Prevention by medroxyprogesterone of perforation in the alkali-burned rabbit cornea: inhibition of collagenolytic activity

David A. Newsome and Jerome Gross

The incidence of perforation and deep ulceration of the alkali-burned rabbit cornea was substantially reduced by topical or parenteral administration of medroxyprogesterone. The mode of action of the hormone is probably at least in part related to its suppressive effects on production of tissue collagenase, as indicated by the considerable reduction in collagenolytic activity by living explants of the treated corneas.

Key words: cornea, alkali-burned, collagenase, medroxyprogesterone, dexamethasone.

Severe alkali burns of the cornea in human beings, as in experimentally burned rabbits, frequently result in deep ulceration, perforation, and loss of the eye. If perforation can be prevented, recovery of sight may be possible through eventual corneal transplantation. It has been well established that destruction of the corneal stroma following injuries such as alkali burns or lacerations is associated with the production of a collagenase capable of destroying corneal and other types of collagen. This enzyme attacks the collagen molecule and tissue fibril under physiologic conditions of pH and temperature in the same specific manner as do collagenases from a wide variety of amphibian and mammalian sources and is correlated with collagen removal in a wide range of physiologic and pathologic states. Also, characteristically, it is inhibited by ethylenediamine tetraacetate (EDTA) and cysteine. Applying these compounds topically to experimentally injured eyes in rabbits, Brown and his associates and Itoi and associates have found that frequent, continuing application begun shortly after burning the cornea with
alkali could prevent the development of deep ulceration and perforations in statistically significant numbers. Similarly, there is some indication that the same therapeutic measure is effective, in part, following alkali burns in man. However, the needed frequency of application, the significantly large numbers of failures, and some evidence of toxicity of these compounds limit their therapeutic usefulness.

If there were a simple way of preventing enzyme formation, as an alternative to blocking enzyme activity, therapy might be more effective and have broader application. This approach might also be effective in other ulcerative conditions involving collagenolytic action. Since Jeffrey and associates and subsequently Halme and Woesner had previously observed that progesterone blocked collagenase formation in cultures of postpartum rat and rabbit uterus, it occurred to us that this hormone might be an effective agent in the prevention of cornea destruction following alkali burns.

Materials and methods

Young adult albino rabbits of both sexes were anesthetized with intravenous sodium pentobarbital. Each eye was mechanically propogated and its cornea covered for 1 minute with a disc of gauge saturated with 1N NaOH. The pledget contacted the entire cornea plus 1 to 2 mm. of perilimbal tissue. Immediately after exposure to the alkali the eyes were flushed with 30 to 50 cc. of 0.9 per cent sterile NaCl and repositioned, and polysporin or erythromycin ointment was instilled as antibacterial prophylaxis. Antibiotic treatment was continued daily. In the single experiments using 4N NaOH, the same procedure was followed, except that the alkali-saturated pledget was in contact only with the central and middle cornea, sparing a 1 mm. rim within the limbus. Conjunctival cultures for bacteria were prepared 3 weeks after burning in three separate experiments.

Experimental design. Three therapeutic modes were tested. (1) A single or twice daily instillation of 1 drop of a 0.5 per cent suspension of medroxyprogesterone in 1 per cent sterile aqueous methylcellulose was made in the subconjunctival sac of one eye and the suspension vehicle alone was similarly administered to the other, control eye. Treatment was routinely begun 24 hours after burning. (2) Subconjunctival depots of 10 mg. of a preparation of medroxyprogesterone (Depo-Provera, 100 mg. per milliliter; The Upjohn Co., Kalamazoo, Mich.) were established in one eye and the vehicle of Depo-Provera (kindly provided by Upjohn) was similarly deposited in the control eye. In one experiment a single depot was used and in others two to four depots were placed and repeated at weekly intervals. (3) Intramuscular injections of 200 mg. of Depo-Provera (400 mg./ml.) into the rear leg at 7 to 8 day intervals were made in experimental animals and the same volume of the vehicle was administered to control animals. All treatments were given by one investigator and the other was kept uninformed. Eyes were closely examined by both investigators regularly and separate records were kept by each, using standard morphologic criteria. The codes of treatment in each experiment were sealed and kept by a disinterested party at the commencement of each experiment.

All experiments were continued for 36 to 46 days, and in one instance animals were carried 120 days. At the conclusion of each experiment both observers independently examined by dissecting stereomicroscope the whole corneas from eyes that had been enucleated and fixed in 10 per cent formalin. Representative halves of the corneas were embedded in paraffin, sectioned, stained with hematoxylin and eosin for histologic study. Before the protocol was revealed to the “blind” observer, data were compared and any disagreements were settled by a mutual reexamination of the corneas in question.

Quantitative measurements of collagenolytic activity for comparison between treated and control eyes. Collagenolytic activity was measured in vitro by explanting six or seven uniform, 1.5 mm. diameter trephined buttons of freshly obtained cornea to the surface of reconstituted guinea pig skin collagen gels. Care was taken to include buttons representing all areas of the cornea from eyes 16 to 21 days after alkali exposure. All explants in any one dish came from the same cornea. Collagen gels were made as described previously by layering 1.0 ml. of cold sterile 0.2 per cent guinea pig skin collagen solution (pH 7.6; Tris Buffer 0.014M and NaCl 0.2M) into 35 mm. plastic Petri dishes and incubating the dishes at 37° overnight. The gels were then allowed to equilibrate with Dulbecco’s Modified Eagle’s Medium (Grand Island Biological Co., Grand Island, N. Y.) before use. Explants were symmetrically spaced on the gel surface without added liquid medium. In those experiments testing the in vitro effects of hormones and inhibitors of protein synthesis, the compounds were mixed with the collagen solution prior to gelation. These compounds included cycloheximide (25 μg per milliliter), dexamethasone 10^-8M and medroxy-
progesterone $^{14}$C-M. It should be noted that although the concentration of solid medroxyprogesterone was high, $10^{4}$M, it is known from the study of Jeffrey and associates$^{12}$ that the concentration of the hormone in tissue is lower by three orders of magnitude. Cultures were maintained at 37° in an atmosphere of 95 per cent CO$_2$ and 5 per cent air at 100 per cent humidity for 10 to 14 days. Visual observations were made with oblique incident light over a black background, and lysis was scored as the number of corneal buttons per dish surrounded by a liquefied zone. Quantitation was obtained by hydroxyproline analysis$^{15}$ of the clear supernatant fluid and sediment after high-speed centrifugation of the contents of each dish. Data were recorded as per cent of total collagen solubilized.

Collagenolytic activity of liquid media was assayed by measuring the lysis of $^{14}$C-glycine-labeled guinea pig skin collagen.$^{14}$ The $^{14}$C-labeled collagen was reconstituted as fibrils in 0.05M Tris-HCl, pH 7.5, with 0.2M NaCl and 0.001M CaCl$_2$. One unit of collagenase activity was defined as the solubilization of 1 $\mu$g of reconstituted fibrils per minute at 37°. Incubations were carried out for 4 hours with a control of 0.01 per cent trypsin (TCPK, Worthington Biochemical Corp., Freehold, N. J.) to check for collagen denaturation. Maximum collagenolytic activity was obtained by incubating tissue in medium containing 5 per cent fetal calf serum and "activating" latent collagenase in the medium by 10 minute incubation at 37° with 0.0006 per cent trypsin (TPCK) followed by 5-fold excess of soybean trypsin inhibitor.

**Cornea collagen extractability and sensitivity to proteases.** Possible structural damage to the corneal collagen in alkali-burned eyes was assessed by (1) measuring collagen extractability in neutral saline and dilute acetic acid, and (2) testing its susceptibility to noncollagenolytic proteases as compared with controls. Burns with 1N and 4N NaOH were produced in the eyes of six rabbits as described above. The corneas from each of two animals were excised, minced, pooled, and extracted in 10 ml of distilled H$_2$O for 24 hours at 4° C. with stirring. The extract was clarified by centrifugation and pH determined. Pellets were then sequentially extracted in two changes of 0.5M NaCl containing 0.02M phosphate buffer (pH 7.7) followed by three changes of 0.5M acetic acid. Pellets from the final acetic acid extract were gelatinized in H$_2$O for 30 minutes in a boiling water bath and separated into supernatant fluid and sediment by centrifugation. Aliquots of the extracts and all final pellets were hydrolyzed and assayed for hydroxyproline. The extracts were examined for collagen breakdown products by sodium dodecyl sulfate (SDS) gel electrophoresis$^{16}$ and by electron microscopy of segment long-spacing crystallites (SLS) preparation.$^{3}$

The susceptibility of normal corneal collagen to pepsin and trypsin was compared with that of alkali-burned corneas, 48 hours after the burn. In triplicate experiments, blocs of tissue ranging from 1.5 to 4.5 mg. wet weight from unburned controls and 1N and 4N NaOH-treated corneas were incubated in either 1 ml of 0.1 per cent crystallized pepsin at pH 2.5 and 4° C with shaking for 24 hours or in 1 ml of 0.1 per cent trypsin pH 7.5 at 37° for 24 hours. The per cent of collagen extracted was determined by hydroxyproline analysis of the supernatant and pellet after centrifugation.

**Results**

Both medroxyprogesterone-treated and control corneas followed a similar course for the first 6 to 8 days after alkali exposure. Freshly burned corneas became cloudy immediately after burning and subsequently turned opaque within 24 hours. Early conjunctival injection and edema resolved by 3 to 5 days after the burn. In about 30 per cent of the cases these signs of inflammation did seem to lessen and disappear 24 hours sooner in the treated as compared to the control eyes. Of 20 control animals, 16 had similar degrees of damage or healing response in each eye. No animals showed widely divergent responses such as one eye healing spontaneously and the other perforating.

First signs of ulceration rarely appeared before 7 days after alkali exposure (range 6 to 18 days) and no new lesions appeared later than about 30 days after the burn. Ulceration appeared characteristically in two locations: as central lesions, or as an excavated ring at the junction of the middle and outer annular thirds of the cornea. Ulcers seemed to have no specific relationship to the blood vessel ingrowth which began 7 to 10 days after alkali exposure. Blood vessel invasion continued until corneas were completely vascularized by 36 to 40 days after the burn. No new lesions appeared after this time in 18 corneas observed for 120 days.

Stromal ulcers, once established, deepened with varying rapidity. In many ulcers
Table I. Effect of subconjunctival depot of 10 mg. of medroxyprogesterone deposited twice, on days 1 and 8 after 1N NaOH burns; control burned eyes received the vehicle alone.

<table>
<thead>
<tr>
<th>Cornea condition</th>
<th>Treated (7 eyes)*</th>
<th>Control (6 eyes)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clinical</td>
<td>Microscopic</td>
</tr>
<tr>
<td>Intact</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Ulcer</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Descemetocoele</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Perforation</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Treated and one control eye perforated after uncontrollable infection and were excluded from table.

Microscopic data obtained by examining the intact, removed, and fixed corneas prior to embedding and sectioning conformed closely with that obtained histologically. Both modes of tissue study were performed on each eye.

Table II. Effect of one daily topical application of 0.5% medroxyprogesterone suspension after 1N NaOH burns; control burned eyes similarly treated with vehicle alone.

<table>
<thead>
<tr>
<th>Cornea condition</th>
<th>Treated (27 eyes)†</th>
<th>Control (27 eyes)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clinical</td>
<td>Microscopic</td>
</tr>
<tr>
<td>Intact</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Ulcer</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Descemetocoele</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Perforation</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

*Combined data from 3 separate complete experiments.
†Three eyes in this category perforated after uncontrollable infection and were excluded from table.

Table III. Effect of intramuscular depot of 200 mg. of medroxyprogesterone deposited weekly after 1N NaOH burns; control animals received the diluent alone.

<table>
<thead>
<tr>
<th>Cornea condition</th>
<th>Treated (12 eyes)*</th>
<th>Control (12 eyes)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clinical</td>
<td>Microscopic</td>
</tr>
<tr>
<td>Intact</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Ulcer</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Descemetocoele</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Perforation</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

*One treated and three control eyes perforated after uncontrollable infection and were excluded from table.

Descemet's membrane developed in 33 per cent of our control animals. Once the entire cornea was vascularized, these ulcers appeared to be filled in somewhat, but the defects never completely disappeared.

Alkali-burned corneas that did not ulcerate remained milky white. The rust-colored advancing tongues of new vessels showed clearly on this background. After about 45 days, the entire cornea was vascularized. After 3 months or so, the prominence of the vessels gradually diminished but the cornea remained opaque with a slightly irregular surface.

Effect of medroxyprogesterone on the clinical course of alkali-burned corneas. Medroxyprogesterone was effective in preventing severe corneal injury in all three modes of administration, i.e., as a single subconjunctival depot (Table I), as a daily topical application (Table II), and as an intramuscular depot (Table III). There was little discrepancy in evaluation of the lesions clinically in vivo, by stereomicroscopy of the removed whole cornea, or by histologic study of specific stained regions.

Among all the experiments in eight groups of rabbits, 49 of 85 control corneas perforated as compared with 8 of 87 medroxyprogesterone-treated eyes (not including those perforating subsequent to blatant infection). Strikingly few, 4 of 85, control corneas healed without serious damage, compared to the 51 of 87 medroxyprogesterone corneas that healed. The number of corneas that ulcerated but did not per-
forate was similar in the two groups, 26 of 85 controls and 21 of 87 medroxyprogesterone-treated.

In the single experiment using a stronger, 4N NaOH burn, all nine control (untreated) corneas perforated but only two of eight treated corneas perforated, and two others developed deep ulcers and a descemetocoele.

The sex of the rabbits did not appear to influence the response to treatment. Seven of the ten animals which sustained serious corneal damage despite hormone treatment were males. Similarly, of the control groups with serious damage about 60 per cent were male. The time of first ulceration, onset, and progress of vascularization was similar for male and female animals.

Bacterial infection preceded corneal damage in very few animals and appeared with about the same incidence in treated and control eyes. In two groups of 12 cultures prepared from conjunctival swabs obtained 3 weeks after alkali exposure, nearly all were negative for bacterial growth. Gross purulent conjunctivitis advancing to severe panophthalmitis with corneal destruction occurred in 5 per cent of both treated and untreated eyes. *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella* species, or other gram-negative bacteria were cultured as pure growths or mixed infections from these eyes.

**Histologic observations.** Histologic examination of treated and control corneas 36 to 46 days after alkali burning revealed that the epithelium covering the stroma in nonulcerated regions was irregularly thickened and not accompanied by an identifiable Bowman's membrane in both types. Some corneas displayed islands of epithelium on an otherwise bare stroma, with collagen destruction occurring only subjacent to the epithelium. The stroma in these areas of destruction always contained some inflammatory cells and smaller numbers of presumed fibroblasts.

Large ulcers were always surrounded by a moderate number of leukocytes. These leukocytes were distributed most heavily at the margins rather than the bases of the ulcers. In the few cases of deep ulcers in medroxyprogesterone-treated corneas we examined in detail, the inflammatory infiltrate was present but less heavy than that usually seen in controls. Quantitative comparisons of the degree of inflammation will require more exhaustive study. Descemet's membrane appeared to be much more resistant to the ulcerative process than stromal collagen: many large ulcers had eroded to a broad acellular base of what appeared to be just Descemet's membrane. Endothelial cells were usually absent.

Perforation was usually grossly obvious as a complete defect in the cornea, often with loose, protruding tissue. In many cases healing had occurred, filling the defect with new tissue. This situation was readily recognized histologically by the presence of a mushroomlike plug of new fibrous repair tissue usually organized perpendicular to the old corneal stroma.

In several rare instances ulceration appeared on the posterior aspect of the cornea, with loss of nearly half the stromal thickness. In such cases, the iris was loosely adherent to the cornea at the site of ulceration.

The completely healed corneas, which include both medroxyprogesterone-treated and the much smaller number of intact controls displayed variable numbers of fibroblast-like cells irregularly distributed among the still relatively well organized stromal collagen bundles. Some areas were obviously thickened with new stroma fairy well arrayed parallel to the old remaining structures. In other regions the old collagen laminae appeared "hyalinized" and acellular. There were scattered groups of inflammatory cells in an apparently random distribution.

**Collagenolysis in vitro.** The effect of medroxyprogesterone on collagenase production in culture was assayed by two separate quantitative methods. Tissue explants to the surface of sterile reconstituted collagen gels gave highly reproducible results by visual criteria and chemical measurements in four complete experiments.
Table IV. Collagenolytic activity in vitro of treated and control alkali-burned cornea explanted to collagen gels (six uniform 1.5 mm. trephined explants per dish)*

<table>
<thead>
<tr>
<th>Treatment of cornea in vivo</th>
<th>Total hydroxyproline (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S  P  %L  S  P  %L  S  P  %L  S  P  %L  S  P  %L</td>
</tr>
<tr>
<td>None</td>
<td>183 20 90 52 263 17</td>
</tr>
<tr>
<td>Freeze-thaw</td>
<td>90 149 38 212 140 61</td>
</tr>
<tr>
<td>Cycloheximide (25 µg/ml.)</td>
<td>141 57 71 18 335 5</td>
</tr>
<tr>
<td>Medroxyprogesterone (0.1 mM)</td>
<td>168 47 78 23 345 6</td>
</tr>
<tr>
<td>Dexamethasone (1 µM)</td>
<td>168 34 83 18 325 5</td>
</tr>
</tbody>
</table>

Gel No.:

1  8 7 17 31 15 71 325 5 60 203 23 16 182 6 0 182 0
2  90 149 38 212 140 61 65 200 25 131 164 45 9 181 5 10 176 5
3  141 57 71 18 335 5 24 235 9 45 168 21 18 180 9 133 88 60
4  168 47 78 23 345 6 49 210 19 21 131 14 33 179 15 16 180 8
5  168 34 83 18 325 5 60 203 23 23 166 12 12 182 6 0 182 0
6  78 170 31 18 335 23 16 180 8 3 179 2 3 158 2

Average % of collagen lysed ± S.E.M.

65 ± 9.4 18 ± 8.2 17 ± 2.5 22 ± 5.1 9 ± 2.0 13 ± 8.4

*Raw data from one experiment. S = Supernatant; P = Pellet; %L = percent lysed.

Table V. Collagenolytic activity (in units) of culture media from explants of alkali-burned corneas (harvested 15 days after alkali exposure)*

<table>
<thead>
<tr>
<th>Rabbit no.</th>
<th>Treated cornea</th>
<th>Control cornea</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.4</td>
<td>2.1</td>
</tr>
<tr>
<td>2</td>
<td>0.6</td>
<td>2.1</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>1.6</td>
</tr>
</tbody>
</table>

*Each value represents average of 2 cultures, all within 15% of each other. Trypsin control value has been subtracted from each datum.

Table IV reports the representative results of one of six similar experiments showing clear-cut inhibition of collagenolysis by cornea explants from medroxyprogesterone-treated eyes as compared with the frequently complete lysis of the gels by untreated, burned tissue explants. The reduction in collagenase activity by the liquid culture medium from explants of medroxyprogesterone-treated corneas as compared with that from explants of control corneas was confirmed by the radioactive collagen fibril assay (Table V). In each of four animals the collagenolytic activity was reduced 3- to 4-fold, with the average specific activity of the control explants 1.94 units and of the treated, 0.48 units. Cycloheximide prevented lysis by untreated alkali-burned corneal tissue, indicating the need for protein synthesis during the culture period. Freeze-thawing had a similar effect, documenting the requirement for living cells. The corticosteroid hormone dexamethasone added to the collagen gel also markedly inhibited collagenolysis (Table IV).

In one of two experiments medroxyprogesterone incorporated into the gel in concentrations of 10^-4M caused considerable inhibition of lysis (Table IV). In the other, results were positive but not as dramatic. The medroxyprogesterone suspension vehicle did not influence the results. Note that some lysis did occur in a few of the medroxyprogesterone-treated- and cycloheximide-containing dishes, probably for technical reasons.

Table VI summarizes the results of all gel lysis experiments. In vivo treatment with medroxyprogesterone of alkali-burned corneas, on the average, reduced their collagenolytic potential to less than one half that of untreated burned corneas.

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Effect of alkali exposure on cornea collagen.

Exposure to strong alkali could possibly alter the structure of the corneal collagen molecule, rendering it susceptible to proteases other than collagenase. We com-
Medroxyprogesterone prevents corneal damage

Table VI. Summary of data from all experiments examining collagenolytic activity in vitro of treated and control alkali-burned cornea buttons explanted to collagen gels

<table>
<thead>
<tr>
<th>In vivo treatment of cornea</th>
<th>Added to collagen substrate</th>
<th>No. of experiments</th>
<th>No. of dishes</th>
<th>% Lysis ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>None</td>
<td>7</td>
<td>56</td>
<td>69.4 ± 2.6</td>
</tr>
<tr>
<td>Vehicle</td>
<td>Medroxyprogesterone</td>
<td>2</td>
<td>13</td>
<td>27.0 ± 6.3</td>
</tr>
<tr>
<td>Vehicle</td>
<td>Dexamethasone</td>
<td>3</td>
<td>17</td>
<td>17.6 ± 3.8</td>
</tr>
<tr>
<td>Vehicle</td>
<td>Cycloheximide</td>
<td>2</td>
<td>12</td>
<td>16.7 ± 1.6</td>
</tr>
<tr>
<td>Vehicle</td>
<td>None (freeze thaw)</td>
<td>1</td>
<td>5</td>
<td>18.7 ± 8.2</td>
</tr>
<tr>
<td>Medroxyprogesterone</td>
<td>None</td>
<td>6</td>
<td>51</td>
<td>34.6 ± 2.1</td>
</tr>
<tr>
<td>Medroxyprogesterone</td>
<td>Medroxyprogesterone</td>
<td>1</td>
<td>6</td>
<td>18.9 ± 4.9</td>
</tr>
<tr>
<td>Medroxyprogesterone</td>
<td>Cycloheximide</td>
<td>1</td>
<td>6</td>
<td>22.5 ± 6.7</td>
</tr>
</tbody>
</table>

pared the extractability in cold neutral salt solutions and 0.5M acetic acid of normal cornea and cornea burned with 1N and 4N alkali. The amount of collagen extracted at neutral pH was similar for both burned and normal tissue, but acid extractability was increased 1.5-fold after 1N alkali and 2-fold after 4N NaOH. Relatively little difference was noted 1, 2, and 5 days after burning. The pH of the water extract of corneas 24 hours after burning with 1N and 4N NaOH was in the neutral range, pH 6.2 to 6.7.

The collagen extracted by cold neutral salt solution and by acetic acid was examined by SDS gel electrophoresis. The migration patterns revealed only intact α-chains and β-components plus higher molecular weight fractions, not different from extracted collagen from normal corneas.

An important measure of disturbance of the covalent structure of collagen is its susceptibility to proteolytic attack. Solubility of collagen after treatment with pepsin was unchanged for 1N NaOH-exposed corneas and greater for those exposed to 4N NaOH as compared with unburned control corneas (Table VII). Trypsin released about twice as much collagen from 1N alkali-burned corneas as from the controls. Note that trypsin solubilized no more collagen at 37°C and neutral pH than did pepsin at 4°C and acid pH. There was a further 30 per cent increase in trypsin solubility of the 4N NaOH–burned corneas (Table VII). Under no conditions was more than about one fourth of the total collagen extracted.

Table VII. Sensitivity of corneal collagen to proteases following alkali exposure (values for duplicate experiments)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Per cent of collagen solubilized</th>
<th>By pepsin*</th>
<th>By trypsin†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16, 16</td>
<td>7, 9</td>
<td></td>
</tr>
<tr>
<td>1N NaOH burn</td>
<td>12, 15</td>
<td>16, 18</td>
<td></td>
</tr>
<tr>
<td>4N NaOH burn</td>
<td>22, 27</td>
<td>21, 24</td>
<td></td>
</tr>
</tbody>
</table>

*0.1% pepsin in 0.5M HAc and HCl, pH 2.5, at 4° for 24 hours.
†0.1% trypsin in modified Eagle's MEM, pH 7.5, at 37° for 34 hours.

Discussion

The clinical data reported here indicate, as predicted, that the progestational hormone medroxyprogesterone is effective in preventing deep ulceration and perforation of the cornea consequent to severe alkali burns in rabbits. It is significant that results of a single implantation of depot medroxyprogesterone 1 day following injury was about as effective as the daily application of a drop in the conjunctival sac. We have no data to indicate how long it takes to exhaust the depot of hormone, nor do we now know the tissue concentrations. It should be noted that, in these initial experiments, we chose very large doses of the hormone so as to be reasonably certain of not missing any effect. Lower doses may also be effective: further extensive dose effect experiments are desirable. It is believed that collagenase activity in burned rabbit cornea reaches its peak about 14 to 21 days after exposure to alkali. It is therefore possible that an inhibition of collagenase production for all or even most of this
period is sufficient to prevent serious collagen destruction. Our observations that alkali-burned corneal tissue treated in vivo with medroxyprogesterone produced significantly less lysis in culture on collagen gels than did control tissue support the idea that suppression of collagenase production during a "critical period" can result in continued lowering of enzyme levels.

We have not demonstrated a physiologic regulatory effect of medroxyprogesterone on collagen metabolism. However, our data are consistent with the idea that the hormone reduces stromal destruction by blocking the production of collagenase; it is not itself an inhibitor of the enzyme.12 There is, also, the alternative possibility that progesterone stimulates the production of collagenase inhibitor or blocks the formation of an activator of procollagenase, or of the latter itself. We note that the reduction in collagenase activity is incomplete and could reflect any of a number of mechanisms. However, since increase or decrease in collagen content depends on a balance between synthesis and degradation, a 50 per cent decrease in the latter could readily prevent net lysis.

In the study of Jeffrey and associates12 on the action of progesterone and medroxyprogesterone on collagenase production by postpartum rat uterus, the hormone was effective on the cultured tissues only within the first 12 hours after delivery. After in vivo hormone administration some regions of the uterus were nonresponsive.19 One might guess that temporal and spatial variations in response to hormones are related to the cellular progesterone receptor mechanism present in uterine cells.20, 21 Are there progesterone receptors, as now detected, in other mesenchymal cells? The available limited evidence restricts them to the uterus and oviduct,22 certain mammary tissues,23 the pancreas,24 and certain central nervous system tissues such as the optic tectum25 and the pituitary gland.26 Recent efforts to detect progesterone receptors in the cytosol of our cultured corneal cells by Richardson and MacLaughlin (unpublished) at this institution have not been successful. However, the assay has not yet been attempted on intact cells.

Could there be other actions of progesterone such as anti-inflammatory effects which limit the number of leukocytes invading the tissue and thus reduce the contribution of leukocytes in treated as compared with untreated corneal tissues? Our limited number of histologic observations of reduced inflammatory infiltrate in medroxyprogesterone-treated corneas as compared with controls suggests such an anti-inflammatory effect. Further, toxicity studies performed for us by Dr. Claes Dohlman (unpublished) showed a mild but definite decrease in anterior chamber inflammation with topical medroxyprogesterone treatment. The only cellular component of the inflammatory reaction known to produce a collagenolytic enzyme is the polymorphonuclear leukocyte.27, 28 However, if the enzyme activity observed was released from leukocytes, its appearance would not have been prevented by cycloheximide or freeze-thawing. We also note that Werb and Reynolds29 recently have claimed, in contrast to a previous report,30 that collagenase or its inactive forms could not be detected either biochemically or immunologically in homogenates of rabbit leukocytes as compared with human leukocytes from which the enzyme was obtained. We conclude that at least the major portion of collagenase was actively synthesized and secreted by living tissue cells repopulating the cornea, and that the inflammatory reaction itself could not alone be responsible for corneal collagen degradation.

In view of the evidence that products of activated lymphocytes can stimulate the production of collagenase by macrophages,30b such a mechanism may be involved here. Aside from a decrease in the number of invading lymphocytes, how medroxyprogesterone might affect such an activation system is unknown.

Medroxyprogesterone blocks the production of active collagenase in ways not yet established. Koob and Jeffrey31 reported a
similar inhibitory activity of cyclic AMP, and Koob and his colleagues\textsuperscript{32} showed that dexamethasone, another steroid hormone, inhibits collagenase production by tissue culture of human skin, rheumatoid synovium, and rat uterus. This corticosteroid has the same effect on collagenolytic activity of the alkali-burned rabbit cornea, shown here. It has been reported that dexamethasone predisposes to perforation in the alkali-burned rabbit cornea\textsuperscript{33} which might reflect a more general anti-anabolic effect depressing collagen synthesis, not shared by progestational hormones. Collagen production and scarring are characteristic features of the aftermath of alkali burns of the cornea. Partial or complete suppression of this phase of repair by dexamethasone could result in perforation even in the face of concurrent reduction of collagenolytic activity. We are exploring this problem in greater depth. Medroxyprogesterone reduction in collagenase may represent a property of several types of steroid hormones, a subject requiring further study.

It is possible that progesterone reduces the levels of other tissue proteases such as the cathepsins. However, there is no reliable evidence that cathepsins will attack native collagen fibrils under physiologic conditions\textsuperscript{34} or that other components of the inflammatory reaction are collagenolytic. We do know that the animal collagenases from a variety of tissues do attack collagen fibrils specifically and that their levels have been well correlated with collagen degradation in physiologic and pathologic processes (see references 4 and 5 for reviews). We also know that collagen is the major structural element of the cornea, providing tensile strength. Other connective tissue components such as proteoglycans may be removed consequent to injury, but removal of these would not in itself cause corneal perforation. Alkali is said to remove proteoglycan from the stroma,\textsuperscript{35,37} thus exposing the fibrils to collagenolytic attack. The partial degradation of collagen by alkali to a stage susceptible to attack by nontropic proteases might also predispose to lysis. Our data on the properties of collagen extractable from burned cornea do not indicate any extensive attack on the covalent structure of the $\alpha$-chains, hence it is likely that the helical structure of the molecules remains intact. Our finding that alkali-exposed corneal collagen is more acid soluble but not more soluble in neutral salt solution than normal collagen supports the idea that the increased solubility after alkali may be in part due to loss of intermolecular covalently cross-linked regions, namely the nonhelical N- or C-terminal peptides. Increased solubility also, in part, probably reflects removal of proteoglycan by alkali.

The collagen gel assay used here is a highly reliable and sensitive index of collagenolytic activity when adequately controlled. It does not provide kinetic data for enzyme release rates as do direct measurements of collagenolytic activity in culture medium as a function of time. Typical reaction products of collagenase activity\textsuperscript{3} have been demonstrated for corneal collagenase in many laboratories, including our own, which validates the specific nature of the enzyme activity (see references 4 to 6 for reviews).

Since we have shown an effective blocking action of medroxyprogesterone on collagenase production and tissue destruction in one nonuterine connective tissue, it is quite conceivable that the clinical applicability of treatment with this hormone might be much broader. Human clinical trials for treatment of corneal ulceration and other ulcerative diseases are planned.

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