An Evaluation of the Role of Leukocytes in the Pathogenesis of Experimentally Induced Corneal Vascularization

II. Studies on the Effect of Leukocytic Elimination on Corneal Vascularization

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Investigations on several experimental models in the past have supported the hypotheses that corneal vascularization is a manifestation of the inflammatory response and that leukocytes perform an essential role in stimulating corneal vascular ingrowth. To evaluate the possible role of leukocytes further in this phenomenon, the effect of leukocyte elimination on corneal vascularization induced by silver nitrate cauterization was investigated. Weanling Fischer albino rats received doses of total body x-irradiation ranging from 1100 to 2100 rads to deplete circulating leukocytes, and corneal silver nitrate cauterization was performed 4 days later. In this model, animals that received 1500 rads or more total body x-irradiation became severely leukopenic within 4 days. As a rule, neither leukocytes nor blood vessels invaded the cauterized corneas, whereas both a leukocytic and vascular invasion occurred at lower doses of irradiation that did not totally eliminate circulating leukocytes. Corneal vascularization ensued if the corneal cauterization was performed immediately after total body x-irradiation with 1500 rads before the leukopenic effect of x-irradiation occurred. Control studies in which the cornea was cauterized 4 days after only the head received 1500 rads x-irradiation ruled out the possibility of irradiation-induced limbal endothelial damage as the explanation for the vascular suppression observed by x-ray treatment. In nonirradiated rats, silver nitrate cauterization of the cornea consistently induced corneal vascularization by 2 to 3 days. In further experiments, methylprednisolone acetate was administered subconjunctivally after corneal cauterization. This corticosteroid inhibited the infiltration of leukocytes and the subsequent vascular invasion into the corneal stroma, if administered immediately after silver nitrate cauterization. However, when the same glucocorticoid was administered 1 day after cauterization, both a leukocytic infiltration and vascular ingrowth occurred but to a less severe degree than in non-glucocorticoid-treated cauterized corneas. These investigations together demonstrated that a vascular ingrowth of the cornea did not follow corneal cauterization with silver nitrate in the absence of leukocytes, and gives further support to the hypothesis that leukocytes serve a crucial function in corneal vascularization. (Am J Pathol 81:531–544, 1975)

THE PHENOMENON OF CORNEAL VASCULARIZATION has concerned investigators for many years, and several theories have been pro-

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posed to account for its pathogenesis. In recent studies in several experimental models, corneal vascularization has been consistently observed by sequential histologic analysis to be preceded by a leukocytic infiltration into the cornea.[1,2] The corneal vascularization in all of these different models was characterized by three distinct phases; an initial leukocytic infiltration (prevascular phase), a period composed of leukocytes and blood vessels within the corneal stroma (vasoproliferative phase), and a later stage where blood vessels persisted without leukocytes (established phase). These experiments supported the hypotheses that corneal vascularization is a manifestation of the inflammatory response and that leukocytes are a prerequisite to corneal vascular ingrowth. These studies also suggested that leukocytes might induce directional vascular growth by the release of chemical mediators.

To further test the hypothesis that leukocytes are a prerequisite to corneal vascularization, the effect of eliminating the leukocytic infiltration by total body x-irradiation or by the subconjunctival instillation of methylprednisolone acetate on corneal vascularization induced in rats by silver nitrate cauterization was investigated.

Materials and Methods

Corneal Cauterization

Normal weanling albino rats (40 to 75 g) of the Fischer strain were anesthetized by ether (E. R. Squibb & Sons) inhalation, and a silver nitrate applicator tip (Graham-Field Surgical Company, New Hyde Park, N.Y.) was applied for 1 to 2 seconds to the central cornea of each eye. Daily observations were made with a Zeiss operation microscope (Carl Zeiss, West Germany).

Irradiation Technique

During irradiation the rats were immobilized in groups of four without anesthesia in close-fitting compartments of a circular Lucite holder measuring 30 cm in diameter and divided by twelve partitions (Figure 4). The heads of experimental animals were positioned in the central portion of the circular holder. Irradiation was performed with a 280 kV x-ray unit (Picker X-Ray Corp., Pittsburgh, Pa.). The half-value layer, the amount of material necessary to reduce the x-ray dosage by 50%, was 1.4 mm copper. To eliminate short wavelength irradiation, a filtration of 0.5 mm copper and 0.25 mm aluminum was used. The target to skin distance was 50 cm, and the field size, 20 cm by 20 cm.

Experimental Groups

Normal Rats

Normal rats were killed at 1, 2, 3, 4, 5, 6, 7, and 14 days after corneal cauterization, and the eyes were enucleated and processed for light microscopic analysis of the sequence of events that preceded and accompanied corneal vascularization.

Total Body and Head X-Irradiation

The entire body and head of groups of 4 rats each were irradiated with 1100, 1300, 1500, 1700, 1900, and 2100 rads. On the day prior to x-irradiation until the termination of the experiment, oxytetracycline hydrochloride (Terramycin soluble powder, animal formula, Pfizer Agricultural Division) in distilled water (7 g/liter) replaced the tap water which was previously given *ad libitum*. From the time of irradiation, the leukocytes in the peripheral blood were counted daily in a Spencer Bright Line Improved Neubauer Chamber (American Optical Company, Buffalo, N.Y.) by the standard hemocytometer technique after dilution with 2.0% acetic acid. Blood was obtained from anesthetized rats by excising approximately 2 mm from the end of the tails, and all counts were determined from freely flowing blood. The corneas of both eyes of all of the aforementioned rats were cauterized with silver nitrate 4 days after the animals were x-irradiated. The corneas of an additional 4 rats that received 1500 rads total body x-irradiation were cauterized immediately after the irradiation.

Total Body X-Irradiation With Shielding of Head

The heads of 10 rats were shielded with lead (1.5 cm thick) while the body was exposed to 1500 rads of x-irradiation—the lowest dose of x-irradiation that produced severe leukopenia within 4 days. The corneas of these animals were cauterized with silver nitrate 4 days later. By shielding the heads in this manner, radiation injury to the endothelial cells of the corneoscleral limbal plexus would be minimized, and these cells would hopefully retain maximum capability to proliferate in response to an injury.

X-Irradiation to the Head With Shielding of the Body

To determine whether or not 1500 rads x-irradiation to the limbal vascular endothelial cells inhibited their capability to proliferate in response to corneal cauterization, the heads of 4 rats were irradiated with 1500 rads while the rest of the body was shielded with lead (1.5 cm thick). On the fourth day after irradiation, the corneas were cauterized with silver nitrate.

Subconjunctival Administration of Glucocorticoids

In 8 rats, immediately after bilateral cauterization of the corneas with silver nitrate, 0.1 ml of methylprednisolone acetate (Depo Medrol, Upjohn) in a concentration of 80 mg/ml was injected into the superior and inferior subconjunctival regions of one eye with a 1-ml tuberculin syringe. Four other rats received subconjunctival glucocorticoid to one eye 24 hours after bilateral corneal cauterization with silver nitrate. This was done to determine whether the administration of methylprednisolone acetate would inhibit the proliferation of endothelial cells of the limbal plexus after the leukocytes had already infiltrated the cornea.

Results

Normal Rats

Within 1 day of corneal cauterization with silver nitrate, the injured site possessed a dark brown discoloration, while the surrounding cornea was opaque and the conjunctival blood vessels were hyperemic. Microscopically at Day 1, a brown region extended through the entire thickness of the cornea at the site of cauterization. At this time numerous poly-

morphonuclear leukocytes could be appreciated in the central and peripheral corneal stroma (Figure 5). Vascularization was invariably evident in the cornea by 2 to 3 days when examined with the operation microscope. Blood vessels were evident in the peripheral superficial corneal stroma on microscopic examination in all ten corneas that were examined at this time. By Days 3 to 4, blood vessels extended into the entire circumference of the cornea (Figure 1), and by microscopic observation, the vascular invasion involved the entire depth of the cornea (Figures 7 and 10). By 5 to 6 days the vessels reached the site of cauterization, and a radiating system of communicating vessels permeated the cornea between the corneoscleral limbus and the region of injury. Vascular channels were still appreciated at 2 weeks.

Total Body and Head X-Irradiation

Three to 4 days after 1500 or more rads total body x-irradiation, rats displayed few, if any, circulating leukocytes in the peripheral blood (0 to 200/cu mm), while those animals exposed to less than this dosage exhibited a milder leukopenia (500 to 3000/cu mm) at this time. The corneal sites of cauterization after total body x-irradiation in all of the doses investigated were indistinguishable from those in normal rats. Of the 4 rats that received 1500 rads of x-irradiation and corneal cauterization 4 days later, 2 died at 2 days after corneal injury. In 1 of these animals a slight vascularization was present in the corneas of both eyes and leukocytes were evident in the corneas central to the zones of vascularization. No corneal vascularization or leukocytic infiltration occurred in either eye of the other rat or in the remaining 2 animals that died 3 and 4 days after cauterization. Leukocytes and blood vessels invaded the corneas of all rats receiving 1100 to 1300 rads x-irradiation in a manner identical to that in the nonirradiated control animals. However, the cauterized corneas of animals receiving 1700 to 2100 rads total body x-irradiation lacked leukocytes as well as blood vessels throughout the time period from cauterization until their deaths within 2 days of corneal injury.

In those studies where the corneas were cauterized with silver nitrate immediately after 1500 rads total body x-irradiation but before the peripheral leukopenia appeared, corneal vascularization ensued in a manner indistinguishable from that in the nonirradiated normal animals.

Total Body X-Irradiation With Shielding of Head

The 10 rats whose bodies were exposed to 1500 rads while their heads were shielded with lead to minimize radiation injury to the limbal vascular plexus responded to corneal cauterization in a manner similar to that in the animals that received total body x-irradiation without shielding of the

head. Neither leukocytes nor blood vessels invaded the corneas (Figures 2, 6, 8, and 11). The mortality of the 10 animals at 1, 2, and 3 days after cauterization was 2, 7, and 1 respectively.

X-Irradiation to the Head and Shielding of Body

The peripheral leukocyte count remained normal (10,000 to 20,000 per cu mm), for the duration of observation (8 days) in rats whose heads were irradiated with 1500 rads, while the rest of the body was shielded with lead. In these animals corneal vascularization followed silver nitrate cauterization, and the sequence of events was identical to that for normal nonirradiated animals with respect to the time of onset of vascularization and the extent of the leukocytic infiltration and subsequent corneal vascularization.

Glucocorticoid Treatment

In glucocorticoid-treated animals the sites of cauterization appeared similar to those in the normal rats—both grossly and microscopically—in terms of pigmentation and the extent of stromal involvement. The sites of subconjunctival injection of methylprednisolone acetate appeared milky white from the time of injection until the termination of the experiment. Of those eyes that received subconjunctival glucocorticoid treatment immediately following corneal cauterization, few if any leukocytes could be identified in the corneal stroma of the corneas by 1 day and thereafter. Blood vessels were also not observed to extend microscopically into the corneas of any of those steroid treated rats during a course of 2 weeks when the observations were terminated (Figures 3, 9, and 12). On the other hand, when methylprednisolone acetate was administered 1 day after cauterization, a corneal vascular ingrowth was evident by Day 2 to 3, and light microscopy disclosed a leukocytic and vascular infiltration which was less severe than in the normal non-glucocorticoid-treated corneas (Table 1).

Discussion

This study gives further support to the hypothesis that leukocytes are a prerequisite to corneal vascularization. Elimination of the leukocytic component of the inflammatory response to silver nitrate cauterization of the cornea by either total body x-irradiation or the subconjunctival instillation of methylprednisolone acetate prevented the subsequent vascular ingrowth. Moreover, corneal vascularization still followed cauterization if the latter was performed after a similar dose of total body x-irradiation but before the peripheral blood became leukopenic.

Neither leukocytes nor blood vessels invaded the corneal stroma

Table 1—Summary of Silver Nitrate Cauterization Experiment

Experimental groups	Dose (rads)	Localization (head/body)	Time of cauterization (PI)	Time of corticoid instillation (PC)	Corneal leukocytes	Corneal blood vessels	Remarks
Normal rats					+	+	
X-Irradiated rats							
	1100–1300	+/+	Day 4		+	+	
	1500	+/+	Day 4				Corneal vascularization and leukocytic infiltration in 1 rat
	1500	/+	Day 4				
	1500	+/	Day 4		+	+	
	1500	+/+	Immediate		+	+	
	1700–2100	+/+	Day 4				All animals died within 2 days of cauterization
Glucocorticoid-treated rats							
				Immediate Day 1	+	+	Leukocytes and blood vessels less than in normal rats

PI = postirradiation, PC = postcauterization.

when methylprednisolone acetate was administered immediately after cauterization. However, this same glucocorticoid did not prevent the invasion of the cornea by blood vessels if it was administered 1 day post-cauterization after leukocytes had already infiltrated the cornea. It is, hence, unlikely that methylprednisolone acetate exerted a direct inhibitory effect on endothelial cell proliferation.

The suppression of corneal vascularization by x-irradiation could not be attributed to any direct inhibitory effect on the endothelial cells of the limbal vascular plexus since vascular invasion, indistinguishable from that observed in normal cauterized corneas, followed corneal injury in those experiments where only the heads of the rats were irradiated with 1500 rads. Also, shielding of the heads with lead failed to prevent the vasoinhibitory effect of x-irradiation–induced leukopenia.

Whereas the lymphopenia caused by total body x-irradiation is a consequence of the direct destruction of lymphocytes in blood and lymphoid tissue,[3-5] the granulocytopenia is believed to be due to an impaired granulopoiesis.[6] The absence of leukocytes in the corneal stroma of those animals that received subconjunctival glucocorticoid immediately following cauterization is in conformity with the well-known antiinflammatory effects of glucocorticoids. Corticosteroids have been shown *in vivo* to inhibit the inflammatory leukocytic infiltration into different tissues including the cornea [7,8] and lung,[9] as well as the *in vitro* migration of polymorphonuclear leukocytes with various experimental models of chemotaxis.[10-12] Corticosteroids also exert a direct cytolytic effect on lymphocytes.[13,14]

Glucocorticoids inhibit corneal vascularization in a wide spectrum of clinical and experimental situations.[15-22] The exact mechanism by which they do this is not entirely clear. However, if leukocytes play a crucial role in corneal vascularization, as these and previous studies suggest,[1,2] then the effect of glucocorticoids on leukocytes can account for the well-established vasoinhibitory effect of steroids.

In the past, several other suggestions have been put forth to explain the corneal vasoinhibitory effect of glucocorticoids. Since these steroids induce vasoconstriction after their topical application, it has been postulated that they inhibit new vessel formation by decreasing the local circulation and, thus, the delivery of metabolites favorable for the proliferation of the endothelial cells of the limbal plexus.[23]

In 1940, Meyer and Chaffee [24] suggested that the avascularity of the normal cornea was due to the presence of mucopolysaccharides and that their enzymatic breakdown by hyaluronidase initiated a vascular ingrowth. For this reason it was once proposed that cortisone might inhibit hyaluronidase and, hence, its effect on corneal mucopolysaccharide degradation, so that vascular invasion would not ensue.[25] This view is, however,

no longer tenable for several reasons. Subsequent studies have failed to demonstrate the presence of hyaluronidase in homogenates of either normal or injured corneas.[26] Moreover, hyaluronidase exerts no action on keratosulfate, the major mucopolysaccharide component of the cornea.[27] Also, the ability of corneal mucopolysaccharides to inhibit vascularization when injected subconjunctivally has not been demonstrated.[28] Other evidence against the possible vasoinhibitory role of mucopolysaccharides is the demonstration by autoradiography that the quantity of labeled mucopolysaccharides is not reduced in the corneal regions with advancing blood vessels.[29]

Corneal vascularization is anteceded and accompanied by local edema,[21,30] which was at one time felt to be the stimulus for the vascular ingrowth.[30] As glucocorticoids inhibit the swelling of the cornea that precedes alloxan-induced corneal vascularization, Langham [21] suggested that the corneal vasoinhibitory effect of steroids resulted from their ability to decrease corneal edema. The role of the consistently observed edema in the pathogenesis of corneal vascularization is, however, questionable. Corneal edema and swelling is not necessarily followed by vascularization.[1,31-33] It seems probable that the edema which precedes the vascular ingrowth reflects mainly the increased vascular permeability component of the acute inflammatory response. That corticosteroids inhibit the corneal leukocytic infiltration (as demonstrated by these and other studies [7,8]) as well as corneal edema [21] conform to this interpretation.

If the factor or factors responsible for corneal vascularization arose in the cornea secondary to tissue necrosis or disorganization, as suggested in the past,[34,35] the vascular invasion of the cornea would be expected to occur independent of the presence or absence of leukocytes. However, the destruction of corneal tissue by silver nitrate cauterization alone was clearly not a sufficient stimulus to provoke vascularization directly. Vascularization in these studies did not occur unless it was first preceded by a leukocytic infiltration. In the present investigation, as well as in past studies,[1] corneal injury alone failed to provoke corneal vascularization.

Numerous observations related to the pathogenesis of corneal vascularization suggest that one or more diffusable factors initiate directional capillary growth in the cornea.[1,22,34,36,37] The observations reported here are consistent with a leukocytic origin of the factor or factors. Both polymorphonuclear leukocytes and lymphoid cells normally invade the cornea following silver nitrate cauterization. As both cell types were eliminated from the cornea by total body x-irradiation or the subconjunctival instillation of methylprednisolone, it is not possible to implicate a particular leukocyte as playing the vasostimulatory role at the present time. Aside from the present study there is evidence that certain cells are capable of stimulating vascularization in other biologic situations, most notably in

embryonic and neoplastic tissue. With neoplasms, diffusible vasostimulatory factors have been isolated from cellular constituents and partially characterized.[38-41]

Since corneal vascularization is a component of the inflammatory response, observations related to the role of leukocytes in wound healing deserve comment In a study of the healing of incised cutaneous wounds in guinea pigs made neutropenic by antineutrophil serum, Simpson and Ross [42] found that the healing process and the formation of granulation tissue, in this normally vascularized tissue, ensued in a manner similar to that in normal animals. On the other hand, subsequent studies with hydrocortisone and antimacrophage serum by Leibovich and Ross [43] indicated that macrophages not only played a role in wound debridement, but seemed to be necessary to stimulate fibroblast proliferation.

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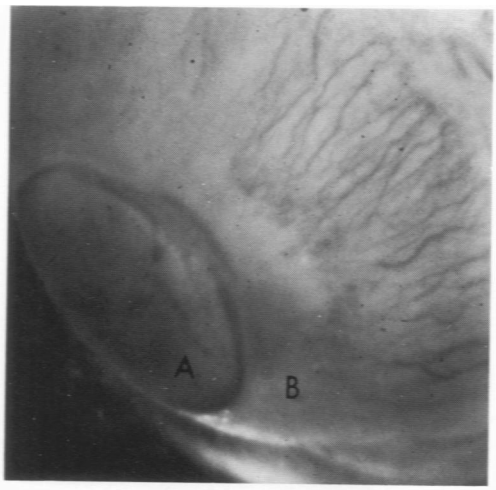
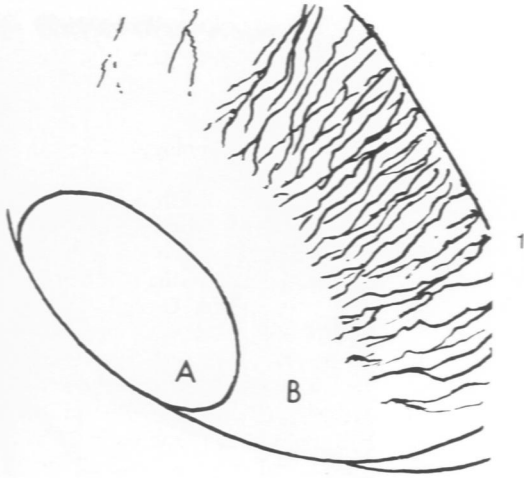
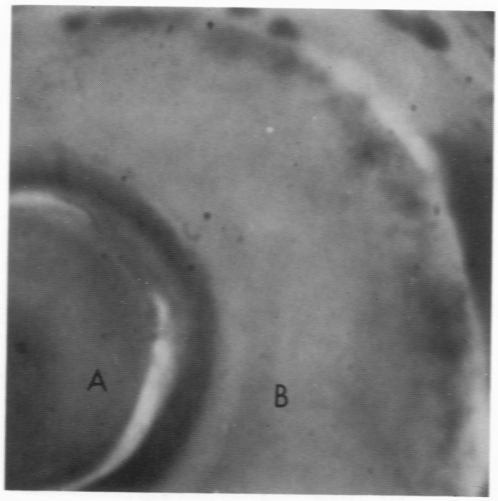
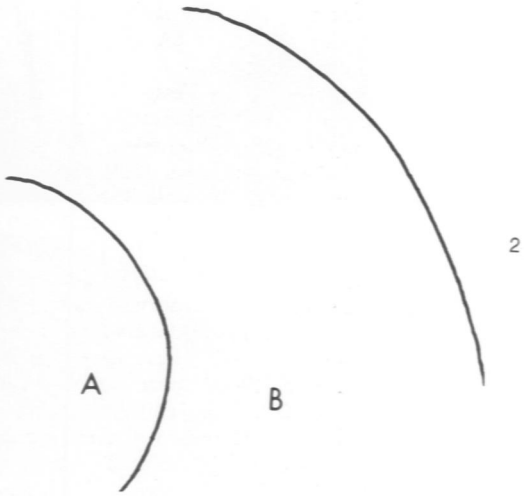
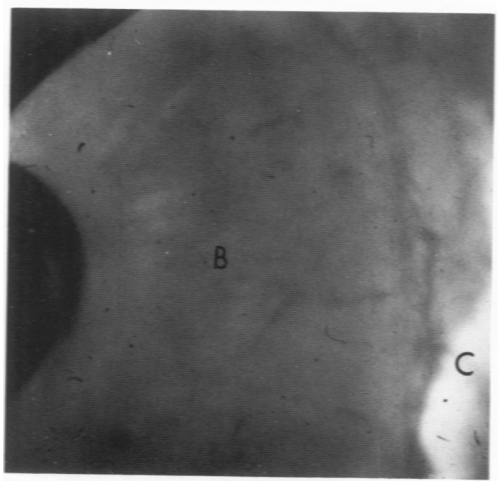
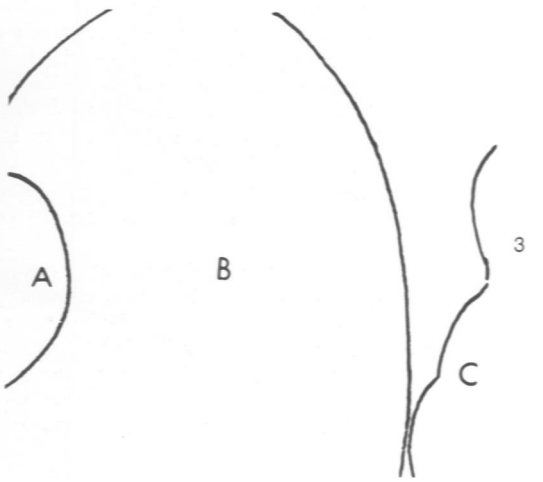




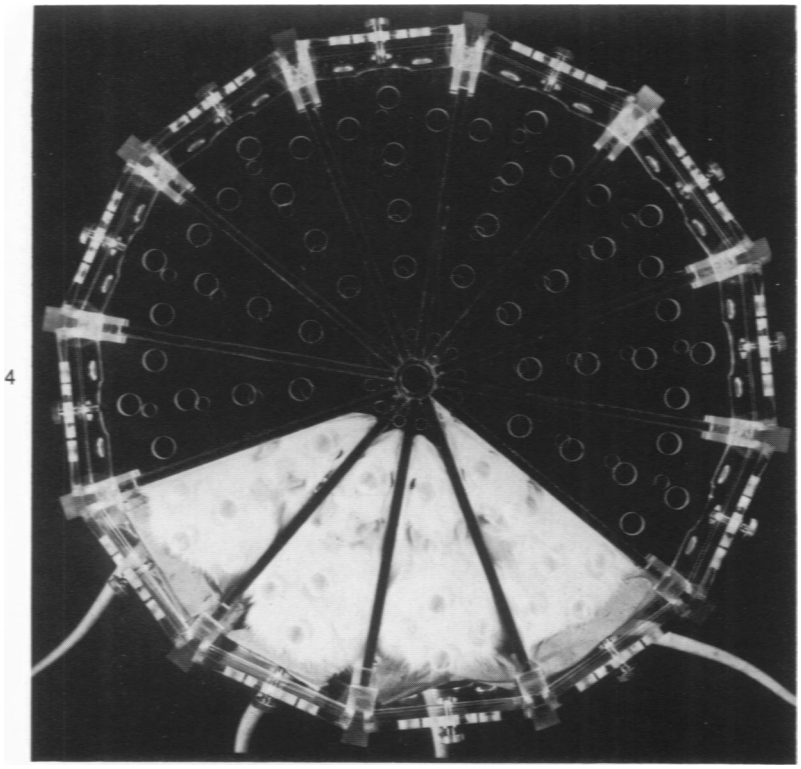








Figures 1-3—Corneas of rats that were cauterized with silver nitrate. The sites of cauterization in the central cornea appear dark (*A*). **1**—In the normal rat, blood vessels extend from the corneoscleral limbus into the cornea (*B*) **2**—In a rat made leukopenic by 1500 rads total body x-irradiation, blood vessels do not extend into the cornea. **3**—The cornea also appears avascular if methylprednisolone acetate (*C*) is injected subconjunctivally immediately after cauterization.



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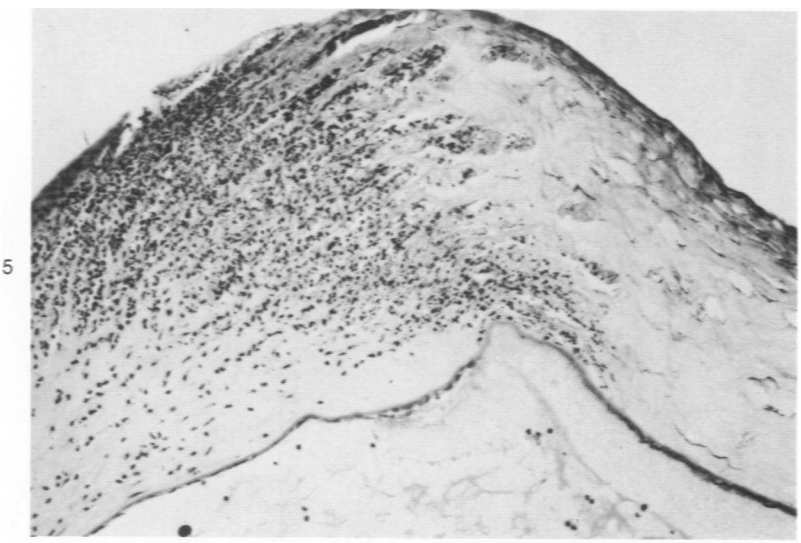

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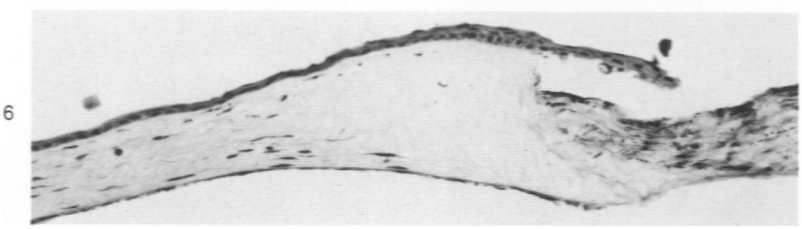

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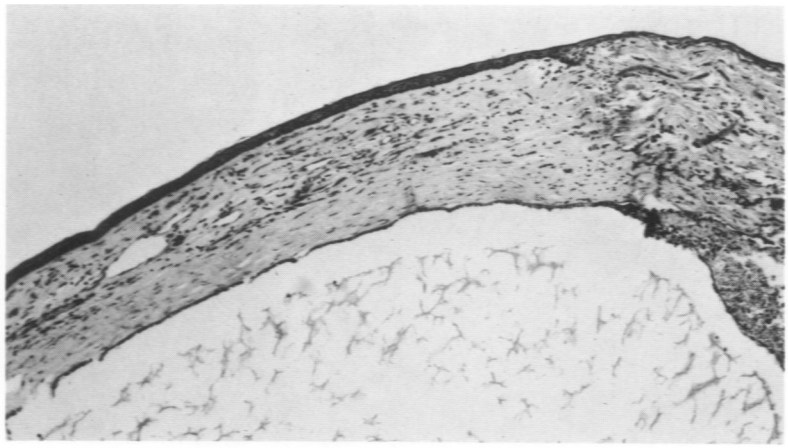
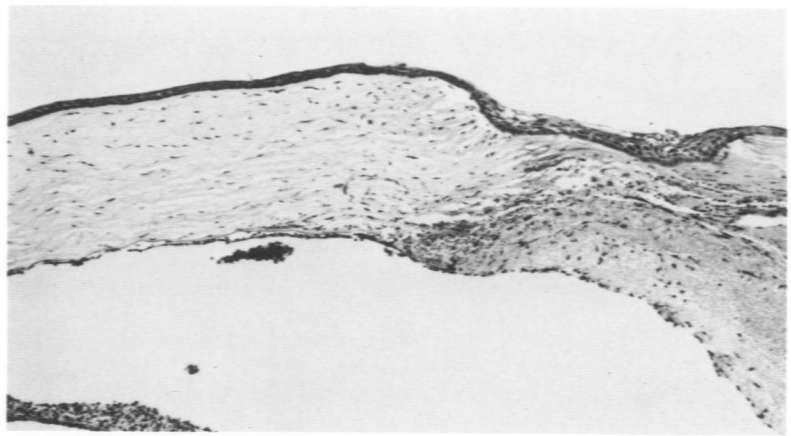
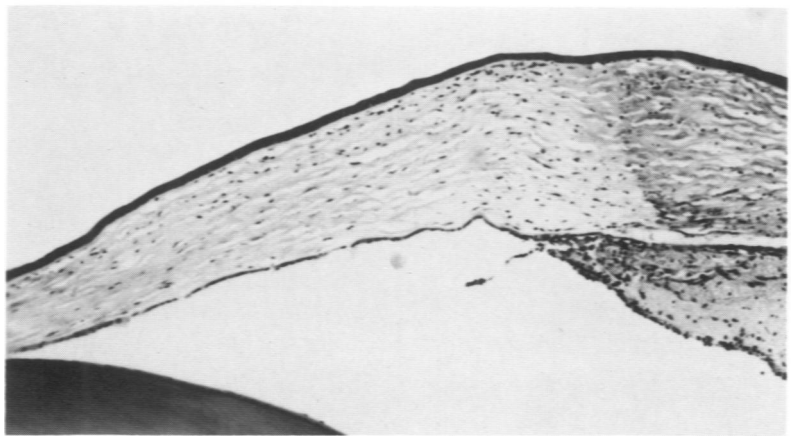
Figure 4—Circular Lucite holder employed in x-irradiation experiments. Rats were immobilized in the triangular compartments with their heads positioned in the central portion of the holder. **Figures 5 and 6**—Photomicrographs of the sites of silver nitrate cauterization from a normal rat (**5**) and a rat made leukopenic by 1500 rads of total body x-irradiation (**6**). The normal rat displays a dense infiltration of leukocytes in the edematous corneal stroma which is absent in the leukopenic rat. (H&E, × 130)



7

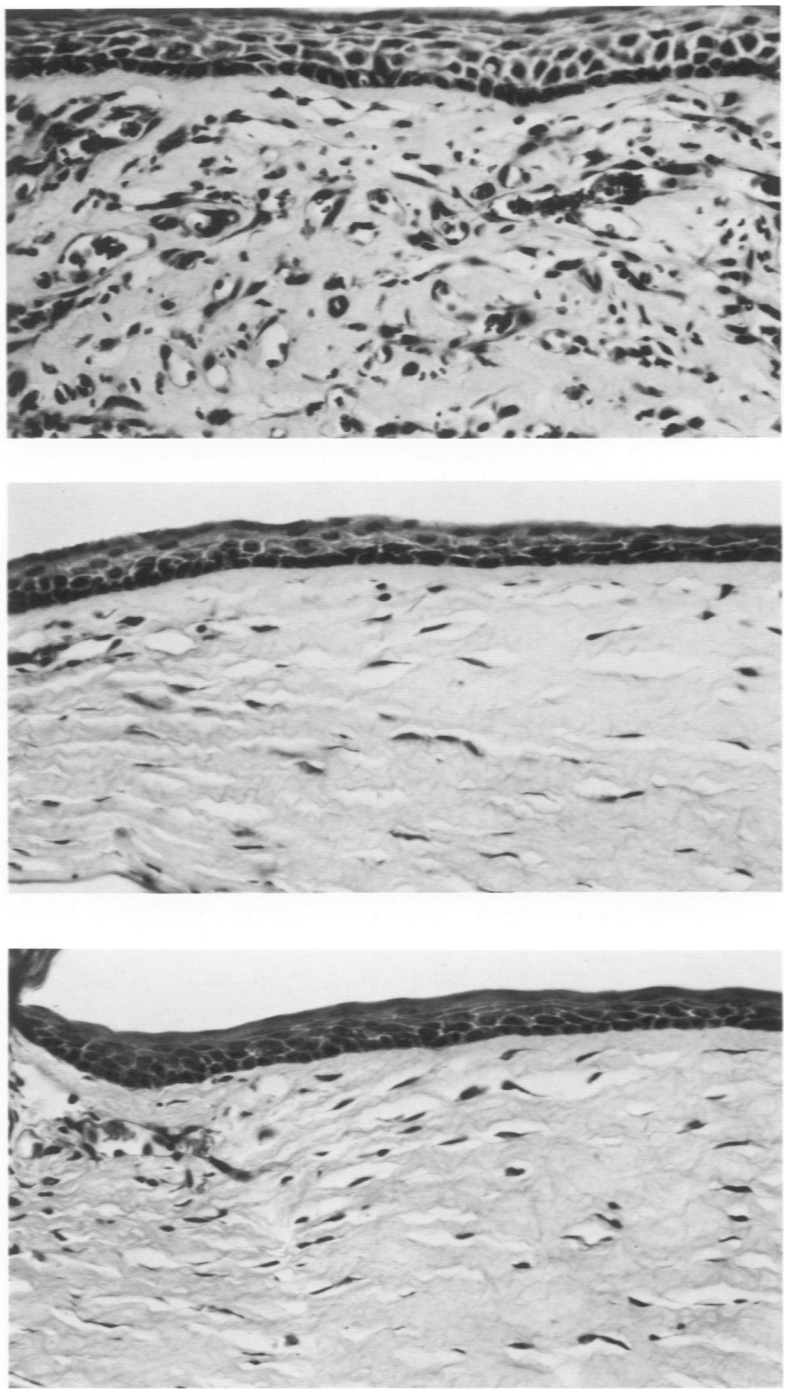


8



9

Figures 7–9—Silver nitrate–cauterized corneas of normal rats (**7**), rats given 1500 rads total body x-irradiat (**8**), and glucocorticoid-treated rats (**9**). The photomicrographs show the sites of silver nitrate cauterization on the right. A leukocytic and vascular invasion are present in the cornea of the normal rat but absent in both the x-irradiated and glucocorticoid-treated corneas. (H&E, × 68)



Figures 10–12—The peripheral cornea 4 days after silver nitrate cauterization in a normal rat (**10**) as well as in a rat made leukopenic by 1500 rads total body x-irradiation (**11**), and in one that received subconjunctival methylprednisolone immediately after cauterization (**12**). The normal rat displays vascular ingrowth unlike the x-irradiated or glucocorticoid-treated rats, which only possess the normal peripheral blood vessels. (H&E × 250)