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THE ROLE OF FREE RADICALS IN PARAQUAT-INDUCED CORNEAL LESIONS

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Paraquat is a synthetic bipyridylium salt widely used as herbicide and defoliant. Enzyme-catalyzed redox-cycling of paraquat generates oxygen radicals. The toxic, even lethal, effects of paraquat are due to free radical-mediated tissue injury. Ocular lesions, sometimes quite severe, have been observed following accidental splashing of paraquat solutions onto the eyes.

These studies were designed to document the generation of paraquat free radicals in corneal tissue, and to describe the histological nature of the corneal injuries in experimental animals (rabbits and monkeys). The EPR spectrum of rabbit corneas, 30 min. after intrastromal injection of paraquat, showed the signal of the free radical of paraquat. Ultrastructural studies of corneas 8 days after intrastromal injections (100μl) of paraquat solutions showed that the initial lesions occur at the epithelium/basement membrane interface. In rabbit cornea, dose dependent lesions were observed, i.e. whereas 50 mM paraquat caused only minimal damage to the epithelial basement membrane, 75 mM caused complete dissolution to the basement membrane with some damage to stromal collagen, and loss of epithelium with stromal ulceration and severe inflammatory response were observed with 150 mM paraquat. Monkey corneas were less susceptible than those of rabbits to the effects of paraquat. No lesions were observed following intrastromal injections of 50 mM or 75 mM paraquat. With higher concentrations of paraquat (100 mM and 150 mM) the primary injuries were to the proximal and lateral plasma membranes of basal epithelial cells; basement membrane alterations were detected only adjacent to areas of significant plasma membrane damage. The underlying Bowman's membrane and stroma were not affected. Anatomical differences between the corneas of rabbit and monkeys as well as possible biochemical differences may account for the species differences observed.

KEY WORDS: paraquat, cornea, free radicals, rabbit, monkey

INTRODUCTION

Paraquat (PQ), also known as methyl viologen, is a bipyridylium compound (N,N′-dimethyl-4,4′-bipyridylium dichloride) widely utilized as herbicide and defoliant.

PQ is highly toxic and fatalities have been reported following accidental ingestion of PQ solutions by human beings[1] and oral as well as parenteral administration to experimental animals.[2] A peculiar proliferative lung condition and lesions to the liver, kidney and mucosa of the digestive tract (esophagus and stomach) have been described.[1,2]

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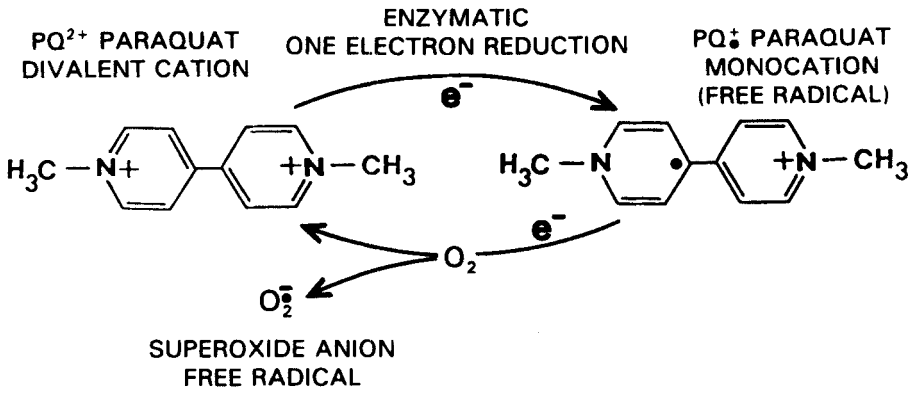

FIGURE 1 Generation of superoxide anion free radical during redox-cycling of paraquat.

Ocular burns resulting from accidental splashing of PQ-containing herbicide solutions onto the eyes have been reported by several investigators. As with alkali eye burns, a single minor PQ splash can also lead to serious ocular burns. However, unlike the fast action of alkali, PQ acts slowly and its full effect may not be apparent for over one week.[3] The severity of the lesions runs from superficial burns to corneal scarring and opacification requiring penetrating keratoplasty.[3,4]

The tissue injuries associated with PQ exposure are caused by free radicals generated during redox-cycling of PQ. The metabolism of PQ involves the enzymatic one-electron reduction[5] of the PQ divalent cation (PQ^{2+}) to the PQ monocation ($PQ^{\cdot+}$) free radical (Figure 1). This is followed by a one-electron transfer from $PQ^{\cdot+}$ to molecular oxygen (O_2) to yield superoxide radical anion ($O_2^{\cdot-}$) with regeneration of PQ^{2+}.[6] Subsequent dismutation of $O_2^{\cdot-}$ yields hydrogen peroxide (H_2O_2) which in the presence of reduced transition metal ions yields hydroxyl radicals ($OH^{\cdot}$) by the Fenton reaction.

These studies deal with the demonstration of the production of PQ free radical in rabbit cornea and with the ultrastructural investigation of PQ-induced corneal lesions in rabbits and monkeys.

MATERIALS AND METHODS

Albino New Zealand rabbits (5–8 lbs. body weight) of both sexes and two female owl monkeys (Aotus trivirgatus) (3 lbs. body weight) were utilized for these studies. The animals were housed individually in stainless steel suspended-floor cages with drip pans in an air conditioned animal facility. The animals were fed ad libitum commercial pellet diets (Purina Rabbit Chow and Purina Monkey Chow, respectively) with free access to drinking water (Hardco Automatic Watering System, Hazelton Systems, Inc., Cincinnati, OH, U.S.A.). The monkeys also received a weekly ration of fresh fruit.

Intrastromal corneal injections were performed under general anesthesia induced in rabbits by intramuscular injection of a mixture of xylazine (5 mg/kg) and ketamine (35 mg/kg). Monkeys received only ketamine (25 mg/kg). The corneas were treated with a few drops of 0.5% proparacaine hydrochloride and the PQ injections (50–100 µl) were given in the central corneal stroma with tuberculin syringes fitted with 30G 1/2 needles under a Topcon Operation Microscope OMS-300 (Tokyo Optical Company, Tokyo,

Japan). All experiments were performed in accordance with The Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23) and following protocols approved by our Institutional Animal Care and Use Committee.

PQ (Methyl Viologen) was purchased from Sigma, (St. Louis, MO). A stock 150 mM solution of PQ (285 mOs/kg) was prepared in deionized water treated with a Milli-Q Water system (Millipore, Bedford, MA). Additional PQ solutions for injection (125 mM, 100 mM, 75 mM and 50 mM) were prepared by diluting the stock solution with sterile 0.9% Sodium Chloride Inj., USP (Abbott, Chicago, IL). The tonicity of these solutions ranged from 283 to 294 mOs/kg. Osmometric measurements were done using a Wescor 5100B Vapor Pressure Osmometer (Wescor, Logan, UT). All PQ solutions were filtered through 0.2 micron Acrodisc (Gelman, Ann Arbor, MI) prior to injection.

For ultrastructural studies, corneal tissue was collected 8 days after PQ injection. The animals were killed under general anesthesia (see above) by intracardial injection (0.1 ml/lb. body weight) of Beuthanasia-D Solution (Schering-Plough Animal Health, Kenilworth, NJ) before corneal harvesting. The corneas were fixed in situ using a solution containing 2% glutaraldehyde and 2% paraformaldehyde in mock aqueous humor.[7] The epithelial and endothelial surfaces of the cornea were treated with fixative by instillation and by perfusion of the anterior chamber, respectively; the corneas were excised and placed in vials with fresh fixative solution. The specimens were treated with the secondary fixative (1% osmium tetroxide in mock aqueous), washed, and dehydrated in graded ethanol solutions. The tissue was then embedded in Spurr epoxy resin and cured overnight before examination by transmission electron microscopy.

For the investigation of free radicals by electron paramagnetic resonance (EPR) spectroscopy only acute PQ effects in rabbit eyes were investigated. The rabbit, under general anesthesia, received 50 μl of 100 mM PQ by intrastromal injection into the cornea of the right eye. Aqueous humor (100 μl) was withdrawn from the anterior chamber of the left eye prior to intracameral injection of 100 μl of 100 mM PQ. Thirty minutes after PQ injection, with the rabbit still under general anesthesia, the anterior chambers were drained and the fluids saved ice-cold. The rabbit was killed as above, and the corneas were harvested, rinsed in saline and kept in ice-cold saline. The EPR spectra were recorded 30–60 min. after collection of the specimens using a Varian E-9 spectrometer (Varian, Palo Alto, CA). Corneal strips were placed in a sealed silica flat tissue holder, whereas the fluid specimens were placed in Pasteur pipettes with a sealed tip, prior to insertion into the cavity of the magnet. All EPR spectra were recorded at room temperature and the spectrometer settings were: Scan Range 100G, Time Constant 3 sec., Modulation Amplitude 2G, Receiver Gain 1.6×10^4, Microwave Power 25 mW, Scan Time 16 min., and Microwave Frequency 9.440 GHz.

RESULTS

The EPR spectrum of a rabbit corneal specimen collected 30 min. after intrastromal injection of PQ showed the signal of the PQ free radical (Figure 2). On the other hand, the corneal specimen from the eye that received a PQ injection in the anterior chamber 30 min. prior to euthanasia, showed no EPR signal (Figure 3). The EPR spectrum of aqueous humor removed prior to PQ injection showed the signal of the ascorbate free radical (Figure 4). Similarly, aqueous humor specimens collected 30 min. after intrastromal or intracameral PQ injection also showed only the ascorbate radical signal (data not shown).

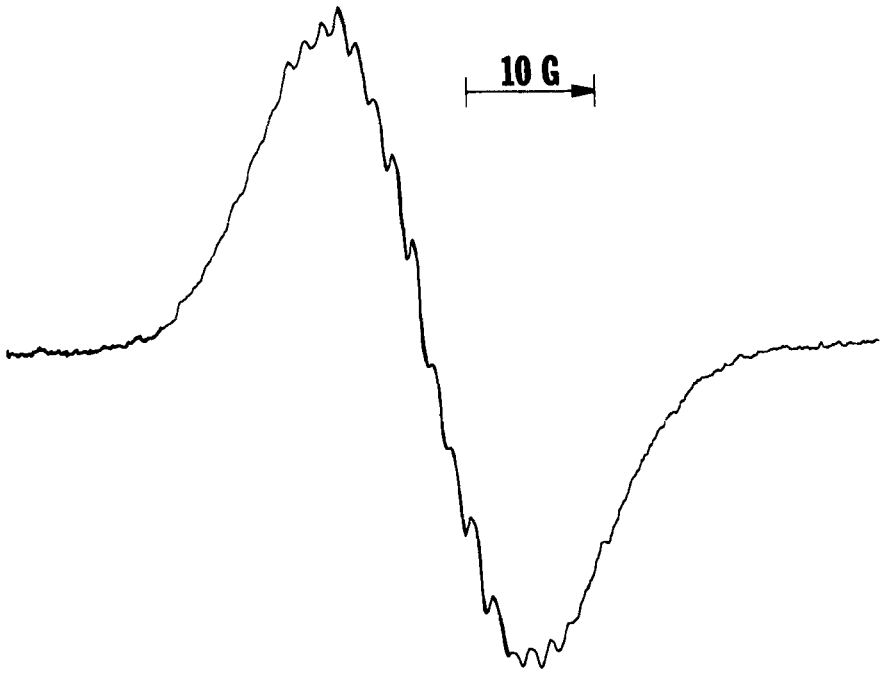

FIGURE 2 EPR spectrum of rabbit cornea 30 min. after intrastromal injection (50 µl) of 100 mM paraquat, showing the signal of the free radical of paraquat.

Ultrastructural studies of corneas harvested 8 days after 100 µl intrastromal PQ injections showed striking differences in the type, site and magnitude of the corneal lesion in the two animal species investigated. Rabbit corneas showed typical dose-related lesions in the epithelial basement membrane. The 50 mM PQ injection caused minimal damage (Figs. 5 and 6). At this PQ concentration, the lamina lucida widened while the lamina densa thinned and was interrupted in some areas. Stromal collagen

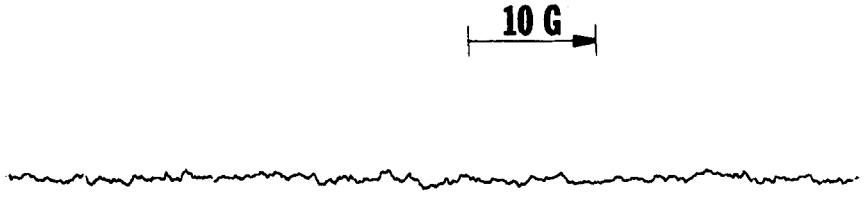

FIGURE 3 EPR spectrum of rabbit cornea 30 min. after injection (100 µl) of 100 mM paraquat into the anterior chamber of the eye, showing no EPR signal.

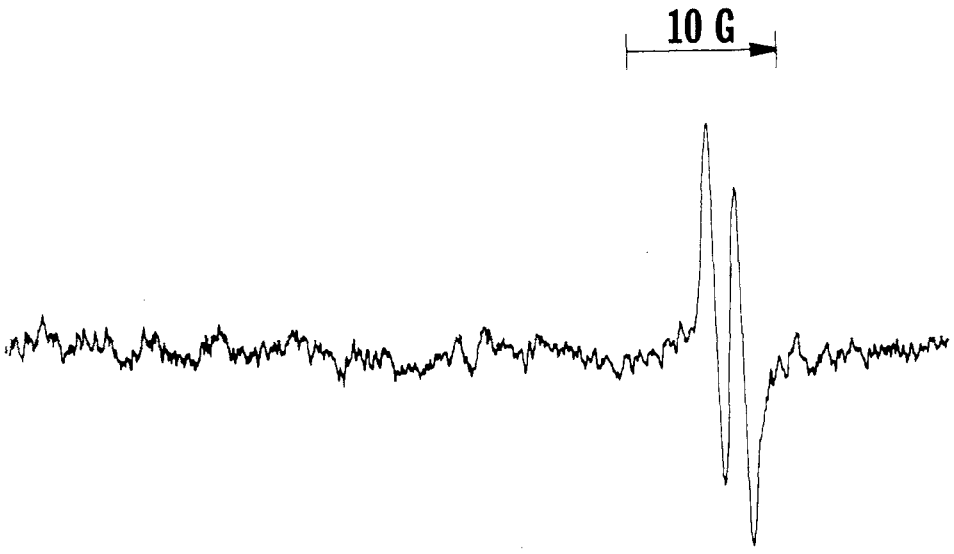

FIGURE 4 EPR spectrum of rabbit aqueous humor showing the signal of the free radical of ascorbic acid.

FIGURE 5 Rabbit corneal epithelial-stromal junction 8 days after intrastromal injection (100 µl) of 50 mM paraquat. The arrow indicates an area of basement membrane damage. The bar equals 300 nm.

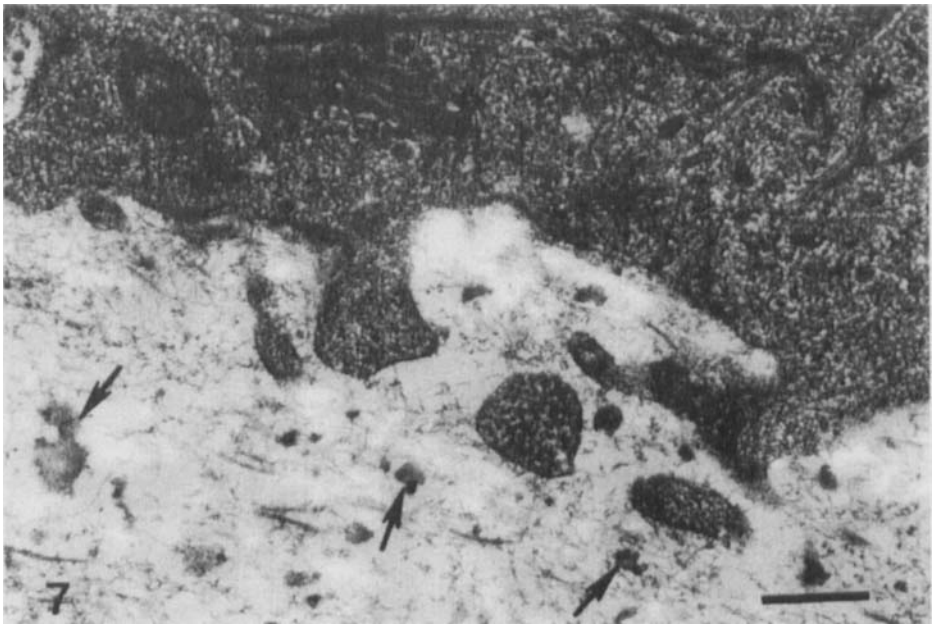

FIGURE 6 Rabbit corneal epithelial-stromal junction 8 days after intrastromal injection (100 µl) of 50 mM paraquat. Note the area of basement membrane injury. The bar equals 300 nm.

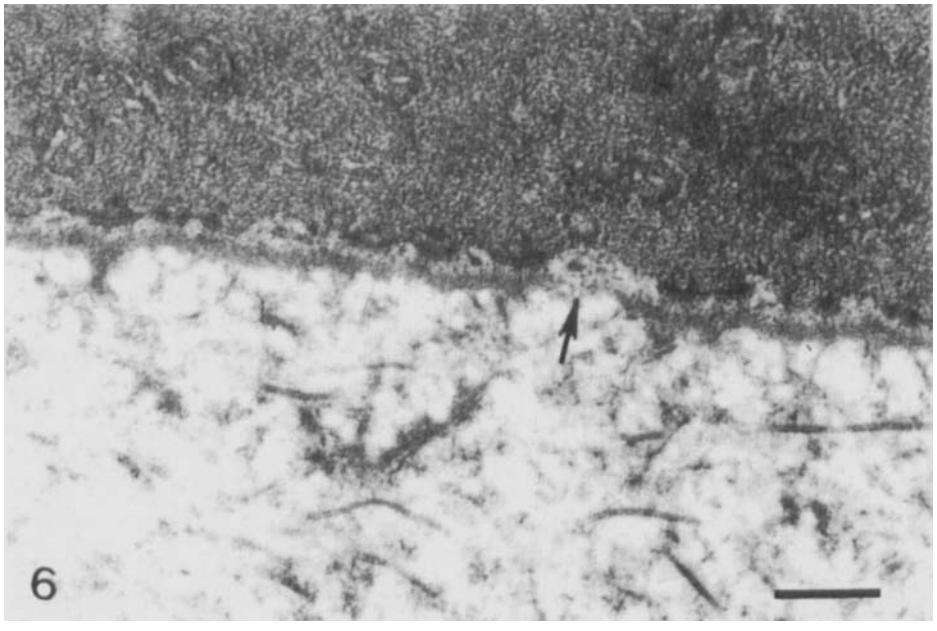

FIGURE 7 Rabbit corneal epithelial-stromal junction 8 days after intrastromal injection (100 µl) of 75 mM paraquat. Note the complete absence of the lamina densa. The arrows indicate lamina densa debris. The bar

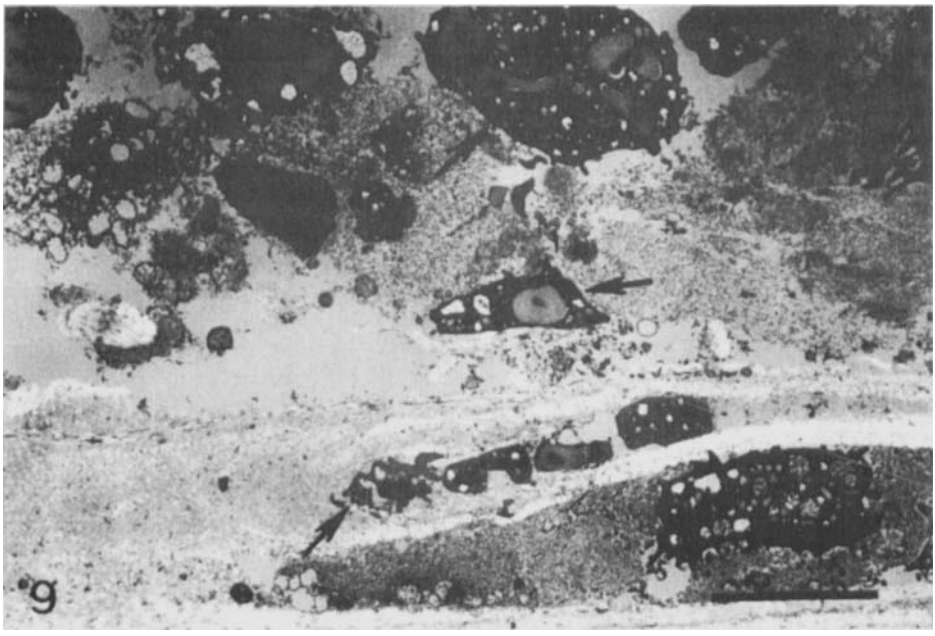

FIGURE 8 Rabbit corneal epithelial-stromal junction 8 days after intrastromal injection (100 µl) of 75 mM

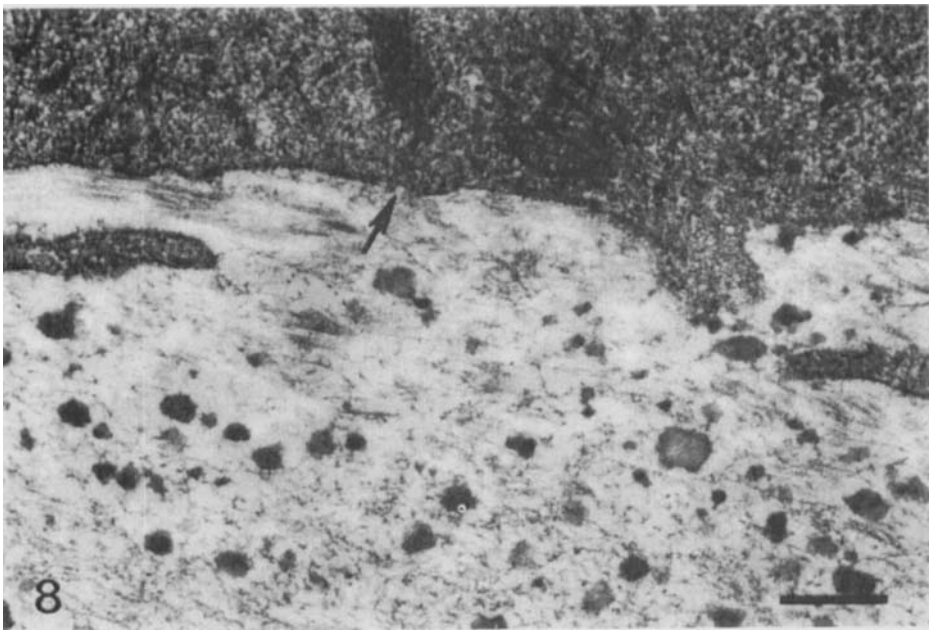

FIGURE 9 Rabbit corneal epithelial-stromal junction 8 days after intrastromal injection (100 µl) of 150 mM paraquat. Almost total loss of epithelium and upper stroma can be observed. The arrows indicate an

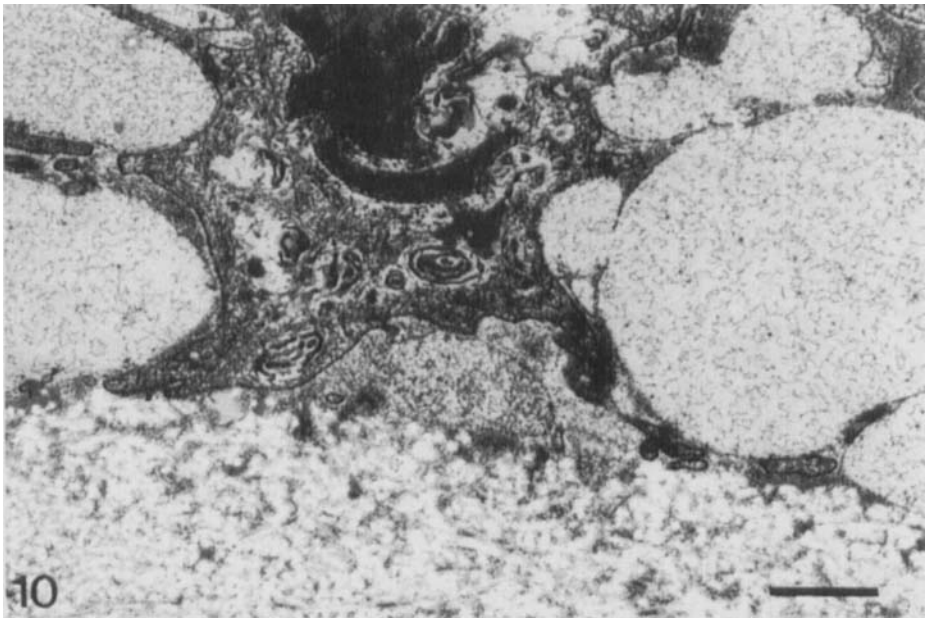

FIGURE 10 Rabbit corneal epithelial-stromal junction 8 days after intrastromal injection (100 µl) of 150 mM paraquat. The epithelium was lost due to dissolution of the basement membrane and upper stroma. This figure shows some of the few remaining adherent epithelial cells which were obviously moribund. The bar equals 600 nm.

did not appear to be affected. Injection of 75 mM PQ resulted in an almost complete dissolution of the basement membrane (Figs. 7 and 8) and significant damage to stromal collagen was also observed. The 150 mM PQ injection resulted in a total loss of the corneal epithelium and a deep stromal ulceration accompanied by a strong inflammatory response (Figs. 9 and 10).

The eyes of the monkeys were much less sensitive to PQ than the rabbit eyes. No corneal damage was observed with 50 mM or 75 mM PQ injections. Injection of 100 µl of 100 mM or 150 mM PQ resulted in similar corneal lesions. The sites of the injury were primarily the proximal and lateral plasma membranes of basal corneal epithelial cells (Figure 11). Basement membrane alterations were only detected adjacent to areas with significant damage in the plasma membranes of basal epithelial cells. No damage to Bowman's membrane or to corneal stroma could be detected.

DISCUSSION

The toxicity of bipyridylium herbicides such as PQ and diquat (N,N′-ethylene-2,2′-bipyridylium dibromide) is of considerable interest because of their widespread use as herbicides and defoliants. The lungs appear to be a very sensitive target since severe lung lesions have been reported in human beings following accidental ingestion of PQ[1] as well as after direct lung exposure through the smoking of PQ-tainted marijuana cigarettes.[8] Among other organs affected in human beings and experimental animals

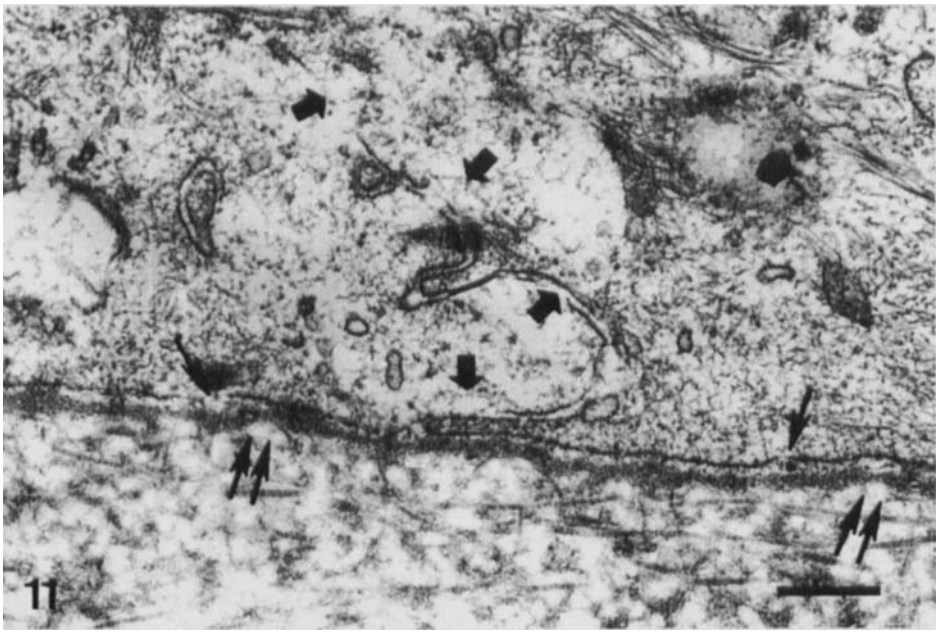

FIGURE 11 Monkey corneal epithelial-stromal junction 8 days after intrastromal injection (100 µl) of 100 mM paraquat. The tissue injury was primarily confined to disruption and dissolution of the proximal and lateral plasma membranes of the corneal epithelium. The thick arrows indicate areas of dissolution of the lateral plasma membranes of adjacent epithelial cells. The single arrows show proximal plasma membrane interruption. The double arrows indicate regions in the basement membrane which have a widened lamina lucida and a narrowed lamina densa. The bar equals 300 nm.

exposed to PQ, lesions to the liver, kidney, digestive tract and skin have been reported.[2,9]

Following reports of ocular lesions resulting from accidental splashing of PQ solutions onto human eyes,[4] experimental direct exposure of animal eyes was investigated. For these studies, solutions of PQ of various concentrations were applied to the conjunctival sac of rabbits.[2,10] In spite of an initial conjunctival inflammatory reaction, no corneal damage was observed at PQ concentrations between 1 mM and 80 mM.[2,10] Minimal corneal damage (diffuse areas of opacification) were observed after application of 0.2 ml of 160 mM PQ.[10] Higher PQ concentrations, 325 mM and 650 mM, caused an increase in the area of opacification and a pannus reaction, whereas the 1.3 M solution resulted in rabbit death within 6 days of ocular application.[10]

A more efficient and sustained effect of PQ would be expected in our experiments not only because of its mode of administration (intrastromal injection) but also because of possible ionic interaction between cationic PQ and polyanionic proteoglycans present in the cornea. Such an interaction would result in a more prolonged retention of PQ. Furthermore, since transition metal ions are known to enhance PQ toxicity *in vivo*[11] as well as the biological damage caused by O_2^- generated *in vitro*,[12] corneal copper[13] could contribute to and amplify the PQ-induced lesions. These considerations may explain the corneal lesions in rabbits injected with 50 mM PQ, whereas previous studies[10] showed no effects with topical 80 mM PQ.

Interestingly, whereas in a previous study with rabbits we showed that the initial corneal damage induced by hydrogen peroxide generated *in situ* involved collagen lysis and disruption of the stromal lamellar structure,[14] the PQ-mediated lesions observed in the present studies with both rabbits and monkeys suggest initial and preferential attack at the level of the epithelium/basement membrane interface. The mechanism underlying this preferential site of attack by PQ has not been studied. However, since bipyridyls are actively accumulated to high concentrations in specific types of lung cells,[15] a similar phenomenon could also operate in cornea.

The absence of PQ radical in the cornea of rabbits given a PQ injection in the anterior chamber supports the concept that the corneal endothelium may be less efficient than the epithelium as a site for the metabolic redox-cycling of PQ. This is also consistent with the absence of damage to the endothelial basement membrane or the endothelium in our rabbits and monkeys as well as in human eyes following accidental splashing of PQ solutions.[4] If the bulk of the PQ free radical observed in the EPR spectrum of the rabbit cornea is produced by the epithelial cells, the O_2^- generated during redox-cycling of PQ could act in conjunction with copper bound to basement membrane collagen type IV to result in a site specific metal mediated attack.[11]

As with the rabbit, the corneal lesions observed in monkeys are also consistent with an active role of the basal epithelial cells in the generation of free radicals by redox cycling of PQ. However, higher doses of PQ were required to elicit corneal lesions in monkeys than in rabbits. Furthermore, the corneal lesions in monkeys were more prominent in the plasma membrane of basal epithelial cells than on the basement membrane. Anatomical differences (e.g. the presence of Bowman's membrane in monkeys but not in rabbits) as well as possible species specific biochemical differences may underly the observed differences both in the susceptibility to PQ, and in the type of corneal lesions observed in monkeys and rabbits.

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