Table I

Case	*Age, color, sex*	*Papilledema (diopters)*	*Visual acuity*	*Intrathecal amphotericin*	*Other diagnosis*
1	35 WM	1	20/400 OU	Yes	Hodgkin's disease
2	2 WF	3	NLP	No	None
3	21 WF	1	J14 OU	Yes	Periarteritis nodosa
4	65 WM	1-2	20/20 OU	Yes	None
5	24 WM	1-2	20/20 OU	Yes	Hodgkin's disease
6	32 WM	1-2	20/20 OU	Yes	Hodgkin's disease

Table II

Case	*Papilledema*	*Decreased visual acuity*	*Cryptococcus in visual pathways*
1	+	Yes	Marked
2	+	Yes	Marked
3	+	Yes	Marked
4	+	No	Slight
5	+	No	Slight
6	+	No	None

decreased visual acuity appears to be related to direct invasion of the visual pathways by the cryptococcal organisms rather than to increased intracranial pressure and attendant papilledema.

The authors wish to acknowledge the advice of Dr. John Bennett in the preparation of this paper.

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Key words: cryptococcal meningitis, visual acuity, increased intracranial pressure.

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Kinetics of corneal epithelial regeneration and epidermal growth factor.

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Purified epidermal growth factor (EGF), isolated from mice submaxillary glands, was used to study regeneration of rabbit corneal epithelium. The progressive decrease in area of standardized 7 mm. central corneal epithelial wounds was determined by serial standardized photography. The projected fluorescein-stained area was measured by planimetry. It has been found that EGF in the concentrations studied (0.05 mg. per milliliter; 0.5 mg. per milliliter; and 2.0 mg. per milliliter) when given topically four times daily, increased the corneal epithelial healing rate compared to saline controls. A 40-fold variation of EGF concentrations failed to effect a statistically significant change in corneal epithelial healing rate. No sign of toxicity was detected clinically and histologically with topical application of EGF on rabbit corneas with intact epithelium and on corneas denuded of epithelium.

Ulcers and erosions of the corneal epithelium are a major cause of ocular morbidity and visual loss. Delayed corneal re-epithelialization may be associated with or follow microbial infections, alkali burns, penetrating keratoplasties, radiation keratoconjunctivitis, toxic keratopathies, dry eyes, and the recurrent corneal erosion syndrome. Prolonged ulceration or erosion of the cornea eventually results in thinning or melting of the corneal stroma. Current conventional therapeutic alternatives for long-standing corneal ulcers or erosions are not uniformly successful and thus far no single therapeutic modality has been demonstrated to have uniform efficacy in the promotion of corneal epithelial repair.

Recently, epidermal growth factor (EGF), first isolated by Cohen from mice submaxillary glands,[1] has been shown to enhance the healing of experimental corneal epithelial wounds.[2, 3] The present studies report our findings on the ocular toxicity of EGF and quantitate the kinetics of corneal epithelial regeneration in rabbits following topical application of highly purified and characterized EGF.[4, 5]

Material and method. Epidermal growth factor.° EGF was isolated from the submaxillary glands of adult male mice and purified by the new procedure of Savage and Cohen[4] involving a two-step column fractionation. It is a white powder soluble in water. EGF solutions of three concentrations (0.05 mg. per milliliter, 0.5 mg. per milliliter, and 2.0 mg. per milliliter) were prepared by dissolving EGF

°EGF was kindly supplied by Dr. S. Cohen at Vanderbilt University School of Medicine.

in normal saline. The solutions were sterilized by passage through sterile Swinnex filters of 0.22 micron pore size, into sterile plastic dropper bottles. The solutions were stored frozen and thawed ten hours before use. Fresh solutions were prepared every three months.

Experimental animals. Forty-eight, pigmented, adult rabbits of both sexes, weighing 3 to 5 kilograms each were examined with a slit-lamp bilaterally to detect any pre-existing eye disease. Only rabbits with both eyes free of disease were used in the experiments. Five rabbits were used in the topical toxicity study and the remaining forty-three rabbits were used in the kinetic studies.

Topical toxicity study. One drop of EGF solution (0.5 mg. per milliliter) was instilled in the cul-de-sacs of the right eyes of five rabbits four times daily for a one-week period. The same schedule was followed with normal saline, instilling it in the cul-de-sacs of the left eyes of the same five rabbits as controls. Both eyes of the rabbits were examined daily with a slit-lamp for evidence of toxicity. Clinical signs were assigned a numerical score which could be converted into a severity gradient for toxicity (Table I). On the eighth day, the rabbits were killed by air embolism. Both the right and the left eyes were enucleated, fixed in 10 per cent formalin, embedded in paraffin, cut into 8 μ sections, and stained with hematoxylin and eosin (H&E) for histologic examination.

Histologic gradings of inflammatory reaction was assessed for toxicity in a similar manner to the method of Aronson.[6] The degree of inflammation was determined histologically in the palpebral and bulbar conjunctiva, limbus, cornea, trabeculum, iris, and ciliary body. For example, the severity of corneal inflammation was graded as follows: 1+, epithelial disarticulation; 2+, PMN infiltration with destruction of epithelial cells; 3+, anterior stromal PMN proliferation; and 4+, total stromal involvement.

Kinetic studies. Rabbits were randomly divided into four groups and only one eye, chosen randomly, of each animal was used: Group A, 13 rabbits receiving saline as controls; Group B, 9 rabbits receiving 0.05 mg. per milliliter of EGF; Group C, 11 rabbits receiving 0.5 mg. per milliliter of EGF; and Group D, 10 rabbits receiving 2.0 mg. per milliliter of EGF. Each of the forty-three rabbits, anesthesized by intravenous injection of sodium pentobarbital (30 mg. per kilogram of body weight), was put under the binocular dissecting microscope. The superior rectus muscle of the right eye was secured with a forcep after a self-retaining eye speculum was positioned. The cornea was irrigated with normal saline and a central corneal area was demarcated with a corneal trephine of 7 mm. diameter and adjusted to cut not deeper than 0.1 mm. The epithelium within this area was denuded with a No. 15 Bard-Parker blade. To facilitate accurate measurement of the wound areas, special efforts were made to produce wounds with round and smooth perimeters. It was impossible to achieve a standard corneal epithelial wound in terms of area because of technical difficulties. Two per cent fluorescein staining was used to insure complete removal of epithelium and uniformity of the wound. The stained area was photographed and a drop of EGF was instilled into the cul-de-sac of the experimental animal at the end of the procedure. Two more drops of the EGF solution were applied at four-hour intervals on the same day starting from recovery from anesthesia. On the following days, EGF drops were given four times daily at four-hour intervals. Control animals observed the same schedule with normal saline replacing EGF. The wound area was stained with 2 per cent fluorescein and photographed four times daily. All photographs were taken under ultraviolet light in a standardized manner using a Franka camera equipped with Kodachrome film, flashlights, and a wire frame extending at a fixed distance and attached to the camera. The date and time of all photographs were recorded. Upon complete healing of the corneal epithelial wound, determined by the absence of corneal fluorescein uptake, the animals were immediately killed with sodium pentobarbital (0.75 Gm. per kilogram of body weight), and the experimental and control eyes were enucleated,

Table I. Assignment of numerical score to clinical slit lamp signs for severity gradient of topical toxicity

Clinical slit lamp signs	*Numerical score*
Conjunctival signs:	
Limbal hyperemia	1
Conjunctival hyperemia	2
Conjunctival chemosis	3
Conjunctival exudate	4
Conjunctival membrane formation	5
Corneal signs:	
Epithelial erosions	1
Epithelial edema	2
Stromal edema	3
Focal infiltration with inflammatory cells	4
Generalized infiltration with inflammatory cells	5
Vascularization	6
Anterior chamber and iris:	
Iris hyperemia	1
Anterior chamber cells and flare	2
Precipitates on the cornea	3
Precipitates on the lens	4
Posterior synechiae	5
Vitreous:	
Inflammatory cells in anterior vitreous	4

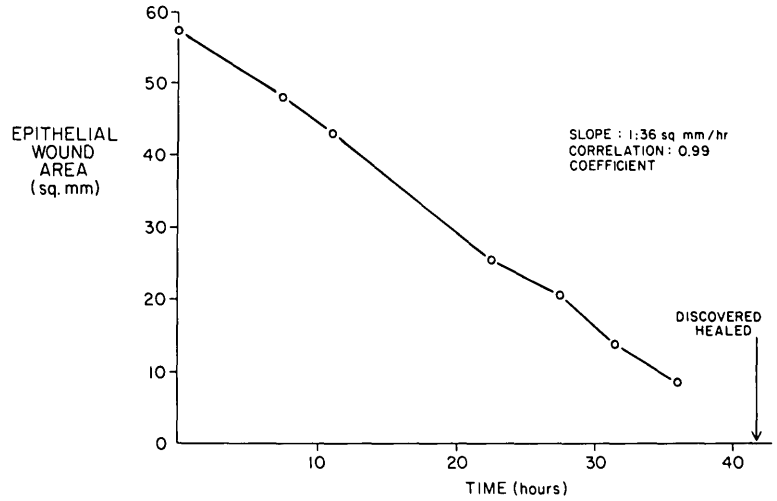

Fig. 1. Time-course of epithelial healing in a rabbit cornea treated with EGF (2 mg. per milliliter) four times a day. The exact time of healing was between the last data point (36 hours) and when first discovered healed (42 hours).

prepared for histologic examination, and stained with H&E in routine fashion. The Kodachromes were projected on a white screen on which the fluorescein-stained areas were measured with the Gelman Planimeter. After standardization with a projected Kodachrome on a piece of graph paper of known dimensions, the projected wound areas were converted to the actual sizes of the corneal wounds. For each animal a healing curve, represented by the time course of decrease in corneal wound area, was obtained by plotting the serial wound area against the time the Kodachromes were taken. Slopes were calculated for all the healing rates individually and the results were averaged for all animals within each of the three experimental groups and the control groups.

Results. *Topical toxicity study.* In all five rabbits under daily slit-lamp examination for a seven-day period, none of the clinical signs listed in Table I were observed in the control eyes as well as the experimental eyes receiving EGF solution on any occasion. The control eyes were of exactly similar appearance as the experimental eyes. On histologic examination the degrees of inflammation in the palpebral and bulbar conjunctiva, limbus, cornea, trabeculum, iris, and ciliary body were given a grade of zero in all five control eyes and in all five experimental eyes. No sign of inflammation was observed. The corneal epithelium in both the EGF-treated and control eyes was of normal four to six layers thickness.

Kinetic studies. Forty-three healing curves were obtained each consisting of five to six experimental coordinate points on the average. The initial area of central corneal wounds ranged from 40 $mm.^2$ to 62 $mm.^2$, equivalent to circles of 7.1 mm. and 8.9 mm. in diameter, respectively. A healing curve of one representative animal is shown in Fig. 1. On inspection, all forty-three healing curves appeared linear, and the slope of each curve was computed by the linear regression method. The slopes, or healing rates, were represented by the rates of decrease in area of epithelial wound in Table II. The linear correlation co-efficients calculated for all healing curves ranged from 0.99 to 0.92. It could be inferred from the linearity of the healing curves that within any one of three EGF-treated groups or the control group, the healing rate of any one rabbit cornea was independent of the original corneal wound size. Also, the healing rate appeared constant throughout the entire time course of corneal re-epithelialization. Furthermore, the Chi-square test indicated that within each of the four groups of rabbits there was no correlation between healing rate and the initial area of corneal wound ($p < 0.01$).

The mean rates of decrease in area of epithelial wounds were also calculated for each of the four groups of animals (see Table II). P-values obtained from two-sample t-tests revealed that the difference in the mean healing rates between the saline-treated group and any of the EGF-treated groups was statistically significant at the 0.001 level. This difference was further illustrated by extrapolating the complete re-epithelialization time of the control and the three EGF-treated groups with their respective mean rates of healing from a corneal epithelial wound of 7 mm. in diameter (Fig. 2). The corneas treated with 2.0 mg. per milliliter of EGF were completely healed within 30 hours. At this same time, about 35 per cent of the scarified surface of an average control cornea was still stripped of epithelium. The mean healing time of control corneas was 47 hours.

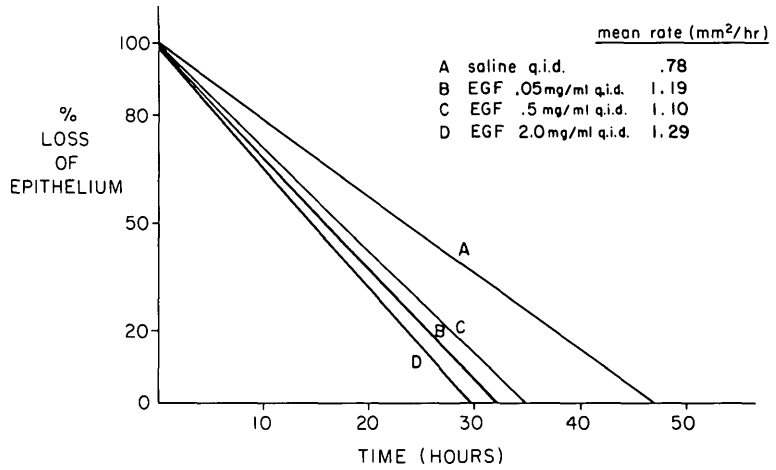

Fig. 2. Mean rates of healing of 7 mm. diameter epithelial wound in rabbit cornea.

Table II. Rate of decrease in area of epithelial wound (square millimeters per hour)*

Group A *Saline control*	*Group B* *EGF* *(0.05 mg./ml.)*	*Group C* *EGF* *(0.5 mg./ml.)*	*Group D* *EGF* *(2.0 mg./ml.)*
0.72 (0.96)	1.16 (0.97)	0.91 (0.96)	1.09 (0.92)
0.86 (0.98)	1.18 (0.99)	1.34 (0.99)	1.36 (0.99)
0.83 (0.99)	1.07 (0.99)	1.19 (0.99)	1.36 (0.99)
0.96 (0.99)	1.09 (0.99)	1.26 (0.99)	1.51 (0.98)
0.88 (0.99)	1.01 (0.99)	1.19 (0.99)	1.57 (0.99)
0.68 (0.96)	1.10 (0.99)	0.88 (0.98)	1.18 (0.98)
0.78 (0.97)	1.78 (0.99)	1.03 (0.99)	1.11 (0.97)
0.72 (0.98)	0.94 (0.98)	1.02 (0.99)	1.14 (0.99)
0.83 (0.99)	1.34 (0.97)	1.10 (0.99)	1.11 (0.99)
0.90 (0.96)		1.28 (0.99)	1.29 (0.99)
0.53 (0.98)		0.89 (0.99)	
0.77 (0.99)			
0.73 (0.99)			
	Mean ± S.D.		
0.78 ± 0.11	1.19 ± 0.25	1.10 ± 0.16	1.27 ± 0.17
	P-value		
—	$p < 0.001$	$p < 0.001$	$p < 0.001$

*Each value listed represents the healing rate obtained from an animal. ( ) signifies correlation coefficient.

Dose-response relationships of EGF and corneal re-epithelialization rate was studied by plotting the mean rate of epithelial healing of the three EGF-treated groups against the logarithm of the EGF concentration (Fig. 3). Analysis of variance of the rates of epithelial healing with the three experimental EGF concentrations used showed no statistical significance at the 0.01 level.

Histologic examination of the eyes enucleated immediately after complete absence of epithelial fluorescein staining revealed no sign of inflammation in the bulbar conjunctiva, limbus, cornea, trabeculum, iris, and ciliary body. In the control eyes, the corneal epithelium was one to two layers in thickness, while in the EGF-treated eyes the corneal epithelium as three to six layers in thickness. A correlation between the EGF concentration and the number of regenerated epithelial cell layers could not be ascertained. It is noteworthy that histologic observations indicate that the presence of one to two layers of corneal epithelium was sufficient to prevent observable fluorescein staining.

Discussion. In this study, the technique of corneal epithelial wound production is essentially similar to that employed by Frati and co-workers,[3] although, in this case the average initial wound size is twice as large and the schedule of topical EGF treatment is also different. The time-course of epithelial regeneration is shown to be linear as exemplified by Fig. 1, and supported by the statistically significant linear correlation co-efficients of each animal shown in Table II. In contrast to this finding is the nonlinear healing

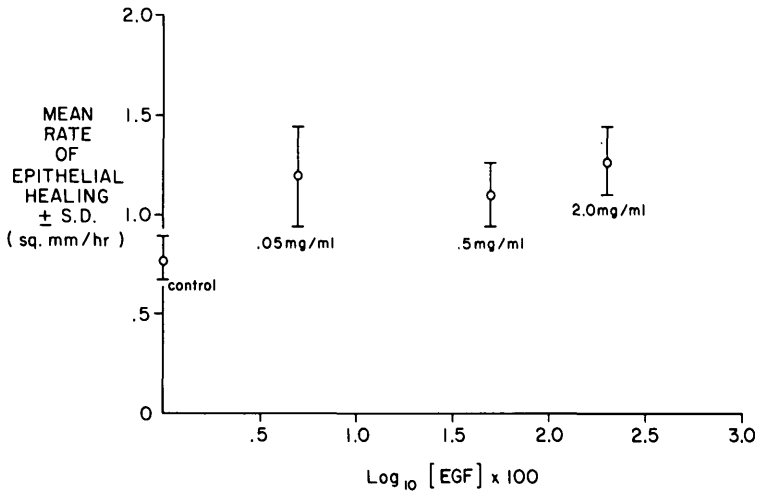

Fig. 3. Dose-response relationship of EGF and corneal epithelial healing. The mean healing rate of saline control is indicated on the ordinate for comparison.

curve reported by Frati and co-workers[3] showing that the rate of re-epithelialization decreases as the wound diminishes in area in the course of healing. Also, as shown in Fig. 2, animals treated with 2.0 mg. per milliliter of EGF healed completely in 30 hours, whereas control animals healed in 47 hours. In the experiments by Frati and co-workers,[3] EGF-treated rabbit corneas healed totally within 48 hours and control corneas healed after four days despite the smaller initial wound area (5.1 mm. in diameter) and application of EGF (2.0 mg. per milliliter) at a regimen of two drops per hour for the first five hours and, subsequently, every four hours. In spite of these differences, it is interesting to note that both this study and that of Frati and co-workers[3] show that at the time of complete epithelial regeneration of the EGF-treated corneal wounds, the control corneas have an epithelial defect which is about 30 per cent of the original area scarified.

The effect of EGF on the kinetics of corneal epithelial regeneration has been studied quantitatively. The data presented shows that EGF treatment definitely increases the rate of wound closure. This finding confirms the observations by Frati and co-workers[3] and by Savage and Cohen.[2] However, in the present studies the only valid quantitative parameter for epithelial regeneration was healing rate (decrease in wound area per hour). Since the above published papers do not present their data in this essential kinetic form, comparisons between the respective results obtained are invalid.

It is also found that in the forty-fold range of EGF concentration, the rate of corneal epithelial regeneration bears no relationship to the variation of EGF concentration. Dose-response relationship could exist outside this concentration range, but the solubility of EGF in normal saline and the availability of purified EGF limits the feasibility of experiments with significantly higher EGF concentrations. On the other hand, experiments with lower concentrations of EGF did not seem practical.

The finding that topically applied EGF has no clinical or histologic toxicity in rabbit corneas, with or without intact epithelium, suggests that clinical trials on humans are indicated. However, since EGF is a polypeptide derived from a xenogeneic source, it could potentially be an immunogen for humans. Further studies addressed to this problem should be conducted before any mass human trials. Certainly, the enhancing effect of EGF on the rate of corneal epithelial regeneration may augment current therapeutic measures for nonhealing epithelial defects and erosions.

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Key words: epidermal growth factor, central corneal epithelial wound, healing rate, dose response, topical toxicity.

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