Enumeration of Autoreactive Helper T Lymphocytes in Uveitis

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In a one-stage, interleukin-2 (IL-2), limiting-dilution analysis, peripheral blood mononuclear cells from patients with uveitis and normal control subjects were assayed for S-antigen specific, tetanus-specific, and in vivo activated helper T cells. Control subjects consistently demonstrated tetanus-specific responses, but neither in vivo activation nor S-antigen specific helper T cell responses were seen. Patients with active forms of diffuse, posterior, and anterior uveitis were found to have significant frequencies of both in vivo activated and S-antigen specific helper T cells in their peripheral blood. These data show that patients with certain forms of uveitis have a measurable frequency of lymphocytes in the peripheral immunologic compartment capable of secreting IL-2 in response to autologous presentation of ocular autoantigen (S-antigen). Limiting-dilution analysis techniques, generating minimal responder cell frequency estimates and distinct IL secretion patterns, may provide an index of disease activity and critical information about the mechanism(s) of ocular inflammation. Invest Ophthalmol Vis Sci 32:2561-2567, 1991

Uveitis is an important cause of visual impairment in the United States. In general, the immunologic mechanisms responsible for ocular inflammation are not well defined. Patients with uveitis often are managed empirically because of our lack of adequate information regarding the status and contribution of the underlying immunologic and inflammatory mechanisms. An accurate index of disease activity and more precise knowledge of the predominant pathophysiologic process governing uveitis are required to evaluate, treat, and follow these diseases effectively.

Several lines of evidence support a dominant role for autoreactive T lymphocytes in mediating ocular inflammation. Animal models of uveitis showed that immunization with retinal S-antigen and interphotoreceptor retinoid binding protein results in a bilateral chorioretinitis. Moreover, this inflammation can be induced by the adoptive transfer of in vivo or in vitro sensitized, antigen-specific T cells to naive recipients. Immunomodulation of T cell function with cyclosporine arrests the inflammation, lending further support to the importance of autoimmune T cells in uveitis.

Histologic and immunohistochemical analysis of eyes from patients with sympathetic ophthalmia, Vogt-Koyanagi-Harada (VKH) disease, and birdshot chorioretinitis show T cell infiltration. Helper T cell subsets appear to dominate the early phases of the disease, and suppressor T cells are found in the chronic phases. In these disorders, in vitro lymphocyte proliferation assays document T cell responsiveness to retinal autoantigens. From these data, it would appear that cell-mediated immunity and, specifically, autoreactive T cells play an important role in mediating certain forms of uveitis.

Most of the information regarding T lymphocyte autoreactivity in uveitis has been qualitative in nature, employing clinical descriptions or immunohistochemistry. Other studies used conventional lymphocyte proliferation assays to establish the presence of cell-mediated immune responses to autoantigens (S-antigen or interphotoreceptor retinoid binding protein (IRBP)). The magnitude of the proliferative response in these assays bears little relationship to the actual numbers of helper T cells required to generate this response. Specific cytokine release also is not determined by proliferation assays. Such assays are influenced by multicellular interactions and represent a bulk response. An accurate method to determine the size and cytokine secretion patterns of the autoreactive T cell pool in patients with uveitis would be clinically important. Antigen-specific forms of immunotherapy and anticytokine drugs could be used based on this information. Disease remission and response to various therapies could be monitored by such a noninvasive, quantitative measure of autoreac-
tivity. Limiting-dilution analysis provides this information by obtaining minimal frequency estimates for a specific T cell population. This system also avoids the "bulk" response problem inherent in conventional lymphocyte proliferation data. By quantifying autoreactive T cells and identifying their antigen specificity, lymphokine secretion patterns, activation status, and unique immunologic compartment differences, limiting-dilution analysis also may provide fundamental information about the immunopathogenesis of uveitis.

We describe a method to enumerate autoreactive helper T cells in the peripheral blood of patients with uveitis. We used limiting-dilution analysis techniques to compare minimal frequency estimates of T cells in the peripheral blood of patients with active uveitis that were capable of secreting interleukin-2 (IL-2) in response to the autologous presentation of tetanus and S-antigen with those of normal control subjects.

**Materials and Methods**

**Culture Media**

Culture media for the limiting dilution analysis consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 1.6 mM L-glutamine, 0.27 mM folic acid, 0.27 mM L-asparagine, 0.55 mM L-arginine, 10 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid buffer, 1.0 mM sodium pyruvate (Gibco, Grand Island, NY); 100 units of penicillin and streptomycin, and 25 μg of amphotericin B (Whittaker, Walkersville MD); 5 × 10⁻⁵ M beta-mercaptoethanol (Sigma, St. Louis, MO); and 10% heat-inactivated human AB serum (Whittaker).

**Mononuclear Cell Preparation**

Peripheral blood mononuclear cells (PMBC) were isolated from heparinized whole blood obtained from patients with uveitis or normal human volunteers after informed consent. Cells were obtained using standard Ficoll-Paque density centrifugation (Pharmacia, Piscataway, NJ) at 400 × g. Monocyte preparations were obtained by subsequent Percoll gradient-density sedimentation (Sigma). T helper cells were defined operationally as lymphocytes capable of secreting IL-2. In vivo activated T cells are lymphocytes spontaneously secreting IL-2 in the absence of specific antigen. Helper T cells secreting IL-2 in response to the autologous presentation of antigen are regarded as antigen-specific T lymphocytes.

**Indicator Cell Line**

CTLL-20 cells, a T cell growth factor-dependent murine cell line, were maintained in complete DMEM supplemented with 10% concanavalin A stimulated rat splenocyte supernatant and 10% heat-inactivated fetal bovine serum. The CTLL-20 cells were washed three times and resuspended in the culture media at a concentration of 4 × 10⁵ cells/ml for a final concentration of 1 × 10⁴ cells/microwell for the IL-2 limiting-dilution analysis. CTLL-20 cells proliferate in response to murine IL-2, murine IL-4, and human IL-2 but not human IL-4. Thus, CTLL-20 cell proliferation is an indirect measure of IL-2 production.

**Test Antigens**

Bovine retinal S-antigen was provided generously by Dr. Robert Nussenblatt (National Eye Institute, Bethesda, MD) and was used at a final concentration of 20 μg/ml in the IL-2 limiting-dilution analysis. Tetanus toxoid (Wyeth-Ayerst, Marietta, PA) was used at a final dilution of 1:999 of stock. Tetanus toxoid was selected as the control antigen because both tetanus and the experimental antigen (S-antigen) are soluble peptides. In addition, most subjects can be expected to be immunized against tetanus and should have effector cells specific for this antigen.

**Limiting-Dilution Assay**

To determine the frequency of antigen-specific, IL-2-secreting helper T cells in the peripheral blood, a limiting-dilution assay was developed as described previously. Briefly, the appropriate serial dilutions of the effecter cell were plated into 96-well, V-bottom microtiter plates (INC/Flow, Los Angeles, CA) resulting in 20 replicate wells each with 20,000, 10,000, 5000, 2500, 1250, or 625 lymphocytes per well. Irradiated (7500 rad) autologous monocytes (5000 cells per well) were added to the wells as autologous, antigen-presenting cells for the protein limiting-dilution analysis. The lymphocyte cultures are incubated for 18 hr at 37°C in 10% CO₂ with the test and control antigens, allowing antigen-specific T cell activation to occur. The plates are irradiated with 2500 rad to prevent mononuclear cell proliferation and overlaid with 1 × 10⁴ CTLL-20 cells/well. After an 8-hr incubation at 37°C, the plates were pulsed with ³H-thymidine (New England Nuclear, Wilmington, DE). The plates then were incubated for 12 hr, harvested, and counted for thymidine uptake by scintillation on a beta counter.

Important controls for the limiting-dilution analysis include CTLL-20 proliferative responses to: (1) control 1—0% IL-2 alone (CTLL-20 negative control), (2) control 2—10% IL-2 alone (CTLL-20 positive control), (3) control 3—monocytes (5000 cells/well) and antigen without effector lymphocytes (stimulator control and negative point for statistics), and (4) control 4—monocytes (5000/well) and lymphocytes (20,000 cells/well) without tetanus (spontaneous, antigen-nonspecific, IL-2 release in vivo activation).
Experimental wells contained limiting dilutions of irradiated, effector PBMC, irradiated autologous monocytes (5000 cells/well), CTLL-20 cells (1000 cells/well), and test antigen.

Critical to the limiting-dilution analysis is the establishment of an optimal limiting-dilution analysis environment and a single dilutional factor. Neither the concentration of antigen nor the number of antigen-presenting cells can be a limiting factor to fulfill the requirements of the limiting-dilution analysis. We found that a concentration of 20 μg/ml for S-antigen, 1:999 dilution for tetanus antigen, and 5000 autologous monocytes per well provided nonlimiting antigen stimuli in the limiting-dilution analysis microculture. Under such conditions, the responder cell number was the only limiting variable in the experimental system.

As a result of spontaneous activation in the patient population, a complete IL-2 limiting-dilution analysis was done without test antigen. This generates a frequency estimate for in vivo activated T helper cells.

Limiting-Dilution Analysis and Statistics

The limiting-dilution analysis generates a minimal frequency estimate for a discrete subset of effector cells in a given population. This frequency is calculated by analysis of the Poisson distribution between the number of responder cells added to the limiting-dilution microwells and the percentage of replicate microwells that do not produce detectable IL-2.\(^1\) Limiting-dilution microcultures are considered positive for IL-2 production if thymidine incorporation by CTLL-20 cells is greater than the thymidine incorporation (mean plus three standard deviations) observed in 20 replicate stimulator control microwells.

Frequency calculations were determined by computer using chi-square minimization analysis as described previously.\(^1\) This analysis yields minimal frequency estimates \((1/f)\), the 95% confidence limits of the frequency estimate, and a chi-square estimate of probability \((P)\). In this system, significance is indicated by \(P > 0.05\). Minimal estimates were reported because of the possibility of multiple effector cells occurring in one microwell and the uncertainty regarding the absolute number of T helper cells required to produce detectable IL-2.

Results

Normal Control Population

To establish normal values for the frequency of tetanus-specific, S-antigen-specific, and in vivo activated helper T cells in peripheral blood, healthy subjects without a history of uveitis were assayed. Figure 1 is a representative IL-2 limiting-dilution analysis for a normal subject. In this example, thymidine incorporation for the 20 replicate wells was plotted against effector cell dilutions. Figure 1A shows an antitetanus response using limiting-dilution analysis. Under the experimental conditions, the percent negative wells for IL-2 secretion was directly proportional to the effector cell concentration. At cell concentrations above 20,000 cells per well, it was common to note a dropoff in both the magnitude of proliferative response and the number of positive wells. This may represent competition for IL-2 by other cells in the microwell environment or the presence of suppressor cell activities at the higher cell concentrations and was comparable to the conditions present in standard lymphocyte proliferation assays. Figure 1B illustrates the absence of a T helper response to S-antigen and represents the normal control population's lack of response to self antigens.

![Graph A: Tetanus LDA](image-url)

![Graph B: S-Ag LDA](image-url)

Fig. 1. Representative IL-2 limiting dilution analysis from a normal control subject. Thymidine incorporation from 20 replicate microwells is plotted against the effector cell dilution. (A) illustrates a strong tetanus response. (B) shows the absence of S-antigen-specific helper T cells in this healthy subject.
Table 1 summarizes these data for the normal control population. In vivo activated helper T cells, spontaneously secreting IL-2 were infrequent (one of eight). Only one subject (12), recovering from an upper respiratory illness, was found to show evidence for this activity with a minimal frequency estimate of 7 T cells in 10^6 peripheral blood lymphocytes (PBL). Most subjects had a significant number of tetanus-specific responder cells with frequency estimates ranging from 25–200 T cells/10^6 PBL. One subject (3) did not have a detectable tetanus response before immunization. Six weeks after a booster shot, the level became greater than 3000 helper T cells/10^6 PBL. As reported by others, limiting-dilution analysis was found to be more sensitive than lymphocyte proliferation assays in detecting immune responses in normal subjects. Several subjects responded to tetanus antigen in limiting-dilution assay but did not respond in a parallel lymphocyte proliferation assay. Only one normal subject (12) responded to S-antigen with a frequency of 8 autoreactive T cells/10^6 PBL. A small percentage of normal subjects have been reported by others to have proliferative responses to this ocular autoantigen.3,14

Unlike standard lymphocyte proliferation assays that detect multicellular interactions, the limiting-dilution assay is designed to recognize and quantify a discrete T cell function, eg, specific cytokine release (IL-2) after autologous presentation of precise antigen. This technique appears to be more sensitive than proliferation assays. This may be due to the elimination of other influencing factors present in bulk culture assays such as competition for cytokines and suppressor cell activity. It also appears to be very sensitive; all healthy control subjects were documented to have a significant frequency of helper T cells committed to tetanus control antigen. Tetanus responses provide an important control for basic T cell function in the subject and patient population and for this assay. Our system also appears to be very specific; only one (1 of 12) normal subject (12) had evidence for autoreactivity.

Uveitis Patient Population

Patients with uveitis were diagnosed by standard criteria of history, extensive review of systems, physical examination, ocular examination, and supplemental laboratory testing. Most patients had been receiving nonspecific antiinflammatory regimens. All patients had active disease and were assayed within several weeks of establishing a diagnosis.

Figure 2 shows a representative tetanus and S-antigen limiting-dilution analysis for a patient (22) with VKH disease. In this example, the S-antigen-specific response was observed with a frequency of 42 helper T cells/10^6 PBMC. Table 2 summarizes the data from 29 patients with active uveitis. Helper T cell frequencies for tetanus-committed cells in these patients were comparable to normal controls and ranged from 5–500 helper T cells/10^6 PBL. One patient (12) did not have a detectable tetanus response.

In contrast to the normal population, patients with active uveitis had both spontaneously activated and S-antigen-committed helper T cells in the peripheral blood (Table 2). Thirteen of our 29 patients (45%) had evidence of spontaneous T cell activation with frequencies ranging from 3–50 activated T cells/10^6 PBL. Spontaneously activated T cells were found in some of the patients in each of the forms of uveitis including idiopathic, presumed infectious (toxoplasmosis and Epstein-Barr virus), Behcet's disease, subretinal fibrosis and uveitis syndrome (SFU), sympathetic ophthalmia, birdshot choridoidopathy, and VKH disease.

Eighteen of the 29 patients with active uveitis (62%) had a distinct subpopulation of T cells capable of secreting IL-2 in response to the autologous presentation of S-antigen. Frequency estimates in these patients for S-antigen-committed helper T cells ranged from 4–43 T cells/10^6 PBL. The diffuse uveitis group (eight of nine) had the highest percent (89%) of S-antigen-positive patients and the highest average number of responder cells (18 T cells/10^6 PBL). Of the patients with posterior forms of uveitis, 58% had evidence for autoreactive T cells specific for S-antigen. As a group, however, the posterior forms of uveitis had a significantly lower average number of responder cells (3 T cells/10^6 PBL) than the diffuse group (P < 0.01).

None of the four patients with intermediate uveitis could be stimulated to produce IL-2 when challenged with S-antigen. The patients with recurrent forms (three of four) of severe, anterior uveitis (histocompat-
Fig. 2. Representative IL-2 limiting dilution analysis from a patient with uveitis (VKH syndrome). This shows both a positive tetanus (A) and S-antigen response (B).

Table 2. Enumeration of autoreactive, cytokine-secreting (IL-2) T cells in patients with uveitis—S-Ag

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The frequency of tetanus, S-antigen, and spontaneously activated, IL-2-secreting T cells is given as cells/10⁶ PBL. All values are significant (P > 0.02) unless noted (NS). Frequencies reported as zero represent undetectable levels of responder cells (less than 3 cells/10⁶ PBL).
Frequency per 1 x 10⁶ PBMC.
tines and suppressor cell activity. By using these techniques, healthy control subjects had a significant frequency of helper T cells capable of secreting IL-2 in response to soluble tetanus antigen without significant in vivo activated or S-antigen-specific effector cells (Table 1). Only one randomly selected subject had evidence of both spontaneous and S-antigen-specific responder cells. Patients with uveitis had comparable tetanus-specific helper T cell frequencies. It would appear that healthy subjects and patients with uveitis have precursor T helper cells in the peripheral blood capable of an anamnestic response to this exogenous antigen when appropriately presented. Ocular inflammation does not appear to affect this unrelated and antigen-specific helper T cell function.

Unlike control subjects, 45% of uveitis patients (13 of 29) had spontaneously activated T cells in their peripheral blood. Activated T cell frequencies ranged from 3-50 cells/10^6 PBMC. Spontaneously activated T cells were found in some of the patients in each of the forms of uveitis including idiopathic, presumed infectious (toxoplasmosis and Epstein-Barr virus), Behcet's disease, SFU, sympathetic ophthalmia, birdshot choroidopathy, and VKH. The frequency of in vivo activated T cells in any autoimmune disease was not previously reported to our knowledge. The presence of activated T cells in the peripheral blood of patients with uveitis is not, however, unexpected and may be secondary to the lack of lymphatic drainage in the eye to regional lymph nodes. The splenomacular route for lymphocyte trafficking and immune processing has been well established for several antigen systems.

Inflammatory cells and antigens have direct access to the central immunologic compartment by aqueous and postcapillary venules. Therefore, activated helper T cells leaving the eye would not encounter regional lymphoid tissue and instead would circulate and be processed in the spleen. Although we did not ascertain the precise antigen specificity of these cells, this activity was not noted in the normal population. It can be postulated that these activated cells are involved in mediating ocular inflammation through interaction with specific antigens found in the eye. Such antigens may include toxoplasmic antigens, viral antigens, or unique uveal autoantigens.

Eighteen of the 29 patients with active uveitis had a distinct subpopulation of T cells capable of secreting IL-2 in response to the autologous presentation of S-antigen (4-43 cells/10^6 PBL). Eighty-nine percent of the patients with diffuse uveitis were reactive and had the highest average number of responder cells (18 T cells/10^6 PBL). All four patients with sympathetic ophthalmia and 80% of patients with VKH (four of five) had finite frequencies of these cells. Of the patients with posterior forms of uveitis, 58% had evidence for autoreactive T cells specific for S-antigen; 67% of patients with birdshot retinochoroiditis were positive. Patients with diffuse uveitis had a significantly higher (P < 0.01) number of responder cells compared with posterior forms of uveitis (3 T cells/10^6 PBL, P < 0.01).

Patients with intermediate uveitis could not be stimulated to produce IL-2 when challenged with S-antigen. It would appear that this form of uveitis is not mediated by IL-2-secreting, S-antigen-reactive T cells. Immunotherapy directed against S-antigen may not be valuable in this disease. Unexpectedly, patients with recurrent forms of severe, anterior uveitis (HLA-B27 associated) also had a population of S-antigen-reactive T cells ranging from 0-33 cells/10^6 PBL. This observation was not reported previously, to our knowledge, and may be a manifestation of the increased sensitivity in the limiting-dilution assay environment.

Although all patients had active clinical disease at the time of the assay, the stage of the disease was less well defined. Recently, investigators emphasized the importance of the stage of the disease to help ascertain initiating and mediating events from unrelated secondary phenomena. Vrabec et al showed the development of S-antigen reactivity in lymphocyte proliferation assays after laser photocoagulation in patients with diabetic retinopathy. In their studies, proliferative responses against S-antigen developed in the absence of clinical uveitis and illustrate the importance of identifying pathogenic T cells mechanisms such as lymphocyte activation and cytokine secretion. Anti-epitope influence also may play an important role in inducing clinical disease. We used bovine S-antigen; limiting-dilution assay responses may be different against other S-antigen peptide moieties.

Of the S-antigen-responsive patients, 50% lacked evidence for in vivo activation. These responder cells therefore represent circulating S-antigen-committed, precursor helper T cells and were not found in the normal population. The other half of the S-antigen-positive patients, however, had evidence of spontaneous activation. These cells may represent circulating S-antigen-committed, activated T helper cells (non-precursor) suggesting in vivo contact with this autoantigen. Given that the normal control population had neither spontaneous nor S-antigen-responder cells, the presence of both subpopulations of T helper cells would support their role in mediating uveitis. The experiments as designed, however, cannot determine the antigen specificity of the spontaneously activated T cells. Therefore, an alternative explanation would be that the activated T cells were directed against another antigen(s).

The cytokine IL-2 is involved with lymphocyte communication, specifically T helper cell function.
An IL-2 assay was chosen to determine the role of this pivotal compound in patients with uveitis. Limiting-dilution analysis techniques can be designed to determine minimal frequency estimates for other exogenous or autologous antigens and used to detect other lymphokines or lymphocyte functions. End-organ responses can be studied to investigate lymphocyte trafficking patterns and distinct immunologic compartment differences in ocular inflammatory disorders. With further experience, frequency estimates may be used to follow the clinical course and responses to therapy and provide a tool to understand the predominant pathophysiologic mechanism(s) governing these diseases better.29

Key words: uveitis, limiting-dilution analysis, helper T lymphocytes, interleukin-2, autoimmune

References