IMMUNOHISTOLOGIC STUDIES ON ANTIGEN-ANTIBODY REACTIONS IN THE AVASCULAR CORNEA*

I. Reactions in Rabbits Actively Sensitized to Foreign Protein‡

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Plates 90 and 91

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It has been generally accepted that the local tissue reaction of anaphylactic hypersensitivity (Arthus reaction) is due to primary damage to blood vessels which serve as the site of antigen-antibody interaction (1–3). This concept has been further refined by the assumption that the precise site of damage is the vascular endothelium. Rich and Follis (4) injected horse serum into the avascular cornea of rabbits previously sensitized by repeated intracutaneous injections of horse serum. They compared the reaction to that occurring in similarly sensitized rabbits in whose corneas vascularization had previously been induced by the local injection of heat-killed tubercle bacilli. The local injection of horse serum in the vascularized corneas evoked severe acute inflammation and hemorrhage in contrast to much milder inflammation without hemorrhage when horse serum was injected into the avascular cornea. It was concluded from this study that blood vessels were necessary for the production of the local tissue reaction of anaphylactic hypersensitivity and it was suggested that the primary site of damage was the vascular endothelium.

Subsequent studies by Rich, Voisin, and Bang (5) indicated that when the reaction of local tissue hypersensitivity was produced in the skin, in addition to vascular damage characterized by thrombosis and hemorrhage there occurred an alteration in the collagen fibrils at the immediate site. The change in collagen fibrils was characterized by swelling as well as by loss of their normal periodicity. Following similar studies, but with the electron microscope Wolpers (6) concluded that there was no essential damage to the collagen fibrils. Their "fibrinoid" appearance was believed

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to be due to surface adsorption of fibrin and "altered ground substance." He suggested that the major disturbance in the connective tissue resided in an alteration of the ground substance in which the fibrils were embedded.

It is apparent that local hypersensitivity reactions involve damage to the connective tissue. However, the precise nature of the injury, whether these changes in the connective tissue are secondary to endothelial damage, whether antigen-antibody interaction affect both the endothelium of blood vessels and connective tissue simultaneously or whether they represent primary injury to the connective tissue is not known. If the primary injury is to the connective tissue, the damage to the endothelium might be due to interruption of the supporting structures of the blood vessels, leading to lysis of the vessel wall. Such a concept is not without precedent; in scurvy, it has been demonstrated that the loss of integrity of the connective tissue leads to capillary hemorrhage.

If it could be shown that tissue damage could be produced by local antigen-antibody interaction in a milieu consisting of pure connective tissue free of blood vessels, the question of the relative role of endothelium and connective tissue damage might be resolved. The normal cornea consists essentially of lamellae of connective tissue comprising four basic elements; fibroblasts, collagen fibrils, reticular fibrils, and amorphous "ground substance," relatively far removed from the blood supply. It was thought, therefore, that an investigation of antigen-antibody interaction in the avascular cornea might produce useful data and provide an experimental tool for characterizing the nature of tissue damage due to hypersensitivity in more basic terms than the traditional histologic approach.

In the present studies, it was found that marked damage to the connective tissue of the avascular cornea could be produced by the intracorneal injection of homologous antigen into rabbits actively sensitized to either bovine serum albumin or bovine gamma globulin.

Materials and Methods

White male rabbits weighing approximately 2 kg were injected subcutaneously weekly, for periods up to 3 months, with either crystalline bovine serum albumin (BSA) or bovine gamma globulin (BGG) incorporated in Freund's adjuvant at a concentration of 20 mg of the respective antigen per ml (7). Animals sensitized by this procedure were injected intracorneally with fluorescein-labeled BSA or BGG. Care was taken to deposit the antigen in the center of the cornea between the connective tissue lamellae. The antigens were labeled by the method of Coons and Kaplan (8), concentrated by lyophylization, and made isotonic by dialysis against phosphate buffered (pH 7.4) 0.15 M saline. The amount of antigen protein in the test injections ranged from 0.05 to 8.0 mg and was usually contained in a volume of 0.05 ml. The corneas were observed, from the time of injection to the time of enucleation, by slit lamp examination and by direct examination with both white and ultraviolet light. The rabbits were sacrificed at varying intervals; the eyes were removed and strips of cornea with adjacent sclera were either formalinized or preserved by rapid freezing at −70°C for future sectioning and study by fluorescence and routine microscopy (9). Preparations were studied first by fluorescence micros-

copy and then stained with hematoxylin and eosin for routine examination. Blood samples were taken by cardiac puncture at the time of sacrifice in order to determine the approximate antibody (Ab) levels (10). With each experiment, several normal rabbits were given an intracorneal injection of the test fluor-antigen. These were examined in the same manner as the sensitized animals.

EXPERIMENTAL RESULTS

I. The Effect of Intracorneal Injection of Fluor-BSA

In the first experiment, 1 mg fluor-BSA was injected into the center of the corneas of 28 sensitized and 9 normal rabbits. This quantity of antigen is capable of producing a marked Arthus reaction in the skin (10). The serum of all of the sensitized rabbits had anti-BSA N in excess of 500 micrograms. The eyes were examined by white and ultraviolet light at 6, 12, 18, and 24 hours and daily thereafter. The rabbits were sacrificed at intervals from 6 hours to 28 days.

A. Sensitized Animals.—During the first several hours after injection, observation with ultraviolet illumination revealed a gradual diffusion of the fluor-antigen from the site of injection toward the limbus. At 6 hours, the cloudiness due to the injection procedure as seen by ordinary light had resolved and the cornea was again clear. As early as 12 hours, a hairline partial or complete ring of opacification, appearing gray under white light, was present in the periphery of the cornea. A clear zone of cornea varying in width from 1 to 3 mm separated the opaque ring from the limbus. At this early stage, the ring was in some cases incomplete and presented merely as a 20° arc. In other cases the ring presented as a complete circle central to the limbus. The position of the arc in the cornea appeared to be related to the area of most intense dilatation of the conjunctival and limbal vascular loops. The arcs or rings observed as lines of gray opacification of the cornea under white light appeared as lines of green fluorescence with ultraviolet light. With the appearance of complete rings, the cornea had the following gross appearance under ultraviolet light: A zone of green fluorescence at the site of injection occupying approximately half the diameter of the cornea, falling off gradually and merging with a zone of weakly or non-fluorescent cornea. External to this zone, the fluorescence became sharply intensified and reached a maximum in the ring of opacification beyond which the narrow zone of clear cornea presented no trace of fluorescence. By 24 to 30 hours, partial or complete rings were evident in 25 of 31 (80 per cent) eyes observed (Figs. 1 and 2).

Acute inflammation of the conjunctiva, characterized by dilatation of blood vessels, edema, focal scleral hemorrhages, and hyperemia of the limbal vascular loops, was present in all animals with high levels of circulating antibody. When limbal inflammation was prominent, the cornea usually exhibited a generalized hazy appearance. Similar injections of antigen in each eye of the same animal did not always result in the formation of a ring in both eyes. In an occasional animal, one eye might show the development of a complete opaque fluorescent

ring while the other eye might show conjunctival and limbal inflammation with or without a partial ring.

After 48 hours, the sharp ring of fluorescence did not migrate outward or broaden appreciably. However, the essentially clear zone between the ring and the limbus became opaque, resulting in a non-fluorescent zone of opacity between the original ring and the limbus. At 4 days, the periphery of the cornea presented ingrown engorged blood vessels readily apparent with the naked eye. On slit lamp examination these were observed to be within the substance of the cornea. At this time the entire cornea had a ground glass appearance.

Between 5 and 13 days, the further course of the reaction in the cornea was variable. In some eyes, the interstitial newly formed blood vessels became less prominent, the cornea clarified, and the ring of fluorescence while remaining stationary showed a progressing diminution of intensity. In others, the ingrowth of corneal blood vessels was progressive and corneal cloudiness persisted up to 2 to 3 weeks (Fig. 3). At the end of 4 weeks, the corneas of all rabbits were normal. The original opacifications and cloudiness had disappeared and the newly formed blood vessels had blanched leaving no sign of corneal damage.

The sequential changes noted by gross observation of the corneas were studied by routine and fluorescence microscopy. By 6 hours the vascular loops of the limbus were surrounded by a mild polymorphonuclear leucocytic infiltrate. Other than a mild structural disruption of the cornea at the site of injection, no changes were observed. The first corneal alterations were seen at 12 hours and were fully developed by 30 hours. The ring of opacification and fluorescence noted in the gross was readily recognized in hematoxylin and eosin-stained sections as a sharp eosinophilic line traversing the cornea from Bowman's to Descemet's membrane (Fig. 4). Frequently the line was incomplete toward the deeper layers of the cornea. The configuration of the line was often such that it was wider toward Bowman's membrane than at the inward extremity. The side of the line nearest the limbus was often serrated. It was invariably accompanied by a moderate infiltration of polymorphonuclear leucocytes which abutted against the line on its limbal side. The leucocytes were concentrated at this point and at the limbus (Fig. 5). It was not possible to distinguish exudate cells from degenerated corneal fibroblasts within the line. The corneal collagen lamellae as they traversed the line appeared to lose their fibrillar structure and became swollen and more eosinophilic. At this time the central area of the cornea and the zone between the line and the limbus were essentially free of inflammatory cells. The only disturbance apparent to the corneal stroma in these areas was a slight separation of the collagen fibers.

Fluorescence preparations of the corneas showed marked fixation of antigen at the eosinophilic line. In these preparations, the greenish fluorescence noted at the site of injection diminished as one moved away from the center of the cornea and then abruptly increased in intensity as one approached the eosino-

philic line, reaching a maximum intensity in the line itself (Fig. 6). The cornea on the limbal side of the line was completely lacking in green fluorescence.

After 48 hours, there was an intensification of the changes just described so that an acute cellular exudate now occupied the major portion of the cornea between the line and the limbus. This microscopic change corresponded with the non-fluorescent opacification between the fluorescent ring and the limbus which had been noted in the gross.

At this time, the eosinophilic line was difficult to distinguish because of the massive infiltration of cells, many of which appeared necrotic and unidentifiable. The line, however, was still recognizable as fragments of deeply eosinophilic swollen masses among the contrasting nuclei. These masses consisted of clumps of amorphous precipitate and fragmented collagen fibers. The line remained intensely fluorescent and the polymorphonuclear leucocytes which had migrated into the line contained clumps of phagocytized fluorescent particles (Fig. 7). The limbus showed dilated vessels, focal hemorrhages, and a rich cellular exudate which now consisted not only of polymorphonuclear leucocytes, but also cells of the lymphoid series.

By the 4th day, plasma cells began to appear at the limbus and thereafter the exudate at the limbus and adjacent cornea changed from one predominating in neutrophiles and lymphoid cells to one consisting almost wholly of plasma cells. The latter accompanied the newly formed capillaries into the cornea. The corneas exhibiting a marked ground glass appearance on gross examination showed extensive edema microscopically as evidenced by considerable separation of the stromal elements. In almost all of these eyes there was gross or microscopic evidence of iritis which probably led to increased anterior chamber pressure. At 10 days the eosinophilic, fluorescent line was no longer evident. The disappearance of the line was due at least in part to removal of the precipitated material by phagocytosis. After 4 weeks, when the eyes were normal in the gross, small, collapsed capillaries surrounded by a few plasma cells were still present in the cornea.

B. Control Animals.—Non-sensitized animals receiving 1 mg of fluor-BSA intracorneally were sacrificed at intervals from 6 hours to 28 days. No gross or microscopic alterations of the cornea were observed during the first 10 days, during which time the reactions in the sensitized animals were at their height. After this time, several of the animals developed the Wessely phenomenon, presumably as a result of sensitization to the intracorneally injected antigen (11).

II. The Influence of Antigen Dosage and Antibody Levels on the Production of the Corneal Reaction

Twelve animals sensitized to BSA were divided equally into two groups. One group received 5 mg of fluor-BSA in the left cornea and 0.5 mg in the right

cornea. The other group received 1 and 0.1 mg fluor-BSA in the left and right corneas respectively. Opaque, fluorescent corneal rings were observed with equal frequency (83, 67, and 83 per cent) in all highly sensitized animals injected with 5, 1, and 0.5 mg of antigen. In similar animals injected with 0.1 mg of antigen no opacifications were seen during the 30 hour period of observation.

The incidence of rings was the same in animals with antibody nitrogen levels between 0.26 and 1.35 mg per ml following injection with at least 0.5 mg antigen. In one animal with a trace of circulating antibody and receiving 0.5 mg of antigen, no ring was observed but striking limbal inflammation was seen on microscopic examination.

III. The Effect of the Intracorneal Injection of Fluor-BGG into BGG Sensitized Rabbits

A total of 19 corneas of BGG-sensitized animals (AbN per ml varying from 0.9 to 1.5 mg) were injected with amounts of fluor-BGG varying from 5 to 0.1 mg. Rings similar to those seen in the BSA group of animals were observed in 9 out of 14 (64 per cent) corneas of the highly sensitized animals receiving 0.5, 1, or 5 mg fluor-BGG. In contrast to the occurrence of a visible ring as early as 12 hours in BSA-sensitized animals, rings were not observed prior to 30 hours after injection of BGG. Furthermore, the location of the rings in the BGG sensitized animals was at a greater distance from the limbus. These differences probably resulted from a slower rate of diffusion of fluor-BGG.

IV. The Specificity of the Corneal Reaction

The specificity of the reaction was demonstrated by the injection of 1 mg fluor-BSA into the left corneas and 1 mg fluor-BGG into the opposing corneas of 8 rabbits, 4 of which had been previously sensitized to BSA and 4 to BGG. Observation of the corneas of these animals by ultraviolet and white light at 24 and 48 hours revealed the presence of fluorescent, opaque rings only in those corneas injected with the homologous antigen.

V. Structural Changes in the Cornea Following Intracorneal Injection of Fluor-BSA into Actively Sensitized Animals Made Leucopenic by Treatment with Nitrogen Mustard

In order to study the nature of the corneal reaction in the relative absence of leucocytes, fluor-BSA was injected into the corneas of sensitized animals made leucopenic by nitrogen mustard. "Mustargen" (Merck, Sharpe, and Dohme) was administered as follows: 5 mg on the first day; 0.3 mg on day 3; 1 mg on day 4; 3 mg on day 5; and 5 mg on day 8. Leucocyte counts ranging from 350 to 2,100 were achieved on the 9th day at which time intracorneal injections of 1 mg of fluor-BSA were made in each cornea. The animals were sacrificed 24 or 48 hours later. Antibody levels (12) were determined on serum obtained at the time of sacrifice. These were found to be of the same order of magnitude as in untreated sensitized controls (AbN per ml ranging from 0.2 to 1.3 mg). The corneas of the 4 sensitized animals not receiving

nitrogen mustard showed gross rings of opacification 24 hours after injection of fluor-BSA. These corneas presented the same alterations as noted in the previous experiments.

Corneal rings of opacification were seen in only 3 of 20 eyes of leucopenic animals. These rings were located closer to the limbus than those that had occurred in untreated animals. Under routine and ultraviolet microscopy the rings of opacification presented as deeply eosinophilic and fluorescent lines or bands almost devoid of leucocytes. Disorganization of the stromal elements and fragmentation of the collagenous lamellae were clearly seen at the zone of antigen precipitation. This damage was easily noted because of the paucity of inflammatory cells (Fig. 8).

Although discrete lines of antigen precipitation were seen within the corneas of only 3 eyes, several corneas showed a definite limitation of the spread of antigen as evidenced by the presence of a broad, sharply demarcated zone of fluorescence. No alterations were noted in the gross in these corneas presumably owing to the small amount of antigen precipitated and the scarcity of cells.

The leucopenic animals did not show the striking conjunctival hyperemia, focal hemorrhage, and edema which occurred in the untreated animals. On microscopic examination, the vascular loops of the limbus appeared less engorged than those of the controls. Only a few inflammatory cells were present, and these were predominately lymphocytes. In some of the animals fluorescence microscopy revealed that the line of antigen precipitation had occurred at the corneoscleral junction. At this location the line was often associated with swelling and necrosis of the limbal vessels and focal hemorrhages (Fig. 9). Ultraviolet microscopy revealed the precipitation of fluorescent antigen within the walls of the affected limbal blood vessels and in the surrounding connective tissue (Fig. 10).

DISCUSSION

The present studies indicate that marked inflammation of the avascular cornea can result from the intracorneal injection of antigen into actively sensitized animals. The inflammatory reaction occurred only in the corneas in which there was sharp localization of the fluor antigen. This localization of antigen was the result of its precipitation with antibody diffusing into the cornea from the limbal blood vessels.

The sequence of events suggested by these observations appears to be as follows; when traces of antigen reach the limbus in a sensitized animal, an allergic inflammatory reaction is provoked leading to dilatation of the capillary loops and the exudation of leucocytes and serum protein. This initial mild inflammatory reaction is followed by a diffusion of antibody into the cornea. This results in the meeting of two diffusing fronts of reactive protein within the cornea and well beyond the limbus. It is presumed that antigen-antibody complexes are formed in the region of the fronts and that when a critical con-

centration of antigen and antibody is reached, precipitation occurs. The fluorescent studies would justify this interpretation. The advancing front of antigen did not always result in a zone of precipitation, although delimitation of the antigen in such cases was found to be quite sharp. Presumably in this instance the concentration of antibody diffusing from the limbus was sufficient to impede the migration of antigen by the formation of soluble complexes but insufficient to produce marked precipitation of antigen. In the control animals, limbal inflammation was insignificant and sharp lines of antigen demarcation were never encountered.

The line of maximal fluorescence is equated with the manifestly visible arc or circle of opacification observed in the cornea central to the limbus in the sensitized animals. The histologic alteration in the cornea was limited to the line of antigen-antibody precipitation and was characterized by heavy infiltration of polymorphonuclear leucocytes closely applied to the line of precipitation. The precise nature of the injury by which antigen-antibody interaction produces inflammation of the corneal stroma is not clear. The collagen fibers that transversed the line of antigen-antibody precipitation appeared swollen, fragmented, and deeply eosinophilic. This was particularly apparent in the leucopenic animals where the change was almost identical with that which has been called "fibrinoid" degeneration. Whether the abnormal appearance of the collagen fibers was solely due to the inclusion of antigen-antibody precipitates could not be definitely established.

Recent work by Cochrane, Dixon, and Weigle (13) indicated that the Arthus response was inhibited in animals made leucopenic by nitrogen mustard. In the present experiments, it was apparent that the appearance of corneal alteration was not dependent on the activity of leucocytes since the basic change was observed in leucopenic animals in which rare polymorphonuclear leucocytes were found at the site of antigen-antibody interaction. It is of interest that marked fibrinoid degeneration of the connective tissues can be seen both in experimental serum sickness and in human periarteritis nodosa in the absence of a cellular inflammatory response.

It is difficult to compare the corneal lesion with lesions of experimental serum sickness, since the pathogenesis of both is poorly understood. Studies by other investigators (14) have demonstrated that large amounts of antigen and gamma globulin (presumably antibody) can be visualized in the lesions of experimental hypersensitivity. From a comparison with these studies, the amount of antigen and antibody required to produce the corneal lesion would not appear to be vastly different.

These experiments would indicate that tissue damage resulting from antigen-antibody interaction can occur in the avascular cornea. However, the same reaction induced in a previously vascularized cornea would appear as an Arthus response. In the experiments of Rich and Follis in which the cornea was

altered so that in effect it acquired a structure similar to that of the skin, it is not surprising that extensive local hemorrhage and thrombosis occurred. The present experiments indicate that since immediate-type hypersensitivity can induce corneal inflammation, the use of the cornea as a site of reaction for the differentiation of immediate and delayed hypersensitivity reactions may be invalid (15).

SUMMARY

The injection of antigen into the center of the avascular cornea of homologously sensitized animals induced a ring of opacification between the center of the cornea and the limbus. This ring of opacification was composed of a line of deeply eosinophilic amorphous material in a matrix of swollen collagen fibers, palisaded by polymorphonuclear leucocytes. By use of fluor-tagged antigen, it was shown that the line of damage in the cornea coincided with the precipitation of antigen presumably by antibody entering the cornea from the limbal vessels. With the passage of time, the antigen-antibody precipitates were removed, at least in part by phagocytosis, and the ring of opacification was replaced by ingrowing blood vessels surrounded by plasma cells.

Treatment of sensitized animals with nitrogen mustard showed that antigen-antibody interaction could injure the corneal stroma in the relative absence of polymorphonuclear leucocytes.

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EXPLANATION OF PLATES

PLATE 90

FIG. 1. Eye of normal albino rabbit injected intracorneally 24 hours previously with 1 mg fluor-BSA in 0.05 ml saline. × 1.5.

FIG. 2. Eye of BSA-sensitized rabbit 24 hours following intracorneal injection of 1 mg fluor-BSA. Note opaque ring in periphery of cornea with clear area between ring and limbus. There is engorgement of limbal and bulbar vessels with petechial hemorrhages. × 1.5.

FIG. 3. Eye of BSA-sensitized rabbit 13 days after intracorneal injection of 1 mg fluor-BSA. Note marked vascularization of cornea at site of previous opaque ring. Central portion of cornea is edematous as indicated by "ground glass" appearance. × 1.5.

FIG. 4. Transverse section of cornea and adjacent limbus of BSA-sensitized rabbit 30 hours following intracorneal injection of 1 mg fluor-BSA. Note eosinophilic line infiltrated by polymorphonuclear leucocytes in cornea distant to limbus, corresponding to section of opaque ring. There is acute inflammation at limbus. Hematoxylin and eosin. × 12.

FIG. 5. Higher magnification, illustrating the palisading by polymorphonuclear leucocytes about the eosinophilic line on side nearest the limbus. Hematoxylin and eosin. × 80.

FIG. 6. Fluorescent preparation of lesion of Fig. 5. Note precipitation of fluor-BSA diffusing from center of cornea. × 65.

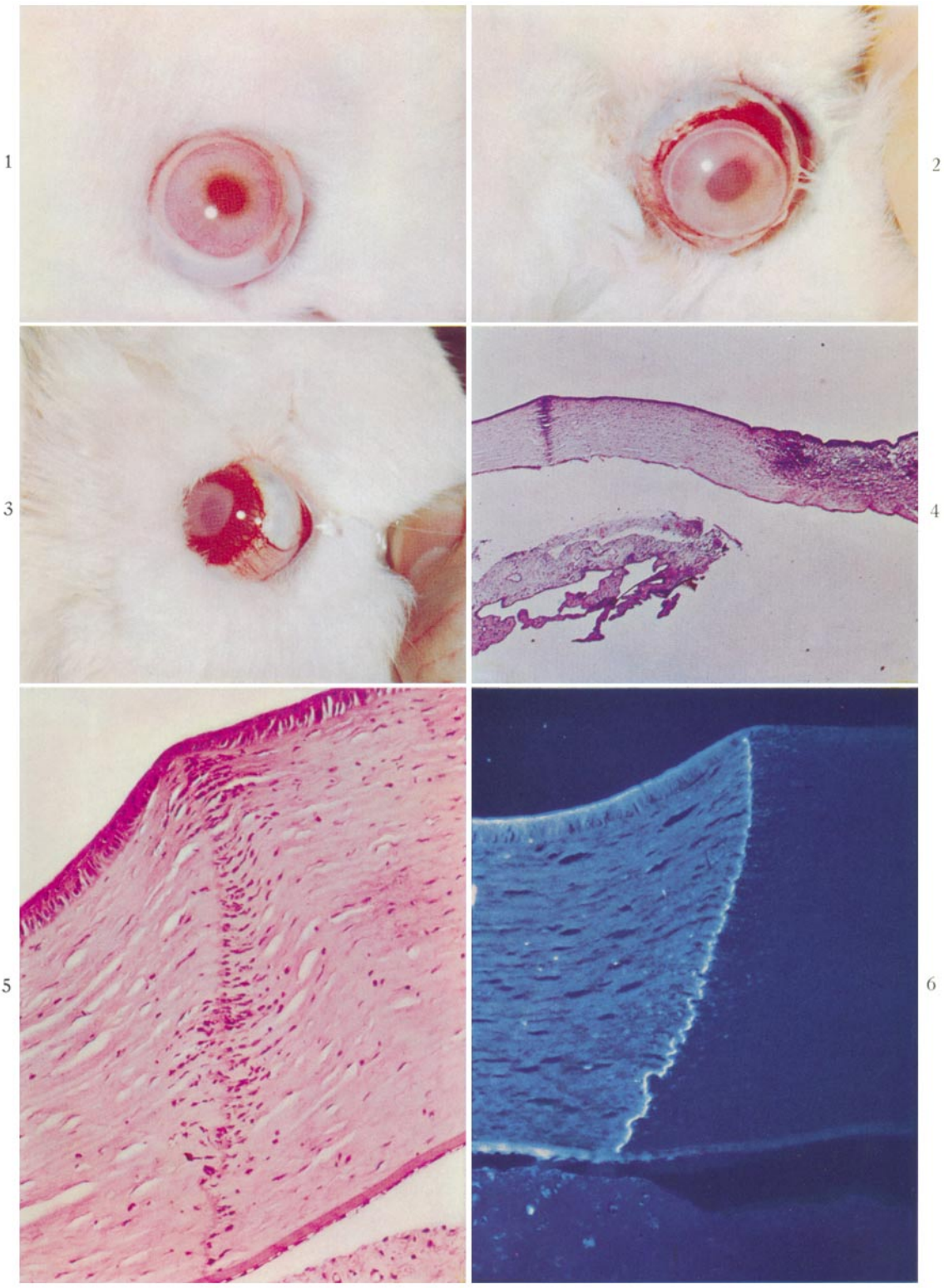

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PLATE 91

FIG. 7. Fluorescent preparation of corneal line (72 hours) showing phagocytosis of fluorescent precipitates. × 90.

FIG. 8. Line of collagen degeneration and slight mononuclear infiltration at site of antigen-antibody interaction in the cornea of a BSA-sensitized animal made leucopenic by nitrogen mustard; 48 hours after injection with fluor-BSA. Hematoxylin and eosin. × 300.

FIG. 9. Miniature Arthus reaction at the limbus, 48 hours after intracorneal injection of 1 mg fluor-BSA in rabbit sensitized to BSA and made leucopenic by nitrogen mustard. Several of the small limbal vessels are thrombosed and there is slight perivascular mononuclear infiltration. Hematoxylin and eosin. × 100.

FIG. 10. Fluorescent preparation of lesion of Fig. 9. Note line of precipitation of fluor-BSA at limbus with concentration of this antigen within and about the walls of blood vessels. × 45.

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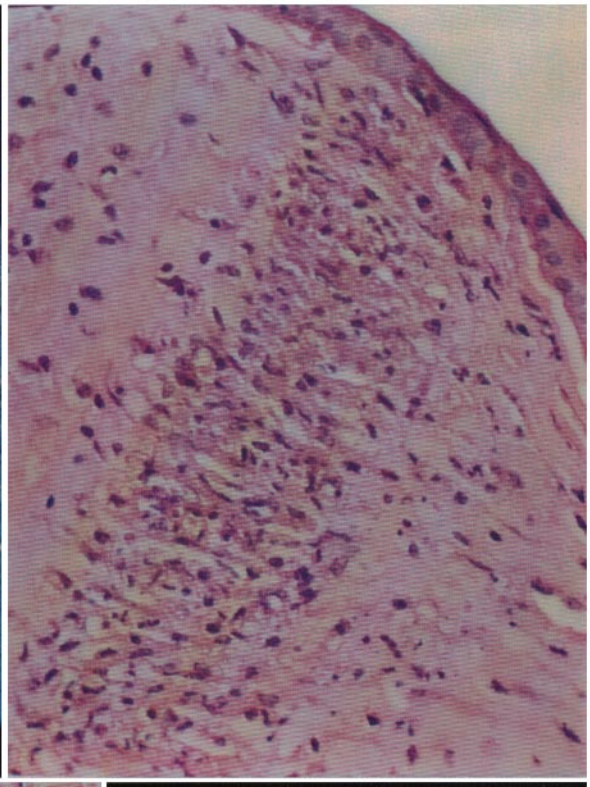

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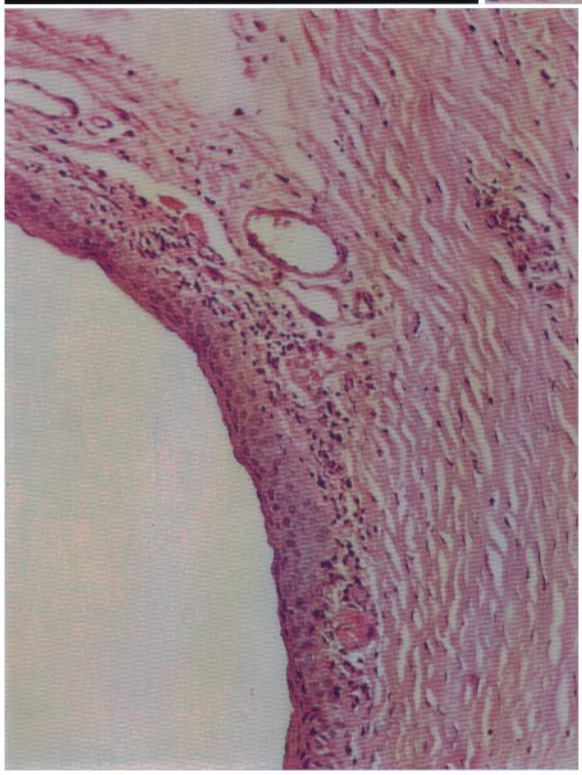

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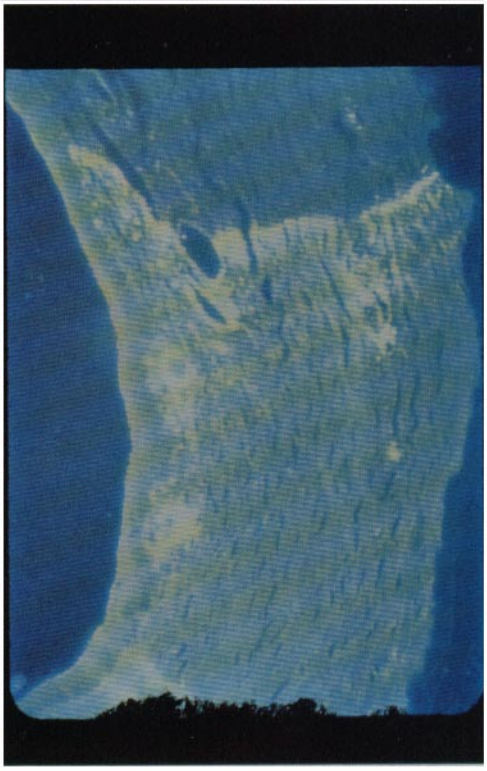

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