A New Method for Studying the Selective Adherence of Blood Lymphocytes to the Microvasculature of Human Retina


Purpose. To develop a sensitive and reproducible technique for measuring the adherence of blood lymphocytes to vessel walls exposed in sections of human retina and for examining the role of lymphocyte and vascular adhesion molecules in these events.

Methods. Cryostat sections of human retina were overlaid with blood lymphocytes from healthy subjects, and experimental conditions were sought by which preferential attachment of the cells occurred to blood vessel walls in the retinal sections. Adherent lymphocytes were identified by staining with methyl green-thionine, and transected blood vessels were identified by their structure and by staining of basement membranes with periodic acid–Schiff. The adherence of enriched preparations of CD4+ (T-helper) and CD8+ (T-cytotoxic) lymphocytes, of interleukin-2 (IL-2)–activated cells, and of lymphocytes from patients with ocular Behçet's disease was examined. The distribution of adhesion molecules on retinal vessel walls was determined by immunohistochemistry, and the contribution of leukocyte integrins to lymphocyte binding was studied by blocking experiments with monoclonal antibodies.

Results. The optimal selectivity of blood lymphocyte attachment to retinal vessel walls occurred when purified lymphocytes were suspended in culture medium with 10% fetal calf serum and overlaid onto retinal sections for 30 minutes at 23°C with gentle agitation. Under these conditions, 92% of the lymphocytes that adhered to the section were confined to the retinal microvasculature, and CD4+ T cells were more adherent than CD8+ T cells (P < 0.01). Prior exposure of normal lymphocytes to IL-2 enhanced their binding to retinal blood vessels, and lymphocytes from patients with Behçet's disease showed supranormal vascular adherence (P < 0.005).

Many transected vessels stained positively for CD31; PECAM (mean 62%), CD54; ICAM-1 (mean 73%), CD62E; E-selectin (mean 35%), CD62P; P-selectin (mean 61%), and CD106; VCAM-1 (mean 42%). However, these vascular adhesion molecules occupied <20% of the area of the blood vessel walls. Lymphocyte adherence to the retinal vessels was more dependent on CD29 (the common chain of the β1 integrins) expression than either CD11a/CD18 or CD49d.

Conclusions. This technique allows measurements to be made of lymphocyte adherence to vascular and nonvascular structures of retina ex vivo. Extension of this approach to the study of leukocyte adherence to sections of pathologic retina may be of clinical and experimental applicability in understanding mechanisms of retinal inflammation. Invest Ophthalmol Vis Sci. 1997; 38:2608–2618.

Leukocytes are considered to play prominent pathogenic roles in several of the retinal vasculitides, either by inducing vascular occlusions or infiltrating the retina to initiate tissue damage. The T lymphocyte is the principal infiltrating leukocyte of the retina in patients with various forms of posterior uveitis, and this cell is important in the initiation, progression, and adoptive transfer of experimental autoimmune uveitis.

Lymphocyte entry into tissue is a multistep process controlled by a sequential interaction of cell adhesion molecules with counterreceptors expressed on various components of blood vessel walls. The initial adhesion to endothelium is dependent on lectin–carbohy-
The study was conducted in three stages. First, the isolation of lymphocytes selectively adhering to blood vessel walls in sections of human retina, to determine whether lymphocytes from patients with retinal inflammation were abnormally adherent for retinal blood vessel walls, and to assess the contribution of known adhesion molecules to lymphocyte–blood vessel wall interaction.

MATERIALS AND METHODS

Experimental Design

The study was conducted in three stages. First, the conditions that permitted the selective adherence of lymphocytes to blood vessel walls in sections of retinal tissue were identified. Second, experiments were performed to evaluate the adherence properties of the major T-lymphocyte subpopulations, of lymphocytes activated with interleukin-2 (IL-2) in vitro, and of blood lymphocytes from 13 patients with ocular Behçet’s disease. Finally, the contribution of known adhesion molecules to lymphocyte attachment was assessed by immunocytochemical staining of adhesion molecules on retinal vessel walls and by antibody blocking experiments.

Clinical Samples

Human eyes were obtained from the Bristol Eye Bank, United Kingdom, and were received within 1 to 3 days of death. The eyes were stored at —70°C. Cryostat sections of retinal tissue (7 um) were cut, transferred to glass slides, and air dried at room temperature for 2 hours. Control blood samples were provided by healthy hospital and medical school staff. All patients with Behçet’s disease fulfilled the clinical criteria for this disorder. Twelve of the 13 patients with Behçet’s disease presented with disease active in one or both eyes, as assessed by cells in the vitreous, vascular sheathing, vascular occlusion, and macular edema. Four of the patients had active systemic disease as well: two had meningitis, one thrombophlebitis, and one active skin lesions. Nine patients were being treated with prednisolone, four of whom were also receiving cyclosporin A; one was receiving azathioprine and three were off treatment. Also included in the study were five patients with active rheumatoid arthritis and two with Sjögren’s syndrome. These patients were under the clinical care of Dr. J. D. Perry, Department of Rheumatology, The Royal London Hospital. Ethical permission for the study was granted by the Ethics Committee of the Guy’s and St. Thomas’ Hospital Trust. The patients who provided blood samples gave informed consent.

Isolation of Lymphocytes

Mononuclear leukocytes were isolated from 40 ml heparinized blood (10 U/ml) by our standard method. Briefly, blood was diluted with an equal volume of 0.9% NaCl and layered onto Lymphoprep (Nycomed, Birmingham, UK) at a ratio of 1 vol Lymphoprep to 2 vol of diluted blood. After centrifugation at 500g for 30 minutes, the mononuclear cell layer was carefully removed and washed twice with Dulbecco’s minimum essential medium (DMEM). Lymphocytes were enriched by suspending mononuclear leukocytes in 2 ml 50% (vol/vol) Percoll solution...
Adherence to Vessel Walls

Lymphocytes and overlaid lymphocytes were identified by staining with anti-B cell antibodies (anti-CD19, Dynabeads, Dynal, Wirral, UK) at a cell to bead ratio of 1:1 with anti-CD8 or anti-CD4 antibodies as described in the above procedure. The percentage of total T cells (CD3) and their CD4+ and CD8+ subpopulations in the initial and final cell preparation was determined using a FACScan flow cytometer (Becton Dickinson, Oxford, UK).

Preparation of CD4+ and CD8+ Lymphocytes

Blood lymphocytes were first depleted of their B lymphocytes by incubation with magnetic beads coated with anti-μB cell antibodies (anti-CD19, Dynabeads, Dynal, Wirral, UK) at a cell to bead ratio of 1:1 with constant mixing at 4°C for 30 minutes. For the enrichment of CD4+ (T-helper) and CD8+ (T-cytotoxic) subsets, lymphocytes were mixed with Dynabeads coated with either anti-CD8 or anti-CD4 antibodies as described in the above procedure. The percentage of total T cells (CD3) and their CD4+ and CD8+ subpopulations in the initial and final cell preparation was determined using a FACScan flow cytometer (Becton Dickinson, Oxford, UK).

Lymphocyte Activation With IL-2

Lymphocytes, resuspended at 1 x 10^6 cells/ml in DMEM plus 10% fetal calf serum, were incubated with recombinant IL-2 (range, 0.02 to 20.0 U/ml) for 24 hours at 37°C. The cells were then washed in DMEM, resuspended in medium, and applied to retinal sections. Control lymphocytes were incubated for 24 hours in the absence of IL-2.

Adherence Assay

Enriched preparations of lymphocytes were suspended in DMEM with 10% fetal calf serum and 25 mM Hepes, and 50 μl was gently pipetted onto each section with gentle agitation on an orbital shaker (Lucasham, Life Sciences Int'l, Basingstoke, UK; 35 rpm/2 cm radius) for 30 minutes over a temperature range of 7°C to 37°C. Nonadherent lymphocytes were removed by gently immersing the slide in phosphate-buffered saline. The sections with their adherent lymphocytes were fixed for 30 minutes in 2% glutaraldehyde in phosphate-buffered saline. Transected blood vessels and overlaid lymphocytes were identified by staining with periodic acid–Schiff and methyl green-thionine, respectively. Adherent lymphocytes were visible as intensely blue-stained cells, appearing under the microscope in a slightly higher plane than the retinal tissue. Lymphocyte adherence to exposed vascular wall was defined as attachment to all layers of the blood vessel wall. All transected vessels, including complete or fragmented ones, were examined for their support of lymphocyte adhesion.

Expression of Adherence Results

To compare the adherent properties of distinct preparations of lymphocytes to blood vessel walls in sections from different retinas, results were expressed as the number of lymphocytes bound per mm^2 of vessel wall. This was achieved by measuring with an eyepiece graticule the total cross-sectional area of all transected vessels (excluding the lumen) and counting the number of lymphocytes bound to every transected vessel (complete or fragmented). Therefore, the lymphocyte adherence to vessel walls (mm^2) equals the total number of lymphocytes adherent to vessel walls divided by the total cross-sectional area of transected blood vessel walls (mm^2).

Nonspecific adherence was evaluated by measuring the area of nonvascular tissue and counting the number of adherent lymphocytes that this tissue supported. Therefore, lymphocyte adherence to nonvascular tissue (mm^2) equals the total number of lymphocytes adherent to nonvascular sites divided by the total cross-sectional area of nonvascular sites (mm^2).

The percentage of lymphocytes bound to the section that selectively adhered to vessel walls was calculated by dividing the number of lymphocytes bound to vessel walls by the number of lymphocytes bound to the whole section, and multiplying by 100.

Immunohistochemical Staining of Adhesion Molecules on Blood Vessel Walls

Retinal tissue sections were stained using the avidin peroxidase–anti-avidin peroxidase method. Briefly, sections were fixed for 10 minutes with 1% paraformaldehyde in phosphate-buffered saline and treated for 20 minutes in a humid chamber at room temperature with normal rabbit serum (Sigma-Aldrich Co., Poole, UK) diluted 1:10 in Tris-buffered saline (pH 7.6) to block nonspecific binding of the second antibody. After washing with Tris-buffered saline, the sections were treated for 30 minutes with a panel of mouse monoclonal antibodies directed against CD54 (R/1/1.1, kindly donated by R. Rothlein, Boehringer-Engelheim, Ridgefield, CT), CD106, CD62E, and CD31 (all purchased from Serotec, Oxford, UK), CD62P (Biodesign International, Kennebunk, ME, and an IgGl isotype control (DAKO, High Wycombe, UK). The sections were then washed for 2 minutes in Tris-buffered saline, and the bound primary antibodies were detected by a 30-minute incubation with bridging antibody (rabbit antimouse immunoglobulin diluted 1:10, DAKO), followed by a further incubation for 30 minutes with a 1:20 dilution of avidin peroxidase–anti-avidin peroxidase complexes (DAKO). Visualization of positive staining was achieved with the use of the substrate naphthol-AS-MX-phosphate and Fast Red (Sigma-Aldrich).
Inhibition of Leukocyte–Retinal Vessel Adhesion

To assess the contribution of leukocyte adhesion molecules in the attachment of lymphocytes to retinal blood vessels, lymphocytes were treated with saturating concentrations of monoclonal antibodies against the adhesion molecules CD11a (Serotec), CD18 (DAKO), CD29 (β chain common to the β1 integrins), and CD49d (both Serotec). Isolated lymphocytes (1 × 10^6 cells) were incubated for 45 minutes at 23°C with 10 μl of antibody diluted in DMEM with 25 mM Hepes and 10% fetal calf serum to a final volume of 100 μl. The lymphocytes were then pelleted by centrifugation, resuspended in the same antibody dilution to 10^6 cells/50 μl medium, and applied to the retinal sections. In all experiments, lymphocytes were also incubated with an indifferent mouse IgG1 antibody as a negative control.

Statistical Evaluation of Results

Results are expressed as the mean ± standard deviation. The Wilcoxon signed rank test for paired data was used to test the significance of differences between the means of test and control groups. Coefficients of variation were calculated for lymphocyte adhesion to cross-sectional areas of transected blood vessel walls in serial retinal tissue sections.

RESULTS

Selectivity of Lymphocyte Adhesion to Retinal Vessel Walls

The selective adherence of overlaid lymphocytes for retinal vessel walls occurred when 1 × 10^6 cells were suspended in 50 μl DMEM with 10% fetal calf serum and applied to retinal sections for 30 minutes with gentle agitation (Fig. 1). Although only 0.01% of the overlaid lymphocytes bound to the total section, >96% were confined to the retinal vasculature. Alteration of any of these parameters generally resulted in an increase in the number of lymphocytes adhering to nonvascular tissue. When the assay was performed at 7°C, few of the overlaid lymphocytes bound to the vessel walls, but increasing the temperature to 23°C resulted in a sixfold increase in attachment and a specificity of adhesion of >96% (in other words, the percentage of lymphocytes that adhered to the retinal section but were confined to vessel walls). At 37°C, the selective adherence of lymphocytes was reduced (mean 81% attachment) because more lymphocytes bound to nonvascular tissue.

Since Stamper and Woodruff reported that fixation of lymphoid tissue before assay preserved tissue structure, sections of retina or overlaid lymphocytes were prefixed with glutaraldehyde or paraformaldehyde. Figure 2 shows that pretreatment of either sections or lymphocytes with paraformaldehyde produced a marked reduction in the number of adherent lymphocytes, and that fixation of lymphocytes with glutaraldehyde also reduced their adherence to vessel walls. The number of lymphocytes adhering to vessel walls in untreated and glutaraldehyde-treated sections was similar (see Fig. 2), but the selectivity of lymphocyte binding to the vasculature was reduced (P < 0.05) by glutaraldehyde fixation (82%) when compared with untreated sections (96%).

In the next stage of our study, we investigated the variability in the adherence properties of vessel walls in serial tissue sections and in sections obtained from different retinas. When lymphocytes from one healthy subject were overlaid onto eight serial sections (7-μm thickness), the mean number of cells binding to vessel walls was 779 ± 152 cells/mm² blood vessel wall, and the coefficient of variation was 15.5%. Figure 3 shows the adherence of lymphocytes from one subject to sections obtained from six retinas. The level of adhesion ranged from 361 cells/mm² to 1093 cells/mm² (mean 662 ± 265 cells/mm²), but the specific adherence for blood vessels was always >96%. From these findings, we decided that all comparative adherence analyses of lymphocytes prepared from different subjects or of lymphocytes subjected to different treatments would be undertaken on sections prepared from the same block of retinal tissue. In addition, each adhesion result would be the mean value derived from reading eight serial sections.

Vascular Adherence of T-Lymphocyte Subpopulations, of Activated Lymphocytes, and of Lymphocytes From Patients With Ocular Behçet's Disease

To determine if the adherence of T lymphocytes differed between CD4⁺ or CD8⁺ cells, enriched preparations of these subpopulations (>75%) were examined for their ability to bind to retinal blood vessels. Figure 4 shows the results of three experiments in which CD4⁺, CD8⁺, and unfractionated lymphocytes from three normal subjects were added to sections prepared from three retinas. In all experiments, the binding of the CD4⁺ cells to vessel walls was similar to that of unseparated lymphocytes, but the CD8⁺ cells were always less adherent than the CD4⁺ cells (P < 0.01).

Lymphocyte migration across the blood–retinal barrier is a property of activated rather than resting cells, and therefore we also investigated the adherence properties of IL-2–stimulated lymphocytes. In the experiment illustrated in Figure 5, activation of unfractionated lymphocytes with 0.02 and 0.2 U/ml IL-2 produced a 27% and 25% increase in adhesion, respectively (P < 0.05). The finding that the adherence of lymphocytes treated with high concentrations of IL-2 (200 U/ml) was similar to that of untreated cells suggests that there is a critical relation between...
FIGURE 1. Selectivity of lymphocyte attachment to blood vessel walls in tissue sections of human retina. Serial sections of retina were either untreated or overlaid with blood lymphocytes as described in the text. The transected blood vessels in an untreated section (A) have no associated lymphocytes, but after incubation with blood lymphocytes (B), the same vessel now supports the binding of several lymphocytes. (C) In a section of another retina, it is the blood vessel wall that selectively supports the attachment of overlaid lymphocytes. Blood vessel walls were identified by staining with periodic acid–Schiff and lymphocytes by staining with methyl green-thionine. Adherent lymphocytes appear as small round cells with intensely staining dark-blue nuclei lying above the plane of the section, and blood vessels are surrounded by a magenta-stained basement membrane. (Magnification, X400)

IL-2 receptor occupancy and the generation of intracellular signals that promote cellular adhesiveness. Similar results were obtained in two further experiments. The above experiments demonstrate that T-lymphocyte attachment to retinal vessel walls is preferentially mediated by CD4+ cells and that adhesion is enhanced after cell activation.

Features common to Behcet's disease include a high preponderance of activated lymphocytes in the circulation18 and the infiltration of the retina by blood lymphocytes.19 A comparative study was undertaken of the adherent properties of blood lymphocytes from 13 patients with Behcet's disease and 13 healthy age- and sex-matched control subjects. In the experiments illustrated in Figure 6, lymphocytes from Behcet's patients exhibited a greater adhesion to vessel walls than the paired control cells (mean 48% increase; \( P < 0.005 \)), and all preparations showed a similar degree of binding specificity for the retinal vasculature (>96%). Only one patient (experiment 4) did not have active disease, but his lymphocytes were also more adherent than the corresponding control cells.

To determine whether the increased adhesiveness of lymphocytes from patients with Behcet's disease was a feature common to autoimmune disorders, binding studies were also undertaken on lymphocytes from five
patients with active rheumatoid arthritis and two patients with Sjögren's syndrome. The lymphocytes from these patients behaved similarly to those of control cells in their attachment to retinal vessel walls (mean 1863 ± 398 versus 2024 ± 872 cells/mm² vessel wall).

The next stage of the investigation determined if the abnormal adherence of Behçet's lymphocytes for vessel walls could be reproduced with sections from nonocular tissue. Blood lymphocytes from four patients with active Behçet's disease and four healthy controls were simultaneously overlaid onto sections of retina and human brain. The patients' lymphocytes were always more adherent than control cells in their binding to retinal vessel walls, whereas supranormal adherence of Behçet's lymphocytes to cerebral vessel walls was seen in only two of four experiments (data not shown).

Adhesion Molecules and the Binding of Lymphocytes

Vessel walls in retinal tissue from seven donor eyes were examined by immunohistochemistry for their expression of CD31, CD54, CD62E, CD62P, and CD106. Most of the vessels in these sections were positive for CD31 (mean 62%), CD54 (mean 73%), and CD62P (mean 61%), and a minority expressed CD62E (mean 35%) and CD106 (mean 42%) (Table 1A). Some of the adhesion molecules were present on the luminal
FIGURE 5. Activation of lymphocytes with interleukin-2 (IL-2) increases their adhesion to vessel walls. Lymphocytes from a healthy subject were incubated for 24 hours with recombinant IL-2 (0.02 to 20.0 U/ml) before being overlaid on serial retinal tissue sections. Control cells were incubated for the same period but in the absence of IL-2. Each result is the mean value of readings from eight serial sections and is expressed as the number of lymphocytes bound per unit area of vessel wall. Lymphocytes activated with 0.02 and 0.2 U/ml IL-2 were more adherent than resting cells (*P < 0.05 compared with untreated cells).

and abluminal sides of blood vessel walls, and occasionally there was considerable variability in their distribution from one retina to another. Examination of serial sections revealed that CD54 was often coexpressed with CD31 and CD62E, but overall no consistent pattern was established regarding the simultaneous expression of adhesion molecules.

At an early stage of the study, it was apparent that the expression of an adhesion molecule in a blood vessel was confined to a small part of its total area. Consequently, four of the retinal samples described in Table 1A were reanalyzed and the results presented as the percentage of total area of vessel walls expressing adhesion molecules. This was achieved by measuring with an eyepiece graticule the total cross-sectional area of every fragmented and complete transected blood vessel wall (excluding the lumen) that was exposed in the retinal section. Using a similar procedure, measurements were made of the areas of vessel walls stained by antiadhesion molecule antibodies. Table 1B shows that only small areas of blood vessel walls were occupied by the adhesion molecules investigated in the study. For example, although CD54 was identified on most blood vessels, its expression was confined to only 19% of their total surface area.

To assess the contribution of lymphocyte adhesion molecules in promoting adhesion, lymphocytes from healthy subjects were treated, before their introduction into the adhesion assay, with antibodies directed against CD11a, CD18, CD29, or CD49d. Figure 7 shows that the anti-CD29 and anti-CD18 antibodies produced a mean 71% inhibition (P < 0.001) and 18% inhibition of adhesion (P < 0.05), respectively. The anti-CD49d antibody did not modify adhesion, and although the anti-CD11a induced a mean 17% inhibition of adhesion, this effect was not significant. The findings demonstrate that CD29 is an important integrin for promoting lymphocyte attachment to vessel walls in retinal sections.

DISCUSSION

This study shows that blood vessels in sections of human retina support the attachment of overlaid lymphocytes. Although only a tiny proportion of the lymphocytes adhered to the sections (1:10,000), >96% of the cells bound were confined to blood vessel walls. The high selectivity of lymphocytes for vessel walls was reproduced in sections obtained from several retinas. Adherent lymphocytes were seen as small, intensely staining cells in sections stained with methyl green-thionine, and blood vessels were recognized by staining of their basement membrane with periodic acid-Schiff. Lymphocyte binding was an active process in that prefixation of the cells or retinal tissue with paraformaldehyde or glutaraldehyde impaired their inter-
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TABLE I. Distribution of Adhesion Molecules on Retinal Blood Vessel Walls

<table>
<thead>
<tr>
<th>Retina</th>
<th>IgG1</th>
<th>CD31</th>
<th>CD54</th>
<th>CD62E</th>
<th>CD62P</th>
<th>CD106</th>
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<tr>
<td>A. Percentage of vessels expressing adhesion molecules</td>
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<td>55</td>
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<td>0</td>
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<td>80</td>
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<td>nd</td>
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<tr>
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<td>60</td>
<td>63</td>
<td>21</td>
<td>83</td>
<td>25</td>
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<tr>
<td>Mean ± SD</td>
<td>0</td>
<td>62 ± 25</td>
<td>73 ± 10</td>
<td>35 ± 19</td>
<td>61 ± 22</td>
<td>42 ± 18</td>
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| B. Percentage of total area of vessel expressing adhesion molecules |
| 4 | 0 | 13 | 20 | 2 | 14 | 4 |
| 5 | 0 | 9 | 18 | 5 | 6 | 2 |
| 6 | 0 | nd | 18 | 6 | 10 | 12 |
| 7 | 0 | 14 | 21 | 4 | 11 | 2 |
| Mean ± SD | 0 | 12 ± 3 | 19 ± 2 | 4 ± 2 | 10 ± 3 | 5 ± 5 |

Retina tissue from seven eyes were examined for their expression of vascular adhesion molecules. In (A) results (mean ± SD) are presented as the percentage of blood vessels expressing adhesion molecules, and in (B) results from four of the samples are presented as the percentage of total area of retinal vessel walls occupied by these same molecules. Serial sections from each of the seven retinas were also treated with a control antibody of the same isotype (IgG-1) as that of the anti-adhesion molecule antibodies. ND = not done.

action with one another. Vascular adhesion molecules were present on retinal vessel walls, and lymphocyte attachment was particularly dependent on CD29 expression.

Comparative analyses revealed that CD4-enriched T cells were more adherent to vessel walls than CD8-enriched T cells, an observation that may relate to the prominent contribution of CD4+ T cells in the initiation and progression of experimental autoimmune uveitis3 and to the large representation of these cells in retinal inflammatory infiltrates of patients with uveitis.2 Few antigen-specific T cells are required to encounter retinal antigens and induce uveitis,20 whose perpetuation thereafter depends on the recruitment into the retina of nonantigen-specific lymphocytes.21 Lymphocyte infiltration precedes blood–retinal barrier permeability,22 and the contribution of CD4+ lymphocytes to ocular inflammation is further highlighted by the findings that it is a subset of memory CD4 lymphocytes that preferentially migrate through endothelial monolayers23 and that CD4+ T cells make up most of the retinal lymphocyte infiltrates in experimental autoimmune uveitis.3

In our assay, the number of lymphocytes adhering to retinal vessel walls was increased after incubation with IL-2; this finding is in keeping with the earlier demonstration that activation of lymphocytes increases their attachment to and migration across endothelial monolayers.24 These observations support the view that migration across the blood–retinal barrier is mediated preferentially by activated rather than resting lymphocytes.18

Obliterative vasculitis is a characteristic of Behçet’s disease, and recurrence of this condition leads to severe visual impairment and irreversible alterations in the retina.25 We found that blood lymphocytes from patients with active Behçet’s disease were more adherent to transected retinal vessel walls than lymphocytes from healthy controls. This group of patients was selected for study because in active disease there is an increased proportion of

FIGURE 7. Effect of antiadhesion molecule antibodies on lymphocyte binding. Lymphocytes from six normal subjects were pretreated for 45 minutes with antibodies against CD11a, CD18, CD49d, and CD29 before being overlaid onto retinal sections. Control cells were incubated for the same period in the presence of isotype control IgG1. Results are expressed as the mean percentage adhesion relative to untreated lymphocytes (control cells). Horizontal lines denote standard deviation of the mean. Lymphocyte adherence was inhibited by the anti-CD18 (*P < 0.05) and anti-CD29 antibodies (**P < 0.001).
both activated T cells\textsuperscript{18} and soluble IL-2 receptors\textsuperscript{26} in the circulation, and because T lymphocytes make up the main population of infiltrating lymphocytes in the posterior chamber of the eye.\textsuperscript{13,14} Due to variations in the adherence properties of vessel walls between different retinas, care was taken to ensure that lymphocytes from one control subject and one patient with Behçet’s disease were always allocated to every other section prepared from the same block of retinal tissue. Furthermore, each result was the mean adherence value from reading eight sections. A similar procedure would be necessary for the introduction of pathologic tissue into the adherence assay and for the study of leukocytes from patients with other forms of ocular disease, either in point-prevalent or longitudinal studies.

The additional observation that blood lymphocytes from patients with rheumatoid arthritis and with Sjögren’s syndrome bound to retinal vessel walls in similar numbers to those of healthy controls suggests that the supranormal adherence properties of Behçet’s lymphocytes may not be a general characteristic of lymphocytes from patients with autoimmune disorders. That the enhanced binding of lymphocytes from patients with Behçet’s disease to vessel walls was more apparent with retinal tissue than with brain tissue could arise from the preferential interaction of lymphocytes with a distinct group or combination of vascular adhesion molecules on retinal vessels or from a degree of organ selectivity. Addressing the latter consideration fully would require an extensive comparative examination of the ability of Behçet’s lymphocytes to adhere to vessel walls in sections from several organs (e.g., lung, kidney, skin).

By immunohistochemistry, we demonstrated that CD54, which is constitutively expressed on resting endothelial cells and upregulated by the action of inflammatory cytokines,\textsuperscript{27} was present on the majority of vessel walls exposed in the retinal sections. High levels of CD54 are present in the eyes of animals with experimentally induced uveitis, and increased expression of CD54 appears to precede retinal inflammatory cell infiltrates.\textsuperscript{7} CD54 is the ligand for the leukocyte integrins CD11a/CD18 and CD11b/CD18,\textsuperscript{28} and evidence for a pathogenetic role in intraocular inflammation is provided by the demonstration that antibodies directed against CD54 or CD11a/CD18 prevent or inhibit experimentally induced uveitis.\textsuperscript{29,30} Many blood vessels in our sections were also stained by anti-CD62P and anti-CD31 antibodies, whereas only a minority of vessels reacted with antibodies against CD106 and CD62E. Closer examination of the retinal vessels revealed that all the adhesion molecules, including CD54, were confined to small areas (<20%) of exposed blood vessel walls. Such a limited expression of vascular adhesion molecules would be in keeping with the normally restricted passage of lymphocytes across the blood–retinal barrier. Adhesion molecules are highly expressed on vessel walls in areas of inflammatory lesions, as seen in multiple sclerosis\textsuperscript{31} and rheumatoid arthritis,\textsuperscript{32,33} and similar findings are likely to apply to the retinal vasculature in inflammatory disorders of the eye, particularly because lymphocytes with a high expression of CD11a/CD18 are located adjacent to CD54\textsuperscript{+} cells in the retinas of patients with posterior uveitis.\textsuperscript{34} Thus, in comparison with normal retina, blood vessels walls exposed in tissue sections from patients with retinal inflammation are likely to support the adhesion of larger numbers of overlaid lymphocytes. Whether the selective accumulation of leukocyte populations within retinal inflammatory infiltrates arises from a recognition of distinct adhesion molecules at the level of the blood–retinal barrier could be investigated by combining the in situ adherence assay with immunostaining techniques.

Lymphocyte–endothelial interaction is governed by leukocyte integrins such as CD11a/CD18 and CD49d recognizing corresponding ligands (CD54 and CD106, respectively) on the endothelial surface.\textsuperscript{6} In our antibody blocking studies, lymphocyte adherence to retinal vessel walls was dependent on the expression of CD18 but not that of CD11a and CD49d. Moreover, lymphocyte attachment was highly dependent on CD29, whose expression is increased on subsets of CD4\textsuperscript{+} memory cells,\textsuperscript{34,35} and also on CD4\textsuperscript{+} T cells that undergo transendothelial migration.\textsuperscript{36} Because CD29 is the \(\beta\) chain common to the \(\beta1\) family of leukocyte integrins that recognize collagen, laminin, and fibronectin,\textsuperscript{28} it is unclear whether in our assay lymphocyte binding to vessel walls is caused by attachment to endothelial cells or by recognition of either the extracellular matrix or traces of its proteins expressed on endothelial cells. This question could be addressed by functional blocking studies with antibodies directed against endothelial adhesion molecules and by performing the current adhesion assay in parallel with the study of lymphocyte adherence to monolayers of retinal-derived endothelial cells and to purified proteins of the extracellular matrix. An important supplement to these investigations would be the ultrastructural localization of extracellular matrix proteins in retinal vessel walls. All of the \(\beta\) integrins and most of the \(\alpha\) subunits are highly expressed on human retinal vessel walls,\textsuperscript{37} introducing the intriguing consideration that these molecules may also have a prominent role in promoting lymphocyte adhesion.

Application of the frozen-section assay to experimental autoimmune uveitis would be advanta-
gendeous for determining, during disease progression, changes in the leukocyte-binding properties of the retinal vasculature and for investigating relations between the expression of adhesion molecules on vessel walls and the entry of distinct subsets of mononuclear leukocytes into the retina. For example, progressive changes in the adherence properties of retinal vessel walls could be monitored by the addition of a standard pool of activated lymphocytes to retinal sections prepared from immunized and nonimmunized animals. It is anticipated that applying retinal sections from patients with posterior uveitis and other forms of intraocular inflammation to the frozen-section assay will advance the understanding of the functional role of adhesion molecules in these disorders.

**Key Words**

adherence, lymphocytes, microvasculature, retina, Behçet's disease

**References**


