

## Polymorphonuclear Neutrophils Delay Corneal Epithelial Wound Healing In Vitro

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Using an in vitro system for measuring epithelial wound healing, we studied the effect of polymorphonuclear neutrophils (PMNs) and PMN lysate on rat corneal epithelial wound healing. After 22 hr of organ culture, epithelial defects that were originally 3 mm in diameter (7.06 mm<sup>2</sup>) measured 0.41 mm<sup>2</sup> (range, 0.17–0.72 mm<sup>2</sup>) in control rat corneas, 2.11 mm<sup>2</sup> (range, 0.81–3.91 mm<sup>2</sup>) in corneas incubated in medium containing  $5 \times 10^6$  PMN/ml, and 2.49 mm<sup>2</sup> (range, 2.21–2.76 mm<sup>2</sup>) in corneas incubated with lysate obtained from an equivalent number of PMNs. Scanning and transmission electron microscopy showed similar morphology in the three groups. PMNs selectively adhered to the leading edge of the wound in corneas incubated with PMNs. These data indicate that PMNs and PMN lysate significantly slow corneal epithelial wound healing in vitro. *Invest. Ophthalmol Vis Sci* 25:1217–1220, 1984

Several clinically important problems arise from lack of epithelial migration. Persistent epithelial defects often can predispose corneas to sterile stromal ulceration,<sup>1,2</sup> particularly when associated with alkali burns,<sup>2</sup> neurotrophic or metaherpetic defects,<sup>1,2</sup> or vitamin A deficiency.<sup>3</sup> Conversely, because reepithelialization often can arrest further stromal ulceration, clinical therapy is directed, in part, to facilitating prompt epithelial recovery.<sup>2</sup> Clinically, persistent epithelial defects seem to be associated uniformly with inflamed eyes.<sup>1</sup> We therefore addressed the effect of polymorphonuclear neutrophils (PMNs) and their products on the retardation of corneal epithelial wound healing in organ culture.

**Materials and Methods.** Epithelial defects were made in healthy corneas of anesthetized, male, Sprague-Dawley rats by outlining a 3-mm diameter (7.06 mm<sup>2</sup>) area with a corneal trephine and removing the epithelium with a Bard-Parker blade. The eyes then were enucleated, and the entire corneas were excised at the limbus.

PMNs were prepared with 82–98% purity from rat peritoneal exudate 18 hr after injection with 10 ml of 2% bovine glycogen in 0.9% saline.<sup>4</sup> Lysate was prepared from a portion of each PMN preparation by suspension of PMNs in 10 ml of medium, 2 min of sonication, centrifugation (3000 rpm for 10 min), and removal of supernatant. The absence of viable or intact cells was confirmed morphologically with trypan blue staining. Trypan blue staining also was performed before and after each experiment on culture containing intact PMNs.

Using previously described organ culture techniques,<sup>5</sup> we incubated groups of four abraded corneas in one of the following media: (1) control medium<sup>5</sup>; (2) control medium containing  $5 \times 10^6$  PMN/ml; and (3) control medium with lysate from  $5 \times 10^6$  PMN/ml. Each experiment was performed four times, so that a total of 16 corneas was studied in each medium. New PMN and lysate preparations were made for each of the four experiments.

After 22 hr in organ culture, the corneas were removed, stained with Richardson stain, and photographed so that the remaining epithelial defects could be measured accurately.<sup>4</sup> Specimens were prepared

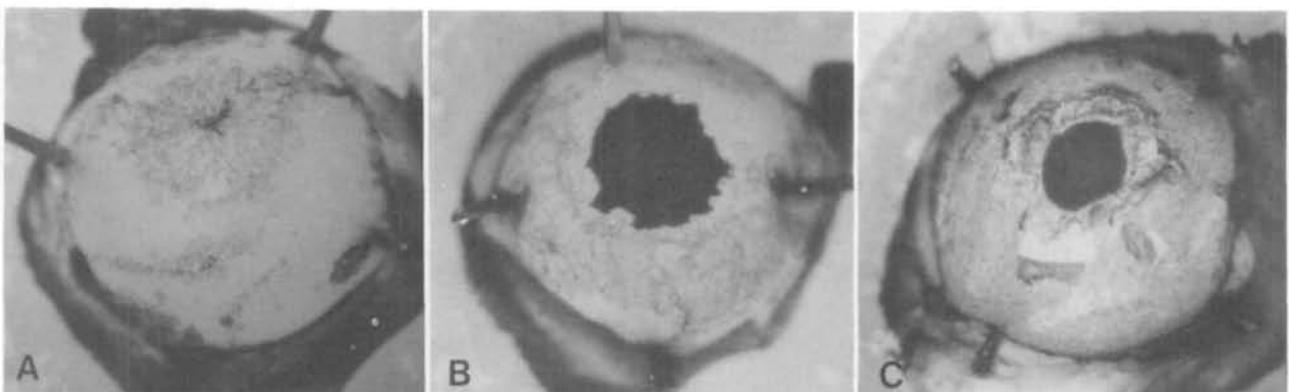
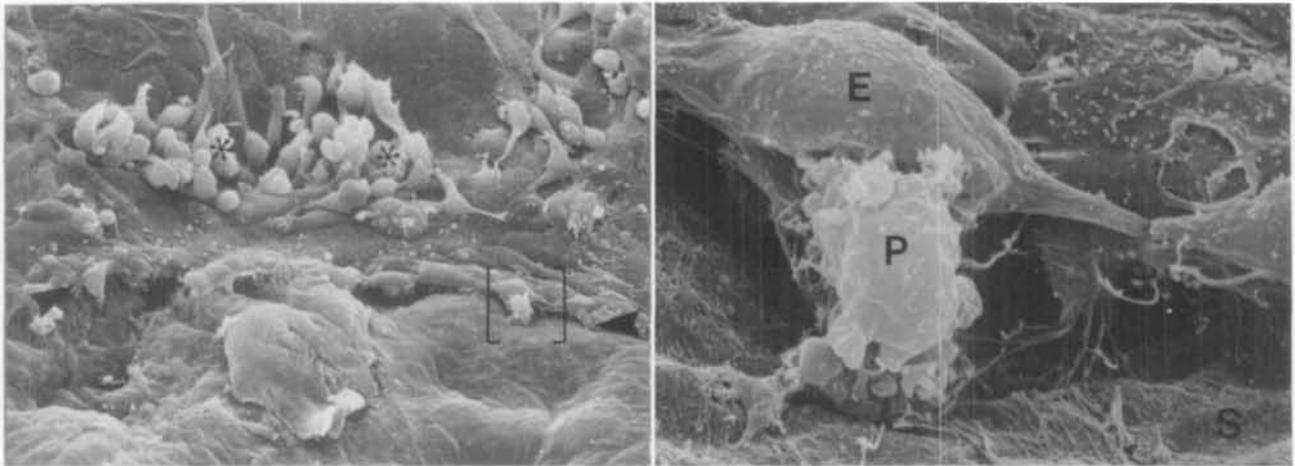


Fig. 1. Typical appearances of corneal epithelial defects after 22 hr incubation with (A) control medium, (B) PMNs, and (C) PMN lysate. Cornea in control medium has almost completely healed defect, whereas corneas in PMN and PMN lysate-containing medium have persistent epithelial defects (dark appearing with Richardson stain,  $\times 3.5$ ).

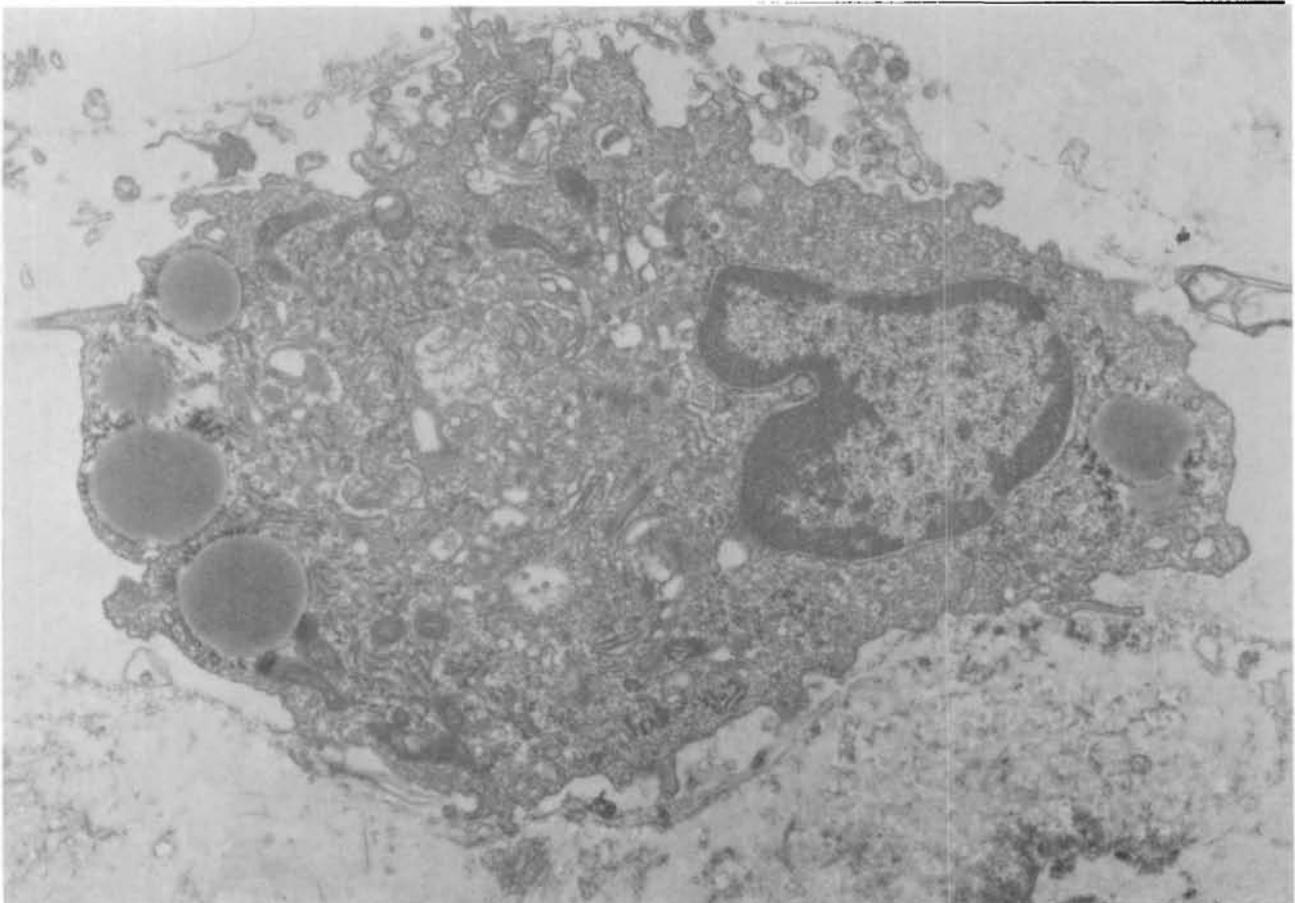


**Fig. 2.** *Left:* Scanning electron micrograph demonstrates multiple PMNs (asterisks) adherent to leading edge (arrowheads) of migrating epithelium ( $\times 400$ ). *Right:* Higher magnification of bracketed area resolves epithelium (E), PMN (P), and exposed stroma (S) ( $\times 4,000$ ).

by routine methods for scanning and transmission electron microscopy.<sup>3</sup> Statistical analysis was performed using the Mann-Whitney test for equality of sample medians.<sup>6</sup>

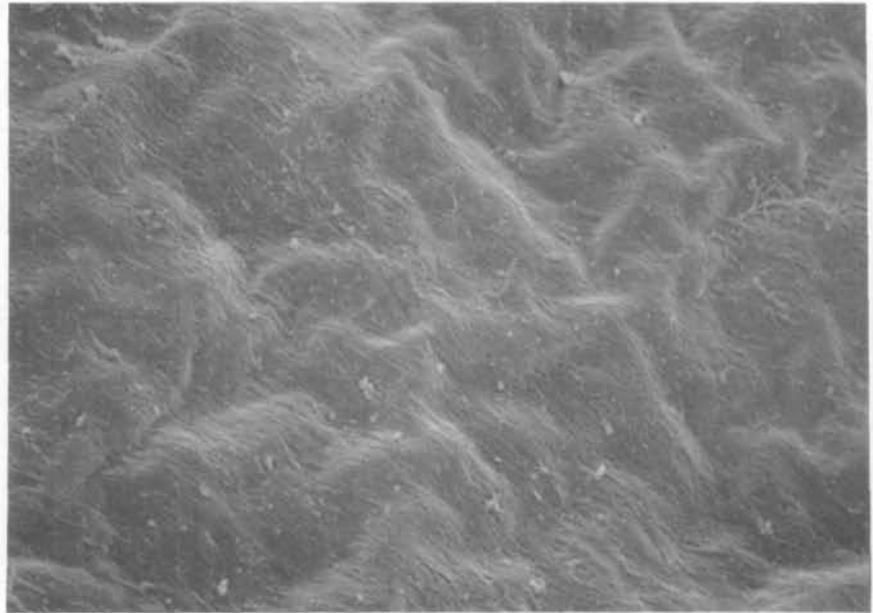
These investigations conformed to the ARVO Resolution on the Use of Animals in Research.

**Results.** Epithelial healing was significantly slowed by PMNs and PMN lysate. The average sizes of the



**Fig. 3.** Transmission electron micrograph confirms the presence of PMN adherent to basement membrane adjacent to leading epithelial edge ( $\times 16,700$ ).

**Fig. 4.** Scanning electron micrograph from area of epithelial defect shows exposed basement membrane with no adhering PMNs ( $\times 2,000$ ).



epithelial defects at 22 hr were  $0.41 \text{ mm}^2$  (median =  $0.31$ ; range =  $0.17\text{--}0.72 \text{ mm}^2$ ) in control corneas;  $2.11 \text{ mm}^2$  ( $2.31$ ;  $0.81\text{--}3.91 \text{ mm}^2$ ;  $P \leq 0.01$ ) in corneas incubated with PMNs, and  $2.49 \text{ mm}^2$  ( $2.58$ ;  $2.21\text{--}2.76 \text{ mm}^2$ ;  $P \leq 0.01$ ) in corneas incubated with PMN lysate (Fig. 1). Among corneas incubated with PMNs, wound healing varied considerably from one experiment to another, but varied little for the four corneas within each experiment. In all four experiments, wound healing with PMNs was slower than in control medium. PMN viability ranged from 97–99% at the beginning of each experiment to 69–72% at the conclusion of each experiment. Scanning and transmission electron microscopy disclosed no morphologic differences in epithelial migration in the three groups. In each case, the picture closely resembled previous, well-documented patterns of epithelial migration and healing.<sup>7</sup> Among corneas incubated with viable PMNs, the PMNs adhered selectively to the epithelium and basement membrane at the leading edge of the wound (Figs. 2, 3), but none adhered to normal epithelium behind the advancing edge or to bare basement membrane within the epithelial defect (Fig. 4).

**Discussion.** Studies on the complex regulatory mechanisms of corneal degradation and repair primarily have addressed PMN enzymatic degradation of the stromal matrix.<sup>8,9</sup> Reports have noted the beneficial effects of PMN inhibitors<sup>10</sup> or tissue adhesives and mechanical barriers to PMNs<sup>2,9</sup> in arresting stromal ulceration. The role of PMNs and their enzymatic products in delaying epithelial wound

healing, and the potential benefits of PMN exclusion from this activity, have not been investigated adequately.

We noted certain differences between results obtained with this model and findings *in vivo*. Although PMNs selectively adhered to the leading edge of the wound *in vitro*, none adhered to bare basement membrane within the epithelial defect, a phenomenon noted in clinical and experimental<sup>1,7,10</sup> studies of eyes that became inflamed *in vivo*. Gipson et al demonstrated significantly higher levels of concanavalin A bound to basement membrane of wounded corneas healing *in vivo* than in organ culture.<sup>11</sup> Fujikawa<sup>12</sup> has suggested that a deposition of fibronectin on basement membrane several hours after corneal injury would account for some of the adherence to basement membrane *in vivo*. Lack of fibronectin could account for the absence of PMNs from basement membrane *in vitro*.

We noted that the rate of epithelial wound healing in the presence of PMNs varied between experiments (but not within the same experiment). Although PMNs showed similar viability following each experiment, we suggest that during each preparation of PMNs for an experiment a different amount of the enzymatic product responsible for retarded healing was released into the medium. Incubation with the PMN lysate, in which levels of cellular enzymes were less variable, produced more consistent and, on average, greater retardation of wound healing. Further work with this model will attempt to identify the specific factor(s) that retard epithelial migration.

**Key words:** polymorphonuclear neutrophil, corneal epithelium, ulceration, wound healing, collagenolysis, electron microscopy

From the Cornea Unit, Eye Research Institute of Retina Foundation\* and the Department of Ophthalmology,† Harvard Medical School, Boston, Massachusetts. Supported in part by NIH grants EY-05718 (MDW), EY-03967 (KRG), EY-03306 (IKG), and the Heed Ophthalmic Foundation (MDW). Submitted for publication: April 9, 1984. Reprint requests: Dr. M. D. Wagoner, Eye Research Institute of Retina Foundation, 20 Staniford St., Boston, MA 02114.

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## *An Improved Method for Restraining Rabbits for Examination of the Eye*

David M. Maurice and Tej Singh

**Rabbits are strapped to a specially shaped platform with their legs dangling over the sides. This system of restraint allows freer access to the eyes for examination than other methods and prevents the animal from injuring itself by kicking. The animals appear to be quite relaxed and show no signs of harm on release. *Invest Ophthalmol Vis Sci* 25:1220-1221, 1984**

When the rabbit's eye has to be observed in the slit lamp or a minor diagnostic procedure such as applanation tonometry must be performed, it is necessary to immobilize the animal and to prevent evasive movements of the head as far as possible. The animal may be put under general anesthesia or be tranquilized heavily, but this can upset the normal metabolism, particularly if the observations or measurements have to be frequent or prolonged. Mechanical restraint is generally preferred for those procedures that are painless but can alarm the animal. The same is true for entering an ear vein with a needle and for minor ocular interventions under local anesthesia.

Two forms of restraint are common. The first is to confine the rabbit in a box with its head protruding from a closure around the neck. This has several disadvantages. First, the box blocks slit-lamp observation of much of the eye; second, if the animal is frightened it will struggle and may injure itself; third, it can jerk its head away when a needle is inserted into an ear vein, which requires reentry into a punctured and bleeding vessel. A more satisfactory procedure is to secure the animal firmly in a bag or some other cloth wrap. This prevents it harming itself and allows better access to the eye for observation. However, it is not easy to wrap the rabbit successfully, and this can be time-consuming, particularly if the animal is recalcitrant. Moreover, it still can jerk its head away on feeling a needle prick. We therefore have developed a system of restraint that goes a long way to avoid these disadvantages.

**Materials and Methods.** The basis of the system is to make the rabbit lie on a narrow platform with its legs straddling it. When its paws have no purchase on a surface, it can only kick into the air, and its