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Effects of Pyrrolidine Dithiocarbamate, a NF- κ B Inhibitor, on Cytokines Expression and Ocular inflammation in Experimental Autoimmune Anterior Uveitis

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ABSTRACT

This study was conducted to investigate effects of pyrrolidine dithiocarbamate (PDTC), a nuclear factor (NF)- κ B inhibitor, on cytokine expressions and suppression of anterior chamber inflammation in experimental autoimmune anterior uveitis. Uveitis was induced in the Lewis rats with the injection of melanin-associated antigen into peritoneum and footpad. At defined time points, cytokine mRNA expressions in the iris/ciliary body were measured by using a semiquantitative polymerase chain reaction method. We found that interferon- γ (IFN- γ) and tumor necrosis factor (TNF)- α mRNA expression reached peak during the active phase of uveitis, whereas interleukin (IL)-10 mRNA increased during the disease resolution. In a separate experiment, PDTC (40mg/kg/day) was administrated intraperitoneally after immunization. We found that PDTC effectively suppressed ocular inflammation, as indicated by reduced clinical scores and inflammatory cells infiltration in aqueous humor and iris/ciliary body. The inhibitory effects of PDTC are mainly resulted from inhibiting the expression of proinflammatory cytokines, TNF- α and IFN- γ but augmenting anti-inflammatory cytokines, IL-10 expression. These findings suggest that application of NF- κ B inhibitors may be a potential therapeutic method for the treatment of acute anterior uveitis.

INTRODUCTION

Uveitis is a major cause of visual defect. The most common form of human uveitis is acute anterior uveitis of unknown etiology (1). The recurrent nature of anterior uveitis may lead to many secondary complications such as cataract, cystoid macular edema and glaucoma. Because the exact mechanism of acute anterior uveitis remains unknown, nonspecific corticosteroids are still the mainstay for the uveitis treatment. However, complications of corticosteroid therapy limit its clinical applications.

Experimental autoimmune anterior uveitis (EAAU) is a well-established animal model for acute anterior uveitis (2). EAAU is induced by immunizing animals with bovine melanin-associated antigen (MAA) (3, 4). The clinical course in EAAU is very similar to that of human AAU (5, 6). Studies on the molecular mechanisms and treatment of EAAU would help us to gain insights into the pathogenesis of human AAU and to develop effective therapeutic drugs that block the activation of inflammatory signaling pathways.

Cytokines were important inflammatory mediators that had been proven to be involved in a variety of inflammatory diseases (7, 8). Cytokines are mainly secreted by helper T (Th) cells. Based on functional characteristics, Th cells are classified into Th1 and Th2 (9). Th1 cells produce IL-2 and interferon (IFN)- γ ; Th2 cells produce IL-4, IL-6, IL-10. Th1 cytokines are known as pro-inflammatory cytokines, because they exacerbate inflammation. On the other hand, Th2 cytokines play an anti-inflammatory role since they inhibit inflammation initiated by Th1 cytokines (10, 11). In many human diseases, Th1 cells are associated with the initiation of diseases, whereas Th2 cells are related to suppression of the diseases (12-14).

The nuclear factor kappa B (NF- κ B) is a transcriptional factor that can regulate the expression of pro-inflammatory genes, including cytokines, adhesion molecules and chemokines (15, 16). NF- κ B has been proven to play a central role in a variety of inflammatory diseases (17). The identification of NF- κ B as a key player in the pathogenesis of inflammatory reactions suggests that NF- κ B-targeted therapeutics might be effective in the treatment of inflammatory diseases.

This study was designed to investigate the expressions of cytokines in iris/ciliary body using a rat model of acute anterior uveitis. The effect of pyrrolidine dithiocarbamate (PDTC), a potent NF- κ B inhibitor (18-20), on the expression of cytokines and ocular inflammation in EAAU was evaluated to gain a better insight into the pathogenesis of acute anterior uveitis.

MATERIALS AND METHODS

Antigen and Induction of EAAU

MAA was prepared as previously described by Borekhuyse and Kuhlmann with a modification (2). The iris and ciliary body were carefully obtained from fresh pigmented bovine eyes. The tissue was gently homogenized and filtered through a wire mesh to remove cellular debris and connective tissue. The

homogenate was centrifuged at $1.2 \times 10^5 g$ at 4°C for 15 minutes and washed once with phosphate-buffered saline (PBS) at pH 7.4. The resulting pellet was suspended in 2% sodium dodecyl sulfate (SDS) (Bio-Rad, Richmond, CA) and incubated at 70°C for 10 minutes. After centrifugation, the pellet was washed three times with water. The insoluble antigen was dried and stored at -20°C.

Lewis rats, 6–8 weeks old and weighing 125–160 g, were used for the experiment. All animals were treated in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. To induce EAAU, the rats were given two separate injections at the same time: 1). 0.05 ml MAA was suspended in PBS, emulsified (1:1) in complete Freund's adjuvant (CFA) and injected into left hind footpad 2). 0.05 ml MAA, emulsified with 1µg purified Bordetella pertussis toxin (PTX) was injected intraperitoneally.

PDTC Treatment

The treatment protocol was conducted in a randomized and double-masked fashion. The rats were divided into two groups as follows:

(1). Group A received intraperitoneal injection of PDTC (40 mg/kg) daily since day 1. (2). Group B received intraperitoneal injection of PBS daily as a control since day 1.

Clinical Activity Scoring

The rats were clinically observed on a daily basis with slitlamp biomicroscopy for clinical signs of ocular inflammation. Disease severity was assessed with a scale ranging from 0 to 4: 0 = normal; 1 = slight iris-vessel dilatation and some anterior chamber cells; 2 = iris hyperemia, with some limitation in pupil dilation, anterior chamber cells, and a slight flare; 3 = a miotic, irregular, hyperaemic, and (sometimes) slightly damaged iris, with a considerable flare and cells (especially with accumulation near the iris); and 4 = a seriously damaged and hyperaemic iris, a miotic pupil often filled with protein, and cloudy gel-like aqueous humor (AqH).

Tissue Preparation

Rats were sacrificed on day 3, 9, 11, 14, 18, 25. The eyes were harvested. The eyes were quickly dissected, and the iris and ciliary body were isolated from the remaining ocular tissue by using an operating microscope.

Preparation of RNA and cDNA

Total RNA was extracted from the iris/ciliary body with Trizol reagent (Life, Gaithersburg, MD). One microgram of total RNA from each sample was annealed for 5 min at 65°C with 300-ng oligo(dT)(Promega, Madison, WI) and reverse transcribed to cDNA by using 80 U Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Gibco, Grand Island, NY) per 50 µg reaction for 1 h at 37°C. The reaction was stopped by heating for 5 min at 90°C.

Polymerase Chain Reaction

The amplification was performed with a thermocycler. (MJ Research, Waltham, MA) The 50- μ l reaction mixture consisted of 5 μ l cDNA, 1 μ l of sense and antisense primer, 200 μ M of each deoxynucleotide, 5 μ l 10 $\times$ Taq polymerase buffer, and 1.25 U Taq polymerase (Promega, Madison, WI). Conditions for amplifying each cytokines were as follows: denaturation, 1 min at 94°C, and elongation, 3 min at 72°C. For the annealing temperature for cytokines, 62°C to 42°C was designed for IL-4, IL-10 and IFN- γ . The temperature then declined at 1°C increments and this was followed by 20 cycles at 55°C. The annealing temperature for TNF- α , IL-6, TGF- β 1 and TGF- β 2 was from 67°C to 50°C, declining at 1°C increment and followed by 21 cycles at 60°C. At the end of amplification, the reaction mixture was heated for 10 min at 72°C and then cooled to 4°C. A 10- μ l sample of each polymerase chain reaction (PCR) product was separated by gel electrophoresis on 2% agarose containing ethidium bromide (Sigma, St. Louis, MO) and then analyzed under ultraviolet light against the DNA molecular length markers. The intensity of the products was analyzed by using an image analyzer (Digital 1D Science; Eastman Kodak, Rochester, NY), and the amount of PCR-amplifiable material in each reverse-transcribed sample was standardized against the amount of a housekeeping gene rat β -actin. The primers sequences were listed in Table 1.

Quantification of Leukocytes in Aqueous Humor

Aqueous humor (AqH) was collected from the eyes using a 30-gauge needle immediately after the animal was sacrificed on day 18. The AqH was pooled in silicon-treated microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA). Two microlitre (2 μ L) AqH from one rat was stained with 0.4% trypan-blue solution and the number of leukocytes was counted under phase-contrast microscopy

Histopathologic Evaluation

On day 18, rats were sacrificed. The eyes were enucleated and embedded in paraffin. Then, 5- μ m sagittal sections were cured and stained with hematoxylin and eosin (H-E)

Statistical Analyses

Differences among the amounts of the cytokines mRNA at different time points were evaluated using Mann-Whitney U-test. Values of $p < 0.05$ were considered as significant.

TABLE 1 Sequences of Rat Cytokines and β -actin Primers Used in RT-PCR

Cytokines	Sequences	Product size (bp)
β -actin	5'-CTGGAGAAGAGCTATGAGCTG-3' 5'-AATCTCCTTCTGCATCCTGTC-3'	246
TNF- α	5'-TACTGAACTTCGGGGTGATTGGTCC-3' 5'-CAGCCTTGTCCTTGAAGAGAACC-3'	555
IFN- γ	5'-ATCTGGAGGAACTGGCAAAAGGACG-3' 5'-CCTTAGGCTAGATTCTGGTGACAGC-3'	287
IL-4	5'-ATGCACCGAGATGTTTGTACC-3' 5'-GGTACAAACATCTCGGTGCAT-3'	230
IL-6	5'-CAAGAGACTTCCAGCCAGTTG-3' 5'-TTGCCGAGTAGACCTCATAGTGACC-3'	683
IL-10	5'-AAGGACCAGCTGGACAACAT-3' 5'-AGACACCTTTGTCTTGGAGCTTA-3'	291
TGF- β 2	5'-CCTGCTGTACCTTCATACCGT-3' 5'-ATCATCGTCATTGTCGTCATA-3'	921

RESULTS

Clinical Scores of EAAU and Cytokine mRNA Expressions in Iris/CB During EAAU

Clinical signs of EAAU showed on day 11 post-immunization. The clinical symptoms reached peak on day 18 and entirely relieved by day 30.

TNF- α mRNA was detected in parallel with the disease progression. As the disease resolved, TNF- α declined to normal control level. IFN- γ started increasing on day 11, concurrent with the disease onset. At maximal clinical activity, however, IFN- γ mRNA dropped abruptly to the control level. IL-10 mRNA increased on day 18 and 25, correlating with remission of the disease. The expression of TGF- β 2 mRNA was remained at a high level. However, when compared with normal control, there was no statistically significant change in TGF- β 2 mRNA during the course of the disease. The mRNA of IL-4 and IL-6 was undetectable throughout clinical evaluation (Figure 1).

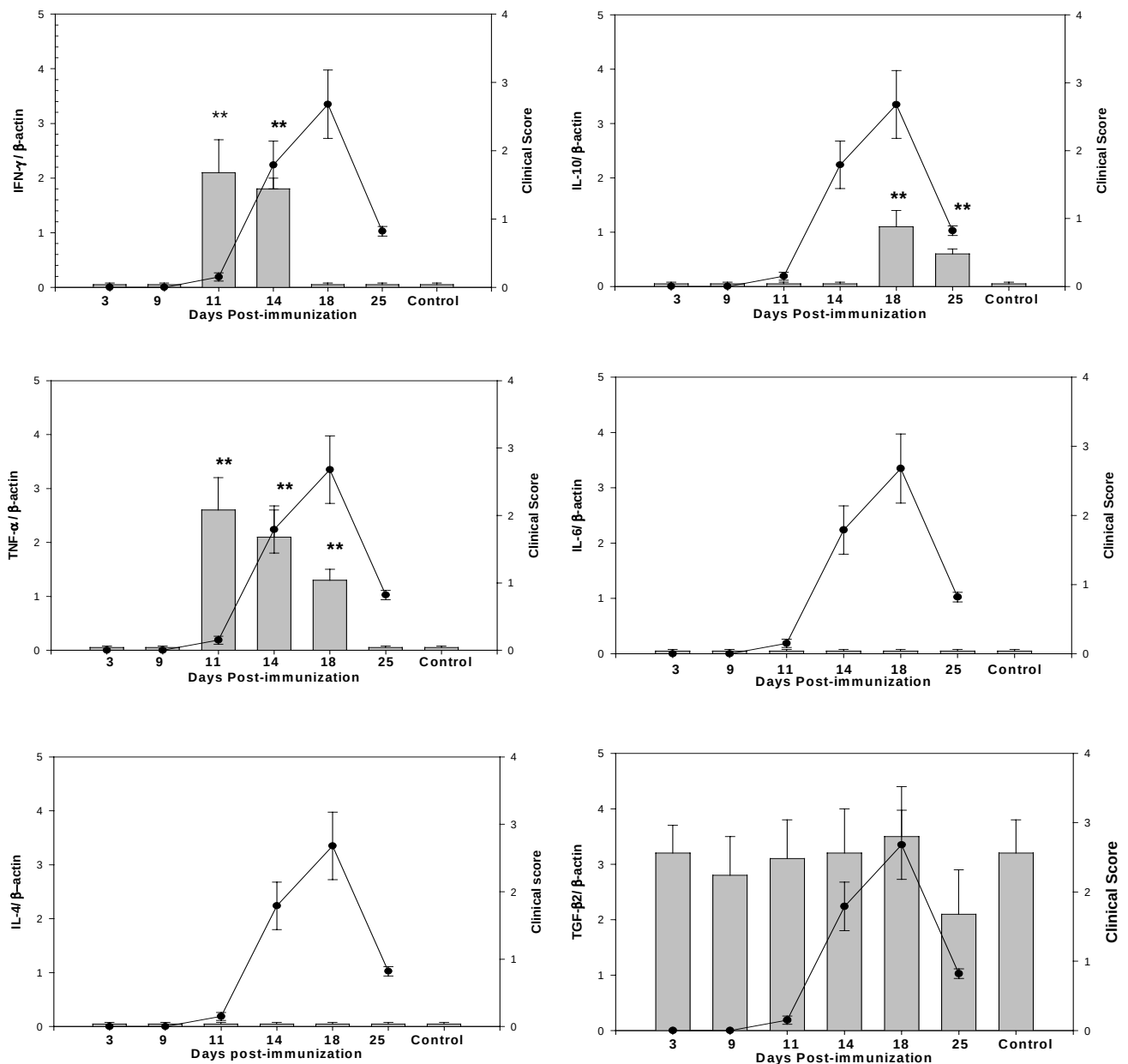


FIGURE 1 Expression of mRNA of cytokines including: TNF- α , IFN- γ , TGF- β 2, IL-4 and IL-10 in the iris/ciliary body from Lewis rats at different time points during the course of EAAU. The control represents the normal rats not being immunized. Bar charts represent the relative intensity of mRNA of cytokines compared to β -actin. Line charts correspond to the clinical scores of the disease. Data are presented as the mean $\pm$ SD in five rats. * $p < 0.05$, ** $p < 0.01$ when compared to normal control

Effects of PDTC on Clinical Activity Scores

Rats treated with PDTC (40 mg/kg) demonstrated a significant reduction in clinical severity than those treated with PBS. The maximal clinical scores in rats treated with PDTC (40 mg/kg) was significantly lower than in those with PBS (Figure 2).

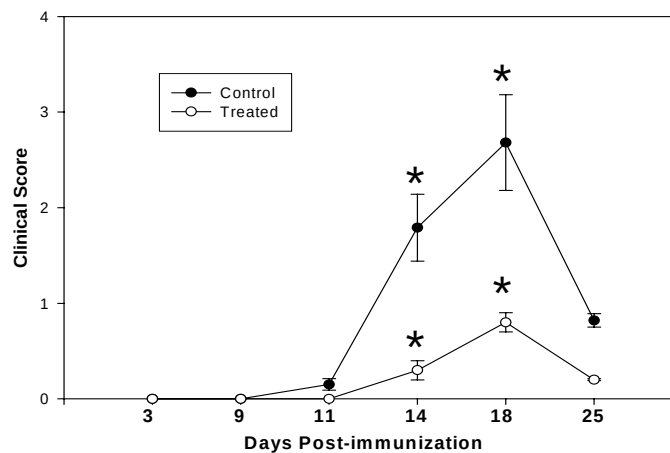


FIGURE 2 Effects of PDTC on clinical scores of Lewis rats with EAAU. Rats treated with PDTC (40 mg/kg) showed a significant reduction in clinical severity than those treated with PBS. * : Mann-Whitney U-test, $p < 0.05$.

Effects of PDTC on Cellular Infiltration in Aqueous Humor

Rats were sacrificed on day 18 and the cells in the anterior chamber were quantified. PDTC treated eyes showed a lower number of cellular infiltrations; the number of infiltrating cells was 960 ± 160 cells/ml in the control rats and treatment with PDTC resulted in a significant reduction of the cell number to 240 ± 30 cells/ml ($p = 0.001$)

Effects of PDTC on Histologic Changes

Leukocytes, mainly lymphocytes, were found to infiltrate in iris/ciliary body of rats on day 18. (Fig. 3, left panel) The number of leukocytes in iris/ciliary body was markedly reduced after PDTC treatment. (Fig. 3, right panel)

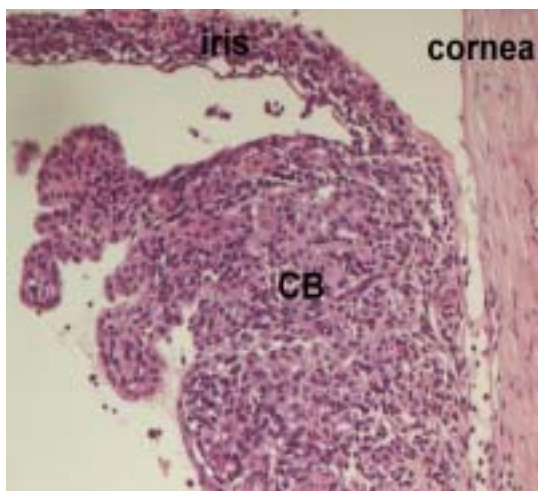


Figure 3 Histological section of eyes on the day 18 after immunization. Rats treated with PBS (left panel); Rats treated with PDTC (40 mg/ml) (right panel). H-E staining; original magnification X 400.

Effects of PDTC of Cytokines mRNA Expression in Iris/Ciliary Body

On day 14, treatment with PDTC demonstrated a significant reduction in IFN- γ mRNA expression in the iris/ciliary body ($p=0.001$). On day 18, treatment with PDTC resulted in significantly reduction in TNF- α mRNA expression ($p=0.001$). In contrast, treatment with PDTC augmented IL-10 mRNA expression when compared with control ($p=0.001$). There was no significant difference in TGF- $\beta 2$ mRNA expression between PDTC treated and untreated groups (Fig 4).

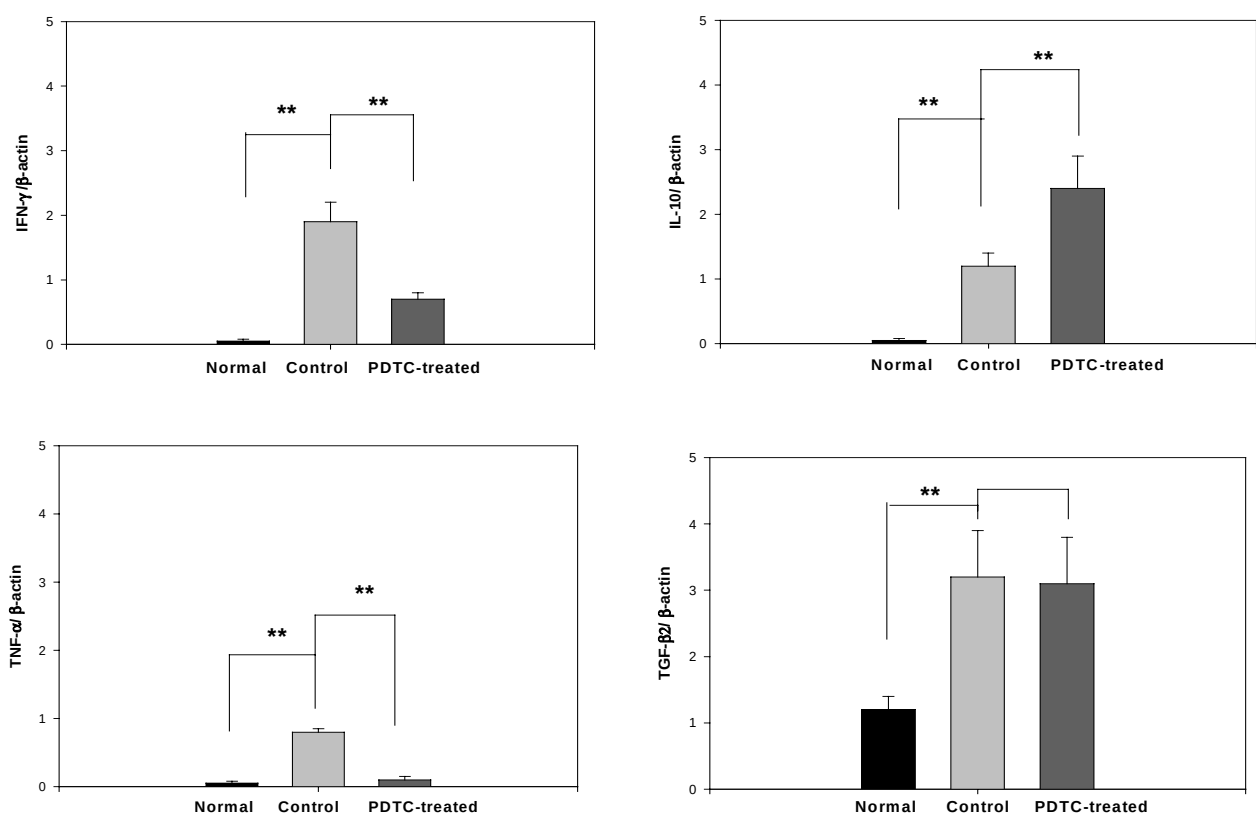


FIGURE 4 Effects of PDTC on cytokine mRNA expression in iris/ciliary body of Lewis rats with EAAU. Data are presented as the mean $\pm$ SD in five rats. **: Mann-Whitney U-test, $p<0.01$.

DISCUSSION

In the present study, we demonstrated that pro-inflammatory cytokines, IFN- γ and TNF- α , increased in parallel with the disease initiation, whereas anti-inflammatory cytokine, IL-10, increased during the

recovery periods of the disease. In addition, we investigated the *in vivo* effects of PDTC, a NF- κ B inhibitor, on the expression of cytokines in rat model of acute anterior uveitis. We found that PDTC effectively suppressed ocular inflammations, as indicated by reduced clinical activity scores, diminished leukocyte infiltration in iris/ciliary body and aqueous humor. These anti-inflammatory effects are mainly by means of decreasing TNF- α , IFN- γ , as well as augmenting IL-10 expression. These results indicated that timely expression of certain cytokines is critical in the pathogenesis of EAAU and PDTC exerted its anti-inflammatory effect by regulating the expressions of specific cytokines.

Previous studies reported that expressions of TNF- α and IFN- γ are correlated with disease progression in several autoimmune diseases (21, 22). TNF- α was reported to play a critical role in the initiation of inflammatory reactions, including the activation of T cells (23), induction of vascular cell adhesion molecule-1 (24), and activation of leukocytes (25), all of which could contribute to the tissue destruction in the autoimmune diseases. IFN- γ enhanced the cytotoxic effect of TNF- α and also activated macrophages (26, 27). These mechanisms presumably mediate the pro-inflammatory roles of TNF- α and IFN- γ in generating uveitis. In our study, decreased IFN- γ and TNF- α expression by PDTC resulted in diminished clinical severity of uveitis, further supporting the pivotal role of IFN- γ and TNF- α in the pathogenesis of uveitis.

In our study, IL-10 mRNA increased in association with the disease resolution, whereas IL-4, IL-6 mRNA did not change during EAAU. Previous report demonstrated that IL-6 was activated to antagonize anterior chamber-associated immune deviation (ACAID) in rat model of endotoxin-induced uveitis (28). The difference between our study and that reported was due to difference in the methods of inducing the experimental uveitis. IL-10 was reported to be a potent suppressor of macrophages, T-cells, and NK cells and suppressed the growth and transformation of Th1 cells (29). Therefore, at disease resolution, IL-10 helps to reduce inflammation and restore ocular immune privilege.

PDTC, a NF- κ B inhibitor, may suppress pro-inflammatory cytokine, TNF- α , expression but enhance anti-inflammatory cytokine, IL-10, expression, thereby reduce inflammations. Nemeth *et al* demonstrated the reduction in TNF- α and increase in IL-10 expression after PDTC treatment in LPS-induced endotoxemia mouse (30). However, since both IL-10 and TNF- α genome contain NF- κ B-like recognition sites (31, 32), it is possible that PDTC may influence the expression of these two cytokines with different mechanisms. Recent studies demonstrated that PDTC could augment protein-1 (AP-1) activation, which was found to be presented in the IL-10 genome (33, 34). We therefore postulated that the enhanced activation of AP-1 by PDTC was responsible for the increased IL-10 levels. However, further studies are required to support this finding.

TGF- β is thought to contribute to immunosuppressive properties and is important for the maintenance of the immune-privileged environment of the anterior chamber (35, 36). Several studies demonstrated reduction of TGF- β activity in aqueous humor of uveitis (37, 38). In the present study, however, we could not find any significant alterations in mRNA expression of TGF- β 2 during EAAU. TGF- β is secreted in a latent complex that needs to be transformed into mature TGF- β to become biologically active (39). In this study, we principally tested the regulation of cytokines at the level of transcription. The level of mRNA detected for

TGF- β may therefore have little relationship to biological function.

PDTC represents a class of antioxidants reported to be a potent inhibitor of NF- κ B (19, 20, 40). In addition, PTDC has been shown to block I κ B- α phosphorylation, precluding the dissociation of NF- κ B from I κ B- α and subsequent NF- κ B translocation from the nucleus in response to inflammatory stimulation. The potential for modulating cell activation suggests that PDTC and its analogs may offer therapeutic benefit in inflammatory conditions in which activation of NF- κ B plays a major role.

There are several limitations in our study, which was designed to be a pilot experiment to examine the expressions of various cytokines and then determines the effects of nuclear factor (NF)- κ B inhibitor, on the expressions of these cytokines. Therefore, we screened the expression and regulation of cytokines only at the transcription level. To obtain more information on the roles of cytokines in uveitis, further study with expressions of major cytokine protein in iris/ ciliary body is needed. Secondly, despite PDTC successfully alleviated the clinical signs of EAAU, PDTC-treated rats showed a retarded body weight gain, suggesting the presence of a toxic action of PDTC (41). The ultimate benefit of such targeted therapy will depend on the delicate balance between suppressing inflammatory and interfering with normal cellular functions. By selectively targeting specific NF- κ B subunits that have a degree of tissue specificity, one might attain therapeutic efficacy and minimize systemic toxicity.

In conclusion, the present study demonstrated the roles of IFN- γ and TNF- α in the disease initiation and proration and IL-10 in resolution of EAAU. PDTC showed important modulatory effects on cytokine expression and effectively suppressed ocular inflammation. These effects are mainly by means of inhibiting the expression of proinflammatory cytokines, TNF- α but augmenting anti-inflammatory cytokines expression. These findings suggest that PDTC may be a valuable therapeutic alternative to uveitis.

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