Central corneal alkali burn injuries were induced in rabbits by applying five NaOH concentrations on uniformly soaked 7 mm filter paper discs. Clinical parameters were evaluated daily by microscopic examination and photography, and corneal myeloperoxidase levels were measured periodically. Satisfactory alkali burn models of corneal inflammation, vascularization and ulceration were developed by manipulating the alkali concentration. Invest Ophthalmol Vis Sci 30:2148-2153, 1989

Corneal inflammation, ulceration and vascularization are often coincident in corneal disease. Several experimental models have been developed to study these individual events. Although their pathophysiology are interrelated, many factors involved in the pathogenesis of these disease states may differ among models. The purpose of this study was to describe the characteristics of central corneal alkali burn injuries produced by standardized filter paper disc application of various concentrations of NaOH in the absence of direct conjunctival involvement.

Materials and Methods
The experiments complied with the ARVO Resolution on the Use of Animals in Research. Experiments were performed on 58 2.5-3.5 kg male New Zealand white rabbits obtained from a single animal breeder. Preliminary experiments had shown that corneal inflammation and ulceration following alkali burns were age-dependent, with younger juvenile rabbits particularly prone to corneal ulceration and animals weighing more than 4.5 kg having less ulceration and corneal neovascularization. The predilection for ulceration in young animals may be due to greater relative injury in the thinner, smaller corneas of immature animals.

Standard Alkali Burning
Anesthesia was induced with intramuscular injections of ketamine hydrochloride, 50 mg/kg and chlorpromazine, 10 mg/kg. Proparacaine hydrochloride, 0.5% was used for topical anesthesia and a wire lid speculum was inserted in the right eye. Surplus moisture was removed with a cotton-tipped applicator. Seven millimeter filter paper discs, produced by standard paper punch, were soaked for 10-20 sec in one of several NaOH solutions: 0.2N, 0.5N, 1N, 2N, or 4N. Whatman #3 filter paper was used, because it easily molded to the cornea when wet. The discs were drained, and excess moisture absorbed (briefly) on tissue paper, with reproducible wetting of ±5% by weight (Table 1). The alkali-soaked discs were applied to the central cornea, centered on the pupil and held gently in position with forceps for 2 min. In one modification using 4N NaOH, 25 μl of alkali was dropped into the center of the disc after 1 min of alkali burning and the disc maintained in position for another min. The cornea was finally irrigated over 60 sec with 15 ml balanced salt solution (BSS: Ocusol, IOLAB Pharmaceuticals, Claremont, CA). Although no topical antibiotic was used, infection was not a problem.

Assessments
Each animal was examined daily by Haag-Streit slit lamp and detailed corneal findings recorded. The radial length of major vessels was measured from the limbus by the height of the spanning slit-lamp illumination beam. The mean length of the three longest vascular tufts was used as a measure of inferior quadrantal vascularization, and superiorly, the mean of two vascular tufts was taken. A daily Neovascular Index (NI) was calculated as the product of the inferior and superior means. Neovascularization also was gauged by the degree (clock-hours) and temporal relationships of neovascular invasion of the circular central corneal burn, and a preterminal count (under general anesthesia) of all visible corneal vessels proceeding radially 0.5 mm internal to the corneal limbus was made using a Zeiss operating microscope and magni-
Table I. Reproducibility of alkali hydration of 7 mm Whatman #3 filter paper discs (n = 10)

<table>
<thead>
<tr>
<th>Range</th>
<th>Mean (±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry weight of filter discs</td>
<td>6.84-7.59 mg</td>
</tr>
<tr>
<td>Wet weight after attempted uniform hydration</td>
<td>30.61-35.31 mg</td>
</tr>
<tr>
<td>Calculated volume of alkali</td>
<td>23.99-27.00 μl</td>
</tr>
<tr>
<td>Percentage wet/dry weight</td>
<td>338-378%</td>
</tr>
</tbody>
</table>

Epithelial Resurfacing

The initial epithelial defect never extended beyond the margin of the opaque stromal burn injury. The corneal epithelial defect healed over the first 2–4 days in all animals with 0.2N (three of three rabbits) and 0.5N NaOH (three of three rabbits) injuries and in all but three animals (23 of 26) with 1N NaOH burns. The epithelial defect failed to close in 33% of 2N (two of six rabbits) and 55% of 4N (11 of 20 rabbits) alkali-burned corneas. In 36 of 42 (86%) animals in which initial epithelial surfacing occurred, there was epithelial breakdown (noted with all alkali concentrations) within 2 to 6 days postburn. Epithelial defects were often recurrent, commonly at different sites in the same eye. All 0.2N and 0.5N NaOH injuries were covered by intact epithelium at day 14, but the incidence of chronic epithelial defects at this time was high with 1N and 2N NaOH alkali injuries (85% and 83%, respectively) and occurred in 100% of the animals following 4N NaOH burns. Areas of surface nonwettability in areas of intact epithelium were common, more so with severe burns; there was no apparent relationship between areas of nonwettability and subsequent epithelial breakdown.

Corneal Edema

On removal of the alkali-immersed disc, the central corneal stromal injury was opaque with a distinct margin. Opacification increased during the postburn BSS irrigation. The burn site rapidly became edematous (Fig. 1A), except following 2N and 4N alkali injury in which corneal swelling was severely restricted (Fig. 1B), particularly during the first week of injury. The disc-shaped stromal wound opacity induced by alkali concentrations of 1N or less tended to recede anteriorly and peripherally over the 14-day period, the wound size becoming somewhat smaller.

The peripheral cornea was edematous to a degree proportional with the alkali concentration. After 2N and 4N alkali burns, peripheral cornea bowed forward, delimiting the central, less swollen area of direct injury. The peripheral corneal edema cleared centripetally from the limbus, beginning clinically 5 to 7 days after mild alkali burning and starting approximately 9 to 10 days after 4N NaOH. Peripheral edema cleared at 1 month, except in the most severe injuries.

Edematous corneal stroma was initially homogenous, but small, clear lacunae developed in the anterior 40% over several days. Superficial bullae were more common at the higher alkali dosages and usually subepithelial. Bullae occurred in the central corneal burn, were uncommon before the second week of injury, were frequently present for only 24 hr
and commonly recurrent, sometimes at differing sites. After 4N NaOH, the bullae tended to occur in peripheral cornea just external to the disc of injury and were often present for several days.

**Corneal Angiogenesis**

The onset of peripheral neovascularization was delayed clinically for 2 to 3 days following central alkali injury. The rates of neovascular ingrowth following 1N NaOH and 4N NaOH burns were not significantly different until day 5; greater rates of new vessel infiltration then occurred after 4N injuries compared to 1N burns. The extent of corneal vascular ingrowth was roughly proportional to the severity of the alkali injury. Vascular proliferation was principally from the superior limbus in eyes with 0.2N and 0.5N alkali burns, but was greater from the inferior than the su-
superior limbus at higher NaOH concentrations. Neovascularization generally occurred within the anterior third of the corneal stroma. However, after 4N alkali burns, neovascular growth was dense, often confluent, and usually also involved deep corneal stroma; the pannus was usually elevated above the corneal surface.

There were between-animal variations in the neovascular response over time, despite the almost identical early appearance of the wounds. Angiogenesis was also nonuniform around the circular injury, and so three measurements were devised to assess the overall neovascularization. The increase in the NI over the first 2 weeks after 1N and 4N alkali burning is compared in Figure 2. The degree of neovascular clock-hour involvement of the central disc of injury was only useful after 2N and 4N NaOH burning because of the limited extent of the neovascular response with lesser injuries. The in vivo counting of corneal (perilimbal) new vessels was practical with injuries induced by ≤1N NaOH, but higher alkali dosages produced neovascularization too florid for individual blood vessels to be counted by this technique.

Inflammation and Ulceration

Acute chemosis and fibrinous discharge were roughly proportional to the concentration of alkali, and settled over 2 to 3 days. Clinically, there was a gradual increase in the optical density of the central burn injury with time; with high alkali concentrations, this was rapid from days 3 to 6 and sometimes associated with a diffuse grey-white infiltration of the peripheral cornea. In a small number of animals after 1N, 2N and 4N alkali burns, an acute, self-limited inflammatory response occurred, roughly between days 3 and 8, that was often associated with a hypopyon and a purulent discharge. These reactions settled spontaneously and were not associated with focal corneal infiltration.

Stromal ulceration did not occur from alkali injuries induced with concentrations below 1N NaOH. With increasing alkali concentrations, stromal loss in the central cornea (when it occurred) tended to arise earlier, and be deeper and more extensive. Early ulceration was difficult to detect after 4N alkali injury because of the relatively thin disc of injury. Ulceration sometimes occurred in relatively quiet (clinically) eyes, but not in neovascularized areas. Submaximal alkali hydration of the filter paper resulted in central ulceration; whereas arcuate ulceration at the margins of the corneal burn were produced by the peripheral pooling associated with greater filter paper hydrations. A very high prevalence of early corneal ulceration (<2 weeks) could be produced within a circular wound by dropping 25 μl of 4N NaOH onto the center of the (submaximally soaked) 4N alkali filter disc after 1 min of the 2 min injury.

In preliminary histologic experiments, a gradual increase in the central corneal infiltrate occurred over the 14-day time period following corneal burning with alkali concentrations of 1N or greater. At 14 days, the corneal infiltrate in the wound area was confined to the anterior two-thirds of the stroma. PMN and macrophages were the prominent inflammatory cells (Figs. 1C, 1D, 3). Keratocytes were more commonly found in close association with macrophages than PMN. Keratocytes were frequently very large (Fig. 1E) and keratocytic mitotic figures common (Fig. 1F). Little MPO activity was detected in 14-day corneas after 1N NaOH; the MPO results in 4N NaOH corneas were highly variable with poor correspondence to the histologic or clinical findings.

Discussion

Rapid growth of knowledge concerning the pathophysiology of inflammation is accompanied by a proliferation of experimental drugs capable of modulant-
ing the inflammatory processes at different points. Accessibility and tissue transparency are valuable attributes of corneal models of inflammation, but most of these models remain unstandardized. The purpose of the study was to develop experimental models of inflammation, neovascularization and ulceration in the clinically relevant context of severe structural damage to the cornea. Uniform alkali injuries are accomplished using alkali-immersed filter discs.

We have developed a standard method of filter-disc hydration with a 5% variability based on Oenberger's alkali-immersed filter paper model. Prolonged soaking of filter paper in 2N and 4N NaOH has to be avoided as the filter disc first becomes less compliant and then eventually disintegrates, due to the mercerization of the cellulose matrix at these concentrations (personal communication, 1988, David Martin, Whatman Paper Ltd., Maidstone, Kent, England). Careful preparatory tissue paper absorption of the excess moisture from the alkali-soaked disc determines the relative homogeneity of each of the models and limits ulceration to the central cornea, thereby avoiding annular ulceration at the edge of the disc of injury from runoff of excess alkali. The alkali injury is limited to the central cornea (and structures of the anterior chamber) and avoids direct injury to the limbus, conjunctiva and episclera, which can sometimes severely complicate corneal inflammation and repair in clinical alkali burns. Alkali does not escape from the confines of the disc of injury. At each alkali concentration, evenly circular corneal injuries are produced in the central cornea with uniform size and appearance (over the first 3 days postburn). The sizes of the initial epithelial defects in each group are also similar.

There has been recent experimental interest in the production of alkali burn injuries using an alkali-filled well applied to the corneal surface. Constant, even pressure needs to be applied to the well to guarantee an intact seal, thereby distorting the cornea. After a period, the well is evacuated by pipette (unavoidably leaving a surface film of concentrated alkali with variable thickness), before the well is removed and the eye irrigated. It remains to be shown whether this method produces as uniform and reproducible lesions as the use of standardized, alkali-immersed filter discs.

The standardized, central discoid burn achieved by our method permits objective clinical evaluation of the corneal neovascular response. Despite the circular shape, uniform size and apparent equal severity of the corneal injury and the near-central corneal position, the angiogenic response was not uniform around 360° of the cornea. Corneal new vessels were derived principally from the superior limbus following 0.2N and 0.5N alkali burning, possibly because the disc of injury was made in reference to the rabbit pupil, which is slightly decentered superiorly. After 1N, 2N and 4N NaOH injury, the inferior limbal angiogenic response was almost always greater than that from the superior limbus. Medial and lateral neovascularization were usually the least extensive. Provisional work using fully alkalized filter discs showed that any spillage was usually superonasal. However, as alkali does not leak outside the disc of injury in the standardized models, the regional neovascular variation suggests genuine differences in the limbal angiogenic responses around the cornea. One possible explanation requiring investigation is whether, in the rabbit, there might be differences in the limbal distribution of potential angiogenic effector cells, such as mast cells.

Furthermore, the regional variability of the neovascular response presents difficulties of measurement. Some animals had relatively even regional angiogenesis, neovascularization in others was characterized by tufts of new vessels, and, in a few animals, a small number of vascular trunks dominated the picture. Computerized quantitation of India ink-suffused corneas provides an objective quantitation of corneal angiogenesis, but the instrumentation is expensive and only postmortem evaluations are possible. Conventional corneal photography is insufficiently sensitive and it is sometimes difficult to obtain limbus-to-limbus views in unanesthetized animals. Subjective scoring on a + to 4+ basis has been commonly used, but is insensitive and difficult to standardize. Calculations based on the area of neovascularization have been used successfully in mice with eccentric lesions. This method might be feasible in the confluent neovascularization of severe alkali burns in the rabbit, but the much larger cornea would necessitate photographic recording and digitization of the involved area; the technique would not be suitable for lesser degrees of injury. The three-part angiogenesis scheme that we have devised assesses an index of radial new vessel length, the temporal and spatial relationships of neovascular infiltration of the central burn, and a premortem count of paralimbal (prebifurcation) corneal vessels proceeding radially. The last method is only useful with lesser degrees of angiogenesis (following ≤1N NaOH burns), but is valuable where there may be marked variability in the new vessel response; it is measured at 14 days or later when peripheral corneal edema has largely settled. Quantitative rather than qualitative characteristics of neovascularization thereby can be addressed.

The corneal swelling response can be assessed in a general way at the slit lamp. A much thinner corneal
injury follows 2N and 4N NaOH compared with less severe alkali injury. There is probably a rapid loss of central corneal substance, particularly from proteoglycan mobilization. However, marked wound "compaction" is seen within the first few hours, suggesting the possible importance of collagen cross-linking as an inhibitor of corneal swelling. The thinnest corneal stroma appear most likely to undergo earlier and more severe corneal ulceration. Clinically, the opacification of the central corneal injury gradually increases in density over the first week following the burn.

Peripheral corneal swelling outside the disc of injury occurs with all alkali concentrations, but is more severe and prolonged after 2N and 4N burns. Stromal edema in the anterior cornea was characterized by the appearance of diffuse lacunae. Kikkawa and Hirayama described uneven corneal swelling in several species, with the reduced swelling of the anterior stroma associated with much greater light scattering, when compared with posterior stroma. Bullous formation was most common after severe injury, generally in the second week; bullae were frequently evanescent.

The onset of ulceration may not be easy to detect in the compacted corneal injuries of 2N and 4N NaOH burns; a suspicion may be confirmed or denied by hindsight. We have noted that the onset of ulceration may be immediately preceded by loss of 1% fluorescein and Richardson's stain binding to the local corneal stroma. A partial localized clearing of the dense opacity of the central disc of injury also was detected in some animals just before stromal loss became apparent.

We have not been impressed by the reproducibility of total corneal MPO assays in these alkali burn models. MPO estimations at 14 days in 1N burns were consistently low, possibly missing a late peak of PMN infiltration described to occur after alkali burning around day 21. It is possible that partial PMN degranulation may influence the assay sensitivity. After 2N and 4N NaOH, there was no clear association between the MPO assay results and either the clinical status (including ulceration) or the PMN infiltration seen on histology. The substantial macrophage population in alkali burn lesions has been inadequately stressed. We suggest that experimental therapeutic trials in alkali-injured corneas should be designed to investigate multiple time points using clinical parameters, changes in corneal structure, quantitative and qualitative aspects of the inflammatory cell infiltration at histology and, possibly, tear mediator levels. Corneal MPO levels may be unhelpful in alkali burn injuries.

Two major events proceed simultaneously in the repair process of many corneal diseases: degradation and removal of necrotic debris and replacement of damaged collagenous matrix and cells. The balance between the two processes determines outcome. Standardized models are required to study these processes with the ultimate aim of controlling them and preserving the structural integrity and normal function of the cornea.

Key words: alkali burn, cornea, myeloperoxidase, ulceration, vascularization

References