# INFLAMMATORY MECHANISMS IN CORNEAL ULCERATION*

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## GENERAL BACKGROUND

THERE ARE NO CLINICAL SITUATIONS MORE FRUSTRATING TO THE CORNEAL SPECIALIST than the relentless ulcerative destruction of the herpetic cornea that has been cleared of virus, the arthritic patient's dry eye that has been copiously lubricated, or the chemically burned cornea that has been carefully encouraged to reepithelialize. This sterile degradation process, involving the stromal collagen and ground substance, is clinically termed corneal "melting." Beyond these conditions encountered frequently in western ophthalmic practice, the extraordinarily rapid corneal melting of keratomalacia as a consequence of vitamin-A deficiency is a major public health problem in developing countries.[1]

Whatever the etiology, sterile corneal ulceration is almost invariably preceded by an epithelial defect and appears associated with an inappropriate inflammatory response or failure of normal wound healing processes, or both. Berman[2] has suggested that corneal ulceration "might be related to the trapping of wound healing in a phase of proteolytic debridement related to a persistent epithelial defect." Such ulceration is known to be mediated through the action of tissue collagenolytic enzymes that perform the initial cleavage of stromal collagen fibrils, with further degradation of collagen and glycosaminoglycans involving proteases, peptidases, and cathepsins.[3-5]

## EPITHELIAL INJURY AND RECOVERY

Since injury and persistent or recurrent defects of the epithelium always precede stromal ulceration, the effect of hormones, biochemical messen-

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gers, and pharmacologic agents on corneal epithelial wound healing has long been of interest. Classic studies of Friedenwald and Buschke[6,7] demonstrated trophic influences on corneal epithelial healing, as topically administered epinephrine would reduce epithelial cell locomotion, mitosis, and wound healing. In other systems, these trophic effects of epinephrine and norepinephrine are mediated by the intracellular second messenger, cyclic 3′-5′ adenosine monophosphate (cyclic AMP or cAMP).[8-12] Acetylcholine and other cholinergic substances have been demonstrated to have opposing effects on cell proliferation and locomotion through the second messenger, cyclic 3′-5′ guanylic acid (cyclic GMP).[13] This hypothesized antagonism between cyclic AMP and cyclic GMP on the growth and turnover of corneal epithelial cells was summarized by Cavanagh et al[14]; who have also shown in abraded rabbit corneas the enhancement of epithelial regeneration by the cholinergic agents, carbachol and phospholene iodide, and the inhibition of epithelialization by dibutyryl cAMP and theophylline.[15,16] The findings of others,[17,18] however, have been less convincing, and so the role of cholinergic and adrenergic drugs and their cyclic nucleotide second messengers in corneal epithelial wound healing is still incompletely resolved.

#### INFLAMMATORY CELLS, INTERACTIONS, AND MEDIATORS

Cells and their enzymatic products are necessary for stromal destruction, since chemically injured stromas do not disintegrate when their acellularity is maintained by freezing[19] or by the use of tissue adhesives.[20] Although explants of epithelium from ulcerating corneas are able to lyse collagen gels,[21,22] pure corneal epithelium alone does not secrete substantial levels of collagenase.[23] Stromal keratocytes have been observed to produce soluble latent collagenase,[24] and recent reports have stressed the synergism between epithelium and fibroblasts in this respect.[25] Our own and others' pathologic and experimental studies[19,26-29] (detailed in Previous Studies By This Investigator), however, have emphasized the role of acute inflammatory cells, particularly polymorphonuclear neutrophils (PMN) and mononuclear macrophages.[30] These cells contain more than a dozen hydrolytic enzymes, both acid hydrolases and neutral proteases (the latter including collagenase, elastase, and cathepsin), within their primary lysosomes[31-36] and are ubiquitous at the site of active stromal ulceration[37] and in the tear film[38] of melting corneas. Recently, the latent collagenase and gelatinase of human PMN have been more completely characterized.[39] These enzymes have the combined capacity to degrade and destroy most phagocytosed substances but also have considerable potential for degradation of connective tissue upon release from the

PMN.[40] Free extracellular granules have also been described in inflamed connective tissue,[41] wound healing sites,[42] delayed hypersensitivity, and Arthus reactions.[43] The activity of PMN enzymes in collagen-vascular disease has been suggested by the demonstration of cartilage proteoglycan degradation by human leukocyte granule proteases.[44]

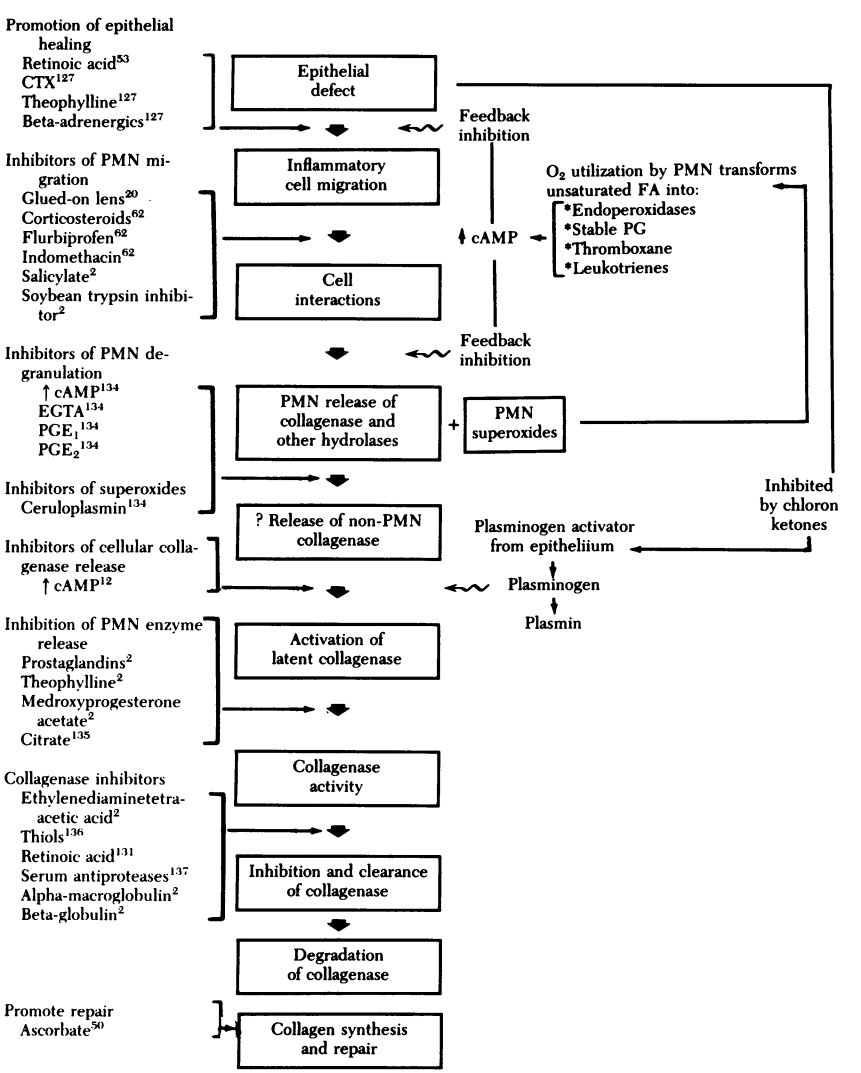
Agents affecting PMN chemotaxis are of great interest. Rivkin et al[12] demonstrated the role of cAMP in the chemotactic responsiveness of rabbit peritoneal PMN; $PGE_2$, $PGA_1$, epinephrine, isoproterenol, and theophylline (all of which elevate cAMP levels in PMN) were found to inhibit neutrophil migration. More recently, Srinivasan and Kulkarni[45] have demonstrated the role of arachidonic acid metabolites in the mediation of the PMN response following corneal injury and have shown the PMN response to be effectively blocked by corticosteroids and nonsteroid antiinflammatory (NSAI) agents. Some prostaglandins also have the capacity to stimulate adenyl cyclase and inhibit the effector function of several inflammatory cell types, including inhibition of selective lysosomal-enzyme release from stimulated PMN.[12,46] For example, the combined use of theophylline (a phosphodiesterase inhibitor that serves to maintain cellular cAMP levels) and $PGE_1$ in adjuvant-induced arthritis prevents inflammation and cartilage damage.[47]

The recent demonstrations[48,49] that plasminogen activator is released by the epithelium, fibroblasts, and PMN of injured corneas suggests the mechanism by which plasmin would alter complement and generate chemotactic factors for PMNs. The finding that plasmin[48] as well as cAMP is also capable of activating latent collagenase from the ulcerating cornea confirms the complexity of interactions among multiple cellular and humoral factors. Several of these interactions are summarized in Table I.

#### STROMAL REPAIR

On the reparative side, the roles of keratocytes and blood vessels are critical. Reactive fibroblasts are dilated cisternae of rough endoplasmic reticulum participate in the secretion of new collagen for wound healing, although their presence within the central corneal ulcerative areas is delayed. Equally apparent from cell culture, however, is that such fibroblasts can simultaneously synthesize both collagen and collagenase, thereby allowing the same cells to participate in both ulceration and repair (reviewed by Berman[2]). Pfister and Patterson[50] observed that the anterior segments of alkali-burned eyes are scorbutic and established the rationale for providing supplementary ascorbate to facilitate the hydroxylation and secretion of collagen. The clinically well-known ability of stromal neovascularization to inhibit ulceration has been further explored by

TABLE I: CELLULAR EVENTS AND INTERACTIONS IN CORNEAL ULCERATION*




*Adapted from Berman.[2]

Conn et al,[51] who found that corneas prevascularized by tumor-angiogenesis factor were less likely to ulcerate following experimental thermal burns. It is likely that both serum antiproteases (such as $\alpha_2$-macroglobulin and $\alpha_1$-antitrypsin) and nutrients (including ascorbate and retinoids) are delivered by vessels to the ulcer area.

#### THERAPEUTIC IMPLICATIONS

Therapeutic approaches to the management of sterile corneal ulcerations are somewhat varied but basically focus on the three phases of (1) determining etiology and initiating primary therapy, (2) promoting epithelial healing, and (3) limiting ulceration and supporting repair.[52]

It is widely appreciated, both clinically and experimentally, that various pharmacologic agents can alter the balance between destructive and reparative processes. Thus, agents that promote epithelial healing have the potential for limiting ulceration. Smolin and Okumoto[53] have shown retinoic acid-derivatives to be effective in the closure of epithelial defects in the rabbit cornea. Tseng and co-workers[54] have recently demonstrated the effect of topical retinoids in healing persistent epithelial defects in human cornea, and agents that elevate cAMP (β-adrenergics, theophylline, cholera toxin) also have this potential. Nishida et al[55,56] have presented experimental and clinical evidence that topically applied fibronectin may be similarly beneficial. The ability of topical corticosteroids to suppress anterior-segment inflammation[57] and thereby promote epithelial recovery and limit inflammatory cell-mediated structural alteration[58] can be highly beneficial. Indeed, corticosteroids do not enhance collagenase activity in vitro[59] and, experimentally, early application of intensive topical corticosteroids does not accelerate the corneal ulceration produced by alkali burns.[60] On the negative side, however, they can suppress repair and neovascularization to the extent of facilitating further ulceration and potentiating infection. In this respect, NSAI agents, such as indomethacin[61,62] and flurbiprofen,[63,64] merit further ocular investigation.

Progestational corticosteroids have a potentially important differential pharmacologic effect, as they may reduce inflammation with less concomitant suppression of wound repair than corticosteroids, and indeed the effectiveness of medroxyprogesterone acetate has been reported in the chemically burned rabbit cornea.[65] Immunosuppressive therapy with antimetabolites or high-dose corticosteroids, or both, is gaining interest for ulcerative disorders with a presumed autoimmune etiology.[66,67] The use of cyanoacrylate tissue adhesives has been extensively practiced for nearly 15 years as an adjunct to the management of corneal perforations.[68]

We have additionally noted that application of tissue adhesive alone to sterile ulcers arrests further stromal loss in the same manner as does a glued-on contact lens.[38] Hormones[69,70] and other specific collagenase inhibitors[71-73] have minimal clinical application; the efficacy, for example, of acetylcysteine[74] in human corneal melting has not yet been convincing. In light of the rather poor prognosis for corneal ulcerations and perforations managed by current techniques, there is need for additional investigations of pharmacologic and mechanical means to restore the ocular surface epithelium and to inhibit stromal ulceration. Certainly the many compounds suggested by the experimental literature to inhibit one or more steps in the progression of epithelial defects to stromal ulceration (Table I) have greater therapeutic potential and deserve evaluation in the melting cornea.

*Summary*

Epithelial defects and stromal ulceration are clinical problems of great significance in cornea- and external-disease practice, as they occur frequently, are difficult to treat, and involve considerable ocular morbidity and sight-threatening sequelae.[52,75-77] The fundamental emphasis of this thesis is to devise appropriate animal models of sterile corneal ulceration and then, using morphologic and biochemical techniques, to extend our knowledge of the pathogenesis of these disorders and to test the inhibition of corneal melting in animal models by pharmacologic agents. By increasing our understanding of the cellular activities and interactions involved in stromal melting, perhaps treatment can be altered to suppress collagenase and other damaging hydrolytic enzymes at the levels of inhibiting inflammatory cell migration and collagenase synthesis or secretion rather than attempting to inhibit extracellular enzymes already actively destructive.

## PREVIOUS STUDIES BY THIS INVESTIGATOR

### EPITHELIAL INJURY

Substantial evidence links erosions of the superficial cornea with anomalies at the junction between the basal epithelial cells and Bowman's layer.[78-82] This junction is composed of the epithelial basement membrane, the associated hemidesmosomes of the basal cell membrane, and the anchoring fibrils of Bowman's layer. In 1968, we demonstrated that the basis of normal adhesion of the rabbit corneal epithelium resides in proper construction of such basement membrane complexes.[83] Our most recent inquiry into epithelial basement membrane abnormalities in hu-

man corneal erosions has involved the clinicopathologic correlation of loosely adherent corneal epithelium debrided from 25 patients with persistent defects, erosions, and ulcerations.[84] By transmission electron microscopy (TEM), in both diabetic and map-dot-fingerprint dystrophy patients, multilaminar basement membranes appeared consistently adherent to the basal epithelial cells. In posttraumatic erosions, in contrast, basal cell rupture, absence of epithelially-adherent basement membrane or segmental new basement membrane was evident. Postinfectious and chemical or thermal burn specimens usually had new basement membrane synthesis but frequently also exhibited amorphous debris adherent to the basal cell surface.

Concern over chemical burns causing abnormal epithelial regeneration and adhesion prompted us to compare in both rabbits and monkeys the effects of central, peripheral, and total corneal burns produced by sulfuric acid, sodium hydroxide, and iodine-cocaine.[85] In addition to establishing the weak acid burn as a potential model of persistent epithelial defect, we also developed a biopsy technique with a 1.5-mm Elliott trephine to facilitate serial morphologic observation of wound healing in the same cornea.[85] In a related study, we determined that chemical damage of basement membrane can delay or inhibit new basement membrane synthesis and also that the anterior stromal "substrate" is important in determing the success or failure of epithelial readhesion.[86] Having subsequently performed the first ultrastructural clinicopathologic correlation of 14 human corneas following chemical injury,[87] we found defective epithelium and basement membrane, anterior stromal scarring, inflammatory infiltration, and vascularization comparable to that produced in these experimental models.

In persistent epithelial defects and ulcerations, it remains unresolved whether the primary failure of healing resides in the epithelium itself or in a faulty basement membrane or stromal substrate. Of potentially great application to this problem is the experimental model of corneal-wound healing in the organ-cultured rat cornea, as developed by Gipson and Anderson.[88] This model, in addition to facilitating excellent morphologic correlation, has proved to be pharmacologically manipulable as, for example, with cytochalasin[89] and with the glycoprotein synthesis inhibitor, tunicamycin.[90] In 1984 we employed this model to demonstrate that PMN and PMN lysate significantly retard corneal-epithelial wound healing[91]; after 22 hours of co-culture with $5 \times 10^8$ PMN/ml (whole or lysed cells), the size of the remaining epithelial defect in control corneas was 0.17 to 0.72 $mm^2$ (mean, 0.41), in PMN co-culture was 0.81 to 3.91 $mm^2$ (mean, 2.21, $P < 0.01$), and in PMN lysate co-culture was 2.11 to 2.76 $mm^2$ (mean, 2.49, $P < 0.01$).

STROMAL INFLAMMATION AND ULCERATION

Evidence for the enzymatic destruction of corneal stromal collagen and glycosaminoglycans and for the several cellular contributions and interactions responsible for such ulceration has been reviewed earlier. Although most previous studies have implicated injured corneal epithelium and keratocytes as the major sources of collagenase, considerable evidence also points to the PMN as the cellular agent of stromal destruction.

In ocular tissues, injection of the challenging antigen into the corneal stroma of sensitized rabbits results in an immune precipitate line containing degranulating PMN, associated with swelling, fragmentation, and dissolution of collagen fibrils.[92,93] We have similarly noted phagocytically stimulated PMN in the corneal stroma of a patient with rapidly progressive bilateral Mooren ulcers.[94] Our ultrastructural observations of four acute melting human corneas showed innumerable stimulated PMN in the zones of stromal matrix degradation.[95] As we and others have found, extensive PMN infiltration of the corneal stroma of the xerophthalmic rat[96,97] and human being[98] accompanies the stromal melting of keratomalacia.

Concern for the cell populations and their interactions in chemically burned corneas prompted our morphologic reevaluation of the alkali-burned rabbit cornea.[20] Most striking was the onset of stromal ulceration coincident with the massive infiltration of PMN that appeared active ultrastructurally, having discharged their enzyme-containing granules extracellularly and acquired phagolysosomes of degraded stromal components. In contrast to these ulcerating corneas, we utilized Dohlman's technique[99] of gluing on a methyl-methacrylate contact lens soon after burning and were able to prevent stromal ulceration completely, coincident with preventing reepithelialization and PMN infiltration. Subsequent partial or complete removal of the lens resulted in epithelial resurfacing of the stroma, PMN infiltration, and subsequent ulceration. The mechanism of the glued-on lens in preventing ulceration, therefore, must depend upon prolonging the acellularity of the stroma. These observations further suggested that the epithelium might stimulate the infiltration of PMN into the corneal stroma after alkali burns, or might influence PMN or other cells, or both, to produce or release collagenase. Prause and associates[100,101] have made similar morphologic findings in melting human corneas treated with tissue adhesive, and they have further correlated this with the presence of PMN collagenase in the tears of these patients. The failure of glued-on rings to prevent ulceration and PMN infiltration, while excluding epithelium, indicated that ulceration can certainly occur in the absence of epithelium and can be mediated by

PMN alone. In these ulcerating corneas, as most of the PMN appeared to have accumulated on the anterior stromal surface, the possible role of PMN released into the tears and chemotactically localizing at the site of ulceration is questioned. Indeed, we subsequently noted in several human melting corneas that examination of tear film cytology revealed innumerable PMN.[38]

We then performed an experiment to study the effect of generalized immunosuppression and selective inflammatory cell-line modification on corneal ulceration. In guinea pigs, total ocular-surface alkali burns produced central stromal ulceration in 86% of control eyes, whereas ulcers developed in only 16% of eyes of animals immunosuppressed with cyclophosphamide. The PMN, the preponderant inflammatory cells in ulcerating corneas, were conspicuously absent from the nonulcerating corneas. Selective PMN suppression by intravenous (IV) administration of a highly specific anti-guinea pig neutrophil serum also suppressed the development of corneal ulcerations in this model; in only 25% of the eyes so treated did ulcers develop after alkali burning. T-lymphocyte or monocyte modifications with similar nonspecific antisera had no effect on the rate of corneal ulceration.[102]

As experimental models of chemical burns of the cornea are often difficult to control and to reproduce, we have also devised a less traumatic, discrete thermal-burn model (to be detailed in this thesis) of sterile stromal ulceration in the rabbit cornea that can be modulated to vary the antagonistic balance between destruction and repair. Utilizing the thermal ulceration model, we have performed several manipulations to examine the effects of possible therapies for stromal melting. We used this model preliminarily in Conn's study of the protective effect of tumor-angiogenesis factor-induced stromal neovascularization in preventing ulceration.[51] We then studied the effect of topical and systemic ascorbate on this thermal burn model, and we noted that the rate and severity of ulceration were accelerated in the ascorbate-treated animals.[103] We hypothesize that this outcome is the result of ascorbate's ability to enhance neutrophil chemotaxis and migration, and we caution against its indiscriminate use in corneal ulcerative disorders other than the chemical burn,[50,104,105] where it is specifically indicated to rectify the alkali-induced scorbutic state. Next, we compared the effects of prednisolone and medroxyprogesterone acetate on stromal wound healing (following linear perforating incision or central trephination) and ulceration (following thermal burn),[106] noting that after thermal burns, when drug applications immediately followed the burn, deep ulceration or perforation developed in 85% of the controls, in none of the prednisolone-treated group, and in

17% of the medroxyprogesterone acetate-treated group. When drug delivery was withheld until day 6, severe ulceration developed in 44% of both treatment groups. In both experiments, stromal neovascularization was markedly suppressed by prednisolone but only moderately decreased by medroxyprogesterone acetate.

Using the vitamin A-deficient rat, collaborations with Prof George Wolf of the Dept of Nutrition and Food Science at Massachusetts Institute of Technology have extended over the past 5 years. Initial TEM and scanning electron microscopy (SEM) morphologic observations of moderate and severe deficiency showed corneal changes consistent with those classically reported by Wolbach and Howe.[107] Experimental efforts to simulate keratomalacia, however, are extremely difficult since severely deficient animals usually die (presumably from infection) before developing corneal ulceration. Since punctate epithelial changes are the earliest corneal alteration preceding xerosis and keratomalacia in human beings,[108] we hypothesized that epithelial or stromal injury, or both, in the vitamin A-deficient cornea might be sufficient to precipitate corneal ulceration at an earlier stage of deficiency when an experimental animal would be relatively healthy and free from severe infection. Accordingly, we applied a mild thermal burn to the central cornea of severely deficient rats and observed rapid sterile stromal ulceration and collagenase release in culture (beginning on day 1 of culture when excised 72 hours after burning) that was histologically correlated with marked PMN infiltration of the stroma.[96] Time-course studies of collagenase release and PMN infiltration suggested that the enzyme released on the first day of culture is derived from the infiltrating PMN. This conclusion is also consistent with Pirie and co-workers[109] finding of PMN in the xerophthalmic rat corneas, which released collagenolytic proteases in culture. Using this same experimental approach, we have further determined that when PMN infiltration after thermal burning was excluded by application of cyanoacrylate tissue adhesive, no ulceration occurred and collagenase activity in the day-1 culture media was negligible.[97] Moreover, if burned and unburned areas of deficient corneas were separated by trephining and cultured separately, then the burned area (containing most of the PMN) was found to have ten times the collagenase activity of the unburned area. Interestingly, trypsin-activation experiments showed little latent collagenase, suggesting that the observed collagenase was fully active and that no collagenase inhibition was involved.

The current status of our experimental approaches to keratomalacia is to ask if an even milder corneal trauma, such as mechanical epithelial abrasion, could precipitate stromal ulceration. Somewhat surprisingly,

preliminary observations of vitamin A-deficient rats following mechanical or heptanol deepithelialization have consistently revealed persistent epithelial defects and rapid progression of ulceration, frequently to perforation.[110] Morphologically, the deficient group exhibited extensive epithelial defects and cellular disorganization with loss of surface microvilli, intense PMN response throughout the stroma and anterior chamber, bacterial forms within the ulcerating stroma, and neovascularization peripherally. Presumably, the factors of abnormal epithelial recovery, acute inflammation, and bacterial infection can also be important in the pathogenesis of keratomalacia in human beings. Our future efforts in this field are directed toward understanding the roles of infection and immunologic compromise in the vitamin A-deficient cornea.

## SCOPE OF THESIS

In this work, the following clinical and experimental pathologic studies will be described:

(A) Clinicopathologic correlations of human corneal melting.
(B) Tear cytology and enzymology in human corneal melting.
(C) Thermal-burn model of sterile corneal ulceration.
(D) Therapeutic manipulations of thermal burn-induced corneal ulceration.
  (1) Cyanoacrylate tissue adhesive
  (2) Colchicine
  (3) Cholera toxin
  (4) Nonsteroidal antiinflammatory agents (flurbiprofen and indomethacin)
  (5) Retinoic acid
  (6) Immunosuppression.

## CLINICOPATHOLOGIC CORRELATIONS OF HUMAN CORNEAL MELTING

### SPECIFIC BACKGROUND

As reviewed earlier, several experimental and pathologic studies have identified the PMN as the predominant cell type in actively ulcerating corneas. Prause and Jensen[37] have recently reported the light-microscopic findings in 50 eyes with corneal ulcers resulting from various etiologies (predominantly postinfectious); they noted frequent PMN in about two thirds of these cases, with about 80% of these containing periodic acid-Schiff (PAS)-positive and amylase-sensitive granules. Corre-

lative TEM suggests that the PAS-positive material corresponds to glycogen, so that the PAS-negative PMN can be considered as phagocytic activated cells that have used their glycogen. This study does not, however, directly correlate the histopathologic findings with the extent of clinically active ulceration.

## MATERIALS AND METHODS

Between 1974 and 1984, I have performed 15 penetrating keratoplasties on 14 patients with active sterile stromal ulceration that had been unresponsive to medical therapy (including tissue-adhesive and pharmacologic agents). During this same interval, eight penetrating keratoplasties were performed on an elective basis in patients who had previously experienced active stromal ulceration that had subsided after medical management or conjunctival flap. The demographic and clinical characteristics of these 21 patients and 23 keratoplasty specimens are detailed in Table II. As expected, herpes simplex virus (HSV) keratitis (eight patients), chemical injury (three patients), and autoimmune or connective tissue disorders (six patients) accounted for the majority of cases. At the time of keratoplasty, the corneal buttons were immediately fixed in Karnovsky's glutaraldehyde formalin, and portions were paraffin embedded for routine light microscopy and plastic embedded for phase contrast and TEM. Histopathologic grading of the cellular constituents within the areas of ulceration (either presently or previously active) were performed on 1-μ semithin sections stained with paraphenylenediamine, and the relative intensity of the cellular response and relative proportions of PMN, monocytic inflammatory cells (lymphocytes, monocytes, and macrophages), and keratocytes were estimated according to the grading system detailed in Table II. For each specimen, TEM was performed in the corresponding area for more precise correlation.

## RESULTS

In corneas undergoing clinically active ulceration, substantial concentrations of PMN were consistently evident in 14 of 15 specimens (Fig 1). Relative to mononuclear cells and keratocytes, PMN were the predominant cell type in 11 specimens, were present in equal proportion with mononuclear cells in 2 specimens, and were less prevalent than mononuclear cells or keratocytes, or both, in 2 specimens. In all cases, a mixed population of PMN, mononuclear cells, and keratocytes was evident, but in no case were epithelial cells substantially present at the site of ulceration. TEM confirms the identity of the involved cellular constituents and further attests to the active participation of the PMN in the stromal

TABLE II: CLINICOPATHOLOGIC CORRELATIONS OF HUMAN CORNEAL MELTING

| SPECIMEN NO | AGE/RACE/ SEX | DIAGNOSIS | CLINICAL STATUS | ULCERATION | HISTOPATHOLOGY | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | PMN | MONOCYTE | KERATOCYTE | COMMENT |
| 1 | 39 BF | Keratoconus, keratitis sicca, S/P PK | Graft reaction, persistent epithelial defect | Active + + + | + + + | + + | + | Descemetocele |
| 2 | 39 BF | Keratoconus, keratitis sicca, S/P PK × 2 | Graft reaction, 6 mo after specimen 1 | Active + + | + + + | + + | + | Mucoid stroma |
| 3 | 74 WF | Sjögren syndrome | Peripheral and paracentral ulcer | Active + + | + + | + | 0 | |
| 4 | 56 WF | Rheumatoid arthritis | Persistent epithelial defect | Active + + | 0 | + | + | Minimal cellular reaction |
| 5 | 41 WM | Alkali burn S/P PK × 2 | Persistent epithelial defect | Active + + + | + + + | * | + | |
| 6 | 26 BM | Alkali burn | Previous tissue adhesive | Active + + | + + | + | 0 | |
| 7 | 12 WF | HSV keratitis | Disciform and interstitial keratitis + corticosteroids | Active + + + | + + | + + | + | |
| 8 | 62 WM | HSV keratitis | No previous therapy | Active + + + | + + + | + | + | Rare HSV within keratocyte |
| 9 | 51 WF | HSV keratitis, recurrent | Perforated descemetocele | Active + + + | + + + | * | + | Iris adherent to Descemet's membrane |
| 10 | 39 WM | HSV, S/P PK | Graft rejection | Active + | + + | + | + | Endothelium intact |
| 11 | 31 BM | HSV, S/P conjunctival flap, then PK | Persistent epithelial defect | Active + + | + + | ± | + | |
| 12 | 84 WF | Pemphigoid | Previous bacterial keratitis | Active + + | + + | + | + | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 13 | 79 WF | Pemphigoid | Persistent epithelial defect | Active + + + | + + | + + | ± | |
| 14 | 34 BF | Erythema multiforme | Immunosuppressed for 2 yrs | Active + | + + | ± | + | |
| 15 | 21 WM | ? Mycotic keratitis | Sterile, persistent epithelial defect | Active + | + | + + | + + | No infectious organisms |
| 16 | 40 BF (compare with specimens 1 & 2) | S/P PK × 3 S/P conjunctival flap | Epithelium intact, no inflammation | Inactive | ± | + | + | |
| 17 | 74 WF | HSV descemetocele | Minimal inflammation | Inactive | 0 | + | + | |
| 18 | 49 WF | Stromal HSV, S/P conjunctival flap | No inflammation | Inactive | + | + + | + + | |
| 19 | 31 WM | Acid burn-ulcer, S/P conjunctival transplant | Epithelium intact | Inactive | + | + | + + | Goblet cells persist in epithelium |
| 20 | 23 WM | *Pseudomonas* sp ulcer | No inflammation | Inactive | 0 | + | + + + | |
| 21 | 35 WM | HSV perforation, S/P adhesive | Stroma thin and vascularized | Inactive | 0 | + | + + | Plasma cells adjacent to vessels |
| 22 | 45 WM | Rosacea ulcer, S/P adhesive | Stroma scarred and vascularized | Inactive | + + | + | + | Neovascularization |
| 23 | 66 WM | Mooren's ulcer unilateral | Epithelium intact, stroma thin | Inactive | ± | + | + | Predominant plasma cells |

*0, absent; ±, minimal; +, mild; + +, moderate; + + +, marked.
HSV, herpes simplex virus; S/P, status after; PK, penetrating keratoplasty.

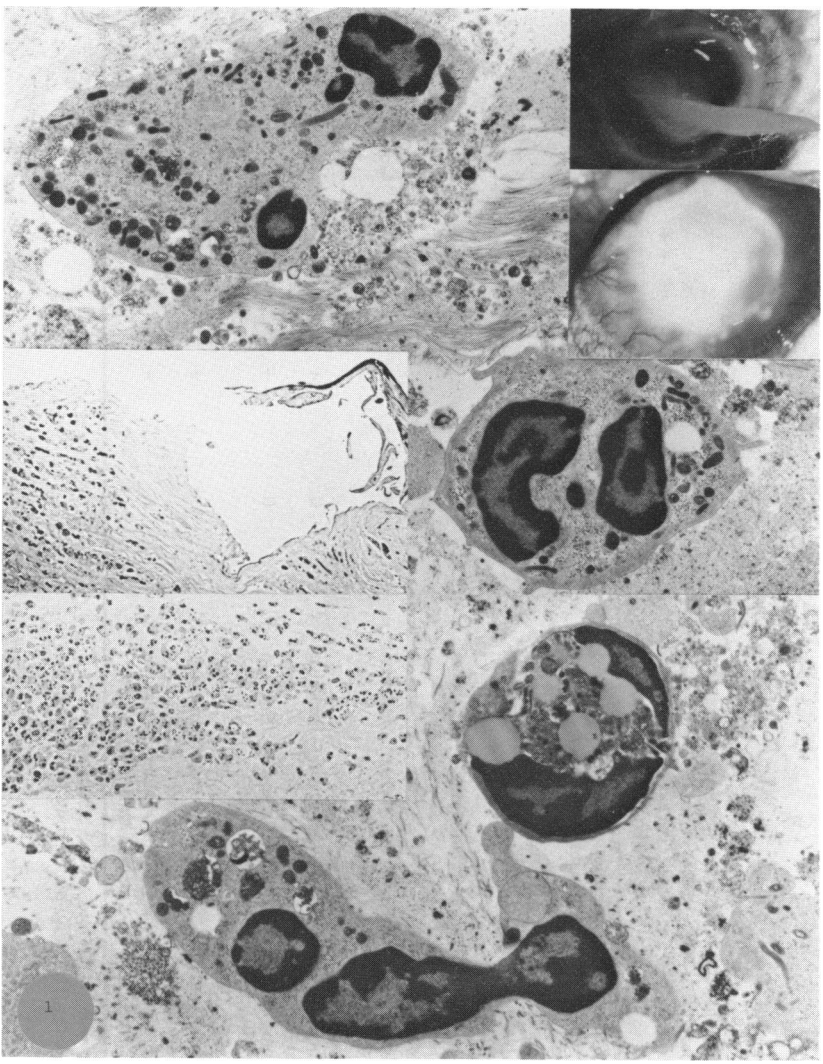


FIGURE 1

Active human corneal ulceration. TOP RIGHT: Case 2. Patient with keratoconus and keratitis sicca. Multiple penetrating keratoplasties all succumbed to massive homograft reactions. Entire stroma of graft appears as mucoid strand adherent to total descemetocele. MIDDLE RIGHT: Case 7. Patient with recurrent, chronic disciform and interstitial herpes simplex virus keratitis. Intense stromal inflammatory infiltration and necrosis are demonstrated. MIDDLE LEFT: Case 5. Phase-contrast microscopy of persistent epithelial defect in chemically burned eye with two previous keratoplasties. Note elevated edge of nonadherent epithelium plus superficial stroma (above) and deep stroma (below) intensely infiltrated by PMN (PPDA, × 300). TOP LEFT: Case 5. TEM of area depicted in phase-contrast photomicrograph. PMN is shown to be partially degranulated; extracellular matrix has disorganized collagen fibrils and cellular debris, possibly from lysed PMN (× 10,000). BOTTOM RIGHT: Case 9. TEM of area of active stromal ulceration demonstrates PMN to be nearly devoid of cytoplasmic granules but contains several phagolysosomes (× 10,000).

degradation process, since PMN could be consistently observed in various stages of having discharged their cytopolasmic granules, either extracellularly or within their numerous phagosomes. Extensive cellular debris was also apparent, much of it seemingly deriving from lysed PMN. Among mononuclear cells, the macrophage was particularly numerous and, in particular, macrophages could be frequently observed to contain not only extracellular debris within their large phagosomes, but also whorls of lipid material that are presumably cellularly derived and, on some occasions, profiles of expended PMN (Fig 2). Lymphocytes and plasma cells were rarely evident. Keratocytes usually appeared quiescent or necrotic with limited evidence of fibroblastic activity.

Among the eight previously ulcerating corneas that were clinically stable at the time of keratoplasty, a different histopathologic picture emerged (Figs 3 and 4). In most specimens, the epithelial cell layer was disorganized and irregular but intact. The PMN were evident in three specimens and either inconspicuous or absent in six. Moreover, ultrastructural survey disclosed these PMN to be relatively quiescent since they had retained their cytoplasmic complement of enzyme-containing granules and glycogen and were usually devoid of phagolysosomes (Fig 5). Chronic inflammatory cells were consistently present among the mononuclear population, but although some macrophages contained lysosomal residual bodies, these cells also appeared rather quiescent and were generally numerically less prevalent than plasma cells and lymphocytes (Fig 4). Keratocytes, in contrast, were predictably numerous and active, as their reactive fibroplasia was evidenced by extensive elaborations of intracytoplasmic rough-surfaced endoplasmic reticulum (RER) cisternae and Golgi apparatus and by extracellular collagen fibrils of variable size and orientation typical of corneal scar tissue (Fig 3). Stromal neovascularization was also a usual occurrence.

## COMMENT

These clinicopathologic observations, although by no means quantitative, nonetheless allow us to develop a consistent morphologic picture of the cellular constituents and events of stromal ulceration and repair. In the interval of active stromal degradation, PMN are the dominant cell both in terms of numbers and activity at the ulcer site. As an end-stage cell programmed for proteolytic debridement, they are chemotactically attracted by damaged tissue (by other PMN and by various cytokines) to discharge their multiple hydrolases, both intracellularly and extracellularly, expending their glycogen content in the process and ultimately undergoing necrosis themselves. Macrophages are also numerous, active pha-

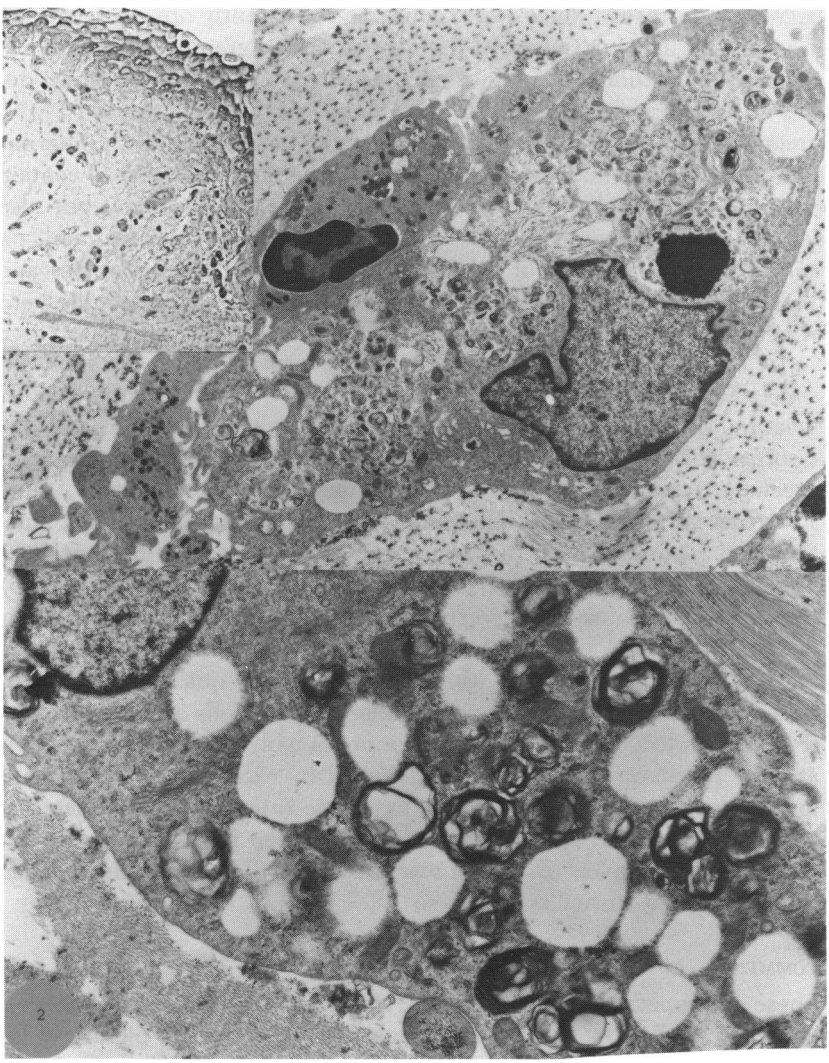


FIGURE 2

Active human corneal ulceration. TOP LEFT: Case 13. Adjacent to area of persistent epithelial defect, phase-contrast microscopy of cornea from patient with ocular cicatricial pemphigoid illustrates thickened epithelium overlying disorganized stroma with numerous mononuclear inflammatory cells (PPDA, × 300). TOP RIGHT: TEM shows large macrophage filled with predominantly lipid materials. Note adjacent PMN (× 10,000). BOTTOM: Case 15. Higher magnification TEM of similar-appearing mononuclear macrophage resolves phagocytosed material consisting of complex phospholipids (electron-dense membranous whorls) and probable neutral lipids (homogeneous electron-lucent globules) (× 20,000).

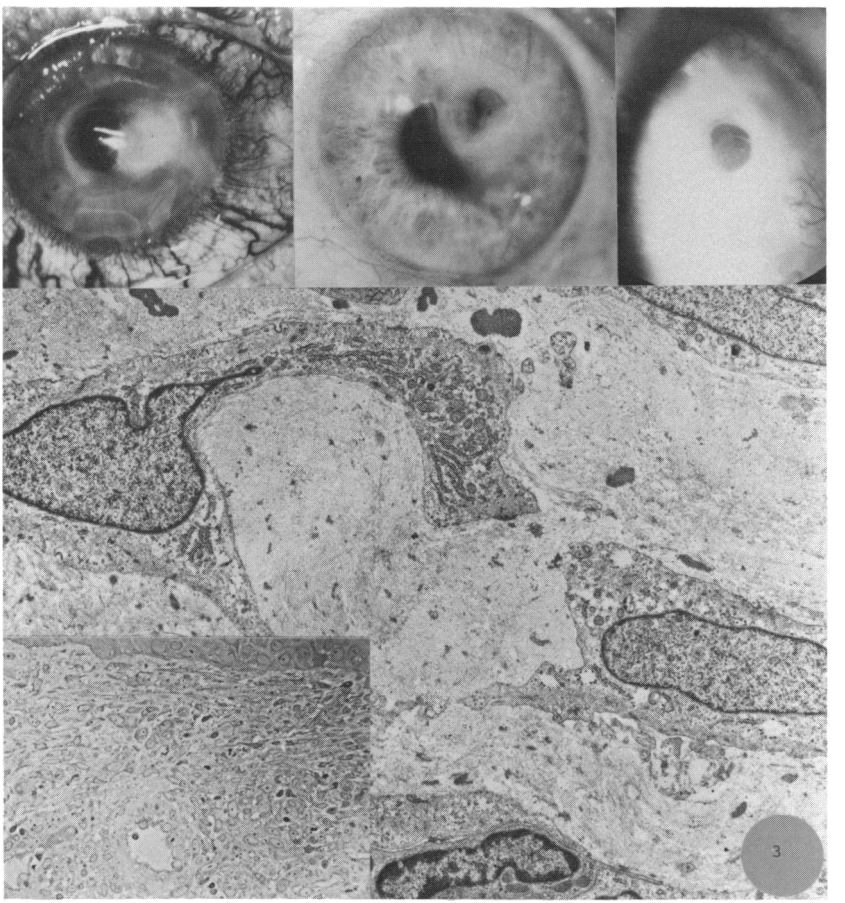


FIGURE 3

Inactive human corneal ulceration. TOP LEFT: Case 20. Young man with prior *Pseudomonas* keratitis. Large central descemetocele has reepithelialized, and stroma is stable with diminished inflammatory infiltration. TOP CENTER: Case 17. Cornea with herpetic descemetocele appears quiescent and suitable for elective penetrating keratoplasty. TOP RIGHT: Case 22. Vascularized cornea with stable descemetocele from rosacea had required tissue-adhesive application. BOTTOM LEFT: Case 18. Phase-contrast microscopy demonstrates abundant monocytes and macrophages, plus neovascularization, within herpetic cornea (PPDA, × 300). BOTTOM RIGHT: TEM finds active-appearing keratocytes with cisternae of rough-surfaced endoplasmic reticulum. Stromal scar is composed of disordered collagen fibrils of variably large diameter (× 10,000).

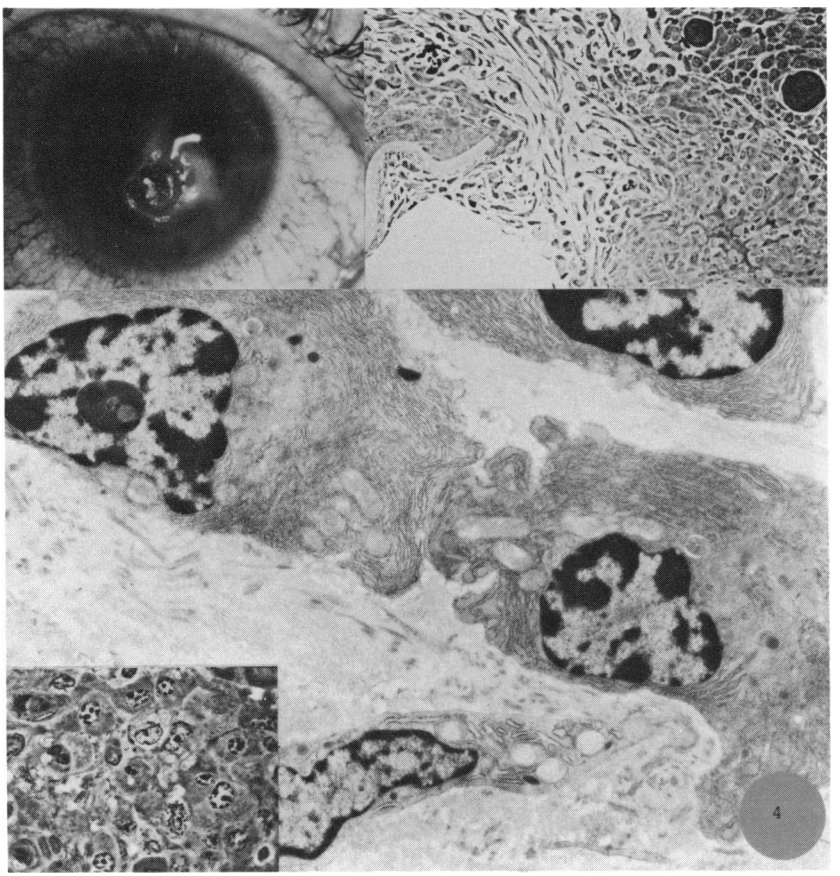


FIGURE 4
Inactive human corneal ulceration. TOP LEFT: Case 21. Tissue adhesive used to manage perforation of herpetic cornea successfully. TOP RIGHT: Phase-contrast microscopy of cornea identifies break in Descemet's membrane (left) to be plugged by abundant fibroblastic cells and chronic inflammatory cells with neovascularization (PPDA, × 300). BOTTOM LEFT: Higher magnification phase-contrast microscopy identifies perivascular inflammatory cells as plasma cells (PPDA, × 1000). BOTTOM: TEM is confirmatory, as cells have classic features of plasma cells (× 10,000).

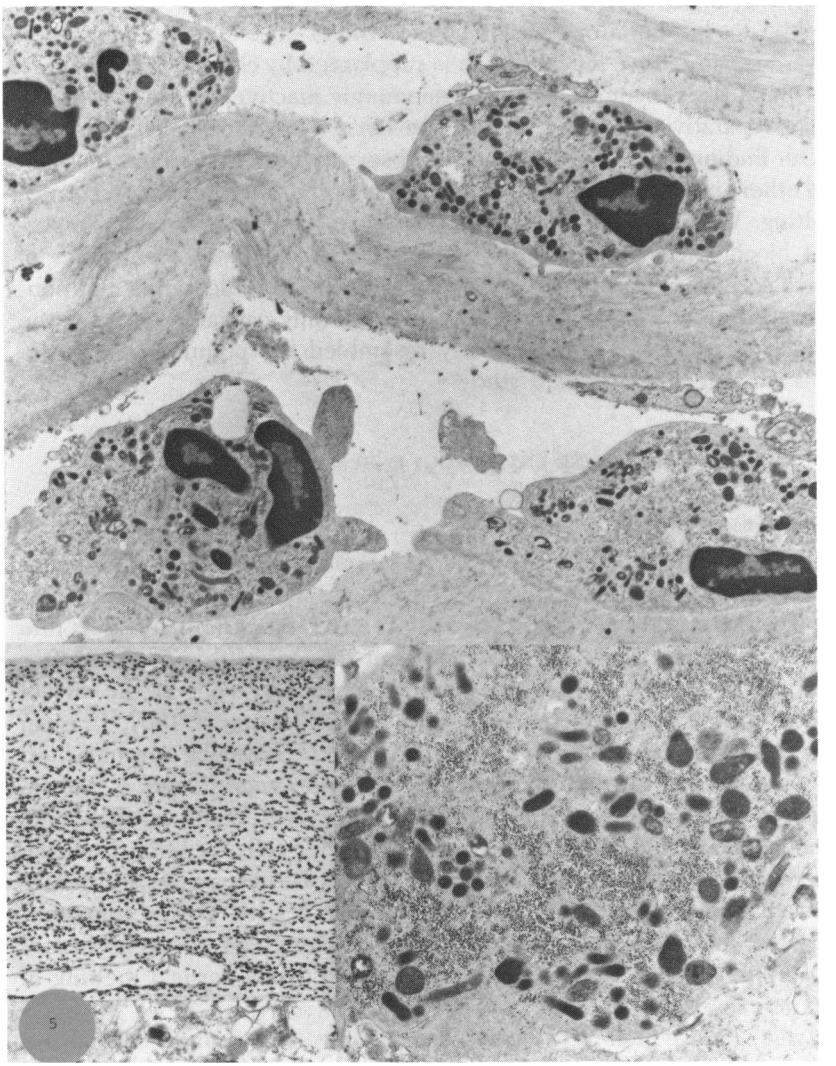


FIGURE 5

Inactive human corneal ulceration. BOTTOM LEFT: Case 22. Vascularized peripheral cornea of rosacea patient. Phase-contrast microscopy illustrates marked inflammatory infiltrate in area of nonulcerated stroma. Neovascular channels are evident at bottom (PPDA, × 300). TOP: Survey TEM of area shows several PMN insinuated between stromal lamellae that are not degraded. Note PMN have retained cytoplasmic granules, have few phagosomes (× 10,000). BOTTOM RIGHT: Higher magnification of cells better illustrates conservation of both lysosomal granules and glycogen particles, indicative of quiescent PMN (× 20,000).

gocytically and (presumably) enzymatically as well, whereas the surviving keratocytes have minimal activity. When the balance of forces has turned toward repair, active inflammation is supplanted by chronic inflammatory cells, and the remaining PMN are seemingly inactive, in contrast to the major reparative efforts of the reactive fibroblastic keratocytes.

Our findings, therefore, extend the observations of Prause and Jensen[37] and others in emphasizing the primary role of the PMN in human corneal melting. With Wagoner and associates,[95] we have also added an important biochemical dimension to this picture, as in four recent cases of active corneal melting, collagenase activities in both ulcerating and nonulcerating areas were markedly elevated, but only partially related to the cellular constituents, which greatly resembled the population distributions evident in the present study.

## TEAR CYTOLOGY AND ENZYMOLOGY IN HUMAN CORNEAL MELTING

### SPECIFIC BACKGROUND

Although the participation of PMN in stromal ulceration has been substantiated, the origin of these PMN has been a source of some controversy. It is known that PMN, presumably released from limbal vessels into the tears, appear in acutely wounded rat corneas within 5 hours after injury.[111-113] These cells are the first inflammatory cells at the site of injury. This mechanism, as suggested by Cohnheim in 1867,[114] is an effective means of conveying PMN to areas of the avascular cornea where they are needed to combat microbes or debride necrotic tissue. Subsequently, PMN infiltrate the corneal stroma, presumably attracted by chemotactic factors elaborated by PMN or other cells, or both, from the ulcer region. Among these factors, PMN proteases are important, as are prostaglandins $PGE_1$, $PGE_2$, and prostacyclin $PG_{12}$.[45] This is consistent with the findings of Fogle et al,[38] who documented the presence of numerous PMN in the tears of patients with active corneal melting of various causes.

Again, Prause et al[115,116] have made the most significant technical and clinical contributions to this area, as they have developed a sensitive micromodification of the immunoelectrophoresis assay for quantification of tear proteins and have determined the tear fluid content of PMN collagenase and other proteins in patients with sterile corneal ulcers in both acute and healing stages.[117,118]

### MATERIALS AND METHODS

Tear samples from a total of 47 patients (85 determinations) were studied.

In most cases, both eyes were entered into the study. Of the 47 patients, at least one eye of 29 patients showed active or recent melting. In 14 of these patients, the fellow eye was normal. Eleven patients (16 eyes) had acute or chronic inflammation, as evidenced by conjunctival hyperemia and tearing, but no evidence of past or active melting. Seven patients had clinically normal eyes. A total of 30 eyes were termed "controls." These included the 16 eyes that were inflamed but did not melt and 14 eyes that were clinically and historically normal.

Tears were collected in 20-μl capillary tubes held at the outer canthus and allowed to fill spontaneously. Tilting the patient's head to the side of collection facilitated entry of tears into the capillary tube by preventing drainage through the punctum. Filled capillary tubes were sealed at each end with plasticene immediately after samples were taken and stored at 4°C until centrifuged for further analysis. Tubes were then emptied into 250-μl centrifuge tubes and spun for 3 minutes in a microfuge. The supernatant was transferred to a separate centrifuge tube and measured, then plunged into a mixture of solid $CO_2$ and absolute alcohol. Immediately upon removal from the $CO_2$-alcohol mixture, the sample was lyophilized for 6 hours, then stored in the same airtight container until protein analysis.

The pellet was resuspended in the remaining tear fluid (2 to 3 μl), spotted onto clean glass slides, and stained with Giemsa stain for PMN-leukocyte counting. The entire slide was studied, and a total of five representative high-powered fields (HPF) were counted. The mean value of these determinations was used as the PMN count per HPF in the sample.

Analysis of protein was carried out by the microimmunoelectrophoresis method of Prause et al.[115] This technique is a micromodification of the Laurell electroimmunoassay and has the advantage of permitting accurate analysis of samples available only in small amounts. Electrophoresis was run in capillary tubes containing 1% agarose gel and immunoglobulin mixture prepared in 0.024 sodium-barbital buffer at pH 8.6. Glass cylinders (500 μl) were fitted to the tops of the capillary tubes with silicone stoppers. A small quantity of test solution was mixed with 500 μl of 0.0002 M sodium-barbital buffer and centrifuged for 15 minutes.[116]

The supernatant was transferred and examined in three dilutions, with 70%, 10%, or 1% of test solution in buffer. Electrophoresis was carried out at 4 V/cm for 12 hours. Gels were gently pushed out onto an agarose-coated glass plate, stained with Coomassie brilliant blue R-250, and read optically, the sharp front border of the immunoprecipitation band being read at a total magnification of × 7. The migration distance was measured

TABLE III: DIAGNOSES OF PATIENTS WITH ACUTE CORNEAL MELTING

| | |
|---|---|
| Herpes simplex keratitis | 6 |
| Mooren's ulcer | 4 |
| Rheumatoid arthritis | 4 |
| Bacterial keratitis (postinfectious) | 2 |
| Keratoconjunctivitis sicca | 2 |
| Erythema multiforme | 2 |
| Alkali burn | 1 |
| Anterior-segment dysgenesis | 1 |
| Herpes zoster | 1 |
| Neuroparalytic keratitis | 1 |
| Wound melt after keratoplasty | 1 |
| Pemphigoid | 1 |
| Rosacea keratitis | 1 |
| Thermal burn | 1 |
| Vernal keratoconjunctivitis | 1 |
| Total | 29 |

to the nearest tenth of an ocular scale unit (OSU), 1 OSU corresponding to a distance of 1.5 mm.[116]

## RESULTS

Eyes with corneal melting were grouped into A, B, or C categories depending on whether at the time of analysis they (A) showed active melting, (B) were 14 days postmelting, or (C) were more than 14 days following their most recent melting episode. Some eyes were studied longitudinally. Various diagnoses were represented in the actively melting group, as shown in Table III.

Twenty-seven normal control eyes had a mean of less than 1 PMN/HPF. Inflamed, nonmelting control eyes (16) had 18.2 PMN/HPF. Actively melting (group A) eyes showed 29.7 PMN, and at 14 days postulceration (group B), 10.5 PMN were seen. Quiescent eyes greater than 14 days postulceration (group C) showed 2.1 PMN/HPF. In actively melting eyes, the mean collagenolytic enzyme level was determined to be 10 OSU/μl. In group B at 14 days postmelting, an average of 5 OSU/μl were found. Group C, at greater than 14 days postmelting, showed 1 OSU/μl of tears. When groups A, B, and C are compared with respect to PMN and collagenolytic enzyme activity, the associated finding of an elevated number of PMN and elevated levels of enzyme activity is apparent (Table IV).

TABLE IV: TEAR PMN AND COLLAGENOLYTIC ENZYME ACTIVITY IN HUMAN CORNEAL MELTING

| | | PMN | MEAN COLLAGENOLYTIC ENZYME ACTIVITY (RANGE) | |
|---|---|---|---|---|
| Control | Normal ($n$ = 14) | < 1 PMN/HPF | 0 OSU/μl | (0) |
| Control | Inflamed, nonmelting (16) | 18.2 | 0 | (0-1) |
| Group A | Active melting (27) | 29.7 | 10 | (7-26) |
| Group B | 14 days postmelting (15) | 10.5 | 5 | (3-10) |
| Group C | > 14 days postmelting (13) | 2.1 | 1 | (0-4) |

#### COMMENT

We demonstrated elevated levels of collagenolytic enzymes coexistent with elevated numbers of PMN in tears of humans with actively melting corneas. Fourteen days after acute melting, both PMN and enzyme concentration had decreased. At greater than 14 days, both numbers of PMN and levels of enzymes had fallen further.

We observed the association of elevated collagenolytic enzymes and increased numbers of PMN in patients with a broad variety of diagnoses, suggesting that regardless of the initiating insult to the cornea, the final result of severe corneal stromal destruction is mediated in the same fashion: the attraction of PMN to the site of injury and the subsequent release of their degradative enzymes. While it is not claimed that tear PMN alone are responsible for degradation of corneal stromal tissue, the association of increased levels of proteolytic enzymes and increased numbers of PMN, both diminishing with decreasing activity of melting, is further evidence that tear-fluid PMN are active in human corneal stromal degradation. These findings support the hypotheses of Fogle et al[38] and Prause et al.[37,118]

### THERMAL-BURN MODEL OF NONINFECTED CORNEAL ULCERATION

#### SPECIFIC BACKGROUND

Several experimental studies of chemical burns of the cornea by Brown et al,[5,19,22] Pfister,[29] and others have histopathologically documented the closely interrelated and often antagonistic factors of inflammation, tissue necrosis, vascularization, and fibroplasia. Given the severity of the usual alkali-induced corneal injury, we sought to devise a less devastating and more discrete model of sterile stromal ulceration that might be predictably modulated to alter the balance between ulcerative and reparative processes. Recalling the clinical observation that thermokeratoplasty for keratoconus can sometimes result in persistent epithelial defects and

even stromal ulceration,[119,120] we began to investigate the mechanisms by which thermal burns cause such destructive events. In developing this model, we attempted to relate clinical and histopathologic correlations with the production of collagenase and collagen degradation.

## MATERIALS AND METHODS

The thermal-cauterization instrument utilized throughout was the thermokeratophore (Frigitronics), as had been developed for thermokeratoplasty (Fig 6). The standard 4.5-mm diameter probe was maintained at 130°, and applications were of approximately 1 second's duration. Adult albino rabbits, following IV pentobarbital and topical proparacaine hydrochloride anesthesia, received either multiple (four) or single corneal burns (Fig 6). Altogether 144 corneas were thermally burned. Immediately after burning, loosely adherent epithelium was gently debrided with a cotton-tipped applicator, and erythromycin ointment was instilled. Eyes of all treatment groups were examined daily, and slit-lamp biomicroscopy was performed on alternate days. The extent of epithelial defects was determined with fluorescein or methylene blue-azure II staining. The extent of stromal ulceration and neovascularization were recorded at each slit lamp examination. Representative animals were sacrificed for histopathologic study at days 3, 7, 10, 14, and 21 after burning. Animals were sacrificed whenever a corneal perforation occurred, and all remaining animals were terminated at day 21. For biochemical studies, 22 rabbits (44 eyes) were sacrificed at day 7 after burning. Thus, clinical observations could be performed on 115 eyes observed for longer than 4 days. Light microscopy and TEM studies were performed on 97 of these corneas.

## RESULTS

The clinical course of 115 eyes receiving either multiple burns (40 eyes) or single burns (75 eyes) is detailed in Table V. Specifically noteworthy is that singly burned corneas received lesions whose edge was either 4, 3, 2, or 0 mm central to the limbus and that the first group (4 mm) essentially received midcorneal burns (Fig 6). In multiply cauterized corneas (Fig 7), only 17% of eyes failed to ulcerate, whereas 48% developed mild-to-moderate stromal loss and 35% progressed to descemetocele formation or perforation, or both. The onset of ulceration was usually within 3 days and often rapidly progressed to perforation within 5 days. In contrast, corneas undergoing single burns centrally placed (3 or 4 mm from limbus) underwent a far milder course with only minimal inflammation, no neovascular-

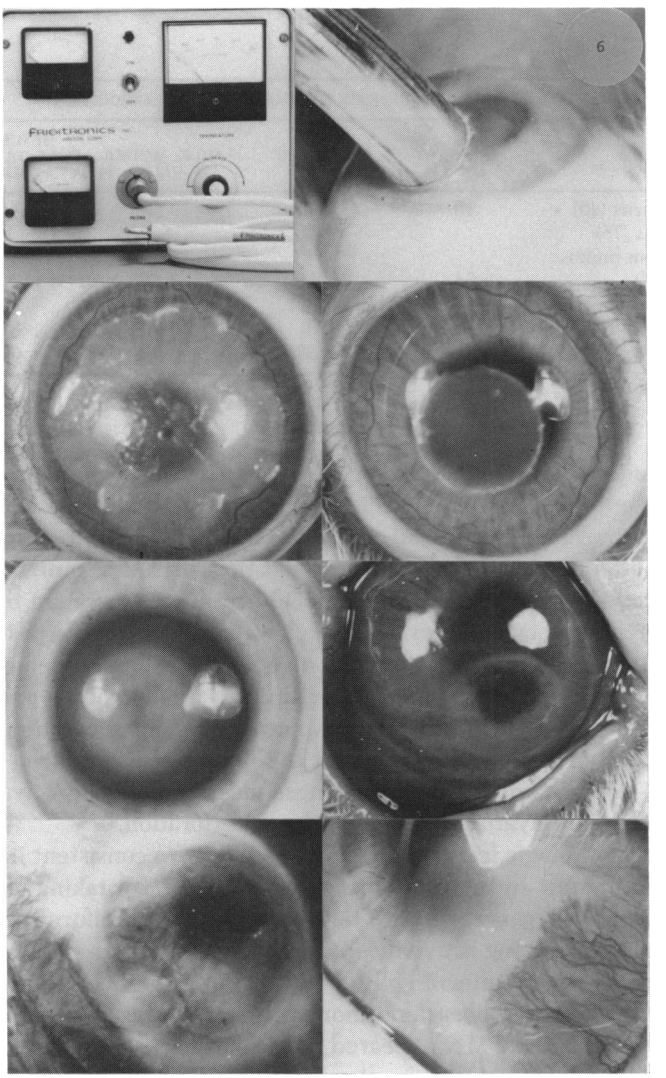



FIGURE 6

Thermal-burn model of corneal ulceration. TOP LEFT: Thermokeratophore probe and control unit. TOP RIGHT: Application of 4.5-mm diameter thermal probe to rabbit cornea, 2 mm from limbus. TOP CENTER (Left): Appearance of multiple thermal burns immediately following application. TOP CENTER (Right): Appearance of single central thermal burn immediately following application. LOWER CENTER (Left): Clinical appearance 14 days after corneal thermal burn shows intact epithelium, stromal leukoma without ulceration and absence of inflammation or neovascularization. LOWER CENTER (Right): Clinical appearance 8 days after burn (2 mm from limbus) includes epithelial defect (stained) with early stromal ulceration. BOTTOM LEFT: Clinical appearance 12 days after burn (2 mm from limbus) illustrates progressive stromal ulceration nearing descemetocele with peripheral neovascularization. BOTTOM RIGHT: Clinical appearance 10 days after burn at limbus shows rapidly progressive neovascularization that will prevent ulceration.

TABLE V: CLINICAL COURSE OF THERMALLY BURNED CORNEAS*

| | NO OF EYES | NO ULCER (%) | MILD, MODERATE ULCER (%) | DESCEMETOCELE OR PERFORATION (%) |
|---|---|---|---|---|
| Multiple burns (40) | 40 | 7 (17) | 19 (48) | 14 (35) |
| Single burns (75) | | | | |
| 4 mm from limbus (central) | 34 | 23 (68) | 9 (26) | 2 (6) |
| 3 mm from limbus | 16 | 11 (69) | 3 (19) | 2 (12) |
| 2 mm from limbus | 16 | 0 (0) | 10 (63) | 8 (37) |
| Limbal | 0 | 3 (33) | 6 (66) | 0 (0) |

*All eyes observed ≥ 4 days.

ization, and slower onset and progression of stromal ulceration despite the persistence of epithelial defects (Fig 7). Among this group of 50 eyes 68% failed to ulcerate at all, and only 8% (4 eyes) advanced to descemetocele or perforation. Single-cautery application placed 2 mm from the limbus produced the somewhat unexpected result of ulceration by day 8 with 100% frequency (16 eyes). Although stromal neovascularization did commence by day 3 in these corneas, ulceration developed before these limbal vessels could extend to the cauterized area. If new vessels did reach the ulcer base, then healing would occur and ulceration would never progress. Single cauterization at the limbus also produced ulceration in six of nine corneas (66%), but these ulcers were shallow, vascularized rapidly, and invariably healed without perforation.

The histopathologic features of these corneas were consistent in the 97 corneas examined by light microscopy or TEM. By taking multiple stepped sections across the entire burned area, it was uniformly evident that in eyes undergoing active ulceration (following either single or multiple burns) the predominant cell type in the areas of ulceration was the PMN, with fewer fibroblasts and mononuclear cells also present (Figs 8 and 9). By TEM, the PMN appeared particularly active as evidenced by their extensive intracytoplasmic vacuolization. In nonulcerating corneas, the opposite picture was apparent, as intense proliferations of fibroblastic cells greatly outnumbered inflammatory cells (Fig 10). An interesting incidental finding was the development of thick fibrocellular layers (retrocorneal fibrous membranes or posterior collagen layers) on the posterior aspect of Descemet's membrane.

To determine if these thermally burned corneas produced collagenase activity and if they degraded their own stromal collagen in vitro, Dr Berman performed tissue cultures of ulcerating and nonulcerating corneas (Fig 11). Assays of collagenase activity in the crude tissue-culture

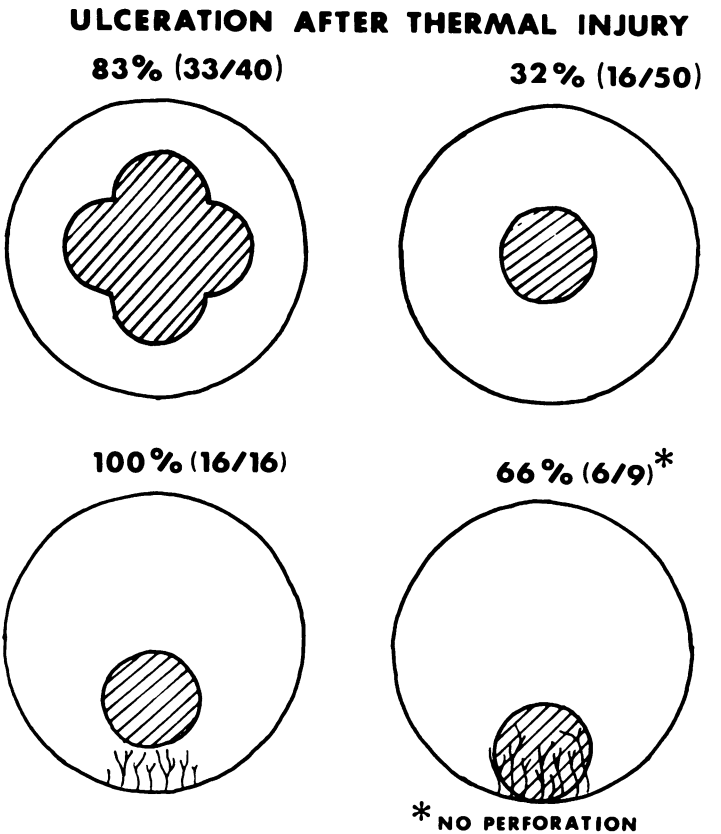



FIGURE 7
Overall proportion of stromal ulceration following thermal injury is dependent upon location of burn.

media were made in a capillary-heat gel system containing calf-tendon collagen as substrate.[121] Both manifest collagenase and latent (ie, trypsin-activatable) collagenase were determined, with total collagenase being the sum of manifest and latent activities. In comparing the collagenase activities of clinically ulcerating *vs* nonulcerating corneas following multiple thermal cauterizations, total collagenase levels were much higher in culture media from the ulcerating corneas. The activities of manifest collagenases, however, were similar in media from ulcerating and from nonulcerating corneas. Media from nonulcerating, singly burned corneas (data not shown) had far lower levels of both total and manifest collagenase.

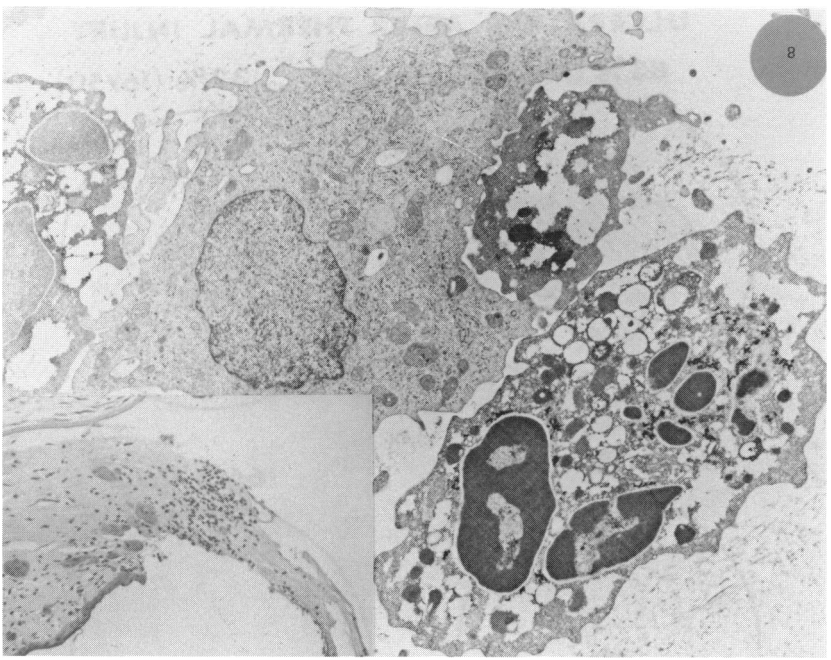


FIGURE 8
Thermal-burn model of corneal ulceration. INSET: Light photomicrograph of ulcerating cornea 12 days after burn, applied 2 mm from limbus, discloses epithelial defect and stroma heavily infiltrated by inflammatory cells (hematoxylin and eosin, × 125). Main figure is TEM of stroma, exhibiting phagocytically active PMN plus monocytes (× 12,000).

In determining the concentrations of solubilized hydroxyproline[122] in the culture media, as evidence of degradation of stromal collagen in vitro, the ulcerating corneas again had greater than twofold higher levels in comparison to nonulcerating corneas (Fig 11). To reconcile this finding with the similar manifest collagenase levels in both ulcerating and nonulcerating cultures, this observation might mean that other noncollagenase proteases contribute to collagen breakdown in the ulcerating corneas. It might also mean that latent collagenase was previously active in culture and was secondarily inhibited by 20,000-MW collagenase inhibitor. If the latter view is correct, then hydroxyproline solubilizations in both instances are approximately proportional to the total collagenase in the media.

That at least part of this observed collagenolytic activity is the result of true tissue collagenases was determined by the cleavage of tropocollagen into typical three-fourths to one-fourth segments, as demonstrated elec-

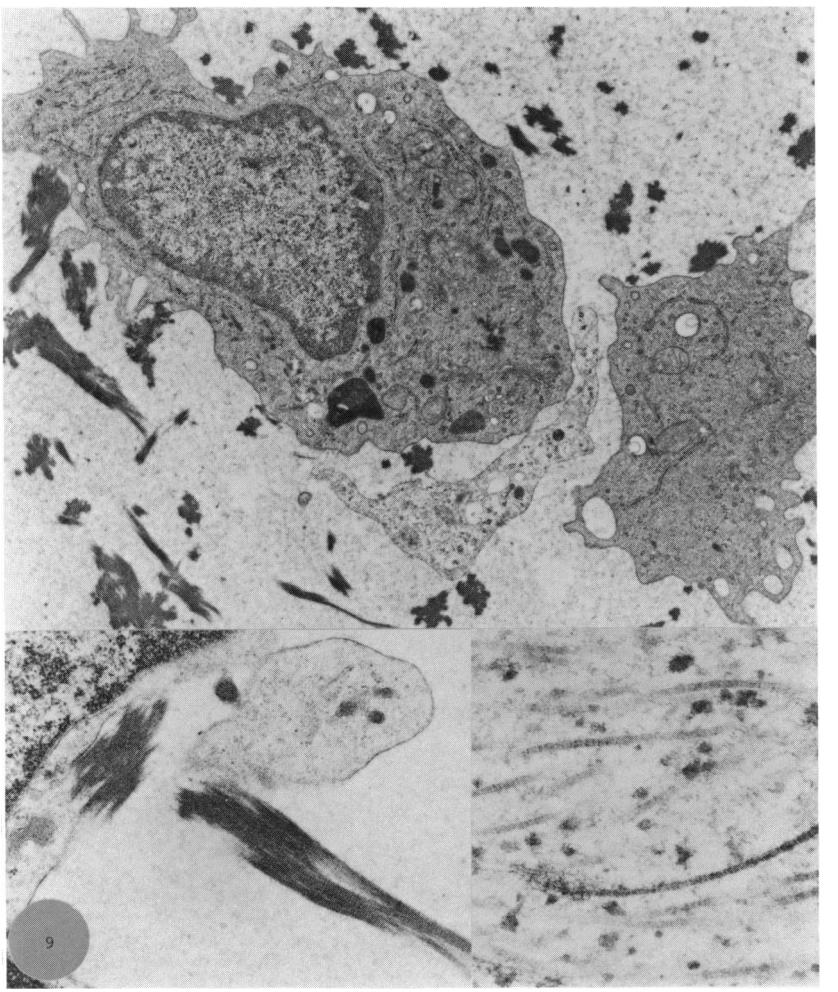


FIGURE 9

Thermal-burn model of corneal ulceration. TOP: TEM of actively ulcerating cornea illustrates collagen-fibril degradation; mononuclear cell is seen within nonfibrillar stromal material having frequent tactoid-body configurations (× 18,700). BOTTOM LEFT: Higher magnification, banding of tactoid is apparent (× 43,400). BOTTOM RIGHT: With high resolution, degraded collagen fibrils are seen to have been frayed into fine filaments (× 80,000).

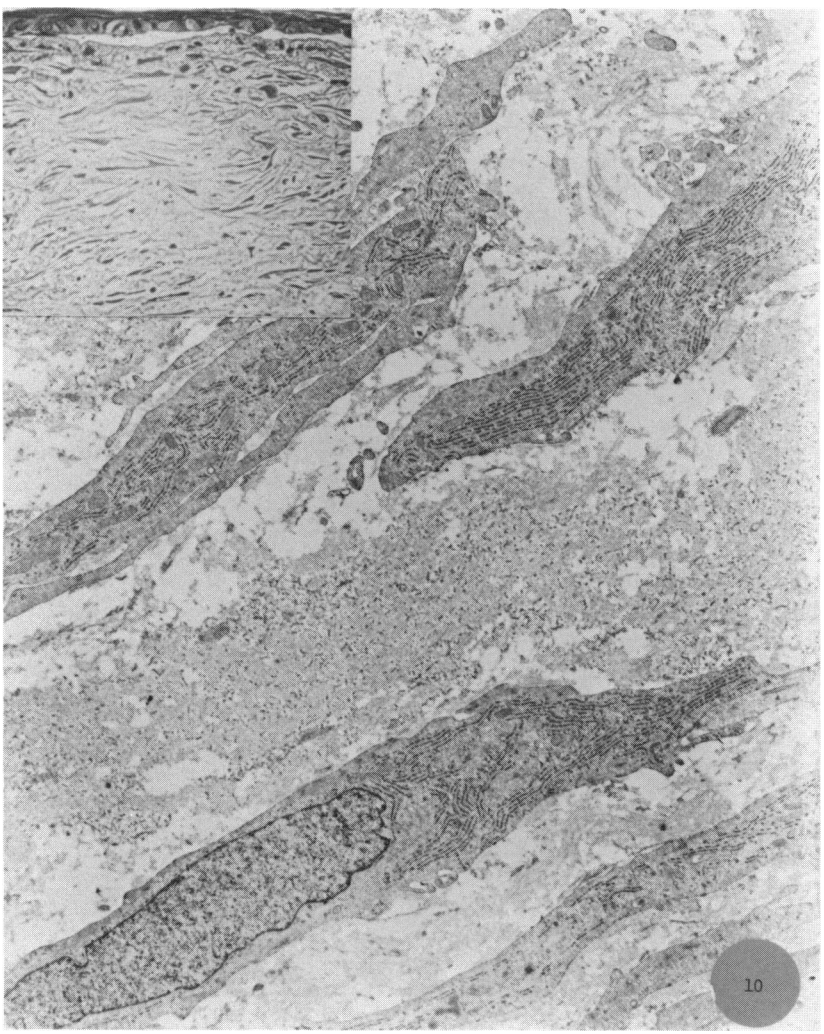



FIGURE 10
Thermal-burn model of corneal ulceration. INSET: Phase-contrast microscopy of central burn at 14 days demonstrates intact epithelium and active fibroblastic proliferation without inflammatory cells (compare with Fig 6) (PPDA, × 200). Main figure shows (by TEM) reactive fibroplasia of keratocytes appearing actively involved in stromal scar formation (× 12,500).

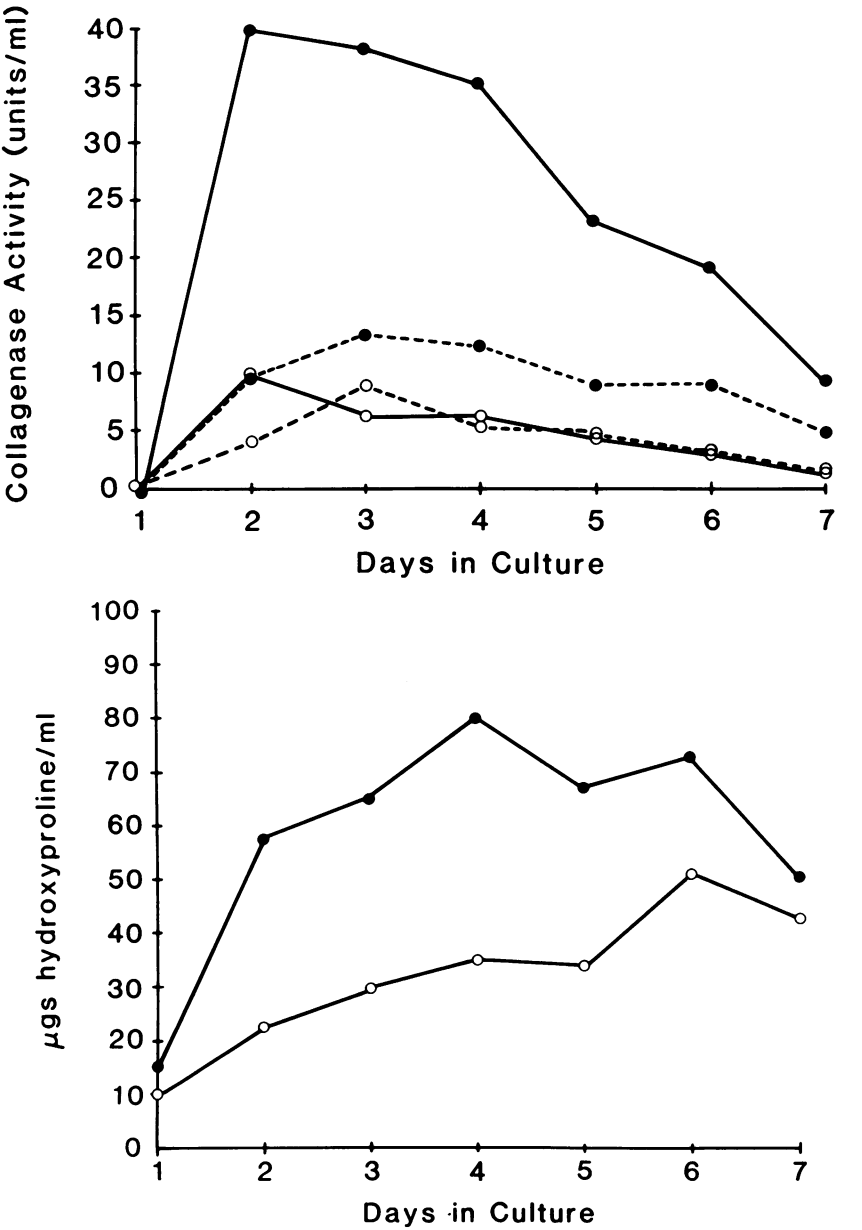



FIGURE 11

Thermal-burn model of corneal ulceration. TOP: Collagenase activity in corneas cultured after thermal burning. In one preparation of seven ulcerating corneas, total collagenase (—●—) is markedly elevated, while manifest collagenase (—○—) is comparable to both total collagenase (– –●– –) and manifest collagenase (– –○– –) apparent in preparation comprising five nonulcerating and one ulcerating cornea. BOTTOM: Solubilization of hydroxyproline within culture media is also greater in ulcerating corneas (—●—) than in nonulcerating corneas (—○—). (Data obtained and prepared by Dr Michael B. Berman.)

tron microscopically in these segment long-spacing crystallites prepared by Dr Peter Davison. Thus, although the contribution of other proteases to the degradation of some collagen denatured by the thermal burn is likely, and although the continued degradation of collagen initially cleaved by collagenase is not ruled out, collagenases would nonetheless appear to play a major role in matrix destruction.

### COMMENT

In summary, either multiple or single thermal-cautery applications were utilized to inflict, respectively, severe or mild thermal burns on rabbit corneas. This variation is reflected in their differing clinical course, with 80% of the former progressing rapidly to ulceration, while epithelial defects without ulceration are more frequent in the latter. Certain histopathologic consistencies are also evident, as PMN leukocytes predominate in ulcerating corneas whereas fibroblasts are more numerous in nonulcerating corneas. Biochemical assays reveal that total collagenolytic activities are highest in ulcerating multiply burned corneas, moderate in ulcerating singly burned corneas, and extremely low in nonulcerating singly burned corneas.

## THERAPEUTIC MANIPULATIONS OF THERMAL-BURN MODEL

### SPECIFIC BACKGROUND

In the preceding section, the clinical, histopathologic, and biochemical characterization of a thermal burn-induced corneal ulceration model is described. We have subsequently utilized this model in several experimental settings to examine potential therapies for stromal melting. First, Conn et al[51] compared the ulceration rate in normal avascular rabbit corneas with that of corneas having tumor angiogenic factor-induced neovascularization, noting the protective effect of stromal vascularization in reducing ulceration. Next, Seng and co-workers[96,97] used a mild thermal burn to precipitate a keratomalacia-like ulceration in the vitamin A-deficient rat cornea. More recently, Phillips et al[106] found similar effects of dexamethasone and medroxyprogesterone acetate on thermal ulceration and wound healing in rabbit cornea. Similarly, Phan and associates[103] demonstrated an enhancement of stromal ulceration following ascorbate treatment in thermally burned corneas. Thus, from a variety of perspectives, we have found this thermal-burn model to be useful to predict and to screen therapeutic modalities for sterile corneal ulceration.

In this section, we additionally explore the effects of the following therapeutic interventions using the thermal-burned rabbit cornea: (1) cyanoacrylate tissue adhesive, (2) colchicine, (3) cholera toxin (CTX), (4) NSAI agents (flurbiprofen and indomethacin), (5) retinoic acid, and (6) immunosuppression.

#### EXPERIMENT 1: CYANOACRYLATE TISSUE ADHESIVE

In a variety of clinical settings, the application of cyanoacrylate tissue adhesive has been found remarkably effective in arresting the progression of sterile stromal ulceration.[38,52] Subsequently, Dohlman et al[99] (and then Zauberman and Refojo[123]) experimentally utilized glued-on polymethyl-methacrylate contact lenses to prevent corneal melting following alkali burns. Although the basis of this effect had been thought to be the result of having excluded damaged ocular-surface epithelium, we[20] later extended Dohlman's experiments and noted that the glued-on hard contact lens maintains stromal integrity, presumably by prolonging its acellularity (notably excluding PMN) following chemical injury.

In this study, we adapted this same approach to tissue-adhesive application in the thermal-burn model. Accordingly, this experiment was comprised of 24 albino rabbits (48 eyes) that received single thermal burns placed 2 mm from the limbus, as had been shown to produce ulceration with nearly 100% consistency. In each animal, both corneas received burns bilaterally, and while one cornea remained untreated (except for daily topical erythromycin), the opposite eye received tissue adhesive (isobutyl 2-cyanoacrylate; Braun, Melsungen, Germany) on either day 1, 3, or 5 (eight corneas on each date) (Fig 12). Clinical observations were performed daily until either a corneal perforating ulcer developed or stromal neovascularization had progressed to involve the entire burned area. After the animals were sacrificed (usually by day 12), most all corneas were prepared for light and electron microscopy.

Among the 16 corneas receiving tissue adhesive on either day 1 or 3, none had begun to ulcerate at the time of adhesive application, and no ulcers subsequently developed prior to termination of the experiment on day 12. Among the eight corneas receiving tissue adhesive on day 5, seven had already developed substantial stromal ulceration. In six corneas of this latter group, the tissue adhesive was retained for the duration of the experiment, and in all of these the preexisting ulcers did not progress. In two eyes, however, the adhesive was repeatedly dislodged, and both of these corneas subsequently perforated by day 10. Overall, among the 24 eyes receiving tissue adhesive, progressive ulceration occurred in only 2

(8.3%), and these corneas might be reasonably considered as technical rather than therapeutic failures. Among the 24 fellow eyes receiving thermal burn only, deep stromal ulceration or perforation, or both, developed in 21 corneas (87.5%). In other respects, specifically conjunctival inflammation and corneal neovascularization, the glue-treated and control groups did not differ. The histopathologic findings in corneas undergoing clinically apparent ulceration (both control groups plus two treatment failures) resembled those previously described for this model, as epithelial defects and marked PMN infiltration of the stroma predominated. Among corneas undergoing gluing on day 1 or 3, the major finding was the continued acellularity of the stroma, except for neovascularization and some fibroblastic activity near the periphery of the burn area (Fig 12). Among corneas ulcerating at the time of tissue-adhesive ulceration on day 5, microscopic study disclosed only inflammatory cell debris (presumably from cells present at the time of adhesive application) with few viable-appearing PMN evident (Fig 12).

In summary, the use of cyanoacrylate tissue adhesive dramatically reduces the incidence of corneal melting (8.3% adhesive treated *vs* 87.5% positive control). This observation is entirely in keeping with human clinical experience of tissue adhesive's ability to arrest corneal melting and also recapitulates our clinical and morphologic findings in alkali-burned rabbit corneas receiving glued-on polymethyl-methacrylate contact lenses. Based on similar histopathologic findings in both studies, we hypothesize that the adhesive prevents degradation of the damaged stroma by excluding the acute inflammatory cells that are primarily responsible for ulcerative destruction. The mechanism of this exclusion is unclear; perhaps the PMN cannot migrate into the hypoxic environment beneath the adhesive or, alternatively, the chemotatic factor(s) responsible for PMN recruitment to the burned area cannot be elaborated or disseminated to attract inflammatory cells to the avascular stromal environment.

#### EXPERIMENT 2: COLCHICINE

Colchicine is a plant alkaloid that binds to tubulin, the protein of which microtubules are composed. As several tubulin-dependent functions (including mitosis and motility) can be selectively interrupted by this drug, we hypothesized that colchicine might suppress PMN migration to the thermally injured cornea and thereby reduce the potential for stromal ulceration. In the rat cornea, for example, Gipson and Keezer[124] have demonstrated colchicine to slow the healing of experimental epithelial abrasions. In other respects, however, colchicine is known not to affect

TABLE VI: CLINICAL COURSE OF THERMALLY BURNED CORNEAS TREATED WITH COLCHICINE (35 EYES)

| | BURN ONLY | COLCHICINE TREATED |
|---|---|---|
| Epithelialization complete | Day 4 | Days 7 to 8 |
| Ulcer onset | Day 4 | Day 7 |
| Ulcer depth — None or healed | 1/7 | 28/28 |
| Ulcer depth — Descemetocele or perforation | 6/7 | 0/28 |
| Vascularization (~ 1 mm) | Days 4 to 5 | Days 7 to 8 |

PMN phagocytosis, and the drug may stimulate or potentiate collagenase production.

In these experiments, 30 rabbits received corneal burns located 2 mm from the limbus (as in experiment 1). Beginning immediately after thermal burn, treated animals received daily colchicine at either low-dose (0.2 mg subcunjunctivally plus 0.35 mg IV) or high-dose (0.4 mg subconjunctivally plus 0.70 mg IV). In 12 corneas, in addition to high-dose colchicine treatment, 0.7 μ latex microspheres were injected intrastromally 72 hours after burn, and these eyes were either followed up in situ or were excised 24 hours following injection and maintained in organ culture to monitor healing according to the method of Gipson.[80-90] Animals were sacrificed according to the criteria previously specified and were prepared for either light microscopy or TEM.

Seven eyes served as positive thermal-burn controls; six (86%) developed deep stromal ulcers or perforations by day 8 and by light and electron microscopy were noted to have numerous intrastromal PMN actively engaged in degranulation and phagocytosis (Fig 13). The eight control eyes receiving either low- or high-dose colchicine (without thermal burn) displayed mild conjunctival injection and mild superficial punctate keratitis (SPK) and stromal haze without clinically significant corneal toxicity; histologically, their epithelium appeared attenuated, and moderate intrastromal infiltration by PMN was distinguished by the absence of degranulation and phagocytosis. Twenty-eight eyes received both thermal burns and colchicine treatment as previously specified. Among those 28 eyes, 17 did not ulcerate at all and 11 corneas developed shallow ulcers that healed, while none progressed to perforation by day 14. However, epithelial recovery was delayed as reepithelialization was complete only by days 7 to 8 (compared to day 4 in thermal-burn controls). Similarly, stromal neovascularization was slowed, as colchicine-treated eyes required 7 to 8 days for neovascularization to progress approximately 1 mm central to the limbus (compared to 4 to 5 days for thermal-burn controls)

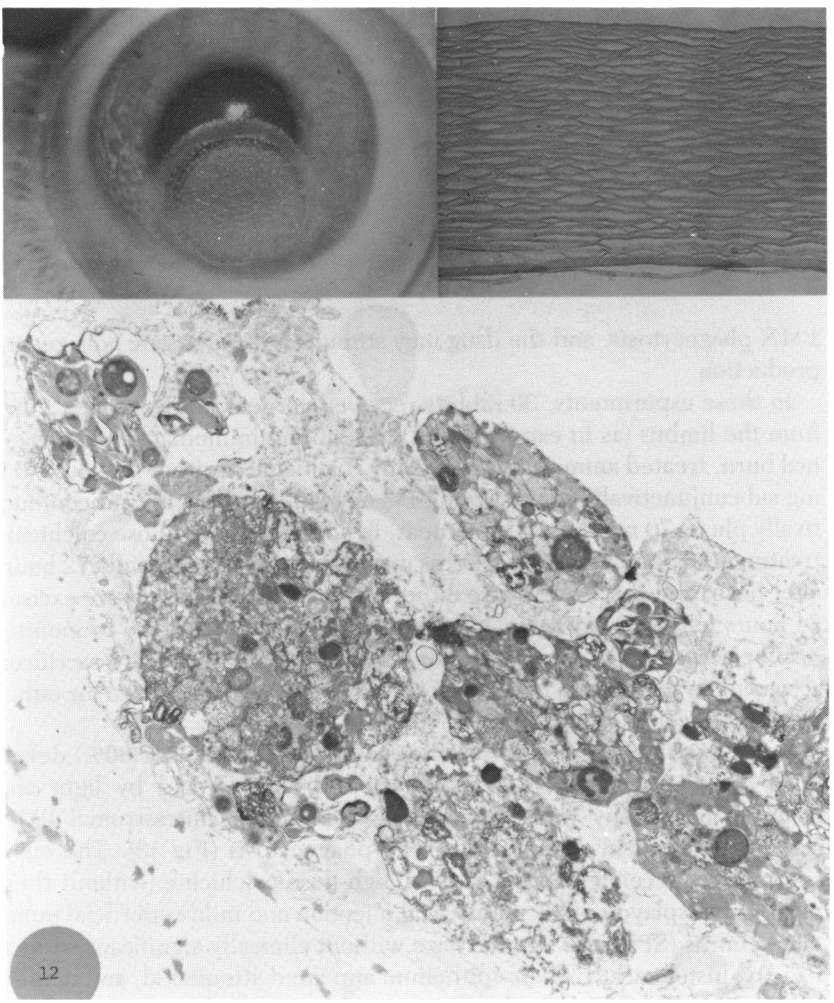



FIGURE 12

Thermal-burn model and cyanoacrylate tissue adhesive. TOP LEFT: Clinical appearance of rabbit eye, having undergone thermal burn 2 mm from limbus, followed immediately by tissue-adhesive application shows retention of adhesive without ulceration on day 8. TOP RIGHT: Light microscopic view of cornea (on day 14 after thermal burn) is remarkable for maintenance of acellular stroma that has not ulcerated (hematoxylin and eosin, × 300). BOTTOM: TEM of thermal-burned cornea having undergone clinically apparent ulceration prior to adhesive application on day 5 and sacrifice on day 18. Note extensive debris of PMN that has presumably infiltrated stroma prior to adhesive application, but is importantly devoid of viable PMN unable to infiltrate beneath adhesive. Several lucent globules of adhesive are apparent (UPPER LEFT) (× 12,600).

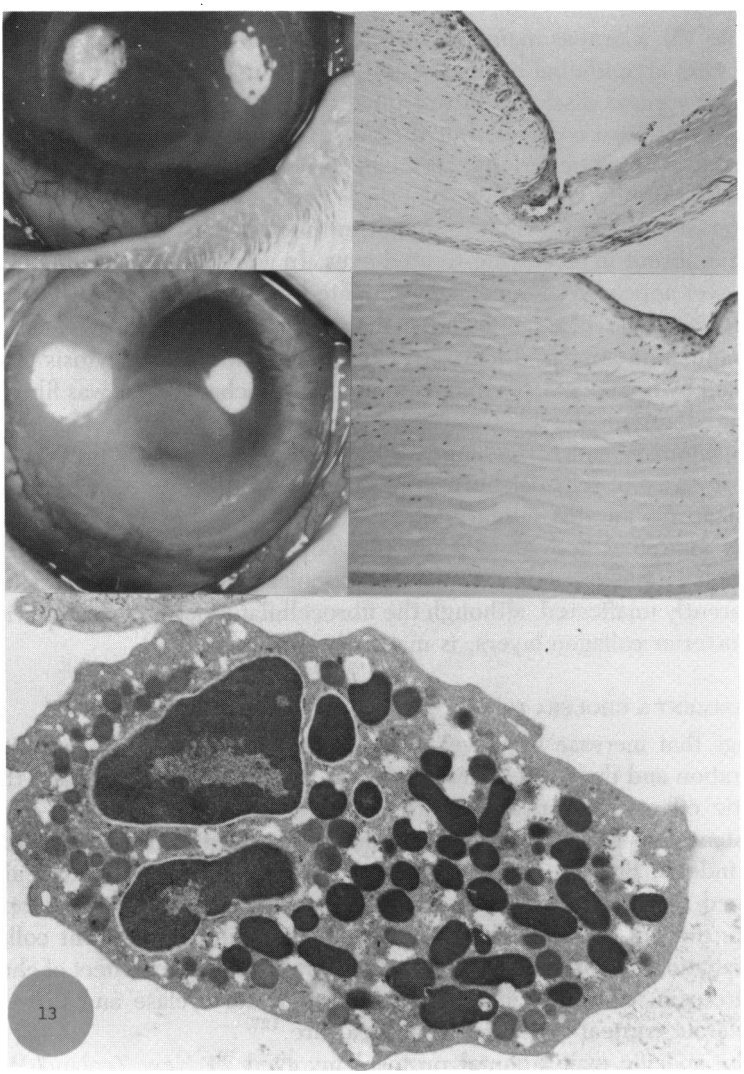


FIGURE 13

Thermal-burn model and colchicine. TOP LEFT: Control eye receiving high-dose colchicine displays mild corneal epithelial haze, limbal neovascularization, and conjunctival injection. TOP RIGHT: Control burn without colchicine shows expected light microscopic findings of stromal ulceration in thermal burned area (note posterior collagenous layer) (hematoxylin and eosin, × 150). MIDDLE LEFT: Clinical appearance 8 days after thermal burn, followed by low-dose colchicine treatment, includes minor epithelial defect without ulceration. MIDDLE RIGHT: Light microscopy of cornea reveals intact epithelium, nonulcerated stroma with few inflammatory cells, and absence of posterior collagenous layer (hematoxylin and eosin, × 150). BOTTOM: TEM in area of thermal burn following colchicine treatment shows quiescent PMN that has retained cytoplasmic granules and has performed minimal phagocytosis (× 18,700).

(Table VI). Corneas maintained in tissue culture similarly displayed retardation of epithelial healing. Light microscopy and TEM of colchicine-treated corneas disclosed intrastromal PMN to be nearly as numerous as in thermal-burn controls, but they displayed seemingly less degranulation and phagocytic activity, although corneas receiving intrastromal latex spheres exhibited phagocytosis of these particles by PMN. Unexpectedly, posterior collagen layers (prominent in thermal-burn controls) were altogether absent in colchicine-treated eyes. In addition, the healing epithelial layer appeared thinned and attenuated with numerous arrested mitotic figures. The PMN infiltration may have been decreased, and PMN degranulation appeared less active, although PMN phagocytosis (as evidenced by uptake of latex microspheres) was unchanged, as was fibroblastic proliferation (Fig 14).

In summary, colchicine appears to decrease significantly the incidence and severity of thermal burn-induced stromal ulceration. Although the mechanisms for this salutary effect are unclear, a possible reduction in PMN migratory activity (but not phagocytosis) is coupled to decreased epithelial recovery and stromal neovascularization. Stromal healing is apparently unaffected, although the fibrocellular reaction, usually evident as posterior collagen layers, is markedly suppressed.

#### EXPERIMENT 3: CHOLERA TOXIN

Drugs that increase the level of cAMP, which is known to inhibit the migration and degranulation of PMN,[125] should have a potentially therapeutic effect on corneal ulceration. Unfortunately, the effect of such substances in a particular tissue is not predictable from in vitro studies for, indeed, Berman and co-workers[126] have demonstrated that in alkali-burned rabbit corneas topical treatment with adenosine 5′-monophosphate did not prevent ulceration despite its ability to prevent collagen degradation in culture. We chose, therefore, to study the effect of cholera enterotoxin, a nonspecific activator of adenylate cyclase and proved to accelerate corneal epithelial wound closure.[127]

The specific experimental protocol involved 24 New Zealand White rabbits who received standard thermal burn (2 mm central to limbus) of both corneas. One hour after burning, one eye of each animal received a 10-minute exposure (via immersion well) of CTX and the other eye received similar exposure to heat-inactivated CTX (ICTX), both at concentrations of 2 μg/200 μl phosphate-buffered saline. (There is no systemic absorption of topically applied CTX.) Animals were examined by slit-lamp biomicroscopy daily and then received repeated daily exposures to CTX or ICTX as above. Animals were sacrificed individually either when the

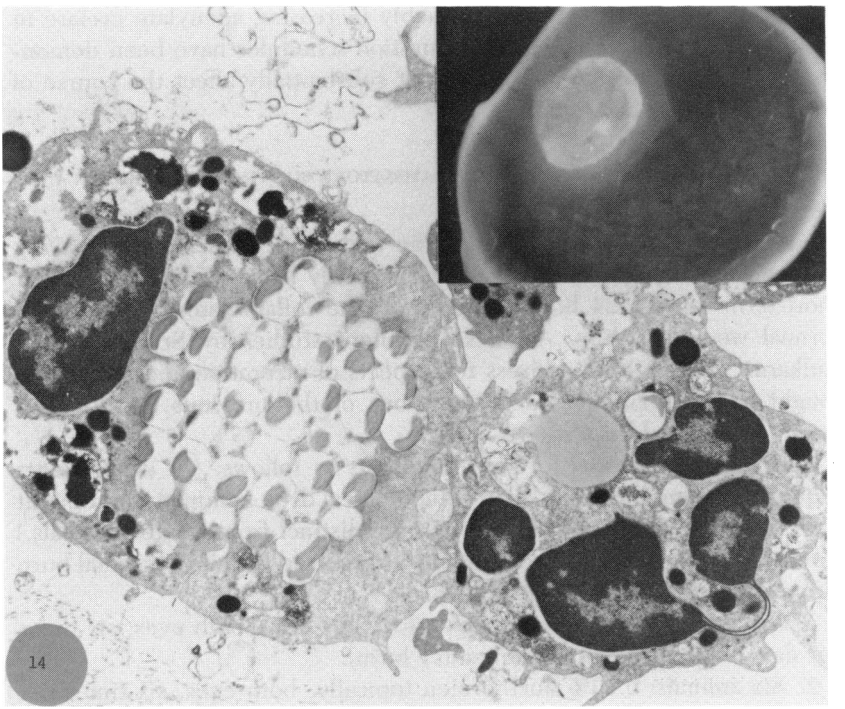



FIGURE 14
Thermal-burn model and colchicine. INSET: Gross photomicrograph of thermal-burned rabbit cornea with latex microspheres injected intrastromally and allowed to heal in organ culture shows retardation of epithelial recovery. Main figure, TEM of thermal-burned cornea, having received intrastromal latex microspheres, confirms phagocytic capability of PMN (× 12,600).

cornea perforated or when both burn areas were healed and vascularized. All corneas were prepared for light or electron microscopy.

In eyes treated with CTX, epithelial recovery was complete by day 4 (no different from untreated controls). The onset of stromal ulceration averaged day 6, and progressive ulceration occurred in 20 corneas (83%), with the remaining corneas developing neovascularization and healing by day 10. The ICTX-treated eyes reepithelialized by day 4; however, 18 (75%) of these corneas also ulcerated or perforated, or both, by day 10. Histopathologically, ulcerating corneas displayed epithelial defects and stromas infiltrated with acute inflammatory cells, and these features were not distinguishable from untreated thermal-burn controls (as previously illustrated).

In summary, CTX, while presumably increasing adenylate cyclase in this model (as similar dosage and duration schedules have been demonstrated in the rabbit cornea), does not substantially affect the course of stromal ulceration or repair, or both.

#### EXPERIMENT 4: NONSTEROIDAL ANTIINFLAMMATORY AGENTS (FLURBIPROFEN AND INDOMETHACIN)

Among the NSAI agents, flurbiprofen and indomethacin, both having prostaglandin inhibitory effects, have recently been evaluated for their short-term effects (24 hours or less) on the inflammatory response to corneal wound healing. As these excellent studies by Srinivasan and Kulkarni[45,61,62] did not address the aspects of ulceration and repair, we sought to complement the observations of the previous investigators using the thermal-burn model.

The specific protocol for flurbiprofen is as follows: (In experiments requiring systemic therapy or in which topical drug administration might have systemic effects, control animals are distinct from treated animals.) Twenty-four New Zealand Albino rabbits received standard thermal burn (2 mm central to limbus), both eyes.

1. Six animals: 0.03% flurbiprofen topically only, both eyes, six times per day (beginning immediately after burn).
2. Six animals: 0.03% flurbiprofen topically, both eyes, six times per day plus 5 mg/kg flurbiprofen intraperitoneally daily (beginning immediately after burn).
3. Six animals: topical vehicle only, both eyes, six times per day (positive control).
4. Six animals: no treatment until onset of ulceration (estimate days 5 to 7), then begin topical and systemic flurbiprofen as above. (The topical dosage of 0.03% flurbiprofen had been determined to be approximately equivalent to 1% prednisolone acetate with respect to corneal wound healing in rabbits.[64,128]) All animals were examined daily with slit-lamp biomicroscopy and were sacrificed individually when either eye of any animal perforated or when both eyes healed and vascularized. All corneas were then prepared for light microscopy or TEM.

Among positive control eyes (group C), 10 of 12 (83%) developed stromal ulceration by day 6, and 8 (67%) of these corneas perforated, while 4 (33%) corneas healed by day 12. Stromal neovascularization reached the edge of the burn area (2 mm from limbus) on the average by day 10. Histopathologic findings were as reported previously.

Among eyes treated topically alone (group A) or in combination with systemic flurbiprofen (group B) beginning immediately after burning,

similar effects were observed, and thus these two groups are reported together. In this group of 24 eyes, conjunctival inflammation was substantially reduced relative to controls. More striking was the effect of flurbiprofen in reducing the incidence and severity of ulcers, as by day 6, only 7 of 24 (29%) had ulcerated and only 2 (8.3%) of these perforated, while by day 12, 22 (92%) had healed. Flurbiprofen also substantially reduced the progression of stromal neovascularization, as by day 10 stromal neovascular ingrowth from the limbus averaged 0.5 mm (compared to 2.0 mm in controls).

Among the 12 eyes in which treatment was delayed (group D), corneal ulceration developed in 10 (83.3%), similar to untreated controls (group C). Treatment with topical and systemic flurbiprofen seemingly was not effective in retarding the progression to ulceration already in progress, as seven (58%) perforated while five (42%) healed by day 12. Neovascularization also progressed at the same rate as controls.

Histopathologically, corneas undergoing ulceration consistently demonstrated epithelial defects and stromal infiltration by acute inflammatory cells with few fibroblasts. At the same time, flurbiprofen-treated eyes that were not ulcerating exhibited abundant fibroblasts and greatly reduced inflammatory infiltrates.

The same protocol was adapted for the indomethacin experiment as follows: again, 24 New Zealand Albino rabbits received standard thermal burns (2 mm central to limbus), both eyes.

1. Six animals: 0.5% indomethacin topically only, both eyes, six times per day (beginning immediately after burn).
2. Six animals: 0.5% indomethacin topically, both eyes, six times per day plus 25 mg/kg indomethacin intraperitoneally daily (beginning immediately after burn).
3. Six animals: topical vehicle only, both eyes, six times per day (positive control).
4. Six animals: no treatment until onset of ulceration (estimate days 5 to 7), then begin topical and systemic indomethacin as above. Clinical and histopathologic examination criteria were as for flurbiprofen experiments.

Among the 12 positive control eyes (group C), 9 (75%) corneas ulcerated by day 6, and 8 (67%) perforated. Stromal neovascularization required an average of 10 days to progress 2 mm from limbus to edge of burn.

As was observed for flurbiprofen, topically treated eyes (group A) or topically and systemically treated eyes (group B), beginning immediately after burning, behaved indistinguishably. In this combined group of 24 eyes, conjunctival inflammation was diminished relative to positive con-

trols. Similarly, indomethacin reduced the incidence and severity of ulcers, as by day 6, 8 of 24 (33%) had ulcerated and only 3 (12.5%) subsequently perforated. Stromal neovascularization by day 10 was also somewhat retarded to 0.7 mm (compared to 2.0 mm in controls). Among the 12 eyes in which indomethacin therapy was delayed (group D), corneal ulceration also developed in 9 (75%) by day 6. Indomethacin therapy did not alter the course of ulceration already in progress, as seven (58%) perforated by day 12. Neovascularization also progressed at the same rate as controls. Histologic studies in these corneas consistently showed that eyes with the deepest ulcers and perforations had the fewest fibroblasts and the most dense PMN infiltration.

In summary, at the dosage concentration and treatment schedules tested, both NSAI agents (flurbiprofen and indomethacin) demonstrated substantial suppression of inflammatory activity (both clinically and histologically) as well as decreasing the frequency and severity of corneal ulceration. This effect was evident only when treatment was instituted immediately after burning, whereas neither drug was effective in halting the progression of established stromal ulceration. These findings are entirely consistent with the observations of Phillips et al,[106] who compared prednisolone and medroxyprogesterone acetate in this same thermal-burn model. Our observations are perhaps in keeping with Donshik and associates,[60] who also observed the suppression of ulceration and inflammation by corticosteroid therapy instituted immediately after alkali burning in the rabbit. Taken together, these results confirm our rationale for utilizing intensive corticosteroid therapy immediately in the management of acute chemical or thermal burns in human corneas, since vigorous suppression of the initial inflammatory response appears to be critically beneficial in avoiding ongoing inflammatory cell recruitment to the injured stroma. Perhaps flurbiprofen or indomethacin, or both, might have similar application in this situation as alternatives to the prednisolone, dexamethasone, and medroxyprogesterone acetate preparations in current use.

#### EXPERIMENT 5: RETINOIC ACID

Retinoids have several clinical and experimental actions that are of therapeutic benefit for sterile corneal ulceration. Specifically, retinoic acid is known (1) to improve corneal xerosis in vitamin A-deficient rats, rabbits, and human beings,[97,108,129,130] (2) to inhibit the collagenase secretion of cultured rheumatoid synovial cells,[131] and (3) to improve the healing rate of experimental epithelial wounds in rabbit corneas.[53,132] Most recently, Tseng et al[54] have demonstrated the effect of topical retinoic acid to

promote healing of persistent epithelial defects in human corneas.

All-*trans* retinoic acid was prepared in vegetable oil to a final concentration of 0.1% and 1.0%. Forty albino rabbits were utilized in this study. All animals received bilateral corneal thermal burns (2 mm central to limbus). One eye of each animal received retinoic acid topically four times daily, beginning immediately after burning (20 eyes at 0.1%, 20 eyes at 1.0%). The opposite eyes received only the oil vehicle four times daily as a positive control. Animals were examined daily by slit lamp and were sacrificed if either eye developed a corneal perforation. Selected corneas were examined by light and electron microscopy.

Among the positive controls, 31 of 40 (77.5%) developed deep stromal ulceration or perforation, similar to previous control series. Among the retinoid-treated eyes, those receiving 0.1% retinoic acid exhibited no difference in conjunctival inflammation relative to controls, whereas those treated with 1.0% retinoic acid were consistently more inflamed. Epithelial healing over the burned stroma proceeded at approximately the same rate in all groups, invariably being complete by day 3. Stromal ulceration seemed less frequent in the treated eyes, as 12 of 20 (60%) eyes treated with 0.1% retinoic acid and 10 of 20 (50%) eyes treated with 1.0% retinoic acid progressed to deep ulceration or perforation; however, these differences were not statistically significant in comparison to positive controls ($P > 0.1$). Stromal neovascularization was somewhat accelerated in treated eyes, as new vessels reached the edge of the burned area (2 mm from limbus) by about day 6 (compared to day 10 for thermal-burn controls). For histologic comparison, we examined paired specimens from the same animal. Notable was the consistent attenuation of the acute inflammatory phase, as PMN were less frequent in retinoid-treated stromas than in matched controls. In addition, treated corneas appeared to enter more rapidly into a healing phase, as increased stromal fibroblastic and neovascular reactions were consistent with their corresponding clinical observations.

In summary, these observations, although preliminary, suggest a beneficial effect of retinoic acid in potentiating stromal wound healing and neovascularization, thereby lessening ulceration in the thermally burned cornea. Further studies are indicated to substantiate these findings.

#### EXPERIMENT 6: IMMUNOSUPPRESSION

Another approach to the hypothesis that inflammatory cells are the effectors of corneal stromal degradation is to suppress leukocyte function locally or systemically and witness its effect on a model corneal wound. Accordingly, as both cyclophosphamide (Cytoxan) and busulfan (Myleran)

are capable of immunosuppression without affecting PMN function,[133] these agents were used to produce pancytopenia in rabbits prior to thermal burning of the cornea.

In these experiments, albino rabbits were given either cyclophosphamide (100 mg/kg) or busulfan (20 mg/kg) intraperitoneally. Peripheral white blood cell (WBC) counts and differentials were obtained every two days. Following development of neutropenia, Bicillin (250 mg subcutaneously) was administered every 5 days as prophylaxis against the increased predisposition for bacterial infections. Prior to administration of cytotoxic drugs, the WBC ranged from 3800 to 9000 with 49% PMN. In cyclophosphamide-treated animals, an initial rebound increase in WBC, often to approximately 20,000, occurred within the first 5 to 7 days, followed by a decrease to less than 2000 and recovery to normal ranges over the next 10 to 14 days. Busulfan-treated animals did not exhibit an initial WBC elevation but instead maintained a pancytopenia of less than 1000 WBC for about 10 days. Cyclophosphamide or busulfan was administered (doses as previously specified) to maintain total WBC at less than 1000, of which approximately 60% were PMN. (Unfortunately, these animals were seemingly extremely susceptible to pneumonitis or other infections and could be maintained only for about 2 weeks in the fully immunosuppressed state without dying from infectious complications. In fact, the overall mortality rate in preparing and maintaining animals for this experiment was nearly 60%.)

Among animals receiving cyclophosphamide, ten rabbits were immunosuppressed, and then all received bilateral corneal thermal burns (2 mm from limbus to edge of burn). Three of these animals died, on days 3, 5, and 12 following thermal injury, and at the time of death no significant ulcers had developed. Among the seven remaining animals, 5 of 14 corneas (36%) ulcerated by day 14, and the onset of these ulcerations tended to be somewhat later (days 7 to 9) than in previous positive controls (usually by day 5). Stromal neovascularization was also delayed, as blood vessels usually reached the wound edge (2 mm from limbus) only by day 10. Histologically, the nonulcerating corneas displayed intact but thin epithelium and nearly acellular stromas, with few inflammatory cells in evidence. In the ulcerating corneas, the epithelial layers were defective, and the stromas showed degradation of the extracellular collagen and ground substance; however, as in the nonulcerating corneas, few acute inflammatory cells were apparent.

Among the rabbits treated with busulfan, 11 animals could be maintained in a neutropenic state for sufficient duration to permit experimentation and follow-up. After receiving bilateral thermal burns (2 mm from

limbus to edge of burn), two animals died on days 5 and 6; at the time of death, no ulcers had developed. Among the nine remaining animals, 5 of 18 corneas (28%) ulcerated by day 14. The retardation in ulcer onset and stromal neovascularization were similar to observations in the cyclophosphamide-treated group. Histologically, the ulcerating corneas exhibited some acute inflammatory cells within the degraded stroma, whereas nonulcerating corneas maintained intact and nearly acellular stromas. Neither clinical nor histologic evidence of infection was present.

In summary, we encountered major difficulty in developing and maintaining rabbits in a systemically immunosuppressed state since these animals are highly susceptible to infection and succumb rapidly, thereby making long-term experiments exceedingly difficult to perform. Nonetheless, in animals that could be maintained for two weeks in the immunosuppressed state, the standard thermal-burn protocol resulted in a highly reduced rate of corneal ulceration (approximately 30%, compared to approximately 90% in previous positive-control series). The paucity of neutrophils was also reflected in the stromas examined histologically. Thus, the reduction in ulceration rate can again be correlated with the absence of acute inflammatory cells. Not readily explainable is the absence of PMN in the stromas of those few corneas that did undergo ulceration. This may point to other endogenous sources of collagenolytic protease and other cellular interactions beyond those involving inflammatory cells. These overall findings are, however, entirely consistent with our previous study[102] of immunosuppression (by cyclophosphamide) and selective inflammatory cell depletion (by antineutrophil serum) in guinea pig, in which alkali burn-induced corneal ulceration was markedly reduced.

## CONCLUSIONS

The mechanisms, interactions, and regulations of sterile corneal ulceration involve complex interactions. The intention of this thesis is to focus on the potential role of the PMN within this framework.

Histologic comparisons of corneal transplant specimens obtained from human patients with diverse clinical diagnoses suggested that, in situations of active corneal melting, the PMN is the predominant cell type at the site of stromal degradation. The PMN are much less prevalent in corneas that had experienced prior ulceration but that had become quiescent at the time of keratoplasty.

Using an immunoelectrophoresis technique to quantitate collagenolytic activity in tears and correlating this with cytology and clinical status in

patients with corneal melting disorders, it was consistently notable that (in tears of subjects with actively melting corneas) both elevated levels of collagenolytic enzymes and PMN were present. With reduction in clinical inflammatory activity, both PMN and collagenolytic enzymes diminished.

A highly reproducible model of sterile corneal ulceration is available utilizing a thermal burn. This model can be manipulated to favor wound repair (by placing the burn in the central cornea) or progressive ulceration (by placing the burn in the midperipheral cornea). Histologic observations of ulcerating corneas revealed numerous PMN at the site of ulceration, and biochemical studies showed these corneas to possess collagenolytic activity and to degrade their own stromal collagen in vitro.

Several manipulations of this thermal-burn model demonstrate its usefulness in testing potential therapeutic interventions for clinical use. Thus, cyanoacrylate tissue adhesive markedly prevents and arrests ulceration in this model, probably by excluding inflammatory cells from the thermal injury site. Topically and systemically administered colchicine, a microtubule inhibitor, also reduced the incidence and severity of thermal burn-induced ulceration, possibly by reducing PMN migratory activity. Topically applied CTX, although presumably stimulating adenylate cyclase, does not substantially affect the course of stromal ulceration and repair. The NSAI agents (flurbiprofen and indomethacin) decreased the frequency and severity of ulceration when applied immediately after burning, but neither drug was beneficial in halting the progression of established stromal ulceration. Topically applied retinoic acid appears to potentiate stromal wound healing and neovascularization, thereby lessening ulceration. Finally, systemic immunosuppression with cyclophosphamide or busulfan is difficult to maintain in rabbits; however, in short-term experiments, a substantially decreased rate of corneal ulceration was correlated with a marked decrease in PMN, both circulating and within the injured corneas.

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