The pathogenetic mechanisms responsible for vernal keratopathy remain enigmatic. Suggested contributory factors include abrasion from giant papillae of the upper lid and toxicity from inflammatory products in the tear film. Eosinophil granule major basic protein (MBP) has previously been advanced as an inflammatory product of putative pathogenetic importance; the protein has been identified in conjunctiva, tear film, contact lenses, and corneal keratoconjunctivitis. The cytotoxic effects of MBP are well documented; MBP has been identified at sites of lung tissue injury in people with asthma and adversely affects organ-cultured respiratory epithelium.

A corneal organ culture system introduced by Gipson and Anderson has made it possible to assess factors influencing corneal epithelial wound healing. The objective of the present study was to investigate the effect of MBP on corneal epithelial migration rates and epithelial metabolism in organ culture to elucidate further the pathogenetic role of MBP in vernal keratopathy.

**MATERIALS AND METHODS**

**Corneal Organ Culture**

The organ culture system used in this study has previously been described in detail. All experimental techniques adhered to the ARVO Resolution on the Use of Animals in Research. Male Sprague-Dawley rats weighing approximately 200 g each were sacrificed by an intraperitoneal injection of sodium pentobarbital. An epithelial abrasion wound, 3 mm in diameter, was created by marking the central cornea with a 3 mm trephine and removing encircled epithelium with a blunted scalpel. Eyes were excised and corneas removed for incubation in organ culture to allow epithelial resurfacing for 18.5 hours in a minimum essential medium (MEM) supplemented with L-glutamine, trace element mix, antibiotic-antimycotic, and nonessential amino acid solution (Gibco, Gaithersburg, MD). All corneas were pinned to rounded paraffin posts in a 60 × 15 mm petri dish.
a single band by polyacylamide gel electrophoresis in sodium dodecyl sulfate. MBP stored in acetate buffer (pH 4.2) was added to organ cultures at concentrations ranging from 10 to 200 µg/ml, which included concentration levels previously identified in patients with vernal keratoconjunctivitis. Corresponding concentrations of acetate buffer were added to control organ cultures. Organ culture medium pH was not affected by the addition of MBP, acetate buffer, or both. Two other basic proteins, lysozyme and protamine (Sigma, St. Louis, MO) were tested in organ culture at concentrations equimolar to 10 µg and 50 µg MBP. Corneas were removed after 18.5 hours in organ culture, stained with Richardson's stain, and photographed so that residual epithelial defect could be measured accurately by image analysis (Zeiss Videoplan 2, Rainin Instruments, Woburn, MA). One set of corneas was incubated in MEM containing MBP (100 µg/ml) for 1 hour only. The MBP containing medium was then removed, and the corneas were washed twice
with MEM before continuing the incubation for an additional 17.5 hours (total incubation of 18.5 hours).

**Histologic Evaluation**

Assigned corneal buttons from all groups were placed in Karnofsky's fixative (1% paraformaldehyde, 1.25% glutaraldehyde, 0.13% sucrose and 25 mM sodium phosphate in 150 mM sodium cacodylate buffer, pH 7.2) stored overnight at 4°C, postfixed in 1% osmium, and embedded in resin (Historesin, LKB-Produkter AB, Bromma, Sweden). Processed tissues were sectioned at 1 to 2 μm thickness and stained with hematoxylin and eosin.

**Immunofluorescence**

Selected corneas from all groups were fixed in formalin and subsequently processed for an indirect immunofluorescence assay to detect MBP as described elsewhere. In brief, tissue sections were mounted to slides and deparaffinized in xylene. After rehydration, sections were treated with trypsin and incubated in 10% normal goat serum. Slides were overlaid with either the negative control serum (normal rabbit IgG) or the test material (affinity chromatography-purified rabbit antibody to human MBP). Sections were subsequently stained with p-phenylenediamine to reduce fluorescent fading. Finally, slides were examined with a fluorescent microscope, and photomicrographs were taken. A formalin-fixed nasal polyp with numerous eosinophils served as a positive control.

**Statistical Analysis**

The Student's t-test of two independent groups was used to compare study corneas to the control. A statistically significant difference in wound size was considered to be $P < 0.05$.

**RESULTS**

**Epithelial Migration**

The highest concentrations (200 μg/ml) of MBP caused a near arrest of epithelial migration, and the lower concentrations produced proportionally less inhibition of migration (Fig. 1). Significant retardation ($P < 0.05$) of corneal epithelial migration was demonstrated in MBP test groups of 10 (N = 4), 25 (N = 4), 50 (N = 12), 100 (N = 13), and 200 μg/ml (N = 7) compared to the control (Fig. 2). The acetate buffer added to the control media did not affect epithelial migration when compared to unaltered organ culture medium. In the experiments testing the need for prolonged exposure to MBP, no difference in migration was observed between corneas incubated with 100 μg/ml of MBP for 1 hour or 18.5 hours (Figs. 1E and 1F). Protamine and lysozyme at concentrations equimolar to MBP produced no significant change in epithelial migration compared to the control medium (results not shown).

**Histology**

Histologic examination of hematoxylin and eosin-stained corneal specimens that had undergone fixation at 18.5 hours revealed an abnormal heaped-up appearance of the leading epithelial edge of cornea exposed to MBP; this finding was more pronounced at the higher MBP concentrations (Fig. 3). There was no apparent abnormality of the basement membrane of the denuded cornea. Amorphous material was seen across the denuded stroma and immediately adjacent to the leading edge of migrating epithelium in all corneas exposed to MBP, including the corneas exposed for 1 hour only.
FIGURE 3. The effect of human MBP on the leading edges of migrating sheets of epithelium in rat corneal wounds. (A) Acetate buffer control for (D) no MBP present, (B) MBP (10 μg/ml). Note the lack of flattening of the basal cells. (C) MBP (50 μg/ml). Note the heaping-up of cells at the leading edge. (D) MBP (200 μg/ml). Note that the basal cells have retained the columnar shape present in unwounded epithelium. Hematoxylin and eosin, original magnification X400.

Immunofluorescence

Corneas subjected to 200 μg/ml MBP had deposition of MBP on the basement membrane of the denuded cornea; little epithelial migration occurred, and no MBP was seen under the epithelium. Corneas subjected to a lower concentration of MBP (100 μg/ml) also had deposition of MBP on the denuded cornea; however, more epithelial migration was evident, and a layer of MBP was present under the migrating epithelium (Fig. 4). MBP was also present in an amorphous mass immediately adjacent to the leading edge of migrating epithelium.

DISCUSSION

Sight-threatening complications of vernal keratoconjunctivitis usually evolve from associated corneal disease, such as corneal ulceration and anterior stromal scarring. Occasionaly, the corneal epithelium fails to resurface ulcerated cornea unless the ulcer bed is removed by a superficial keratectomy, suggesting an inhibitory factor lodged in the affected cornea. The presence of MBP in tears and corneal ulcers of patients with vernal keratoconjunctivitis lends credence to the hypothesis that MBP may adversely influence corneal epithelium and pathogenetically contribute to vernal keratopathy. Previous in vitro experiments exploring the effect of MBP on cultured respiratory epithelium have shown that MBP concentrations as low as 10 μg inhibited the ciliary activity of structurally intact respiratory epithelium and produced epithelial sloughing.

In contrast to the previous investigations of intact respiratory epithelium, the present study assesses the effect of MBP on the healing process of corneal epithelium subjected to a standardized abrasion wound. This well-characterized corneal epithelial wound healing model allows for the study of various influences on epithelial cell migration and wound closure. The epi-
FIGURE 4. Immunofluorescence for MBP in corneas exposed to 100 μg/ml MBP for 18.5 hours. (A) Section stained with hematoxylin and eosin showing deepithelialized cornea defect (left) and intact cornea epithelium (right). (B) Immunofluorescence-stained section serial to A, identifying MBP on the deepithelialized cornea (left) and under the leading corneal epithelial edge (right). Original magnification X400.

The present study offers no detailed information on the mechanisms by which MBP may retard epithelial migration. However, the presence of MBP on denuded stroma suggests that MBP may interfere with adhesion of the migratory sheet to its underlying matrix. The amorphous mass of MBP near the leading edge of migrating epithelium might suggest an attempt by the cells to remove MBP. Hypothetically, interference with focal adhesion formation and fibronectin deposition could slow or arrest epithelial migration. When corneal epithelial basal cells become migratory, hemidesmosomes are dismantled and focal adhesions form, which provides a provisional adhesion junction. The focal adhesion consists of an actin bundle inserted into an electron-dense plaque along the cell membrane; αβ3 and αβ6 integrins are present in the focal adhesion membrane. Fibronectin, which appears on the wound bed surface shortly after injury, may induce the assembly of the provisional adhesion junction. It is conceivable that MBP could retard epithelial migration by adversely affecting the provisional adhesion mechanisms of migrating epithelium. The demonstration of MBP deposition on the basement membrane of denuded cornea and under the migrating edge of healing epithelium suggests that MBP may influence epithelial anchoring mechanisms. Conceivably, MBP may inhibit integrin binding, but no detailed information is available on this possibility. Alternatively, MBP may prevent the binding of basic fibroblast growth factor to its receptor with a mechanism similar to another small basic protein, namely protamine sulfate. Because basic fibroblast growth factor has been shown to enhance migratory rates, it is possible that blocking its action could slow migration. Our data indicate that, unlike MBP, protamine and lysozyme do not slow migration, suggesting that MBP is
not functioning via this mechanism. Further investigations, however, will be necessary to better define the effects of MBP on corneal epithelial healing.

**Key Words**

eosinophil granule major basic protein, corneal epithelial wound healing

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**References**