The Effects of Complement Depletion on Corneal Inflammation in Rats

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There are indications that complement activation may be involved in inflammatory processes of the cornea. To investigate the role of the complement system in experimental keratitis, rats were depleted of their plasma complement by treatment with cobra venom factor (CVF). Intraperitoneal injection of CVF resulted in undetectable complement serum activity for 6 days when measured by a hemolytic assay. The corneal inflammatory response, induced by a single intracorneal injection of heterologous serum into nonsensitized CVF-treated rats, was suppressed significantly. The onset of the clinical symptoms of keratitis was delayed, and the severity was reduced. In addition, analysis of the serum antibody titers showed impaired antibody synthesis in the CVF-treated group. When keratitis was induced by an intracorneal antigen challenge in sensitized rats, no difference was observed when comparing the clinical signs of keratitis of CVF-treated animals with sham-treated animals. In addition, CVF treatment did not alter the course of lipopolysaccharide (LPS)-induced corneal inflammation. These findings suggest that the role of plasma complement activation in antigen- and LPS-induced keratitis appears to be limited. The implications for the immunopathologic mechanisms underlying keratitis are discussed. Invest Ophthalmol Vis Sci 33:273-279,1992

The complement system is an important mediator of acute inflammation, and activation can lead to severe tissue damage. It consists of a complex series of proteins, many of which have enzymatic activity. The complement system is activated by various immunologic and nonimmunologic stimuli, of which antigen-antibody complexes, bacterial products (eg, lipopolysaccharide [LPS]), burns, and ischemia are the most important. Two separate routes of activation are known: the alternative and classic pathway. Activation follows a cascade-like manner, during which many biologic active substances are formed. The effects generated by these proteins and peptides are diverse and may modulate immune responses. They include: adherence of leukocytes to vascular endothelium; chemotaxis; activation of neutrophils, monocytes, basophils, mast cells, and lymphocytes; solubilization of immune complexes; opsonization; and cytolyis.

In previous reports, several investigators have postulated that complement activation may be involved in inflammatory processes of the human cornea. Marginal keratitis secondary to systemic vasculitis may be the result of formation or deposition of immune complexes and subsequent complement activation by the classic pathway.1 In keratitis induced by gram-negative bacteria, activation of the alternative pathway of the complement system by LPS has been suggested.2,3 Experimental immunologic disorders, closely mimicking human corneal diseases, have been described. Immune corneal rings which develop in rabbits after an intracorneal injection of antigen are composed of leukocytes, immune complexes, and possibly complement activation products4 or are (at least in part) the result of complement activation.5 Intracorneal injection of C5a induces a reaction that resembles the inflammation produced by an intracorneal antigen challenge in immunized rabbits.6 Furthermore, complement activation product C3c can be detected in the rat cornea at the site of stromal immune complexes.7 Other observations made in rabbits suggest that bacterial endotoxin can stimulate the alternative complement pathway in the cornea.8

One way to analyze the role of the complement system in corneal inflammation further is to deplete animals of complement by treatment with cobra venom factor (CVF). This technique has been useful in several experimental models, including allergic uveitis, endophthalmitis, and corneal infection.9-13 Studies by others12,13 using CVF have demonstrated that C3 plays a central role in a murine model for pseudomonal corneal infection, while C5 is not im-

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portant. The modes of action of CVF are well understood. This factor can be regarded as a C3b analogue. The formation of a complex between CVF and factor B results in activation of the alternative pathway, but this CVF-Bb complex is insensitive to the native mammalian inhibitor. The persistence of CVF-Bb finally leads to exhaustion of the complement system, resulting in depletion of complement components C3 and C5-9.

We describe the effects of complement depletion in experimentally induced keratitis in the rat. In antigen-induced keratitis, immune complex formation and subsequent complement activation (by the classic pathway) is thought to trigger the corneal inflammatory response.\(^4^5\) Activation of the alternative pathway of the complement system may mediate LPS-induced keratitis and might occur during gram-negative bacterial keratitis in humans.\(^8\)

**Materials and Methods**

**Experimental Animals**

Inbred male Brown Norway (BN) rats (weight range, 175-250 g) were used. They were purchased from HSD/CPB (Harlan Sprague-Dawley, Central Institute for the Breeding of Laboratory Animals, Zeist, The Netherlands), housed at our institute under standard conditions, and given food and water ad libitum. Treatment of the animals adhered to the ARVO Resolution on the Use of Animals in Research.

**Chemicals**

Phenol-extracted LPS from *Salmonella minnesota* and partially purified anticomplementary protein (CVF) from *Naja naja kaouthia* were purchased from Sigma (St. Louis, MO). Rabbit anti-sheep erythrocyte serum (hemolytic amboceptor) was a kind gift from Professor M. R. Daha (Leiden, The Netherlands). Normal rabbit serum (NRS) was obtained from the Dr. Van Haeringen Laboratory (Wageningen, The Netherlands).

**Buffers**

Sterile phosphate (10 mM)-buffered saline (PBS, 0.15 M, pH 7.4) was obtained from our hospital pharmacy. The solution DGVB\(^{2+}\) was prepared by mixing barbital sodium (5 mM)-buffered saline (0.15 M), pH 7.4, containing 0.15 mM Ca\(^{2+}\), 0.5 mM Mg\(^{2+}\), 0.1% gelatin, and 0.01% azide (GVB\(^{2+}\)) with an equal volume of 3% D-glucose (DW\(^{2+}\)) in distilled water, containing 0.15 mM Ca\(^{2+}\) and 0.5 mM Mg\(^{2+}\).

**Erythrocytes**

Sheep blood in Alsever's solution (114 mM citrate, 27 mM glucose, and 72 mM NaCl, pH 6.1) was obtained from the RIVM (Bilthoven, The Netherlands). The sheep erythrocytes were washed three times in PBS and resuspended in DGVB\(^{2+}\). The cell number was adjusted to 2 \(\times\) \(10^8\)/ml. The erythrocytes were sensitized by adding heat-inactivated, rabbit anti-sheep erythrocyte serum. The optimal antiserum dilution, which is subagglutinating, was found to be 1:500. The mixture of sheep erythrocytes and antiserum was incubated subsequently for 30 min at 37°C, 30 min at 0°C, and centrifuged. Sensitized erythrocytes were resuspended in DGVB\(^{2+}\) to a final concentration of 2 \(\times\) \(10^8\)/ml.

**Hemolytic Complement Assay**

Overall complement activity (CH50) of rat serum was determined using a colorimetric method described previously\(^1^4\) with minor alterations. In short, rat serum was 1:40 prediluted in DGVB\(^{2+}\). Duplicate serial dilutions were made in U-welled microtiter plates (no. 650160; Grenier, Nürtingen, Germany). To each well 50 μl of sensitized sheep erythrocytes in DGVB\(^{2+}\) (\(2 \times 10^8\)) was added (final volume, 150 μl). The plates were covered with tape and incubated for 60 min at 37°C in a water bath. The cells were spun down (8 min, 600 \(\times\) g), 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 absorbance at 405 nm (E\(_{405}\)) was read. The percentage of lysis (\(Y\)) was calculated using the following equation:

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

in which I refers to the average E\(_{405}\) value of the test sample. The 0% lysis control was obtained by incubating sensitized erythrocytes with buffer and the 100% lysis control, by incubating sensitized erythrocytes with distilled water. To calculate the CH50, serum dilutions were plotted logarithmically against the curve obtained (ie, the x-axis intercept). Finally, complement activity was expressed as units per milliliter of serum.

**Complement Consumption Assay**

Complement consumption by LPS was assessed in an inhibition assay. Checkerboard titrations were done in which serum was diluted in one direction and LPS (0.06-2.0 mg/ml) in the other. Plates were incubated for 30 min at 37°C. The remaining comple-
ment activity was determined by adding sensitized sheep erythrocytes and subsequent incubation for 60 min at 37°C. The E_{405} was read as described. Inhibition (%) was calculated by the equation:

\[
\% \text{ inhibition} = \left[ 1 - \frac{[\text{CH50 (preincubated with LPS)}]}{[\text{CH50 (preincubated without LPS)}]} \right] \times 100\% \quad (1)
\]

**Measurement of Antibody Titers**

Flat-bottomed 96-well microtiter plates (no. 655101; Greiner) were coated for 1 hr at room temperature (RT) with 100 µl of 1:20,000 diluted NRS (in 0.05 M carbonate buffer, pH 9.6). Plates were washed three times with PBS containing 0.1% Tween 20 (PBS-Tw) and incubated for 1 hr at RT with duplicate serial dilutions (100 µl) of rat serum in PBS-Tw. Unbound material was washed away, and plates were incubated with 1:2000 in PBS-Tw diluted, peroxidase-labeled goat anti-rat immunoglobulin G (GaRa/IgG (H + L)/PO; Nordic, Tilburg, The Netherlands) for 1 hr at RT. After another wash, bound peroxidase was visualized using tetramethylbenzidine (TMB) as the chromogen (100 µM TMB in 0.1 M sodium acetate/citric acid, pH 5.5, containing 0.03% hydrogen peroxide). The reaction was stopped after 10 min by adding 50 µl of 2 N H₂SO₄. The E_{450} was read.

Antibody titers were defined as the reciprocal of the last dilution giving rise to an extinction value at least 2.5 times the background value.

**Induction of Corneal Inflammation**

Four different models of corneal inflammation were applied. In the first experiment (group 1), keratitis was induced essentially as described previously. In short, BN rats were anesthetized systemically by an intramuscular injection of 1 mg of fentanyl citrate (0.1 ml Hypnorm; Janssen, Goirle, The Netherlands) and topically by 0.5% tetracaine hydrochloride (Bournonville Pharma, Almere, The Netherlands). The eye was fixed with a forceps, and 5–15 µl of undiluted, heat-inactivated NRS was injected using a gas-tight microliter syringe (1705 LT; Hamilton, Bonaduz, Switzerland) and sterile 30-gauge needle. The procedure was considered successful if a bleb developed during the intracorneal injection. In the next series of experiments (groups 2 and 3), rats were sensitized by an intracorneal injection of undiluted, heat-inactivated NRS. After the inflammatory response had subsided (day 30), rats were challenged in the contralateral cornea (group 2) and in the ipsilateral cornea (group 3). In group 4, rats were injected intracorneally with 5–15 µl LPS (2 mg/ml).

**Induction of Complement Depletion**

The CVF was dissolved in distilled water containing 10 mM ethylenediaminetetraacetic acid and 1% glycerol to a final concentration of 20 µg/ml and stored at 4°C. We administered 1 ml of CVF (ie, 20 µg/animal) intraperitoneally daily for 6 days. Sham-treated rats were handled similarly but received an equivalent volume of the appropriate diluent without CVF. In group 1 (the nonsensitized animals), administration of CVF started on the fifth day after the intracorneal injection of NRS. The CVF treatment of groups 2, 3, and 4 started 3 days before the intracorneal injection. Serum CH50 levels were measured 1 day before CVF treatment, daily during treatment, and 1 day after treatment.

The inoculated animals were examined regularly by slit lamp by an observer unaware of the treatment given. Chemosis, hyperemia of the conjunctival or episcleral vessels, corneal opacification, corneal neovascularization, epithelial or stromal edema, and hemorrhage were the clinical parameters used. Each parameter was scored on a scale from 0 to +3 with 0, no signs; +1, involvement of 1–2 clock hours; +2, involvement of 3–6 clock hours; and +3, involvement of 7–12 clock hours. The keratitis score was the sum of the six parameters. The maximum score for any animal was therefore 18.

**Statistics**

The results are expressed as the arithmetic mean of n individual determinations ± the standard error of the mean. Statistical comparison of the CH50 and the clinical severity of CVF-treated rats versus the control rats was done with the Mann-Whitney U test. Differences with P values < 0.05 were considered statistically significant.

**Results**

**The Effect of CVF Treatment on Serum Complement Activity**

The serum CH50 levels of normal untreated BN rats (n = 5) was 2852 ± 102 U/ml (Fig. 1). It was observed that, 24 hr after the initial CVF dose, serum complement activity was undetectable (detection level, 40 U/ml). The CH50 remained below the detection level until day 7, when levels rose to approximately 50% of the normal value. On day 8, CH50 levels were restored. Additional injections of CVF were ineffective.

**The Effects of Complement Depletion on NRS-Induced Keratitis**

When keratitis was induced by an intracorneal injection of NRS, the corneal inflammation started at
Fig. 1. Effect of CVF administration on serum CH50 levels. Treatment started on day 0. BN rats (n = 5) were injected ip every day for 6 days with 20 μg CVF/animal (▲) or with the appropriate diluent (●).

day 6 and reached its maximum score at day 11 (Fig. 2A). CVF treatment gave a delayed onset of keratitis (maximum at day 13). The keratitis score, which includes the six parameters described, was reduced significantly at days 6–11 and 13. Furthermore, the antibody production, in response to intracorneal injected NRS, was impaired in the CVF-treated animals. Antibody titers differed significantly at days 9 and 10 (Fig. 2B). To circumvent this difference in antibody level, a second series of experiments was done with rats already sensitized to NRS. A difference was made between untreated corneas (group 2) and corneas that had already undergone NRS-induced keratitis (group 3). Due to presensitization, the intracorneal challenge with NRS resulted in keratitis with immediate onset in both groups (Figs. 3A–B). The kinetics of the inflammatory response differed, however, between the groups. Corneas that underwent an inflammatory reaction for the first time displayed a more prolonged period of inflammation (Fig. 3A) compared with corneas undergoing a secondary inflammatory response. The latter reacted more severely, particularly during the first 2 days (Fig. 3B). Systemic complement depletion did not alter the inflammatory response in either of the groups.

The Effect of Complement Depletion on LPS-Induced Keratitis

The biologic activities of LPS preparations of different origins may vary substantially. To ensure that phenol-extracted LPS from S. minnesota was able to activate rat complement, an in vitro complement consumption assay was done. Preincubation of rat serum with graded amounts of LPS caused a dose-dependent depletion of overall complement activity (Fig. 4). A dose of 1 mg/ml gave a 47% decrease of hemolysis. Intracorneal injection of LPS in the rat cornea induces an immediate inflammatory response. The eyes showed total corneal edema, severe conjunctival hyperemia, and chemosis. On day 3, neovascularization started; this resulted in a dense vascularized cornea, with blood vessels extending toward the center of the cornea. When comparing the clinical signs of the CVF-treated group with the sham-treated group, no statistical significant difference was observed (Fig. 5).
can reduce or even abrogate the inflammatory response in several kinds of disorders, including allergic uveitis, and protect animals from tissue damage.\textsuperscript{9,10}

By contrast, complement depletion can lead to reduced numbers of polymorphonuclear cells infiltrating the cornea, which enables bacteria to persist and may lead to loss of the eye. This was observed in pseudomonal keratitis, using mice normally able to restore corneal clarity.\textsuperscript{12,13} In our study, the effects of complement depletion on antigen- and LPS-induced keratitis in the rat were analyzed. Using rats instead of rabbits enabled us to do experiments with inbred strains and less CVF. In addition, intracorneal injections were easy to do compared with those in mice. The CVF treatment resulted in a 6-day period during which serum complement activity was undetectable. Additional injections of CVF to prolong the state of complement depletion were ineffective. It has been demonstrated that CVF elicits an immune response that results in enhanced clearance of CVF by neutralizing antibodies and restoring normal serum CH50 levels.\textsuperscript{16} Effects of CVF treatment on keratitis were observed only after a single intracorneal dose of antigen in nonsensitized animals. This was accompanied by impaired antibody synthesis. The observed differences in onset and severity of keratitis therefore could be the result of low antibody titers. The finding that impaired antibody synthesis is observed after CVF treatment agrees with several other studies.\textsuperscript{17,18} It was shown that CVF suppressed thymus-dependent antibody production in mice.\textsuperscript{17} This effect was attributed to depletion of C3. It has been hypothesized that C3 or its fragments have regulatory effects on immune responses, in particular on the induction of antibody

Discussion

The type of immune response elicited by a particular antigen may determine whether a clear cornea is restored or permanent visual loss is induced after an inflammatory reaction. The complement system may play an important role in these mechanisms. Activation can be both protective and injurious. This ambiguity has been demonstrated using complement-depleted animals or animals genetically deficient of complement components. Complement depletion

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{The influence of CVF administration on the severity of corneal inflammation of BN rats after an intracorneal challenge with NRS. In panel (A), rats had been sensitized by intracorneal injection of NRS in the opposite eye, 1 month earlier. These corneas had therefore not been subject to inflammation previously. In panel (B), corneas that had been previously injected with NRS and had subsequently undergone keratitis were reinjected. The shaded area represents the period in which serum CH50 levels were decreased or undetectable in the CVF-treated rats ($n = 6$) ($\triangle$). Control rats ($n = 6$) ($\bullet$) were injected with diluent.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{Dose-dependent complement consumption by LPS in the hemolytic complement assay, after preincubation of rat serum with graded amounts of LPS for 30 min at 37°C.}
\end{figure}
Fig. 5. The influence of CVF administration on the severity of corneal inflammation of BN rats after intracorneal injection of LPS. The shaded area represents the period in which serum CH50 levels were decreased or undetectable in the CVF-treated rats (n = 5) (▲). Control rats (n = 5) (●) were injected with diluent.

synthesis. This was more plausible after C3-deficient guinea pigs and dogs were used in experiments.19,20 A tenable explanation, therefore, is that the differences of corneal inflammation were a result of suppression of the immune response, rather than actual complement depletion at the site of the antigen–antibody interactions. Furthermore, the results obtained using sensitized rats sustain this explanation. In rats with antigen-specific antibodies, CVF treatment did not interfere with the kinetics and severity of the keratitis.

Previous investigators hypothesized that antigen-induced keratitis is a true immune-complex and complement-mediated reaction in rabbits; this was not confirmed in rats and should be reevaluated. Our results suggest that plasma complement is a nonessential component of antigen-induced keratitis in rats. However, local complement sources should be considered. We cannot exclude participation of corneal complement components in the inflammatory response. Others showed that the human and rabbit cornea contain the major complement proteins and that corneal fibroblasts in vitro are able to synthesize complement factors.21,22 Little is known about the effect of CVF treatment on complement activity present in extravascular spaces. In mice, intraperitoneal administration of CVF results in complete complement depletion in the bloodstream and the peritoneal cavity. Intravenous administration of CVF results in undetectable levels of complement activity in the bloodstream, whereas complement levels in the peritoneal cavity are reduced only partially.23 Based on these findings, corneal complement may have been (partly) unaffected by CVF treatment. Another possibility is that there was local production of complement components by mononuclear phagocytes.24 Immune complexes have various immunoregulatory properties through the Fc region of the immunoglobulin. Immune complex-induced nonspecific lymphocyte activation and release of soluble factors by Fc receptor-bearing cells may trigger inflammatory responses.25 A study on the histologic features of antigen-induced keratitis in rats reported massive infiltration of the cornea by macrophages and, to a lesser extent, by polymorphonuclear cells.15 In addition, T-lymphocytes of the helper phenotype were found in the cornea, and B-lymphocytes and plasma cells were absent. Antibodies to complement-activation product C3c showed faint staining. These data (in particular, the abundance of macrophages, the presence of helper T-cells, and the presence of only minute amounts of C3c) support the idea that antigen-induced keratitis may be mediated by cellular immune mechanisms.

The observation that CVF treatment has no effect on LPS-induced keratitis may be less surprising. Others have found that CVF treatment did not inhibit endotoxin-induced uveitis in rats and rabbits.26,27 Apart from these explanations for the ineffectiveness of the CVF treatment, LPS causes many biologic effects. Besides activation of the complement system, LPS is known to activate macrophages, which, in response, release cytokines and arachidonic acid metabolites.28 Moreover, LPS is an immunologic adjuvant, induces T-cell proliferation, is mitogenic for B-cells (in mice), enhances antibody synthesis, and has chemotactic properties. In conclusion, the data we presented support the idea that the antigen-induced keratitis models in the rat are primarily cell mediated and that complement activation plays a minor or negligible role.

Key words: rat, cornea, keratitis, complement depletion, cobra venom factor, lipopolysaccharide

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