Measurement of Corneal Sensitivity to Mechanical and Chemical Stimulation with a CO₂ Esthesiometer

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PURPOSE. To develop an instrument to measure corneal sensitivity. Mechanical stimulation was performed with increasing air flow. Chemical stimulation consisted of local pH decreases induced by a mixture of air and CO₂ at different concentrations.

METHODS. Air and 98.5% CO₂ were mixed with an electronic, proportional-direction control valve to obtain gas mixtures from 0% to 80% CO₂. The regulated outflow of gas was carried to a probe mounted on a slit lamp holder, where it was warmed and its CO₂ concentration monitored. An electronic valve directed gas pulses of controlled duration to the cornea. Corneal stimulation was performed in 17 young human subjects. The intensity of the experienced sensation was recorded in a continuous visual analog scale (VAS). To obtain threshold values and intensity-response curves, 3-second pulses were applied. For mechanical stimulation, air pulses of increasing flow were used. For chemical stimulation, gas mixtures of increasing CO₂ concentration at subthreshold flow and CO₂ in stepped increases of 5% was applied.

RESULTS. Mechanical and chemical stimulation of the cornea evoked a brief sensation of irritation. Mechanical threshold (flow values) varied among subjects but were reproducible within each subject and were higher with warmed air. The magnitude of the sensation increased proportionally to the flow of air. The mean chemical stimulation threshold (CO₂ concentration) was 25% ± 3%. Increases in CO₂ concentration from 10% to 80% augmented proportionally the intensity of the evoked sensation.

CONCLUSIONS. The gas esthesiometer, which combines variable air flow and CO₂ concentrations, permits application to the cornea of mechanical stimuli of controlled force and pH reductions of increasing magnitude. This instrument may be useful in a separate exploration of mechanical and chemical sensitivity of the cornea in human subjects. (Invest Ophtalmol Vis Sci 1999;40:513-519)

Sensations of irritation evoked by a brief mechanical stimulation of the cornea are commonly used to evaluate corneal sensitivity in clinical and experimental conditions.¹² Other forms of energy (thermal, chemical) that also elicit conscious sensations of irritation and pain when applied to the human cornea³⁵ have not commonly been used in the clinics to quantify corneal sensitivity.

Corneal sensations result from the activation of sensory nerve afferents, which are the peripheral branches of various types of trigeminal nociceptive neurons. In the cat's eye, some of the corneal sensory nerve fibers (approximately 20%) are activated only by mechanical forces and are called mechanosensory. The majority of corneal nerve fibers (approximately 70%) also respond to chemical irritants and to noxious heat and thus are classified as polymodal nociceptive fibers. Finally, a small group (approximately 10%) respond preferentially to cooling of the corneal surface and are called "cold" fibers (for review see Ref. 6).

The contribution of these different classes of sensory afferents to corneal nociceptive sensations is largely unknown, but it is thought to depend on the modality of stimulus acting on the cornea. Sensations evoked by mechanical stimuli are elicited by activation of mechanosensory and polymodal fibers. Other corneal pain sensations, such as those after corneal injury and inflammation, are preferentially mediated by polymodal nociceptors that are sensitive to inflammatory agents released by damaged tissues.⁶ Therefore, polymodal fiber excitation provides the main peripheral sensory input for corneal pain.

Acid is an effective stimulus for polymodal nociceptive fibers. In the cat, acetic acid solutions produce a discharge of nerve impulses in corneal polymodal fibers that is proportional to the pH of the solution.⁷ Corneal pH can also be reduced by application of a CO₂ stream to the corneal surface. CO₂ combines readily with water to form carbonic acid, thus producing a decrease in local pH. When CO₂ is applied as a gas stream to the cornea of cats, it evokes a discharge of nerve impulses in polymodal fibers that is proportional to its concentration in the gas mixture. Similarly, in humans, a jet of air containing increasing concentrations of CO₂ causes a sensation of pain when the mean CO₂ concentration is approximately 40%.⁸

These observations suggest that CO₂ may be used as a controlled acidic stimulus for polymodal nociceptive fibers of the human cornea. The purpose of this work was to develop an instrument to stimulate corneal nerve terminals mechanically by using increasing flow through the air jets or chemically by applying pulses of air and CO₂ at defined concentrations. This instrument could be used to assess corneal sensitivity in human subjects.
METHODS

Human Subjects

Human volunteers of both sexes (8 men and 9 women) 20 to 27 years of age (mean, 22.5 ± 0.1 years) were recruited among medical students. The research followed the tenets of the Declaration of Helsinki. The subjects signed an informed consent to a protocol approved by our institute and were free to interrupt the session at any time. They received financial compensation for their participation in the study. None of them had a history of corneal or ocular disease. Three wore eye glasses (< ±2 D) to correct myopia or hypermetropia.

Esthesiometer

The device used for corneal stimulation is represented schematically in Figure 1. One cylinder containing air and another 98.5% CO₂ were each connected through a pressure regulator (adjusted to 1 bar) and an unidirectional caudal regulator to an electronic proportional directional control valve (PCV) that adjusted the flow of air and CO₂ separately, thus generating at its output gas mixtures with a controlled proportion of CO₂ and air.

The final flow of the gas mixture was adjusted with a flowmeter (FWM; Fig. 1) and carried to a probe (PB) mounted in a slit lamp holder. The probe contained a temperature-controlling device composed of a thermode, a servoregulator, and a Peltier cell (°C) that warmed or cooled the flowing gas to an adjustable temperature value and a three-way solenoid valve to direct the output of gas (EV). When no stimulation was performed, the gas flowing continuously through the valve was diverted away to the back of the probe and entered a CO₂ meter (model PM3; Analytical Development, Herts, UK) where the CO₂ concentration of the gas mixture was monitored. During stimulation, the gas was transiently directed to the tip of the probe (diameter, 0.8 mm) by means of a pulse generator that changed the direction of flow from the electronic valve during a pre-established period of 1 to 10 seconds. This produced a short pulse of gas in the tip of the probe, with a defined CO₂ concentration, temperature, and flow rate. In preliminary experiments performed in two rabbits anesthetized with 30 mg/kg intravenous sodium pentobarbital, the

![Figure 1](image1.png)

**Figure 1.** Schematic drawing of the CO₂ esthesiometer.

![Figure 2](image2.png)

**Figure 2.** Effect of air flow on corneal surface temperature. The changes of corneal surface temperature were plotted against the flow rate of stimulating air pulses with air warmed at various temperatures (room temperature, 25°C). Every point is the mean of 4 measurements performed in both eyes of two rabbits and in six measurements performed in one eye in three human subjects.
corneal surface temperature was measured with a 0.1-mm-diameter thermistor (model IT-23; Physitemp Instruments, Clifton, NJ), and the corneal temperature decline caused by changes in air flow rate was calculated for air warmed up to temperatures of 25°C and 50°C (Fig. 2).

An estimate of the pressure exerted by the gas jet on the cornea was obtained by placing the tip of the probe at a distance of 5 mm perpendicular to the dish of a precision balance (model 2462; Sartorius, Göttingen, Germany) and applying 1-minute pulses of increasing flow rate. The equivalent force values (in milligrams) calculated for the flow rates applied to experimental subjects were as follows: 33 ml/min, 0.43 mg; 58 ml/min, 1.08 mg; 83 ml/min, 1.82 mg; 110 ml/min, 2.96 mg; 138 ml/min, 4.37 mg; 170 ml/min, 6.35 mg; 208 ml/min, 9.06 mg; and 264 ml/min, 14.00 mg.

**Corneal Thermography**

Infrared thermography was performed in humans to determine intensity and extension of corneal temperature changes. Thermographic pictures were taken at a frequency of 3 Hz and a resolution of 155 × 128 pixels (model 870; Thermo-Vision, Agema, Sweden). A close-up lens (20°SW; Thermo-Vision) was used to record the cornea selectively in an area of 3.5 × 3 cm resulting in a spatial resolution of approximately 0.3 mm. Thermal resolution of the system was 0.1°C. Series of 24 pictures were taken with a baseline of 2 seconds followed by a stimulation period of 3 seconds and a control period of 3 seconds. Recordings were transferred to a desktop computer through an interface (IR-SAVE; Gesotec, Darmstadt, Germany) and analyzed by dedicated software.

**Experimental Procedure**

The esthesiometer was calibrated at the beginning of the experiment to check CO2 concentrations and temperature of the gas produced by the instrument at the output of the probe. Temperature (25°C) and humidity (52%) of the room were maintained constant. Experiments were always performed between 4 PM and 8 PM.

The subject was seated comfortably in front of the slit lamp table, with the head supported by the holder. The probe was brought near the eye with slit lamp table commands, and the tip was placed perpendicular to the center of the cornea, 5 mm from the corneal surface, measured with a transparent ruler. The subject was asked to blink immediately before the stimulus. The subject identified the onset of the stimulus by the click produced by the opening of the valve in the probe. At the end of each stimulating pulse, the subject marked in the VAS the intensity of the sensation (see later description).

**Determination of Mechanical Threshold.** The mechanical threshold was determined using the method of levels. Briefly, a series of 3-second pulses of increasing flow were applied, starting with 45 ml/min. After each pulse, the subject was asked whether any sensation was experienced. If not, the intensity of the next pulses was increased by 25 ml/min steps until the response was positive. Then, the following pulse was applied at a flow of 12.5 ml/min lower. If this pulse was suprathreshold, a further stimulus was applied with an intensity value of 6.25 ml/min lower; alternatively, if the pulse was subthreshold, a pulse 6.25 ml/min higher was applied. The lowest positive value was taken as threshold flow for mechanical stimulation.

**Mechanical Stimulation.** To test mechanical sensitivity, eight pulses at different flow rates (33–264 ml/min) were randomly applied with 2-minute pauses between pulses. Pulses of zero flow were interspersed to detect false-positive results. The subject was free to blink between stimuli.

**CO2 Stimulation.** For CO2 stimulation, pulses of subthreshold mechanical intensity (6.25 ml/min below mechanical threshold) were used. Pulses with CO2 concentrations of 10% to 80% were randomly applied with 2-minute intervals between pulses. The subject was free to blink between stimuli. Pulses of zero flow were interspersed between stimuli.

Test to obtain intensity–response curves for mechanical and CO2 stimulation were always performed on different days. After completion of the stimulating series, no signs of epithelial damage were noted under slit lamp examination.

**Psychophysical Measurements**

A 10-cm horizontal, continuous VAS without marks, on which 0 was assigned to "no sensation" and 10 to "maximum sensation," was used to quantify the magnitude of the sensation. The subjects were instructed to signal the intensity of the sensations experienced at the end of each stimulating pulse. They were also asked to describe in their own words the quality attributes of the sensation.

**Data Analysis**

Intensity–response curves were obtained averaging the data from all subjects. Suprathreshold intensity values were normalized by subtracting the threshold flow within subjects. The averaged data were plotted in a log-log scale to convert the intensity–response power function in a straight line and calculate its exponent.

Data were expressed as mean ± SEM. Statistical differences were determined with parametric or nonparametric tests for paired or unpaired data. Significance was set at P < 0.05.

**RESULTS**

In human subjects, the temperature of the stimulated point in the corneal surface decreased 1.89 ± 0.65°C (n = 6; range, −5.05°C to −0.85°C) when 3-second air pulses at room temperature (25°C) and flow below mechanical threshold were applied. In human and rabbit corneas this temperature reduction was not noted when the same air jet was warmed up to 50°C in the tip of the probe (Fig. 2, 3). Thermography images showed that temperature changes induced by air at room temperature were always restricted to the center of the cornea and did not extend to the corneal borders.

**Mechanical Stimulation**

**Threshold.** Detection threshold for pulses of air at a neutral corneal temperature (33°C) in the center of the cornea, ranged from 46 ml/min to 165 ml/min (average, 56 ± 11 ml/min; n = 34; Fig. 4A; median, 93 ml/min; Fig. 4B, arrow). The stimulus was described as a brief, mild irritating sensation. The thresholds varied among subjects, although values obtained on 4 days from the same subject did not differ significantly (one-way repeated measures analysis of variance; Fig. 4A). Also, no significant differences were found between left and right eyes within subjects (paired t-test).

**Intensity–Response Curve.** The flow–response curve obtained from 17 volunteers in whom mechanical pulses of
variable flow were randomly applied to both eyes is shown in Figure 4C. A correlation was found between flow of air and subjective intensity of the evoked sensation, which reached its maximum at flows of more than 208 ml/min. When stimulus-response data were normalized and represented in a log-log plot (Fig. 4C, inset) a straight line was obtained, the exponent (0.58) of which characterized the growth of the intensity of sensation with increasing stimuli.

In six subjects, the effect of gas temperature on mechanical sensitivity was explored, by applying series of air pulses at room temperature (25°C) or at a temperature of 50°C, which eliminated the cooling effect on the cornea (see earlier description). The mechanical threshold was significantly lower when air at room temperature was used (at 25°C, 73 ± 7 ml/min; at 50°C, 103 ± 14 ml/min; n = 6; P = 0.04; paired t-test; Fig. 5A). The power exponent of the intensity-response curve obtained with air at room temperature corrected for threshold was higher than that at 50°C (at 25°C, 0.49; at 50°C, 0.31), but no statistical differences between curves at both temperatures were found (Fig. 5B, inset).

**Chemical Stimulation**

**Threshold.** The threshold CO₂ concentration was determined in 14 subjects. Threshold sensation was described as a mild stinging sensation. Threshold ranged from 10% to 45% CO₂, with an average of 25% ± 3% CO₂ (median, 25% CO₂; percentiles 10 and 90, 10% and 45% CO₂, respectively; Fig. 6A).

**Intensity—Response Curve.** A correlation was also found between the subjective intensity and the magnitude of the applied stimulus. Figure 6B shows the VAS data obtained when a series of pulses of CO₂ at different concentrations were applied to the cornea in random manner in 17 subjects. The exponent of the power function when represented in a log-log plot was 0.63 (Fig. 6B, inset).

**Discussion**

The results show that it was possible to perform selective mechanical or chemical stimulation of the cornea in humans using a noncontact esthesiometer that produced controlled...
FIGURE 4. Threshold responses and correlation between air flow and corneal sensation in human subjects. (A) Values of mechanical threshold obtained with the method of levels (see the Methods section) in the same subjects at different days. Symbols represent flow values at which sensation was reported by each subject in three to four separate experiments. Horizontal lines show the mean value of the day (days 1-3, n = 17; day 4, n = 7). (B) Incidence of the positive response to air pulses at increasing concentration. Arrow represents the median of the threshold flow at 50°C (large arrow) and at 25°C (small arrow). (C) Mean VAS ratings of intensity of pulses of air at different flows applied to the center of the cornea at 50°C (filled circles) and at 25°C (open circles). Inset: Log-log plot of the VAS as a function of the intensity corrected for threshold. Data are mean ± SEM; n = 17.

FIGURE 5. Correlation between air flow and corneal sensation in human subjects. (A) Frequency distribution of the positive responses to two series of air pulses at 50°C (continuous line) or at 25°C (dotted line). Arrows represent the medians of the threshold flow at 50°C (large arrow) and at 25°C (small arrow). (B) Mean VAS ratings of intensity of pulses of air at different flows applied to the center of the cornea at 50°C (filled circles) and at 25°C (open circles). Inset: Log-log plot of the VAS as a function of the intensity corrected for threshold. Data are mean ± SEM; n = 6.

Pulses of air or air plus CO2 at variable concentrations. Previous attempts to develop instruments for clinical evaluation of corneal sensitivity have been restricted to mechanical stimulation. Contact instruments based on the force exerted by a probe of known surface, are either highly unreliable, as is the Cochet-Bonnet esthesiometer, or require high-precision, complicated mechanical devices. Moreover, with these instruments, there is a risk of corneal injury in hypoesthesic eyes. To avoid these problems, noncontact esthesiometers have been developed more recently that use an air jet to apply pressure to the corneal surface. Although the accuracy of corneal stimulation obtained with air jets has been questioned, careful control of the air flow and the distance between the tip of the probe and the corneal surface seems to provide well-localized and highly reproducible mechanical stimuli. However, during mechanical stimulation of the cornea with an air stream at room temperature, cooling also takes place to a degree that is related to the air jet temperature and flow, confirmed by our thermographic measurements. Therefore, the stimulus in con-
FIGURE 6. Relationship between CO₂ concentration and corneal sensation. (A) Frequency distribution of the positive responses to pulses of conventional noncontact air esthesiometers is not only the mechanical force exerted by the gas jet, but in an unknown measure, the decrease in temperature on the corneal surface.

In our instrument, mechanical stimulation was performed using pulses of air of pre-established duration and flow rate. In addition, the temperature of the air stream was controlled, in an effort to reduce the types of stimulating energy acting on the cornea. Our thermographic measurements show that the temperature of the air jet can be adjusted so that temperature changes on the corneal surface are negligible in a broad flow range.

In the present work, the threshold for detection of the mechanical stimulus varied among subjects. Threshold force values in our experiments seemed to be lower than those found by other investigators. Comparisons are difficult because of the inaccuracy associated with the transformation of flow levels into force units per surface area. However, differences in mechanical thresholds between our data and those of previous investigators could be attributable to differences in the size of the stimulated area (with larger stimulated areas, mechanical threshold decreases); also such differences may be linked to the corneas of young subjects having a lower mechanical threshold.

As could be expected, the subjective intensity of the sensation evoked by mechanical stimulation was directly related to the magnitude of air flow in the pulse. Stimulus-response curves were highly repeatable in the same subject, and the slopes were similar from one subject to another. Slopes were linear between 53 ml/min and 208 ml/min and saturated above the latter value. For human psychophysical studies stimulus–response curves are often represented as power functions that are straight lines when plotted in a log–log scale. The slope of the line is given by the exponent of the power function and describes the growth of the perceived intensity with increasing magnitudes of the stimulus. The value of the exponent in this work was similar to that reported for olfaction and slightly lower than that for irritation sensations elicited by CO₂ application to the nasal mucosa and cornea. The contribution of corneal cooling to the sensation evoked by mechanical stimulation was revealed by the lower mechanical threshold and by the subject’s use of the descriptor “cooling” added to “irritating” when air at room temperature was applied to the cornea. Heating the air to 50°C prevented this cooling effect in a broad range of flow rates.

Confirming previous reports, CO₂ was notably effective in evoking a sensation of pain from the cornea. CO₂-air mixtures were warmed and applied at flow rates below mechanical threshold, thus excluding a contribution of mechanical or thermal stimulation to the reported sensations. This was confirmed by the absence of a positive warming or cooling component among the descriptors used by the subjects to describe the sensation experienced with CO₂ pulses. Elevations of 5% in CO₂ concentration were discriminated as increasingly painful. Mean threshold was 25% CO₂ and exhibited some variability among subjects. Chen et al. reported higher CO₂ concentration thresholds; however, they began their testing with 35% CO₂, thus excluding the possibility of threshold levels at lower CO₂ concentrations.

Mechanical indentation of the cornea by an air jet may stimulate both mechanosensory and polymodal nociceptive fibers. Corneal cooling resulting from convection and evaporation, additionally excites “cold” sensory fibers. In our instrument, the use of warm air possibly prevented the activation of this last category of sensory afferents. That there was no cooling component in the sensation evoked by mechanical stimulation with warmed air speaks in favor of this interpretation. The difference between thresholds with air at room temperature and with warm air probably reflects the contribution of the population of corneal “cold” fibers to the final sensation when air at room temperature is used.

The effect of CO₂ is to produce a transient pH change in the corneal surface, which selectively excites polymodal nociceptive terminals proportionally to the magnitude of the pH decrease within the corneal epithelium. Local pH changes induced by CO₂ are dependent on its concentration in the gas mixture. Therefore, higher CO₂ concentrations applied to the cornea are likely to produce more pronounced local pH reductions. This pH change is presumably transient because of the short duration of the pulse and the buffering capacity of the epithelial cells but suffices to excite polymodal nociceptive fibers in a measure proportional to the pH decrease.
augmented firing and progressive recruitment of corneal polymodal fibers by increasing acidity explains the growing irritation evoked by increasing CO2 concentrations.

Conventional noncontact air esthesiometers use air at room temperature and are expected to recruit all types of sensory nerve fibers present in the cornea. In our instrument, warm air pulses presumably reduce or eliminate activation of corneal "cold" sensory units, thus restricting their stimulating action to the sensory fibers that are preferentially involved in signaling pain—that is, mechanosensory and polymodal nociceptive fibers. Moreover, stimulation with a warmed (50°C) CO2 gas mixture at a flow below mechanical threshold allows limiting of neuronal activation to corneal polymodal nociceptive fibers, the main group of sensory afferents involved in peripheral pain associated with local inflammation.6

The CO2 esthesiometer described herein permitted control of the temperature, flow, and composition of the gas stimulating the cornea and, through the selection of the type of stimulus applied, separate activation of the various populations of corneal sensory fibers. An instrument with these characteristics may be useful in evaluating corneal sensitivity in normal and pathologic conditions with improved accuracy.

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References


Cell Surface Regulators of Complement, 512 Antigen, and CD59, in the Rat Eye and Adnexal Tissues

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PURPOSE. Cell surface complement regulatory proteins have been identified in high levels in ocular tissues, but no experimental model is available for examining their physiological roles. To develop such a model, the distribution of 512 antigen, a protein possessing the functions of the human decay-accelerating factor (DAF [CD55]) and membrane cofactor protein (MCP [CD46]), and rat inhibitory protein (CD59), the homologue of the human membrane inhibitor of reactive lysis (MIRL [CD59]) were characterized in the rat eye and ocular adnexal structures.

METHODS. After euthanasia of female Wistar rats, followed by orbital exenteration, eyelids and orbital tissue including the lacrimal gland were separated from the globes and immediately snap-frozen in liquid nitrogen at −70°C. Tissues then were sectioned at −20°C and examined immunohistochemically for 512 antigen and rat CD59.

RESULTS. Both molecules were found to be present in high levels in multiple sites. Corneal and conjunctival epithelia showed moderate to intense labeling for both regulators. Fibroblasts in the corneal stroma, conjunctiva, and sclera labeled similarly. Corneal endothelial cells showed intense labeling for rat CD59 but not for 512 antigen. The iris and ciliary body showed intense labeling for both proteins. The retina showed labeling at multiple levels, with that of rat CD59 being more intense than that of 512 antigen. The lacrimal gland labeled for both regulators. Vessels, muscle, and nerves in the orbit labeled intensely for both antigens. In the eyelid, conjunctiva, sebaceous glands, and muscle and nerve tissues labeled moderately to intensely for both molecules, whereas skin epithelium labeled less intensely.

CONCLUSIONS. 512 antigen and rat CD59 are expressed in high levels and distributed similarly in the rat eye and lacrimal gland to DAF, MCP, and MIRL in the human eye and lacrimal gland. These findings establish the rat ocular surface as a model for studying the role of cell surface
complement regulators in this site. This first identification of copious expression of these proteins in eyelid structures, which also participate in protection of the ocular surface, further suggests an important role for surface complement regulatory proteins in this location. (*Invest Ophthalmol Vis Sci.* 1999;40:519-524)

The ocular surface must maintain the functional homeostasis that is required for clear vision while defending itself from foreign substances to which it is continuously exposed. Among the effector systems that participate in this defense is the complement cascade. Components of the classical and alternative pathways are present on the ocular surface, and activation fragments are generated not only in pathologic states but also physiologically in the closed-eye environment. During complement activation, host tissues must be protected from bystander damage. This protection is afforded by cell surface regulatory proteins that inhibit the activation sequence at multiple steps. In humans, these regulators (reviewed in reference 4) include the decay-accelerating factor (DAF [CD55]), the membrane cofactor protein (MCP [CD46]), and the membrane inhibitor of reactive lysis (CD59). DAF and MCP inactivate the classical and alternative pathway C3 and C5 convertases, the central amplification enzymes of the cascade, while CD59 prevents the formation of lytic membrane attack complexes. Each of these factors has been identified on the ocular surface. DAF and CD59 differ from most other surface proteins in that they are attached to cells by posttranslationally added sialylphosphatidylinositol anchor; 512 antigen is attached by a protein initially termed the rat inhibitory protein, the rat homologue of human CD59. Although similar to human DAF and CD59, rat CD59 is linked to the cell membrane by a glycosylphosphatidylinositol anchor; 512 antigen is attached by a conventional transmembrane polypeptide. In tissue surveys, the two proteins have been shown to be widely distributed but vary greatly in their levels like the human regulators. 512 antigen and rat CD59 have been shown to be present on the rat cornea. However, within ocular tissues, the two regulators have not been further studied. In view of the existence of many established models of immunologic ocular disease in the rat and the potential for using the rat as an animal model to investigate further the importance of restriction of autologous complement activation on ocular tissues, we sought to analyze comprehensively the distribution of these two proteins on the rat ocular surface and lacrimal gland as well as in eyelid structures that also participate in defense of the ocular surface, the eye, and orbital tissues.

**METHODS**

Female Wistar rats (200-250 g) underwent thiopental euthanasia. The eyes, eyelids, and lacrimal glands were removed, immediately frozen in liquid nitrogen, and stored at -70°C. Tissues were mounted in OCT medium at -20°C, and 5-μm sections were prepared concurrently. Sections were examined by immunohistochemical labeling. As described, sections were fixed in cold acetone for 10 minutes. They were then washed, incubated for 18 hours at 4°C with primary antibody, washed, incubated for 1 hour at 20°C with secondary biotinylated horse anti-mouse IgG (1:200; Vector, Burlingame CA), and then washed and incubated for 20 minutes at 20°C with peroxidase-conjugated streptavidin (1: 200; Vector). Labeling was visualized by incubation with diaminobenzidine-hydrogen peroxide. Control samples, in which no primary antibody and nonrelevant primary antibody were used, were prepared concurrently. Sections were examined by two authors (DSB, CC) in a masked manner, and labeling intensity of each structure was graded semiquantitatively with a scale of: 0, no label; 1+, mild label; 2+, moderate label; and 3+, intense label.

Murine monoclonal antibodies to 512 antigen (512) and to rat inhibitory protein/rat CD59 (TH9) were obtained as previously described. In western blot analysis of whole-cell extracts, the two antibodies were shown to be monospecific, the former recognizing two 512 isoforms of 55 kDa and 65 kDa and the latter a single rat CD59 band of 21 kDa. Nonrelevant RT1A and anti-ciliary neurotrophic factor monoclonal antibodies were purchased from Pharmingen (San Diego, CA). Primary antibody concentrations were 1 μg/ml for 512, 1 μg/ml for rat CD59, 10 μg/ml for OK18, and 5 μg/ml for ciliary neurotrophic factor. All studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**RESULTS**

For each protein, the eyes from three animals were studied. With any given monoclonal antibody preparation and dilution, all specimens showed similar labeling. The data are summarized in Tables 1 and 2. For both 512 antigen and rat CD59, corneal epithelium showed intense or moderate to intense labeling (Figs. 1A, 1B). Control sections showed no label. Corneal kerocytes and fibroblasts in the conjunctival substantia propria similarly showed moderate to intense labeling for both regulators. In contrast, corneal endothelium showed intense labeling for rat CD59 but no labeling for 512 antigen. Conjunctival epithelium...
**Table 1.** Labeling of Ocular and Ocular Surface Tissues for 512 Antigen and Rat CD59  

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* 1, mild; 2, moderate; 3, intense; 0, no labeling.

showed moderate labeling for 512 antigen (Fig. 1C) and intense labeling for rat CD59, whereas trace labeling occurred with the control antibody (not shown). The iris and ciliary body showed intense labeling for both proteins. Again, control sections (Fig. 1D) showed no or only trace labeling.

Within the retina, the plexiform layers showed mild labeling for 512 antigen and moderate labeling for rat CD59. Labeling of the nuclear layers was seen for rat CD59 (Fig. 2). The choroid showed moderate labeling for 512 antigen and rat CD59, whereas retinal pigment epithelium labeled intensely for both antigens. Again, some nonspecific trace labeling was seen with control antibody.

Within the orbit, lacrimal gland acinar cells showed intense labeling for rat CD59 (Fig. 3) and lesser labeling for 512 antigen with both antigens present in an identical distribution (not shown). Extraocular muscles showed intense labeling for both proteins. Sheaths of orbital nerves showed moderate labeling for both proteins. Vessels within the soft tissues and the eye showed consistent intense endothelial labeling for both proteins (data not shown).

Within the eyelid, an identical distribution of labeling for 512 antigen (not shown) and rat CD59 (Fig. 4) was seen. There was differential labeling of epidermal and mucous membrane squamous epithelia with more intense labeling in the conjunctiva. Sebaceous glands within pilosebaceous units, and multilobular meibomian glands, both of which contribute to the tear film, showed intense labeling. The membranes of striated extraocular muscle cells showed labeling with both antibodies, similar to that observed for these cells in humans.

**DISCUSSION**

Previous work unexpectedly has shown that human corneal and conjunctival cells express high levels of DAF, MCP, and CD59 on their surfaces, with DAF expressed at levels among the highest in the body.8 It additionally has shown that DAF and CD59 are expressed on the surfaces of corneal endothelium, stromal keratocytes, fibroblasts, and lacrimal gland acinar cells,5,6 and that the two proteins are expressed in the retina.5,7 In a prior study, Funabushi et al.10 reported that the rat anterior segment resembles that of humans, in that 512 antigen, possessing the combined functions of DAF and MCP, is present on corneal epithelium, whereas the rat homologue of CD59 is present on corneal epithelium and endothelium.

In the present study directed at developing an experimental model to examine the physiological function of cell surface complement regulators in the eye, we carried out a systematic analysis of individual components of rat ocular and orbital tissues using monoclonal antibodies specific for the rat proteins. We confirmed previous observations that 512 and rat CD59 are present on corneal epithelium, and rat CD59 is present on corneal endothelium. In semiquantitative analyses we showed that they were expressed in high levels. We found that keratocytes labeled strongly for both proteins, and that fibroblasts in the conjunctival substantia propria as well as sceral fibroblasts showed labeling similar that in to keratoocytes, suggesting that 512 antigen and rat CD59 proteins are present in significant quantities on all ocular fibroblasts.

Inside the rat eye, we found that all components of the uveal tract (i.e., the iris, ciliary body, and choroid) labeled intensely for the two regulators. Although in the choroid such labeling could reflect the predominance of vascular structures, in the iris and ciliary body, blood vessels comprise only a small part of the tissue. The uvea is a common target in a broad range of inflammatory, infectious, and autoimmune processes. Teleologically, it therefore is a site where protection from autologous complement injury to host tissues would be needed.

In the retina we found intense labeling of the pigment epithelium for 512 antigen and rat CD59. In humans, our previous studies5 similarly showed intense labeling of retinal pigment epithelium for DAF, whereas studies by others7 described intense CD59 labeling of all retinal structures but minimal labeling of the photoreceptor outer and inner segments for MCP.

The rat lacrimal gland showed moderate to intense labeling for rat CD59 compared with moderate labeling of 512 antigen. This pattern differs slightly from that in humans where DAF and CD59 are present in moderate amounts on lacrimal gland acinar cells.5,6

**Table 2.** Labeling of Eyelid and Orbital Tissues for 512 and Rat CD59

<table>
<thead>
<tr>
<th>Tissue</th>
<th>512</th>
<th>Rat CD59</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eyelid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epidermis, upper</td>
<td>0*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Epidermis, basal</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Pilo-sebaceous unit</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Muscle</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Meibomian glands</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Tarsal fibroblasts</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Conjunctival epithelium, upper</td>
<td>2-3</td>
<td>2-3</td>
<td>0</td>
</tr>
<tr>
<td>Conjunctival epithelium, basal</td>
<td>2-3</td>
<td>2-3</td>
<td>0</td>
</tr>
<tr>
<td>Orbit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lacrimal gland</td>
<td>1-2</td>
<td>2-3</td>
<td>0</td>
</tr>
<tr>
<td>Extraocular muscle</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Orbital nerves</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

* 1, mild; 2, moderate; 3, intense; 0, no labeling.
The differences between our findings for 512 antigen in the rat and previous findings for DAF and MCP in humans in the lacrimal gland, cornea, and retina could relate to the fact that the tissues analyzed in the present study were frozen immediately, in contrast to studies of human tissue, in which donation-associated delays can lead to tissue degradation after enucleation. Alternatively, the differences could point to the presence of other rat regulators in these sites. The fact that in the mouse direct homologues of DAF and MCP in the rat has been obtained using antibodies against the human proteins, although CD59 was not detected. The respective physiological roles in rodents of Crry and 512 antigen and of DAF and MCP, which seemingly have overlapping functional activities, remain as yet undetermined.

This study includes the first description of cell surface complement regulators in the eyelid. The eyelid is of importance in ocular surface phenomena for several reasons. It contains, on its inner surface, palpebral conjunctiva, and eccrine accessory lacrimal glands. These glands are similar to the main lacrimal gland, which labels for CD59 in rats and for DAF and CD59 in humans. In addition, intense labeling was detected in the sebaceous glands. The large multilobular meibomian glands in the tarsal plate in the posterior lamella of the eyelid and the solitary glands in pilosebaceous units secrete their sebum into the tear film. The palpebral conjunctiva is a common site for pathologic conditions thought to have an autoimmune basis and to involve complement, such as giant papillary conjunctivitis. Understanding how activation of the cascade is regulated in this site could help clarify the pathogenesis of these conditions.

Intrinsic membrane regulators of complement play a physiologically essential role in maintaining host cellular integrity while allowing defense against foreign agents. Although the absence of these proteins has been shown to play a central role

**Figure 1.** (A) Cornea labeled for 512 antigen: Moderate to intense labeling is seen in epithelium and keratocytes but no labeling in endothelium. Labeling of the basal cell membranes is evident. (B) Cornea labeled for rat CD59: Intense labeling is seen in epithelium, keratocytes, and endothelium. (C) Anterior segment of eye labeled for rat CD59: Conjunctival epithelium and keratocytes show moderate to intense labeling; the iris and ciliary body similarly show intense labeling. The corneal endothelium is labeled. (D) Anterior segment of eye labeled with nonrelevant antibody: Trace labeling is seen in iris and ciliary body. Other structures are unlabeled. Original magnification, (A, B) X400; (C, D) X100.

**Figure 2.** (A) Retina and choroid labeled for 512 antigen: Labeling of retinal pigment epithelial cells (arrow) is moderate to intense, as is labeling of the choroid. Plexiform and nerve fiber layer labeling is mild. Nuclear layer labeling is mild to slight. (B) Retina and choroid labeled for rat CD59: Retinal pigment epithelial and choroidal labeling is moderate to intense. Nuclear layer labeling is moderate and stronger than mild plexiform layer and nerve fiber layer labeling. (C) Retina and choroid labeled with nonrelevant antibody: Slight labeling is present in retinal pigment epithelium. Other tissues show no label. Original magnification, X100.
in paroxysmal nocturnal hemoglobinuria and their expression on cutaneous and ocular melanomas has been shown to correlate with susceptibility to complement-mediated cytosis, their physiologic role on the ocular surface and possible involvement in ocular disease is not yet known. However, this study demonstrates that all the ocular and adnexal tissues that comprise the ocular surface or secrete material onto it express these proteins in high levels. Similarly, within the eye, the layers most commonly involved in inflammatory and autoimmune processes (the uvea and retinal pigment epithelium) also express these proteins, suggesting a role for them in protecting the functionally delicate visual system from complement-associated damage. Presumptive evidence for upregulation of the proteins has been obtained in experimental iritis.

This study presents the first definitive description of the distribution of intrinsic C3 and C9 complement surface regulators in the ocular and orbital tissues of an experimental animal. Although differences exist between the regulatory system in the rat and that in the human, the presence of numerous models of ocular inflammation in the rat, the well-characterized functionality of the regulators in the rat, and the results of this study provide a basis for using the rat as an experimental model to study the physiological importance of complement regulation in this location.

**References**

Cellular Response in Subretinal Neovascularization Induced by bFGF-Impregnated Microspheres

Hideya Kimura,1,3 Christine Spee,1 Taiji Sakamoto,1 David R. Hinton,2 Yuichiro Ogura,3 Yasubiko Tabata,4 Yoshito Ikada,4 and Stephen J. Ryan1

PURPOSE. To determine the sequence of cellular changes associated with a new rabbit model of subretinal neovascularization (SRN) induced by subretinal injection of basic fibroblast growth factor (bFGF)-impregnated microspheres.

METHODS. bFGF-impregnated gelatin microspheres, prepared by forming a polyion complex between gelatin and bFGF, were subretinally implanted into rabbit eyes. The eyes were studied by immunochemistry at 3 days to 8 weeks after implantation. Antibodies to CD4, CD8, cytokeratin, CD31, glial fibrillary acidic protein (GFAP), and RAM11 were used.

RESULTS. Cytokeratin-positive retinal pigment epithelial (RPE) cells appeared on day 3 and continued to increase in number in the subretinal space throughout the growth of the SRN membrane, becoming the predominant cell type. Macrophages (RAM11-positive) appeared early, but most disappeared within 7 days. GFAP-positive Müller cells were evident early in the retina but migrated into the subretinal space after 7 days; the gliotic adhesion formed between the retina and the SRN membrane was prominent at 8 weeks. CD31-positive endothelial cells were first evident at 14 days and formed neovascular channels that were still present for up to 8 weeks. CD4- and CD8-positive lymphocytes appeared in the early stages but were few in number.

CONCLUSIONS. SRN membranes are primarily composed of RPE cells and vascular endothelial cells. The membrane adheres to the retina by a gliotic band. The cellular components involved in the membrane of this model resemble those found in SRN membranes removed from patients with age-related macular degeneration. (Invest Ophthalmo Vis Sci. 1999;40:524–528)

Subretinal neovascularization (SRN) is often associated with severe visual impairment, especially in age-related macular degeneration (ARM). The pathogenesis of SRN is not fully understood; however, the new vessels of SRN are of choroidal origin. Recent studies of surgically excised choroidal neovascular membranes (CNVMs) have provided some pathologic information on the mechanism of CNVM formation.1-3 However, data obtained from these studies have often been limited to late stages in the evolution of the CNVMs. Early stages of CNVMs can only be studied in an animal model.

We have developed a new model of SRN in the rabbit based on implanting basic fibroblast growth factor (bFGF)-impregnated gelatin microspheres under the retina.4 Approximately 80% of eyes that receive bFGF-impregnated microspheres show fluorescein leakage from the CNVM 2 weeks after implantation. These CNVMs are largely involuted within 8 weeks after microsphere implantation.

In this study we examined the time course of cellular response into the subretinal space after initiation of the bFGF SRN model using cell-specific antibodies. The results provide insight into pathogenic mechanisms involved in this lesion and demonstrate relevance to human CNVMs.

METHODS

Preparation of bFGF-Impregnated Microsphere Suspension

bFGF-impregnated microspheres were prepared by forming a polyion complex as previously described.4 Briefly, 2.5 mg of cross-linked gelatin microspheres were placed in 100 μl of distilled water containing 25 μg of bFGF at 37°C for 1 hour, after which 400 μl of phosphate-buffered solution (PBS; pH