Dominance of Activated T Cells and Interleukin-6 in Aqueous Humor in Vogt-Koyanagi-Harada Disease

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**Purpose.** To determine the immunopathologic role of the lymphocytes and lymphokines in aqueous humor (AH) of patients with Vogt-Koyanagi-Harada disease (VKH).

**Methods.** The distribution of leukocyte subsets in the peripheral blood and AH was examined using fluorescein isothiocyanate-conjugated monoclonal antibodies. The levels of lymphokines, such as interleukin-2 (IL-2) and interleukin-6 (IL-6), in the sera, AH, and cerebrospinal fluid from the patients with VKH were determined using an enzyme-linked immunosorbent assay.

**Results.** T cells constituted the majority of lymphocytes within AH. The value for CD4+ cells (helper/inducer T lymphocytes) in AH was 51.7% ± 14.9% (mean ± SD) and that for CD8+ cells (cytotoxic/suppressor T lymphocytes) was 31.1% ± 13.0%. The percentage of HLA-DR+ cells (B lymphocytes, monocytes, macrophages, and activated T lymphocytes) in AH (50.8% ± 24.9%) significantly exceeded (P < 0.001) that in blood (13.1% ± 4.2%). The percentage of CD8+ cells in AH from three patients with the delayed type of VKH rose during their clinical course. The level of IL-6 was significantly elevated in AH from the patients with VKH. The level of IL-6 in AH correlated with the number of lymphocytes in AH, and it reflected the severity of the inflammatory response in AH of patients with VKH. The level of IL-2 in the sera, AH, and cerebrospinal fluid was in the normal range.

**Conclusions.** Aqueous humor lymphocytes from the patients with VKH were more activated than were peripheral blood lymphocytes. IL-6 may play an important role as an inflammatory mediator in VKH. It may be useful to analyze the lymphocyte subsets and the levels of lymphokines, especially of IL-6, at the site of inflammation in uvea to improve the criteria for assessing the prognosis of VKH.


Vogt-Koyanagi-Harada disease (VKH) is characterized by bilateral uveitis, meningitis, alopecia, perceptive deafness, and vitiligo. VKH is believed to be a systemic disorder affecting various organs containing melanocytes. Recent studies of lymphocytes at the sites of inflammation, including in cerebrospinal fluid (CSF), have provided important insights into the mechanisms of diseases, confirming that those that infiltrate the target organs may differ from those in peripheral blood. The distribution of such lymphocytes and their roles in VKH are little known. Characterization of the lymphoid infiltrates in the eyes of patients with VKH is a first step toward a better understanding of this disease.

We analyzed the surface markers of the lymphocyte subsets and T cell receptor (TcR) at the intraocular site of inflammation, i.e., aqueous humor (AH) by staining the cells with monoclonal antibodies (MAbs).

Cytokines play an important role during inflammation, primarily by regulating the diverse functions of lymphocytes and monocytes. Interleukin-6 (IL-6) is believed to be a potential mediator of inflammation in rats with endotoxin-induced uveitis, and it may mediate inflammation in uveitis in humans. Interleukin-2 (IL-2) has been found in the human eye in the presence of inflammatory and autoimmune diseases. Therefore, in this study, we measured the levels of IL-6 and IL-2 in the sera, AH, and CSF from patients...
with VKH. We used an enzyme-linked immunosorbent assay (ELISA) to assess the function of the T cells present at the site of inflammation.

**MATERIALS AND METHODS**

**Subjects and Specimens**

From 1988 to 1992, we studied 11 patients with VKH; 8 patients were diagnosed with Harada’s type and 3 with Vogt-Koyanagi type. Inflammation in the anterior chamber recurred more than 6 months in 5 patients (delayed type). They lived in Matsumoto and its surrounding areas in Japan. Specimens of AH were obtained by anterior chamber paracentesis, performed via the limbus using a tuberculin syringe and a 27-gauge needle. A sample of 0.2 to 0.3 ml of AH was removed for analysis. At the same time, peripheral blood lymphocytes (PBL) were isolated by Ficoll-Conray gradient centrifugation as previously described in detail. In some patients, anterior chamber paracentesis was performed twice to examine the correlation of the IL-6 level in AH and the clinical course of VKH. Aqueous humor cells were collected by centrifugation in Eppendorf micro-test tubes (Eppendorf, Hamburg, Germany) at 1,000 rpm for 10 minutes. Supernates were stored at −80°C until assayed by ELISA.

Specimens of CSF were obtained by spinal tap from these patients during the acute phase of the illness. Supernates of CSF were obtained by centrifugation at 1,000 rpm for 10 minutes. All the initial specimens of both AH and CSF were collected before steroid administration. Patients 4 and 5 had received local steroid therapy (dexamethasone eyedrops) during the clinical course. The steroid therapy was ceased 3 days before the second collection of sampling taken at 93 (patient 4) and 120 (patient 5) days after the initial sampling.

Peripheral blood lymphocytes from 50 age-matched controls were similarly analyzed. Control specimens of sera for ELISA were obtained from 36 age-matched healthy subjects. Control specimens of AH were obtained for ELISA from 10 patients undergoing extracapsular cataract extraction for senile cataracts. Control specimens of CSF for ELISA were obtained from 10 patients undergoing spinal anesthesia. Tenets of the Declaration of Helsinki were followed.

**Grading of Iritis**

Grading of iritis has been defined by the number of infiltrating cells in AH. The number of infiltrating cells in AH was counted by using the highest slit lamp magnification and the smallest spot size. Iritis was graded on a scale of 1+ to 4+, where 1+ was defined as occasional cells, 2+ as 1–7 cells, 3+ as 8–14 cells, and 4+ as >15 cells.

**Distribution of Cell Population**

The distribution of leukocyte subsets in the peripheral blood and AH from the 11 patients was examined using fluorescein isothiocyanate (FITC)-conjugated MAbs. We used MAbs to the surface antigens of human leukocytes: OKT3 (CD3: pan T lymphocytes); OKT4 (CD4: helper/inducer T lymphocytes); OKT8 (CD8: cytotoxic/suppressor T lymphocytes); B1 (CD20: B lymphocytes); HLA-DR (B lymphocytes, monocytes, macrophages and activated T lymphocytes); Leu 11a (CD16: natural killer cells); and TcRαβ and TcRγδ to determine the distribution of the leukocytes in the peripheral blood and AH in the 11 patients. After staining, cells in AH were washed and resuspended onto slides for examination by immunofluorescence microscopy because the number of lymphocytes recovered from AH was too small to analyze by flow cytometry. The frequency of lymphocyte subsets found in PBL was measured by immunofluorescence microscopy and flow cytometry. There were no significant differences between the frequency of lymphocyte subsets found in PBL by microscopic examination and by flow cytometric analysis. Therefore, PBL were resuspended for sorting on an EPICS V cell sorter (Coulter Immunology, Hialeah, FL) and FACScan (Becton-Dickinson, Mountain View, CA). B1 MAb was purchased from Coulter Immunology. OKT3, OKT4, and OKT8 MAbs were purchased from Ortho Diagnostic Systems (Raritan, NJ). HLA-DR and Leu 11a MAbs were purchased from Becton-Dickinson (Sunnyvale, CA), and TcRαβ and TcRγδ MAbs were purchased from T Cell Sciences (Cambridge, MA).

**Assay for ELISA**

Sera from the patients and the controls were separated from clotted whole blood by centrifugation at 3,000 rpm for 10 minutes. Sera and supernates of AH and CSF were stored at −80°C until assayed for lymphokines. We used commercial double-sandwich ELISA test kits to quantify IL-2 (Otsuka, Tokyo, Japan) and IL-6 (Toray-Fuji Bionics, Tokyo, Japan). Because of the limited amount of AH, it was diluted two to five times for measurement by ELISA.

IL-2 was measured by a sandwich ELISA assay. Before the assay, the MAb-coated 96 well microplates were washed three times with Dulbecco’s phosphate buffered saline (−) containing 0.05% Tween 20. Two hundred μl of the samples or the diluted standards were put into each well and allowed to react overnight at 37°C. After washing three times, 100 μl of rabbit anti-human IL-2 polyclonal antibody was added and
allowed to react for 2 hours at room temperature. After washing once again, 100 µl of horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was added. After incubation, 100 µl of substrate solution (1 mg/ml o-phenylenediamine) was added. The reaction was terminated by adding 100 µl of 1.0 N H₂SO₄. The final absorbance was measured at 490 nm using an ELISA autoreader (TOSO, Tokyo, Japan). The lowest detectable concentration of IL-2 is 10 pg/ml.

IL-6 was also measured by an ELISA assay. Samples were incubated with a monoclonal anti-human IL-6 antibody (clone IG61)¹⁰ that was bound to a plate to a plate for 2 hours at room temperature. After completion of the antigen-antibody reaction, the test specimens were removed with an aspirator. Then, biotinylated F(ab')₂ of a monoclonal antibody to human IL-6 (clone IC67)¹⁰ was added and incubated for 1 hour at room temperature. Next, avidin-horseradish peroxidase conjugate was used to bind to immune complexes. Finally, a substrate (o-phenylenediamine) was added. The reaction was stopped by adding 100 µl of 2.0 N H₂SO₄. The absorbance was measured at 490 nm using an ELISA autoreader. The lowest detectable concentration of IL-6 is 10 pg/ml.

Statistical Methods
Data were analyzed by Student’s t-test and Wilcoxon’s rank sum test, with P < 0.05 accepted as statistically significant.

RESULTS
Distribution of Leukocytes
The percentages of CD3⁺, CD20⁺, CD4⁺, CD8⁺, HLA-DR⁺, CD16⁺, TcRαβ⁺ and TcRγδ⁺ cells in the patients’ peripheral blood were all within normal ranges (Table 1). The majority of lymphocytes in the inflammatory AH was T cells. The percentages of the CD3⁺, CD20⁺, CD4⁺, CD8⁺ cells in AH did not differ significantly from those of PBL. The percentage of HLA-DR⁺ cells in AH (50.8% ± 24.9%) was significantly higher (P < 0.001) than that in blood (13.1% ± 4.2%). We analyzed double fluorescein studies of the surface markers of the lymphocyte subsets in AH by staining the cells with FITC-conjugated MAbs and phycoerythrin-conjugated MAbs (CD4 and HLA-DR, CD8 and HLA-DR). Although a small percentage of CD4⁺HLA-DR⁺ cells were monocytes, the greater part of CD4⁺HLA-DR⁺ cells were lymphocytes according to the cell size and granule patterns (data not shown). In addition, the percentage of CD20⁺ cells in AH is 7.0% ± 4.4%. These data suggest that activated T cells infiltrate the AH in these patients. There was no significant difference between the CD4⁺/CD8⁺ ratio in AH and that in the peripheral blood. The percentage of TcRαβ⁺ cells in AH was significantly higher (P < 0.02) than that in blood. The percentage of TcRγδ⁺ cells in AH and in PBL did not differ significantly.

In three patients with the delayed type of VKH, one with Harada’s type (patient 4) and the other two with Vogt-Koyanagi type (patients 5 and 11), the percentage of CD8⁺ cells increased during the course of their diseases (Table 1).

The percentage of CD16⁺ cells in AH (4.5% ± 3.0%) was significantly lower (P < 0.05) than that in blood (14.0% ± 8.9%).

Measurement of IL-2 and IL-6
The level of IL-6 in AH from the patients with VKH was 814.2 ± 1775.8 pg/ml (Table 1). Statistical analysis showed a significant difference in IL-6 level in AH from the patients with VKH versus controls (P < 0.01), and a similar inclination was also observed in the sera (P = 0.05) (Fig. 1). In contrast, CSF samples from all but one patient (patient 10) showed a low level of IL-6. The CSF sample from patient 10, taken in the more acute phase of the illness, showed a relatively high level of IL-6 (25.4 pg/ml), although serum IL-6 level (51.3 pg/ml) of the patient exceeded that in CSF. This patient’s CSF leukocyte cell count was extremely high (505 per mm³).

A significant level of IL-2 is defined as more than 50 pg/ml. No significant level of IL-2 was detected in the sera, AH, and CSF from the patients with VKH or the controls (data not shown).

IL-6 Level and Clinical Course
We analyzed the correlation of the levels of IL-6 in AH and the degree of iritis of patients with VKH (Fig. 2). The inflammatory activity of iritis correlated with the level of IL-6 in AH. The level of IL-6 in AH from patients 4 and 7 declined with the lessening of the inflammation. Although the level of IL-6 and the number of leukocytes in AH of patient 7 with Harada’s type of VKH were exceedingly high during the acute phase, they decreased after 190 days. The level of IL-6 and the number of leukocytes in AH of patients 5, 10, and 11 increased during the clinical course. These findings indicate that the level of IL-6 in AH correlates with the number of leukocytes in AH, and it reflects the severity of the inflammatory response in AH of patients with VKH.

DISCUSSION
We found that the percentage of HLA-DR⁺ cells in AH was increased over that in PBL (P < 0.001), although the percentage of CD3⁺, CD4⁺, CD8⁺, and CD20⁺ cells in AH from the patients with VKH did not differ significantly from that in PBL. The percentage of HLA-DR⁺ cells in AH from the patients with sarcoidosis was also increased over that in PBL (manu-
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Control PBL (n = 50), sera (n = 36), and AH (n = 10)

1 Leukocyte subsets were analyzed by immunostaining methods as described in Materials and Methods. The percentages of these phenotypes of PBL were analyzed by counting immunostained 1 x 10⁶ cells with EPICS V or FACScan. Data of the leukocyte subsets were expressed as percent positive cells. Statistical significance was analyzed by Student’s t-test and Wilcoxon’s rank sum test.

* Harada’s type of VKH.
† Vogt-Koyanagi type of VKH.
‡ Delayed type (recurrent inflammation more than 6 months)
§ Student’s t-test.
¶ Wilcoxon’s rank sum test.

1 See materials and methods section.

Days, number of days between disease onset and the day of sample collection.

NT, not tested; NS, not significant.
Activated T Cells and IL-6 in Aqueous Humor in VKH

Activated lymphocytes and IL-6 were measured in aqueous humor (AH) from patients with VKH. Levels of IL-6 were determined by double-sandwich ELISA system. Statistical analysis was done by Wilcoxon's rank sum test. Figure in parenthesis indicates the concentration of IL-6 (pg/ml).

- VKH; O = control
- NS, not significant.

In a patient with sympathetic ophthalmia that manifested itself 10 weeks after surgery and was followed by enucleation 10 weeks later, Kaplan et al\(^{12}\) demonstrated that a majority of the cells was CD3\(^+\), with a predominance of the CD4\(^+\) helper/inducer subset. However, in a case of sympathetic ophthalmia that manifested itself 17 months after surgery and was followed by enucleation 4 weeks later, Jakobiec et al\(^{13}\) showed that the choroidal infiltrate was composed predominantly of CD8\(^+\) cells. Sakamoto et al\(^{14}\) reported two patients with VKH; the first died of uterine cancer 32 months after the onset of VKH; the other died of pulmonary fibrosis after 7 years. In these patients, the CD4\(^+\) cells outnumbered the CD8\(^+\) cells in the choroid. Chan et al\(^{15}\) and Lightman et al\(^{16}\) demonstrated a relatively higher ratio of helper/inducer T cells in the choroid and subretinal fibrous tissue in the sympathetic ophthalmia of shorter duration; the percentage of suppressor/cytotoxic T cells rose as the inflammation progressed. In the present study, we observed that the percentage of CD4\(^+\) cells was higher than that of CD8\(^+\) cells in AH from the patients with VKH in the acute phase. However, the percentage of CD8\(^+\) cells in three cases of the delayed type increased during the course of their disease. The local administration of steroids did not influence the cell populations in AH.\(^{17}\)

We therefore assume that CD8\(^+\) cells predominate in AH during the chronic phase of VKH. Ariga et al\(^{18}\) reported that an increase of CD4\(^+\) cells in early stages of VKH and an increase of CD8\(^+\) cells in later stages by the immunohistopathologic examination of limbal biopsies. Nonaka et al\(^{19}\) reported that suppressor/cytotoxic lymphocytes become prominent in the iris during the chronic stage of uveitis and that they may suppress the recurrence of inflammation. Our data appear to agree with the results of Ariga et al\(^{18}\) and Nonaka et al.\(^{19}\) However, the origin of T cells in AH and uveal tissues, or a possibility of translocation of T cells between AH and uveal tissues, remains to be studied.

The percentage of CD16\(^+\) cells in AH was significantly less than it was in PBL. The relative number of

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933177/)  
**Figure 1.** IL-6 levels in sera, aqueous humor (AH), and cerebrospinal fluid (CSF) from patients with VKH. Levels of IL-6 were determined by double-sandwich ELISA system. Statistical analysis was done by Wilcoxon's rank sum test. Figure in parenthesis indicates the concentration of IL-6 (pg/ml).

- VKH; O = control
- NS, not significant.

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933177/)  
**Figure 2.** The correlation of the level of IL-6 in aqueous humor and the degree of iritis of patients with VKH.

*Delayed type (recurrent inflammation more than 6 months).  
†See Materials and Methods section.
of VKH.

The intraocular release of IL-6 from the infiltrating cells in AH, are involved in local IL-6 production. IL-6 level correlated with the severity of the iritis acute phase. Our data demonstrate that the aqueous sarcoidosis than in the sera of patients with sarcoidosis ocular cells lining the anterior chamber, as well as T cells, macrophages, monocytes, fibroblasts, and endothelial cells. IL-6 promotes the production of antibodies, fibrinogen, and so on, and it can induce the production of acute-phase proteins such as CRP. Despite a low level of IL-6 in sera, the IL-6 level in AH of patients with Fuchs' heterochromic cyclitis and sera from the patients with sarcoidosis. The IL-6 level was significantly higher in the AH of patients than in controls ($P < 0.01$). IL-6 is a multifunctional cytokine that is produced by a wide variety of cell types, among them, T cells, macrophages, monocytes, fibroblasts, and endothelial cells. IL-6 promotes the production of antibodies, fibrinogen, and so on, and it can induce the production of acute-phase proteins such as CRP. Despite a low level of IL-6 in sera, the IL-6 level in AH of patients with Fuchs' heterochromic cyclitis and Toxoplasma uveitis rose, indicating that the increased level of IL-6 found in the AH of patients with uveitis did not result from serum leakage but from its local production. We also measured the IL-6 level in AH and sera from the patients with sarcoidosis. The IL-6 level was significantly higher in the AH of patients with sarcoidosis than in the sera of patients with sarcoidosis and in the AH of controls (manuscript in preparation). The intraocular release of IL-6 from the infiltrating cells in the uvea, rather than leakage from the serum, is associated with the development of uveitis. Our results show that a high IL-6 level in the AH of patients with VKH did not result from the breakdown of the blood-aqueous barrier and subsequent serum leakage. Recently, it was reported that retinal pigment epithelial cells can produce IL-6 in vitro after stimulation with IL-12 and TNF-α. It is possible that resident ocular cells lining the anterior chamber, as well as T cells in AH, are involved in local IL-6 production. Identification of IL-6-producing cell type(s) in AH remains to be answered. The highest level of IL-6 in AH was found in patient 11, whose iritis was severe at the acute phase. Our data demonstrate that the aqueous IL-6 level correlated with the severity of the iritis of VKH.

Vogt-Koyanagi-Harada disease is believed to be a systemic disorder that affects various organs containing melanocytes. Patients with VKH show a “sunset sky” fundus, indicating the hypopigmentation of the choroid during convalescence from the disease. A recent report demonstrated that IL-6 inhibits the growth of melanoma cells and influences the adhesion-molecule expression of melanocytes and melanoma cells. IL-6 significantly upregulates the intercellular adhesion molecule-1 (ICAM-1) expression of melanocytes and melanoma cells. IL-6 released within inflammatory uvea may induce ICAM-1 expression on melanocytes and render them susceptible as targets for cytotoxic T cells and natural killer cells. Thus, these cells may destroy melanocytes and lead to postinflammatory hypopigmentation. It may be useful to analyze the lymphocyte subsets and the levels of lymphokines, especially of IL-6, at the site of inflammation in uvea to improve the criteria for assessing the prognosis of Vogt-Koyanagi-Harada disease.

Key Words
Vogt-Koyanagi-Harada disease, IL-6, aqueous humor, cerebrospinal fluid, T lymphocyte subsets

References
Activated T Cells and IL-6 in Aqueous Humor in VKH


