MORPHOLOGICAL AND FUNCTIONAL CHANGES IN CORNEAL ENDOTHELIUM CAUSED BY THE TOXIC EFFECTS OF INFLUENZA AND NEWCASTLE DISEASE VIRUSES*

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VIRUS-INDUCED toxicity has been defined as " the production of lesions in the absence of multiplication adequate to account for them " (Burnet, 1955). During the past 15 or 20 years this term has come to be associated specifically with a group of more or less related pathological states experimentally induced by the inoculation of high concentrations of a suitable virus into animals. The observed lesions commonly include haemorrhage, vascular congestion and oedema singly or in combination depending upon the route of injection of the virus (Rake and Jones, 1944 ; Henle and Henle, 1946 ; Ginsberg, 1951). Such observations suggest that alterations in the capillary endothelium may present a factor of general significance in virus-induced toxicity. The possibility that this tissue represents the primary " target " of the virus remains to be tested.

The introduction of large amounts of certain viral agents into the anterior chamber of the rabbit eye elicits the development of corneal opacity without a concomitant rise in virus titre (Evans and Rickard, 1945 ; Evans and Bolin, 1946 ; Bolin, Anderson, and Leymaster, 1950). It seems likely that this opacity is the result of a movement of fluids into the corneal stroma in response to an alteration in the permeability of the endothelial lining of the anterior chamber. The unique architecture of the anterior chamber, around which a single layer of endothelial cells forms an almost continuous membrane, provides a favourable situation for observing the effect of toxic concentrations of virus upon these cells.

It is the purpose of this paper to present evidence which indicates that certain viruses are indeed capable of reacting directly with corneal endothelium in such a fashion as to destroy the physiological and morphological integrity of this membrane.

MATERIALS AND METHODS

Virus preparations

The Beaudette strain of Newcastle disease virus (NDV) obtained from Dr. Seymour Levine and Influenza A virus (PR-8 strain) obtained from the American Type Culture Collection were employed. Undiluted allantoic fluid suspensions were used to elicit the toxic reactions.

Tests of endothelial permeability

The rabbits used in these studies were injected with 0·2 ml. of NDV into the right eye and an equal amount of normal allantoic fluid into the opposite eye. Eighteen to 24 hr.

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later a unilateral toxic reaction was well developed. At this time ^{131}I-labelled human albumin obtained from Abbott Laboratories, Chicago, Illinois was injected either intravenously or directly into the anterior chamber of both the toxic and control eyes. At intervals thereafter the animals were sacrificed and samples of aqueous humour, blood and cornea were assayed for ^{131}I content with a well-type scintillation counter. Changes in endothelial permeability were estimated by a comparison of the distribution of labelled material in the normal and the toxic eye of each animal.

Virus and antibody titrations

NDV infectivity was assayed by the plaque-count technique on monolayers of cells derived from chick embryos (Dulbecco, 1952). Influenza infectivity was determined by egg titration, the 50 per cent infectious titre being calculated by the method of Reed and Muench (1938). Haemagglutination (HA) titrations were carried out by a modification of the Salk pattern test designed to minimize the effect of HA inhibitors often present in biological materials (Granoff, 1955). Anti-NDV antibody concentrations are expressed as haemagglutination inhibition (HI) units (Salk, 1944) or as serum equivalents; one serum equivalent is defined as that concentration of antiserum which reduced the infectivity of a given amount of virus to e^{-1} or 37 per cent (Dulbecco, Vogt and Strickland, 1956). The effect of non-specific inhibitors was eliminated by the use of heat inactivated serum (56° for 30 min.) in all titrations for neutralizing antibody.

Corneal preparations

At appropriate intervals following injection of the virus into the anterior chamber the animals were killed and the corneal endothelium was prepared for cytological examination by gently scraping the posterior surface of the cornea with a blunt scalpel and spreading the detached endothelial cells on a glass slide. These preparations were immediately fixed in methanol and stained by Wolbach's variation of the Giemsa stain. Whereas this method resulted in good preparations for evaluating individual cell changes it gave little information concerning the integrity of the entire endothelial lining and thus no quantitative data on the extent of endothelial damage resulting from the toxic reactions.

To follow the initiation, spread, and extent of the endothelial lesions, the endothelial cell borders were stained while still on the excised cornea by a 30-sec. exposure to a 0·5 per cent solution of $AgNO_3$. To prevent the interference of naturally occurring chlorides, the corneas were rinsed briefly in isotonic sucrose both before and after staining. They were then fixed in methanol. By careful dissection the endothelial layer was teased free from the underlying cornea and small fragments were transferred to glass slides. This permitted the application of various stains (Hansen's iron trioxyhaematin, Giemsa) to the same endothelial preparation for more precise cytological studies.

RESULTS

The morphology of the toxic reaction

The normal endothelium, when stained with silver nitrate, revealed a regular mosaic pattern of polyhedral cells (Fig. 1). The application of a nuclear stain showed a single, very large, bean-shaped nucleus within each of these cells (Fig. 2). Occasionally 2 or 3 nuclei were observed within the apparent limits of a single cell.

Within 2 hr. following the injection of $1—2 \times 10^8$ plaque-forming particles (PFP) of NDV the normal mosaic pattern of the endothelial cells was altered. This change was characterized by a progressive failure of the silver nitrate to delineate cell boundaries, resulting in what appeared to be a fusion of adjacent cells (Fig. 3). Initially only widely scattered pairs of adjacent cells were involved; however, by the fourth hour numerous areas could be found which appeared to represent the fusion of 6 or more cells (Fig. 4). Superimposed nuclear stains at this stage revealed apparently normal nuclei within these areas. The obliteration

of silver staining boundaries progressed until, 6–8 hr. following the introduction of virus, only an occasional line of deposited silver could be observed on the endothelial surface (Fig. 5 and 6). Up to this time preparations stained with Giemsa or trioxyhaematein revealed little evidence of cellular damage. Subsequent to this, however, the development of cytoplasmic granularity (Fig. 7 and 8) and vacuolization in similarly stained preparations were interpreted as an early sign of cytonecrosis. Eighteen to 24 hr. following the introduction of virus the posterior surface of the cornea was usually devoid of endothelial cells and appeared to consist of Descemet's membrane littered with debris and leucocytes.

Ordinarily a faint corneal opacity became grossly apparent within 6 hr. This reached a maximum within 24 hr. (Fig. 9) and, after a variable period, a slow return to normal transparency often occurred. Depending upon the amount of virus injected, any degree of reaction between complete irreversible opacity and slight cloudiness could be obtained.

Inasmuch as it is well established that an intact endothelium is necessary for the maintenance of corneal transparency (Thomas, 1955), recovery of previously opaque eyes was considered to be evidence of endothelial regeneration. This was substantiated by the observation that every recovered eye suitably examined was found to possess a regenerated corneal endothelium.

Microscopic examination of corneas exhibiting only mild toxic reactions revealed the simultaneous presence of normal, degenerating and regenerating cells. On the other hand, examination of corneas with severe reactions demonstrated what appeared to be a complete loss of corneal endothelium prior to the establishment of the regenerative process.

Regenerating cells were characteristically larger and more irregular in shape than normal endothelial cells (Fig. 10 and 11). It appeared that these cells were incapable of restoring the normal physiological status to the cornea since corneal transparency returned only after such cells reverted to an essentially normal appearance. It was frequently noted that the morphological appearance of the cells directly underlying opaque patches in otherwise transparent eyes was typical of regenerating endothelium whereas the endothelium underlying the transparent areas of the same cornea was normal in appearance.

Frequently the opacity associated with severe toxic reactions proved to be irreversible. Examination of these eyes indicated that the posterior surface of the cornea had become permanently repopulated by cells which were extremely irregular in shape, resembling those seen in Fig. 10.

The morphological changes accompanying the reaction induced by influenza virus were identical to those just described for NDV but developed more slowly.

The fate of NDV in the anterior chamber of the rabbit eye

An accurate appraisal of the fate of virus introduced into the anterior chamber of the eye was difficult. Several factors contributed in varying degrees to the loss of virus from this area. First among these was the variable amount of virus lost through back-leakage of the inoculum following withdrawal of the needle from the eye. Another factor of importance was the increasing ability of the aqueous humour to reduce both the haemagglutinating activity and the infectivity of the virus. This became evident within 10 min. following the trauma associated with intraocular injection and was presumably due to the influx of non-specific virus inhibitory components from the serum (Karzon, 1956). The escape of virus in

conjunction with the rapid circulation of the aqueous humour (Kinsey, Grant and Cogan, 1942*a*; Kinsey, Livingood and Curtis, 1942*b*) was probably of less importance. The observation that convalescent animals rarely possessed even small amounts of specific antiviral antibody makes it doubtful that appreciable amounts of virus escaped into the circulation.

Duplicate experiments were undertaken to study the fate of NDV in the rabbit eye. In each, several animals were injected intraocularly with $1{\cdot}5 \times 10^8$ plaque-forming particles (PFP) of virus in a volume of 0·2 ml. At appropriate intervals the animals were sacrificed and 0·1 ml. of aqueous humour was withdrawn, diluted and assayed for plaque-forming particles. In addition, the *in*

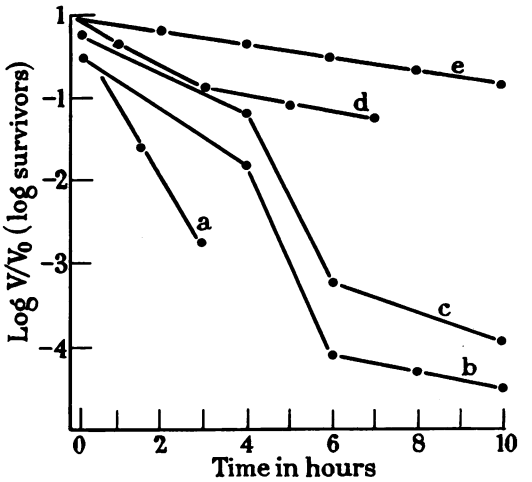


FIG. 12.—The *in vivo* and *in vitro* decline of NDV infectivity. (*a*) *In vitro* inactivation of NDV in aqueous humour withdrawn 4 hr. after corneal puncture. (*b*) and (*c*) *In vivo* decline of NDV in aqueous humour (2 experiments). (*d*) *In vitro* inactivation of NDV in aqueous humour. (*e*) *In vitro* inactivation of NDV in a balanced salt solution.

vitro inactivation of NDV at 37° in a balanced salt solution, normal aqueous humour and aqueous humour withdrawn 4 hr. after traumatizing the cornea by a single needle puncture was followed. The results of these assays are plotted in Fig. 12. The rapid drop in the *in vivo* titres observed during the first 10 min. was largely due to leakage which, however, soon ceased to be a factor. The increased rate of virus inactivation between 4 and 6 hr. following the injection of virus was attributed to the accumulation of non-specific virus inhibitory material in the aqueous humour. In support of this concept it is interesting to note that the rate of inactivation of NDV *in vitro* by aqueous humour removed 4 hr. after traumatizing the cornea paralleled that of the *in vivo* tests between the fourth and sixth hours. After the maximum effect of non-specific inhibitor had been attained the rate of virus disappearance decreased and, from the sixth hour onward, closely paralleled the rate of thermal inactivation of NDV in a balanced salt solution.

As has been reported previously for influenza virus (Evans and Rickard, 1945), no evidence of virus multiplication could be detected. Examination of aqueous humour and endothelial lysates (prepared by alternate freezing and thawing) at

intervals up to 30 hr. following injection of the virus failed to reveal any evidence of an increase in either non-infectious haemagglutinin (NIH) or infectious virus (PFP). It must be remembered, however, that the concentration of non-specific virus inhibitory substances in the aqueous humour may have been sufficient to mask any signs of multiplication.

Alterations in endothelial permeability following the development of corneal opacity

Rabbits exhibiting fully developed unilateral toxic reactions, induced by the injection of NDV into the right eye, were employed in a series of experiments in which equal amounts of ^{131}I-labelled albumin were introduced into the anterior chambers of both the toxic and opposite control eyes of the same animals. At intervals of 30 min., 3 hr. and 24 hr. following the introduction of the labelled material, rabbits were killed and the concentrations of ^{131}I in aqueous humour, blood and cornea were determined. It was readily apparent that the corneas of the toxic eyes accumulated more radioactive material than did those of the opposite control eyes (Table). This was presumably due to the destruction of the endothelial lining of the toxic corneas resulting in the removal of a barrier to the passage of albumin from the aqueous humour into the corneal stroma. With regard to this, it is interesting to note that the water content of the toxic corneas averaged 88 per cent by weight as compared to 75 per cent for the opposite control corneas. At the same time it was noted that the loss of labelled material from the aqueous humour of the toxic eyes was less rapid than that from the control eyes ; a factor which undoubtedly contributed somewhat to the elevated levels of isotope found in the toxic corneas. It would appear that the normal drainage of aqueous humour from the anterior chamber was impaired.

TABLE.—*The Distribution of ^{131}I-Labelled Albumin After Injection into the Anterior Chambers of Rabbit Eyes Previously Treated with NDV*

		Cornea			Aqueous humour		
		Specific activity†		Ratio§ Toxic/	Activity‡		Ratio Toxic/
Animal	Interval*	Toxic	Control	Control	Toxic	Control	Control
1	30 min.	316	33	9·6	1515	354	4·3
2		421	67	6·3	1286	1159	1·1
3		248	30	8·4	1465	328	4·5
4		640	137	4·7	2247	2834	0·8
5	3 hr.	287	16	17·9	1326	10	132·6
6		292	7	41·7	822	33	24·9
7	24 hr.	42	12	3·5	35	3	11·7
8		147	47	3·1	38	4	9·5

† Specific activity = counts/min./mg. dry wgt.
‡ Activity = counts/min./mg. wet wgt.
* Time from injection of labelled albumin to test for activity. Albumin was injected 24 to 48 hr. after virus. All toxic eyes showed fully developed reactions.
§ Numbers are numerical values of the ratio of the numbers in the 2 columns to the left.

The effect of a toxic corneal reaction on passage of protein from the blood stream into the eye was also examined. With respect to this it was noted that following the intravenous injection of labelled albumin the aqueous humour of

toxic eyes accumulated radioactive material more rapidly than did that of the control eyes. It is not unlikely that the mild inflammatory response often evident at this time was responsible for this concentration of labelled material in the anterior chamber of the toxic eye. Despite the fact that the blood levels of isotope following intravenous injection of labelled material were from 10 to 100 times greater than those achieved following intraocular injection, corneal radioactivity averaged less than one-tenth of that of the intraocularly injected animals. Since there appeared to be no relationship between blood and corneal levels of labelled material it seems probable that the movement of labelled albumin from aqueous humour into corneal stroma was, for the most part, direct.

Challenge of the regenerated endothelial membrane with toxic amounts of homologous virus

A transitory period of refractoriness to challenge with the homologous virus following a sublethal toxic reaction has been shown to occur in several systems (Wagner, 1952 ; Dougherty and Groupé, 1957*a*). It was not surprising, therefore, that the challenge of a partially or completely regenerated corneal endothelium with the same virus used to elicit the original reaction often failed to initiate a second reaction. In 7 rabbits challenged in this manner with NDV there was neither gross nor microscopic evidence of a toxic reaction. This ability to resist challenge remained complete for at least 2 weeks following the initiation of the original toxic reaction. Challenge of different animals after this period revealed an increasing degree of endothelial damage and opacity until ultimately the eye regained its normal susceptibility. Although variable, this return to normality occurred most typically at 3 to 5 weeks following the original injection of virus.

The sera of both resistant and normal rabbits were shown to contain a heat-stable haemagglutination inhibitor (Hirst, 1942) and a heat-labile inhibitor of virus infectivity (Karzon, 1956) both of which are non-specific in nature. Only occasionally could the sera of resistant animals be shown to contain even very low levels (1 in 4) of specific neutralizing antibody.

Aqueous humour removed from both normal and resistant rabbit eyes just prior to injection of virus showed no evidence of either specific or non-specific virus inhibitory activity. Following the trauma of intraocular injection, however, the presence of appreciable levels of heat-stable haemagglutination inhibitor (40 to 80 HI units) and heat-labile virus inhibitor could be demonstrated. It is important to note that the presence of similar inhibitor levels produced by traumatizing normal eyes was not sufficient to prevent a toxic reaction following the injection of standard amounts of NDV.

In order to assess the contribution of specific circulating antibody to the refractory state, 3 normal rabbits were inoculated twice weekly by alternating intravenous and intramuscular injections of allantoic fluid NDV for a total of 6 weeks. During this period the serum haemagglutination-inhibition titres rose from an average value of 1 in 128 to 1 in 1536. This latter value corresponds to 1100 serum equivalents per ml. Seven days following the last immunizing injection these animals were injected in both eyes with NDV, as were 2 normal control animals of the same size and age. Within 24 hr. every eye, including those of the controls, exhibited a marked toxic reaction.

As an alternate approach to this problem 7 normal animals were injected with NDV in the right eye only. Within 24 hr. 6 of these animals showed gross toxic

reactions in the treated eyes. Seventeen days later recovery of transparency was complete in 2 of the rabbits, partial in 3 and had not occurred in one animal. At this time both the treated and untreated (right and left) eyes were challenged with NDV. As was expected, 6 of the 7 treated eyes were resistant to challenge and no further signs of toxicity could be detected either grossly or microscopically following sacrifice; however, all 7 of the untreated control eyes showed a gross toxic reaction within 24 hr.

The results of these 2 experiments are interpreted as evidence that the resistance to homologous challenge shown by eyes following a toxic reaction is not due to humoural antibody.

DISCUSSION

Clarke and Fox (1948), working with typhus rickettsia in mice, first suggested that a seriously altered vascular permeability was the major factor underlying the toxic reaction. The frequency with which haemorrhage and oedema have been associated with virtually every type of virus-induced toxic reaction (Cox, 1953) adds to the probability that endothelial tissue represents a " primary target " in reactions of this type. The data presented in this paper indicate that certain viruses are indeed capable of acting more or less directly upon at least one type of " endothelial " cell in such a way as to destroy the functional integrity of the membrane which these cells compose. Further studies will be required to determine to what extent the mechanisms responsible for the corneal reaction are of general significance.

The cell-virus interaction resulting in destruction of the endothelium may well represent that of an abortive infection. This concept of the nature of viral toxicity was first advanced by Schlesinger (1950), who observed the production of NIH accompanying the neurotoxic action of influenza virus. More recently Henle, Girardi and Henle (1955) observed a similar effect *in vitro* following the treatment of HeLa cells with influenza virus. In view of our failure to demonstrate any degree of virus multiplication, it is important to bear in mind that recent evidence indicates the virus synthetic sequence may be blocked or diverted prior to the appearance of either NIH or complement fixing antigen (Prince and Ginsberg, 1957); therefore, the absence of readily detectable virus components does not rule out infection of this type.

It is necessary to consider the possibility that endothelial destruction is the result of a surface reaction similar to the haemolysis of erythrocytes, a well known property of certain of the myxoviruses (Burnet and Lind, 1950; Chu and Morgan, 1950). This does not seem likely, however, in view of the fact that influenza virus has no such haemolytic ability and yet is fully as active as NDV in producing the corneal reaction.

Because of the uncertainty regarding the structural and functional basis of the silver staining reaction, the progressive changes demonstrated early in the toxic reaction by utilizing this technique are difficult to evaluate. The fact that the silver stain reveals early morphological changes not made apparent by other techniques underlines the desirability of determining the nature of this reaction. This may well provide an important clue to the nature of the functional damage caused by the virus.

The establishment of a state refractory to further challenge coincided with the recovery of the toxic eye. Refractory states have also been reported in other

more or less similar systems (Wagner, 1952; Dougherty and Groupé, 1957*a*). Unlike the more transient immunity associated with these other virus-induced toxic reactions (Khoobyarian and Walker, 1957), the resistance of the newly regenerated corneal endothelium persists for periods in excess of 2 weeks. Preliminary experiments indicate that this state of immunity is not related to the production of humoural antibody. This is in agreement with the conclusions reached by other workers utilizing a different system (Dougherty and Groupé, 1957*b*). In view of these observations at least 3 different mechanisms must be considered as possible bases for this immunity: (*a*) An autointerference mechanism may be operative although the immunity seems unduly prolonged for this; (*b*) some sort of local antibody response may occur (Witmer, 1955); (*c*) the endothelial cells may be resistant as a result of physiological changes associated with regeneration and recovery of normal ocular function.

SUMMARY

Corneal toxicity, induced by the intraocular injection of either Newcastle disease virus (NDV) or influenza A (PR-8) virus, is associated with a progressive destruction of the corneal endothelium. By employing a silver staining technique it was possible to observe very early morphological changes in this membrane, not made apparent by other methods. Studies utilizing ^{131}I labelled human albumin indicated that the endothelial changes produced an altered exchange relationship between the corneal stroma and aqueous humour; as a result, corneal oedema and opacity developed. Although the cell-virus interaction resulted in cellular destruction, there was no evidence for virus multiplication as determined by infectivity and haemagglutination assay.

The regeneration of the corneal endothelium was followed by a regression of corneal oedema and opacity. It could be shown that regenerating or recently regenerated endothelial cells were refractory to challenge with toxic doses of the

EXPLANATION OF PLATES

Fig. 1, 3, 4, 5, 6 and 10 represent corneal endothelium stained with silver nitrate. Both silver nitrate and iron haematoxylin were used to stain the corneal endothelium shown in Fig. 2, 7, 8 and 11.

FIG. 1.—Normal rabbit corneal endothelium. × 200.

FIG. 2.—The same endothelium as shown in Fig. 1 stained to show nuclei. × 475.

FIG. 3.—Corneal endothelium 2 hr. following the injection of NDV into the anterior chamber. × 200.

FIG. 4.—Corneal endothelium 4 hr. following injection of NDV. × 200.

FIG. 5.—Corneal endothelium 6 hr. following injection of NDV. × 200.

FIG. 6.—Corneal endothelium 8 hr. following injection of NDV. × 200.

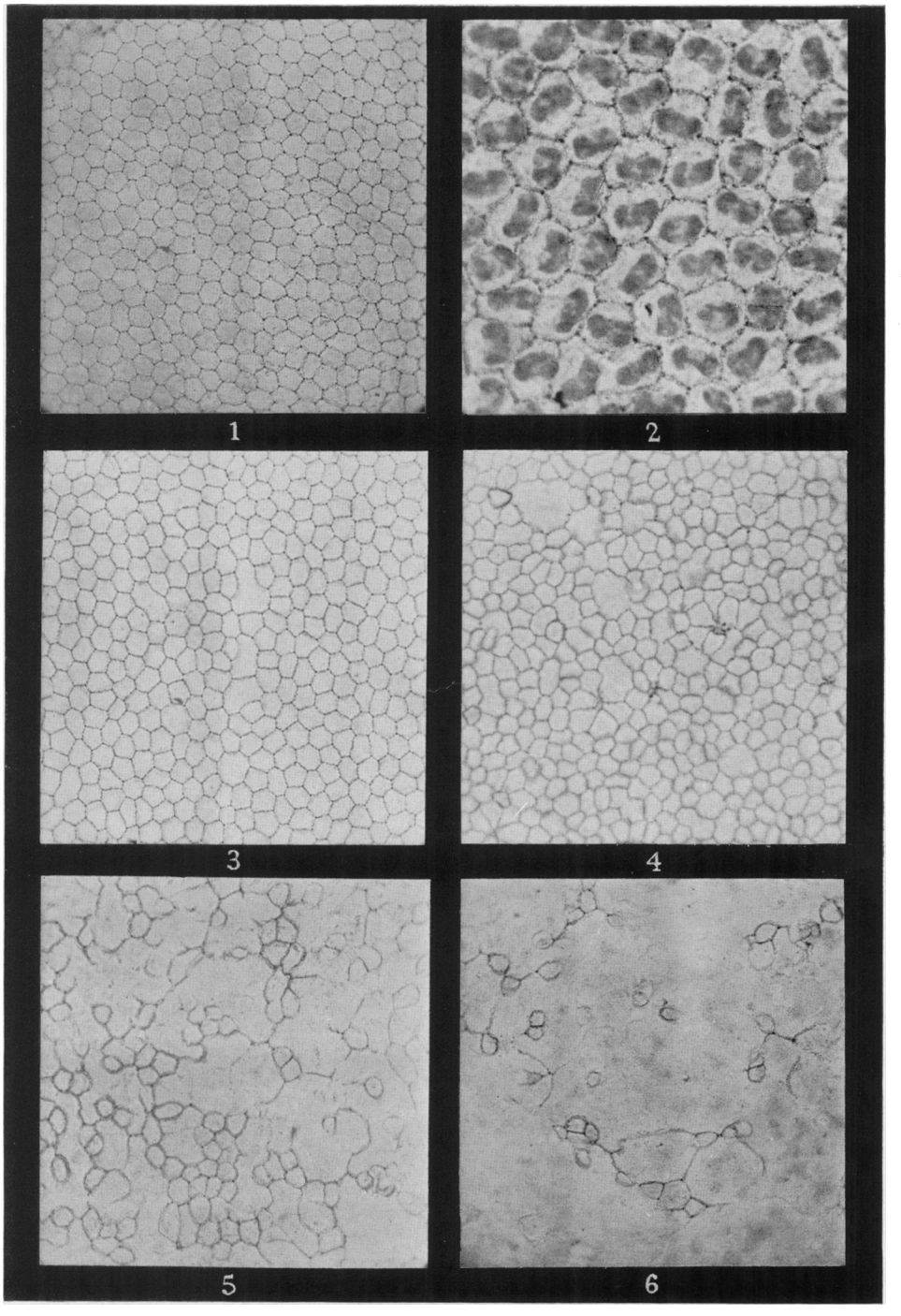
FIG. 7.—The same endothelium as shown in Fig. 5 (6 hr. post injection) showing early nuclear changes. × 475.

FIG. 8.—The same endothelium as shown in Fig. 6 (8 hr. post injection) showing increased granularity and nuclear changes. × 475.

FIG. 9.—Corneal opacity following the injection of NDV into the anterior chamber of the rabbit's eye.

FIG. 10.—Regenerating corneal endothelium 7 days after injection with NDV. Note the large size of these cells. × 200.

FIG. 11.—Regenerating corneal endothelium 14 days after injection with NDV. The typical mosaic is beginning to reform. × 200.



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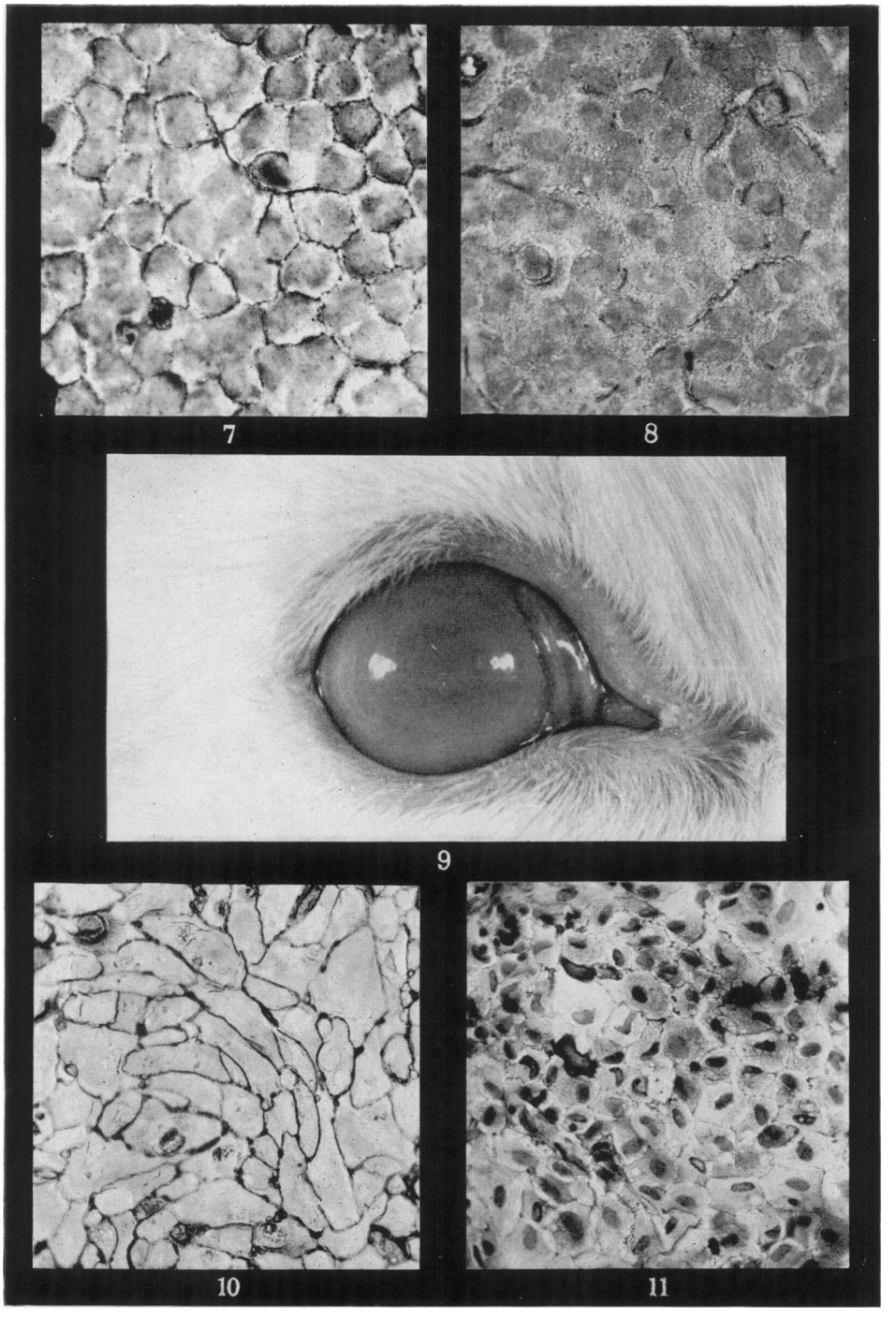

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homologous virus for periods up to several weeks. This transitory immunity appeared to be unrelated to humoural factors.

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