Analysis of Aqueous Humor Immunoglobulin G in Uveitis by Enzyme-Linked Immunosorbent Assay, Isoelectric Focusing, and Immunoblotting

P. I. Murray,* R. Hoekzema, L. Luyendijk, S. Konings, and A. Kijlstra

Immunoglobulin G (IgG) in aqueous humor from patients with various uveitis syndromes was analyzed using a number of immunologic techniques. Sixty-five percent of patients with Fuchs' heterochromic cyclitis (FHC), 70% of patients with other forms of uveitis, and 44% of controls showed local synthesis of IgG, as demonstrated by an elevated IgG:albumin relative concentration ratio. Using an enzyme-linked immunosorbent assay to measure the concentration of IgG subclasses 1-4, a relative excess of IgG1 was found in the aqueous compared with the serum in FHC. Isoelectric focusing and immunoblotting studies revealed oligoclonal IgG bands in the aqueous of 13 of 23 (57%) patients with FHC, most being of the IgG1 subclass. Oligoclonal bands were not found in 18 patients with other types of uveitis or 13 patients undergoing surgery for senile cataract. These findings indicate intraocular production of IgG of restricted specificity in FHC, providing further evidence for local immune dysfunction in this condition. As yet the antigenic stimulus for this oligoclonal B-cell response has not been identified. Invest Ophthalmol Vis Sci 31:2129-2135, 1990

Uveitis is a complex group of diseases in which, in most cases, the etiology is unknown. Immunologic mechanisms are thought to play an important role in pathogenesis.1 Disturbances of humoral immunity were reported in some uveitis syndromes.2-8 Systemic humoral immune abnormalities include raised levels of immune complexes5,6 and the presence of ocular specific autoantibodies, eg, antilens8 and antiretinal antibodies.4 Other workers, however, detected these autoantibodies in the same frequencies in normal subjects.9,10 Nonspecific autoantibodies do not occur more often in uveitis except for antigen-specific antibodies in juvenile chronic arthritis-associated uveitis,11 and previous studies do not show any association between levels of serum immunoglobulins (Ig) and uveitis.12-15 Local humoral immune abnormalities occur in Fuchs' heterochromic cyclitis (FHC).5,16-18 Apart from increased levels of immune complexes in the aqueous,6 many plasma cells have been found in the aqueous16 and iris,17 and there is evidence for local IgG production.18

Various immunologic mechanisms may theoretically give rise to intraocular inflammation. Among them is expansion of B-lymphocytes in the eye producing antibodies that react with intraocular antigens. Polyclonal activation has been proposed as the reason that autoantibodies are produced during autoimmune disease,19 but if the local humoral immune response in uveitis is triggered by one or a few intraocular (self) antigens, we can expect an oligoclonal B-cell response instead of the polyclonal response seen after a nonspecific trigger. This oligoclonal response may result in a different distribution of IgG subclass. Oligoclonal Ig synthesis is seen in other diseases, and one of the hallmarks of multiple sclerosis (MS) is the occurrence of oligoclonal IgG bands in the cerebrospinal fluid (CSF).20 This is not specific for MS; these bands have been found in the CSF in other neurologic disorders including acute idiopathic facial nerve palsy,21 vascular dementia,22 lymphocytic meningoradiculitis,23 human T-cell leukemia/lymphoma virus (HTLV) type I associated myelopathy,24 acute cerebral malaria,25 cerebral systemic lupus erythematosus,26 and various acute psychiatric disorders.27 Oligoclonal bands have also been detected in the sera of HTLV-III-positive healthy individuals28 and lung cancer patients undergoing cytotoxic therapy.29 There are also reports of oligoclonal bands in synovial fluid of arthritis patients30.
and in tuberculous pleural effusions.\textsuperscript{31} Oligoclonality can be demonstrated by the techniques of isoelectric focusing (IEF) and immunoblotting, and quantitative determination of IgG subclass can be analyzed with an enzyme-linked immunosorbent assay (ELISA).

Although the localization of IgG in the eye has been well documented,\textsuperscript{32,33} there are few studies on aqueous humor IgG in uveitis.\textsuperscript{18,34-38} We examined the intraocular clonal B-cell response, with reference to IgG and its subclasses, seen in various types of uveitis. The findings may shed more light on the immunopathogenesis of FHC and other uveitis syndromes.

### Materials and Methods

Aqueous humor samples were collected from 23 patients (15 men and eight women, aged 23–72 yr) with FHC who were undergoing either extracapsular cataract extraction or trabeculectomy, and 18 patients (eight men and ten women, aged 56–82 yr) without uveitis who were undergoing surgery for senile cataract (controls). Aqueous was also collected from 23 patients (14 men and nine women, aged 22–62 yr) with various types of uveitis (14 toxoplasmosis, five posterior, and four panuveitis all of unknown etiology) for diagnostic purposes. Great care was taken to ensure that the aqueous did not become contaminated with blood and that there was no damage to the corneal endothelium, iris, or lens. Ten milliliters of venous blood was drawn from each patient at the time the aqueous was collected. All our investigations were done on aqueous humor and corresponding serum samples of each patient. Serum and aqueous samples were stored at –30°C until use.

Informed consent was obtained before undertaking this study.

**IgG and Albumin Estimation**

The amount of IgG and albumin in the aqueous and serum samples of every patient was measured by standard single radial immunodiffusion techniques. The IgG:albumin relative concentration ratio (RCR) for each patient was calculated using the following equation:\textsuperscript{18}

\[
\text{Aqueous IgG/Serum IgG} / \text{Aqueous Albumin/Serum Albumin}
\]

(all values mg/ml)

**IgG Subclass ELISA**

Flat-bottomed 96-well microtiter plates (No. 655101; Greiner, Alphen a/d Rijn, The Netherlands) were coated overnight at 4°C with a 1:1 mixture of two monoclonal antibodies against human Ig kappa light chains and against human Ig lambda light chains (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) at a concentration of 10 μg/ml in phosphate-buffered saline (PBS) pH 7.4. After three washes with PBS containing 0.1% Tween 20 pH 7.4 (PBS/T), dilutions (titrated twofold) of human standard serum containing 6.1 mg/ml IgG1, 3.3 mg/ml IgG2, 0.47 mg/ml IgG3, and 0.62 mg/ml IgG4 were added as a calibration curve. Aqueous and serum samples were added in duplicate at dilutions which gave values on the linear part of the calibration curve for each IgG subclass. The plates were then incubated for 1 hr at 37°C. After another three washes with PBS/T, peroxidase-conjugated monoclonal antibodies against human IgG1, 2, 3, and 4 (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service) 1:300 in PBS/T were added, and the plates incubated for 1 hr at 37°C. After three more washes with PBS/T, substrate solution containing 0.05 M acetate buffer, pH 5.5, 100 μg/ml of tetramethyl benzidine, and 0.003% H₂O₂ was added. The reaction was stopped after 10 min by the addition of 4 N H₂SO₄. The plates were then read on a Tiertek Multiskan Plus microplate reader (Flow, Ayrshire, Scotland) at a wavelength of 450 nm.

By comparing values obtained in each sample with values from the calibration curves, the concentration of each IgG subclass was calculated. Samples whose values were not on the linear part of a calibration curve were retested at the appropriate dilution. The data were analyzed statistically with the Mann-Whitney U Test.

**IEF and Immunoblotting**

The IEF was done with the PhastSystem (Pharmacia, Uppsala, Sweden) according to the manufacturer’s instructions. One μl of undiluted aqueous humor and 1 μl of serum diluted 1:150 in distilled water (centrifuged for 10 min at 2000 × g) from each patient was applied in duplicate to PhastGel IEF pH 3–9 gels (Pharmacia). After electrophoresis, each gel was equilibrated in transfer buffer (TB) of 25 mM Tris, 192 mM glycine, and 20% methanol (final pH 8.3) for 1 min. After being wetted in 100% methanol for a few seconds, soaked in distilled water for 2 min, then equilibrated in TB for 5 min, a 0.45-μm polyvinylidene difluoride blotting membrane (Immobilon, Millipore, Bedford, MA) was applied carefully to the gel surface, and diffusion blotting was done for 20 min at room temperature (RT). The membrane was then removed and blocked with 1% normal rabbit serum (NRS) in PBS containing 0.05% Tween 20 pH
7.4 (T/PBS) for 30 min at RT to prevent nonspecific binding. After two washes of 5 min each in T/PBS, the membrane was incubated with peroxidase-conjugated rabbit anti-human IgG (Dakopatts, Copenhagen, Denmark) 1:300 in T/PBS containing 2.5% NRS for 2 hr at RT. After four more washes of 7.5 min each in T/PBS, Ig bands were visualized using a substrate of 0.05% 3,3'-diaminobenzidine (Sigma, St. Louis, MO) and 0.01% hydrogen peroxide. After 5 min the reaction was stopped by washing with distilled water. All incubation and washing procedures were done on a rocker platform.

The isoelectric point (pl) range of the samples was determined from the migration distances of standards of known pI (pH 5-10.5; Pharmacia). Both IEF and immunoblotting to detect IgG subclasses were done under these conditions except: (1) the blocking solution used was 1% bovine serum albumin (BSA) in T/PBS and (2) the conjugates used were peroxidase-conjugated monoclonal antibodies against human IgG1, 2, 3 and 4 (Central Laboratory of the Netherlands Red Cross Transfusion Service) 1:300 in T/PBS containing 2.5% BSA.

Results

IgG and Albumin

The range of serum and aqueous humor IgG and albumin values for each group are shown in Table 1. Individual aqueous IgG and albumin values are depicted in Figure 1.

The RCR values of the FHC, uveitis, and control groups are shown in Figure 2. Local IgG production is said to occur if the RCR is >0.65.18 Fifteen of 23 (65%) FHC patients, 16 of 23 (70%) uveitis patients, and eight of 18 (44%) controls had RCR values of >0.65.

IgG Subclass ELISA

Ten FHC patients, ten uveitis patients, and ten controls were examined. The proportion of each IgG subclass in the serum and aqueous of each group is shown in Table 2. Using the actual concentrations determined, the aqueous humor:serum (AH:S) ratio of each subclass for every patient and control was calculated. The values obtained for each subclass in the FHC and uveitis groups were compared with those found in the control group. There was a significant statistical difference (P < 0.01) between the FHC and control group for the subclass IgG1, indicating a relative increase in IgG1, in the aqueous humor in FHC.

IEF and Immunoblotting

Twenty-three FHC patients, 18 uveitis patients, and 13 control subjects were examined. Oligoclonal IgG bands (pI range, 6.8-8.8) were detected in the aqueous (but not the serum) of 13 of 23 (57%) patients with FHC (Figs. 3, 4). These bands could be confirmed using the following peroxidase-labeled conjugates: rabbit anti-human IgG gamma chains (Dakopatts) and Protein G (Calbiochem, San Diego, CA). A simple grading system (from +1 to +4) was used to assess the intensity of the bands, and no statistical correlation between band intensity and either the RCR or aqueous IgG concentration of the patient could be demonstrated (Table 3). In six of 13 samples the IgG subclass of the bands was determined; four samples contained IgG1 only, and the other two were a combination of IgG1 and IgG4. Oligoclonal bands could not be found in the aqueous or serum of any other patient or control tested.

Discussion

The major Ig in serum and aqueous,18,39,40 IgG normally is not produced locally in the quiescent eye.32,41 However, 44% of controls in this study had a RCR >0.65 implying intraocular synthesis of IgG. This figure differs from Dernouchamps18 value of 17% for his control group. It is difficult to explain the difference in the two studies; both used the same technique to measure IgG and albumin, and both used patients with senile cataract as a controls. This im-

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum</th>
<th>Aqueous</th>
<th>Serum</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 18)</td>
<td>7.0-21.0</td>
<td>0.007-0.116</td>
<td>27.4-56.4</td>
<td>0.02-2.08</td>
</tr>
<tr>
<td></td>
<td>(13.23)</td>
<td>(0.03)</td>
<td>(42.26)</td>
<td>(0.27)</td>
</tr>
<tr>
<td>FHC (n = 23)</td>
<td>7.5-16.6</td>
<td>0.009-3.2</td>
<td>36.8-74.2</td>
<td>0.03-1.5</td>
</tr>
<tr>
<td></td>
<td>(11.8)</td>
<td>(0.20)</td>
<td>(49.47)</td>
<td>(0.42)</td>
</tr>
<tr>
<td>Uveitis (n = 23)</td>
<td>7.1-20.9</td>
<td>0.01-2.2</td>
<td>33.8-71.2</td>
<td>0.02-1.5</td>
</tr>
<tr>
<td></td>
<td>(12.23)</td>
<td>(0.20)</td>
<td>(51.58)</td>
<td>(0.62)</td>
</tr>
</tbody>
</table>

Values are range in mg/ml with the mean in brackets.
plies that there may be other factors influencing the RCR. An increase in aqueous IgG has been associated with aging by some authors, although others have been unable to confirm this. Age appears not to be a factor in our study because no difference in the age distribution between controls above and below the 0.65 cutoff point could be detected. The type of cataract may influence the RCR. The exact type of cataract is not known in our control group, but Dernouchamps' group was composed only of patients with senile immature cataract. Both studies showed evidence of intraocular IgG synthesis in FHC, but very high RCR values were seen in some of our patients compared with Dernouchamps whose RCR values were never >2.0. Again, cataract may be implicated as Dernouchamps did not mention whether aqueous was taken from his FHC patients at the time of cataract extraction or at an earlier stage in the disease, when there may have been only a small amount of cataract present. Cataract cannot account for the high RCR values seen in some of our uveitis group; it was not a feature in these patients. The IgG:albumin RCR appears not to be a reliable method for measuring intraocular IgG production. It is of limited value because it gives no details about the absolute amount of IgG synthesized and may also

Table 2. Proportion of IgG subclass in serum and aqueous

<table>
<thead>
<tr>
<th>Group</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td>Controls (n = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>68</td>
<td>22</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Aqueous</td>
<td>56</td>
<td>29</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>FHC (n = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>59</td>
<td>30</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Aqueous</td>
<td>76</td>
<td>18</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Uveitis (n = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>71</td>
<td>18</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Aqueous</td>
<td>63</td>
<td>20</td>
<td>11</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are mean % of the total of the IgG subclasses.
Fig. 3. IEF immunoblots of aqueous humor. (A), (B), (C) are patients with FHC showing oligoclonal IgG bands of different intensity in the cathodic (high pi) range of the blot. The bands are graded +4, +3, +1 for (A), (B), (C) respectively. (D) shows the normal aqueous humour IgG profile.

give false-negative results. This has been shown by three FHC patients with aqueous oligoclonal bands, all with a RCR of <0.65, ie, a low RCR does not exclude intraocular synthesis of IgG. In serum, IgG1 constitutes the largest proportion (approximately 65%) of the IgG subclasses, followed by IgG2 (approximately 25%), then about equal amounts of IgG3 and 4,44 which is consistent with our findings. The IgG concentration of a given individual appears to be related to the Gm allotype which indicates that genetic factors are one variable that determines the overall IgG subclass concentration.45 This study could find no abnormality in IgG subclass distribution in the serum of the FHC, uveitis, or control groups. Although there was a similar distribution of IgG subclass in the aqueous of all groups, by calculating the AH:S ratio for each subclass in each group, a relative increase in aqueous IgG1 was found in FHC compared with controls.

Oligoclonal IgG bands were detected in the aqueous humor in 57% of FHC patients. The oligoclonal bands seen were of different intensities and were graded according to Table 3. Although the intensity of the bands did not correlate statistically with either the RCR or aqueous IgG concentration, the three FHC patients with a RCR <0.65 all had low intensity bands. No relationship between band intensity and duration of disease could be found. Since these discrete IgG bands were not found in the corresponding sera, they indicate intraocular stimulation of a small number of B-cells, present in the vicinity of the anterior chamber. This would result in a selective increase in aqueous humor IgG of restricted heterogeneity, ie, restricted antigenic specificity. Several possibilities have to be considered regarding the antigen(s) recognized by these particular antibodies. They could either be part of an as yet unidentified infectious agent that may be involved in the pathogenesis of FHC, or they could be intraocular self-antigens, indicating an autoimmune factor in this disorder. Finally, they could be unrelated antigens that are not involved in the development of FHC. Antibodies against such antigens have been called "nonsense" or "mis-

<table>
<thead>
<tr>
<th>Patient</th>
<th>RCR</th>
<th>Aqueous IgG oligoclonality</th>
<th>Aqueous IgG concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.92</td>
<td>2+</td>
<td>0.29</td>
</tr>
<tr>
<td>2</td>
<td>0.26</td>
<td>1+</td>
<td>0.062</td>
</tr>
<tr>
<td>3</td>
<td>0.77</td>
<td>1+</td>
<td>0.035</td>
</tr>
<tr>
<td>4</td>
<td>0.49</td>
<td>2+</td>
<td>0.035</td>
</tr>
<tr>
<td>5</td>
<td>8.35</td>
<td>3+</td>
<td>0.079</td>
</tr>
<tr>
<td>6</td>
<td>1.65</td>
<td>4+</td>
<td>0.096</td>
</tr>
<tr>
<td>7</td>
<td>1.78</td>
<td>3+</td>
<td>0.037</td>
</tr>
<tr>
<td>8</td>
<td>0.98</td>
<td>4+</td>
<td>0.042</td>
</tr>
<tr>
<td>9</td>
<td>1.75</td>
<td>3+</td>
<td>0.38</td>
</tr>
<tr>
<td>10</td>
<td>1.03</td>
<td>2+</td>
<td>0.029</td>
</tr>
<tr>
<td>11</td>
<td>0.73</td>
<td>3+</td>
<td>0.069</td>
</tr>
<tr>
<td>12</td>
<td>0.37</td>
<td>1+</td>
<td>0.009</td>
</tr>
<tr>
<td>13</td>
<td>1.66</td>
<td>1+</td>
<td>0.067</td>
</tr>
</tbody>
</table>

Table 3. Relative concentration ratio (RCR), oligoclonal band intensity and aqueous IgG concentration in 13 patients with FHC.
sense antibodies and could result from dysregulation of B-cell clones normally present in the eye. In the other types of uveitis examined, there is polyclonal B-cell stimulation. The B-cells react without clonal restriction, and because they come from the general circulation with their immunologic memory, the synthesized IgG are indistinguishable from those found in the serum. Most oligoclonal bands analyzed for IgG subclass were of the IgG1 type, in agreement with the finding of a relative excess of aqueous IgG1 in FHC. This may have some particular significance regarding the immunopathogenesis of FHC because IgG1 offers the major tissue protection against bacterial proteins and binds to cell-surface Fc receptors, eg, FcR-I on monocytes. However, in two patients, IgG4 bands were seen; this was unexpected as the actions of IgG4 differ greatly from IgG1. The former does not activate complement, and IgG4 antibodies appear to inhibit immune precipitation and binding of Clq to IgG1 in complexes containing IgG1 and IgG4. Although three discrete IgG bands of pl range 8.4–8.5 (Fig. 3D) were always detected in the blots of normal aqueous and serum, they could easily be distinguished from oligoclonal bands. The significance of these bands is unknown.

To our knowledge this is the first documented report of aqueous oligoclonal IgG production in an ocular disease. This finding, combined with an excess of aqueous IgG1, clearly distinguishes FHC from the other forms of uveitis studied by us. Both IEF and immunoblotting of aqueous humor were previously obtained since only trace amounts were detectable on immunoblots. 51-54 The significance of these bands is unknown.

To realize a rapid and appropriate antibody production by B-lymphocytes, complex regulatory signals and interactions are required that are provided by T-lymphocytes. When B-cells encounter antigens complementary to their surface IgG and are exposed to the activity of T-helper cells (Th), they may develop into memory B-cells or further differentiate into antibody-secreting plasma cells. During this antigen-dependent stage of B-cell maturation, most mature B-lymphocytes undergo an Ig heavy chain switch leading to plasma cells that secrete only one Ig isotype. The signals that B-cells require during the stages of activation (activation, proliferation, and differentiation) are produced by soluble mediators known as cytokines (interleukins). Activation requires interleukin-1 produced by antigen presenting cells and Th factors, such as interleukin-4. Proliferation requires further Th participation through interleukins-2, -4, and -5. Differentiation into antibody-forming cells is also under T-cell regulation, and interleukin-6 is probably the most important cytokine involved.52 Our group recently detected raised levels of interleukin-6 in the aqueous but not in the serum of patients with FHC and active ocular toxoplasmosis.53 The possibility that the local production of polyclonal and/or oligoclonal IgG in these uveitis conditions is related to this intraocular release of interleukin-6 is currently under investigation.

Thus, IEF and immunoblotting provide a valuable tool for investigating an abnormal regulation of Ig synthesis in the eye. Oligoclonal IgG bands are a potential clue to the pathogenesis of FHC, but as yet their particular antigenic target is unknown.

Key words: aqueous humor, heterochromic cyclitis, isoelectric focusing, immunoblotting, IgG oligoclonal bands

Acknowledgments

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References