Ulceration Is Correlated with Degradation of Fibrin and Fibronectin at the Corneal Surface

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Although ulceration of the corneal stroma after alkali burns is known to be correlated with persistent epithelial defects, the relationship between a defect and the mediators thought to contribute to stromal destruction (plasminogen activator, plasmin, collagenase) has not been understood. This report demonstrates that fibrin and fibronectin appear on the stromal surface after an alkali burn, and that those substratum, matrix components disappear in correlation with the appearance of plasminogen activator on the stromal surface, re-surfacing by the epithelium and a persistent epithelial defect. The facts that epithelium releases plasminogen activator1 and that plasmin, generated from plasminogen by an activator, can degrade both fibrin1 and fibronectin,2 as well as the laminin3 component of the subepithelial basement membrane, would suggest that the plasminogen activator-plasmin system effect degradation of those macromolecules, thus initiating the events that lead to eventual, frank stromal ulceration. It is hypothesized that stromal ulceration is initiated by the chronic secretion from an epithelium with a persistent defect of a protease (plasminogen activator) involved in wound healing. Invest Ophthalmol Vis Sci 24:1358–1366, 1983

It has been hypothesized previously that corneal ulceration after alkali burns results from the failure of wound healing to progress beyond a phase of proteolytic debridement related to a persistent epithelial defect.4,5 Although it now seems probable that the plasminogen activator (PA)-plasmin system initiates both stromal destruction and repair (ie, vascularization), after alkali burns, the relationship between the mediators involved in stromal destruction and a persistent epithelial defect has not been understood.

Fujikawa et al7 recently have reported that removal of epithelium by scrape injury of the normal rabbit cornea results in the appearance on the stromal surface of fibrin(ogen) and fibronectin. These findings suggested to us the possibility that those same macromolecules also would polymerize on the stromal surface after alkali burns; and that, as suggested by Fujikawa et al, corneal reepithelialization might involve interaction between epithelium and a subjacent fibrin-fibronectin matrix during corneal resurfacing. The current studies demonstrate that fibrin and fibronectin, presumably derived by extravasation from limbal blood vessels, do appear on the anterior stromal surface after an alkali burn, and that stromal ulceration is correlated with a persistent epithelial defect, the appearance of plasminogen activator on the stromal surface, and with the loss of subepithelial fibrin and fibronectin.

Materials and Methods

Male albino rabbits (3–4 lbs) were anesthetized systemically with intravenous sodium pentobarbital and topically by proparacaine (see Addendum). Alkali burns were made by placing 8-mm diameter discs of Whatman #40 filter paper, saturated with 4 N NaOH, on the surface of the cornea for 2 min. Burns of this severity produce ring-shaped ulcers between 5 and 10 days post-burn, in virtually 100% of corneas so treated (Fig. 4A). In this model, the cornea usually is resurfaced completely by epithelium by 3 to 5 days post-burn; later, however, a persistent epithelial defect develops, followed by the stromal ulceration.

Immunohistologic Studies

For localization studies, animals were sacrificed by an overdose of intravenous pentobarbital and topical proparacaine (see Addendum). Alkali burns were made by placing 8-mm diameter discs of Whatman #40 filter paper, saturated with 4 N NaOH, on the surface of the cornea for 2 min. Burns of this severity produce ring-shaped ulcers between 5 and 10 days post-burn, in virtually 100% of corneas so treated (Fig. 4A). In this model, the cornea usually is resurfaced completely by epithelium by 3 to 5 days post-burn; later, however, a persistent epithelial defect develops, followed by the stromal ulceration.

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Supported by NIH Grant No. EYO3879-03 and a general Departmental Grant from Research to Prevent Blindness, Inc.

Submitted for publication November 29, 1982.

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0146-0404/83/1000/1358/$1.25 © Association for Research in Vision and Ophthalmology
Tek Cryostat (Miles Scientific, Naperville, IL) and were mounted on gelatin-coated microscope slides. For immunohistologic staining (direct method), slides were soaked for 10 min at room temperature in phosphate-buffered saline (PBS), pH 7.3, drained, and incubated for 30 min with fluorescein-labeled antibody (diluted in 0.1% bovine serum albumin) in a moist environment at room temperature. Stained slides were washed gently in several changes of pH buffered saline (PBS), for 30 min with fluorescein-labeled antibody (diluted for 30 min with fluorescein-labeled antibody (diluted

Antisera

Fluorescein-labeled goat IgG anti-human fibrinogen fraction was obtained from Kallestad Laboratories (Austin, TX). Fluorescein-labeled sheep IgG anti-rabbit fibrinogen was obtained from Cappel Laboratories (Malvern, PA). Although antibodies against fibrinogen, and not solubilized fibrin, were used in the current study, it is assumed, based on the known insolubility of fibrin and its ability to cross-link to fibronectin,2 that the material identified as “fibrin” is, indeed, fibrin and not fibrinogen. Fluorescein-labeled goat IgG anti-human plasma fibrinogen and fluorescein-labeled goat IgG anti-rabbit plasma fibrinogen were obtained from Cappel Laboratories.

Since fibrinogen and fibronectin commonly co-purify,2 control absorptions were performed with each protein to insure the specificity of staining of tissue sections by fluorescinated antibodies. Anti-human fibrinogen was incubated with Sephadex® beads (Pharmacia Fine Chemicals, Uppsala, Sweden) to which human fibronectin had been coupled (1 X absorption). After incubation, the beads were centrifuged down in a Beckman® Microfuge B (Beckman Instruments, Inc., Fullerton, CA) (~10,000 x g) and the adsorbed supernatant divided into two aliquots: one aliquot was incubated with frozen sections from an ulcerating cornea (14 days post-burn); and the other aliquot was incubated with Sephadex beads to which human fibrinogen had been coupled (2 X absorption). After incubation and centrifugation, the latter absorbed supernatant was incubated with other sections from the same ulcerating cornea. Staining by both fractions was compared with that obtained by non-absorbed antibody.

In comparable fashion, fluorescein-labeled antibodies to human fibronectin were incubated with, sequentially, beads to which human fibrinogen or human fibronectin had been coupled. Initial studies with absorbed anti-human fibrinogen (0.54 mg IgG/ml) and anti-human fibronectin (1.5 mg IgG/ml) produced the same immunohistologic patterns as those with anti-rabbit fibrinogen (1.25 mg IgG/ml) and anti-rabbit fibronectin (0.84 mg IgG/ml), respectively, except that staining with anti-rabbit antibodies was more intense at the titers used. Subsequent work with rabbit corneas was done using anti-rabbit fibrinogen and anti-rabbit fibronectin. Studies with human corneas were done with anti-human fibrinogen and fibronectin IgG fractions. Fluorescein-labeled non-immune sheep or goat IgG (Cappel Laboratories) at concentrations greater than or equal to those used for fluorescein-labeled anti-fibrinogen and anti-fibronectin, respectively, did not stain the anterior surface of the 14-day post-burn ulcerated rabbit cornea. Corroboration of the immunohistochemical identification of fibrin is provided by electron microscopy which showed (Fig. 7) banded fibrin on the corneal surface 30 hours after the alkaline burn.

Fluorescein-labeled goat IgG anti-human plasminogen was obtained from Atlantic Antibodies. A goat IgG antibody preparation to the 33,000 MW heavy chain of urokinase (kidney plasminogen activator) was obtained through the courtesy of Dr. Kenzo Tanaka, Kyushu University, Japan. The anti-UK preparation gave a single precipitin band against highly purified urokinase and against activator in normal human tears (data not shown). Localization of urokinase-like activator in sections of alkali-burned, ulcerating corneas was obtained by the indirect method: sections were incubated with anti-UK antibodies (3.6 mg IgG/ml) as under conditions used for the localization of fibrin and fibronectin; subsequently, the sections were rinsed in PBS, stained with fluorescein-labeled F(ab)2 rabbit anti-goat IgG (0.94 mg IgG/ml) (Cappel Laboratories), rinsed again, mounted in Elvanol and inspected. As controls for activator localization, normal rabbit corneas showed no activator in the subepithelial basement membrane region, using indirect staining; and ulcer corneas treated with non-immune goat IgG (3.6 mg/ul) followed by fluorescein-labeled F(ab)2; rabbit antigoat IgG (0.94 mg IgG/ml) or treated with the latter reagent alone showed no staining of the corneal surface.

Results

Normal rabbit corneas (Fig. 1A) demonstrated no staining with anti-fibrin (Fig. 1B) or anti-fibronectin (Fig. 1C) antibodies in the subepithelial basement membrane region, although Descemet’s Membrane did stain with anti-fibronectin (1C, inset) antibodies but not for fibrin. These results are thus in agreement with those of Fujikawa and colleagues.7 At 30 hours post-burn, all cells of the burned cornea appeared dead histologically (Fig. 2A), and the entire epithelium-free, anterior surface of the stroma was covered with fibrin (Fig. 2B; Fig. 7) and fibronectin (Fig. 2C).

At seven days post-burn, by which time the cornea had developed a persistent epithelial defect and a superficial stromal ulcer (Fig. 3A, inset), staining for fibrin...
HEMATOXYLIN ANTI-FIBRIN ANTI-FIBRONECTIN

Fig. 1. Normal rabbit cornea. A. Histologic detail; B. Anti-rabbit fibrinogen; no fibrin was detected in the subepithelial, basement membrane zone, (anterior stromal surface) or associated with Descemet's Membrane; C. Anti-rabbit fibronectin: although the subepithelial zone did not stain for fibronectin, Descemet's Membrane (DM) did (inset). Ep, epithelium. S, stroma (×160). (Note: Sections from normal corneas (1B, 1C) were exposed for a much longer period (3 min) than were those from alkali-burned corneas (1 min) since, with a 1-min exposure, the non-stained sections from normal corneas could not be seen at all.)

Fig. 2. Thirty hours post-burn. A. Histologic detail: the alkali burn has caused death through all layers of the cornea; B. Anti-rabbit fibrinogen: fibrin was present on the whole anterior surface of the stroma; C. Anti-rabbit fibronectin: fibronectin was present on the whole anterior surface of the stroma (×160).

Fig. 3. Seven days post-burn (superficial stromal ulcer). A. Histologic detail: loosely adherent epithelium adjacent to border of stromal ulcer has been pulled away from the cornea. Inset: superficial ulcer in vivo (arrow); B. Anti-rabbit fibrinogen: fibrin was present on the anterior surface of the stroma in the epithelial defect region and subjacent to epithelial cells bordering the defect. Arrow indicates attenuated staining. Ep, epithelium; C. Anti-rabbit fibronectin: fibronectin also was present in the defect region and under the epithelial cells bordering the defect; and fibronectin, like fibrin became increasingly attenuated (arrow) under epithelium increasingly more distal from the defect. Ep, epithelium (×160).

Fig. 4. Fourteen days post-burn (deep stromal ulcer). A. Histologic detail: a persistent epithelial defect was present superficial to the stromal ulcer containing PMN. Inset: deep ulcer, in vivo (arrow); B. Anti-rabbit fibrinogen: fibrin was present between the borders of the defect superficial to the stromal ulcer and under the epithelial cells bordering the defect. It became increasingly attenuated then undetectable (arrow) more distal from the defect/ulcer region. Ep, epithelium; C. Anti-rabbit fibronectin: fibronectin was present between the margins of the defect superficial to the stromal ulcer and under the epithelial cells bordering the defect. It became undetectable (arrow) under epithelium more distal from the defect/ulcer region. Field shown includes epithelium just distal to the defect region. Ep, epithelium (×160).
(Fig. 3B) and fibronectin (Fig. 3C) was diminished beneath the epithelium adjacent to the defect region, and not detected more distal from the defect region (not shown); it was detectable extending over the surface of the stroma between the borders of the epithelial defect. Fibronectin continued to be demonstrable in Descemet's membrane. Fibrin also was identified on the outermost aspect of the membrane.

By 14 days post-burn, a deep stromal ulcer had developed (Fig. 4A, inset). Fibrin (Fig. 4B) and fibronectin (Fig. 4C) were again identifiable in the defect region on the surface of the stromal ulcer and under the leading edges of epithelium. However, fibrin and fibronectin became attenuated and were not identifiable further away from the defect region.

That the identifications of fibrin and fibronectin are valid is supported by the results of the absorption controls (Figs. 5A–5C, 6A–6C) and, for fibrin, the electronmicroscopic evidence (Fig. 7). Urokinase-like plasminogen activator (Fig. 8) was found to be co-extensive with fibrin and fibronectin, and staining for PA was found to be diminished when specific staining for those proteins also was diminished.

Results from studies with a human ulcer caused by an alkali burn were in accord with the observations in the rabbit model. The non-ulcerating human cornea had, unlike the normal rabbit cornea, detectable fibronectin in the subepithelial basement membrane (Fig. 9A). As in the normal rabbit cornea, fibronectin also was present in Descemet's Membrane (Fig. 9A,
Fig. 7. Thirty hours post-burn: electron microscopy of the edge of the burned corneal surface. Fibrin (within inset), characterized by a banding period of ~260 Å, was associated with the epithelium and (not shown) was present as irregularly-shaped aggregates in association with the basement membrane over the whole surface of the cornea. Ep, epithelium; F, fibrin (X16,000).

inset). Fibrin was not detectable in either the subepithelial region or stroma of the normal human cornea. In the case of an ulcer after an alkali burn, however, fibrin (Fig. 9C) and fibronectin (Fig. 9B) were detected both on the surface of the cornea adjacent to the ulcer region and in the stromal ulcer region itself (Fig. 9D). Moreover, plasminogen that was not detectable in the normal human corneal stroma (using the direct staining method) was apparent in the stroma of the ulcerating cornea (Fig. 9E). Fibrin also was detected in association with the corneal endothelium (Fig. 9C, inset) as observed after alkali burns of the rabbit cornea.

Discussion

The correlation of persistent (or recurrent) epithelial defects with subsequent stromal ulceration after alkali burns has prompted clinicians to exert great effort to get those defects to heal. Such attempts include the use of soft (bandage) lenses which, sometimes, permit the epithelium to cover the stroma under the lens and to develop adhesive structures so that the defect does not recur. It has been hypothesized previously that after alkali burns, unlike after simple injuries like scrape-debridement, wound healing goes awry such that epithelium is trapped in a phase of proteolytic debridement. This hypothesis was based on the observation that stromal ulceration begins superficially in association with the epithelial defect and on the assumption that re-surfacing epithelium secretes proteases involved in wound healing/debridement. Indeed, Weimar had reported that epithelium of injured rat cornea releases a serine protease that is chemotactic for polymorphonuclear leukocytes (PMN); and Pandolfi and Astrup had suggested in an earlier study on corneal plasminogen activator that "... fibrinolytically active corneal epithelial cells could assist in tissue repair by providing the surfaces of the avascular cornea with sufficient plasminogen activator to prevent excessive or permanent deposition of fibrin following exudation after injury. . . ."

The fact that epithelium from an alkali-burned, ulcerating cornea releases plasminogen activator, that plasmin is known to be able to degrade fibrin and fibronectin, and the current observation that subepithelial fibrin and fibronectin apparently are resorbed in regions where PA is located, would suggest that PA initiates the system that mediates the disappearance of the fibrin and fibronectin. Although direct assay of
PA production by cells adjacent to a defect has not yet been made, it is hypothesized that PA is secreted chronically from epithelial cells adjacent to a defect, and perhaps in response to fibrin and fibronectin that are polymerizing continuously on the stromal surface of the deepening ulcer. It may be suggested further that the PA/plasmin wound-healing proteolytic system normally is controlled after corneal injury such that frank loss of basement membrane and stromal matrix do not occur. After an alkali burn, in the presence of a persistent epithelial defect and after the subepithelial basement membrane also is degraded, plasminogen activator-plasmin-dependent pathways could, in this hypothesis, also initiate the events that lead to stromal destruction and eventual repair (Fig. 10).

In a recent study of wound healing after simple scrape injuries of the rabbit cornea, Fujikawa and colleagues have reported that fibrin and fibronectin, which appear on the stromal surface after the scrape injury, disappear gradually over several weeks after complete closure of the epithelial defect. By inference, the fibrin-fibronectin substrate is considered to be possibly necessary for successful re-epithelialization. This hypothesis is supported by work in other systems that suggests that fibronectin can interact with non-epithelial cells to mediate their attachment to substrata.
Fig. 10. Hypothetical sequence of wound-healing proteolytic activities that relate a persistent epithelial defect with stromal ulceration. Plasminogen activator (PA) is thought to be secreted chronically by cells at the leading edge of corneal epithelium adjacent to a persistent defect. Activator activates plasminogen, adsorbed to the subjacent fibrin-fibronectin matrix, to plasmin.1 Plasmin, in turn, degrades the fibrin-fibronectin matrix, and component(s) of the subepithelial basement membrane, subsequent to which it acts at the fibroblast cell surface to stimulate secretion of plasminogen activator and latent collagenase. Plasmin then activates the latent collagenase to a form that can degrade collagen, and plasmin possibly generates chemotactic fragments for PMN from complement.17 PMN then appear to degranulate in the ulcer region.16 PA itself, is angiogenic and results in the enhanced entrance of antiproteases and nutrients into the stroma and the eventual arrest of ulceration.

In the current work, alkali-burned corneas were not examined at the stage when complete epithelial closure had just occurred and prior to the secondary epithelial breakdown. Thus, the results of the current study do not permit a determination of the need for fibrin/fibronectin for re-epithelialization. The finding of fibrin/fibronectin under a completely resurfaced epithelium would not, of course, mean that those macromolecules are required for resurfacing. Had fibrin/fibronectin been found to be no longer present under such an epithelium, the case could be made that complete closure of the defect does not require a fibrin/fibronectin substratum. For a given cornea, however, it is not known if such a substratum had been present but had been resorbed quickly. Moreover, although initial epithelial closure might appear to have occurred in the absence of a fibrin/fibronectin substratum, secondary epithelial breakdown occurs in virtually all of the corneas in this model, and the epithelial breakdown still would be correlated with absence of fibrin/fibronectin. Whether or not fibrin/fibronectin are required for re-epithelialization, fibrin is reported10 to stimulate the secretion of PA in various systems, and the continued presence of (ie, fibrin/fibronectin) might be expected to stimulate corneal epithelial cells to secrete PA chronically. Although perhaps not required for re-epithelialization, it may be suggested that the fibrin/fibronectin substratum would need to be removed in order for strong, hemi-desmosomal adhesive structures to be formed between epithelium and the anterior stroma.

Although scraped corneas were not studied in parallel with alkali-burned corneas in the current work, the observation that following an alkali burn fibrin and fibronectin apparently are resorbed much more quickly (ie, within days rather than within weeks of injury) than after simple scrape-debridement of the normal cornea (Figs. 3B, 3C, 4B, 4C) would suggest that the resurfacing epithelium after an alkali burn secretes higher levels of plasminogen activator than does epithelium after the simple scrape injury. Indeed, the secondary epithelial breakdown and defect (Figs. 3B, 3C) after an alkali burn might result from the shearing forces of the lids5,12 on an epithelium whose raised plasminogen activator activity has resulted in fewer and/or weaker cell attachments to fibrin-fibronectin in the substratum.

Following the development of a defect in the epithelium, ultrastructural changes in the subepithelial basement membrane are observed, and then the basement membrane becomes no longer detectable by electron microscopy.13 Subsequently, stromal ulceration is seen to have occurred.13 This sequence of morphologic changes is consistent with the clinical impression that once the subepithelial basement membrane is no longer present, the stroma ulcerates quickly.1,4 One can hypothesize that, in addition to plasmin which degrades the laminin component that binds epithelial
cells to type IV collagen\textsuperscript{14} of the basement membrane,\textsuperscript{3} collagenases from the epithelium directed against type IV and V collagens also contribute to basement membrane destruction.

Previous work has indicated that the plasminogen activator-plasmin system also can initiate matrix-destructive sequences in the corneal stroma.\textsuperscript{1,4,5} Plasminogen activator activity has been detected previously in the periphery of the normal or ulcerating rabbit corneal stroma by the gel lysis method.\textsuperscript{1} In the current study, however, no specific staining for activator has been observed in the corneal stroma using antiserum to UK. Since culture media from both normal and ulcerating corneas contain activator of approximately 72,000 MW (the MW of vascular-like activators) in addition to activator species of approximately 46,000 MW and 35,000 MW\textsuperscript{15} (UK-like), and since antisera to UK do not react with vascular-like activators, it seems possible that activator in the peripheral cornea is vascular-like and, therefore, not detectable with the anti-UK antibodies used in the present study. Alternatively, peripheral UK-like activator might have been eluted out of the stroma and, thus, was not detectable. Preliminary studies, however, using sections from ulcer corneas that had been fixed with cold glacial acetic acid or ethanol, did not show activator in the corneal periphery after staining with anti-UK antibodies.

To test the hypothesis that the PA/plasmin system has an important role in stromal ulceration, plasminogen was added in previous studies,\textsuperscript{1} to organ cultures of ulcerating rabbit corneas. After the addition of plasminogen to such cultures, plasminogen was found to have been converted to plasmin by plasminogen activator secreted by the corneas;\textsuperscript{1} PA was secreted earlier, and collagenase was secreted by fibroblasts in increased amounts. Plasmin was found to activate collagenase that was secreted in a latent form; and the increased levels of active collagenase resulted in increased degradation of stromal collagen.\textsuperscript{1} Plasmin also has been found to degrade cell-associated fibronectin and to stimulate the secretion of collagenase by corneal fibroblasts in cell culture in keeping with the model reported here (M. Berman, unpublished observations).

In addition to fibroblasts, polymorphonuclear leukocytes (PMN) also are thought to participate in stromal ulceration.\textsuperscript{16} PMN have been found previously to be active phagocytically in stromal ulceration after alkali burns; and a glued-on methylmethacrylate lens which prevents re-epithelialization of the cornea also prevents PMN infiltration of the stroma as well as the stromal ulceration.\textsuperscript{16} Based on these observations,\textsuperscript{16} the data of Weimar\textsuperscript{3} that suggest that a corneal epithelial serine protease causes PMN infiltration of the cornea after injury, and the reports\textsuperscript{17} that plasmin cleaves the third component of complement to produce fragments chemotactic for PMN, it has been hypothesized that the PA/plasmin system is also responsible for attracting PMN into the corneal stroma.\textsuperscript{16} Eventual arrest of ulceration is thought to result from neovascularization of the stroma in response to PA which is angiogenic\textsuperscript{6} (Fig. 10). Thus, the PA/plasmin system is thought, possibly, to initiate the resorption of fibrin and fibronectin on the corneal surface after injury. After an alkali burn, regulation of the system is thought, for unknown reasons, to go awry such that increased secretion of PA results in weaker and/or fewer attachments of epithelium to the subjacent fibrin/fibronectin and the consequent formation of an epithelial defect. (PA species from rabbit corneal epithelial cells and fibroblasts have since been found to be secreted as latent precursors, activatable by plasmin or trypsin.)\textsuperscript{15} Continued secretion of PA is thought to result in the degradation of the subepithelial basement membrane, in the infiltration of the stroma by PMN and in the activation of fibroblasts, both of which cell types contribute to later stromal destruction.\textsuperscript{1,16} While some components of this working hypothesis remain to be established experimentally (eg, a requirement for fibrin/fibronectin for re-epithelialization; the direct demonstration that epithelium from the alkali-burned cornea secretes more PA than does epithelium from the re-surfacing, scraped cornea; and the direct demonstration that PA/plasmin generate signals chemotactic for PMN in the corneal system), the data to date are consistent with the hypothesis that the PA/plasmin system has important roles in corneal ulceration; and the hypothesis provides a framework within which further experimentation can be performed.

**Key words:** alkali burns, plasminogen activator, plasmin, collagenase, fibrin, fibronectin, wound healing, ulceration

**Addendum**

Animals whose corneas are to be alkali burned are first anesthetized systemically by sodium pentobarbital (approximately 20–25 mg/kg) and topically by 0.5% proparacaine hydrochloride to ensure that they are not caused pain by the alkali burns. Animals once adequately anesthetized, are alkali burned unilaterally (the contralateral eye is not burned) by the application for 2 minutes of an 8-mm filter disc saturated with 4 N NaOH. After the burn is made, the cornea is irrigated copiously with 0.9% NaCl; and antibiotic ointment (erythromycin) is instilled in the lower fornix immediately post-burn and daily to prevent corneal infection. Animals treated in this way show no signs of ocular pain either during the administration of the topical burn or during the post-operative period. Animals are killed by the intravenous injection of sodium pentobarbital. The methods of anesthesia used and care are approved by the University Veterinarian, pursuant to the requirements of the Animal Welfare Act,
Amended Public Law 94-279, April, 1976, and those of the
Guide for the Care and Use of Laboratory Animals, 1978,
National Research Council.

Acknowledgments

The authors wish to thank Dr. Harold Dvorak, Beth Israel
Hospital, Boston, for technical advice and Dr. George Waring,
Emory University Medical School, Atlanta, for human ulcer
issues. They also thank Dr. Kenzo Tanaka, Kyushu Uni-
versity, Japan, for a sample of anti-UK antibodies, and Mr.
Donald Boyle and Mr. Thomas Fletcher, Emory University
Medical School, for help with photographic methods.

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