Generation of Complement Membrane Attack Complex in Normal Human Corneas

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Purpose. To determine whether complement-derived SC5b-9, the soluble nonlytic-fluid phase of the membrane attack complex, can be generated in normal human corneas when they are injured with lipopolysaccharide (LPS), ribitol teichoic acid (RTA) immune complexes, acid, or alkali.

Methods. The experimental cornea of each donor pair was injected with 50 μL of sterile saline containing 0.5 mg of LPS or 50 μL of sterile saline containing 250 μg of RTA immune complexes. Other experimental corneas were treated topically for 35 seconds with either 200 μL of 1N HCl or 2N NaOH. The control cornea of each donor pair was injected with 50 μL of sterile saline or was treated topically for 35 seconds with 200 μL of sterile saline. After injury, all corneas were incubated in medium 199 for 6 hours at 37°C in 5% CO₂, then eluted for 24 hours in phosphate-buffered saline with 10 mM ethylenediaminetetracetic acid. Each corneal eluate was collected and stored at −70°C until assayed for SC5b-9 by an enzyme immunoassay.

Results. Compared with control corneas, SC5b-9 levels were increased significantly in corneas injected with LPS or RTA immune complexes. However, when compared with controls, SC5b-9 levels were decreased significantly in corneas treated with HCl or NaOH.


As a result of activation of the complement system by either the classical or the alternate pathway, the membrane attack complex (MAC) is generated by the assembly of complement components C5b through C9. The MAC is a stable supramolecular complex that mediates the irreversible target cell membrane damage associated with complement activation. The complex is formed in five main reaction steps. Initially, limited proteolysis of C5 by C5 convertase produces the fragments C5α and C5b. Secondly, C5b and C6 fuse into a bimolecular complex. In the third step, binding of C5b-6 and C7 yields the trimolecular C5b-7 complex that transiently expresses a membrane binding site. During the fourth and fifth steps, respectively, C8 binds to C5b-7 and then C9 binds to C5b-8, after which the C5b-9 complex acquires membranolytic activity. However, in the absence of target membranes, a regulatory serum protein, the S-protein, binds at the C5b-7 stage of assembly. The result is a soluble, nonlytic-fluid phase complex of MAC referred to as SC5b-9 (Fig. 1).

The five precursors of MAC are hydrophilic glycoproteins, with molecular weights ranging from 70,000 to 180,000. The fully assembled complex contains one molecule of C5b, C6, C7, and C8 and multiple molecules of C9. The end product consists of the tetramolecular C5b-8 complex (MWt 560,000) and of tubular poly C9 (MWt 1,100,000). Evidence suggests that poly C9 represents the major complement transmembrane channel and that C5b-8 represents the unit that catalyzes C9 polymerization.

Normal human donor corneas have been shown to contain functional complement. Our previous experiments have demonstrated that when normal hu-
Membrane Attack Complex in Corneas

CLASSICAL PATHWAY
Activation via immune complexes

ALTERNATE PATHWAY
Activation via LPS

CR1, DAF, MCP

C5

C5 convertase

C5b, C6

C7

C5b-7

S Protein

C5b-8

MIRL, HRF

C9

SC5b-9

MIRL, HRF

SC5b-8(poly 9)

C5b-7

C9

FIGURE 1. Diagram of the assembly of membrane attack complex (MAC) and control by regulatory serum protein (S-protein). Included are membrane inhibitors of C5 convertase: complement receptor type 1 (CR1), decay accelerating factor (DAF), and membrane cofactor protein (MCP). Inhibitors of C9 polymerization are membrane inhibitor of reactive lysis (MIRL) and homologous restriction factor (HRF). Solid arrow indicates activation; dashed arrow indicates inhibition.

dried with sterile gauze. Next, the scleral rims and limbal tissues were cut away, and the corneas were rinsed in 10 mM PBS, dried, and weighed.

Lipopolysaccharide Injection Experiment

Lipopolysaccharide (LPS) 50 mg from *Escherichia coli* (serotype 0127:B8; Sigma, St. Louis, MO) was dissolved in 5 ml of sterile 0.85% saline. A 1-ml tuberculin syringe with a 28-gauge needle was filled with the LPS solution. The experimental cornea of each donor pair was injected with 50 μl of the solution containing 0.5 mg of LPS. The control cornea of each donor pair was injected with 50 μl of sterile 0.85% saline. 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, sodium bicarbonate, Earle’s salts, and L-glutamine (Gibco, Grand Island, NY) and incubated at 37°C in 5% CO₂ for 6 hours. To avoid a potential source of complement, serum was not added to the tissue culture medium. After the incubation period, each cornea was rinsed in 10 mM PBS to remove medium and blot dried with sterile gauze. Each cornea was minced into small pieces using a sterile razor blade and placed in a test tube containing 1.0 ml of sterile 10 mM PBS, pH 7.4, with 10 mM ethylenediaminetetraacetic acid (EDTA). The EDTA was added to prevent further complement activation. The corneas were eluted at 37°C in 5% CO₂ for 24 hours. Afterward, the eluate from each test tube was collected, aliquoted, and stored at −70°C until assayed. Elutions for longer times did not increase the yield of SC5b-9.

Immune Complex Injection Experiment

The antigen for the immune complexes was ribitol teichoic acid (RTA) and purchased from Meridian Diagnostics (Cincinnati, OH). This is the major antigenic determinant of *Staphylococcus aureus*. Antibodies for the immune complexes were obtained from human antiserum against RTA. The RTA immune complex was selected because it contains human antibodies and bacterial antigens relevant to human corneal disease. The equivalence point of the antigen–antibody 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.

Using a 1-ml tuberculin syringe with a 20-gauge needle, each experimental cornea was injected with 50 μl of sterile 0.85% saline containing 250 μg of RTA immune complex (10 μg of RTA and 240 μg of antibody). Control corneas were injected with 50 μl of sterile 0.85% saline. After these injections, the experimental and control corneas were incubated and

MATERIALS AND METHODS

Donor Corneas

Forty-eight pairs of normal human corneas in Optisol media (Chiron, Irvine, CA) were obtained from the Jules Stein Eye Bank. Donor ages ranged from 31 to 93 years. The donor corneas, with their scleral rims, were harvested, placed in Optisol within 24 hours of donor death, and stored at −70°C until used. On the day of experimentation, each pair of corneas was allowed to thaw at room temperature, then rinsed with 10 mM phosphate-buffered saline (PBS), pH 7.4, and

man donor corneas are subjected to various forms of immunologic or chemical injury, the complement system is activated, and the anaphylatoxins C3a, C4a, and C5a are produced. In our current experiments, we subjected normal human donor corneas to similar kinds of immunologic or chemical injury to determine whether the terminal pathway of the complement system could be activated and the SC5b-9 complex generated.
processed as described for the corneas injected with LPS.

**Acid Injury Experiment**

An 8-mm trephine was placed on the epithelium of the experimental cornea of each donor pair and inserted superficially into the stroma. A volume of 200 μl of 1N HCl (pH 1.93) was applied to the epithelial surface within the trephine for 35 seconds, after which the cornea was rinsed in 10 mM PBS for 1 minute and blot dried. For the control cornea of each donor pair, an 8-mm trephine was placed on the epithelium and inserted superficially into the stroma. Then 200 μl of 0.85% saline was applied to the epithelial surface within the trephine for 35 seconds. The control cornea was rinsed in 10 mM PBS for 1 minute and blot dried. The experimental and control corneas were placed in sterile 60 × 15-mm petri dishes, covered with 10 ml of medium 199 with 25 mM Hepes buffer, sodium bicarbonate, Earle’s salts, and L-glutamine, and they were incubated at 37°C in 5% CO2 for 6 hours. During the 6-hour incubation period, pH readings were similar in media containing experimental and control corneas and averaged 7.4 because of the buffering capacity of Hepes buffer and Earle’s salts. After the incubation period, each cornea was rinsed in 10 mM PBS to remove medium and was blot dried. A volume of 200 μl of 2N NaOH (pH 12.04) was applied to the epithelial surface within the trephine for 35 seconds, after which the cornea was rinsed in 10 mM PBS for 1 minute and blot dried. For the control cornea of each donor pair, an 8-mm trephine was placed on the epithelium and inserted superficially into the stroma. Then 200 μl of 0.85% saline was applied to the epithelial surface within the trephine for 35 seconds. The control cornea was rinsed in 10 mM PBS for 1 minute and blot dried. Then the tissue culture medium samples were incubated at 37°C in 5% CO2 for 6 hours without a cornea. After the incubation period, the tissue culture medium samples were collected, aliquoted, and stored at −70°C until assayed for SC5b-9.

**Tissue Culture Media Experiment**

The media used in our study, Optisol and medium 199, were assayed for SC5b-9. In addition, the same quantity of LPS, immune complex, acid, alkali, or saline that were used to treat the corneas was added to the tissue culture medium. These treated tissue culture medium samples were incubated at 37°C in 5% CO2 for 6 hours without a cornea. After the incubation period, the tissue culture medium samples were collected, aliquoted, and stored at −70°C until assayed for SC5b-9.

**Enzyme Immunoassay of SC5b-9**

SC5b-9 was measured in corneal eluates using commercially available enzyme immunoassay kits (Quidel, San Diego, CA). Briefly, this assay used a microassay plate with wells coated with a mouse monoclonal antibody specific for human SC5b-9. After the SC5b-9 in the standards, controls, or test specimens was bound to the anti-SC5b-9 antibody, a wash cycle removed any unbound material. Horseradish peroxidase (HRP)-conjugated antibodies to antigens on SC5b-9 were added to each test well. The HRP-conjugated antibodies bind to SC5b-9 antibodies bound on the surface of the microassay wells. After a 1-hour incubation, a wash cycle removed unbound conjugate. Then the chromogenic enzyme substrate [0.7% 2-2’-azino-di(3-ethylbenzthiazoline sulfonic acid) diammonium salt] was added to each microassay well. After a 1-hour incubation, 250 mM oxalic acid was added to each well to stop color development. The standard, control, and test specimen absorbances were measured spectrophotometrically at 405 nm after automix in a Thermomax microplate reader (Molecular Devices, Menlo Park, CA). The intense green of the reaction mixture was proportional to the concentration of SC5b-9. The concentration of SC5b-9 in each test specimen was determined by interpolation from a standard curve. This enzyme immunoassay is a highly specific procedure that permits quantitation of SC5b-9 in nanograms per milliliter. Experimental and control corneas of each donor pair were assayed on the same day with the same reagents.

Levels of SC5b-9 in corneal tissues were calculated as described previously for corneal tissue using the following formula:

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Ca = \frac{W + V}{W} \times Cb
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where \(Ca\) is the concentration of SC5b-9 in ng/g of corneal tissue, \(Cb\) is the concentration of SC5b-9 in ng/ml of eluting fluid, \(W\) is the weight of the tissue in grams, and \(V\) is the volume of the eluting fluid in milliliters.5,10
RESULTS

Compared with control corneas, SC5b-9 levels were increased significantly in corneas injected with LPS (3570 ng/g versus 755 ng/g; \( P < 0.02 \)) or RTA immune complexes (1713 ng/g versus 695 ng/g; \( P < 0.01 \)). Compared with control corneas, SC5b-9 levels were much lower in corneas treated with acid (68 ng/g versus 653 ng/g) or alkali (82 ng/g versus 535 ng/g) (Fig. 2). In fact, the control corneas generated SC5b-9 levels that were significantly greater than the HCl-treated corneas (\( P < 0.01 \)) and the NaOH-treated corneas (\( P < 0.001 \)). SC5b-9 levels could not be detected in Optisol or in medium 199 with or without the addition of LPS, RTA immune complexes, HCl, NaOH, or saline.

DISCUSSION

For the study of corneal injuries, this model has certain advantages. First, the human cornea can be studied in isolation without the influence of adjacent structures, such as the limbus, sclera, and conjunctiva, or adjacent fluids, such as tears and aqueous humor. Additionally, the results are not influenced by inflammatory cells invading the cornea. Second, 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 SC5b-9 in corneas from different donors, such as age of donor, time between death and harvesting, and time between harvesting and processing. Third, analysis of acutely injured corneas from living patients would be virtually impossible because corneal transplantation rarely is performed under such circumstances. Elgebaly and associates used a similar approach with an isolated bovine cornea cup model to study leukocyte-mediated injury of the cornea.

Our results suggest that immunologic corneal injuries induced by LPS generate complement-derived SC5b-9. When Escherichia coli LPS was injected into experimental corneas, the SC5b-9 levels produced were more than four times greater than saline-injected control corneas (3370 ng/g versus 755 ng/g). Other in vitro studies have demonstrated that LPS can activate classical and alternate pathways of serum complement. Activation of the classical pathway depends on the lipid A region of the endotoxin molecule and results in direct binding and activation of C1, the first complement component. Alternate pathway activation depends on critical determinants embodied in the polysaccharide region of the endotoxin molecule. Vukajlovich et al have documented that both polysaccharide determinants (1-glycerol-d-mannoheptose) and O-antigen polysaccharide independently contribute to the alternate pathway activation mechanism.

Corneas injured with RTA immune complexes also generated SC5b-9. When 250 \( \mu \)g of RTA immune complexes was injected into experimental corneas, the SC5b-9 levels produced were almost 2.5 times greater than saline-injected control corneas (1713 ng/g versus 695 ng/g). The RTA immune complexes were prepared in slight antigen excess. The RTA antibodies were obtained from human antisera and were predominantly immunoglobulin G (personal commun-
culation Meridian Diagnostics, 1995). Immunoglobulin G immune complexes can initiate the activation of the classical complement pathway by binding to C1 (the first complement component), specifically to Clq. In addition, teichoic acids have been shown to activate complement. Using a preparation of Staphylococcus aureus purified cell wall, Wilkinson et al demonstrated consumption of C5 to C9 by isolated teichoic acids.

Chemical injuries with acid or alkali did not generate SC5b-9 in corneal tissue. Acid-treated corneas produced significantly lower levels (P < 0.01) of SC5b-9 than saline-treated corneas (68 ng/g versus 653 ng/g). Of the complement components in the complex, the hemolytic activity of C6 is known to be destroyed rapidly at acidic pH. In the alkali-injury experiment, the NaOH-injured corneas produced significantly lower levels (P < 0.001) than saline-treated corneas (62 ng/g versus 535 ng/g). Under the conditions used, exposure to 2N NaOH might denature all or some of the SC5b-9 components. Work by Kolb et al have shown that purified C6 retains only 20% hemolytic activity when incubated at 37°C for 1 hour at pH 12.

In this study, SC5b-9 was generated in immunologically, but not chemically, injured normal human donor corneas. This result was not totally unexpected because normal human corneas contain functional complement components. When corneas were immunologically injured with LPS or immune complexes, the SC5b-9 levels obtained were significantly higher than control levels. However, when corneas were chemically injured with acid or alkali, the SC5b-9 levels were significantly lower than control levels. The harsh pH treatment of the acid or alkali-injured corneas resulted in the alteration of physical properties of C6 and probably other components in the complex. In contrast, our previous work demonstrated the production of the anaphylotoxins C3a, C4a, and C5a in normal human donor corneas when injured by LPS, RTA immune complexes, NaOH, and HCL. Our current experiments indicate that the final phase of complement activation, which yields SC5b-9, the fluid counterpart of MAC, can be produced in normal human corneas when injected with LPS or RTA immune complexes. These findings suggest that the human cornea has the potential to harness the entire complement cascade against infectious agents. However, the human cornea also possesses surface regulatory proteins that protect it from autologous complement-mediated injury. Decay-accelerating factor (DAF or CD55), which dissociates C5 convertase, and membrane inhibitor of reactive lysis (MIRL or CD59), which prevents polymerization of C9, have been localized in human corneal endothelium and stromal keratocytes, respectively. These and other inhibitors, such as complement receptor type 1 (CR1), which possesses decay-accelerating activity for C5 convertase and homologous restriction factor, which inhibits C9 polymerization, can restrict MAC generation (Fig. 1).

Several nonocular complement studies provide evidence that although production of complete transmembrane channels (C5b-poly 9) can be inhibited by these regulatory proteins, the formation of sublytic complexes C5b-8 and C5b-9 can mobilize several inflammatory activities, such as the release of arachidonic acid, prostaglandins, and leukotrienes, in-duction of IL-1 synthesis, and activation of platelets. In addition, signal transduction studies indicate that C5b-7 increases the generation of the transmembrane messengers, diacylglycerol and ceramide, which participate in cell activation during inflammation and repair. Further corneal research is needed to determine the precise roles of the anaphylatoxin-derived acute inflammatory response and the MAC-derived cytolytic and sublytic reactions during the immune response to bacteria and other ocular immunogens.

Key Words: complement, corneal injury, immune complexes, lipopolysaccharide, membrane attack complex

References


