Intraocular Antibody Synthesis during Experimental Uveitis

J. C. M. von der Voet, A. Liem, A. J. Otto, and A. Kijlstra

Determination of intraocular antibody synthesis against certain microorganisms is a diagnostic aid in identifying the causative agent in clinical uveitis. Little is known, however, concerning the kinetics and specificity of antibodies produced during intraocular inflammation. To investigate this subject we induced uveitis in rabbits by injecting small amounts of human serum albumin (HSA) into the vitreous. Aqueous humor and serum were taken before and after the induction of uveitis and levels of total IgG, rabbit albumin and anti-HSA-IgG were determined. The anti-HSA-IgG was quantitated using immunofinity purified anti-HSA-IgG as a standard. Six weeks after intravitreal HSA injection, high levels of total IgG (4.7 mg/ml) and albumin (15.4 mg/ml) were observed in the aqueous as compared to control eyes (IgG: 0.12 mg/ml; albumin: 0.48 mg/ml). Using albumin to correct for blood aqueous barrier breakdown we calculated that only 0.6% of the locally synthesized IgG was directed against intravitreally injected HSA. Two months after the intravitreal injection of HSA the main signs of the uveitis had subsided. A recurrent uveitis was subsequently induced by an intravenous HSA injection. This resulted in a marked increase of total IgG (14.3 mg/ml) and albumin (24.6 mg/ml) in the aqueous humor of the uveitis eyes. It was remarkable that the mean anti-HSA-IgG level (0.62 mg/ml) in the uveitis eyes was higher than that seen in serum (0.41 mg/ml). After this secondary uveitis, 9% of the locally synthesized antibodies were directed against HSA. The development of uveitis and intraocular antibody production was much milder in “Dutch” strain rabbits than in “Chinchilla” rabbits, which suggests that a genetic background may play an important role in the development of intraocular inflammation. The data obtained in this experimental model show that current methods to assess intraocular antibody production in clinical uveitis have a number of shortcomings. Newer techniques may provide future tools to further develop this powerful diagnostic method.

Uveitis has been the subject of numerous clinical and experimental studies. This interest is justified by the seriousness of this disease with its recurrences in some cases and the difficulty in determining its cause in many. A diagnostic aid in identifying a causative agent is the paired aqueous humor and serum sampling to assess intraocular production of antibodies. This can be determined by calculating the Goldmann-Witmer coefficient, which is based on the comparison of the level of specific antibodies to total immunoglobulin, in both aqueous humor and serum.

Although it is a practical method in certain clinical cases, the validity of the Goldmann-Witmer coefficient has been questioned. During ocular inflammation it is relevant to establish whether the immunoglobulins found in the eye are the result of local production or whether they represent leakage from the circulation through the blood-aqueous barrier. By comparing immunoglobulin levels in aqueous and serum with those of a protein which is not synthesized in the eye, such as albumin, it is theoretically possible to correct for the blood-aqueous leakage.

Little is known concerning the kinetics of local antibody production in the eye during intraocular inflammation. It is also not yet known which part of the antibodies synthesized in the eye are specific for the triggering antigen. To investigate these questions we have employed the experimental uveitis model in rabbits previously described by Silverstein. Using quantitative techniques it was shown that only a small percentage (less than 10%) of the antibodies produced in the eye are directed against the intravitreally injected antigen.

Materials and Methods

Rabbits

Sixteen male rabbits (2.5–3 kg) were used, eight Dutch rabbits (Broekman Institute, Someren, the Netherlands).
Netherlands) and eight Chinchillas (TNO, Zeist, the Netherlands). They formed two groups of eight rabbits. Before experimenting they were checked for general health and preexisting eye diseases. The rabbits were treated according to the ARVO Resolution on the Use of Animals in Research.

Induction of Uveitis

*Antigen:* A sterile, endotoxin-free 20% human serum albumin (HSA) solution (CLB, Amsterdam, the Netherlands) was employed.

*Intravitreal injection:* After anesthesia with Hypnorm (Janssen Pharmaceutica, Beerse, Belgium) i.m. and local tetracaine HCl (2%) eyedrops, the eye was proptosed and a sterile 27-gauge needle (Becton Dickinson, Dublin, Ireland) was inserted at 2 to 3 mm from the corneoscleral junction through the pars plana at an angle slightly backwards. Caution was taken to avoid perforating the posterior lens capsule. The antigen was administered to both eyes (12 mg HSA in 60 μl: Dutch rabbits) or to one eye only with the other eye receiving the same amount of phosphate buffered saline (PBS) (10 mg HSA in 50 μl: Chinchilla rabbits).

*Intravenous injection:* Part of the ear was shaved and disinfected with alcohol. HSA was injected into the marginal ear vein, with a sterile 1 ml tuberculin syringe and a 25-gauge needle, 8 weeks after the intravitreal injection.

Aqueous Humor and Blood Sampling

Aqueous humor and serum samples were taken 12 days prior to the intravitreal injection (pre-uveitis) and 6 weeks after the intravitreal injection (post primary uveitis). Samples of aqueous humor and serum were also taken 7 days after the induction of secondary uveitis. Aqueous humor and blood samples were taken at the same time to determine the concentrations of albumin, total immunoglobulin G and anti-HSA immunoglobulin G. The rabbits were anesthetized with Hypnorm and locally with tetracaine. Corneal paracentesis was performed with a sterile 1 ml tuberculin syringe and a 30-gauge needle. Caution was taken to avoid touching the lens or iris. Approximately 100 μl aqueous humor was aspirated. Blood was obtained from the central ear artery after shaving and disinfection, using a venoject system (Terumo, Leuven, Belgium). Blood was allowed to clot, then centrifuged and the sera distributed over small aliquots. Aqueous humor and serum were stored until use at −20°C.

**Albumin and IgG Quantitation**

The concentrations of albumin and total immunoglobulin G were determined by single radial immuno-
Table 1. Albumin, total IgG and anti-HSA-IgG concentrations in serum and aqueous humor before uveitis and after primary uveitis and secondary uveitis

<table>
<thead>
<tr>
<th></th>
<th>Albumin*</th>
<th>Total IgG</th>
<th>Anti-HSA-IgG</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-uveitis</td>
<td>45.84 (2.81)</td>
<td>4.85 (2.47)</td>
<td>&lt;0.0001</td>
<td>8</td>
</tr>
<tr>
<td>Post primary uveitis</td>
<td>53.22 (8.44)</td>
<td>9.09 (2.77)</td>
<td>0.05 (0.02)</td>
<td>6</td>
</tr>
<tr>
<td>Post secondary uveitis</td>
<td>50.79 (5.65)</td>
<td>20.24 (3.07)</td>
<td>0.41 (0.29)</td>
<td>5</td>
</tr>
<tr>
<td>Aqueous humor, uveitis eyes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-uveitis</td>
<td>0.29 (0.08)</td>
<td>0.03 (0.02)</td>
<td>&lt;0.0001</td>
<td>8</td>
</tr>
<tr>
<td>Post primary uveitis</td>
<td>15.37 (6.2)</td>
<td>4.72 (2.14)</td>
<td>0.03 (0.02)</td>
<td>6</td>
</tr>
<tr>
<td>Post secondary uveitis</td>
<td>24.60 (12.2)</td>
<td>14.34 (7.18)</td>
<td>0.62 (0.53)</td>
<td>5</td>
</tr>
<tr>
<td>Aqueous humor, control eyes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-uveitis</td>
<td>0.26 (0.11)</td>
<td>0.03 (0.01)</td>
<td>&lt;0.0001</td>
<td>8</td>
</tr>
<tr>
<td>Post primary uveitis</td>
<td>0.48 (0.13)</td>
<td>0.12 (0.02)</td>
<td>0.0002 (0.0001)</td>
<td>6</td>
</tr>
<tr>
<td>Post secondary uveitis</td>
<td>0.51 (0.13)</td>
<td>0.26 (0.05)</td>
<td>0.0022 (0.0018)</td>
<td>5</td>
</tr>
</tbody>
</table>

* Data are expressed as mean values (mg/ml) with the standard deviation within the parenthesis.

Calculation of Coefficients

The Goldmann-Witmer coefficient for the determination of anti-HSA-IgG production was calculated using the following formula:

\[
\frac{\text{anti-HSA-IgG-aqueous}}{\text{total IgG aqueous}} : \frac{\text{anti-HSA-IgG-serum}}{\text{total IgG serum}} (1)
\]

Intraocular production of anti HSA IgG using the albumin coefficient was determined with the following formula:

\[
\frac{\text{anti-HSA-IgG-aqueous}}{\text{albumin aqueous}} : \frac{\text{anti-HSA-IgG-serum}}{\text{albumin serum}} (2)
\]

Total IgG production in the eye was calculated with the albumin coefficient as follows:

\[
\frac{\text{total IgG-aqueous}}{\text{albumin aqueous}} : \frac{\text{total IgG serum}}{\text{albumin serum}} (3)
\]

Statistical Analysis

Statistical analysis of the data was performed using a two-sided student t-test.

Results

Primary Uveitis

Uveitis was induced in eight Chinchilla rabbits by an intravitreal injection of 10 mg human serum albumin (HSA) in one eye (uveitis eye). The other eye (control eye) received the same volume (50 μl) of phosphate buffered saline (PBS). All rabbits responded with a severe primary uveitis, which resulted in a phthisis bulbi in two rabbits. These rabbits were excluded from further experimentation due to the fact that aqueous humor sampling became complicated. Five other rabbits developed posterior synechiae and cataract. In one rabbit the uveitis subsided without complications. After 6 weeks the cells and fibrin in the anterior chamber had disappeared. The uveitis was considered to be over after these 6 weeks.

No effect of the intravitreal injection with PBS could be observed in the control eyes of most rabbits. In one rabbit a mild uveitis developed in the control eye which lasted 2 weeks.

Analysis of Aqueous Humor and Serum after Primary Uveitis

Aqueous humor and serum samples were taken 12 days prior to the intravitreal injection (pre-uveitis) and 6 weeks after the intravitreal injection (post primary uveitis). After aqueous humor sampling the eyes showed a mild irritation which was no longer visible after 1 day. All samples were analyzed for total IgG, anti-HSA-IgG and albumin (Table 1). Intravitreal injection of HSA resulted in the appearance of 0.03 mg/ml of anti-HSA antibodies in the aqueous humor of the uveitis eyes as compared to 0.0002 mg/ml in the control eyes (P < 0.01). The level of anti-HSA antibodies in the circulation was 0.052 mg/ml.

After primary uveitis, total IgG was 4.7 mg/ml in the uveitis eyes as compared to 0.1 mg/ml in the control eyes (P < 0.001), while pre-uveitis the aqueous humor contained 0.03 mg/ml total IgG. Of interest was the observation that the intravitreal injection of HSA in the rabbits also resulted in an almost two-fold increase of total serum IgG, whereas serum albumin was not significantly increased. Albumin levels were also markedly increased in the uveitis eyes: 0.5 mg/ml before uveitis and 15.4 mg/ml after primary uveitis (P < 0.001). In the control eyes the albumin level was 0.26 mg/ml pre-uveitis and 0.48 mg/ml after intravitreal injection of PBS.
Table 2. Intraocular production of immunoglobulin G after primary uveitis

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Intraocular production of anti-HSA-IgG</th>
<th>Intraocular production of total IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Goldmann-Witmer coefficient*</td>
<td>Albumin coefficient†</td>
</tr>
<tr>
<td>Uveitis eye</td>
<td>Control eye</td>
<td>Uveitis eye</td>
</tr>
<tr>
<td>1</td>
<td>2.90 0.62</td>
<td>5.31 0.89</td>
</tr>
<tr>
<td>2</td>
<td>0.85 0.25</td>
<td>0.73 0.47</td>
</tr>
<tr>
<td>3</td>
<td>0.56 0.34</td>
<td>0.61 0.33</td>
</tr>
<tr>
<td>4</td>
<td>0.61 0.10</td>
<td>1.25 0.13</td>
</tr>
<tr>
<td>5</td>
<td>0.61 0.24</td>
<td>2.30 0.40</td>
</tr>
<tr>
<td>6</td>
<td>1.28 0.16</td>
<td>3.54 0.46</td>
</tr>
</tbody>
</table>

Mean (SD) 1.14§ (0.91) 0.29 (0.18) 2.29§ (1.85) 0.45 (0.25) 2.06§ (1.08) 1.70 (0.69)

* See equation (1) in "Calculation of Coefficients."
† See equation (2) in "Calculation of Coefficients."
‡ See equation (3) in "Calculation of Coefficients."
§ P < 0.05.
† Not significant.

Local Production of Immunoglobulins after Primary Uveitis

Local antibody production was calculated using three different methods (Table 2). When anti-HSA antibody production was calculated using the Goldmann-Witmer coefficient, a significant difference was observed between uveitis eyes (C = 1.14) and control eyes (C = 0.29). When local anti-HSA antibody production was calculated using albumin to correct for blood-aqueous leakage, a coefficient of 2.29 was seen for the uveitis eyes as compared to 0.45 for the control eyes. Calculation of total intraocular antibody production was performed by comparing total IgG and albumin levels in aqueous humor and serum. For the uveitis eyes this coefficient was 2.06 and for the control eyes 1.70. Pre-uveitis this coefficient calculated for all eyes was 1.45 (SD 0.90, data not shown).

Although the coefficients mentioned above indicate that local antibody synthesis occurs, they do not reflect how much antibody is produced. The exact amount of locally produced antibody was calculated by subtracting serum-derived IgG from the IgG level in aqueous humor. The serum-derived IgG was determined by assuming that IgG and albumin behave identically in their passage through the blood-aqueous barrier.7

The absolute amount of anti-HSA-IgG produced in the uveitis eye was 0.014 mg/ml as compared to undetectable levels in the control eye (Table 3). Total IgG produced in the eyes before intravitreal injection of HSA was less than 0.01 mg/ml. After intravitreal injection the total amount of IgG produced in the uveitis eyes was 2.12 mg/ml as compared to 0.04 mg/ml in the eyes which had received PBS only. These findings show that only 0.6% of the locally synthesized IgG is directed against HSA.

Secondary Uveitis

Two months after the induction of the primary uveitis a secondary uveitis was induced by the intravenous injection of 20 mg HSA. Within 4 hr after the injection signs of recurrent uveitis were present in the previously affected eyes of all rabbits. The uveitis was characterised by conjunctival hyperemia, and flare, cells and fibrin in the anterior chamber. The secondary uveitis was less severe than the primary and started to subside slowly after 2 days. The control eyes only reacted with a mild flare in the aqueous which disappeared after 1 day.

Analysis of Aqueous Humor and Serum after Secondary Uveitis

Samples of aqueous humor and serum were taken 7 days after the induction of the secondary uveitis. From one rabbit no aqueous humor and serum samples could be obtained. The results of the analysis of total IgG, anti-HSA-IgG and albumin are presented in Table 1. Most striking is the enormous increase of anti-HSA-IgG in the aqueous humor of the uveitis eyes (mean 0.62 mg/ml) as compared to that seen after primary uveitis (0.03 mg/ml). It is remarkable that the mean anti-HSA-IgG level in the uveitis eyes was higher than that seen in serum. The anti-HSA-IgG level in the control eyes was also increased but was still very low (0.002 mg/ml).

The secondary uveitis was accompanied by an increase of total IgG and albumin levels in the uveitis eyes (14.34 mg/ml and 24.60 mg/ml, respectively). In the control eyes total IgG was increased two-fold as compared to primary uveitis, whereas albumin levels remained the same. Both albumin and IgG in the uveitis eyes were much higher than in the control eyes.
Table 3. Absolute amount of intraocularly produced IgG in experimental uveitis

<table>
<thead>
<tr>
<th></th>
<th>Pre-uveitis</th>
<th>Post primary uveitis</th>
<th>Post secondary uveitis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uveitis eye</td>
<td>Control eye</td>
<td>Uveitis eye</td>
</tr>
<tr>
<td></td>
<td>0.014 (0.024)</td>
<td>-0.0003 (0.0003)</td>
<td>0.42 (0.38)</td>
</tr>
<tr>
<td>Anti-HSA-IgG*</td>
<td>-</td>
<td>-</td>
<td>2.12 (2.13)</td>
</tr>
<tr>
<td>Total IgG</td>
<td>-0.0003 (0.0003)</td>
<td>-0.002 (0.001)</td>
<td>4.67 (4.45)</td>
</tr>
</tbody>
</table>

Mean (SD), concentrations in mg/ml.

* aHSA-IgG (AH) = (alb (AH)/alb (Se) x aHSA-IgG (Se)).
† Total IgG (AH) = (alb (AH)/alb (Se) x total IgG (Se)).

Local Production of Immunoglobulins after Secondary Uveitis

Table 4 presents the determinations of the Goldmann-Witmer coefficient, the albumin coefficient for local anti-HSA-IgG production and the albumin coefficient for total IgG production after secondary uveitis. The two coefficients for local anti-HSA-IgG production both showed a difference between uveitis and control eyes, while no difference was observed for the coefficient for total IgG production. This latter finding may be explained by the fact that IgG production occurs both in the uveitis as well as in the control eyes. Although the absolute amount of IgG produced in the uveitis eyes is much larger than that seen in the control eyes (Table 3), this difference is masked when calculating the albumin coefficient. For the uveitis eyes both the Goldmann-Witmer coefficient (C = 2.47) and the albumin coefficient for local anti-HSA-IgG production (C = 3.57) were higher after secondary uveitis than primary uveitis.

The absolute amount of intraocular produced IgG was calculated as before (Table 3). After secondary uveitis 0.42 mg/ml anti-HSA-IgG was produced in the uveitis eye as compared to negative results in the control eye. The anti-HSA-IgG production in the uveitis eye was significantly higher after secondary uveitis than after primary uveitis. Intraocular production of total IgG was 4.67 mg/ml in the uveitis eye and 0.07 mg/ml in the control eye. Secondary uveitis resulted in an increase of total IgG production in the uveitis eye while in the control eye no significant increase was observed. These data show that during secondary uveitis 9% of the locally synthesized IgG is directed against HSA.

Primary Uveitis in Dutch Rabbits

Eight Dutch rabbits received 12 mg HSA in the vitreous of both eyes. All rabbits responded with a uveitis, varying from mild to severe, which developed after 8 days. Much lower levels of anti-HSA-IgG were found in the eyes of these rabbits (0.0006 mg/ml) as compared to the Chinchilla rabbits (0.03 mg/ml) 2 months after the intravitreal injection of HSA. Both total IgG and albumin levels in the aqueous of the Dutch rabbits were also much lower as compared to the Chinchilla rabbits. These findings are in agreement with the observation that the uveitis in the Dutch rabbits developed later, after intravitreal HSA injection, and was much milder than that seen in the Chinchillas.

Discussion

The study presented here shows that intravitreal injection of an antigen induces a marked local anti-
body synthesis in the rabbit eye. Several authors have already described the appearance of antibodies in the aqueous humor of the rabbit after intravitreal injection of small amounts of a foreign protein. In agreement with others, we also found that rabbits vary considerably in their response to intravitreal injection of a foreign protein. Clinical uveitis in the animals correlated with their immune response to the injected antigen as measured by local antibody production.

Most investigators have employed indirect techniques focused on the antibody-producing cells or semiquantitative methods to measure antibody titers. In our study sensitive quantitative techniques were used to investigate antibody production in the eye. Only a small portion (less than 10%) of the synthesized antibodies were directed against the antigen employed to induce the uveitis. These data are in agreement with earlier histological studies by Silverstein and coworkers showing that less than 10 to 20% of the plasma cells in the uveal tissue were specific for the antigen employed to induce the experimental uveitis.

The percentage of specific antibodies was very low (0.6%) in the aqueous after primary uveitis but increased to 9% after recurrence of the uveitis. The low levels of total IgG and antibody observed after primary uveitis could be due to the large time interval (6 weeks) between the intravitreal injection of the antigen and aqueous sampling. In an earlier study, Denouchamps showed that the peak of intraocular antibody production occurred 3 to 4 weeks after intravitreal antigen injection, after which antibody synthesis rapidly dropped. After a recurrence of the uveitis was induced by an intravenous antigen injection, the percentage of specific antibodies in our experiments increased to 9%. It is possible that renewed parenteral administration of the antigen might even lead to a higher specific response. It is obvious, however, that during this experimental uveitis in the rabbit, the inflammatory response is accompanied by a marked polyclonal antibody synthesis. If similar mechanisms are involved during uveitis episodes in humans, the analysis and evaluation of local antibody synthesis for clinical purposes should be reconsidered.

Most clinical uveitis studies in which local antibody production is determined to confirm a diagnosis make use of the Goldmann-Witmer coefficient calculation. This method is based on the comparison of the level of specific antibodies to local immunoglobulins in both aqueous humor and serum. If polyclonal antibody production also occurs during clinical uveitis, this would lead to false positive results, but also to false negative results.

The occurrence of a false negative result was encountered in our experiments, whereby very low Goldmann-Witmer coefficients for the anti-human serum albumin response were observed after primary uveitis due to the relatively large amount (99%) of nonspecific antibodies produced in the eye, leading to an overcorrection of blood-aqueous barrier leakage.

Another great disadvantage of expressing local antibody synthesis with the Goldmann-Witmer coefficient is the fact that this number gives no information on the absolute amounts of specific antibodies synthesized.

Whether the experimental uveitis model in rabbits is an adequate model to study ocular antibody synthesis is a matter of debate. There are a number of differences between the aqueous humor protein content of rabbits and humans during uveitis. First of all, the sieving function of the blood-aqueous barrier is completely disrupted during experimental uveitis in rabbits; there is no selective passage of small proteins (albumin) as compared to larger ones (IgG). In humans, on the other hand, permeability of the blood-aqueous barrier is increased during uveitis, but the molecular sieve properties are still maintained.

Intraocular antibody synthesis in the rabbit exceeds that seen in clinical uveitis many-fold. Whereas total protein levels in the aqueous of uveitis patients rarely exceeds a value of 10 mg/ml, the aqueous of rabbits with uveitis may contain between 20 to 30 mg/ml of protein. The extreme breakdown of the blood-aqueous barrier in the rabbit during inflammatory conditions is well known. The polyclonal antibody synthesis induced during a uveitis episode may represent a prophylactic intraocular defense mechanism against further external ocular attacks in this species. The extreme results obtained in this experimental model, however, do show that current methods to analyze local antibody production in uveitis patients have a number of shortcomings. Newer immunochemical techniques may provide future tools to further develop this powerful diagnostic method.

Key words: uveitis, antibody synthesis, rabbit, blood-aqueous barrier

References

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