

# Cytokine Production by T Cells Infiltrating in the Eye of Uveitis Patients

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**Abstract:** The capacity of T cells to produce cytokines was investigated using T-cell clones (TCCs) established from infiltrating cells in the aqueous humor (AH) or peripheral blood mononuclear cells (PBMC) of patients with Vogt-Koyanagi-Harada (VKH) disease or sarcoidosis. The cytokines produced and tested in the study were interleukin (IL)-1 $\alpha$ , IL-6, IL-8, interferon (IFN)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , and granulocyte monocyte colony stimulating factor (GM-CSF). All TCCs ( $n = 9$ ) from AH of VKH patients spontaneously produced significantly larger amounts of IL-6, IL-8, and IFN- $\gamma$  than TCCs from healthy donor PBMC. All TCCs ( $n = 9$ ) from AH of the sarcoidosis patient spontaneously produced significantly larger amounts of IL-1 $\alpha$ , IL-6, and IL-8 than TCCs from healthy donor PBMC. In addition, the effects of antiinflammatory drugs on the cytokine production by the TCCs were investigated. Hydrocortisone significantly suppressed the production of IL-6, IL-8, and GM-CSF by TCCs from AH of VKH patients. Tacrolimus also significantly suppressed the production of IL-8 and GM-CSF by the TCCs. FTY720, an experimental drug, suppressed only GM-CSF production by TCCs from AH of VKH patients. Diclofenac failed to suppress the production of any cytokines by any TCCs. All tested drugs did not suppress the production of cytokines by TCCs from the sarcoidosis patient. These results thus suggest that cytokines produced by T cells infiltrating in the eye may play an important role in the pathogenesis of uveitis. **Jpn J Ophthalmol 1998;42:262–268** © 1998 Japanese Ophthalmological Society

**Key Words:** Cytokines, FTY720, hydrocortisone, sarcoidosis, T-cell clones, tacrolimus, Vogt-Koyanagi-Harada disease.

## Introduction

We previously reported that T-cell clones (TCCs) established from ocular infiltrating cells of patients with human T lymphotropic virus type 1 (HTLV-I) uveitis were capable, without any stimulation, of producing large amounts of cytokines, such as interleukin (IL)-1 $\alpha$ , IL-3, IL-6, IL-8, tumor necrosis fac-

tor (TNF)- $\alpha$ , interferon (IFN)- $\gamma$ , and granulocyte monocyte colony stimulating factor (GM-CSF).<sup>1,2</sup> We also demonstrated that TCCs from ocular infiltrating cells of Behçet's disease patients produced IL-8 and TNF- $\alpha$ .<sup>3</sup> Because cytokines are known to play significant roles in immunologic and inflammatory responses, it would be important to clarify whether the profile of the cytokine produced by ocular infiltrating cells might differ among different clinical entities of uveitis. The present study was, therefore, aimed at examining the cytokine production by TCCs derived from patients with Vogt-Koyanagi-Harada (VKH) disease and sarcoidosis, and making a comparison with the production by Behçet's disease and HTLV-I uveitis TCCs. This study also investigated the effects of antiinflammatory drugs on cytokine production by these TCCs.

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## Materials and Methods

### Subjects

Following are brief clinical histories of the three patients whose aqueous humor (AH) and peripheral blood mononuclear cells (PBMC) were used in this study. This study was approved by the Internal Ethical Reviewer Committee of Kurume University, and informed consent was given by the patients before the samples were collected. The research followed the tenets of the Declaration of Helsinki.

**Patient 1.** A 75-year-old woman was referred to Kurume University Hospital Eye Clinic on August 3, 1994. She complained of sudden onset of ocular floaters and blurring of vision in both eyes. Ocular examinations disclosed mild iridocyclitis, multifocal exudative retinal detachment, and hyperemia and edema of the optic disc. Systemic examinations disclosed sensorineural deafness and aseptic meningitis with pleocytosis in the cerebrospinal fluid. The patient's HLA was A2, A24, B35, B54, Cw1, DR4, DR9, DQ3, DQ4. Based on these ocular and systemic findings, the diagnosis of VKH disease was made. Samples of AH (0.1 mL) and heparinized peripheral blood (20 mL) were taken during the therapy with topical and systemic corticosteroids.

**Patient 2.** A 58-year-old man was referred to Kurume University Hospital Eye Clinic on July 4, 1994, complaining of sudden onset of metamorphopsia in both eyes. Ocular examinations disclosed iridocyclitis, multifocal exudative retinal detachment accompanied by sensorineural deafness, and pleocytosis in the cerebrospinal fluid. The patient's HLA was A2, A11, B51, B62, Cw4, DR4. The patient was diagnosed as having VKH disease. Samples of AH (0.1 mL) and heparinized peripheral blood (20 mL) were taken during the treatment with corticosteroids.

**Patient 3.** A 20-year-old woman was referred to Kurume University Hospital Eye Clinic on July 26, 1993, for the ocular examinations for systemic sarcoidosis. Ocular examinations disclosed iridocyclitis with mutton-fat keratic precipitates, snowball opacities in the vitreous body, and chorioretinal exudates. Bilateral lymphadenopathy was detected by chest radiography and positive histologic findings by transbronchial lung biopsy. The patient's HLA was A31, A33, B35, B51, Cw4, DR4. Samples of AH (0.1 mL) and heparinized peripheral blood (20 mL) were taken before the treatment with corticosteroids.

### Establishment of TCCs

T cells were expanded by the limiting dilution methods reported previously.<sup>1</sup> Briefly, cells from AH or PBMC of the two patients with VKH disease or one patient with sarcoidosis, or PBMC from two healthy donors (males, 27 and 31 years old, negative for HTLV-I, hepatitis virus type -A, -B, and -C, and human immunodeficiency virus) were diluted and placed at a concentration of one cell per well in 96-well U-bottom tissue culture plates (Falcon, Lincoln Park, NJ, USA) in the presence of  $2 \times 10^5$ -irradiated (50 Gy) allogeneic PBMC obtained from healthy volunteers (negative for HTLV-I, hepatitis virus type-A, -B, and -C) as the feeder cells. The medium used for the culture was RPMI-1640 medium (GIBCO Laboratories, Grand Island, NY, USA) supplemented with 100 U/mL penicillin G, 50  $\mu$ g/mL streptomycin, 10% heat-inactivated fetal bovine serum (FBS) (Bioserum, Parkville, Victoria, Australia), and 100 U/mL human recombinant IL-2 (rIL-2, Shionogi Pharmaceutical, Osaka). The plated cells were incubated in humidified 5% CO<sub>2</sub> in air at 37°C. The feeder cells were added to each well along with 100 U/mL rIL-2 every 7 days until an outgrowth of cells was observed. The proliferating cells were maintained with the feeder cells and rIL-2 in 24-well tissue culture plates (Falcon) and used for further studies.

### Cytokine Assay

The TCCs were washed three times with RPMI 1640 medium, then cultivated at  $5 \times 10^5$ /mL in RPMI 1640 medium supplemented with 10% FBS and antibiotics in 48-well tissue culture plates (Falcon) for 22 hours with or without 10  $\mu$ g/mL of PHA-P (*phytohemagglutinin*) (Difco Laboratories, Detroit, MI, USA) or drugs (1  $\mu$ mol/L). The drugs tested in this study were tacrolimus (Fujisawa Pharmaceutical, Osaka), hydrocortisone (Sigma Chemical, St Louis, MO, USA), diclofenac sodium (Sigma), and FTY720 (Yoshitomi Pharmaceutical, Osaka). Cell-free culture supernatant from each TCC was obtained by centrifugation of the cell supernatant and stored at  $-80^\circ\text{C}$  until use.

The enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN, USA) were used to detect cytokines. The sensitivity of each assay kit was as follows: IL-1 $\alpha$  > 0.3 pg/mL, IL-2 > 6.0 pg/mL, IL-6 > 0.7 pg/mL, IL-8 > 6.0 pg/mL, TNF- $\alpha$  > 4.4 pg/mL, IFN- $\gamma$  > 3.0 pg/mL, and GM-CSF > 1.5 pg/mL.

**Table 1.** Spontaneous Production of Cytokine by T Cell Clones

Disease	Source	Cytokine (pg/mL)						
		IL-1 $\alpha$	IL-2	IL-6	IL-8	TNF- $\alpha$	IFN- $\gamma$	GM-CSF
Vogt-Koyanagi-Harada	AH	1.9 $\pm$ 1.4	2.4 $\pm$ 4.6	42 $\pm$ 26*	1088 $\pm$ 548**	48 $\pm$ 46	49 $\pm$ 42*	22 $\pm$ 15
	PBMC	2.0 $\pm$ 1.3	16 $\pm$ 24	17 $\pm$ 11	140 $\pm$ 61*	100 $\pm$ 71*	91 $\pm$ 89*	81 $\pm$ 98
Sarcoidosis	AH	8.5 $\pm$ 2.9**	2.6 $\pm$ 3.7	60 $\pm$ 25**	983 $\pm$ 604**	44 $\pm$ 49	18 $\pm$ 21	15 $\pm$ 6.4
	PBMC	2.2 $\pm$ 2.5	5.4 $\pm$ 5.5	22 $\pm$ 17	37 $\pm$ 15	24 $\pm$ 21	69 $\pm$ 102	29 $\pm$ 37
Healthy donor	PBMC	3.0 $\pm$ 3.0	7.4 $\pm$ 3.6	13 $\pm$ 5.0	73 $\pm$ 29	16 $\pm$ 14	0 $\pm$ 0	12 $\pm$ 4.0

IL-1 $\alpha$ : interleukin-1 $\alpha$ . IL-2: interleukin-2. IL-6: interleukin-6. IL-8: interleukin-8. TNF- $\alpha$ : tumor necrosis factor-alpha; IFN- $\gamma$ : gamma interferon. GM-CSF: granulocyte monocyte-colony stimulating factor. AH: aqueous humor. PBMC: peripheral blood mononuclear cells.

\* $P < .05$ . \*\* $P < .005$ , versus respective cytokine of healthy donor PBMC.

### Statistical Analysis

Results are given as the mean  $\pm$  standard deviation of cytokine levels or as percentage of controls. A statistical analysis was performed by a paired or nonpaired *t*-test. The difference between the two groups compared was determined to be statistically significant, when the *P* value was smaller than .05.

## Results

### Spontaneous Production of Cytokines by TCCs

Spontaneous production of various cytokines without any stimulation was investigated in nine TCCs (all CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> clones) from AH and five TCCs (all CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> clones) from PBMC of patients with VKH disease or sarcoidosis. The results are summarized in Table 1. Among the cytokines examined, IL-8 was produced in the largest amount by all the TCCs, particularly the TCCs from AH of VKH disease and sarcoidosis patients (mean: 1088 and 983 pg/mL, respectively.) Small but significant amounts of IL-1 $\alpha$ , IL-6, TNF- $\alpha$ , IFN- $\gamma$ , and GM-CSF were produced by the majority of the TCCs from AH and PBMC of VKH disease or sarcoidosis patients. Conversely, IL-2 was minimally produced by only a small proportion of these TCCs. In contrast to TCCs from uveitis patients, those from healthy donor PBMC (five clones, all CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> clones) produced only small amounts of cytokines.

As for the cytokine production by VKH disease TCCs, all TCCs from AH or VKH patients produced significant amounts of IL-6, IL-8, and IFN- $\gamma$ , and the difference between the levels of these cytokines and those from healthy donor PBMC was statistically significant (see Table 1). In addition, the IL-8 production by TCCs from AH was significantly higher than that from PBMC of the same patients (1223 pg/mL vs. 128 pg/mL,  $P < .05$ ). The majority of TCCs from AH also produced TNF- $\alpha$  and GM-CSF, but the

amounts were small and the difference was not statistically significant compared to those from donor PBMC.

As for the cytokine production by sarcoidosis TCCs, all TCCs from AH produced detectable levels of IL-1 $\alpha$ , IL-6, and IL-8, which were significantly higher than those from healthy donor PBMC (see Table 1). The IL-8 production by TCCs from AH of the sarcoidosis patient was significantly higher than that from PBMC of the same patient. The majority of TCCs from AH of the sarcoidosis patient also produced some other cytokines, such as TNF- $\alpha$ , IFN- $\gamma$  and GM-CSF, but the amounts of these cytokines were small and the difference was not statistically significant compared to those from healthy donor PBMC.

### Cytokine Production by PHA-Stimulated TCCs

T-cell clones from AH and PBMC of uveitis patients and those from healthy donor PBMC were examined after stimulation with PHA-P (10  $\mu$ g/mL). The results are summarized in Table 2. Phytohemagglutinin stimulation increased the capacity of TCCs from AH of VKH patients to produce almost all cytokines tested. A statistically significant increase was recorded in the production of IFN- $\gamma$  and GM-CSF by PHA-stimulated TCCs from AH of VKH patients as compared to nonstimulated TCCs. Very similar results were found in TCCs from PBMC of VKH patients. In contrast, PHA stimulation failed to cause significant changes in the production of any of the cytokines produced by TCCs from AH and PBMC of the sarcoidosis patient, or from healthy donor PBMC.

### Drug Effects on Cytokine Production by TCCs

Three therapeutic drugs (hydrocortisone, tacrolimus, and diclofenac) and one experimental drug (FTY720) were tested for their capacity to modulate

**Table 2.** Phytohemagglutinin-Stimulated Production of Cytokine by T Cell Clones

Disease	Source	Cytokine (pg/mL)						
		IL-1 $\alpha$	IL-2	IL-6	IL-8	TNF- $\alpha$	IFN- $\gamma$	GM-CSF
Vogt-Koyanagi-Harada	AH	3.3 $\pm$ 3.0	13 $\pm$ 17	134 $\pm$ 194	1223 $\pm$ 440	381 $\pm$ 453	186 $\pm$ 207*	537 $\pm$ 456*
	PBMC	1.1 $\pm$ 1.3	23 $\pm$ 23	25 $\pm$ 8.0	128 $\pm$ 70	291 $\pm$ 310	246 $\pm$ 208*	705 $\pm$ 440*
Sarcoidosis	AH	7.9 $\pm$ 2.0	9.0 $\pm$ 14	60 $\pm$ 25	984 $\pm$ 563	21 $\pm$ 13	46 $\pm$ 70	22 $\pm$ 17
	PBMC	1.7 $\pm$ 1.1	31 $\pm$ 30	13 $\pm$ 11	36 $\pm$ 10	74 $\pm$ 49	45 $\pm$ 41	60 $\pm$ 56
Healthy donor	PBMC	5.0 $\pm$ 4.0	4.2 $\pm$ 4.3	22 $\pm$ 10	82 $\pm$ 39	42 $\pm$ 46	6.7 $\pm$ 11	38 $\pm$ 21

IL-1 $\alpha$ : interleukin-1 $\alpha$ . IL-2: interleukin-2. IL-6: interleukin-6. IL-8: interleukin-8. TNF- $\alpha$ : tumor necrosis factor-alpha; IFN- $\gamma$ : gamma interferon. GM-CSF: granulocyte monocyte-colony stimulating factor. AH: aqueous humor. PBMC: peripheral blood mononuclear cells.

\* $P < .05$ , as compared with spontaneous production of respective cytokine shown in Table 1.

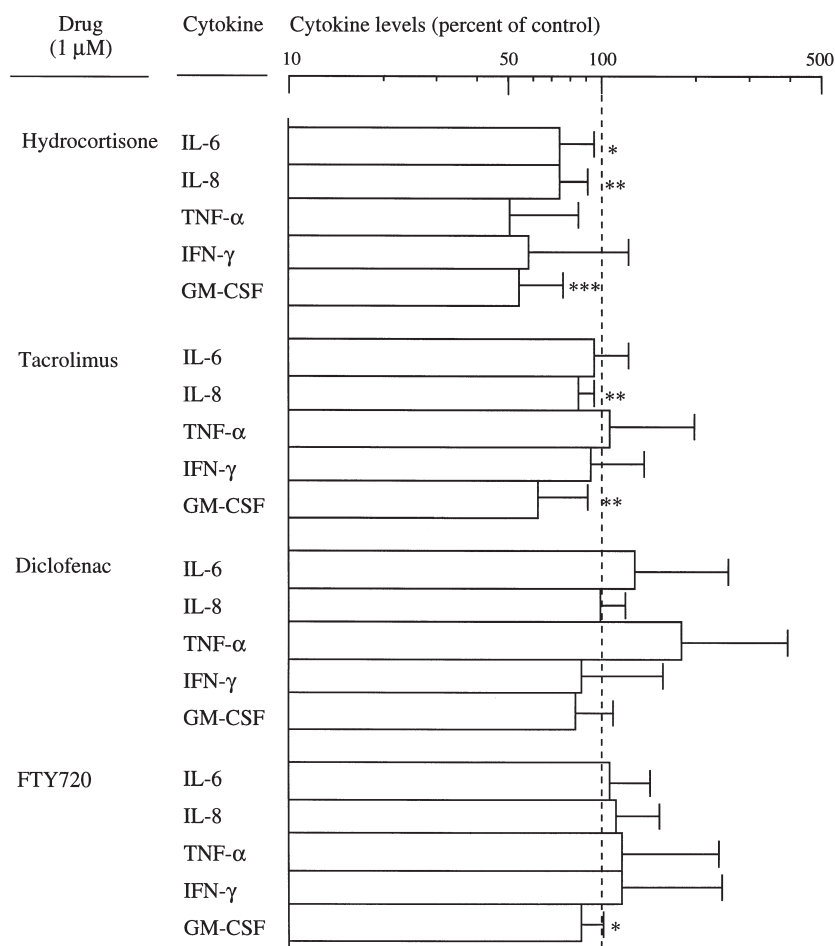
the cytokine production by TCCs from AH of the VKH and sarcoidosis patients. T-cell clones from AH of VKH patients were sensitive to the tested drugs (Figure 1). Hydrocortisone significantly suppressed the production of IL-6, IL-8, and GM-CSF. Tacrolimus caused significant suppression in the production of IL-8 and GM-CSF. FTY720 significantly suppressed GM-CSF production. Conversely, TCCs

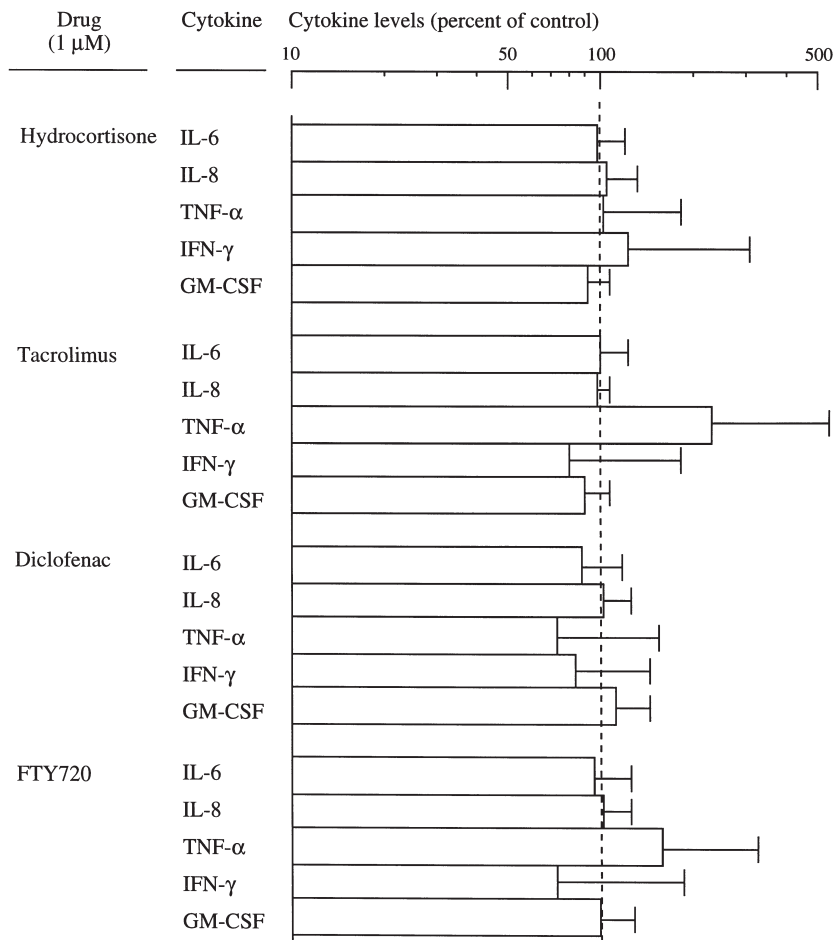
from AH of the sarcoidosis patient were not affected by any of these drugs in their capability to produce cytokines (Figure 2).

### Discussion

The role of cytokines in the pathophysiology and pathogenesis of uveitis has been studied in experi-

**Figure 1.** Drug effects on cytokine production by T-cell clones (TCC) from aqueous humor of Vogt-Koyanagi-Harada patients. Results are given as mean  $\pm$  standard deviation of percentage of control (cytokine production by TCCs from healthy donor peripheral blood mononuclear cells). Statistical analysis was performed by paired  $t$ -test. \* $P < .05$ . \*\* $P < .005$ . \*\*\* $P < .0005$ .





**Figure 2.** Drug effects on cytokine production by T-cell clones (TCC) from aqueous humor of sarcoidosis patient. Results are given as mean  $\pm$  standard deviation of percentage of control (cytokine production by TCCs from healthy donor peripheral blood mononuclear cells). Statistical analysis was performed by paired *t*-test.

mental animals<sup>4-10</sup> and some patients with uveitis.<sup>11-14</sup> However, no studies have analyzed cytokines produced by infiltrating cells in the uveitis eye. Therefore, we have devised a method to establish TCC from ocular infiltrating cells and to analyze the production of cytokines by these TCCs. Using this method, we previously reported on cytokines produced by TCCs from HTLV-I uveitis<sup>1,2</sup> and Behçet's disease<sup>3</sup> patients. This study further analyzes the cytokine production by TCCs of two other clinical entities of uveitis; that is, VKH disease and sarcoidosis.

The present study demonstrated that TCCs from AH of VKH patients spontaneously produced large amounts of IL-8, small but significant amounts of IL-6 and IFN- $\gamma$  in all TCCs examined, and moderate amounts of TNF- $\alpha$  and GM-CSF in the majority of tested TCCs (Table 1). Similarly, TCCs from AH of the sarcoidosis patient produced large amounts of IL-8, small but significant amounts of IL-6 and IL-1 $\alpha$  in all tested TCCs, and moderate amounts of TNF- $\alpha$ ,

IFN- $\gamma$ , and GM-CSF in the majority of tested TCCs. In our previous studies,<sup>1,2</sup> HTLV-I-infected TCCs established from ocular infiltrating cells of patients with HTLV-I uveitis were shown to produce large amounts of all tested cytokines except IL-4 (IL-1 $\alpha$ : 12699 pg/mL, IL-2: 61 pg/mL, IL-6: 8358 pg/mL, IL-8: 1268 pg/mL, TNF- $\alpha$ : 272 pg/mL, IFN- $\gamma$ : 5095 pg/mL, GM-CSF: 2886 pg/mL), whereas HTLV-I-noninfected TCCs from the disease failed to produce these cytokines. We also demonstrated that TCCs from ocular infiltrating cells of Behçet's disease produced large amounts of IL-8 (556 pg/mL); small but significant amounts of IL-1 $\alpha$  (7 pg/mL), IL-6 (15 pg/mL), and TNF- $\alpha$  (76 pg/mL) in the majority of TCCs examined; and smaller amounts of IFN- $\gamma$  and GM-CSF in one-half the TCCs tested.<sup>3</sup> The present study, along with the previous studies, indicates that significant amounts of cytokines are spontaneously produced by infiltrating T cells in the eyes of uveitis patients, and that the cytokine profile produced by

ocular infiltrating T cells appears to vary among different clinical entities of uveitis.

The extremely high activity of HTLV-I-infected T cells in producing various cytokines in the HTLV-I uveitis eye can be attributed to the molecular transactivation mechanisms of the virus. Tax 1, a viral protein encoded by the tax gene of HTLV-I, is known to transactivate the gene expression of various cytokines in the virus-infected T cells such as IL-1 $\alpha$ , IL-2, IL-6, INF- $\gamma$ , and GM-CSF.<sup>15</sup> Conversely, the infiltrating T cells of endogenous uveitis, such as Behçet's disease, VKH disease, and sarcoidosis, produce selective cytokines in much smaller amounts. Our studies have demonstrated that the common cytokine produced by TCCs from the three entities of endogenous uveitis was IL-8. Interleukin-8 is well known to have chemotactic and activating properties on neutrophils<sup>16-18</sup> and to be produced by a variety of cells including T cells.<sup>19,20</sup> It was reported that the amounts of IL-8 in the vitreous fluid from patients with various entities of uveitis were larger than those in control patients.<sup>11</sup> It is of note that the amounts of IL-8 produced by TCCs from ocular infiltrating cells in VKH disease were significantly larger than those produced by TCCs from PBMC in the same patients (Table 1). This was also true in the sarcoidosis patient. The mechanisms that cause the IL-8 production of infiltrating T cells in the eye to be higher than in the PBMC is unknown, but the significantly higher production of IL-8 in the eye might be one of the factors that contribute to the selective accumulation of neutrophils and other inflammatory cells in the eye, resulting in the augmented inflammatory process in the uveitis eye.

Other cytokines produced spontaneously by TCCs from ocular infiltrating cells in significantly larger amounts were IL-6 and INF- $\gamma$  in VKH disease, and IL-1 $\alpha$  and IL-6 in sarcoidosis (Table 1), and TNF- $\alpha$  in Behçet's disease. These cytokines are potent cytokines capable of mediating immune reaction and inflammation. Interleukin-1 $\alpha$  is a basic mediator of intercellular communication and is involved in the pathogenesis of autoimmune diseases and inflammation.<sup>21</sup> Moreover, injection of IL-1 $\alpha$  into the vitreous of rabbits caused anterior uveitis in the injected eye.<sup>4</sup> Interleukin-6 is a typical multifunctional cytokine with numerous biologic activities, including hemopoiesis and the acute phase response of inflammation.<sup>22</sup> In addition, IL-6 can induce inflammation when it is injected into the eye of experimental animals.<sup>5-8</sup> Tumor necrosis factor- $\alpha$  appears to induce inflammation when it is injected in the eye of experimental

animals.<sup>9,10</sup> Gamma interferon is a strong mediator of immune responses with multi-regulatory effects, which include expression of MHC class I and II antigens, induction of phagocytosis by macrophages, and enhancement of cytotoxicity in macrophages, cytotoxic T cells and natural killer cells.<sup>23</sup> Therefore, it is suggested that IL-1 $\alpha$ , IL-6, TNF- $\alpha$ , and INF- $\gamma$  produced by infiltrating T cells in the eye play a significant role in the inflammatory process of the diseases. Our studies also found that there were some differences in the profile and amounts of cytokines produced by infiltrating T cells among the different entities of uveitis. The differences in the cytokine production by ocular infiltrating T cells might be attributed to the different immunopathogenic mechanisms of the diseases, and might contribute to the different ocular manifestations among the diseases.

Phytohemagglutinin stimulation caused a significant increase in the levels of a few cytokines (INF- $\gamma$  and GM-CSF) produced by TCCs of VKH origin (Table 2). However, none of cytokines produced by TCCs of sarcoidosis and healthy donor origin were affected by PHA stimulation. Therefore, the effects of various drugs on cytokine production by TCCs were investigated without PHA stimulation (Figures 1 and 2). Drugs having different modes of action on the immune responses were tested for their capacity in suppressing cytokine production. Tacrolimus is known to inhibit the expression of the early phase of T cell activation genes, such as mRNAs of IL-2, IL-3, IL-4, INF- $\gamma$ , TNF- $\alpha$ , and GM-CSF.<sup>24-29</sup> Hydrocortisone is known to suppress many stages of the immune process, including inhibition of the synthesis of various cytokines.<sup>30-32</sup> Diclofenac belongs to a group of non-steroidal antiinflammatory drugs that suppress the cyclooxygenase pathway, although their activities in the immune and inflammatory processes are less potent than corticosteroids or tacrolimus. FTY720 is a new immunosuppressive agent isolated from the culture broth of *Isaria sinclairii*.<sup>33</sup> The agent induces lymphocytopenia without myelotoxicity, prolongs the allograft survival in animals, and suppresses the induction of experimental allergic encephalitis.<sup>34</sup> Among the four drugs tested, hydrocortisone had the most intense activity in suppressing the cytokine production by TCCs from AH of VKH patients; there was statistically significant suppression in the production of IL-6, IL-8, and GM-CSF. Tacrolimus also significantly suppressed the production of IL-8 and GM-CSF by the TCCs. FTY720 suppressed only GM-CSF production. In contrast, diclofenac failed to suppress production of any of the cytokines tested. These data

thus suggest that the therapeutic effects of corticosteroids and tacrolimus on uveitis can be attributed, at least in part, to the immunosuppressive properties of these drugs on the cytokine production of the infiltrating T cells in the uveitis eye.

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