Multiplex Bead Analysis of Vitreous Humor of Patients with Vitreoretinal Disorders

Sounath Banerjee, Vijay Savant, Robert A. H. Scott, S. John Curnow, Graham R. Wallace, and Philip I. Murray

PURPOSE. Vitreoretinal disorders are frequently characterized by increased vitreous levels of cellular mediators, including cytokines, chemokines, and growth factors. The study was conducted to investigate whether multiplex bead analysis could identify disease-specific profiles of these mediators in a variety of vitreoretinal diseases.

METHODS. Levels of 19 mediators were measured: the cytokines IL-6, IL-10, IL-12, IL-13, IL-15, IL-17, TNF, IFN-γ, granulocyte-macrophage--colony-stimulating factor (GM-CSF), and granulocyte-stimulating factor (G-CSF); the chemokines CCL2, CCL3, CCL4, CCL5, CCL11, and CXCL8; and the growth factors epidermal growth factor (EGF), FGF, and VEGF, by using multiplex bead analysis of vitreous humor of 58 eyes undergoing vitrectomy for a variety of vitreoretinal disorders.

RESULTS. The predominant mediators detected were IL-6, CXCL8, and CCL2. The most complex pattern of mediators was seen in patients with proliferative vitreoretinopathy (PVR) and included a mixture of cytokines, chemokines, and growth factors. Patients with chronic uveitis showed a limited mediator pattern that did not suggest either a Th1 or Th2 response. By comparison, patients with lens-induced uveitis (LIU) showed significantly greater levels of cytokines than did patients with chronic uveitis, including IFN-γ and IL-12, with a trend toward an acute Th1 inflammatory response. Moreover, in samples from patients with LIU, CXCL8 inversely correlated with time after initial surgery and duration of treatment.

CONCLUSIONS. Multiplex bead analysis allows the measurement of multiple mediators from a single vitreous sample. The data confirm patterns of mediators previously described in different vitreoretinal conditions. In addition, LIU mediator levels correlate with duration of treatment and time after cataract surgery. (Invest Ophthalmol Vis Sci. 2007;48:2203–2207) DOI:10.1167/iovs.06-1358

Posterior uveitis (PU) is an increasing cause of visual morbidity, especially in young individuals of working age. PU is caused by a group of inflammatory diseases characterized by breakdown of the blood-retinal barrier, with leukocytic infiltration of the retina and macular edema. Some cases are associated with a systemic disease, such as sarcoidosis, Behcet’s disease, and multiple sclerosis. In endogenous posterior uveitis (EPU) there is no apparent association with inflammatory disease outside of the eye. It is generally considered to be an autoimmune, cell-mediated, organ-specific disease based on the findings of autoreactive T cells and antibodies in patients, the response of the disease to immunosuppression, and the existence of experimental models that share many of the clinical and immunologic features of the human disease. Moreover, the phenotype of many of these conditions overlap, and no specific biomarkers are available to aid in diagnosis. Lens-induced uveitis may be characterized as acute uveitis, as it develops after lens trauma or surgery. Posterior dislocation of lens material into the vitreous cavity is an infrequent complication of cataract surgery. In patients with retained lens fragments, uveitis, increased intraocular pressure, corneal edema, cystoid macular edema, and retinal detachment may develop.

Changes in the vitreous humor occur in proliferative vitreoretinal disorders. Such changes include proliferative diabetic retinopathy (PDR) caused by retinal ischemia, which induces the production of vasoproliferative factors and results in the formation of aberrant blood vessels that can ultimately destroy retinal architecture, and PVR, which is characterized by inflammatory and vascular components that arise from the migration of retinal pigment epithelial cells into the vitreous cavity and the subsequent release of inflammatory mediators.

In all these inflammatory and proliferative potentially sight-threatening conditions, the infiltration of cells into the vitreous cavity is a common feature. Whatever the underlying cause, it is increasingly apparent that such effects are induced by and lead to profound alterations in various molecules including cytokines, chemokines, and growth factors. Although vitreous humor specimens, compared with aqueous humor, provide a reasonable sample volume for analysis, in previous studies only a limited number of cytokines have been measured in each patient. In most studies, comparisons were made by measuring a few cytokines in each sample or by examining patients in different disease cohorts. Recently, multiplex-bead–based immunoassays have been established that allow the identification of many molecules in a single small sample volume. They have already been successfully used to measure cytokines in serum, tears, culture supernatants, and aqueous humor. More recently, this technique has been used to measure CXCL8, VEGF, and angiogenin in the vitreous humor of diabetic patients.

The purpose of this study was to analyze the inflammatory response in the vitreous of patients with a variety of vitreoretinal disorders by using multiplex bead analysis to determine whether specific patterns of cytokines, chemokines, and growth factors could be identified for each condition. The findings may provide a greater insight into the intraocular environment in these conditions and into their underlying pathogenic mechanisms.

METHODS

Patients were recruited from the tertiary referral vitreoretinal unit of the Birmingham and Midland Eye Centre. Vitreous samples were taken from 50 patients undergoing vitrectomy for either inflammatory or proliferative vitreoretinal disease: PDR (n = 10), PVR (n = 8), chronic
Chronic uveitis (n = 8; four idiopathic panuveitis, four intermediate uveitis [three idiopathic/pars planitis, and one sarcoidosis]), LIU (n = 15), idiopathic choroidal neovascular membrane (CNVM; n = 6), CNVM-punctate inner choroidopathy (CNVM-PIC; n = 3), and eight patients undergoing surgery for idiopathic epiretinal membrane (ERM) who acted as the normal control group. All patients with chronic uveitis except two were receiving systemic immunosuppression: three with prednisolone alone; one with prednisolone and methotrexate; one with prednisolone and mycophenolate mofetil; and one with prednisolone, methotrexate, and infliximab. The LIU group comprised patients who, within the previous 14 days, had undergone complicated phacoemulsification cataract surgery in which the lens nucleus had inadvertently been dropped into the vitreous cavity resulting in an acute form of uveitis. Of the 15 patients with LIU, 12 were receiving topical corticosteroid, and none were receiving systemic anti-inflammatory or immunosuppressive therapy at the time of vitrectomy.

An undiluted vitreous sample was taken at the beginning of surgery, snap frozen in liquid nitrogen, and stored at −80°C until analysis. Briefly, a standard three-port pars plana vitrectomy was set up. A vitreous sample was obtained with the vitreous cutter before the infusion was performed. A minimum volume of 0.5 mL was taken in each patient. Ethics approval and the patients’ consent were obtained, and the research adhered to the tenets of the Declaration of Helsinki.

### Table 1. Vitreous Levels of Cytokines

<table>
<thead>
<tr>
<th></th>
<th>IL-6 (0.1)</th>
<th>IL-10 (0.1)</th>
<th>IL-12 (0.9)</th>
<th>IL-13 (0.2)</th>
<th>IL-15 (1.0)</th>
<th>IL-17</th>
<th>TNF (0.5)</th>
<th>IFN-γ (0.8)</th>
<th>GM-CSF (1.0)</th>
<th>G-CSF (7.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDR (n = 10)</td>
<td>32 (0–686)</td>
<td>ND</td>
<td>14 (0–48)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PVR (n = 8)</td>
<td>975 (0–14182)</td>
<td>7 (0–55)</td>
<td>15 (0–88)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>12 (0–31)</td>
<td>5 (0–40)</td>
<td>ND</td>
</tr>
<tr>
<td>ERM (n = 8)</td>
<td>6 (0–25)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CNVM (n = 6)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CNVM-PIC (n = 3)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Chronic uveitis (n = 8)</td>
<td>145 (0–4019)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LIU (n = 15)</td>
<td>5000 (65–1770)</td>
<td>3 (0–19)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>14 (0–158)</td>
<td>ND</td>
<td>1166 (0–9465)</td>
</tr>
</tbody>
</table>

Levels are expressed as the mean (range) picograms per milliliter, with the lower limit of detection below each mediator.

uveitis (n = 8; four idiopathic panuveitis, four intermediate uveitis [three idiopathic/pars planitis, and one sarcoidosis]), LIU (n = 15), idiopathic choroidal neovascular membrane (CNVM; n = 6), CNVM-punctate inner choroidopathy (CNVM-PIC; n = 3), and eight patients undergoing surgery for idiopathic epiretinal membrane (ERM) who acted as the normal control group. All patients with chronic uveitis except two were receiving systemic immunosuppression: three with prednisolone alone; one with prednisolone and methotrexate; one with prednisolone and mycophenolate mofetil; and one with prednisolone, methotrexate, and infliximab. The LIU group comprised patients who, within the previous 14 days, had undergone complicated phacoemulsification cataract surgery in which the lens nucleus had inadvertently been dropped into the vitreous cavity resulting in an acute form of uveitis. Of the 15 patients with LIU, 12 were receiving topical corticosteroid, and none were receiving systemic anti-inflammatory or immunosuppressive therapy at the time of vitrectomy.

An undiluted vitreous sample was taken at the beginning of surgery, snap frozen in liquid nitrogen, and stored at −80°C until analysis. Briefly, a standard three-port pars plana vitrectomy was set up. A vitreous sample was obtained with the vitreous cutter before the infusion was performed. A minimum volume of 0.5 mL was taken in each patient. Ethics approval and the patients’ consent were obtained, and the research adhered to the tenets of the Declaration of Helsinki.

### Cytokine, Chemokine, and Growth Factor Analysis

Multiplex bead analysis was performed on all vitreous samples using the Beadlyte Human Multi-Cytokine Detection System (Upstate Biotechnology, Lake Placid, NY) for the following molecules: the cytokines IL-6, IL-10, IL-12, IL-13, IL-15, IL-17, tumor necrosis factor (TNF), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte stimulating factor (G-CSF), and interferon (IFN)-γ; the chemokines CCL2, CCL3, CCL4, CCL5, CCL11, and CXCL8; and the growth factors VEGF, endothelial growth factor (EGF), and FGF.

For measurements, vitreous samples, diluted with an equal volume of PBS, 1% BSA, and 0.05% Tween-20, were incubated with monoclonal antibody-coated capture beads (2 × 10^5 for each cytokine) for 2 hours at 20°C. Washed beads were further incubated with biotin-labeled polyclonal anti-human cytokine antibody (2 μg/mL) for 2 hours followed by streptavidin-phycocerythrin (Upstate Biotechnology) at 40 μg/mL for 30 minutes. Samples were analyzed with a bioassay analyzer (model 100; Luminex, Austin, TX). Standard curves of known concentrations of recombinant human cytokines (R&D Systems, Minneapolis, MN) were used to convert fluorescence units to cytokine concentration (picograms/milliliter). Results were calculated from standard curves prepared on each plate and are expressed as picograms per milliliter.

### Statistical Analysis

Cytokine, chemokine, and growth factor levels between different disease groups were analyzed using the Mann-Whitney test. Correlation between CXCL8 and time after surgery was analyzed by the Spearman coefficient.

### Results

The results of the study are summarized in Tables 1, 2, and 3. Although IL-6, CXCL8, and CCL2 were found in most of the samples, IL-13, IL-15, IL-17, CCL3, and EGF were not detected in any sample. In vitreous from patients with idiopathic CNVM and CNVM-PIC, only CCL2 was present in detectable levels. The findings were almost the same in the control patients with idiopathic ERM, except that IL-6 and CXCL8 were also found, but at very low levels.

Vitreous of patients with PDR had detectable levels of VEGF; however, an inflammatory component was also suggested by the presence of the cytokines IL-6, and IL-12, and the chemokines CXCL8, CCL2, CCL4, and CCL5. Vitreous of patients with PVR showed a more complex phenotype with IL-6, CXCL8, and CCL2 in most samples, but with IL-10, TNF, IFN-γ, CCL3, CCL4, CCL5, G-CSF, and FGF being found in some samples. Low levels of CCL11 was found in five (62.5%) of eight PVR samples. Vitreous samples taken from patients with chronic uveitis showed the presence of IL-6, CXCL, and CCL2, but no inflammatory (IL-12, IFN-γ) or regulatory (IL-10) cytokines or inflammatory chemokines (CCL3, CCL4, and CCL5).

### Table 2. Vitreous Levels of Chemokines

<table>
<thead>
<tr>
<th></th>
<th>CCL2 (17.1)</th>
<th>CCL3 (29.0)</th>
<th>CCL4 (&lt;1.0)</th>
<th>CCL5 (0.8)</th>
<th>CCL11 (0.8)</th>
<th>CXCL8 (0.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDR (n = 10)</td>
<td>1310 (0–7918)</td>
<td>ND</td>
<td>4.5 (0–33)</td>
<td>3 (0–26)</td>
<td>ND</td>
<td>22 (0–335)</td>
</tr>
<tr>
<td>PVR (n = 8)</td>
<td>6101 (128–8777)</td>
<td>ND</td>
<td>22 (0–135)</td>
<td>6 (0–25)</td>
<td>ND</td>
<td>65.5 (0–977)</td>
</tr>
<tr>
<td>ERM (n = 8)</td>
<td>449 (54–1228)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>5 (0–27)</td>
</tr>
<tr>
<td>CNVM (n = 6)</td>
<td>426 (40–941)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CNVM-PIC (n = 3)</td>
<td>235 (122–364)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Chronic uveitis (n = 8)</td>
<td>635 (0–3102)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>36 (0–100)</td>
</tr>
<tr>
<td>LIU (n = 15)</td>
<td>5539 (1350–13688)</td>
<td>ND</td>
<td>15 (0–41)</td>
<td>ND</td>
<td>ND</td>
<td>99 (0–682)</td>
</tr>
</tbody>
</table>

Levels are expressed and mean (range) in picograms per milliliter, with the lower limit of detection below each mediator. See Table 1 legend for disease states. ND, Not detected.
were found, whereas TNF (31 pg/mL) was found in only one sample.

Of the most interest were patients with LIU. Vitreous samples from these patients contained significantly greater levels of IL-6, compared with those with chronic uveitis ($P < 0.001$), PVR ($P = 0.01$), or PDR ($P = 0.0001$), and higher levels of CXCL8 compared with patients with chronic uveitis ($P = 0.03$) or PDR ($P = 0.01$; Figs. 1 and 2). Moreover, two samples from patients with LIU also had detectable levels of G-CSF, CCL4, and VEGF, with IFN-γ. The increased inflammatory response in LIU was confirmed, as vitreous levels of CXCL8 correlated inversely with the time from initial surgery to vitrectomy (Spearman coefficient, $-0.72$, $P = 0.002$; Fig. 3).

**DISCUSSION**

During an inflammatory or proliferative insult, blood- or retina-borne cells enter the vitreous cavity in response to chemokines and growth factors. Once in the vitreous, these cells secrete mediators such as cytokines. In this study, we have applied multiplex bead technology to analyze multiple mediators in vitreous from patients with a range of vitreoretinal conditions.

In terms of cytokines, patients with LIU had the most active disease, with the highest levels of CXCL8, IL-6, and CCL4. In particular, these mediators were significantly higher in LIU than in chronic uveitis. Moreover, proinflammatory cytokines, including IFN-γ and G-CSF, were found only in vitreous samples from patients with LIU, although IFN-γ was present at only low levels. This result may be a reflection of treatment, as patients with chronic uveitis were on established immunosuppressive drug regimens, whereas most patients with LIU had only recently started topical corticosteroid and therefore would be likely to have a more acute inflammatory response, particularly in the vitreous. The reduction in levels of CXCL8 over time in those patients with LIU who were receiving treatment supports this contention. Second, LIU studies in animal models showed a major role for humoral immunity and autoantibody production, although a recent study has suggested a cellular component.16,17

In support of our findings in patients with PDR, a previous study showed significantly higher levels of CXCL8 and CCL2 in the vitreous of patients with PDR than in those with nonproliferative conditions (NPCs).4 In another study, vitreous of patients with PDR also had significantly increased levels of CCL2, but CCL3 and CCL4 were undetectable.18 Moreover, CCL2 levels in patients with PDR were significantly higher than those in control subjects with NPCs, and multivariate analysis.

---

**TABLE 3. Vitreous Levels of Growth Factors**

<table>
<thead>
<tr>
<th>Disease</th>
<th>VEGF (pg/mL)</th>
<th>EGF (pg/mL)</th>
<th>FGF (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDR (n = 10)</td>
<td>246 (0–3193)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PVR (n = 8)</td>
<td>975 (0–14182)</td>
<td>ND</td>
<td>28 (0–2555)</td>
</tr>
<tr>
<td>ERM (n = 8)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CNVM (n = 6)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CNVM-PIC (n = 3)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Chronic uveitis (n = 8)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LIU (n = 15)</td>
<td>286 (0–3161)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Levels are expressed and mean (range) in picograms per milliliter, with the lower limit of detection below each mediator. See Table 1 legend for disease states. ND, Not detected.

---

**FIGURE 1.** Vitreous IL-6 levels in PDR, PVR, chronic uveitis, and LIU groups.

**FIGURE 2.** Vitreous CXCL8 levels in PDR, PVR, chronic uveitis, and LIU groups.

**FIGURE 3.** Correlation of vitreous CXCL8 levels with days after initial surgery in 15 patients with LIU.
showed a significant association between vitreous CCL2 levels and the degree of proliferative membrane and a negative association with the extent of preoperative retinal photocoagulation.\textsuperscript{19} Recently, Hernandez et al.\textsuperscript{20} reported elevated vitreous levels of CXCL8 and CCL2 in PDR, and these correlated with disease activity. The same study showed very low IL-10 levels, and we were unable to detect this cytokine in our patients with PDR. Other investigators have also found elevated vitreous CXCL8 and IL-6 levels in patients with PDR.\textsuperscript{21,22} In one study, IL-6 was found in much higher levels in patients with PDR than in those with PVR.\textsuperscript{23} The presence of VEGF in vitreous samples and plasma from 30 patients with PDR has been shown to correlate with severity of PDR and levels of vitreous endostatin.\textsuperscript{24} Using multiplex bead analysis, Maier et al.\textsuperscript{15} recently showed elevated CXCL8, VEGF, and angiogenin levels in vitreous humor samples from 13 diabetic patients compared with levels in samples from nondiabetic patients.

The profile of vitreous from our patients with PVR showed a complex mix of cytokines, chemokines, and growth factors. Detectable levels of IL-6, TNF, IFN-γ, IL-10, CCL3, CCL4, CCL5, and VEGF support a role for an inflammatory response in this condition. A similar inflammatory profile has been reported with TNF, IL-6, and IFN-γ expression in PDR membranes, suggesting a complex interaction with resident ocular cells and infiltrating leukocytes.\textsuperscript{25} Also, the presence of FGF has been reported and linked to a wound-healing response in PVR.\textsuperscript{26} Raised vitreous CCL2 levels have been reported in several studies,\textsuperscript{4,18} and in one study correlated with the severity of proliferation in patients with PVR and with vitreous IL-6 levels.\textsuperscript{5} A possible role for CCL2 in PVR was suggested in an in vitro wound-healing model.\textsuperscript{27} CCL2 stimulated RPE cells growth in a dose-dependent manner—a response that was blocked by dexamethasone. The data suggest that CCL2 stimulates RPE cell migration and regulates development of PVR in the initial stage. By comparison, CXCL8 was detected in vitreous of patients with PVR,\textsuperscript{5,21} but did not correlate with the grade of PVR or the duration of symptoms.\textsuperscript{7} Similar to our data, mRNA and protein levels of IL-6 were significantly higher in vitreous of patients with PVR compared with control patients with noninflammatory samples or macular hole.\textsuperscript{21,25,26} However, one study also reported the presence of vitreous IFN-γ in patients with PVR, it was undetectable in our patients.\textsuperscript{28} It is not clear why there is a difference between the results in these studies, as the levels reported would have been detected by our system. This discrepancy should be addressed in future studies.

Recently, connective tissue growth factor (CTGF) was measured in the vitreous of patients with various vitreoretinal disorders including PVR and PDR. CTGF levels correlated significantly with the degree of fibrosis in the various vitreoretinal disorders studied and the investigators concluded that CTGF may be a therapeutic target for vitreoretinal scarring.\textsuperscript{29} There are potential sources of bias in this study that relate to the patient population. First, this was a retrospective study of stored vitreous samples, and there were differences in the timing of sample collection that may have affected some of the mediators analyzed. Second, it is not possible to obtain longitudinal samples to address clearly the effect of treatment. However, regardless of these caveats this is the first study in which multiplex bead analysis has been used to measure a large number of mediators in one vitreous sample.

The identification of cytokine and chemokine profiles potentially allows for analysis of the link between such molecules and disease type or status. Recent studies have shown a link between vitreous levels of VEGF and IL-6 with disease progression in patients with PDR after surgery.\textsuperscript{30} Similarly, vitreous levels of IL-6 and VEGF correlate with the extent of retinal ischemia in patients with branch retinal vein occlusion.\textsuperscript{31}

In conclusion, this data shows that multiplex bead analysis of cytokines, chemokines, and growth factors in vitreous humor is possible; that the results support previous data; and that the method provides new opportunities for analyzing correlations between multiple factors in ocular fluids. The differences shown between LIU and chronic uveitis are of particular importance and define similarities in the role of inflammatory mediators in both diseases, while suggesting differences in response to therapy.

\textbf{References}


