Vitreous Macrophage Elicitation: Generation of Stimulants For Pigment Epithelium In Vitro

Janice M. Burke*† and Sally S. Twining*‡

Vitreous and macrophage samples were tested for the ability to stimulate proliferation and cell migration in cultured rabbit retinal pigment epithelium (RPE). A macrophage invasion was elicited by the intravitreal injection of latex particles in rabbits and after 3 days the vitreal macrophages were collected. The macrophages themselves, macrophage-conditioned culture medium, and macrophage-incubated vitreous had modest effects on RPE proliferation, but significantly stimulated RPE migration. A portion of the migration activity may be due to macrophage-derived proteases acting on normal vitreous. Mitogenic and additional migration-stimulating activity may also arise from adjacent tissues or from a breakdown of the blood-vitreous barrier that accompanies a macrophage invasion. A macrophage ingress into the vitreous may provide part of the stimulation for the migration and proliferation of RPE in conditions such as proliferative vitreoretinopathy. Invest Ophthalmol Vis Sci 28:1100-1107, 1987

Normal vitreous from rabbit1,2 and other species3 has been shown to stimulate the proliferation of RPE in vitro. Vitreal mitogenic activity for cultured RPE1 and retinal glia3 increases if the vitreous contains experimentally-induced macrophages. Further, intravitreal cellular membranes composed of RPE likewise undergo increased proliferation in vivo following an induced macrophage invasion.1 The observation of increased growth in RPE, both in vitro and in vivo, in the presence of vitreous that has been exposed to macrophages suggests that a macrophage invasion contributes to the vitreous growth-promoting activity.

Human vitreous taken from patients with proliferative vitreoretinopathy (PVR) has been shown to contain elevated growth-promoting4 and chemotactic activity4,5 when tested on cultured RPE cells. The source(s) of these activities is unknown. The stimulatory activities could conceivably arise from adjacent injured tissues or from a breakdown of the blood-vitreous barrier. The activities may also be derived in part from vitreal macrophages since macrophages are frequently found in tissue membranes removed at vitrectomy from patients with PVR.6 The intent of this study was to determine if vitreal macrophages are potential contributors to the mitogenic and chemotactic activities in pathologic vitreous. To address this question, we used a combined in vivo-in vitro approach in which a macrophage invasion was elicited into rabbit vitreous. The macrophages and the vitreous into which the elicited macrophages had migrated were then tested for bioactivity on cultured rabbit RPE.

Materials and Methods

Macrophage Elicitation and Sample Preparation

The animals used in these experiments were treated in compliance with the ARVO Resolution on the Use of Animals in Research. A macrophage invasion was elicited into rabbit vitreous by procedures that have been previously described.1,3,7 Briefly, 1.0 μm latex particles were injected into the midvitreous using a 1:20 dilution of the commercial suspension (Polysciences, Warrington, PA) in a volume of 50 μl sterile saline. After 3 days, the vitreous from four injected eyes were pooled and centrifuged to pellet the macrophages. The day 3 vitreous supernates were retained for testing as indicated below. The cells were washed with phosphate-buffered saline, then resuspended for hemacytometer counts. Aliquots of the macrophage suspension were (1) taken for direct test
ing (mac); (2) homogenized to generate a lysate (lys); or (3) placed in culture to produce a macrophage-conditioned medium (cond med). For both proliferation and migration testing, mac, lys and cond med samples were prepared in Minimum Essential Medium (MEM) (Gibco, Grand Island, NY) supplemented with antibiotics.

The methods for preparation of the macrophage samples are as follows: (1) for direct testing of the macrophages (mac) for growth and migration, the cells were suspended in culture medium at $3 \times 10^5$ macrophages/ml; (2) to prepare macrophage lysates (lys), 3 to $9 \times 10^5$ cells were suspended in 1 ml culture medium, homogenized with a glass homogenizer, frozen and thawed, then clarified by centrifugation as previously described; (3) macrophage-conditioned medium (cond med) was also prepared as described. Briefly, macrophages were suspended in culture medium and plated in 35-mm plastic dishes. Cells non-adherent after 1 hr of incubation were discarded and the culture medium was replaced. The medium conditioned by a 24-hr exposure to the macrophages was used for analysis. In the reported experiments, macrophage numbers used to generate conditioned medium ranged from 3 to $9 \times 10^5$ cells/ml of medium.

The methods to prepare vitreous samples for growth and migration testing were as follows: to prepare normal, freshly-isolated vitreous, vitreous from four normal rabbit eyes were pooled and the liquid vitreous was used for analysis (vit, fresh). To determine if exposure of normal vitreous to macrophages altered the mitogenic and/or migration activity of vitreous, aliquots of fresh vitreous were incubated for 24 hr at 37°C in the absence (vit no mac, incub) or presence of macrophages (vit + mac, incub). For the latter incubation, macrophages, prepared as described above, were suspended in vitreous for the 24-hr incubation, then removed by centrifugation. In the reported experiments, incubations were conducted with macrophage numbers equivalent to those used to generate macrophage lysates and condition medium (3 to $9 \times 10^5$ cells/ml vitreous). In addition to samples prepared from normal vitreous, the supernatant vitreous from which the day 3-elicited macrophages were taken was also retained for analysis (d3 vit). The d3 vit potentially contains products released by macrophages in vivo as well as materials released from adjacent tissues or from blood vessels as a result of the macrophage elicitation. An aliquot of the d3 vit was incubated for an additional 24 hr in vitro, using the procedure described above for the normal vitreous, to generate a d3 vit, incub sample. Table 1 summarizes the experimental groups.

Protein concentrations in the normal vitreous and the day 3 vitreous were determined by the method of Bradford, using bovine serum albumin as a standard.

For the proliferation assay, all vitreous samples (vit, fresh; vit no mac, incub; vit + mac, incub; d3 vit; d3 vit, incub) were added in a concentration of 10% by medium volume in the test wells. For the migration assay, vitreous samples were tested undiluted.

### Table 1. Experimental groups and their sample designations

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample designation</th>
<th>Sample description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mac</td>
<td></td>
<td>living macrophages used in coculture</td>
</tr>
<tr>
<td>2 cond med</td>
<td></td>
<td>macrophage conditioned medium</td>
</tr>
<tr>
<td>3 lys</td>
<td></td>
<td>macrophage lysate</td>
</tr>
<tr>
<td>4 vit, fresh</td>
<td></td>
<td>normal, freshly-isolated vitreous</td>
</tr>
<tr>
<td>5 vit no mac, incub</td>
<td></td>
<td>normal vitreous incubated for 24 hr in the absence of macrophages</td>
</tr>
<tr>
<td>6 vit + mac, incub</td>
<td></td>
<td>normal vitreous incubated for 24 hr in vitro of macrophages</td>
</tr>
<tr>
<td>7 d3 vit</td>
<td></td>
<td>vitreous from which macrophages were removed 3 days after elicitation</td>
</tr>
<tr>
<td>8 d3 vit, incub</td>
<td></td>
<td>d3 vit incubated for 24 hr in vitro</td>
</tr>
</tbody>
</table>

Proliferation and Migration Assays

Cultures of rabbit RPE were used that were propagated by published procedures and passaged fewer than four times by trypsin treatment. Quantitation of proliferation was by a modification of a previously-described radioautographic method. RPE cells were plated into wells of eight-chamber tissue culture chamber slides (Lab-Tex, Naperville, IL) at a sub-confluent density of $7.5 \times 10^4$ cells/cm² in MEM containing 10% fetal bovine serum (FBS). After 24 hr, the medium was replaced by serum-free MEM for an additional 24 hr to slow cell growth. The medium was removed and macrophage or vitreous samples were then added to the cultures. The samples were tested in medium that was supplemented with a basal concentration of FBS. The amount of FBS used as the basal level ranged from 1% to 5% in the reported experiments. In each experiment a basal serum control group and a stimulated control group (10% FBS) were also included. After 24 hr, 0.5 $\mu$Ci $^3$H-thymidine was added to each well and cultures were labeled for 8 additional hr. Radioautographs were prepared using Kodak (Rochester, NY) NTB2 liquid emulsion with an exposure period of 6 days. All test and control samples were analyzed in quadruplicate wells and labeling rates were assessed by determining the percentage of labeled nuclei in 300 to 500 cells per well.
For migration testing, 48-well micro chemotaxis chambers were used. Lower wells contained the macrophage, vitreous or control samples. The basal control was serum-free MEM with 0.2% lactalbumin hydrolysates (LH); stimulated controls were MEM-supplemented with 5% and 15% FBS. Upper wells contained the RPE plated at a density of $7.5 \times 10^4$ cells/cm$^2$ in serum-free MEM containing 0.2% LH. Upper and lower wells were separated by an 8-$\mu$m pore gelatin-coated Nucleopore membrane (Neuroprobe, Cabin John, MD). Chemotaxis chambers were incubated at 37°C for 6 hr. Following incubation, chemotaxis membranes were air-dried, stained with hematoxylin and eosin and counted. Four wells were established for each test or control sample and the total number of migrated RPE cells was determined in six fields at 400 per well. Reported data are means ± SD of the quadruplicate tests. Checkerboard tests to distinguish chemotaxis from chemokinesis were not routinely employed but were conducted on some day 3 vitreous samples.

**Evaluation of Macrophage-Derived Proteases**

To determine if macrophages proteolytically alter the composition of the vitreous, macrophage lysates were prepared in serum-free medium as described above, then used as the enzyme source for incubation with normal, freshly-dissected vitreous. The incubation mixture contained $50 \mu$l vitreous, $20 \mu$l macrophage lysate, and $50 \mu$l buffer at pH 3.0 (100 mM formate), pH 5.5 or 7.5 (both 100 mM Tris maleate). Incubations were for 30 min at 37°C and the reaction was stopped by the addition to the reaction mixture of an equal volume of sample buffer for electrophoresis, followed by boiling for 3 min. Polyacrylamide slab gel electrophoresis was done by the method of Laemmli,

\[ \text{Laemmli, 9 using a 7.5% separating gel and loading 75} \]

\[ \mu\text{l of the reaction mixture per well.} \]

Some vitreous samples that were prepared by the methods used to generate samples for growth and migration testing were also analyzed by electrophoresis. Aliquots of the following were analyzed: freshly-isolated normal vitreous, vitreous incubated for 24 hr with or without macrophages, day 3 vitreous, and day 3 vitreous that had been incubated for an additional 24 hr in vitro. The vitreous samples were mixed 1:1 with electrophoresis sample buffer and loaded on a gel in a volume of 50 $\mu$l/well for electrophoresis as described above.

As indicated in the results, incubation of normal vitreous with macrophages and additional in vitro incubation of day 3 vitreous appeared to result in increased migration activity. To determine if proteolysis contributed to the increase, the incubations and migration assays were conducted in the presence of protease inhibitors that had been previously shown to inhibit the activity of enzymes derived from vitreal macrophages. For these experiments, the incubation of normal vitreous with macrophages and the 24-hr incubation of day 3 vitreous were conducted in the presence or absence of the following inhibitor cocktail: 5 $\mu$M leupeptin, 1 $\mu$M pepstatin and 1 $\mu$M phenylmethylsulfonylfluoride (to inhibit thiol, carboxyl, and serine proteases, respectively). Inhibitors were not removed following the incubations and samples were directly tested for migration activity as described above. Migration assays of the basal control medium (MEM with 0.2% LH) and of the macrophages were also conducted in the presence and absence of the protease inhibitors.

**Results**

**Proliferation**

Figure 1 shows the results of a proliferation assay in which macrophage-derived and vitreous samples were tested on RPE cells in basal medium containing 1% FBS. In this experiment, stimulation of RPE was observed in cocultures with macrophages and also in the presence of macrophage lysates, but not in macrophage-conditioned medium. In accordance with published observations, all vitreous samples stimulated RPE proliferation. Day 3 vitreous taken from eyes following a macrophage elicitation showed more activity than normal vitreous. Incubation of normal vitreous in vitro for 24 hr in the presence or absence of macrophages did not significantly alter its growth-promoting activity. Similarly, additional in vitro incubation of day 3 vitreous elicited no significant change in activity.

To demonstrate which test samples showed reproducible stimulatory activity, the results of several proliferation experiments are summarized in Table 2. Coculture with macrophages consistently showed stimulation over the basal controls (group 1). Activity above basal levels was not detected in conditioned media (group 2) nor was activity routinely observed in macrophage lysates (group 3), with the exception of the experiment shown in Figure 1. All vitreous samples were stimulatory in all experiments (groups 4–8).

To show which treatments altered vitreous mitogenic activity, the relative growth-promoting activities of the different vitreal samples are summarized in Table 3. The day 3 vitreous samples were consistently more stimulatory than both normal vitreous and normal vitreous incubated in vitro with macrophages (groups 3, 4). Incubation of vitreous in general had no effect on RPE proliferation (groups 1, 2 and 5).
GROWTH AND MIGRATION ACTIVITY OF VITREOUS MACROPHAGES / Burke and Twining

**Table 2.** Summary data from several separate proliferation experiments

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample</th>
<th>Number of experiments stimulation observed above basal control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>mac</td>
<td>4 (of 4)</td>
</tr>
<tr>
<td>2</td>
<td>cond med</td>
<td>0 (of 3)</td>
</tr>
<tr>
<td>3</td>
<td>lys</td>
<td>1 (of 3)</td>
</tr>
<tr>
<td>4</td>
<td>vit, fresh</td>
<td>4 (of 4)</td>
</tr>
<tr>
<td>5</td>
<td>vit no mac, incub</td>
<td>4 (of 4)</td>
</tr>
<tr>
<td>6</td>
<td>vit + mac, incub</td>
<td>4 (of 4)</td>
</tr>
<tr>
<td>7</td>
<td>d3 vit</td>
<td>4 (of 4)</td>
</tr>
<tr>
<td>8</td>
<td>d3 vit, incub</td>
<td>4 (of 4)</td>
</tr>
</tbody>
</table>

**Table 3.** Summary data from several separate proliferation experiments showing the relative activities of vitreous samples following different treatments

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample comparison</th>
<th>Number of experiments stimulation observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>vit no mac, incub &gt; vit fresh</td>
<td>0 (of 4)</td>
</tr>
<tr>
<td>2</td>
<td>vit + mac, incub &gt; vit no mac, incub</td>
<td>1 (of 4)</td>
</tr>
<tr>
<td>3</td>
<td>d3 vit &gt; vit, fresh</td>
<td>4 (of 4)</td>
</tr>
<tr>
<td>4</td>
<td>d3 vit &gt; vit + mac, incub</td>
<td>4 (of 4)</td>
</tr>
<tr>
<td>5</td>
<td>d3 vit, incub &gt; d3 vit</td>
<td>1 (of 4)</td>
</tr>
</tbody>
</table>

Table 4 summarizes the results of several migration experiments. Macrophages and conditioned media, but not lysates, consistently stimulated RPE migration. Vitreous samples were also consistently stimulatory; although freshly-isolated vitreous and vitreous incubated without macrophages showed marginal stimulatory activity.

The relative migration activities of the vitreal preparations are summarized in Table 5. Day 3 samples had consistently higher activity than preparations of normal and incubated normal vitreous (groups 3, 4). In contrast to tests for proliferation activity, incubation of normal vitreous, especially with macrophages.

**Fig. 2.** Assay showing RPE migration in the presence of serum (FBS), macrophage-derived or vitreous samples. Macrophage samples were assayed in serum-free medium; vitreous samples were assayed undiluted. The basal control is indicated by the dotted line.
Table 4. Summary data from several separate chemotaxis experiments

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample comparison</th>
<th>Number of experiments sample stimulatory above basal control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>mac</td>
<td>3 (of 3)</td>
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<tr>
<td>4</td>
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</tr>
<tr>
<td>7</td>
<td>d3 vit</td>
<td>5 (of 5)</td>
</tr>
<tr>
<td>8</td>
<td>d3 vit, incub</td>
<td>5 (of 5)</td>
</tr>
</tbody>
</table>

(group 2), as well as incubation of day 3 vitreous (group 5), generated additional migration activity. Although tests for chemokinesis were not routinely conducted, some day 3 vitreous samples were assayed in varying concentration gradients in a standard checkerboard assay. The analysis indicated that the samples stimulated directed migration (chemotaxis) in RPE (data not shown).

The electrophoretic profile of vitreous proteins incubated in the presence and absence of macrophage lysates is shown in Figure 3. Some minor alterations in protein band density and position result from the incubation of vitreous even in the absence of macrophage-derived lysates (compare lane 2 with lanes 6, 11 and 15). When vitreous is incubated in the presence of macrophage lysates, however, other changes in protein banding pattern are observed. For example, at pH 5.5 and 7.5, alterations are seen in the region of the 45-K dalton molecular weight standard (compare the profiles in lanes 8 and 9, and compare lanes 12 and 13).

Electrophoretic analysis of vitreous samples prepared as for growth and migration testing is shown in Figure 4. Alterations in the protein profile are seen following incubation of vitreous with macrophages (compare lanes 3 and 6). Alterations are also observed following additional in vitro incubation of the day 3 vitreous samples (compare lanes 4 and 5). Some, but not all, of the changes in protein banding in both the normal and the day 3 incubated vitreous samples were inhibited by incubation with protease inhibitors (not shown). An equal volume (25 μl) of vitreous was added to all wells in the gel shown in Figure 4. The day 3 vitreous samples (lanes 4 and 5) contain significantly more protein than the samples prepared from normal vitreous (lanes 3, 6 and 7). Measurements of protein concentration indicated a three- to fivefold increase in vitreous protein following a macrophage elicitation. Since much of the additional protein in these samples is in a single band (probably albumin), samples were loaded on the gel in a volume equivalent (rather than protein equivalent) manner in order to visualize alterations in patterns of the minor components.

Table 5. Summary of data from several separate chemotaxis experiments showing the relative activities of vitreous samples following different treatments

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample comparison</th>
<th>Number of experiments stimulation observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>vit no mac, incub &gt; vit fresh</td>
<td>3 (of 5)</td>
</tr>
<tr>
<td>2</td>
<td>vit + mac, incub &gt; vit no mac, incub</td>
<td>5 (of 5)</td>
</tr>
<tr>
<td>3</td>
<td>d3 vit &gt; vit fresh</td>
<td>5 (of 5)</td>
</tr>
<tr>
<td>4</td>
<td>d3 vit &gt; vit + mac, incub</td>
<td>5 (of 5)</td>
</tr>
<tr>
<td>5</td>
<td>d3 vit, incub &gt; d3 vit</td>
<td>4 (of 5)</td>
</tr>
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</table>
vitreous sample incubated with macrophages and in
the incubated day 3 vitreous was significantly re-
duced by including protease inhibitors during the in-
cubation and assay.

Discussion
Pathologic human vitreous from patients with
PVR has been shown to contain growth and chemotaxis stimulants active on RPE, but the origin(s) of
the activities is unknown. This investigation was un-
dertaken to determine if vitreal macrophages are a
potential source of either or both activities. Macro-
phages are a common cellular constituent of PVR
membranes both in specimens removed at vitrec-
motomy and in the animal model of PVR in which cells
are injected into the rabbit vitreous. It is reasonable
to consider ocular macrophages as a source of mito-
genic and/or migration activities because macro-
phages from other sources have been shown to pro-
cduce growth factors such as platelet-derived growth
factor (PDGF) and fibroblast growth factor, and
chemoattractants such as PDGF and fibronectin. PDGF and fibronectin have been shown to be che-
motactic for RPE, and PDGF has also been
shown to facilitate RPE proliferation. Macrophages from vitreous have not, however, been shown to pro-
cduce these or other mitogenic or chemotactic activi-
ties.

The data presented here suggest that macrophages
are not a major source of growth-promoting activity
for RPE in conditions in which macrophages invade
the vitreous. Neither the contents of macrophages re-
leased on lysis nor macrophage-conditioned culture
medium consistently significantly stimulated RPE
proliferation in vitro. Some stimulation was routinely
observed, however, when macrophages were cocul-
tured with the RPE. The demonstration of activity in
cocultures but not in macrophage-conditioned me-
dium may have resulted from better access in the
cocultures to products of macrophages or to the asso-
ciation of the RPE with the macrophages on the cul-
ture substrate.

Although macrophages are not themselves a major
source of mitogenic activity for RPE, the elicitation

Fig. 4. Electrophoresis of aliquots of macrophage-derived and
vitreous samples that were prepared for growth and migration anal-
ysis. The positions of molecular weight standards are given at the
left.

Fig. 5. Assay showing RPE migration in samples that were pre-
pared and tested in the presence (+) or absence (−) of protease
inhibitors. The basal control was serum-free medium. The macro-
phage samples were assayed in serum-free medium; the vitreous
samples were assayed undiluted.

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of a macrophage invasion into vitreous increases vitreal mitogenicity. Other potential sources of the elevated vitreous mitogenic activity that might be considered are blood components and/or adjacent tissues. The macrophage elicitation appears to lead to a breakdown of the blood-vitreous barrier as indicated by the elevated protein levels in the vitreous after macrophage invasion. Blood components may thus be available to potentiate RPE growth. Other active agents may originate from necrotic retina following a macrophage invasion. We have previously shown that macrophages transmigrate the retina. The flux of cells through the retina could cause focal retinal injury and the release of retina-derived growth factor activity, such as acidic and basic fibroblast growth factors.

The data presented here indicate that vitreal macrophages secrete activity which stimulates RPE migration. Both macrophage-conditioned medium and the cells themselves promoted RPE migration. The migration activity also appears to be secreted by the macrophages into the vitreous; part of the elevated activity in the vitreous after macrophage elicitation (d3 vit) and in fresh vitreous samples incubated with macrophages (vit + mac, incub) presumably arises from this source. Macrophages also generate additional migration activity by virtue of their ability to secrete or release proteolytic enzymes that act on vitreal components. When the incubation of vitreous with macrophages is conducted in the presence of inhibitors that have been shown to inhibit macrophage-derived proteases, the generation of additional migration activity is partially inhibited. Similarly, the generated migration activity by additional in vitro incubation of the day 3 vitreous is also partially inhibited by the protease inhibitors, perhaps due to the elevated levels of macrophage-derived enzymes in the day 3 vitreous. Electrophoretic analysis of vitreal proteins before and after incubation with macrophage proteases indicated that the composition of the vitreous is altered by enzymes active predominantly at pH 5.5 to 7.5. The pH of the vitreous following macrophage invasion is approximately 6.2, suggesting that the macrophage-derived proteases may be active in vivo.

Macrophages appear not to be the only source of migration activity in the day 3 vitreous, based upon the observation that the migration activity of the day 3 vitreous appears significantly greater than can be attributed to the macrophages and to the products of incubation of vitreous with macrophages. Invasion of the vitreous by macrophages follows retinal injury, the intravitreal introduction of foreign substances including blood components, and as part of the pathologic processes in PVR. The data in this report indicate that the composition of the vitreous is significantly altered by events accompanying a macrophage invasion, resulting in a vitreous that stimulates both the migration and proliferation of RPE. The macrophages themselves are a potential source of stimulating activity, especially of migration stimulants. Not only do vitreal macrophages produce a migration activity for RPE, but they may also secondarily generate additional activity by proteolytic alteration of vitreal proteins.

**Key words:** proliferation, chemotaxis, macrophages, blood-vitreous barrier, retinal pigment epithelium, proliferative vitreoretinopathy

**Acknowledgments**

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**References**

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