Demonstration of Inflammatory Mediator-Induced Inflammation and Endothelial Cell Damage in the Anterior Segment of the Eye

FREDERICK N. ROWLAND, PhD,
MATTHEW J. DONOVAN, BA,
MICHAEL LINDSAY, BS, WALTER I. WEISS, MS,
JAMES O'ROURKE, MD,
and DONALD L. KREUTZER, PhD

From the Department of Pathology and the Division of Ophthalmology, University of Connecticut Health Center, Farmington, Connecticut

Although some investigations have demonstrated the ability of inflammatory mediators, including vasopermeability and chemotactic factors, to induce acute inflammatory reactions *in vivo*, little is known about the response of various elements of the anterior segment to the direct effects of inflammatory mediators. These studies were initiated to develop models for the investigation of inflammatory responses in this region of the eye. Acute inflammatory reactions were induced within the rabbit anterior chamber by intracameral injection of 50 μl of various inflammatory mediators and were evaluated by clinical grade, leukocyte influx into the aqueous humor, and morphologic changes in the corneal endothelium. Peak responses were recorded following injection of 10^{-4} M formyl-methionyl-leucyl-phenylalanine (fMLP); 5 ED_{50} C5fr; 0.5 mg/ml C5; undiluted anti-red blood cell (RBC) serum; and 10^{-5} M histamine. The number of leukocytes per milliliter of aqueous humor induced by each mediator was quantitated by comparison with the number of leukocytes induced by buffer instillation into a separate group of rabbits (mediator-induced influx/buffer-induced influx). Comparisons were made 24 hours after instillation of mediators. The results of these studies were as follows: buffer alone, 1.0; fMLP, 3.1; C5fr, 61.0; C5, 8.7; anti-RBC, 91.0; and histamine, 24.0. Clinical grades correlated well with these ratios. In addition, differences were noted when the time kinetics of acute responses induced by two different mediators (10^{-4}M fMLP, a synthetic preformed chemotactic factor; and a 1:5 dilution of anti-RBC, which binds to vascular and corneal endothelial cells) were directly compared over 48 hours. Responses induced with fMLP peaked between 5 and 8 hours and resolved rapidly, whereas anti-RBC–induced responses peaked between 8 and 12 hours and resolved very slowly. Histopathologic analysis indicated that both fMLP and anti-RBC induced a similar sequence of changes in the corneal endothelium. Within 2–3 hours after instillation of either mediator, the endothelial cells exhibited prominent vacuolization/retraction phenomena. At the peak of leukocyte influx PMNs filled these vacuoles, then migrated back into the aqueous humor within several hours. Normal morphologic features were recovered following clearance of leukocytes from the anterior chamber. We believe that these models will be useful in identifying the roles of individual mediators in acute and chronic endocular inflammation and in the injury of corneal endothelium. (Am J Pathol 1983, 110:1–12)

INFLAMMATORY PROCESSES, whether initiated by infectious, immunologic, or environmental factors, represent extremely explosive and potentially destructive biologic responses. When uncontrolled within the limited confines of the eye, inflammation can unrelentingly ravage the small amounts of tissues on which vision depends.

In general, inflammatory reactions are thought to be initiated and amplified by chemical mediators, including vasopermeability and chemotactic factors. Although extensive *in vitro* studies have implicated these and other mediators and modulators in inflammatory reactions, relatively few *in vivo* studies have demonstrated their specific biologic roles and mechanisms. For the most part, these studies have focused on the roles of the mediators in lung and kidney disease, with only limited application to studies of ocular inflammation. Hence, much of our present knowledge of mediators of ocular inflammatory reactions has been obtained by indirect means and is largely based on the presumption that immunologic mechanisms which are known to be active at other tissue sites be-

Supported in part by NIH Grants EY 00123, EY 05586, and EY 10431, and HL 25015.

Dr. Kreutzer is an Established Investigator of the American Heart Association.

Accepted for publication July 21, 1982.

Address reprint requests to F.N. Rowland, Department of Pathology, University of Connecticut Health Center, Farmington, CT 06032.

0002-9440/83/0107-0001$01.40

have in a similar manner in the eye. However, the unique anatomy and function of the eye suggest that it has unique proinflammatory and antiinflammatory mechanisms to maintain its critical functions. We initiated the present series of experiments to begin a systematic evaluation of mechanisms and mediators of inflammation, injury, and healing within the anterior segment of the eye. This region involves the key functions of corneal and lens transparency, pupillary motion, and aqueous humor flow.

The tissues of the anterior chamber are susceptible to all types of immunologic responses, including 1) acute immune-complex–mediated and IgE-mediated allergic responses, 2) cell-mediated responses, and 3) corneal graft and tumor rejection.[1] In addition, it has become evident that immune-complex deposition within ocular tissues may be the cause of several forms of uveitis, most notably those associated with rheumatoid spondylitis, Behçet's syndrome,[2] and granulomatous inflammations.[3,4] A number of early experimental studies have implicated immune complexes in uveitis and vascular changes seen in uveitis.[5–9] More recent studies have implicated immune complex deposition in uveitis by the demonstration of complexes within the aqueous humor.[10] In other body tissues, immune complexes are known to be highly effective in triggering the development of inflammation by activation of the complement system, which in turn generates inflammatory mediators, including vasopermeability regulators and chemotactic factors,[11–13] as well as activation of coagulation systems (fibrinogenesis, fibrinolysis, and kinin generation). Previously, the measurement of direct biologic effects of these mediators *in vivo* has been difficult and was only recently shown to be quantifiable in lung.[14] The studies described below focus on delineating the ability of known inflammatory mediators such as chemotactic and vasoactive factors to directly induce acute responses within the anterior segment as well as the consequences of those inflammatory reactions. Their purpose is the evaluation of the potential mechanisms, consequences, and role of these inflammatory mediators in the initiation and amplification of ocular inflammation and tissue injury.

Materials and Methods

Experimental Animals and Method of Ocular Instillation

The experimental animals used were 10–12-week-old male and female New Zealand white rabbits. Inflammatory mediators and control substances (50-μl samples) were instilled into the anterior chamber of the left eye by intracameral injection, using a 30-gauge lancet-pointed needle mounted on a microsyringe.[15]

Evaluation of Ocular Inflammation

The endocular inflammation was quantitated at various times (1–48 hours) after instillation and clinically graded according to the basic outline of Aronson et al,[16] ie, clinical grading ranging from 1+, representing mild irideal hyperemia and aqueous humor flare, to 4+, representing the presence of fibrinous exudate within the anterior chamber, dense opacity of the cornea, and severe chemosis of the limbic area. At various times after instillation, the rabbits were anesthetized with ketamine (2 mg/kg) plus Acepromazine maleate (Fort Dodge, Iowa) (0.2 mg/kg), and an aliquot of the aqueous fluid (0.1–0.2 ml) was removed from each eye by intracameral tap. Total cells within this fluid were determined by direct hemocytometer count and the relative percentage of polymorphonuclear leukocytes determined following cytocentrifuge preparation and hematoxylin–eosin staining. Following the intracameral tap, the animals were exsanguinated by cardiac puncture. The eyes, both experimental and control, were enucleated, and the anterior segment was removed, fixed in 10% buffered formalin (pH 7.4), and analyzed in paraffin-infiltrated, hematoxylin–eosin-stained 4–5-μ sections by light microscopy.[14]

Histopathologic studies included examination of sections for irideal vascular congestion and edema; cellular and fibrinous exudate in the anterior chamber; the presence of cells at the corneal–irideal angles or within the trabecular meshwork—Schlemm's canal area; and, particularly, morphologic changes of the corneal endothelial cells, which included the retraction/vacuolization of these cells as well as the appearance of polymorphonuclear leukocytes (PMNs) within the endothelial monolayer.

Immunofluorescence

Corneas were frozen in O.C.T. Compound (Lab-Tek products, Naperville, Ill) immediately following enucleation, and 5-μ sections were cut and mounted on gelatin-coated slides. These sections were stained by indirect immunofluorescence techniques with the use of goat anti-rabbit red blood cell antiserum (anti-RBC) (1:20 dilution) and developed with fluorescein-isothiocyanate-conjugated rabbit anti-goat IgG (1:40 dilution) (Cappel Laboratories, Cochranville, Pa).

Inflammatory Mediators and Antibodies

The synthetic chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP) was obtained from Sigma (St. Louis, Mo) and prepared as described previously.[17] The leukocyte chemotactic factor derived from the fifth component of human complement (C5fr) was generated from zymosan-activated human serum containing ε-amino-caproic acid and partially purified by gel filtration.[18] Chemotactic activity was quantitated with rabbit neutrophils and was expressed as ED_{50s}/ml.[17] The fifth component of human complement was purified from human serum by hydrophobic chromatography and hydroxyapatite chromatography[19] and stored frozen. Histamine was diluted from a stock solution of clinical grade histamine phosphate (Eli Lilly, Indianapolis, Ind). Anti-RBC and normal goat serum were obtained from Cappel Laboratories (Cochranville, Pa) and used undiluted or at 1:5 or 1:10 dilutions in Hanks' balanced salt solution (HBSS).

For general evaluation and comparison of results, the reagents used were divided into the following categories of mediators of acute inflammatory mediators: 1) preformed chemotactic factors (fMLP and C5fr); 2) precursors of inflammatory mediators (C5); or 3) agents presumed to act by causing direct damage to anterior segment tissues (anti-RBC) or vasopermeability changes (histamine) in the anterior chamber.

Quantitation of Leukocyte Influx

Leukocyte influx into the aqueous humor was quantitated as the mean (± standard error of the mean [SEM]) number of leukocytes per milliliter of fluid withdrawn from 2–5 rabbits per group. The different groups were compared by expression of the data as a ratio of the average number of leukocytes in mediator-instilled eyes at 24 hours after instillation over the number of leukocytes per milliliter in buffer-instilled eyes. Ratios greater than one thus represent the induction of greater leukocyte influx than that due to buffer alone.

Results

Morphologic and Histopathologic Features of Mediator-Induced Inflammations in the Anterior Chamber of the Eye

We performed the initial experiments to determine the ability of known inflammatory mediators to induce acute inflammatory reactions within the anterior chamber. These reactions were evaluated 24 hours after instillation. Leukocyte influx and clinical grading is summarized in Table 1 and is correlated with the histologic data, which follows.

Control Injections

Histologically the uninstilled eyes exhibited a typical thin monolayer of corneal endothelial cells (Figure 1) with no leukocytes detected within the trabecular meshwork or aqueous humor of the eye.

The instillation of a control buffer (HBSS) led to a low but measurable inflammatory response accompanied by a small influx of leukocytes into the anterior chamber (Table 1). These eyes also exhibited a slight accumulation of PMNs at the corneal–irideal angles and within the trabecular area. Generally, the corneal endothelial cells maintained their normal structure except for small areas that showed slight retraction or vacuolization.

Human serum albumin (HSA) injections induced a slight clinical response that was not significantly different from that induced by buffer alone. In subsequent studies, the effects of inflammatory mediators on cellular influx into the anterior chamber was quantitated by our computing the ratio of the number of cells in aqueous humor of mediator-instilled eyes to the number of cells in buffer-instilled eyes (legend of Table 1).

Instillation of normal goat serum (NGS) (1:5 dilution in HBSS) induced a mild inflammatory response and histologic evidence of moderate irideal hyperemia and small amounts of fibrinous exudate in the anterior chamber. Accumulations of PMNs were seen at the angles and within the trabecular meshwork. In several rabbits given injections of NGS a few PMNs appeared within the corneal endothelial layer, and approximately 40% of the endothelial cells exhibited vacuolization.

Preformed Chemotactic Factors

fMLP

The clinical endocular inflammatory response to the synthetic chemotactic peptide fMLP was mild, as shown by grading at 24 hours after injection, and required much higher doses of chemotactic factor (10^{-4}-10^{-5} M) than can be measured *in vitro* (10^{-9}–10^{-11} M).[17] When lower doses (10^{-6}–10^{-7} M) were used in preliminary experiments, they induced responses indistinguishable from those of buffer-instilled eyes (data not shown). At 10^{-4} M and 10^{-5} M fMLP the clinical grade correlated with the number of leukocytes present in

Table 1—Leukocyte Influx and Clinical Grade 24 Hours Post-instillation of Inflammatory Mediators

Treatment	Cells/ml ($\times 10^{-5}$) ± SEM* of aqueous humor	Experimental† /control	Clinical grade‡
Uninstilled	0		0
Controls			
HBSS only (control)	0.30 ± .04	1.0	1+
Human serum albumin 25 μg/ml (2.5 μg/ml)§	0.25 ± 0.11	0.8	1+
Normal goat serum	1.18 ± 0.31	3.9	1-2+
Chemotactic factors			
f-MLP¶			
10^{-4} M (10^{-5} M)	0.93 ± .27	3.1	2+
10^{-5} M (10^{-6} M)	0.53 ± N.D.**	1.7	1+
C5fr			
5 ED_{50}	18.3 ± 2.4	61.0	3+
15 ED_{50}	14.2 ± 3.2	47.3	3+
25 ED_{50}	1.6 ± 0.9	5.3	2+
Precursors/agents of direct damage			
C5			
1 mg/ml (100 μg/ml)	0.79 ± .01	2.6	1+
0.5 mg/ml (50 μg/ml)	2.62 ± .23	8.7	2-3+
Anti-RBC serum			
0 (1/10)	27.30 ± 11.6	91.0	4+
1:5 (1/50)	7.20 ± 0.29	24.0	4+
1:10 (1/100)	3.12 ± 1.40	10.4	2-3+
Histamine			
10^{-3} M (10^{-4} M)	0.72 ± .15	2.4	2-3+
10^{-4} M (10^{-5} M)	0.71 ± ND	2.4	4+
10^{-5} M (10^{-6} M)	7.21 ± .37	24.0	2-3+

* The average number of leukocytes per milliliter of aqueous humor withdrawn 24 hours after instillation of the inflammatory mediator. Each number represents the mean of leukocytes per milliliter in 2-5 rabbits ± standard error of the mean (SEM).

† Data is expressed as the ratio of the average number of leukocytes per milliliter in aqueous humor withdrawn from mediator-instilled eyes over the number of leukocytes per milliliter in humor withdrawn from eyes instilled with buffer alone.

‡ Clinical grading was accomplished with the use of the 1+ -4+ grading system of Aronson, et al[15] as described in Materials and Methods.

§ Number in parentheses represents the estimated concentration of mediator in the aqueous humor assuming a total aqueous volume of 0.5 ml.

¶ 10^{-6} M and 10^{-7} M fMLP were also instilled into individual rabbits. The leukocyte influx was no different from that in eyes instilled with buffer only.

** Not determined.

the aqueous humor. Histologically, fibrinous exudate appeared within the anterior chamber with accompanying leukocytes. The leukocytes, primarily PMNs, were massed at the corneal-irideal angle and within the trabecular meshwork (Figure 2). The corneal endothelial cells exhibited some retraction or vacuolization (Figure 3), which resembled the irideal vascular endothelial changes noted by Howes and McKay.[8] It is unclear at this time whether these vacuoles are subendothelial or intra-endothelial, ie, whether they directly interface with the aqueous humor.

C5fr

Instillation of 5 and 15 ED_{50}s of C5fr led to a severe inflammatory response accompanied by a large influx of leukocytes (up to 60 times buffer-instilled eyes). The reaction was often accompanied by moderate swelling and opacity of the cornea. At high doses (25 ED_{50}s) the response was greatly diminished when measured both by clinical grade and cellular influx. This may be due to the process of desensitization of leukocytes at very high doses of chemotactic factors.[20] Histologically, 24 hours after C5fr injection (5–15 ED_{50}s), the irideal vessels exhibited hyperemia. An extensive fibrinous exudate was seen in the anterior chamber along with many neutrophils and red blood cells accumulated at the corneal-irideal angle (Figure

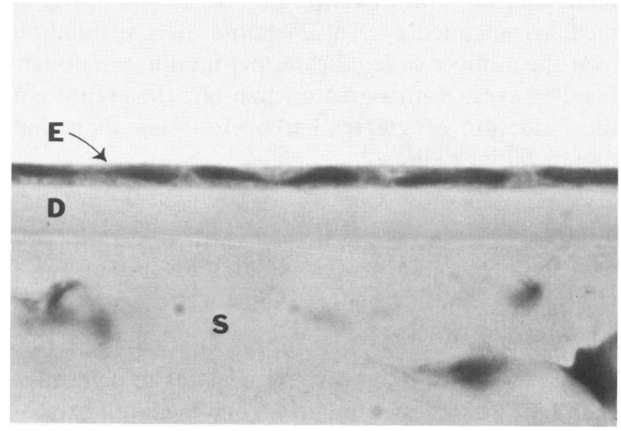

Figure 1—Normal cornea showing flat confluent layer of endothelial cells (*E*). *D*, Descemet's membrane; *S*, Corneal stroma. (H&E, × 1320)

4). Most of the corneal endothelial cells were vacuolated, and some PMNs bound to the luminal surface of the endothelium.

Precursor of Mediator

Whole C5

The instillation of whole human C5 (0.5–1.0 mg/ml) also resulted in a clearly measurable response with occasional opacity and swelling of the cornea. Histologically, there was a buildup of PMNs at the angles, but very little corneal endothelial damage. Only one series of sections from this group showed corneal endothelial vacuolization, and this eye also showed RBCs and fibrinous exudate in the anterior chamber. Similar to fMLP and C5fr doses, high doses of C5 (1 mg/ml) induced a lower clinical grade and leukocyte influx than lower amounts of mediators.

Agents of Direct Tissue Damage and Vasopermeability Changes

Anti-RBC

Indirect immunofluorescence of frozen sections of cornea demonstrated the presence of RBC antigen(s) on rabbit corneal endothelial cells (Figure 5), indicating their similarity to vascular endothelial cells, which exhibit RBC antigens.[21] Anti-RBC was instilled into the anterior chamber in an attempt to create direct cytotoxic injury, as well as immune complex formation *in situ*. A very striking dose response of cellular influx was seen with the use of various dilutions of antiserum (Table 1). At high concentrations this antiserum led to complete opacity and distortion of the cornea and severe chemosis of the limbic vasculature (Figure 6). Following injection of a 1:5 dilution, there was a major buildup of leukocytes at the corneal-irideal angles and large numbers within the trabecular meshwork. A large amount of fibrinous exudate was apparent within the anterior chamber (present at 12 hours [Figure 7] and still at 48 hours [Figure 11B]). At 24 hours after injection, most of the corneal endothelial cells were vacuolized, and many had PMNs adherent to their luminal surface.

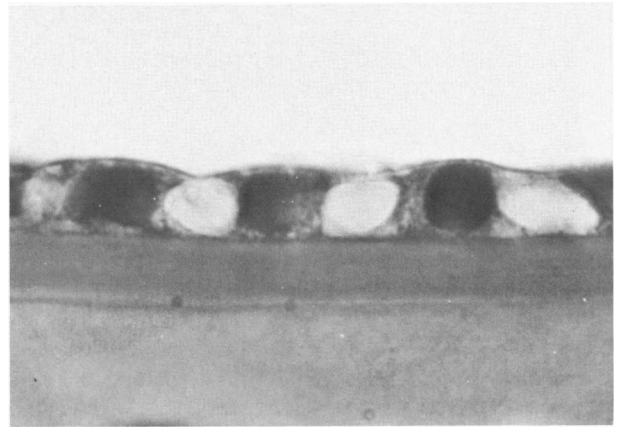

Figure 3—fMLP-treated eye 24 hours after instillation. Note the retraction/vacuolization of the endothelial cells. It is not known at this time whether these are subendothelial spaces or intraendothelial vacuoles. (H&E, × 1320)

Histamine

Histamine was included as a test mediator in these experiments because of the prominent role vasoactive amines play in other immune-complex-mediated reactions.[22] Injections (10^{-5} M) into the anterior chamber led to hyperemia of the irideal vessels and hemorrhage into the chamber. Hence, a large number of red blood cells were found throughout the

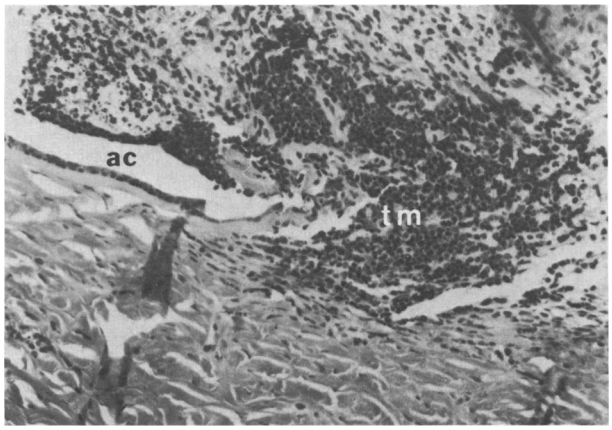

Figure 2—fMLP-treated eye 12 hours after instillation showing moderate accumulation of leukocytes at the corneal-irideal angle and within the trabecular meshwork. *ac*, anterior chamber; *tm*, trabecular meshwork. (H&E, × 320)

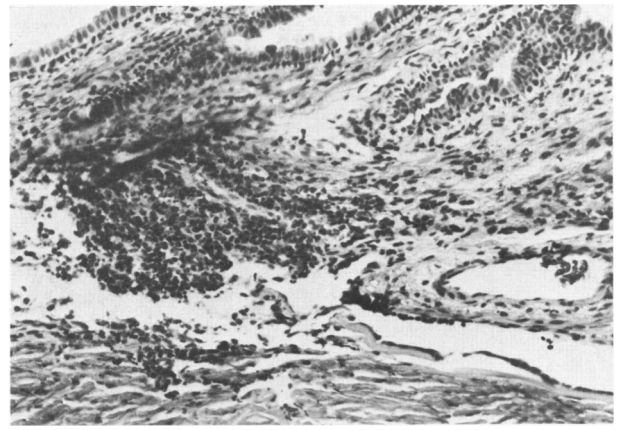

Figure 4—Accumulation of leukocytes and red blood cells in the trabecular meshwork 24 hours after instillation of C5fr. (H&E, × 320)

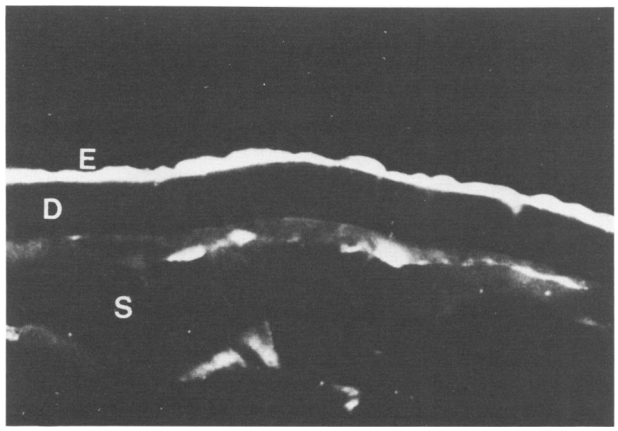

Figure 5—Frozen section of normal cornea stained by indirect immunofluorescence with anti-rabbit RBC. *E*, endothelium; *D*, Descemet's membrane; *S*, corneal stroma.

chamber, accumulating at the corneal irideal angle, and within the trabecular meshwork. In some sections, it appeared that the entire chamber was filled with a fibrin clot. In most sections, almost all of the corneal endothelial cells showed vacuolization, although no PMNs were found attached to or beneath the endothelium. Higher doses of histamine (10^{-4}–10^{-3} M) induced a smaller leukocyte influx, indicating again a possible desensitization process.

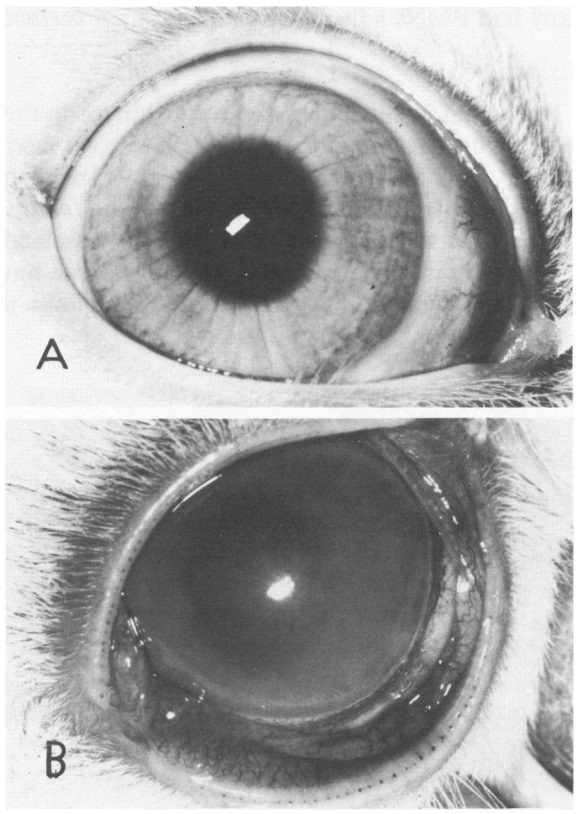

Figure 6A—Normal, uninstilled eye. **B**—Clinical appearance 24 hours after instillation of anti-RBC serum. Note the opacity of the cornea and severe conjestion of the limbal vasculature.

Kinetics of fMLP and Anti-RBC-Induced Endocular Inflammation

By clinical examination of reactions allowed to continue beyond 24 hours, it was apparent that most resolved within 48 hours (see below). Also, in the case of preformed chemotactic factors, and of direct or indirect precursors of same, it appears that observations limited to 24 hours might not permit an accurate description of total changes involved in these reactions. We therefore selected two different types of mediators for a time-kinetic analysis of the endocular response: 1) fMLP (10^{-4} M), the synthetic chemotactic peptide, which acts directly as a chemotactic factor; and 2) anti-RBC antiserum (diluted 1:5), since the response would probably derive from combined tissue damage, complement activation, and other intermediate reactions. Figures 8 and 9 summarize the data on cellular influx in these experiments.

Very early in the course of these reactions, independent of the type of inflammatory effector injected, there was an accumulation of leukocytes (mostly PMNs) at the corneal–irideal angle and within the trabecular meshwork, Schlemm's canal area. This accumulation was also evident in histologic sections made beyond the time of apparent clinical regression of the inflammatory response.

fMLP

Injections of 10^{-4} M fMLP into the eyes induced a moderate clinical reaction as early as 3 hours after instillation, which only rarely progressed to the stage

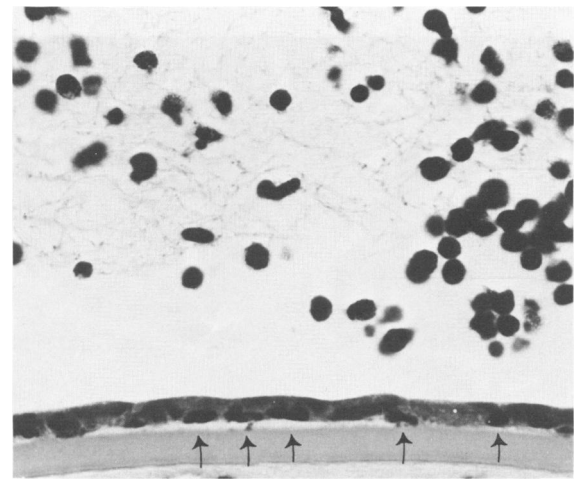

Figure 7—Cornea and anterior chamber 12 hours after instillation of anti-RBC serum. Note the extensive fibrinous/cellular exudate within the anterior chamber and the leukocytes infiltrated into the endothelial layer (*arrows*). (H&E, ×535)

of corneal opacity. Cellular influx (Figure 8), which was acute and began between 1 and 2 hours after instillation, showed a peak level between 5 and 8 hours and rapidly resolved, with very few leukocytes remaining in the aqueous humor at 24 hours. In histologic preparations 1 hour after intracameral injection, there was no evidence of morphologic alterations in the irideal vessels or corneal endothelium. At 2 hours, leukocytes and a few RBCs appeared at the corneal-irideal angles. The corneal endothelium exhibited some subendothelial vacuolization, with PMNs adherent to the endothelial cells (Figure 10A). In moderate inflammatory responses, the endothelial cells appeared by light microscopy to maintain confluency, even when virtually all were associated with vacuoles. As the reaction proceeded to 3–4 hours, there was an increase in endothelial vacuolization, which seemed to begin near the corneal-irideal angle and extended to the central cornea, and an increase in the numbers of leukocytes (mostly PMNs). A fibrinous exudate appeared by 3 hours and increased in amount until the inflammatory reaction peaked, then slowly dissipated. At 8 hours, virtually all of the endothelial cells exhibited vacuoles (Figure 10B), exudate filled the anterior chamber, and a large number of leukocytes were present. Significantly, virtually all the endothelial vacuoles contained PMNs. Except for an occasional RBC, no other cell types have been seen within these spaces. At 12 hours, exudates still filled the anterior chamber, PMNs were still prevalent, and all of the endothelial cells remained vacuolized; however, PMNs were found at the luminal surface of the endothelium, not in the vacuoles (Figure 10C). By 24 hours, the inflammation was decreasing, the exudate was partially cleared, and leukocyte numbers were decreased, but the endothelial cells had not yet regained their normal morphologic features. At 48 hours there was little histologic evidence of the inflammatory response, except for a small amount of fibrinous exudate on the anterior surface of the iris.

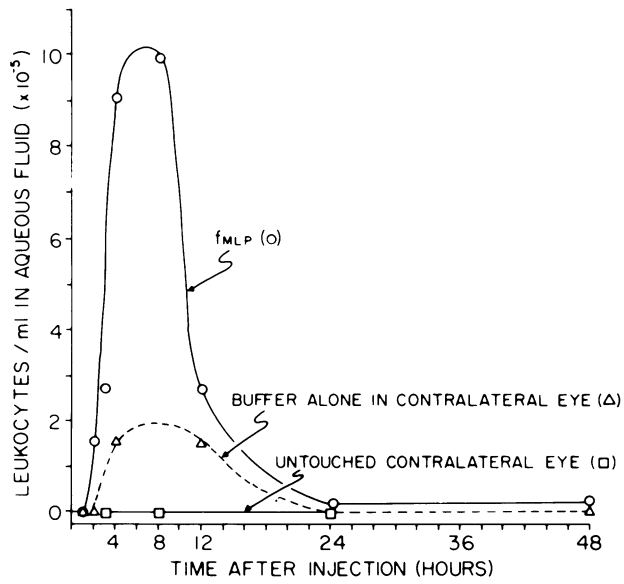

Figure 8—Time kinetics of leukocyte influx into anterior chamber following fMLP instillation.

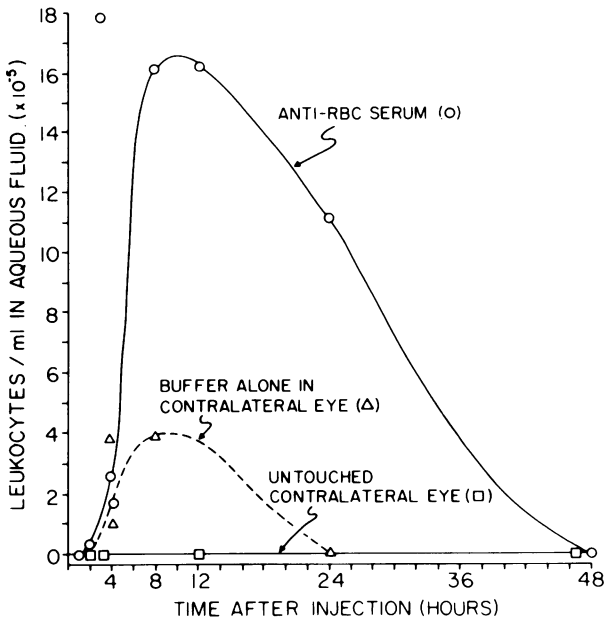

Figure 9—Time kinetics of leukocyte influx into anterior chamber following anti-RBC serum instillation.

Anti-RBC

As in the case of fMLP, inflammation resulting from anti-RBC (1:5) injection was clinically detectable within 3 hours of instillation but progressed in many cases to complete opacity of the cornea by 24 hours (Figure 6). There was also an apparent secondary glaucoma, as determined by distortion of the corneal curvature. Figure 9 shows that the cellular influx in response to anti-RBC peaked at 8–12 hours, slightly later than that following fMLP injection. In general, the anterior chamber of anti-RBC–instilled eyes was much more slowly cleared of leukocytes than that of eyes in which fMLP was instilled.

The histologic changes observed in eyes in which anti-RBC was instilled generally paralleled those seen after fMLP injection, with a few significant differences: 1) endothelial vacuolization was more variable, appearing to occur in patches rather than all the way across the cornea; 2) by 8 hours only a few PMNs appeared adherent to endothelial cells, and massive migration of PMNs into the endothelial layer was not seen until 12 hours after injection (Figure 11A); 3) at 24 hours after instillation, the endothelial cells re-

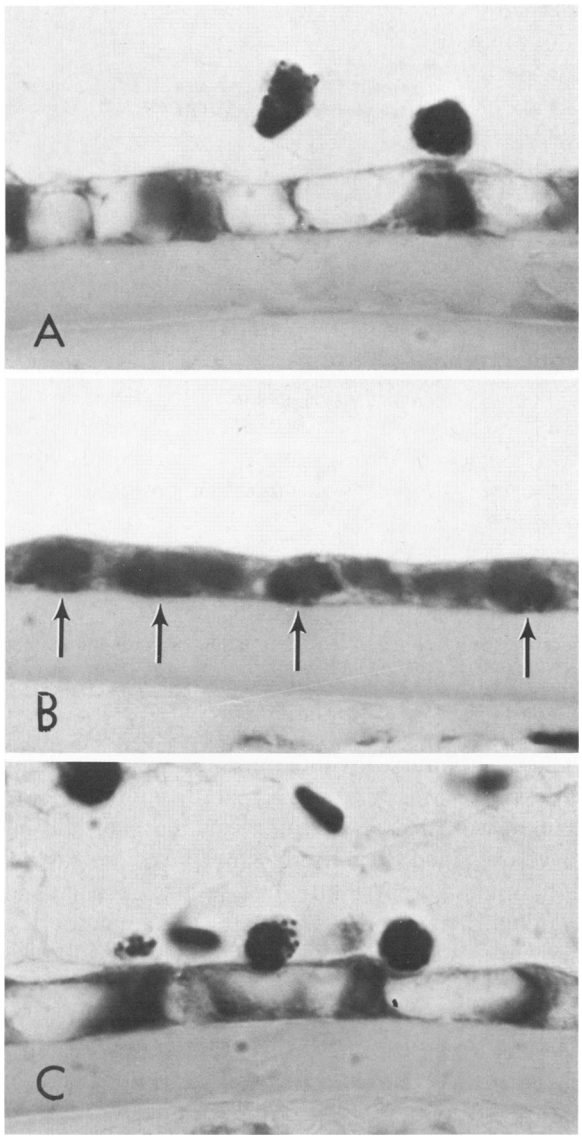

Figure 10—Sequence of corneal endothelial changes during the course of acute inflammatory responses induced by intracameral instillation of fMLP into the anterior chamber. **A**—Two hours following instillation, the endothelial cells have lost their normal flat, confluent appearance and appear to have become vacuolated or retracted from one another. Leukocytes are seen attached to endothelial cell surface at this timepoint. However, the endothelial changes appear prior to the appearance of large numbers of leukocytes. **B**—Eight hours after instillation, at the peak of leukocyte influx, virtually all of the endothelial vacuoles are occupied by leukocytes (*arrows*). **C**—Twelve hours after instillation, shortly following the peak of the inflammatory response, the leukocytes have migrated back out of the endothelial layer. By 48 hours after instillation of fMLP the endothelial layer has regained its normal morphologic characteristics. (H&E, ×1320)

mained vacuolated, but these vacuoles appeared empty except for an occasional leukocyte; and 4) fibrinous exudate remained extensive and was not cleared completely after 48 hours (Figure 11B).

Contralateral Eye Controls

In the time-kinetic experiments the eye contralateral to that in which mediator was instilled remained untouched ("untreated control") or received an injection of control buffer ("buffer-treated control"). Leukocyte influx into these "control" eyes is also shown in Figures 8 and 9. Untreated control eyes demonstrated no leukocyte influx in these experiments. In contrast, buffer-treated control eyes in these two experiments demonstrated a definite influx of leukocytes, with the time kinetics of influx resembling those of fMLP-treated eyes. While the number of leukocytes in buffer-treated control eyes was small, when contralateral to mediator-instilled eyes, it was 6–12-fold greater than seen when a buffer-treated control was contralateral to an untreated control eye (Table 1). These observations suggest the possibility of reflexive vascular communication between the eyes, similar to that which has been suggested to exist between the lobes of the lungs.[14] This type of communication was further suggested in experiments (data not shown) in which a severe inflammatory reaction was induced in eyes contralateral to untreated control eyes. Leukocyte influx was seen in the untreated control but never exceeded 2×10^3 cells per milliliter of aqueous humor.

Discussion

We initiated the present studies in order to begin a systematic delineation of the processes influencing acute and chronic anterior chamber inflammation and tissue injury, ie, corneal endothelium. The use of inflammatory mediators that result in PMN-rich inflammatory reactions at other tissue sites allows one to develop a set of reproducible qualitative measures of acute inflammatory reactions in the eye. Thus, we believe these studies confirm and extend two recent reports of endocular inflammatory activity induced by synthetic and preformed chemotactic factors.[23,24]

These studies provide *in vivo* and correlated *in vitro* evidence that endocular inflammation may be induced by individual preformed chemotactic factors, suggesting that at least some forms of anterior segment inflammation may develop via inflammatory mechanisms common to other body tissues. This model promises to be very useful for planned studies of the action and control of endocular inflammatory responses. This anterior segment model may also provide an ideal tissue site for the *in vivo* study of the action of other categories of biologic mediators that are not easily quantifiable in other tissues.

Previously, biologically relevant chemotactic factors were believed to be largely derived from vascular elements, the complement system (C3 and C5 split-products), or from antigen-stimulated lymphoid cells.[25] For example, C5a and C5a desArg/helper factor appear to represent some of the C5-derived chemotactic factors (C5fr) found in serum.[13] Recently chemotactic activity has been associated with certain

split, degradation, and denaturation products of fibrin, hemoglobin, collagen, and arachidonic acid.[26] Also, cell-derived chemotactic factors have been described, including those elaborated from alveolar macrophages. Macrophage-derived chemotactic factors are thought to play a role in the induction of acute pulmonary inflammation.[14] The most direct *in vivo* evidence that chemotactic factors induce important biologic consequences is from our experiments in which intrapulmonary instillations of preformed chemotactic factors consistently induced acute local cellular inflammatory reactions within the alveoli of experimental animals.[14]

Preformed Chemotactic Factors and Complement

The synthetic peptide fMLP, thought to be an analog of the naturally occurring bacterial factor produced by *Escherichia coli*,[27] is active in the induction of acute inflammatory reactions *in vivo*.[14,24] The usefulness of this peptide in the study of inflammatory responses is confirmed in the anterior chamber in the above experiments by the acute influx of leukocytes and the similarity of the morphologic changes to that induced by the immune complex type of reaction to anti-RBC injection.

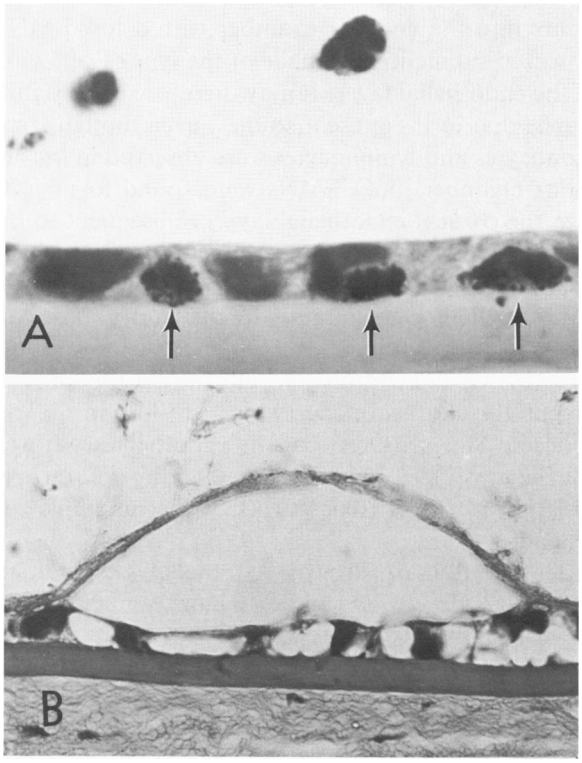

Figure 11—Corneal endothelium following anti-RBC instillation. **A**—Cornea 12 hours after instillation. Alterations of corneal endothelial cells follow the same sequence as in instilled eyes except that leukocyte migration into the endothelial vacuoles occurs slightly later (12 hours versus the 8-hour peak seen in Figure 10B). (H&E, × 1320) **B**—Forty-eight hours after instillation. The endothelial changes induced by inflammatory responses to anti-RBC serum are longer-lasting than those induced by preformed chemotactic factors, as seen here by the remaining fibrinous exudate and endothelial damage. (H&E, × 525)

The latter response presumably involves the activation of the complement cascade, including C3 and C5 cleavage to their chemotactic products, C3a and C5a. The involvement of complement in endocular inflammation has been implicated in a number of studies of human aqueous humor and cornea by the demonstration of both classical and alternate pathway complement components.[28,29] The potential of C5 to induce or enhance endocular inflammation is shown by both C5 and C5fr instillation in the present studies. C5fr may act by the direct induction of leukocyte influx and leads to a vast number of leukocytes within the aqueous fluid at 24 hours after instillation (2–20 times that seen with fMLP at 24 hours), suggesting that C5fr may be much more active in the eye *in vivo* than the synthetic peptide fMLP. However, as suggested by the time-kinetic data of fMLP and anti-RBC, this difference may be due to differences in influx kinetics over time. This emphasizes the need to examine the time kinetics of inflammatory responses induced by various inflammatory mediators, especially when one is attempting to compare the role each plays in the reaction. Peak response time differences may also aid in the dissection of the mechanisms involved in these responses and may be critical in terms of the development of therapeutic approaches to decrease the endocular damage resulting from these individual responses.

C5

The C5-induced inflammatory response may suggest the possible presence of enzymatic activities within the anterior segment of the eye with the potential to cleave C5 into its active chemotactic fragment, C5a. This type of tissue-derived specific C5-cleaving activity has been suggested in the lung.[14] It is also known that leukocytes contain an enzyme, elastase, which will cleave C5, yielding chemotactic factors.[30,31] Thus an early leukocyte influx initiated by the trauma of injection may lead to C5 cleavage. It is also possible that damage to resident cells of the anterior segment (the endothelial cells?) leads to the production or release of C5-cleaving enzymes or alternately to the production of other chemotactic mediators. With the use of this and other models under development, these possibilities should be discernible.

Anti-RBC and Histamine

The presence of RBC antigen on the corneal endothelial cells (Figure 5) allows one to induce a cytotoxic or immune-complex reaction within the anterior

chamber without the necessity of antigen challenge and rechallenge. This facilitates the direct comparison of antibody-mediated reactions with reactions induced by preformed inflammatory mediators within the anterior chamber. The use of anti-RBC antiserum is proposed as an alternate model of inducing inflammation within the anterior chamber. The clinical changes observed in our experiments are similar to those seen in uveitis of several causes. In addition, intracorneal and intravitreal injection of fMLP and C5a induce similar gross morphologic changes when compared with ovalbumin injection in sensitized rabbits.[24] While these experiments[24] did not examine the anterior chamber or corneal endothelial cells, the time kinetics of response corresponded closely with our own.

While anti-RBC antiserum probably acts by binding to antigen on the corneal endothelial cells and activating the complement cascade with subsequent direct cytotoxicity, antibody-dependent cell-mediated cytotoxicity may also be involved. However, there also may be no absolute requirement for complement activation. Direct interaction of antibody with the endothelial cells may induce the production or secretion of endothelial cell-derived chemotactic factors. Whatever the specific mechanisms of induction and inflammation and endothelial cell alteration in this anti-RBC model, the clinical and morphologic changes were in general quite similar to those induced by preformed chemotactic factors, suggesting that the mechanisms underlying endothelial damage may be similar.

In antibody-induced reactions, as well as in reactions induced by direct injection of C5 or C5fr, damage may also be enhanced by the potential stimulation of histamine release from mast cells,[32] resident in the limbic area. Such a release of histamine, a potent vasoactive amine with actions on both vascular smooth muscle cells and endothelial cells,[22] may lead to a flooding of plasma proteins and blood cells into the anterior chamber. Histamine may also directly affect the retraction of the corneal endothelial cells. This is suggested by the increased corneal edema and opacity observed following direct histamine injections. It is unlikely that this is the only reaction, since retraction alone would not account for the percentage of polymorphonuclear leukocytes in the cellular influx (up to 97% PMNs at 24 hours after histamine injection), suggesting that alternate chemotactic factors may be generated during this reaction.

The mode of action of histamine in these responses may be investigated further by simultaneous instillation of H_1 and H_2 receptor antagonists. The model can also be used to evaluate vascular changes—ie, irideal vasculature permeability changes versus alterations in corneal endothelial cells (discussed below).

Morphologic Alterations

The changes seen in corneal endothelial cells by light microscopy appear to follow an ordered sequence that is independent of the mediator instilled. Early in the course of the reaction these cells lose their smooth, flat appearance and become vacuolated. This occurs before the appearance of a large number of leukocytes in the anterior chamber, suggesting that this reaction may not be due to toxic effects of leukocyte products, but this possibility cannot be excluded. As the reaction proceeds, leukocytes appear adherent to the corneal endothelial cells, and at the peak of leukocyte influx many are found within the endothelial vacuoles. It has been shown *in vitro* that leukocytes do not penetrate Descemet's membrane.[33] Cellular migration into the corneal endothelial layer is not limited to acute inflammatory responses, as seen in the published micrographs of lymphokine-induced anterior segment inflammation.[34] These lymphokine reactions are reported to induce a mononuclear cell influx into the anterior chamber with a few PMNs; however, no mention is made of the type of cell within the endothelial layer. It may therefore be of significance that in the present studies, even though some monocytes and lymphocytes were observed in the anterior chamber, only PMNs were found to migrate into the corneal endothelial layer. Subsequent to the peak of leukocyte influx, they appear to migrate back out of the endothelial layer and are removed from the anterior chamber apparently by the natural flow pattern of aqueous humor. No evidence was seen of destruction of the endothelial cells during this process except for short stretches which are lost in 4+ responses. This suggests that the endothelial cells are, for the most part, able to retain viability and recover function after the toxic effects of the inflammatory reaction.

This pattern of corneal endothelial morphologic changes, followed by leukocyte influx and efflux and endothelial recovery, suggests that while the corneal endothelial cells may be damaged (as demonstrated by the edema and opacity of the cornea), they may also be activated in response to the inflammatory process. This activation may include the production of both fibrinogenic and fibrinolytic activities, tissue-produced chemotactic factors or inhibitors, or other specific endocular mediators that aid rapid restoration of corneal endothelial function.

Implications and Future Uses of This Model System

Using this and similar models, we may delineate the role played by specific inflammatory mediators and processes in anterior segment responses easily and quantifiably. This anterior chamber inflammation model, along with the more complicated constant perfusion model of Battacherjee et al,[23] should be extremely useful in numerous studies of inflammatory mediators. The normal absence of leukocytes in the aqueous humor and the ease with which samples of fluid may be obtained make these models relatively easy to quantify.

Models of anterior chamber inflammation may be particularly useful in elucidating the factors involved in the development of vascular permeability changes and tissue edema due to the nature of the tissues surrounding this chamber, ie, the vascular tissue of the ciliary body and iris and the nonvascular endothelial layer of the cornea. Use of our model may specifically allow further definition of the "two-mediator hypothesis" of increased vascular permeability,[35] which proposes a leukocyte-dependent mediator of endothelial permeability change coupled with a mediator of vascular dilation through actions on the vascular smooth muscle cells. One of the leukocyte-dependent mediators is thought to be C5a, whereas the enhancing factor, or smooth muscle mediator, may be an arachidonic acid product such as PGE_2.[35] Evidence in favor of the two-mediator hypothesis was obtained by the influx of leukocytes into the anterior chamber in response to fMLP and leukotriene B_4 instillation.[23] Arachidonic acid and PGE_2, however, induced an increase in intraocular pressure that was suggested to be due to effects on vascular permeability.[23]

In our model, mediators that affect the vascular endothelial cells may be found to cause similar alterations of the corneal endothelial cells. Morphologically, the changes in corneal endothelial cells in the present studies appear to be similar to those seen in vascular endothelium in inflammatory processes.[36] In addition, the morphologic damage of corneal endothelial cells appeared prior to the detection of leukocyte attachment to the endothelial layer, suggesting that direct contact of PMNs and endothelial cells may not be necessary for endothelial permeability change. This is in contrast to the hypothesis of Wedmore and Williams, who suggest that direct PMN contact may be necessary[35] but does not eliminate the possibility of "direct-action mediators" such as histamine or bradykinin.[35] Using the corneal endothelial cells as analogs of the vascular endothelial cells, our model may thus allow the analysis of factors mediating endothelial change with confluent endothelial cells on their normal basement membrane, Descemet's membrane, in the absence of the smooth muscle effects accompanying vascular tissue. At the same time, vascular permeability effects may be monitored by the appearance of intravenously administered radiotracers, such as ^{125}I-albumin, in the aqueous humor, and correlated with corneal endothelial changes. Depletion of inflammatory components such as complement, fibrinogen, and leukocytes, and inhibition of the cyclooxygenase and lipoxygenase pathways, may be done prior to intracameral induction of responses to evaluate the role of these components in vascular permeability and endothelial changes.

In addition to the use of our model system in elucidating the mechanisms and mediators of induction of inflammatory processes, the model should also prove valuable in the analysis of processes involved in recovery and healing of anterior segment tissues. In particular, it may be possible to evaluate the role played by growth factors and connective tissue proteins in this process by either instilling these factors along with inflammatory mediators or looking for their appearance in the aqueous humor during the healing process. The presence of fibronectin in the aqueous humor has been demonstrated,[37] although its function still remains speculative. Use of our anterior segment model to evaluate changes in the levels of connective tissue proteins during inflammatory processes may provide an easily quantifiable system that could lead to significant insights into the mechanisms involved in healing processes, not only of corneal endothelial cells, but also of the general processes that occur at sights that are not as accessible.

In summary, we have developed a simple, quantifiable model for evaluation of acute inflammatory responses in the anterior segment. The model includes the correlation of clinical observations with quantitation of inflammatory responses by leukocyte influx, and, in addition, with corneal endothelial cell morphologic change or damage. This model should prove valuable in the elucidation of mediators and mechanisms involved in inflammatory responses in general in an easily accessible tissue site, as well as the dissection of mechanisms that may be unique to the eye.

References

1. Friedlander MH: Allergy and Immunology of the Eye. Hagerstown, Harper & Row, 1979
2. Williams BD, Lehner T: Immune complexes in Behçet's syndrome and recurrent oral ulceration. Br Med J 1977, 1:1387-1389

3. Zubler RH, Nydegger U, Perrin SH, Fehr K, McCormick J, Lambert PH, Miescher PA: Circulating and intra-articular immune complexes in patients with rheumatoid arthritis. J Clin Invest 1976, 57:1308–1319
4. Howell SB, Epstein WV: Circulating immunoglobulin complexes in Wegener's granulomatosis. Am J Med 1976, 60:259–268
5. Waksman BH, Bullington SJ: A quantitative study of the passive Arthus reaction in the rabbit eye. J Immunol 1956; 76:441–453
6. Silverstein AM, Zimmerman LE: Immunogenic endophthalmitis produced in the guinea pig by different pathogenetic mechanisms. Am J Ophthalmol 1959, 48: 435–447
7. Levine RA, Ward PA: Experimental acute immunologic ocular vasculitis. Am J Ophthalmol 1970, 69: 1023–1031
8. Howes EL, McKay DG: Circulating immune complexes: Effects on ocular vascular permeability in the rabbit. Arch Ophthalmol 1975, 93:365–370
9. Gamble CN, Arsonson SB, Brescia FB: Experimental uveitis: II. The pathogenesis of recurrent immunologic (Auer) uveitis. Arch Ophthalmol 1970, 84:331–341
10. Dernouchamps JP, Michiels J: Circulating antigen-antibody complexes in aqueous humor, Immunology and Immunopathology of the Eye. Edited by AM Silverstein, GR O'Connor. New York, Masson, 1979, pp 40–45
11. Johnson KJ, Ward PA: Acute immunologic pulmonary alveolitis. J Clin Invest 1974, 54:349–357
12. Scherzer H, Ward PA: Lung and dermal vascular injury produced by preformed immune complexes. Am Rev Respir Dis 1978, 117:551–557
13. Johnson KJ, Anderson TP, Ward PA: Suppression of immune complex-induced inflammation by the chemotactic factor inactivator. J Clin Invest 1977, 59:951–958
14. Desai U, Kreutzer DL, Showell H, Arroyave CH, Ward PA: Acute inflammatory pulmonary reactions induced by chemotactic factors. Am J Pathol 1979, 96:71–83
15. O'Rourke J, Lindsay M, Reddy S: Use of needle crimping to seal limbal punctures technical note. Ophthal Res 1980, 12:57–63
16. Aronson SB, Howes EL, Fish MB, Pollycove M, O'Day DN: Ocular blood flow in experimentally induced immunogenic uveitis. Arch Ophthalmol 1974, 91:60–65
17. Kreutzer DL, O'Flaherty JT, Orr W, Showell HJ, Ward PA, Becker EL: Quantitative comparisons of various biological responses of neutrophils to different active and inactive chemotactic factors. Immunopharmacology 1978, 1:39–47
18. Fernandez HM, Henson PM, Otani A, Hugli T: Chemotactic response to human C3a and C5a anaphylatoxins: I. Evaluation of C3a and C5a leukotaxis *in vitro* and under simulated *in vivo* conditions. J Immunol 1978, 120:109–115
19. Tack BF, Prahl JW: Third component of human complement: Purification from plasma and physiochemical characterization. Biochemistry 1976, 4513–4521
20. Becker EL, Henson PM: *In vitro* studies of immunologically induced secretion of mediators from cells and related phenomena. Adv Immunol 1973, 17:94–193
21. Szulman AE: The histological distribution of blood group substances A and B in man. J Exp Med 1960, 111:785–800
22. Majno G, Shea SM, Leventhal M: Endothelial contraction induced by histamine-type mediators. An electronmicroscopic study. J Cell Biol 1969, 42:647–672
23. Bhattacherjee P, Hammond B, Salmon JA, Stepney R, Eakins KE: Chemotactic response to some arachidonic acid lypoxygenase products in the rabbit eye. Eur J Pharmacol 1981, 73:21–28
24. Ben-Zvi A, Rodriguez MM, Gery I, Schiffmann E: Induction of ocular inflammation by synthetic mediators. Arch Ophthalmol 1981, 99:1436–1444
25. Ward PA, Cochrane CG: Bound complement and immunologic injury of blood vessels. J Exp Med 1965, 121:215–234
26. Leukocyte Chemotaxis: Methods, Physiology and Clinical Implications. Edited by JI Gallin, PG Quie. New York, Raven Press, 1978
27. Schiffman E, Corcoran BA, Wahl SM: N-formylmethionyl peptides as chemoattractants for leukocytes. Proc Natl Acad Sci USA 1975, 72:1059–1062
28. Mondino BJ, Rattajczak HU, Goldberg DB, Schanzlin DJ, Brown SI: Alternate and classical pathway components of complement in normal cornea. Arch Ophthalmol 1980, 98:346–349
29. Martenet A-C, Bischoff PM, Grob PJ, Fierz W: Immune complexes and uveitis. In: Immunolgy and Immunopathology of the Eye. Edited by AM Silverstein, GR O'Connor. New York, Masson, 1979, pp 55–57
30. Ward PA, Hill JH: Biologic role of complement products. Complement-derived leukotactic activity extractable from lesions of immunologic vasculitis. J Immunol 1972, 108:1137–1145
31. Brozna JP, Senior RM, Kreutzer DL, Ward PA: Chemotactic factor inactivators of human granulocytes. J Clin Invest 1977, 60:1280–1288
32. Grant JA, Settle L, Wharton EB, Dupree E: Complement-mediated release of histamine from human basophils: II. Biochemical characterization of the reaction. J Immunol 1976, 117:450–456
33. Basu PK, Minta JO: Chemotactic migration of leukocytes through corneal layers. An *in vitro* study. Canad J Ophthalmol 1976, 11:235–240
34. Chandler JW, Heise ER, Weiser RS: Induction of delayed-type sensitivity-like reactions in the eye by the injection of lymphokines. Invest Ophthalmol 1973, 12: 400–409
35. Wedmore CV, Williams TJ: Control of vascular permeability by polymorphonuclear leukocytes in inflammation. Nature 1981, 289:646–650
36. Howes EL, McKay DG: Ocular changes in the generalized Schwartzman reaction. Arch Ophthalmol 1973, 90:218–224
37. Reid T, Kenney MC, Waring GOI: Isolation and characterization of fibronectin from bovine aqueous humor. Invest Ophthalmol Vis Sci 1982, 22:57–61