Human Corneal Extract Enhances Serum Complement Activity

Cora Verhagen,* Rick Hoekzema,† and Aize Kijlstra*†

**Purpose.** The concomitant presence of complement-activation products in the cornea during inflammation is well described. The present study was undertaken to analyze the complement activity of normal human corneal tissue and to assess the presence of complement-modulating activities.

**Methods.** Human corneal tissue was extracted in veronal-buffered saline. The overall complement (C) activity of the human corneal extract (HCE) and the effect of HCE on serum C-activity were investigated using a hemolytic assay. Anion-exchange chromatography and gel filtration were applied for biochemical analysis of HCE. Monoclonal anti-human C3 was used to detect corneal C3 and to remove C3 from HCE by immunoadsorption.

**Results.** It was found that C-activity of HCE was less than 200 U/g tissue. Experiments to test whether HCE exhibited inhibitory activity led to an unexpected result: When added to human serum dilutions, HCE caused a significant, dose-dependent increase of C-activity. Pretreatment of HCE at 56°C abolished the effect. Analysis of HCE by anion-exchange chromatography revealed two C-enhancing peaks. One peak was identified as C3 whereas the identity of the other protein peak remained unknown.

**Conclusions.** Results indicate that the human cornea contains an as yet unidentified heat sensitive factor(s) able to enhance complement activity of serum. It is postulated that this factor(s) may play an important role in corneal physiology and pathology.

The complement (C) system is a key element of our immunologic defense mechanism. Activation by immune complexes and bacterial products makes the C system part of the adaptive as well as the innate immune system. The cornea as a site for complement activation has been a subject of controversy. For a long time, it was thought that the avascularity of the cornea precluded the development of an Arthus reaction, i.e., deposition of immune complexes and complement-mediated inflammation.1 However, a large number of studies have furnished evidence that the complement system may be involved in the immunologic defense mechanisms of the cornea. Early studies by Germuth and colleagues suggested that antigen-antibody-complement complexes might be involved in corneal injury.2,3 More recently, several reports by Mondino and associates have demonstrated that extracts of normal corneal tissue contain substantial amounts of C1 to C7 and properdin.4-6 The concentration of C components in the cornea appeared to be dependent on the molecular weight of the proteins,5 and for each component a limbus-to-center gradient of decreasing concentration was observed.6 The presence of complement components in normal corneal tissue suggests a possible role in immunologic defense mechanisms of the cornea. Keratitis caused by fungi or gram-negative bacteria in man or rabbits is thought to be associated with complement activation.7,8 In fact, complement depositions in the human and rabbit cornea have been demonstrated in these types of keratitis.9-11 In addition, complement activation products can be demonstrated at the site of precipitated immune complexes in the rat cornea.12 In other animal models, it has been shown that intracorneally injected C5a is a potent inducer of keratitis.13

In this study, human corneal extract (HCE) was analyzed for the presence of complement (-associated) activity. Although convincing evidence exists implicat-
ing the cornea as an extravascular site of C proteins, little information is available on the functionality of the corneal complement system. The hemolytic activity of several individual corneal complement proteins has been demonstrated. A recent study by Pleyer et al demonstrated hemolytic complement levels in guinea pig corneas. The question that remained is, does HCE cause cytolysis by itself, or, to put it differently, does the cornea contain a functional complement system? Furthermore, because the cornea contains the C-regulatory proteins C1 inhibitor, C3 inactivator (factor 1), and beta-1H, it was of interest to know if corneal extract exhibits inhibitory activity. The present study was undertaken to address these questions. Results demonstrate that HCE significantly augments the hemolytic activity of serum, whereas HCE alone causes little hemolysis.

MATERIALS AND METHODS

Human Corneal Extract Preparations

Human eyes were obtained from Eurotransplant and processed within 24 hours of death at the Eye Bank of the Netherlands Ophthalmic Research Institute. The donated corneas had no history of disease but were found unacceptable for transplantation because of senile changes of the endothelium. Corneas were excised using an 11-mm trephine, minced and placed in a preweighed test tube containing either 500 µl GVB°, isotonic veronal (5 mM) buffered saline (150 mM), pH 7.4 containing 0.1% gelatin, or GVB° containing 0.1 mM ethylenediamine tetra-acetic acid (GVB°-EDTA). Tubes were reweighed to determine the net weight of the corneal tissue, and tissue was incubated for 48 hours at 4°C by rotation. Next, tubes were centrifuged (30 minutes, 14,000g), and supernatant (HCE) was collected and stored at −70°C. Protein concentrations were measured with folin phenol reagent, using dilutions of bovine serum albumin (Sigma Chemical Company, St. Louis, MO) as references (0.25 to 2.0 mg/ml).

An HCE preparation free of complement component C3 was prepared by immunoadsorption. One hundred µg mouse IgG1 monoclonal antibody against human C3 was coupled covalently to 5 mg CNBr-activated sepharose according to the manufacturer’s instructions (Pharmacia LKB Biotechnology, Woerden, The Netherlands). Sepharose beads coupled to a nonrelevant mouse IgG1 monoclonal antibody (anti-human interleukin 2-receptor; Dakopatts, Glostrup, Denmark) or incubated in all media except monoclonal antibody served as negative controls. HCE (0.5 ml; 2 mg protein/ml) was incubated for 16 hours at 4°C with the sepharose beads by shaking. Beads were removed by centrifugation.

The effectivity of the immunoadsorption on HCE C3-levels was evaluated by means of an anti-human C3 enzyme-linked immunoadsorbent assay (ELISA). Serial dilutions of immunoadsorbed HCE in phosphate (10 mM) buffered saline (150 mM) (PBS), pH 7.4, were coated to microtiter ELISA plates (655101, Greiner, Nürtingen, Germany). The plates were incubated for 2 hours at room temperature, washed three times with PBS containing 0.1% (v/v) Tween 20 (PBS-Tw), and subsequently incubated for 2 hours at room temperature with 100 µl of diluted monoclonal mouse anti-human C3 (1:1000 in PBS-Tw). Next, the plates were rinsed three times in PBS-Tw and incubated for 1 hour at room temperature with 100 µl peroxidase-conjugated rabbit anti-mouse IgG (Dakopatts), diluted 1:2000 in PBS-Tw, containing 0.01% (w/v) human IgG. Bound antibodies were visualized using a mixture of 0.4 mM tetramethylbenzidine and 0.03% hydrogen peroxide in 0.1 M sodium acetate buffer, pH 5.5 as chromogenic substrate. The absorbance was read at 450 nm (E 450).

Chromatography of HCE

Corneal extracts were analyzed by gel filtration using Sephacryl S-300 High Resolution (Pharmacia). The gel filtration column (1.7 × 46 cm) was equilibrated in GVB°-EDTA, and 2 ml HCE (2.0 mg/ml) was applied. Separation was carried out at room temperature, with a flow rate of 2.5 ml/cm² · hr⁻¹. The absorbance was read at 280 nm (E 280), and 1.5 ml fractions were collected.

A Protein Pak, DEAE 5PW high-performance liquid chromatography (HPLC) column (Waters, Etten-Leur, The Netherlands) was used to separate HCE based on charge differences. The column was equilibrated in 10 mM Tris/HCL, pH 7.5. Two ml HCE (2.0 mg/ml) was diluted 1:10 in 10 mM Tris/HCl, pH 7.5, and loaded onto the column. Proteins were eluted with a linear salt gradient of 0 to 500 mM NaCl in 10 mM Tris/HCl, pH 7.5. The absorbance was read at 280 nm (E 280), and 1.5-m1 fractions were collected. The DEAE column was also run in a linear salt gradient (0 to 500 mM NaCl) without proteins to study the effect of increasing amounts of salt on hemolysis.

Single fractions obtained after gel filtration and anion-exchange chromatography were tested for C-enhancing activity in the below described hemolytic complement assay, using 1:300 diluted normal human serum (NHS). The presence of C3 was assessed by the anti-human C3 ELISA, as described. In addition, fractions obtained by gel filtration were assessed for the presence of human serum albumin and human-IgG by radial immunodiffusion using polyclonal rabbit antibodies. A human standard serum, based on relevant World Health Organization International Standards, was included in each assay to calculate concentrations.
Hemolytic Complement Assay

Normal human serum, stored at −70°C within 2 hours of sampling, was used as a complement source. Overall complement activity (CH50) was determined using the colorimetric assay described by Klerx and colleagues18 with minor alterations. Sheep erythrocytes (National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands) were washed three times in PBS and resuspended (2 × 10^8/ml) in DGVB²⁺; half isotonic veronal buffered saline, pH 7.4, containing 0.05% gelatin, 1.5% D-glucose, 0.5 mM Mg²⁺, and 0.15 mM Ca²⁺. Cells were then incubated with hemolytic amboceptor (final dilution, 1:500) for 30 minutes at 37°C and subsequently put on ice for 30 minutes. The sensitized sheep erythrocytes (ShEA) were again washed, resuspended in DGVB²⁺ (2 × 10^8 cells/ml), and kept on ice. Duplicate serial dilutions of the samples were made in round-bottomed microtiter plates (no. 650160, Greiner). Then 50 µl ShEA was added to each well (final volume, 150 µl). The plates were covered with tape and incubated for 60 minutes at 37°C in a water bath. The cells were spun down (8 minutes, 600g), and 50 µl of the supernatant from each well was transferred to a flat-bottomed microtiter plate (no. 655101, Greiner). Distilled water (200 µl) was added to these wells, and the E₄₀₅ was read. The percentage of lysis (Y) was calculated using the following equation:

\[ Y = \frac{E(I) - E(0\%)}{E(100\%) - E(0\%)} \times 100\% \]

in which I refers to the average E₄₀₅ value of the test sample. The 0% lysis control was obtained by incubating ShEA with 100 µl DGVB²⁺, and the 100% lysis control was obtained by incubating ShEA with 100 µl distilled water. To calculate the CH50, sample dilutions were plotted logarithmically against log

\[ \frac{Y}{100 - Y} \]

The amount of sample giving 50% lysis

(1 unit: log \( \frac{Y}{100 - Y} = 0 \))

was read from the curve obtained, i.e., the x-axis intercept. Finally, overall complement activity (CH50) was expressed as U/ml serum or U/g tissue.

The overall C-activity of corneal tissue (extracted in GVB⁹) was determined by incubating graded amounts of HCE with ShEA as described. Mg²⁺ and Ca²⁺ were added to a final concentration of 0.5 mM and 0.15 mM, respectively, in each well. To assess C-enhancing activity, a fixed amount of either heat-treated HCE (30 minutes, 56°C) or untreated HCE was added to a serial dilution of NHS. A dose-response curve of the C-enhancing effect of HCE was obtained by incubating graded amounts of HCE with NHS diluted 1:500 in DGVB²⁺. Fractions obtained by gel filtration and anion-exchange chromatography were mixed with an equal volume 3% D-glucose, 1.0 mM Mg²⁺, and 0.4 mM Ca²⁺. Normal human serum was added to a final concentration of 1:200 or 1:300. Serial dilutions of these preparations, made in DGVB²⁺ containing 1:200 or 1:300 diluted NHS, were then tested in the hemolytic complement assay.

RESULTS

The initial objective of this study was to assess overall complement activity of HCE. When varying doses of HCE were incubated with ShEA, hemolysis was observed only at high concentrations (50 µg HCE/well). The percentage of lysis did not exceed 15%, which precluded calculation of the CH50. Thus, the overall hemolytic activity of HCE was below the detection level (200 U/g corneal tissue) of our assay.

Another potential characteristic of HCE, the ability to inhibit complement-mediated cell lysis, was studied next. This was done by adding HCE to a titration of human serum. The results of the hemolytic complement assay are presented in Figure 1. Instead of inhibition, enhancement of serum C-activity was observed. The serum CH50 increased from 2280 to 8040 U/ml. This threefold increase of the CH50 was observed repeatedly and on separate occasions using different batches of HCE (n > 5). The same results were obtained when rat corneal extract was incubated with normal rat serum (data not shown). Incubation of the HCE for 30 minutes at 56°C before adding to NHS, abrogated the enhancing effect (Fig. 1). In the next series of experiments, the serum CH50 was determined after adding increasing amounts of HCE to 1:200 diluted NHS. The C-enhancing effect was found to be dose dependent (Fig. 2). The addition of 1 µg HCE per well to 1:200 diluted NHS was still able to enhance serum C-activity.

To analyze the C-enhancing activity in more detail, HCE was submitted to gel filtration and anion-exchange chromatography. After HCE components were separated by gel filtration, fractions were assessed for C-enhancing activity and the presence of C3 (Fig. 3). In addition, the albumin and IgG concentration of each fraction was determined. The hemolytic profile showed that fractions 20 to 32 had a mild inhibitory effect on the hemolytic activity, while fractions 33 to 37 displayed C-enhancing activity. The anti-human C3 ELISA demonstrated that C3 was present in fractions 32 to 35. The highest concentration of IgG
Human Corneal Complement

**FIGURE 1.** The effect of HCE on human serum complement activity. A serial dilution of NHS was incubated in the absence (squares) or presence (circles) of 50 μg/well HCE. In addition, NHS was incubated with heat-treated (30 minutes, 56°C) HCE (triangles). The CH50 was calculated as described in Materials and Methods.

and albumin was found in fractions 35 and 38, respectively. When comparing these results, it was observed that the peaks of C-enhancement and C3 overlapped but were not superimposed. Maximum C-enhancement was observed in fraction 35 whereas, according to the ELISA, the highest C3 level was found in fraction 34. The same results were obtained when the separation procedure was repeated. These results suggest that C3 could in part be responsible for the C-enhancing activity of HCE. However, the observation that maximum C-enhancement coeluted with IgG suggests that a molecule of 150 kDa might be responsible. It was therefore decided to separate HCE components by other means. Human corneal extract fractions obtained by anion-exchange chromatography gave a different hemolytic profile (Fig. 4). Two peaks of C-enhancing activity were observed, present in fractions 17 to 19 and 25 to 27, respectively. The second peak corresponded to C3, as judged by ELISA. The C-inhibitory activity present in fractions 29 to 40 was also observed in the fractions obtained after a DEAE run without HCE (not shown). The latter finding suggests that inhibition was due to high salt concentrations.

**FIGURE 2.** Dose-dependent enhancement of serum complement activity by HCE. The CH50 of 1:300 diluted NHS was determined after adding different amounts of HCE (10 to 50 μg/well) to the incubation mixture.

**FIGURE 3.** Hemolytic assay and presence of immunoreactive C3 of HCE fractions obtained after separation by gel filtration on a Sephacryl S-300 HR column. C-enhancing activity (cross-hatched bars) and C3 (dotted line) eluted as single peaks. Arrows indicate human-IgG (1) and human serum albumin (2) containing fractions.

**FIGURE 4.** Hemolytic assay and presence of immunoreactive C3 of HCE fractions obtained after separation by anion-exchange chromatography on a DEAE-HPLC column. C-enhancing activity (cross-hatched bars) eluted as two discrete peaks. C3 (dotted line) was eluted as a single protein peak, corresponding to the small C-enhancing peak. HPLC, high-performance liquid chromatography.
When HCE was incubated with mouse anti-C3 sepharose and tested subsequently in the hemolytic assay, more than 90% of the C-enhancing activity was still present. The specificity of the immunoadsorption was evaluated with an ELISA. As shown in Figure 5, HCE incubated with anti-C3 sepharose displayed low extinction values when compared to HCE incubated with sepharose coupled to a nonrelevant mouse IgG1 monoclonal antibody or with uncoupled sepharose.

**DISCUSSION**

It has previously been shown that human donor corneas possess hemolytic activity. This was demonstrated for several individual C proteins (C1 to C7). In this study, evidence is provided for the existence in the cornea of a complement-enhancing factor. The identity and mechanism of action of this factor(s) has still to be determined. It is demonstrated that an extract of normal human corneal tissue causes a dose-dependent increase of the overall C-activity of serum and that the material is heat labile. The results obtained after submitting HCE to gel filtration suggested that a molecule of about 150 kDa was responsible for the C-enhancing effect. It was observed that the early elution fractions displayed inhibition of the hemolytic activity. This inhibition might be attributed to aggregated IgG, which is able to activate the classical complement route, thereby competing with the ShEA in the hemolytic assay.

Anion-exchange chromatography of HCE resulted in two peaks of C-enhancing activity. The fact that one of the peaks was identified as C3 was not surprising. It is known that in humans the serum CH50 correlates with the serum C3 concentration. Furthermore, the normal cornea is known to contain substantial amounts of C3. Immunoadsorption of HCE with sepharose coupled to anti-human C3 could not influence the C-enhancing effect substantially. It is therefore postulated that HCE contains an as yet unidentified factor(s), other than C3, able to enhance complement activity of serum. We are currently investigating if immunoadsorption of HCE for C2 or C5 has any effect on the C-enhancing activity and whether HCE has an enhancing effect on the alternative activation route of the complement system. With regard to the former, it seems unlikely that immunoadsorption has any effect because the molecular weights of C2 and C5, 102 and 180, respectively, do not correspond to the molecular weight of the C-enhancing factor (150 kDa).

The mechanism by which serum complement enhancement occurs remains to be established. The factor described in this paper may affect the normal C-regulatory proteins, thereby stabilizing one of the C convertases. A mechanism like this has been described for C3 nephritic factor: an autoantibody directed against the Bb portion of the alternative pathway C3 convertase (C3bBb). Binding of the autoantibody to C3bBb renders C3 convertase resistant to the normal regulatory proteins (factor H and I). Its prolonged half-life promotes sustained activation of C3, resulting in hypocomplementemia. Whether this applies to the human cornea, which would explain the low CH50, remains to be clarified.

The observation that the guinea pig cornea contains a measurable CH50 suggests the existence of differences in C levels between species. A comparative study on serum complement levels of various sources reported that guinea pig serum has five times more CH50 than human serum. It is generally accepted that corneal complement proteins are supplied via the limbal vessels, such as immunoglobulins. It has been reported that the concentration of C proteins in the cornea correlates with serum C protein levels. The difference in CH50 between guinea pigs and human serum might explain the low CH50 of HCE compared with the guinea pig cornea. Because guinea pig corneal C-activity was determined by a method different from ours, a full comparison cannot be made.

In summary, a low CH50 of human corneal tissue could reduce the possibility of potentially harmful activation of the complement system. It is postulated that the human cornea contains a factor(s) responsible for the low CH50. On the other hand, this factor(s) might be of biologic importance during keratitis, when...
Human Corneal Complement

functional complement, provided by a serous exudate or corneal neovascularization, reaches the cornea. One could postulate that continued activation of the complement system at the site of corneal injury reflects a more efficient defense mechanism, providing better protection of the cornea.

Key Words
cornea, human, CH50, C3, complement inhibitory factor

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