The Significance of Complement in Proliferative Vitreoretinopathy

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Complement is the principal effector arm of antibody-mediated allergic response and plays a central role in the pathogenesis of many immunologic disorders. The possible pathophysiologic importance of complement was examined in the development of proliferative vitreoretinopathy (PVR). Vitreous aspirates from patients with idiopathic PVR (n = 21) and traumatic PVR (n = 15) were examined for total vitreal protein (TVP) and complement components C3, C3d, C4, and C1q-fixed immunoglobulins using enzyme-linked immunosorbent assay (ELISA), sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and western blotting. The TVP, C3 components, and factor C4 were elevated significantly in diseased vitreous. The C3-TVP and C4-TVP ratios showed no difference between traumatic and idiopathic PVR. A C4 index to estimate the rate of intraocular C4 synthesis had a mean value of 3.2 (n = 15). The increased relative amount of C3d reflected complement activation in diseased vitreous. The negative values in normal human serum and plasma and in patient plasma samples (n = 15) indicated a local reaction in the eye. The authors found C1q-fixed immunoglobulin G; this may be the cause of complement activation by the classic pathway. These findings support the opinion that the cause of PVR may be based partly on an autoimmune reaction against ocular structures. Invest Ophthalmol Vis Sci 32:2711-2717, 1991

The pathogenesis of proliferative vitreoretinopathy (PVR) is characterized by cell dispersion into the vitreal cavity and the development of periretinal membranes followed by traction retinal detachment.2 The initiation of this pathologic process seems to be the traumatic or surgical alteration of blood–ocular barriers. If the disease follows rhegmatogenous detachment, we call it idiopathic PVR. The development of traction retinal detachment probably is caused by a regenerative cellular activity related to the proliferative and fibroblastic phase of physiologic wound healing. That the eye has potential autoantigens, such as proteins from the lens, uvea, and retina and that previous studies support an immunologic promotion of PVR could indicate the existence of a morphologic substrate in the diseased eye that may come into contact with the immune organs and induce the formation of autoantibodies. Such antibodies would form an immune complex with the specific antigen. The complex can incite tissue damage by virtue of its ability to trigger the activation of comple-

The activation of the complement system, which consists of a group of high molecular weight proteins (β1- and β2-globulin fraction), is amplified by a cascade phenomenon, ie, the product of one reaction is the catalyst for the next. This system can be activated by two convergent pathways: classic and alternative. The promotion of the classic pathway is associated with immune complexes and their capacity to bind the subcomponent C1q which associates with the proenzymes C1r and C1s to yield C1. The alternative pathway activation is primarily antibody independent. Both pathways culminate in the cleavage of C3 and trigger the common pathway (C5-C9), leading to the formation of the “membrane attack complex,” a self-associating multimolecular complex that acquires the capacity to attach to and subsequently lyse membranes.6

**Materials and Methods**

**Samples**

Central vitreous from donor eyes for keratoplasty was aspirated within 6 hr of death and stored immediately at −20°C. Vitreous aspirates for the determination of complement components were obtained from patients with idiopathic (n = 21) and traumatic PVR (n = 15) by needle aspiration from the center of the vitreal cavity before vitrectomy. The time between
trauma and surgery in traumatic PVR samples ranged from 1–6 months in most cases. Pooled human serum (catalog number S-2257; Sigma, Deisenhoven, Germany) and human plasma of individuals without recognizable systemic diseases were tested for normal concentrations of complement components. Preoperative (1 day before surgery) plasma of patients with idiopathic (n = 13) and traumatic (n = 2) PVR also was tested.

Buffers and Reagents

The following buffers and solutions were used. Enzyme-linked immunosorbent assay (ELISA) coating buffer contained 50 mM sodium carbonate and 0.02% sodium azide, pH 9.6. We also used phosphate-buffered saline (PBS, pH 7.3), PBS-Tween containing 0.1% (ELISA) or 0.05% (blot-staining) Tween20 (37470; Serva, Heidelberg, Germany), substrate buffer for ELISA (415286; Boehringer Mannheim, Mannheim, Germany), and polyethylene glycol 6000 (3074919; Serva).

ELISA

The ELISA was done as described previously. Optical densities were read with a micro-ELISA reader (MR 5000; Dynatech, Denkendorf, Germany) with a dual wavelength mode (test filter, 405 nm; reference filter, 450 nm).

C3 measurements: To obtain a general view of the participation of the complement system in idiopathic and traumatic PVR, we chose a double-sandwich ELISA composed of a polyclonal antibody against the intact C3 molecule and a polyclonal antiserum against the C3c determinant found on the native C3 component and the split products C3b, C3bi, and C3c but not on the fragments C3a and C3d.

Micro-ELISA plates (M124A; Dynatech) were coated with monospecific anti-human complement C3c (1:1000, C-6025; Sigma). Standards and samples were diluted serially in triplicate over rows of eight wells in steps of 1:2. The top of the ELISA sandwich consisted of anti-human complement C3 (C-7761; Sigma) diluted 1:2000. Further labeling was achieved by the use of anti-goat immunoglobulin G (whole molecule) conjugated with alkaline phosphatase (AP, 1:1000, A-7650; Sigma). The activity of AP was determined by enzymatic conversion of p-nitrophenyl phosphate (905 VV516050; Merck, Darmstadt, Germany) diluted 1:1000 in substrate buffer. This also measured C3 split products and therefore reflected the presence of the C3 components independently of degradation of the molecule. The human complement C3 standard used to plot a standard curve was measured at the initial dilution of 1:400 (0.625 μg/ml) to a final dilution of 1:51,200 in eight double steps.

C3d measurements: We regarded the C3d level in plasma as the complement activation indicator of choice, and we used an antibody-capture ELISA to estimate its relative amounts in idiopathic PVR, traumatic PVR, and controls. We used a monoclonal antibody (24.132.76; Janssen, Beerse, Belgium) that recognizes an epitope localized on C3b, C3bi, and C3d but not on native C3 (1:1000). To intensify the reaction a biotin–streptavidin system was used, consisting of anti-mouse immunoglobulin (1:1000, E 418 01; Dakopatts, Hamburg, Germany) and streptavidin-AP (1:500, D 396 21; Dakopatts). Controls in human serum and plasma resulted in normal concentrations. To get a standard curve, the C3d standard (C3035; Sigma) was diluted from 1:500 (2 μg/ml) to 1:64,000 in eight double steps.

C1q-binding assay: The C1q-binding assay was based on the complement-binding property of immune complexes. It is generally agreed that the anti-
body component must consist of an immunoglobulin G or M subclass to activate the complement system by the classic pathway. To detect immune complexes fixed by the Clq component, we used a double-sandwich ELISA that detects the Clq attached to the immune complex and the immunoglobulin component. Micro-ELISA plates were coated with a monoclonal goat-derived antibody against human complement Clq (1:1000, C-3900; Sigma). After incubation with vitreous samples, we used a goat-derived anti-human immunoglobulin G (γ-chain specific) AP conjugate (1:1000, A-5403; Sigma) for immunoglobulin G detection. For immunoglobulin M detection, biotinylated anti-human immunoglobulin M from rabbits (1:1000, A 425 01; Dakopatts) and streptavidin-AP (1:500, D 396 21; Dakopatts) were used.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

The specificity of the primary antibodies against C3, C3c, C3d, and C4 was confirmed by SDS-PAGE of human plasma complement components and subsequent western blot analysis. These techniques also were used to examine normal and diseased vitreous.

Protein Measurements

Total vitreal protein (TVP) was determined according to Bradford’s method.

Controls and Statistical Analysis

Blanks in double-sandwich assays consisted of wells containing the first antibody, second antibody, labeled antibody, and substrate but not the test sample. Blanks in antibody-capture assays consisted of wells having the first antibody, labeled antibodies, and substrate but no test sample. The values were recorded as the mean of three test samples minus the mean of three blanks. Statistical analysis was done using the Mann-Whitney U test.

Results

Evaluation of C3 Components

The levels of C3 components in control samples and pathologic vitreous are presented in Table 1 and Figure 2. Compared with the control samples, the levels of C3 components in traumatic and idiopathic PVR were increased markedly. Statistical analysis did not show any difference between the two proliferative disorders with regard to the central tendency.

Evaluation of C4 Component

Similar to the C3 measurements, the concentration of C4 was elevated in pathologic vitreous compared with physiologic samples (Table 1, Fig. 2). Here, too, no significant difference between the two proliferative disorders could be found.

Evaluation of TVP and Percentages of C4 and C3 Components

Compared with physiologic human vitreous, both proliferative disorders had higher levels of TVP and complement percentages (Table 1). Significant differences between traumatic and idiopathic PVR were not observed.

C4 Index

We calculated a C4 index analogous to the immunoglobulin G index of routine cerebrospinal fluid chemistry using plasma samples, obtained 1 day before surgery, from patients:

| Table 1. (A) Mean values and spread for C3 components, factor C4, and total vitreal protein (TVP) in physiologic and pathologic human vitreous; (B) mean values and spread for percentages of C4 and C3 components of total vitreal protein in traumatic and idiopathic PVR |
|---------------------------------|--------------------|--------------------|--------------------|
|                                 | Physiologic        | Traumatic PVR       | Idiopathic PVR      |
| A                               |                    |                    |                    |
| C3 components (µg/ml)            | 5.8 (n = 13)       | 315.2 (n = 14)     | 330.7 (n = 21)     |
| C4 component (µg/ml)             |                    |                    |                    |
| TVP (µg/ml)                      | 1040 (n = 8)       | 6800 (n = 15)      | 8900 (n = 20)      |
|                                | (350–2100)         | (1160–27000)       | (380–28400)        |
| B                               |                    |                    |                    |
| Percentage of C3 components      | 0.7% (n = 10)      | 3.5% (n = 14)      | 3.4% (n = 20)      |
|                                | (0.2–1.9)          | (0.6–5.5)          | (0.3–11.9)         |
| Percentage of C4 component      | 0.7% (n = 9)       | 2.0% (n = 13)      | 2.5% (n = 19)      |
|                                | (0.1–2.1)          | (0.6–3.0)          | (0.1–6.5)          |
The C4 index levels of PVR patients ranged from 0.39–9.37 (mean, 3.2; median, 2.0; n = 15). Because the physiologic vitreous was taken from donor eyes for keratoplasty, we could not obtain plasma samples from these same persons. For the calculation of the C4 index, we therefore measured vitreal C4 and TVP levels and took the normal plasma levels from the literature. We used the lowest normal C4 level (0.2 g/l) and the highest normal protein level (80 g/l) to prevent overestimation of the significance of our findings. The C4 index for physiologic eyes ranged from 0.29–8.1 (mean, 2.8; median, 2.3; n = 9).

Relative Amount of C3 Fragment C3d

The relative amount of the C3 split product C3d in pathologic vitreous was elevated markedly. Only 1 of 10 eyes with traumatic PVR and 6 of 18 eyes with idiopathic PVR were negative at the lowest assay dilution (1:40). Negative results were found in seven of nine physiologic vitreous samples (Fig. 3), in standard serum (Sigma), and in normal human plasma. The examination of 15 vitreous aspirates and the preoperative (1 day before surgery) plasma samples of the same patients showed a C3d elevation only in the vitreous in contrast to negative results in the plasma.

Detection of C1q-Fixed Immunoglobulins

Figure 4 shows the presence of C1q-fixed immunoglobulin G in idiopathic and traumatic PVR vitreous samples. The C1q-fixed immunoglobulin M was negative both for physiologic vitreous and patients with PVR.

Correlation Between C3d and C1q-Fixed Immunoglobulin G Concentration

Figure 5 presents the amount of C3d in relation to C1q-fixed immunoglobulin G. High levels of C1q immunoglobulin G correlated with a high value of C3d component.
Discussion

The pathogenesis of PVR is the focus of attention of many reports and has many unanswered questions. We wanted to concentrate our attention on the question whether the morphologic and biochemical changes in vitreous were related to a disruption of the blood–ocular barrier or to another pathologic mechanism.

The significantly elevated amount of TVP in patients with PVR (Table 1) and the levels of complement components rising together with the vitreal protein may be the consequence of a perioperative breakdown of the blood–ocular barrier. Our examination of C3–TVP and C4–TVP ratios showed no difference between the percentages of complement components in idiopathic and traumatic PVR. This suggested that there was no different pathologic mechanism of these disorders with regard to the alteration of the barrier. Nevertheless we introduced the C4 index to estimate the importance of the disruption of the blood–ocular barrier. We used it similarly to the immunoglobulin G index of routine cerebrospinal fluid chemistry, which is employed to determine intrathecal immunoglobulin G synthesis and is similar to the Goldmann-Witmer coefficient. It was considered to indicate local production of antibodies in the aqueous humor. Theoretically a simple alteration of the blood–ocular barrier can lead to values below or equal to 1. Values greater than 1 suggest a selective dysfunction of the barrier and/or a significant local intraocular synthesis of substances. The examination of 15 patients with idiopathic and traumatic PVR showed a C4 index with a mean value of 3.2. This value was similar to the mean value of 2.8 in physiologic vitreous. Thus we could not show a specific elevation of C4. However, the existence of elevated complement factors in pathologic specimens does not prove the participation of the complement system in proliferative ocular disorders. Therefore, we examined the activation of the complement cascade by determining C3d, the most sensitive and reliable indicator of activation, both during acute and chronic complement activation. The higher C3d levels in pathologic vitreous gave unequivocal evidence of complement activation. Furthermore we found negative results in normal human plasma and serum and in preoperative plasma of the same patients, suggesting that the C3d component in the vitreous cavity was not the consequence of a dysfunction of the blood–ocular barrier, but rather was the result of a local reaction.

The activated complement system can induce an acute inflammatory response; anaphylatoxins like C3a formed during complement activation can alter vessel permeability, produce edema, stimulate influx of leukocytes and macrophages, and facilitate phagocytosis and the release of secondary mediators such as thromboxane, histamine, and interleukin-1. These biologic effects may show a significant role for the complement system at least in the initial phase of PVR. Whether this activation of the system is a normal and physiologic part of an inflammatory process, possibly caused by surgical intervention, or the consequence of a more specific promoter is controversial. A specific
trigger is the humoral sensitization of the complement system.

Some studies reporting elevated levels of immunoglobulin G in vitreous, large amounts of immunoglobulins and complement factors in epiretinal membranes, complement and immunoglobulin G deposits underlying the basal pole of pigment epithelium in pars plana specimens, the presence of lymphocytes and macrophages, and abnormal expression of human leukocyte locus A system antigens on pigment and nonpigment epithelial cells suggest that the immune system plays a role in the progression of PVR.

The complement cascade is a coherent functional system. We therefore examined whether the activation of the C3 component and the common pathway is the result of the activated classic pathway. These studies were based on examining complement factors C4 and C1q, both characteristic for the classic pathway.

The evaluation of the C4 component did not give a clear reflection of the activation of the complement system by the classic pathway. Normally the complement activation leads to a consumption of native complement factors and production of split products, but because of the behavior of the complement factors as acute-phase reactants, their levels can be normal or increased. Thus an increased synthesis, e.g., in liver cells or macrophages, compensates for the accelerated catabolism of these factors. Furthermore, the extravascular localization of the vitreous enables it to be in equilibrium with the intravascular compartment. This explains why our levels do not reflect reliably complement consumption with a concomitant decrease of native C4.

Our examination of the complement component C1q was not based on the quantitation but was a practical application of the singularity of this factor. It is the first component of the classic pathway and, therefore, involved in the recognition and binding of activators. Our C1q-binding assay found C1q-fixed immunoglobulin G; this may be the activator. Handling and storage procedures may lead to false C1q-fixed immune complex results as a consequence of immunoglobulin G aggregation. Because these aggregates cannot be distinguished from “real” immune complexes, false-positive results may be obtained. Our examination of our findings with regard to the storage time of the samples showed an outcome independent of this factor (unpublished results).

These results and the correlation between relative C3d and C1q-fixed immunoglobulin G increasing together (Fig. 5) support our opinion that the C1q-fixed immunoglobulin G could be the antibody component of an antibody–antigen aggregate that triggers complement activation and causes progression of PVR. Finally, the detection of serum antibodies to retinal structures in experimentally induced PVR and in 60% of patients with retinal detachment, the demonstration of serum antibodies against vitreal antigens, and reports suggesting cellular immunity in PVR development caused us to investigate this question further. These studies, currently in progress, are concerned with analyzing C1q-fixed immunoglobulin G and its probable function as an antibody.

Key words: traumatic PVR, idiopathic PVR, complement factors, autoimmunity, C1q-fixed immunoglobulin G

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References

13. Perrin LH, Lambert PH, and Miescher PH: Complement breakdown products in plasma from patients with membrano-


