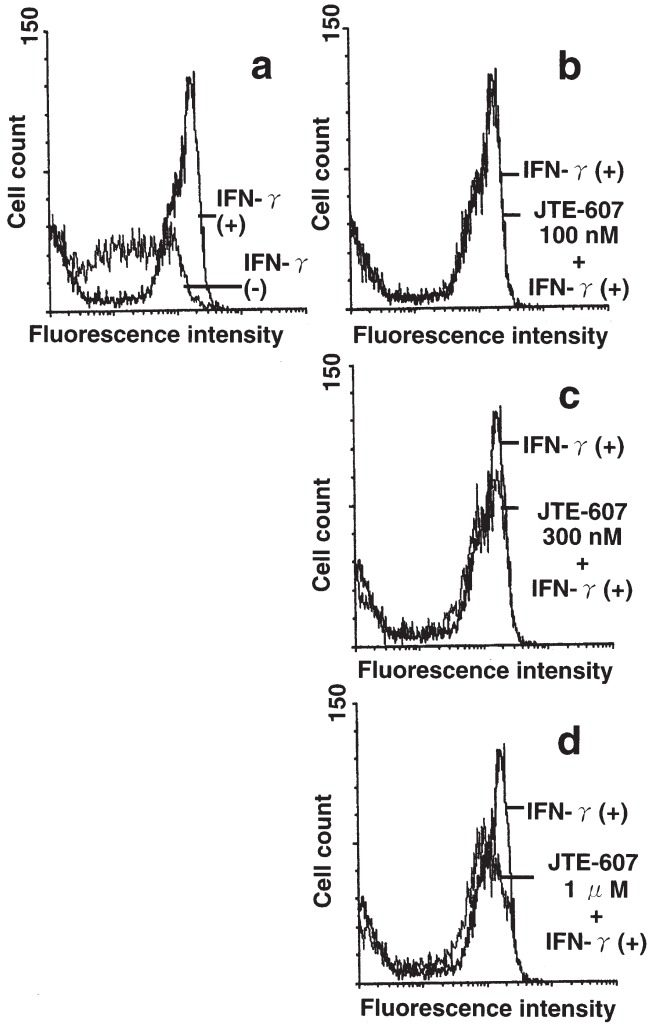

Fig. 3. Effect of JTE-607 on IFN-γ stimulated MHC class II expression. Human PBMCs were incubated for 1 h with vehicle (DMSO, 0.5% final concentration (a)), or JTE-607 (100 nM (b), 300 nM (c) and 1000 nM (d)) prior to IFN-γ stimulation (100 JRU/ml). Cells were cultured for 24 h, and collected with 0.2% EDTA in PBS. After washing 2 times with PBS, FITC labeled anti-human HLA-DR antibody was added. Cells were analyzed by flow cytometry. One representative experiment is shown (n = 3, different donors).

Table 5. Effect of JTE-607 on reactive oxygen species production from LPS-stimulated human PBMCs

	Luminal intensity (x e^6)
Control	23.4 ± 6.8 → 19.1 ± 6.8
JTE-607	19.1 ± 8.6 → 20.1 ± 7.7

Human PBMCs were incubated for 5 min with luminol solution and JTE-607 (1 μM) or vehicle (DMSO, 0.5% final concentration) prior to LPS stimulation (10 μg/ml). Luminal intensity was measured at 37°C for 60 min. Each data represents mean ± SEM (n = 3).

act as a control system in inflammatory reactions. In the present study, JTE-607 inhibited inflammatory cytokine production from LPS-stimulated human PBMCs with an IC_{50} of approximately 10 nM. This activity was much stronger than that of prednisolone, a well known anti-inflammatory drug and cytokine synthesis inhibitor. IC_{50} values for JTE-607 in LPS-stimulated IL-8 production from monkey and rabbit PBMCs, and TNF-α production from mouse and rat PBMCs were 59, 780, 1600 and 19000 nM, respectively. These results indicate that the inhibitory activity of JTE-607 is specific for human. We also demonstrated that JTE-607 is not a simple LPS antagonist. As shown in the experiment of IL-8 production from TNF-α stimulated human PBMCs, JTE-607 inhibited production with a similar IC_{50} value as LPS-stimulation. In addition, inflammatory cytokine mRNA expression in LPS-stimulated human PBMCs was diminished by JTE-607. It is suggested, therefore, that JTE-607 is an inhibitor of cytokine production at a signaling pathway not restricted to LPS.

We next tested cell specificity of JTE-607 in various human cell types, since immunocytes are not the only source of inflammatory cytokines. For example, IL-8 is also secreted from fibloblasts, HUVEC and mesangial cells [23–25]. We observed that IC_{50} values for JTE-607 in IL-8 production from LPS or TNF-α stimulated fibloblasts, HUVEC and mesangial cells were much higher than in PBMCs. Although the PBMCs include T-lymphocytes, B-lymphocytes and monocytes, T-lymphocytes do not express CD14, the receptor for complex formation of LPS and LPS-binding protein, and thus show minimal response to LPS. In the case of B-lymphocytes, these cells produce few inflammatory cytokines. In contrast, LPS-stimulated monocytes secrete large quantities of inflammatory cytokines. These findings suggest that JTE-607 may be a highly selective cytokine synthesis inhibitor for human monocytes.

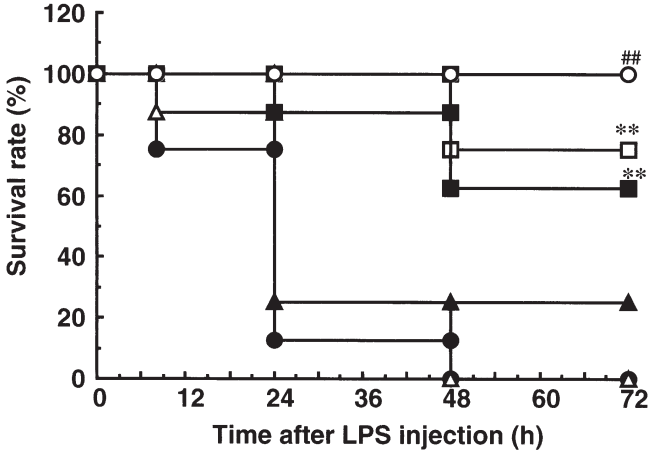

Fig. 4. Effects of JTE-607 and prednisolone on LPS induced mortality in *C. parvum* sensitized mice. Vehicle (●), JTE-607 (0.3 mg/kg; △, 1 mg/kg; ▲, 3 mg/kg; □, 10 mg/kg; ■) and prednisolone (1 mg/kg; ○) were administered intravenously 10 min before the LPS challenge. Mortality was observed at 24 h and 48 h after the LPS challenge. Data represent mean ± SEM (n = 8). ## $p < 0.01$ (Wilcoxon test), ** $p < 0.01$ (Log-Rank test) compared to vehicle control.

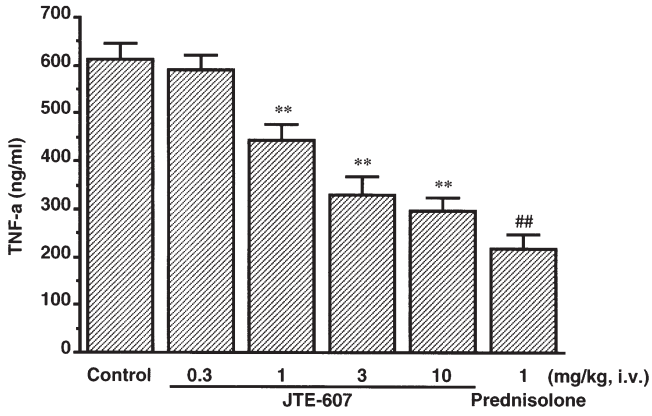

Fig. 5. Effects of JTE-607 and prednisolone on LPS induced plasma TNF-α level in *C. parvum* sensitized mice. Vehicle, JTE-607 (0.3 mg/kg, 1 mg/kg, 3 mg/kg, 10 mg/kg) and prednisolone (1 mg/kg) were administered intravenously 10 min before the LPS challenge. Plasma was obtained at 1 h after the LPS challenge. Data represent mean ± SEM (n = 8). ## $p < 0.01$ (Student's *t* test), ** $p < 0.01$ (Dunnett's test) compared to vehicle control.

Cytokines that are produced in inflammatory diseases are classified into two groups, the pro-inflammatory cytokines (TNF-α, IL-1β, IL-8, IL-6, etc.), and the anti-inflammatory cytokines (IL-10, IL-1RA, TGF-β, sTNFR, etc.). It has generally been believed that pro-inflammatory cytokines rather than anti-inflammatory cytokines are the central cause of these diseases. For example, it is observed that the concentration of pro-inflammatory cytokine is elevated in patients with lethal septic shock, systemic inflammatory response syndrome, cachexia, transplant rejection and various inflammatory diseases [3, 4, 26, 27]. In animal models, the administration of pro-inflammatory cytokine inhibitors (anti-TNF-α monoclonal antibody, anti-IL-8 monoclonal antibody, recombinant IL-1RA and so on) result in inhibition of both acute and chronic inflammatory responses [28–30]. However, complete block of only one cytokine by an antibody may cause a contrary effect. That is the case with anti-TNF-α antibody: while it decreases mortality in LPS shock model, it increases the mortality in murine cecal ligation and puncture model [31]. Furthermore, clinical trials of these antibodies have revealed insufficient effects on mortality of septic patients [10, 11]. The cytokine network of pro- and anti-inflammatory cytokines is considerably complex, and it may be important that a balance of pro- and anti-inflammatory cytokines is maintained [15, 32]. JTE-607 apparently inhibited both pro- and anti-inflammatory cytokines in vitro, and it was shown that JTE-607 inhibited mortality and elevated plasma TNF-α level induced by LPS challenge in *C. parvum*

sensitized mice in vivo, which is a TNF-α dependent model [33]. Although it should be necessary to prove that the multiple cytokine inhibition by JTE-607 is much more efficacious than anti-cytokine antibody treatment in a study of cecal ligation and puncture model for example, it is possible that JTE-607 may produce an anti-inflammatory effect by maintaining balance of the cytokine network.

Glucocorticoids are known to be both inflammatory cytokine synthesis inhibitors and anti-inflammatory drugs. They also inhibit T-lymphocyte derived cytokines such as IL-2 and IFN-γ [16, 17], however, suppress bacteria phagocytosis and the secretion of ROS from macrophages [18, 19]. IL-2 and IFN-γ are known to be type 1 T helper cytokines. IL-2 induces activation and proliferation of T-lymphocytes, while IFN-γ acts as one of the central cytokines in resistance to viral and bacterial infection [35–37]. These functions are important in the host defense system to clear bacterial pathogens. Treatment with these drugs, therefore, may increase the risk of infection [21, 22]. For example, most animal models of *P. carinii* pneumonia have relied on glucocorticoid-induced immunosuppression to activate latent *P. carinii* infection in rats [38]. In contrast to glucocorticoids, the inhibitory activity of JTE-607 on IL-2 and IFN-γ production from T-lymphocytes stimulated with a combination of anti-CD3 and anti-CD28 antibodies, was much less than that on TNF-α, IL-1β, IL-8 etc. from LPS-stimulated PBMCs. Moreover, JTE-607 did not suppress microbead phagocytosis or ROS production in LPS-stimulated PBMC at 1 μM, which is a 100-fold higher concentration than the IC_{50} value of cytokine production. Therefore, JTE-607 may be able to suppress inflammatory diseases without increasing the risk of infection and lymphoproliferative disorders.

We conclude that JTE-607, a novel cytokine synthesis inhibitor, should be more useful in the treatment of diseases mediated by inflammatory cytokines than currently existing anti-inflammatory drugs.

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