

Immunomodulatory Effect of Pentoxifylline in Suppressing Experimental Autoimmune Myocarditis

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Although a recent clinical study reported the beneficial effects of pentoxifylline (PTX), a phosphodiesterase inhibitor, on both symptoms and cardiac function in dilated cardiomyopathy (DCM), the precise mechanism of the drug has not been delineated. This study examined the efficacy of PTX in the treatment of experimental autoimmune myocarditis (EAM), as a model of the autoimmune mechanism involved in DCM. Oral PTX, or saline as control, was administered to Lewis rats at 150 mg/kg body weight per day bid daily from 5 days before immunization with cardiac myosin until death on Day 21. Histological examination of the hearts showed PTX significantly reduced the severity of EAM. mRNA expression of tumor necrosis factor (TNF)- α , interleukin (IL)-4, IL-6, and IL-10 was significantly reduced in peripheral blood mononuclear cells, but expression of IL-4 and IL-6 was upregulated in heart tissue. PTX in vitro could suppress T cell proliferation and inhibit TNF- α and interferon- γ production. In conclusion, the immunomodulatory effects of PTX had a significant therapeutic result in EAM. This is the first report to describe such an effect of PTX in a specific animal model for DCM. (Circ J 2002; 66: 499–504)

Key Words: Autoimmune disease; Cytokines; Dilated cardiomyopathy; Myocarditis; Pentoxifylline

Biologically active molecules are important in the pathogenesis of heart failure and in addition to neurohormones, current interest centers around understanding the role of proinflammatory cytokines in disease progression.¹ Increased plasma concentration and myocardial expression of tumor necrosis factor α (TNF- α) has been reported in patients with advanced heart failure,^{1,2} and TNF- α has been found to contribute to myocardial injury and dysfunction;^{3,4} thus, it may be predictive of disease severity and the clinical outcome of heart failure.^{2,5}

Dilated cardiomyopathy (DCM) is a representative disease with severe heart failure and a set of heterogeneous diseases of myocardial dysfunction with unknown etiology. One of the possible causes of DCM is myocarditis, which is the result of progressive autoimmune injury;^{6,7} but it is difficult to prove clinically the contribution of an autoimmune mechanism to the disease process. Unique forms of experimental autoimmune myocarditis (EAM) have been reported in mice and rats after immunization with cardiac myosin^{8,9} and by repeating the immunization with cardiac myosin in the recovery phase of inflammation, we have shown that autoimmune myocarditis leads to DCM in a model using the Lewis rat.¹⁰

Clinically beneficial effects of pentoxifylline (PTX), a xanthine-derived agent known to inhibit the production of TNF- α , have been reported in patients with DCM,¹¹ but the

mechanism of the improvement in both symptoms and left ventricular systolic function remains unclear. We designed the present study to provide evidence of the ability of the immunomodulatory effects of PTX to ameliorate EAM in rats.

Methods

All experimental procedures and protocols conformed to institutional guidelines for the care and use of animals in research.

Immunization and Treatment

Female 7-week-old Lewis rats were purchased from Charles River (Tsukuba, Ibaraki, Japan) and maintained in the institute's animal facilities. Cardiac myosin was prepared from the ventricular muscle of porcine heart as described,¹² and the method of induction of EAM has also been reported.¹³ Briefly, rats were immunized subcutaneously in the rear footpad with 0.4 mg whole cardiac myosin emulsified with an equal volume of complete Freund's adjuvant supplemented with 0.1 mg Mycobacterium tuberculosis H37RA (Difco) under ether anesthesia. Oral PTX (n=30), or an equal volume of saline as control (n=31), was administered to the animals by a gavage needle at 150 mg/kg body weight per day bid daily from 5 days before the immunization (Day -5) until death (Day 21).

Histological Assessment

All rats were killed under ether anesthesia at Day 21. The weight of the heart was determined by measuring the cardiac ventricles, excluding the atria and other tissues, and the macroscopic findings were classified into 5 grades: 0, no inflammation; 1, presence of a small discolored focus; 2, diffuse discolored areas not exceeding a total of one-third

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Table 1 Histological Findings

	Macroscopic grade					Microscopic score affected area ratio (%)
	0	1	2	3	4	
PTX (n)	1	0	9	11	9	40.1±4.1
Saline (n)	0	0	4	6	21	50.2±2.8

of the cardiac surface; 3, diffuse discolored areas exceeding one-third of the cardiac surface, but not exceeding a total of two-thirds of the cardiac surface; 4, diffuse discolored areas totaling more than two-thirds of the cardiac surface. For microscopic grading, transverse sections through the middle of the heart specimens were sliced into 3 equal portions and fixed in 10% formalin. Paraffin-embedded tissues were cut and stained with hematoxylin–eosin for histological examination. The area of the entire heart and of the regions affected by myocarditis (ie, regions showing inflammation with inflammatory cells and myocardial necrosis) were determined using a personal computer (CAMAC-300, NEC) and the ratio of affected area per entire area was calculated by 2 blinded observers and the inter- and intra-observer variance was less than 5%.

RNase Protection Assay

Peripheral blood mononuclear cells (PBMC) were isolated at the time of death by Nycoprep (Nycomed Pharma) gradient centrifugation. RNA was isolated from both the PBMC and homogenized heart tissue specimens using the lysis buffer ISOGEN (Nippon gene) according to the manufacturer's method and the total RNA estimated by spectrophotometer at 260 nm. The rCK-1 multi-probe template sets (Pharmingen) were used for multiple cytokine gene RNase protection assays. The *in vitro* transcription was carried out using the template set with [³²P] UTP: 1 µg of the total RNA was hybridized with the riboprobes and digested by the RNase mixture according to the manufacturer's method. The protected fragments were electrophoresed on 5% acrylamide urea-denaturing gel (L32 was used as internal controls) and the dried gel was exposed to an imaging plate for 5 h. The detection and quantification of the mRNA bands were performed with an image analyzer (FLA 2000, FUJIFILM). The gel was exposed again for 30 min to measure the density of the L32 bands. Messenger RNA (mRNA) expression of cytokines was standardized by dividing by the density of L32, and each of the standardized mRNA expression levels in the saline group was expressed as 100 because of the scattered values among the experimental series.

Effect of PTX in Vitro

Myocarditogenic T cell lines were established according to a modified method of Ratcliffe et al.¹⁴ In brief, draining lymph nodes were harvested on Day 9 from Lewis rats immunized with synthetic immunodominant peptide CM2 (Sawady Tech Co), residues 1,539–1,555 of rat cardiac myosin.¹⁵ The cell suspension was cultured in complete Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% autologous rat serum, non-essential amino acids, glutamine, L-asparagine, sodium pyruvate, penicillin–streptomycin, and 2-mercaptoethanol for 2 days together with 5 µg/ml of CM2. T-cell blasts were then continued in culture in complete medium with 10% horse serum and 10% concanavalin A (Con A) supernatant for 5–7 days. The T cell line underwent repeated cycles of

antigen-supplemented stimulation medium containing irradiated (5,000 rad) syngeneic thymocytes and then growth medium with Con A supernatant. These CM2-specific T-cell lines were phenotypically characterized as Th1 type CD4⁺ T cells using a fluorescent activated cell sorter and an enzyme-linked immunosorbent assay (ELISA) for cytokine production, and were shown to cause myocarditis in all of the transferred rats (data not shown).

CM2-specific T cell (3×10⁴ per well) were cultured with irradiated syngeneic thymocytes (1×10⁶ per well) in a round-bottomed 96-well tissue culture plate (Sumitomo Bakelite Co) supplemented with 0.2 ml DMEM per well. Antigen (CM2 5 µg/ml) or mitogen (Con A 2.5 µg/ml) and PTX in different concentrations (1, 10, 100 µg/ml) were added to triplicate wells and the culture was incubated at 37°C in a humidified 10% CO₂ incubator for 48 h. T cell proliferation was determined by adding ³H-thymidine (18.5 kBq (0.5 µCi) per well; NEN Life Science Products) for the following 18 h by standard liquid scintillation technique. We ran 3 series for *in vitro* T cell proliferation assay and the results were expressed as mean counts per minute (cpm); the standard error of the mean was always less than 10% except for very low values.

Together with the proliferation assay, aliquots of supernatant were collected after incubation for 48 h and immediately stored at –80°C. Serum concentrations of TNF- and interferon-gamma (IFN-) were determined by Cytoscreen ELISA kit (BioSource International) according to the manufacturer's instructions. We ran 2 series for the *in vitro* cytokine assay and the results were expressed as the mean of the triplicate assays and the standard error of the mean was as before.

Statistical Analysis

Data are presented as mean±SD. Macroscopic scores were analyzed using the chi-squared test. Other results were statistically evaluated by Student's unpaired *t* tests using the Stat View program (Abacus Concepts). A *p* value less than 0.05 was considered significant.

Results

Reduction of Severity of EAM by PTX

During the observation period, only 1 rat died in each treatment group. There was no significant difference in the change in body weight between the 2 groups, with each group showing steady weight gain (data not shown). The sum of the mean macroscopic scores of diseased hearts from 5 separate experiments demonstrated that the macroscopic disease severity of myocarditis was significantly lower in the PTX-treated group than in the saline-treated group (Table 1). There was much less pericardial effusion in the PTX-treated rats than in the saline-treated ones. There was no significant difference in the heart weight-to-body weight ratio between the 2 groups (data not shown).

Histological observation showed that mononuclear cells and polymorphonuclear cells, including multinucleated

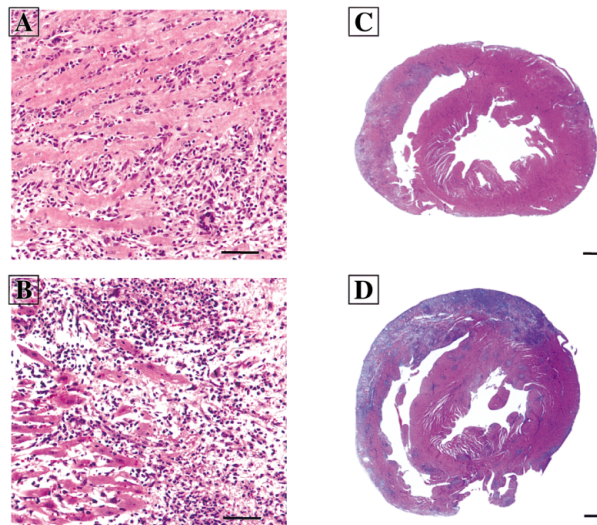


Fig 1. Representative photomicrograph of histopathologic sections of the heart with experimental autoimmune myocarditis (EAM). (A,C) PTX-treated rat; (B,D) saline-treated rat (H&E; A,B: original magnification $\times 200$, bar = $10\mu\text{m}$; C,D: original magnification $\times 1$, bar = 1mm).

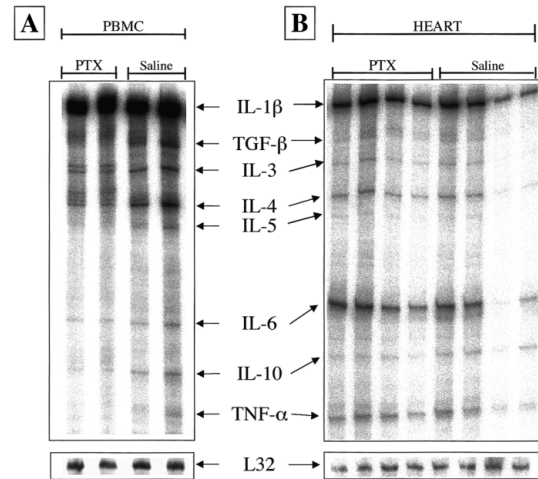


Fig 2. Representative findings of mRNA expression of cytokines in peripheral blood mononuclear cells (PBMC) (A) and hearts (B) using RNase protection assay.

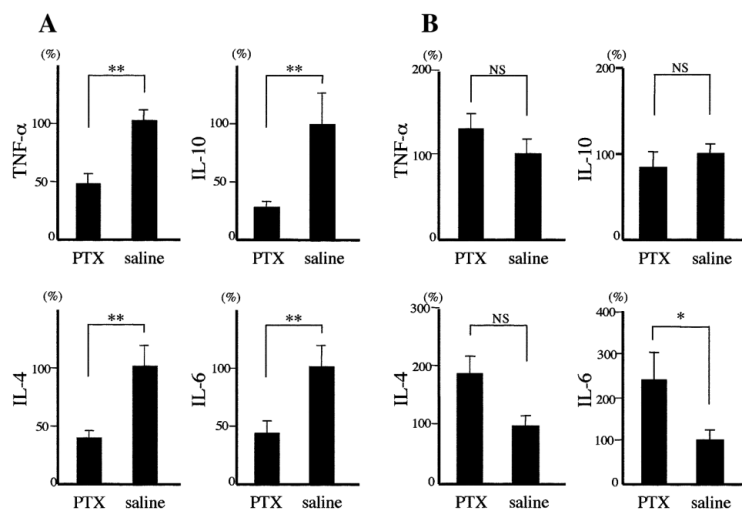


Fig 3. mRNA expression of TNF- α , IL-4, IL-6, and IL-10 standardized with L32 as internal control in peripheral blood mononuclear cells (PBMC) (A) and hearts (B). This is a representative result of 3 experimental series. The average of the standardized ratio of cytokine expressions in saline treated group is indicated as 100%. * $p < 0.05$; ** $p < 0.01$.

giant cells, destruction of myocardial fibers and interstitial edema were observed in both groups. Although the number of infiltrating cells appeared to be equal, the area of affected myocardium was significantly lower in the PTX-treated rats than in the saline-treated ones (Fig 1).

Immunomodulatory Effects of PTX on Cytokine Production in Hearts and in Peripheral Cells

The results of the RNase protection assays of cytokines in PBMC and heart specimens from rats with EAM are shown in Figs 2 and 3. Interleukin (IL)-2 and IFN- γ mRNA were not detected with this method. On Day 21, mRNA expression of TNF- α , IL-4, IL-6, and IL-10 in PBMC was significantly depressed in the PTX group compared with the saline group. On the other hand, mRNA expression of IL-4 and IL-6 was upregulated in heart tissue at the time of death in the PTX group. There were no significant differences in the mRNA expression of TNF- α and IL-10 in heart tissue between the 2 groups.

Suppression of Myocarditogenic T Cell Proliferation in Vitro by PTX

To study the suppressive effect of PTX on T-cell-triggered immune responses, antigen-specific CD4 $^{+}$ T cells were stimulated with their respective nominal antigen or with mitogen in the presence of increasing concentrations of the drug (Fig 4). Without PTX, the T cells proliferated with the various concentrations of antigen, almost in a dose-dependent manner, and also with the mitogen. PTX concentrations as low as $1\mu\text{g/ml}$ had a marked inhibitory effect on antigen-driven T cell activation in each setting. The inhibitory effect of PTX was dose dependent: concentrations of $1\mu\text{g/ml}$ resulted in approximately 10% reduction of T cell proliferation and to achieve 50% inhibition, nearly $100\mu\text{g/ml}$ PTX/ml was required. The inhibition was not caused by toxic effects on cell integrity, as assessed by trypan blue staining (data not shown). A difference in the inhibitory effect of PTX on T cell proliferation was not obvious between the CM2- and Con A-stimulations.

The CM2-specific CD4 $^{+}$ T cell line used in this study

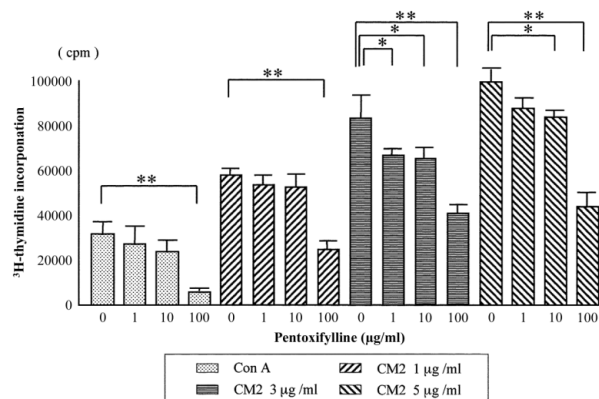


Fig4. T cell proliferation of myocarditogenic T cell lines specific to CM2 (aa 1,539–1,555) of cardiac myosin with nominal antigen (CM2) or mitogen (concanavalin A (Con A)) and PTX in different concentrations. This is a representative result of 3 experimental series. * $p < 0.05$; ** $p < 0.01$.

was shown to be capable of producing very high amounts of IFN- γ and TNF- α , as 'Th1 cytokines', but not IL-4, a 'Th2 cytokine' (data not shown). In conjunction with the T cell proliferation assay, we studied the effects of PTX on the production of TNF- α and IFN- γ by the Th1-like CD4 $^{+}$ lymphocytes using the supernatant of the culture medium co-stimulated with the antigen. As shown in Fig5, TNF- α and IFN- γ production was strongly downregulated in a dose-dependent manner and the dose dependency of the inhibitory effect on cytokine secretion was much more pronounced compared with T cell proliferation.

Discussion

This is the first report to demonstrate the efficacy of PTX in suppressing a cardiac disorder in a specific animal model, as well as analyzing its mechanism. We observed inhibitory effects of PTX on the induction of rat EAM as well as on myocarditogenic T cell proliferation in vitro. As to the immunomodulatory effects of PTX in vivo, a different modulation of the cytokine profile was seen in the diseased hearts and PBMC.

Beneficial Effects of PTX in EAM

PTX, 3, 7-dimethyl-1-(5-oxohexyl) xanthine, is a widely used vasoactive drug with proven clinical efficiency in microcirculatory disorders. In recent years, the potential use of PTX as an antiinflammatory drug has gained increasing interest because in-vitro and in-vivo studies have shown that PTX suppresses or reduces the production of TNF- α , and beneficial effects have been reported in disorders such as graft-versus-host disease,¹⁶ cerebral malaria¹⁷ and acute hepatitis,¹⁸ in which TNF- α has an important role. It has been reported that PTX can inhibit the onset or severity of other organ-specific autoimmune diseases (eg, experimental autoimmune encephalomyelitis, which has been regarded as analogous to human multiple sclerosis)^{19,20} We demonstrated histologically the inhibitory effect of PTX on myocardial inflammation, although there was no difference in the clinical status and survival between the PTX group and the control group.

Smith et al demonstrated that anti-TNF- α antibody reduced the severity of EAM only when given before, not after, antigen immunization²¹ which suggested that TNF-

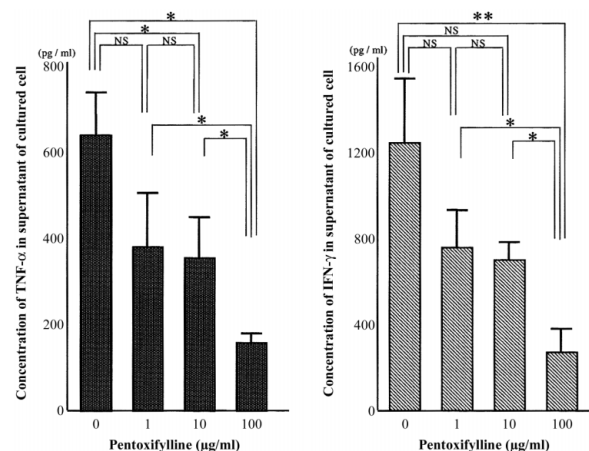


Fig5. Cytokine production in the supernatant of TNF- α (A) and IFN- γ (B) together with CM2-specific T cell stimulation. This is a representative result of 2 experimental series. * $p < 0.05$; ** $p < 0.01$.

is an important early mediator in the pathogenesis of inflammation. In addition, that report showed that the administration of PTX could suppress the onset or disease severity only when given in the induction phase; that is, from pre-immunization, whereas it was merely effective when given in the effector phase only.²² The immunomodulatory effects of PTX are considered to be dose-dependent in vivo;²³ as well as in vitro, as confirmed by the present study. Because an acute and subacute toxicity test in rats showed that less than 400 mg/kg per day of oral PTX had no adverse effects;²⁴ we decided to use the oral administration route and a dosage of 150 mg/kg per day bid from the induction phase.

Immunomodulatory Mechanisms of PTX in EAM

We previously reported that cytokines relating to inflammation are involved in the induction and resolution of EAM;²⁵ and in the current study we used heart specimens to demonstrate that Th1 type cytokines (IL-2 and IFN- γ), mainly produced by the infiltrating lymphocytes, and proinflammatory cytokines (IL-1 and TNF- α), mainly produced by the infiltrating macrophages, were detected only in the inflammatory phase (Day 19–25) of EAM and were subsequently followed by expression of a Th2 cytokine, IL-10, as well as transforming growth factor- β 1 in the recovery phase. We used the mRNA levels, not protein levels, of cytokines to investigate their contribution to the pathogenesis for 2 reasons. First, the plasma concentrations of the representative cytokines, TNF- α and IFN- γ , could only be detected within established normal values throughout the disease course with the commercial ELISA kit we used in the T cell proliferation assays. Second, a previous study had demonstrated that the inhibitory effect of PTX on TNF- α production is exerted at the transcriptional level, by elevating the intracellular level of the second messenger cAMP.^{17,26}

Interestingly, we found that cytokine mRNA expression in the heart after PTX treatment is dissociated from that in the peripheral cells. The levels of all cytokines examined were significantly decreased in PBMC, but those of IL-4 and IL-6 were significantly increased in the heart specimens. PTX exerts a suppressive effect mainly on Th1-associated cytokine production, without affecting²⁰ or even increasing²³ Th2-associated cytokines. On the other hand,

it has been reported that PTX at high concentration suppressed Th2 cytokine production with a corresponding suppression of Th1 cytokines.²⁷ Rott et al demonstrated in a compact tissue that the systemic effects of a systemically administered drug were more robust than the regional effects, presumably because of differences in tissue distribution with oral PTX administration,²⁰ which would explain our finding of the different cytokine expression in the heart and the PBMC. Furthermore, it might be that the inhibitory mechanism of PTX in actively immunized EAM derives mainly from suppression of antigen-priming in the peripheral cells, not from regional defensive effects in the heart against the cellular infiltration.

Although we have proposed a mechanism of the effect of PTX based on the findings of the present study, there may be other explanations. For example, PTX has been found to inhibit apoptosis in different human cell types,²⁸ so it may prevent the apoptotic cell death of cardiomyocytes and lymphocytes involved in the pathogenesis of EAM.

Clinical Implications

Although a causal link between viral myocarditis and DCM has long been recognized, the number of transplant cases with viral infection is less than expected.²⁹ Instead, it is probable that an episode of subclinical viral myocarditis can initiate an autoimmune reaction that culminates in DCM.^{7,30} In ventriculectomized cardiac tissue specimens of human DCM, cell infiltrates could be seen in the myocardium,³¹ which supports the involvement of the inflammatory process, including autoimmune mechanisms, in human DCM and suggests that it is rare for the autoimmune mechanism to be elicited from the very beginning in DCM. The animal model of EAM used in the present study can produce DCM by repeat immunization with cardiac myosin and taken together, these facts indicate that the immunomodulatory effects of PTX even in the induction phase of the autoimmune mechanism are involved in human DCM. However, further investigation of the clinical efficacy of PTX on disease progression or the chronic phase is necessary.

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