Chapter 7

A Novel Method for the Determination of T-cell Proliferative Responses in Patients with Uveitis

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A novel method for the determination of T-cell proliferative responses in patients with uveitis

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Abstract Standard proliferation assays using autoantigens such as S-Ag have given erratic responses when studied with human peripheral blood mononuclear cells. This erratic response is a reflection of the low number of circulating cells in the peripheral blood capable of generating a response as well as the presence of competing cells for the available cytokines in culture. The present study compares the standard proliferation assay with a novel technique in which multiple short-term cell lines are established to S-Ag in medium enriched in helper cytokines. After 12-14 days of culture, these lines were tested for their response to S-Ag. A significant difference was found between patients and controls in the ability to generate responsive cell lines. This translated to a frequency of responsive cells of 0-4 per 107 peripheral blood mononuclear cells (PBMC) in normal individuals and 0-200 per 107 PBMC in patients. This novel technique may provide a means of determining the number of responsive cells to specific autoantigens in the peripheral blood of patients and the ability to follow the response over time.

Key words S-antigen; lymphocytic proliferation; uveitis; autoimmunity

Introduction Chronic posterior uveitis is an inflammatory disease of unknown origin which may lead to serious visual impairment. It is often seen in the context of systemic inflammation such as sarcoidosis, vasculitic syndromes such as in Behçet’s disease, or in purely ocular inflammatory syndromes such as sympathetic ophthalmia. T cells are felt to play an important role in these disorders. In addition, a form of uveitis that is similar to many human conditions can be induced in laboratory animals by injection of partially purified retinal proteins, such as S-antigen (S-Ag) or interphotoreceptor retinoid binding protein (IRBP). In patients, the presence of T cell infiltration in pathologic eye specimens

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points to the central role played by these cells in the inflammatory process. Several reports have demonstrated that patients with uveitis have a greater cellular immune reactivity to S-Ag and to IRBP than control individuals. The presence of a response is indicative of the existence of sensitized cells within the peripheral blood that have the ability for involvement in the disease process. However, this assay is qualitative and does not quantify the number of reactive cells present within the peripheral blood. One reason is the limited number of cells with the appropriate specificity circulating at any one time.

To circumvent this problem, one can study a statistically significant number of wells rather than the limited number usually studied or perform limiting dilution assays. Previous work has also shown that the magnitude of the response per well has little relationship to the actual number of helper T cells present in a given culture well. This response is a function of the microenvironment in which the T cells are growing. One can partially correct for this phenomenon by establishing a large number of short-term T cell lines in the presence of cytokines such as IL-2. The procedure used in these experiments involves culturing human peripheral blood mononuclear cells for two weeks in a medium enriched with T cell growth factors. These short-term lines can then be tested for a proliferative response to a target antigen. This simple approach shows promise as a method for the enumeration of autoreactive helper T cells in the peripheral blood of patients with uveitis.

Materials and methods

Patient selection Patients with posterior pole uveitis were taken from the pool of patients seen in the uveitis clinic of the National Eye Institute. All patients had a history and evidence of prior disease involving the retina, choroid, and/or the vitreous. At the time of testing, they were controlled on a combination of cyclosporine and prednisone. The patients tested had one of the following diagnosis: Behçet's disease, pars planitis, ocular sarcoidosis, or the Vogt-Koyanagi-Harada syndrome. The basis for the diagnosis of each disease is outlined elsewhere. Control individuals were either clinic patients who did not have uveitis or normal volunteers. Controls were age- and sex-matched with patients. All patients signed an informed consent approved by the Investigational Review Board of the National Eye Institute. The study abides by the guidelines set forth in the Declaration of Helsinki as well as the U.S. Code of Federal Regulations as it pertains to research on human subjects (45 CFR 46).

Mononuclear cell preparation and culture Peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood obtained from patients or normal controls. Cells were obtained on standard Isolymp l gradients (Gallard-Schlesinger, Carle Place, NY, USA) by centrifugation at 4000g. In the standard proliferation assay, 2x10⁶ cells were incubated in flat bottom 96-well microtiter plates for 5 days in 200 μl RPMI-1640 medium with HEPES (GIBCO, Grand Island, NY, USA), supplemented with glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml) and 10% heat-inactivated human AB serum (Biocell Laboratories, Carson, CA, USA). Bovine S-Ag, prepared according to

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the method of Dorey and associates. was used as a test antigen at 4 μg/ml and 20 μg/ml in triplicate wells; phytohemagglutinin was used as a positive control. For the last 16 hours, the cells were pulsed with 3H-thymidine (3H-TdR, New England Nuclear, Boston, MA, USA; 0.5 μCi per 10 μl/well), then harvested and counted on a scintillation counter. Results are reported as a stimulation index (S.I.), which is calculated by dividing the average count for the wells containing antigen divided by the average count for the wells containing only medium.

DETERMINATION OF THE FREQUENCY OF RESPONSE IN SHORT-TERM LINES In the frequency analysis assay, the cells were incubated at 37°C with bovine S-Ag for 1 hour at a cell density of 1x10⁶. Then, the cells were diluted to a concentration of 1x10⁶/ml and plated at 200 μl per well into 480 wells on five 96-well round-bottom microtiter plates (Costar, Cambridge, MA, USA). Starting on the third day and every three days thereafter, half of the medium was replaced with fresh medium containing 5% T cell stimulant (Collaborative Research Inc., Bedford, MA, USA) and 2 U ml⁻¹ human rIL-4 (Genezyme, Boston, MA, USA).

On days 12 to 14, each short-term T-cell line (480 in total) was analyzed for its reactivity to bovine S-Ag. Each microwell was washed and centrifuged twice with medium. The concentration of viable cells was determined following resuspension using a Neubauer chamber. Aliquots of 10,000 cells from each T-cell line were placed in presence of 10,000 irradiated autologous mononuclear cells used as antigen-presenting cells (APC). For each T-cell line, four wells of a 96-well round-bottom microtiter plate were seeded with the appropriate number of T cells; S-Ag-stimulated APC were added to two wells, while unstimulated APC were added to the other two. Plates were incubated for 72 hours and pulsed with [3H]-thymidine during the last 18 hours of culture. The APC were prepared by pulsing 1x10⁶ APC per ml in media with or without bovine S-Ag for one hour at 37°C. Lines were considered positive for bovine S-Ag if the stimulation index (S.I.) was above 3.0 and if the coefficient of variation for each duplicate culture was less than 30%.

Results The response to bovine S-Ag was measured in patients and controls using the standard five-day assay as well as by means of short-term lines (analysis of frequency). The new assay was tested in five controls (Table 1) and seven patients (Table 2). The diagnosis in the seven patients tested is shown in Table 2. All patients had ocular involvement for at least two years with visible sequelae in the posterior pole. All patients were on prednisone and/or cyclosporine, but none had been treated with cytotoxic agents. All patients had been free of ocular inflammation for at least two months when they were tested.

Establishing short-term cell lines in both normal individuals and in patients was easily performed using the culture conditions described above. All wells showed signs of strong proliferation at the end of 12 days of culture with an apparent doubling or tripling of the number of cells per well. However, upon challenge with bovine S-Ag, only a limited number of wells gave a positive response with an S.I. above 3.0. A lower number of responding wells was observed in controls when compared to patients (Tables 1 and 2). No direct correlation was seen between the
Table 1. Stimulation index to bovine S-Ag and percentage of positive wells in the short-term cell lines in normal subjects.

<table>
<thead>
<tr>
<th>Standard proliferation assay (S.I.)</th>
<th>Short-term cell lines Percentage of positive wells</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 µg/ml</td>
<td>4 µg/ml</td>
</tr>
<tr>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>1.8</td>
<td>1.0</td>
</tr>
<tr>
<td>3.0</td>
<td>1.6</td>
</tr>
<tr>
<td>0.7</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*Only wells with an S.I. above 3.0 were considered positive and included in the calculation.

Table 2. Stimulation index to bovine S-Ag and percentage of positive wells in short-term cell lines in patients with uveitis.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Standard proliferation assay (S.I.)</th>
<th>Analysis of frequency Percentage of positive wells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 µg/ml</td>
<td>4 µg/ml</td>
</tr>
<tr>
<td>Behçet</td>
<td>2.7</td>
<td>1.5</td>
</tr>
<tr>
<td>Behçet</td>
<td>9.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Behçet</td>
<td>1.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Behçet</td>
<td>1.1</td>
<td>1.3</td>
</tr>
<tr>
<td>VKH</td>
<td>2.4</td>
<td>2.6</td>
</tr>
<tr>
<td>Pars planitas</td>
<td>4.0</td>
<td>3.7</td>
</tr>
<tr>
<td>Sarcoid</td>
<td>1.8</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*All patients were inactive at the time of testing.

Discussion  Little information is available regarding the number of circulating T cells with immune memory for ocular autoantigens. Such knowledge would be useful to monitor autoreactivity in patients, and is necessary if one wants to understand the interplay between the eye and the peripheral immune system. This is particularly true if one wants to monitor levels of ocular inflammation by performing in-vitro assays. However, standard assays are unable to provide such quantitative information for several reasons. The number of circulating cells capable of generating a response may be very low, such that responsive cells are not present in each of the test wells. In addition, the number of T cells required to generate a response is highly variable and dependent on factors such as the level of suppressor activity within the culture and competition between cultured cells for those cytokines available in the culture medium. Increasing in-vitro concentrations of helper cytokines can compensate for both of these limiting factors. To compensate for the low precursor frequency, the easiest approach is to increase the number of cultured cells per well or to increase the number of wells being cultured to a statistically significant number. We decided to use the second approach, particularly since it also gave us the possibility of establishing cell lines

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to S-Ag with a minimum of effort. In past experiments, we had noticed that patients with active disease had a lower proliferative response in the standard assay. We had postulated, that in addition to the factors mentioned above, the inactivity could be due to margination of cells into the eye thus decreasing the pool of responsive cells in the peripheral blood. It is for this reason that we chose to test only those patients who did not have active ocular disease at the time of the in-vitro study.

As shown in Table 1, responsive cells to S-Ag can be found in the peripheral blood of normal controls. This is in keeping with previous studies where patients and controls were found to have both T- and B-cell responses to S-Ag. In our own study, up to 30% of normal individuals have a proliferative response to bovine S-Ag. However, there appears to be a quantitative difference in the number of responsive cells between patients and controls, as suggested by our results in Table 2.

Given the low number of positive wells that we identified, one can assume that each well contains only one reactive cell. That this assumption is true is further supported by work on MBP-responsive lines cultured using an analogous method, and by TCR analysis where over 98% of the wells contained only one rearrangement. Thus, the clonal nature of the cell lines generated by our culture technique allows us to estimate the number of responsive T cells present in the peripheral blood of both patients and controls. If one assumes that all positive wells contain only one responsive T cell and that all reactive cells have generated a response, the number of responsive cells in the peripheral blood is in the range of 0-4 per 10^7 lymphocytes in normal individuals and 0-200 per 10^7 lymphocytes in patients. These numbers are comparable to that calculated by Opremcak et al. using a limiting dilution assay: they had found a range of 0-40 per 10^6 lymphocytes for patients with uveitis.

This culture technique shows considerable promise for the future. In addition to giving us an estimate of the number of circulating reactive cells, it may allow us to determine several characteristics of the responding cell population. Since each positive well represents the clonal expansion of one autoreactive cell, it should allow us to identify the epitopes of S-Ag to which cells are responding as well as the pattern of TCR rearrangement present in these autoreactive cells. In addition, by testing patients on a repeated basis during different stages of disease or immune activation, it should be possible to gain some insight into the cellular trafficking of autoreactive cells in peripheral blood.

References

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