Generation of Complement-Derived Anaphylatoxins in Normal Human Donor Corneas

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Complement-derived anaphylatoxins (C3a, C4a, and C5a) are potent, stable mediators of acute inflammation. Because human corneas contain functional complement, the authors subjected normal human donor corneas to various forms of immunologic or chemical injury to determine if the complement system could be activated and anaphylatoxins generated. The experimental cornea of each donor pair was injected with lipopolysaccharide (LPS) or immune complexes or injured by application of acid or alkali. The remaining cornea of each donor pair served as a control. After incubation of corneas in tissue culture media for 6 hours and elution in phosphate-buffered saline for 24 hours, C3a, C4a, and C5a were measured in corneal eluates by radioimmunoassay. Compared with control corneas, C3a levels were significantly increased in corneas injected with LPS or immune complexes and in corneas injured with acid or alkali. C4a levels were significantly elevated in corneas injected with immune complexes and in corneas injured with acid or alkali but not in corneas injected with LPS. C5a levels were detectable only in corneas injured with acid or alkali. These results suggest that immunologic reactions in the human cornea may activate the classic or alternative complement pathways and generate anaphylatoxins. Additionally, chemical injuries with acid or alkali generate anaphylatoxins in the cornea. Anaphylatoxins may participate in the acute inflammatory response of the human cornea to chemical or immunologic injury. Invest Ophthalmol Vis Sci 31:1945-1949, 1990

Activated complement is a prime mediator of tissue inflammation and is important in host defense against infection and autoimmune tissue injury.1 Activation of the complement system after tissue injury or during immunologic damage generates three anaphylatoxins: C3a, C4a, and C5a. These anaphylatoxins have similar functions with C5a the most active and C4a the least active.2 Anaphylatoxins induce vasodilation, smooth muscle contraction, and an increase in capillary permeability. They trigger mast cells and basophils to release histamine and neutrophil chemotactic factor and synthesize leukotriene B4.3 A potent chemotactic factor for neutrophils, C5a triggers them to release lysosomal proteases and produce leukotriene B4.2 C3a has significant but lower chemotactic activity.2 Anaphylatoxins also have been shown to stimulate prostaglandin synthesis and release in tissues.2

Normal human donor corneas contain functional complement.4,5 Acutely injured human corneas are rarely available for analysis of complement activation. We subjected normal human donor corneas to various forms of immunologic or chemical injury to determine if the complement system could be activated and anaphylatoxins generated.

Materials and Methods

Donor Corneas

Fifty-two pairs of normal human donor corneas in K-Sol media (Cooper Vision, Huntington, West VA) were obtained from the Jules Stein Eye Bank. Donor ages ranged from 13–81 years. The donor corneas with their scleral rims were harvested and placed in K-Sol within 24 hr of donor death. Within 24 hr, the corneas were removed from K-Sol, rinsed with sterile 0.01 M phosphate-buffered saline (PBS) at pH 7.4, blot dried with sterile gauze, and stored at -70°C until used. On the day of experimentation, each pair of corneas was allowed to thaw at room temperature, rinsed in PBS, and dried with sterile gauze.

Lipopolysaccharide Injection Experiment

An 8-mm trephine (Katena, Denville, NJ) was used to excise the central portion of each cornea. Lipopolysaccharide (LPS) 100 mg from Escherichia coli (serotype 0127:B8; Sigma, St. Louis, MO) was dissolved in 5 ml of sterile 0.9% saline containing 1% triethylamine (Kodak, Rochester, NY). Triethylamine was used to dissolve the LPS. A 1-ml tuberculin syringe with a 28-G needle was filled with the LPS.

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solution. The experimental cornea of each donor pair was injected with 50 µl of the solution, containing 1.0 mg of LPS. On the other hand, the control cornea of each pair was injected with 50 µl of sterile 0.9% saline with 1% triethylamine. Each experimental and control cornea was placed in a sterile 60 x 15-mm Petri dish (Falcon, Oxnard, CA), covered with 10 ml of medium 199 with 25 mM Heps buffer, Earle's salts, and L-glutamine (Gibco, Grand Island, NY), and incubated at 37°C and 5% CO₂ for 6 hr. Serum was not added to the tissue culture media to avoid a potential source of complement. After the incubation period, each cornea was rinsed in 0.01 M PBS to remove any media and blot dried with sterile gauze. Each cornea was minced into small pieces using a sterile razor blade and placed in a preweighed test tube containing 1.0 ml of sterile 0.01 M PBS with 10 mM ethylenediaminetetraacetic acid (EDTA). The EDTA was added to prevent further complement activation. The tubes were reweighed to determine the net weight of each cornea, and the corneas were eluted at 37°C and 5% CO₂. Twenty-four hours later, the eluate was collected from each test tube and stored at -70°C before anaphylatoxin assays. Elutions for longer times did not increase the yield of anaphylatoxins.

**Immune Complex Injection Experiment**

The antigen for the immune complexes was ribitol teichoic acid (RTA), which was purified from the cell walls of *Staphylococcus aureus* according to the method of Umeda and associates. Antibodies for the immune complexes were obtained from human antiserum against RTA (Meridian, Cincinnati, OH). We chose this immune complex because it contains human antibodies and a bacterial antigen which may be relevant to corneal disease. The equivalence point of the antibody-antigen reaction was determined by quantitative precipitin reactions according to Knox and Wicken. Immune complexes precipitated in slight antigen excess were used for corneal injections because they cause destructive immunologic injury of tissues through complement activation.

An 8-mm trephine was used to excise the central portion of each cornea. Experimental corneas were injected with 50 µl of sterile 0.9% saline containing 300 µg of immune complex (40 µg of RTA and 260 µg of antibody); control corneas were injected with 50 µl of sterile 0.9% saline. After these injections, the experimental and control corneas were processed as described for the corneas injected with LPS.

**Acid Injury Experiment**

An 8-mm trephine was placed on the epithelial surface of each experimental cornea and inserted into the superficial stroma. A volume of 200 µl of 1 N HCl was applied to the epithelial surface within the trephine for 35 sec, after which the cornea was rinsed in 0.01 M PBS for 2 min and blot dried. For the control cornea of each donor pair, an 8-mm trephine was placed on the epithelium and inserted into the superficial stroma. Afterwards, 200 µl of 0.9% saline was applied to the epithelial surface within the trephine for 35 sec. The control cornea was rinsed in 0.01 M PBS for 2 min and blot dried. The central 8 mm of experimental and control corneas were excised and placed in sterilized dishes, covered with 10 ml of medium 199 with 25 mM Heps buffer, Earle's salts, and L-glutamine, and incubated at 37°C and 5% CO₂ for 6 hr. During the 6-hr incubation period, pH readings were similar in media containing experimental and control corneas and averaged 7.4 because of the buffering capacity of the Earle's salts and Heps buffer. After the incubation period, each cornea was rinsed in 0.01 M PBS to remove any media and blot dried with sterile gauze. Then each cornea was minced into small pieces and placed in a preweighed test tube containing 1.0 ml of sterile 0.01 M PBS with 10 mM EDTA. The tubes were reweighed to determine the net weight of each cornea, and the corneas were eluted at 37°C and 5% CO₂ for 24 hr. The pH readings were similar in the eluting fluid containing experimental and control corneas and averaged 7.4. At 24 hr, eluate was collected from each tube and stored at -70°C before complement assays.

**Alkali Injury Experiment**

An 8-mm trephine was placed on the epithelial surface of each experimental cornea and inserted into the superficial stroma. A volume of 200 µl of 2 N NaOH was applied to the epithelial surface within the trephine for 35 sec, after which the cornea was rinsed in 0.01 M PBS for 2 min and blot dried. For the control cornea of each donor pair, an 8-mm trephine was placed on the surface and inserted into the superficial stroma. Afterwards, 200 µl of 0.9% saline was applied to the epithelial surface within the trephine for 35 sec. The control cornea also was rinsed in 0.01 M PBS for 2 min and blot dried. The central 8 mm of experimental and control corneas were excised and processed as described in the acid experiment. The pH readings averaged 7.4 in the incubating media and eluting fluid for both experimental and control corneas.

**Tissue Culture Media Experiment**

The K-Sol and the tissue culture media used in our study were assayed for anaphylatoxins. Additionally,
the same quantity of LPS, immune complex, acid, or alkali that we used was added to our tissue culture media or K-Sol, which were then incubated for 6 hr without a cornea before anaphylatoxin assays.

Radioimmunoassay of C3a, C4a, and C5a

C3a, C4a, and C5a were measured in corneal eluates using commercially available radioimmunoassay kits (Amersham, Arlington Heights, IL). The radioimmunoassays for C3a and C5a follow methods described by Hugli and Chenoweth,\textsuperscript{1} and the radioimmunoassay for C4a follows methods described by Gorski.\textsuperscript{10} Radioimmunoassay is an extremely sensitive technique that permits quantitation of anaphylatoxins in ng/ml. The experimental and control corneas of each donor pair were assayed on the same day with the same reagents.

Levels of C3a, C4a, and C5a in corneal tissues were calculated as described previously for corneal tissues\textsuperscript{4,5} using the following formula:

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Ca = \frac{W + V}{W} \times Cb
\]

where \(Ca\) is the concentration of anaphylatoxins in ng/g of corneal tissue, \(Cb\) is the concentration of anaphylatoxins in ng/ml of eluting fluid, \(W\) is the weight of the tissue in g, and \(V\) is the volume of the eluting fluid in ml.

Fig. 1. Mean C3a levels ± SEM (ng/g corneal tissue) in experimental corneas that were subjected to chemical or immunologic injury versus control corneas. Statistically significant differences between experimental and control corneas were present for immune complexes (\(P < 0.02\)), alkali (\(P < 0.05\)), and acid (\(P < 0.01\)), but not LPS. \(N\) refers to the number of pairs of corneas in each experiment.

Statistics Analysis

The paired t-test was used to evaluate differences in anaphylatoxin levels between experimental and control corneas in each injury experiment. This test emphasizes differences between the experimental and control cornea of each donor pair and minimizes variability between corneas from different donors such as age of donor, time between death and harvesting, and time between harvesting and processing.

Results

Compared with control corneas, C3a levels were significantly increased in corneas injected with LPS or immune complexes and in corneas injured with acid or alkali (Fig. 1). Compared with control corneas, C4a levels were significantly elevated in corneas injected with immune complexes and in corneas injured with acid or alkali (Fig. 2). On the other hand, corneas injected with LPS did not show significantly elevated levels of C4a. C5a levels were measured only in six of 14 corneas injured with acid and four of 13 corneas injured with alkali. Mean C5a levels ± standard error of the mean were 41 ± 20 ng/g in acid-injured corneas and 14 ± 6 ng/g in alkali-injured corneas.

Anaphylatoxins could not be shown in the tissue culture media used in this study or in K-Sol with or
without the addition of LPS, immune complexes, acid, or alkali.

Discussion

Our model for the study of corneal injury has certain advantages. First, the human cornea can be studied in isolation without the influence of adjacent structures, such as the sclera and conjunctiva, and adjacent fluids, such as the tears and aqueous humor. Moreover, the results are not influenced by inflammatory cells invading the cornea. Second, a precisely defined area of the cornea—the central 8 mm—was used throughout the study. Third, studies of human corneas in this model can be conducted in a controlled, prospective manner. In other words, each injured cornea has a corresponding control cornea from the same donor pair. This approach enhances the chance of detecting a difference between experimental and control corneas and minimizes other factors that might influence complement levels and the generation of anaphylatoxins in corneas from different donors such as age of donor, time between death and harvesting, and time between harvesting and processing. Last, analysis of acutely injured corneas from living patients would be virtually impossible since corneal transplantation is rarely done under such circumstances. A similar approach was used previously by Elgebaly and associates who used an isolated bovine corneal cup model to study leukocyte-mediated injury of the cornea.

Our results suggest that immunologic and chemical injury to the cornea generates complement-derived anaphylatoxins. The hallmark event of complement activation is the conversion of C3 into C3a and C3b. Immune complexes and LPS generate C3a in corneal tissue as they do in serum. Also, immune complexes generate C4a in corneal tissue as they do in serum because immune complexes activate the classic pathway of complement which results in the generation of both C3a and C4a. On the other hand, LPS activates the alternative complement pathway which results in the generation of substantially more C3a than C4a in cornea and serum. Our results suggest that anaphylatoxins can be generated in normal human donor corneas as the result of activation of either the classic or alternative complement pathways.

Chemical injuries with acid or alkali also generate C3a and C4a in corneal tissue. Changes in pH are known to activate complement in serum. For example, acid activates C1 and enhances alternative pathway activity. To the best of our knowledge, acid or alkali injuries have never been shown to activate complement in tissues. Of further interest, anaphylatoxins are extremely resistant to denaturation by extremes of temperature or pH (over a range of 0–13). Thus, acid and alkali generate anaphylatoxins in corneal tissue, and these molecules are stable in this environment.

Anaphylatoxins were found in control and experimental corneas in this study. This is not surprising since normal human donor corneas contain functional complement. Complement in the cornea, like that in the serum, undergoes spontaneous breakdown with the generation of anaphylatoxins while incubated for 6 hr at 37°C. Nevertheless, the various forms of injury we studied generated significantly elevated levels of anaphylatoxins compared with their respective controls.

Of all the forms of corneal injury in this study, acid and alkali generated the highest levels of C3a and C4a. In fact, C5a could be measured only in corneas injured with acid or alkali. C5a levels are the most difficult to correlate with complement activation. First of all, there is less C5 in the serum and cornea than C3 or C4. Moreover, C5a binds well to cells such as neutrophils and monocytes making it unavailable for assay. In all of our studies of complement activation in aqueous humor and vitreous humor, C5a was the most difficult anaphylatoxin to detect.

Anaphylatoxins are extremely potent mediators of inflammation with physiologic effects mediated in the nanomolar or lower range. The small molecular weight of the anaphylatoxins (approximately 10,000) and their stability in extreme conditions of pH should allow them to diffuse easily throughout the corneal stroma in their intact form. These stable and potent mediators of inflammation may participate in the acute inflammatory response of the human cornea to chemical and immunologic injury.

Key words: complement, anaphylatoxins, corneal injury, alkali, acid, immune complexes, lipopolysaccharide

References