Flow Cytometric Detection of Lymphocyte Proliferation in Eyes With Immunogenic Inflammation

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Identification and analysis of antigen-activated lymphocytes from sites of ocular inflammation is important to understanding of the role of infiltrating immune effectors in ocular inflammatory disease. Unfortunately, few assays can distinguish activated lymphocytes from the antigen-irrelevant lymphocytes that randomly migrate to sites of inflammation. The authors describe a method that allows the quantitative recovery and identification of activated lymphocytes from mouse eyes. Recovered subconjunctival lymphocytes were simultaneously stained for specific cell-surface markers (with fluorescein-labeled antibodies) and for DNA content (with propidium iodide), then analyzed by flow cytometry. For any subpopulation of lymphocytes, the relative percentage and absolute number of cells in each phase of the cell cycle was determined. The activated subset of T and B cells was quantitated by determining the number of proliferating cells (ie, the number of cells in the S + G2 + M phases of the cell cycle). Using this method, T cell proliferation was found to be a specific component of the subconjunctival immunogenic inflammatory response induced by a local graft-versus-host reaction and by subconjunctival delayed hypersensitivity reactions. In addition, proliferating T cells could be distinguished from nonproliferating B cells produced by the subconjunctival inoculation of a T cell tumor.

Eyes undergoing immunogenic inflammation are infiltrated by numerous types of leukocytes, especially lymphocytes.1,2 However, whether lymphocytes contribute to the inflammation in an antigen-specific manner has been difficult to demonstrate and/or quantitate. Since lymphocyte proliferation is one of the consequences of activation by specific antigen,3 assays which measure proliferation of lymphocytes in target tissues undergoing inflammation begin to approximate this goal. For instance, methods using systemic administration of radiolabeled nucleotides found that cellular proliferation is a specific component of cutaneous delayed hypersensitivity reactions.4 However, this method has several limitations: radioisotopes are expensive, pose a significant health hazard, and cannot be used in human research. In addition, current radioisotope methods cannot identify the precise cellular population that is proliferating.

Recently, techniques have been developed for the identification and quantification of the proliferating population among cultured cells by flow-cytometric analysis of DNA content and cell cycle kinetics.5 Certain fluorescent dyes, such as propidium iodide (PI), bind double-stranded DNA (or RNA) with high affinity and specificity.6 Therefore, the amount of fluorescence emitted by the cell is proportional to its DNA content. A resting cell (ie, in G0/G1 phases of the cell cycle) contains the normal amount of DNA present in all diploid cells and can be recognized as a discrete peak on a DNA histogram by flow cytometry. Any cell containing more than this amount of DNA can be identified by its increased fluorescence and is considered to have entered into S, G2, or M phases (ie, is proliferating).7,8 By simultaneously staining the cells for identifying surface markers with fluorescein isothiocyanate (FITC)-labeled antibodies, dual labeling of cells can be used to analyze the proliferation of discrete lymphocyte subsets.

Several investigators adapted this technique to analyze proliferation in cell populations harvested from live animals.9,10 We were interested in characterizing the role of lymphocyte proliferation in rat and mouse eyes undergoing immunogenic inflammatory reactions. However, previously described techniques for DNA analysis are only relevant for cell populations, such as bone marrow,9 that are not contaminated by cells obtained from tissues of disparate embryonic derivation and vast morphologic heterogeneity. Unfortunately, the analysis of infiltrating lymphocytes in whole mouse eyes would require the separation of lymphocytes from the other resident cell types form...
ing the ocular tissues. Even the physical dissection of the murine eye into anterior segment or conjunctival infiltrates would not separate the tissues adequately and would result in a very low yield of lymphocytes.

To that end, this report demonstrates that simultaneous analysis of DNA content and cell-surface markers by flow cytometry can be adapted for the analysis of lymphocyte proliferation recovered from the whole eyes of mice. We found that this method can identify T cells proliferating in a local subconjunctival graft-versus-host induced inflammatory reaction and in subconjunctival delayed hypersensitivity reactions. Additionally, we were able to distinguish proliferating T cells from nonproliferating B cells in eyes infiltrated by a T cell tumor.

Materials and Methods

Animals

Female, inbred C57BL/6, BALB/c or (C57BL/6 × BALB/c) F1 mice, of 6–12 weeks of age, were maintained in our own colony and used in these experiments. All investigations adhered to the ARVO Resolution on the Use of Animals in Research.

Cell lines

DG11 is a T cell hybridoma created by the fusion of an alloreactive (anti-laδ) T cell clone and a lymphoma-cell line.11 This hybrid proliferates spontaneously and maintains numerous T cell characteristics, including the Thy 1.2 surface molecule and the ability to secrete interleukin-2 (IL-2) when stimulated with an laδ antigen-presenting cell (APC). DG11 is maintained in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS; Hyclone, Logan UT), HEPES (10 mM, Gibco), L-glutamine (2 mM, Gibco), penicillin (100 units/ml, Gibco), and streptomycin (10 μg/ml, Gibco).

Ocular Inoculation

Subconjunctival inoculations were done using a blunt 30-G needle attached with plastic tubing to an automated Hamilton syringe. For each injection, 2 μl of air followed by 2 μl of cell suspension was drawn into the tubing. The blunt needle was inserted into the subconjunctival space through the inferior bulbar conjunctiva of the right eye, and a small bleb of fluid and air was raised.

Local Graft-Versus-Host Reaction

Local graft-versus-host reactions were used as a model for localized cell-mediated immunogenic inflammation.12-13 Splenocytes from C57BL/6 mice were separated from erythrocytes by passage over a Lymphoprep density gradient (Nycomed, Oslo, Norway). Then 100 × 10⁶ parental C57BL/6 splenocytes were mixed with 100 × 10⁵ irradiated F1 splenocytes for 3 days in a 25-cm² culture flask to produce a mixed lymphocyte reaction containing activated parental graft-versus-host effectors. Later 1 × 10⁶ cells from this culture were inoculated in 2 μl into the subconjunctival space of eyes of (BALB/c × C57BL/6) F1 mice. Clinical examination revealed significant inflammation within 24–48 hr (Cousins and Streilein, in preparation). Pilot experiments revealed that 48 hr was optimal for analysis of proliferation.

Local Adoptive Transfer of Delayed Hypersensitivity

Subconjunctival delayed hypersensitivity reactions were produced by the local adoptive transfer of primed lymph node cells into the subconjunctival space. C57BL/6 mice were primed to Mycobacterium tuberculosis (10 mg/ml in mineral oil; Difco, Detroit, MI) at the base of the tail. Two months later, inguinal and perineal lymph nodes were harvested and dispersed in a single-cell suspension. Naive splenic adherent cells were pulsed with 50 μg/ml of microparticulate mycobacterial antigenic preparation and were used as APCs. For the experimental group, naive recipients were injected with a mixture of 1 × 10⁶ (primed) lymph node cells plus 2 × 10⁵ pulsed APCs. For the controls naive recipients were injected with 1 × 10⁶ (naive) spleen cells plus 2 × 10⁵ antigen-pulsed APCs. Pilot studies revealed that 48 hr was optimal for evaluation of proliferation, and clinical inflammation was still apparent.

Clinical Examination of the Eye

A clinical grading scale was developed for the subconjunctival sites using a slit lamp or dissecting microscope. The clinical scoring of subconjunctival inflammation was not very precise due to few specific clinical signs. Scoring was based upon the amount of infiltration (tan or gray cellular infiltrate) and the presence or absence of obvious nodular thickening. A semiquantitative score was assigned to each eye according to the following scale: 0.5, chemosis or hemorrhage without infiltration; 1, mild infiltration; 2, moderate infiltration; and 3, marked infiltration. All clinical examinations were done in a masked fashion without knowledge of treatment or immune status of the animal.

Radiolabeled Cells

Radiolabeled cells were produced by growing DG11 in media containing 37 kmCi of 125I-iododeoxycytidine (New England Nuclear, Boston, MA) for 24
hr, followed by two washes with normal media. After inoculation of these cells into the subconjunctival space, the eyes were enucleated 30 min later. Whole or processed specimens were counted for gamma emissions on a LKB gamma scintillation counter (Turku, 10, Finland).

For in vivo 125I-iododeoxyuridine labeling of cells in sites of inflammation, the 125I-iododeoxyuridine was used to identify proliferation at sites undergoing immune-mediated inflammation as previously described. Briefly, 48 hr after adoptive transfer of local graft-versus-host or delayed hypersensitivity reactions, mice received an intravenous injection of 5-fluorodeoxyuridine. Thirty minutes later, mice received 0.5 µCi of 125I-iododeoxyuridine intravenously. Eyes were removed 24 hr later, after pilot studies revealed that maximal cell-associated radiolabel was present, and extracellular and intravenous radiolabel had been cleared. Whole eyes were counted on a LKB gamma scintillation counter. Because of high variability of radiolabel uptake among animals, results are expressed as a ratio between the counts per minute (CPM) measured in the injected eye to the CPM measured in the contralateral normal (unmanipulated) eye.

Antibodies

Rat anti-mouse Thy 1.2-FITC-labeled antibody (rat monoclonal IgG2a isotype; Becton Dickinson, Mountain View, CA) was used to identify T cells. Goat anti-mouse immunoglobulin (Ig) G, A, M, and antigen-binding fragments (Fab2) (Cappell goat polyclonal) were used to stain surface Ig-positive lymphocytes (ie, B cells). In initial experiments appropriate control antibodies were used (an isotype matched control for Thy 1.2 and goat anti-rat IgG, A, and M for B cells), but additional experience showed these to be unnecessary since clear separation of positive populations from negative populations was regularly achieved in the control.

Recovery and Analysis of Ocular-Infiltrating Lymphocytes

Eyes for analysis were removed en bloc with adherent adnexa and conjunctiva by exenteration of the entire orbital contents. The eyelid margin and glandular tissue were carefully dissected away. The residual conjunctival tissue and globe were placed in individual wells of a 24-well plastic plate. A collagenase/dispase mixture was made by adding the appropriate concentration of collagenase/Dispase (Boehringer Mannheim, Indianapolis, IN) and crude collagenase (Sigma, St. Louis, MO) in normal media to give a final concentration with collagenase activity of 150 units/ml and Dispase activity of 0.5 units/ml. Then 500 µl of the collagenase/dispase mixture was added, and the eyes were crushed with the plunger of a 5-ml syringe and minced with a sharp scalpel. The eyes were incubated for 2.5 hr at 37° C after which the residual gelatinous tissue was filtered through a Nylon mesh. Then 750 µl of 10% bovine serum albumin (BSA) was used to rinse the residual adherent cells through the mesh and to dilute the suspension.

After microcentrifugation through 10% BSA, FITC-labeled antibody was added to the pellet at a 1:5 concentration (normal concentration was 1:20), allowed to react for 30 min on ice, and 950 µl of 2% Tween 20 (Sigma) in phosphate-buffered saline containing PI (50 µg/ml, Sigma) was added. The suspension was allowed to stand at room temperature for 1 hr and then analyzed by flow cytometry.

Flow Cytometry

Flow cytometry was done using a Becton Dickinson FACScan Flow Analyzer, equipped with a 15-mW argon ion laser tuned for peak excitation at 488 nm with the filter system over the photodetectors set for 530 nm (green) and 650 nm (red). Hewlett Packard FACScan (Becton Dickinson, San Jose, CA) software was used to collect and analyze the data. Instrument parameters for acquisition mode were set according to the criteria established in the Results section. Dual light-scatter gates were not used. Instead, events were acquired by collecting only PI fluorescence-positive cells. The PI acquisition gates were set by arbitrarily choosing a channel halfway between threshold and the G0/G1 peak. Compensation was set according to routine methods, and 7500 to 10,000 PI-gated events were usually collected.

Quantitative cell concentrations were determined by the following formula:

Total cell number per ml = No. events per sec/flow rate per sec.

The number of events and run time per sample were automatically calculated by the instrument for each sample. The flow rate was determined by measuring the weight of water removed by the input stem of the instrument from a known sample over a 5-min period; then this was converted to volume uptake per second.

For the analysis of the flow data, two controls were run for each experiment. First, whole splenocytes were stained with FITC-labeled antibody and PI to guarantee that the reagents were working normally. Second, 1 X 10^6 splenocytes were added to a normal eye, processed as described, and stained with FITC-labeled antibody and PI. This control was used to set the electronic analysis gates.

The FITC gates were determined by identification of the channel location that separated the FITC-pos-
tive population from the FITC-negative population of the controls and electronically gating the positive population. If inadequate separation of the two populations in the control occurred, the experiment was discarded. These settings were arbitrarily applied to all experimental samples equally to determine the percentage of FITC-positive cells in each eye.

The percentage of proliferating cells was determined by electronically setting analysis gates for DNA content as determined by PI fluorescence. The G0/G1 peak of the control population was electronically gated from the S + G2 + M population by arbitrarily determining the endpoint of the G0/G1 peak on the DNA histogram of the control. All events representing an increase in fluorescence (ie, to the right of this gate) were considered to represent cells that had entered the proliferative phase of the cell cycle. Again, this control-determined gate was arbitrarily applied to all experimental samples to calculate the percentage of cells in S + G2 + M phase of the cell cycle for each eye. The number of proliferating cells in an individual eye was calculated by the formula:

\[
\text{No. proliferating cells/eye} = \text{total no. cells recovered} \times \% \text{FITC positive.}
\]

Finally, proliferation of subsets was determined by activating both gates simultaneously. Proliferation was quantitated by the formula:

\[
\text{No. FITC positive cells} = \text{Total cells recovered} \times \% \text{FITC positive in S + G2 + M.}
\]

Values for no. FITC positive, % proliferating, and no. proliferating were calculated for each individual eye, then each category was averaged for ten samples per experiment.

Statistics

Where appropriate, experimental and control populations were compared using the student two-tailed t-test.

Results

We used flow cytometry to quantitate proliferation of lymphocyte subsets obtained from immunogenically inflamed mouse eyes. We attempted to achieve the following four goals: (1) quantitative recovery of lymphocytes from whole eyes excluding debris and nonlymphoid cells; (2) quantitative determination of cell concentration per eye sample by flow cytometry; (3) cell surface staining with FITC-labeled antibodies while enabling penetration of PI into the nucleus of all cells for DNA analysis (avoiding artifacts induced by clumping or aggregation of cells); and (4) development of a simple method for determination of DNA content and proliferation by flow cytometry.

Quantitative Recovery of Lymphocytes From the Eye

Initial attempts to recover lymphocytes from inoculated eyes revealed wide disparities in cell yield. Two major sources of artifact were detected. These were: (1) loss of cells at inoculation and/or enucleation and (2) loss during processing of the enucleated sample. Loss of cells during removal of the eye was eliminated by using an exenteration technique which removed the globe, bulbar conjunctiva, palpebral conjunctiva, and adnexa en bloc. After inoculation of \(1 \times 10^6\) radiolabeled cells into the subconjunctival space of normal eyes, 91% of the inoculated radioactivity was recoverable when eyes were removed with this method.

Loss of cells during processing of the eye sample was minimized by insuring complete digestion with collagenase and Dispase. However, complete digestion resulted in the production of large amounts of debris, which complicated analysis by flow cytometry. Centrifugation of the cell suspension through 10% BSA markedly reduced the debris, but also reduced the cell yield from 3-4 \(\times\) \(10^6\) to less than 1 \(\times\) \(10^5\).

Nevertheless, experiments adding known numbers of splenocytes or T cell hybridomas to a suspension extracted from normal eyes, followed by this process, revealed that 75%-90% of added lymphocytes were recovered in the pellet; the wash selectively retained large squamous epithelial cells but relatively few lymphocytes in the supernatant. Thus, the estimated recovery of cells in the final suspension compared with the initial subconjunctival inoculum was 67%-81% (91% recovery of inoculated cells \(\times\) 75%-90% recovery from processing). This yield was confirmed by determining that 68% of inoculated radioactivity was recoverable in the final cell suspension.

Quantitative Determination of Cell Yield From Enucleated Eyes

To define the cell population for analysis, we chose to acquire data for the whole population of cells in the suspension. Thus, we did not set dual light scatter acquisition gates, but instead included any event that was considered to be PI positive by the flow cytometer (and therefore represented only nucleated cells, Appendix A). Figures 1A and B show how this method excluded debris and identified a cell population with an acceptable pattern by dual light scatter without actually setting the gates.

Using PI positivity as the gating criterion, we were
Fig. 1. Dual light scatter (DLS) characteristics for the ungated population (A) and with acquisition gates set to detect only the propidium iodide fluorescence positive events (B) of a cell suspension from a normal eye. The small, dense collection of material in the lower left of (A) represents debris, not present in (B). Note that the DLS characteristics of (B) resemble the customary tight pattern normally obtained when DLS gates are manually set.

able to utilize the flow cytometer to calculate the cell concentration in a given suspension of cells. To insure the accuracy of this method, we assayed the concentration of suspensions of serial dilutions of known concentration of fluorescent microbeads. Over a concentration range of $0.250 \times 10^6$–$4 \times 10^6$ microbeads, the measured cell number correlated well with the predicted concentration, and the calculated flow rate varied less than $\pm 7\%$ from the directly measured flow rate. Identical results were obtained when PI stained DG11 T cell hybridomas or splenocytes were used for the same type of measurement (Appendix B).

Staining Ocular Cell Suspensions With Antibody and PI

We next developed a simple method for dual staining of lymphocytes with PI and FITC-labeled antibody. The dilemma we faced was that the cell membrane must be made permeable with detergent to allow entrance of PI into the cytoplasm. However, this step dissolves away the cell membrane which contains interesting surface markers.

Eventually, we settled on a simple one-step staining procedure that balanced optimal preservation of cell surface staining, cell permeability, and access of PI to the cell nucleus but induced minimal additional cell loss, clumping, or doublet formation. Staining the cell surface with higher-than-usual antibody concentrations (1:5) followed by resuspension of cells in 1%–2% Tween 20 mixed with PI resulted in adequate preservation of FITC staining which remained stable for at least 3 hr (Figs. 2A–C, Appendix C). Additional washes to remove excess unbound antibody did not alter the surface staining or background but resulted in loss of cells and clumping.

Determination of DNA Content in Cell Suspensions

After experimentation with various analyses of the DNA histograms in the determination of DNA content, we decided to estimate the location of the end of the first G0/G1 peak on the control histogram (Figs. 3A–D). An electronic analysis gate was determined that divided the control histogram into two phases: (1) containing those cells representing the G0/G1 phase of the cell cycle (nonproliferating population) and (2) containing those cells representing the S + G2 + M phases (proliferating population). The percentage of cells in S + G2 + M was used to calculate the percentage of proliferating cells (Appendix D). This same gate was then applied to all experimental samples.

Application of the Technique to the Analysis of T and B Cell Proliferation in Normal and Tumor-Infiltrated Eyes

These procedures were then used in several different types of experimental systems. Table 1 illustrates...
the recovery of T and B lymphocytes from normal mouse eyes. As can be seen, approximately equal numbers of each type of lymphocyte were recovered.

For each, a relatively high percentage of cells was found to be in the proliferative phase of the cell cycle, although the B cells had a much higher percentage of

![Fig. 2](image1.png)

**Fig. 2.** The fluorescence intensity histograms for FITC-Thy 1.2 stained cells within various ocular preparations: (A) represents the control suspension resulting from the addition of $1 \times 10^6$ splenocytes to a suspension made from a normal eye; (B) represents the FITC-Thy 1.2 populations from a normal eye; (C) represents the FITC-Thy 1.2 positive population of an eye inoculated with $1 \times 10^6$ graft versus host effector lymphocytes 48 hours previously.

![Fig. 3](image2.png)

**Fig. 3.** The DNA histograms generated by the DNA content of various ocular cell populations as determined by propidium iodide fluorescence: (A) illustrates the DNA histogram for the total cellular population obtained from a normal mouse eye; (B) represents the histogram as constructed from only the FITC-Thy 1.2 positive (ie, T cell) subset from (A); (C) shows the DNA histogram for all the cells from an eye undergoing a local graft versus host reaction; (D) demonstrates the DNA histogram for only the Thy 1.2 positive cells from (C). In both (B) and (D), a marked increase in S + G2 + M phases was measured as compared to the value obtained from the whole eye. Note that the greatest percentage of T cell proliferation occurred within the eye undergoing a graft versus host immune response (D).
dium iodide (PI). The cells were simultaneously analyzed by flow cytometry digested in collagenase/dispase for 2.5 hours. The resultant single-cell sus-

Table 1. T and B lymphocyte proliferation from normal mouse eyes

<table>
<thead>
<tr>
<th>Lymphocyte type</th>
<th>Number of FITC-positive cells (×10^6)</th>
<th>% Proliferating cells</th>
<th>Number of proliferating cells (×10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>13.7 ± 0.9</td>
<td>22.4 ± 1.5</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>B</td>
<td>12.6 ± 1.3</td>
<td>51.0 ± 5.0</td>
<td>5.1 ± 0.4</td>
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</table>

Ten normal eyes and their adnexa were removed en bloc (exenterated) and digested in collagenase/dispase for 2.5 hours. The resultant single-cell sus-
pension was stained for T cells (Thy 1.2-FITC) or B cells (anti-IgG, A, M-FITC), then resuspended in PBS with 2% Tween 20 containing propi-
dium iodide (PI). The cells were simultaneously analyzed by flow cytometry for the percentage of FITC-positive cells and DNA content by PI fluores-
cence. The number of proliferating lymphocytes within each individual eye was calculated by the formula: (Number of Proliferating Lymphs) = (Total Cells Recovered) × (% FITC-positive cells) × (% of FITC-positive cells in S + G2 + M Phase of Cell Cycle).

All values are expressed as mean ± SEM of the ten individual eyes.

Table 2. T and B lymphocyte proliferation from eyes inoculated subconjunctivally with a T cell neoplasm

<table>
<thead>
<tr>
<th>Lymphocyte type</th>
<th>Number of FITC-positive cells (×10^6)</th>
<th>Percentage of proliferating cells</th>
<th>Number of proliferating cells (×10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>309.1 ± 30.2</td>
<td>34.4 ± 0.8</td>
<td>106.4 ± 27.8</td>
</tr>
<tr>
<td>B</td>
<td>30.3 ± 10.4</td>
<td>28.0 ± 3.6</td>
<td>9.8 ± 1.9</td>
</tr>
</tbody>
</table>

Ten eyes received subconjunctival injections with 200 × 10^6 DG11 T cell hybridoma cells. Five days later, an infiltrative tumor mass was present. The eyes were prepared as described previously. The T lymphocytes represent mostly DG11 tumor cells. All values are expressed as mean ± SEM.

proliferating cells. We presume that these proliferat-
ing cells represent the resident activated-lymphocyte population in the conjunctival-associated mucosal lymphoid tissues.14 Our analysis does not permit us to distinguish intraocular from extraocular lymphoid cell populations in mouse eyes. We found that peripheral blood and spleen contain less than 2% prolifer-
erating lymphocytes (data not shown). Therefore, circulating lymphocytes in the ocular vascular compartments probably contributed little to the prolifer-
ating cell population in our ocular samples.

Next, we wanted to know if we could distinguish which lymphocyte subsets were proliferating in individual eyes. We inoculated 200 × 10^3 cells of the T cell hybridoma (DG11) in the subconjunctival space of C57BL/6 mice; 5 days later an infiltrative tumor mass was observed. When the eyes were removed, quantitative analysis revealed that 309 × 10^6 cells were recovered, 34% (106 × 10^6) of which were prolifer-
ating (Table 2). In the same eyes, the number of proliferating B cells was only slightly increased from the normal background, showing that flow cytometry can distinguish among proliferating subsets of lymphocytes. Interestingly, the percentage of proliferating B cells was less than that found in the normal eye, suggesting that nonactivated blood-borne B cells may have been recruited to the tumor-inoculation site.

Application of Technique to the Analysis of Inflammation Mediated by Subconjunctival Local Graft-Versus-Host Reactions

We tried to determine if we could detect a specific increase in the number of proliferating T cells in a site undergoing a cell-mediated immunologic reaction. Local graft-versus-host reactions are T cell mediated, antigen-specific immunogenic inflammatory reac-
tions that occur when parental lymphocytes are inoc-
ulated into local tissues of semiallogeneic recipients.12,13 In this response, the T cells from one parent-

Table 3. Comparison between clinical exam and in situ proliferation as measured by uptake of 125I-iododeoxyuridine for subconjunctival local graft versus host reactions

<table>
<thead>
<tr>
<th>Type of inoculum</th>
<th>Clinical score</th>
<th>125I-Udr uptake cpm injected eye/ cpm normal eye</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graft versus host</td>
<td>1.00 ± 0.38</td>
<td>1.45 ± 0.21</td>
</tr>
<tr>
<td>Syngeneic</td>
<td>0.50 ± 0.19*</td>
<td>1.11 ± 0.06*</td>
</tr>
</tbody>
</table>

Graft versus host effector cells were generated by culturing C57BL/6 splenocytes with (BALB/c × C57BL/6) F1 stimulators for three days; 1 × 10^6 of these cells were inoculated into the subconjunctiva of the F1 recipients, while the control group received the same number of unstimulated F1 splenocytes. Seven eyes from each group were examined clinically at 48 hours. Five mice from each group received an intravenous injection of 125I-UdR, and both injected and normal eyes were harvested 24 hours later. 125I-UdR uptake was measured in counts per minute (cpm) by gamma scin-
tillation for each eye.

All values are expressed as mean ± SEM. * NS by the student t-test.
shown, the ratio of CPM of the injected versus normal eye was greater in mice receiving graft-versus-host effectors (1.45) than in those receiving syngeneic cells (1.11), but that this difference was not statistically significant.

Additionally, the radiolabeling method was unable to identify the proliferating cell population. Therefore, we used flow-cytometric analysis to determine if T cell proliferation occurred in the graft-versus-host reactions. Table 4 illustrates the flow-cytometric analysis of F1 eyes that received a subconjunctival inoculation of 1 x 10^6 parental lymphocytes 48 hr previously. Although eyes in the experimental group contained slightly greater numbers of total proliferating cells than did the eyes in the control group (20.5 x 10^3 versus 16.1 x 10^3), the difference was not statistically significant.

However, analysis of the T cell subset revealed important differences between the two groups. In mice receiving a subconjunctival inoculation of syngeneic effectors, a large Thy 1.2-positive T cell population was present (56.7 x 10^3) in the subconjunctival space, but only a small number (8.9 x 10^3) were proliferating. Eyes that received parental strain lymphocytes contained a slight, but not significant, increase in the number of retained T cells (74 x 10^3). More importantly, the percentage and number of proliferating T cells in these eyes was significantly increased. Thus, subconjunctival delayed hypersensitivity (DH) reactions were produced by the local adoptive transfer of DH effectors into the subconjunctival space of naive C57BL/6 mice. Experimental recipient mice received 1 x 10^6 Mycobacterium tuberculosis primed lymph node cells plus 2 x 10^5 antigen-presenting cells (splenic adherent cells) that had been pulsed with 50 ug/ml of microparticulate Mycobacterial antigenic preparation. Naive control recipients were injected with 1 x 10^6 naive spleen cells plus antigen-pulsed APCs. Seven eyes in each group were examined clinically and five mice in each group received administration of ^125I-UdR as described. All values are expressed as mean ± SEM.

An analogous series of experiments was done to compare clinical examination, ^125I-iododeoxyuridine labeling, and flow cytometry in the evaluation of delayed hypersensitivity induced by the local adoptive transfer of antigen-primed lymphocytes into the subconjunctival space. The local adoptive transfer of lymphocytes to cutaneous sites is a well-accepted model for evaluating delayed hypersensitivity effector function. The adoptive transfer of 1 x 10^6 mycobacterium-primed lymphocytes (recovered from nodes draining the base of the tail previously primed to M. tuberculosis in mineral oil) mixed with 2 x 10^5 APCs (mycobacterial antigen-pulsed splenic adherent cells) into the subconjunctival site produced an inflammatory nodule of moderate intensity which persisted for at least 48 hr. Compared with mice receiving a subconjunctival inoculum of unprimed splenocytes, a significantly greater inflammatory reaction was observed in mice receiving primed cells (Table 5).

### Table 4. Cellular proliferation in eyes undergoing subconjunctival graft versus host reactions

<table>
<thead>
<tr>
<th>Type of inoculum</th>
<th>Number of cells recovered (x10^3)</th>
<th>Percentage of proliferating cells (%)</th>
<th>Number of proliferating cells (x10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total proliferating population</td>
<td>Graft versus</td>
<td>431 ± 60.2</td>
<td>4.76 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Syngeneic</td>
<td>420 ± 50.0†</td>
<td>4.01 ± 0.3†</td>
</tr>
</tbody>
</table>

**Type of inoculum**

<table>
<thead>
<tr>
<th>Type of inoculum</th>
<th>Number of FITC-positive cells (x10^3)</th>
<th>Percentage of proliferating cells (%)</th>
<th>Number of proliferating cells (x10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells only</td>
<td>Graft versus</td>
<td>74.0 ± 10.0</td>
<td>22.1 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Syngeneic</td>
<td>56.7 ± 9.4†</td>
<td>15.3 ± 0.6</td>
</tr>
</tbody>
</table>

Subconjunctival local graft versus host reactions were produced as described previously. After 48 hours in situ, eyes were harvested, and a single cell suspension of the infiltrating cells was obtained. Flow cytometric analysis for proliferating cell subsets (by propidium iodide fluorescence) and T cells (by Thy 1.2-FITC positive staining) was performed. All values expressed as mean ± SEM for 10 individual eyes; note that the number of proliferating cells was calculated for each individual eye before averaging into the mean.

* P < 0.01  
† NS by the student t-test.

### Table 5. Comparison of clinical exam and in situ proliferation as measured by uptake of ^125I-iododeoxyuridine in eyes undergoing subconjunctival delayed hypersensitivity reactions

<table>
<thead>
<tr>
<th>Type of inoculum</th>
<th>Clinical score</th>
<th>I-UdR uptake cpm injected eye/ cpm normal eye</th>
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<tbody>
<tr>
<td>Primed</td>
<td>1.92 ± 0.26</td>
<td>1.67 ± 0.25</td>
</tr>
<tr>
<td>Naive</td>
<td>0.68 ± 0.24*</td>
<td>1.29 ± 0.13†</td>
</tr>
</tbody>
</table>

Subconjunctival delayed hypersensitivity (DH) reactions were produced by the local adoptive transfer of DH effectors into the subconjunctival space of naive C57BL/6 mice. Experimental recipient mice received 1 x 10^6 Mycobacterium tuberculosis primed lymph node cells plus 2 x 10^5 antigen-presenting cells (splenic adherent cells) that had been pulsed with 50 ug/ml of microparticulate Mycobacterial antigenic preparation. Naive control recipients were injected with 1 x 10^6 naive spleen cells plus antigen-pulsed APCs. Seven eyes in each group were examined clinically and five mice in each group received administration of ^125I-UdR as described. All values are expressed as mean ± SEM.

* P < 0.01  
† NS by the student t-test.

**Application of Technique to the Analysis of Inflammation Mediated by the Local Adoptive Transfer of Subconjunctival Delayed Hypersensitivity Reactions**

An analogous series of experiments was done to compare clinical examination, ^125I-iododeoxyuridine labeling, and flow cytometry in the evaluation of delayed hypersensitivity induced by the local adoptive transfer of antigen-primed lymphocytes into the subconjunctival space. The local adoptive transfer of lymphocytes to cutaneous sites is a well-accepted model for evaluating delayed hypersensitivity effector function. The adoptive transfer of 1 x 10^6 mycobacterium-primed lymphocytes (recovered from nodes draining the base of the tail previously primed to M. tuberculosis in mineral oil) mixed with 2 x 10^5 APCs (mycobacterial antigen-pulsed splenic adherent cells) into the subconjunctival site produced an inflammatory nodule of moderate intensity which persisted for at least 48 hr. Compared with mice receiving a subconjunctival inoculum of unprimed splenocytes, a significantly greater inflammatory reaction was observed in mice receiving primed cells (Table 5). Similarly, ^125I-iododeoxyuridine uptake showed that greater uptake of radioactive label occurred in the experimental versus the control site (Table 5). As in...
the case of the graft-versus-host reaction, the difference was not statistically significant.

Flow cytometric analysis of cells recovered from eyes undergoing subconjunctival delayed hypersensitivity were similar to those obtained with local graft-versus-host reactions (Table 6). The experimental group had a significantly increased number of total proliferating cells (22.1 × 10³ versus 8.1 × 10³) and proliferating T cells (13.3 × 10³ versus 4.8 × 10³). Interestingly, fewer total T cells were recovered from the control group. Thus, flow-cytometric analysis was more sensitive than ¹²⁵I-iododeoxyuridine uptake and gave more useful information.

Discussion

We wanted to develop a technique to quantitate proliferation of lymphocyte subsets, especially T cells, in eyes undergoing immunogenically mediated inflammation. To that end, we used DNA analysis by flow cytometry to identify proliferating subsets in cells harvested from mouse eyes. Our results reveal that flow-cytometric analysis can readily identify proliferating T and B cells in normal eyes and can distinguish proliferating T cells from nonproliferating B cells in eyes infiltrated with a T cell neoplasm. More importantly, we used flow cytometry to compare the results obtained with our technique to those obtained by clinical examination and ¹²⁵I-iododeoxyuridine uptake in two different models of subconjunctival inflammation. We found that all three methods can be used to measure quantitatively various aspects of local inflammation. However, only the value obtained by flow cytometry was statistically significant in both model systems. In addition, only flow cytometry can analyze the contribution of specific proliferating cell populations.

Clinical examination is useful because it assesses the overall clinical severity of an inflammatory process. However, the external surface of the mouse eye is difficult to examine accurately, and few quantifiable clinical findings are identifiable. Subtle, but important, immune-mediated events may escape clinical observation (such as cytotoxic T cell activation during graft-versus-host reactions). Flow cytometric analysis was more sensitive at detecting T cell proliferation than the clinical examination. Furthermore, using antibodies that identify the common leukocyte antigen, flow cytometric analysis might be capable of quantifying the severity of inflammatory infiltrate with great precision.

Uptake of the radioactive label in the DNA of proliferating cells at the site of inflammation is also capable of quantifying certain aspects of cellular proliferation induced by the inflammatory process. However, statistically significant differences between experimental and control eyes were not obtained in this study despite many attempts to optimize this technique. By contrast, flow cytometry was able to quantify the number of total proliferating cells from individual eyes better. The disadvantages of the ¹²⁵I-iododeoxyuridine method were considerable. The reagent is expensive and has a short shelf life. Once injected, the mice required shielding in a special area. Disposal of radioactive carcasses and cage materials required special procedures and was costly. Of greater concern was the difficulty in interpreting the radiolabeling data. The delivery and uptake of label at the inflammatory site was extremely variable among animals, requiring the use of a ratio to standardize the data. Finally, the identity of the proliferating cell population was unknown. Others have shown that in cutaneous delayed hypersensitivity that much of the radioactive uptake occurs in the proliferating basal layers of the epidermis and not in the immune cells inducing the inflammation.⁴

The most important use of flow cytometric analysis of immunogenic inflammation is that it makes it possible to analyze the contribution of proliferating lymphocyte subsets, especially T cells, to both immune-mediated and nonspecific inflammation. In both graft-versus-host reactions and delayed hypersensitivity reactions, we found T cell proliferation to be significantly above control values. We are currently exploring modifications of our technique that

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**Table 6. Cellular proliferation from eyes undergoing subconjunctival delayed hypersensitivity reactions**

<table>
<thead>
<tr>
<th>Type of inoculum</th>
<th>Number of cells recovered (×10³)</th>
<th>Percentage of proliferating cells (%)</th>
<th>Number of proliferating cells (×10³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total proliferating</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primed cells</td>
<td>767 ± 268</td>
<td>3.77 ± 0.8</td>
<td>22.1 ± 3.8</td>
</tr>
<tr>
<td>Naive cells</td>
<td>538 ± 81.8f</td>
<td>1.39 ± 0.2†</td>
<td>8.1 ± 2.4†</td>
</tr>
<tr>
<td>T cells only</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primed cells</td>
<td>83.0 ± 10.0</td>
<td>17.9 ± 2.2</td>
<td>13.3 ± 1.5</td>
</tr>
<tr>
<td>Naive cells</td>
<td>29.1 ± 3.3*</td>
<td>13.7 ± 1.9†</td>
<td>4.8 ± 1.2*</td>
</tr>
</tbody>
</table>

Local adoptive transfer of subconjunctival delayed hypersensitivity was induced as described. After 48 hours, eyes were harvested and analyzed for proliferating cell populations by flow cytometry. Each value represents the mean of five individual eyes; the number of proliferating cells represents the mean value of five eyes when the value for each eye is calculated independently.

All values are expressed as mean ± SEM.

*P < 0.01.
†P < 0.05.
‡NS by the student t-test.
will allow us to distinguish proliferation of donor T cells from host T cells. We are hopeful that this technique will permit us to determine the contribution of lymphocyte proliferation to immunogenic inflammation and allow us to distinguish immunogenic reactions that are predominantly mediated by activated T cells subsets from those mediated by activated B cells.18

This technique offers distinct advantages over other methods that have been used to quantitate the contribution of proliferating lymphocytes to local inflammation. Most methods utilize the systemic administration of halogenated thymidine analogues.4 As discussed, radiolabeled nucleotide-dependent methods have limited utility. Other techniques with halogenated thymidine analogues, such as bromodeoxyuridine, have been given to humans and animals, and their uptake in proliferating tumors can be detected by staining cells with a monoclonal antibody specific for the nucleotide analogue.19,20 However, this method requires the administration of potentially toxic agents before analysis, is limited by the kinetics of distribution and clearance of the molecule, and requires qualitative assessment of frozen sections or complicated three-color flow cytometric analysis.

DNA analysis also offers advantages over other types of flow cytometry for evaluation of lymphocyte activation. Methods using the identification of "activation" markers in the cell nucleus or on the cell surface have well-known limitations. For instance, identification of an IL-2 receptor- or transferrin receptor-bearing population can detect some of the activated lymphocytes; however, these markers are rapidly down regulated shortly after their expression, are expressed on non-T cell populations, or are present on many circulating lymphocytes.21,22 Since only 1%–2% of circulating T cells are in the S/G2/M phase versus 17%–22% of the T cells in sites of immune-mediated inflammation, DNA analysis might more precisely identify site-specific activation than would the analysis of IL-2 receptor expression. Other markers, such as the Ki-67 antigen, are species specific or not yet well characterized.23

The disadvantages of flow-cytometric DNA analysis lie in the methodologic difficulty in extracting lymphocytes without producing clumps and aggregates. Moreover, the investigator must have access to a flow cytometer. The major advantages of this technique are that it avoids the use of radioisotopes and uses widely available reagents for identification of cell subsets. Therefore, the same method can be used for the analysis of effector lymphocytes obtained from uveitis patients. In addition, this method may be useful beyond its immunologic applications. For instance, we showed that it is possible to quantitate the number of proliferating tumor cells in the conjunctiva infiltrated by a T cell neoplasm. In addition, this method can be used for the analysis of the contribution of proliferating cells in other diseases, such as in proliferative vitreoretinopathy24 or epithelial ingrowth.25

Since lymphocyte recruitment can be a component of any inflammatory reaction, distinguishing between lymphocytes that are merely trafficking through an inflamed site versus those that are actually activated and participating has been difficult.1,2 We hypothesize that T cell proliferation in sites of inflammation is a specific consequence of antigen-specific activation. It is known that certain T cell clones and lines can be induced to proliferate by certain cytokines in the absence of specific antigen. In addition, some evidence suggests that activation with the CD2 receptor by its specific ligand might activate T cells through an antigen receptor-independent mechanism. However, most evidence suggests that freshly isolated T cells (and probably T cells in vivo) require at least two signals for the induction of sustained activation and proliferation: (1) activation of the T cell receptor (by antigen, mitogen, or anti-receptor antibody) and (2) additional cytokine signals (especially IL-2).3 Therefore, we hypothesize that T cell proliferation in an inflamed eye should reflect the antigen-driven component of the ocular inflammatory process, although we cannot exclude the possibility that antigen-independent proliferation might also occur.

Others found that only a small number of challenge antigen-specific T cells (ie, alloantigen-specific in graft-versus-host or mycobacterium-specific in delayed hypersensitivity) are present in lymph nodes and spleens (and presumably inflamed sites).26 Preliminary data suggest that only 0.02% of the T cells in the delayed hypersensitivity adoptive transfer inoculum are specific for mycobacteria. Thus, we expect that many of the proliferating T cells found in these lesions represent lymphocytes activated to other antigens besides the original challenge antigen. For instance, local presentation of autoantigens or exogenous antigens by local APCs are two possible mechanisms for antigen-specific T cell activation. The capacity of nonlymphoid tissue sites to down regulate T cell activation and proliferation might be an important regulatory mechanism in suppressing immunogenic inflammation. Our technique will allow us to test these hypotheses.

Currently, we are using DNA analysis by flow cytometry to gain insight into the immunopathology of uveitis. It has been postulated that sites of chronic inflammation are improperly converted into tissues resembling lymph nodes.27 With DNA analysis, it will be possible to determine if T cell functions,
normally confined to the lymphoid tissue (ie, activation, proliferation and differentiation), inappropriately occur in inflamed eyes. The results of this study support the conclusion that T cell proliferation does occur in the subconjunctiva during local graft-versus-host inflammatory reactions and delayed hypersensitivity reactions. Preliminary evidence suggests that lymphocyte proliferation is occurring outside of the conjunctival-associated lymphoid tissue. Further analysis of these and other models for ocular immunogenic inflammation will allow us to distinguish between two important possibilities: (1) the contribution of clonal expansion of activated cells in the eye or (2) the recruitment of differentiated T cells from the circulation (which have previously undergone expansion and differentiation in the peripheral lymphoid tissues). This determination will influence treatment strategies for local antiinflammatory therapy.

**Key words:** flow cytometry, DNA analysis, lymphocytes, immune response, lymphocyte proliferation, graft-versus-host disease, delayed hypersensitivity

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### Appendices

#### Appendix A

In conventional flow cytometry, the operator usually determines the population of cells for study by setting electronic acquisition gates based on the morphologic appearance of the cells (ie, forward-angle and side-scatter characteristics of the population, also called dual light scatter parameters). We chose not to do this for two reasons. First, the high concentration of debris made it difficult to distinguish cells from particles (Fig. 1A). Second, we desired to achieve a quantitative determination of cells in our suspension. Setting gates would necessarily exclude some cells and include some debris.

#### Appendix B

The concentration of Tween had a profound effect on the intensity of antibody staining and access of PI to the cell. Concentrations of Tween lower than 1% made less than 75% of the cells permeable, resulting in falsely low estimates of cell yield. Concentrations of 1% or greater made 90%–95% of cells permeable in 1 hr. Conversely, concentrations of Tween greater than 2% produced unacceptable loss of cell surface staining.

#### Appendix C

The isotype and nature of the antibody was important for brightness of staining and to minimize doublet formation. Both IgM isotypes and indirect antibody methods induced unacceptable doublet formation. Directly fluoresceinated IgG2a antibodies provided satisfactory fluorescence intensity (Figs. 2A–C) and minimal doublet formation. Fab fragments produced no artificial clumping but were the most susceptible to loss during the solubilization procedure. The addition of fixatives to stabilize antibody staining introduced unacceptable clumping and doublet formation. RNase treatment was not necessary in these experiments but could have been added during the solubilization step.

### Appendix D

In conventional DNA analysis, histograms are generally analyzed with commercially available software programs that fit a mathemetic curve to the DNA histogram. However, in many cases, we recovered an inadequate number of lymphocytes to allow this type analysis with our specimens (Fig. 3B). Comparison of our gating method to the Hewlett Packard commercial software method that used a sum of broadened squares method for calculating the cell-cycle kinetics showed that the estimates of S + G2 + M for both methods were comparable when an adequate number of events was available.

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