THE EFFECT OF BACTERIAL ENDOTOXINS ON TOXIC CORNEAL LESIONS PRODUCED BY INFLUENZA AND NEWCASTLE DISEASE VIRUSES

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It was reported previously that a massive dose of Newcastle disease virus (NDV) or influenza virus injected into the anterior chamber of the rabbit eye results in a toxic reaction. This is characterized by corneal opacity which is caused by stromal edema; the edema, in turn, is the result of lesions of the corneal endothelium with characteristic microscopic features. The reaction does not depend upon the multiplication of the infectious virus in the cornea.[1,2] In recent experiments gram negative bacterial endotoxins were found to suppress the NDV-induced corneal toxic reaction.[3]

The anterior chamber of the eye is a cavity surrounded anteriorly by a single layer of corneal endothelial cells and posteriorly by the highly vascularized iris and by a portion of the lens. An intact and functioning corneal endothelium is essential for proper control of stromal hydration. Normally, the anterior chamber is filled with clear watery aqueous humor which, like cerebrospinal fluid, contains practically no protein and few, if any, wandering cells. The aqueous humor originates from the ciliary processes of the iris, and drains at the angle of the anterior chamber. The intravenous or intra-ocular injection of endotoxin was reported to affect both the ciliary process [4] and the aqueous humor. The latter becomes rich in protein [5] and contains many leukocytes.

Thus the cornea provides a unique site for the study of the changes induced in an endothelial membrane by either locally or systemically administered bacterial endotoxin. The resistance to the toxic effects of NDV is one useful indicator of such change.

This paper is concerned with (1) further characterization of the corneal lesions produced by NDV and influenza virus, (2) different effects of bacterial endotoxins on the production of the corneal lesions, (3) experimental evidence suggesting an increased permeability of

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"blood-aqueous barrier" of the iris as an essential step for the induction of corneal resistance to NDV.

Material and Methods

Rabbits. Young, male, New Zealand white rabbits, weighing 4 to 5 lbs were obtained from the University of British Columbia animal farm.

Endotoxin. Alcohol-precipitated, ether-extracted endotoxins of *Salmonella typhosa* (TO-901) and *Salmonella enteritidis* were kindly donated by Dr. E. Neter (Buffalo, N.Y.) and Dr. R. L. Anacker (Hamilton, Mont.) respectively. *Escherichia coli* (055:B5) endotoxin, prepared according to the phenol extraction method of Westphal, was obtained from Difco Laboratories, Inc. (Detroit, Mich.). Stock solutions were made by dissolving endotoxins in non-pyrogenic sterile normal saline (Baxter, Alliston, Ontario, Canada) following passage through a Swinny filter and stored at −20° C. Dilutions to the desired concentration were made with non-pyrogenic sterile saline immediately prior to use.

Virus. The L-Kan 1948 strain of NDV was obtained from Dr. H. Rubin (Berkeley, Calif.), the PR 8 strain of influenza A from the American Type Culture Collection. Both viruses were cultivated in 10-day-old embryonated white Leghorn eggs by inoculating 10^4 plaque-forming units (PFU) of NDV or 10^4 50 per cent egg infectious dose (EID_{50}) of influenza virus into the allantoic cavity. The allantoic fluid was harvested at 48 hours, pooled and centrifuged at 3000 rpm for 5 minutes. The supernatant was dispensed into tightly sealed glass tubes and stored at −70° C. The NDV titer was 2×10^9 PFU; that of influenza virus, 10^9 EID_{50} per ml NDV infectivity was determined by the plaque count technique using a monolayer of cells derived from chick embryos.[6]

Glassware. All glassware was washed as for tissue culture, then sterilized in a hot air oven at 180° C for 2 hours.

Evans blue solution. A 1 per cent solution of Evans blue for intravenous injection was prepared in double distilled water and autoclaved at 15 lbs for 30 minutes.

Intra-ocular injection. Rabbits were anesthetized with intravenous sodium Nembutal®, and a drop of Pontocaine HCl (Winthrop Labs., N.Y.) was topically applied to the cornea. The technique for injection of the inoculum into the anterior chamber of the eye was that described previously.[2]

Preparation of the endothelium for microscopic examination. After staining with silver nitrate, the entire sheet of endothelium was separated from the cornea and mounted on a slide. The dried sheet was then stained with Harris' hematoxylin. Details of the technique have been described elsewhere.[2]

Determination of protein and Evans blue content in aqueous humor. Aqueous humor was collected from both eyes of each rabbit with a sterile tuberculin syringe and was pooled in a tube containing potassium oxalate. It was centrifuged and the supernatant collected. The protein content in the supernatant was measured by the method described by Lowry, Rosebrough, Farr and Randall.[7] The dye concentration was determined at 620 mμ with a Coleman junior spectrophotometer with an ultramicrocell.

Detection of leukocytes in aqueous humor. Aqueous humor (0.2 ml) was aspirated with a tuberculin syringe by inserting a 26-gauge needle through the corneal tissue 2 mm from the limbus. Drops of aqueous humor were placed on a slide, air dried and stained with Wright's solution. All drops were examined microscopically with a final magnification of 430 and with oil immersion lens for confirmation. The number of leukocytes was graded as indicated in Table I.

Results

Effects of Intravenous Injection of Endotoxin on the Production of Corneal Opacity by NDV and by Influenza Virus. A gross toxic reaction

TABLE I

EFFECTS OF VARIOUS TREATMENTS ON THE PRODUCTION OF CORNEAL OPACITY BY NDV OR INFLUENZA VIRUS AND ON AQUEOUS HUMOR

Pretreatments	Aqueous humor		Resistance to *	
	Evans blue †	WBC ‡	NDV	Influenza
S. typhosa endotoxin				
10–100 μg IV injection	+ or ++	+	+ §	−
10–100 μg to conjunctival sac	−	± or −	−	−
1 μg IV injection	−	−	−	−
1 μg into anterior chamber	+++	+++	+	−
E. coli endotoxin				
1–10 μg IV injection	+ or ++	+++	+	−
0.1 μg IV injection	−	−	−	−
S. enteritidis endotoxin				
0.1–10 μg IV injection	+ or ++	± or −	+	−
0.01 μg IV injection	−	−	−	−
Histamine acid phosphate				
100 μg IV injection	−	± or −	−	−
400 μg to conjunctival sac	+ or −	± or −	+ or −	−
Compound 48/80				
100 μg IV injection	−	ND	−	ND
100 μg to conjunctival sac	−	ND	−	ND
Nitrogen mustard (HN_2)				
Two 3 mg-IV injections	+ or −	−	+ or −	−
Single 3 mg-IV injection	−	−	−	ND
Paracentesis of anterior chamber (ant. ch.)	+++	+	+ §	−
Two 3 mg HN_2 IV injections and paracentesis of ant. ch.	+++	−	+	−
Normal saline (non-pyrogenic)				
IV injection	−	−	−	−
Into anterior chamber	++	−	+ §	ND
Anti-NDV immunization ¶	−	−	−	ND

* Resistance to the production of corneal opacity by virus: −, no resistance; +, significant increase in resistance.

† −, no blue color; +, pale blue; ++, intensity between + and +++; +++, deep blue, outline of pupil cannot be seen.

‡ 0.1 ml of aqueous humor was used for smear: −, no WBC on entire smear; +, occasional WBC on entire smear; ++, many WBC on entire smear; +++, many WBC in every microscopic field.

§ Resistance was observed for more than 4 days after the treatment.

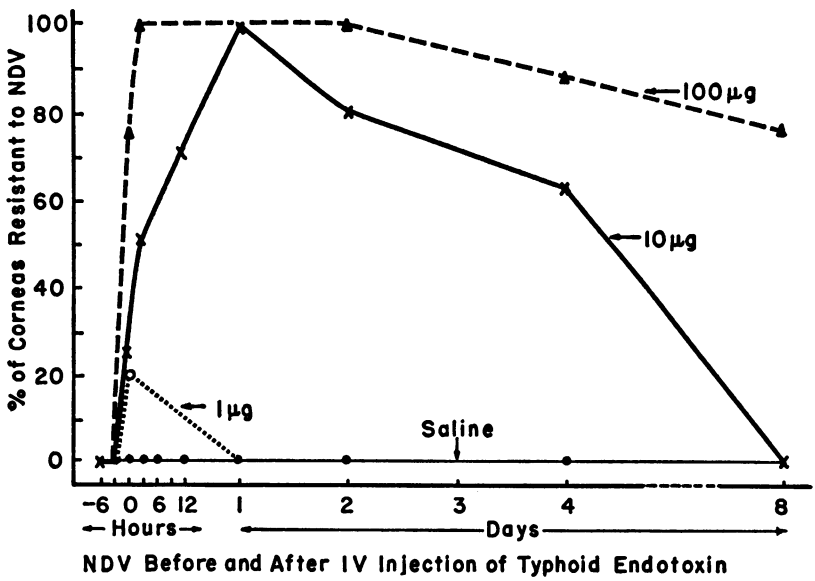
¶ Titers of antibody to NDV in sera ranged from 800 to 2000 HI/ml at the time of virus injection into the eye.

ND, not done.

in the cornea was characterized by an opacity which could be seen 24 and 48 hours after injection into the anterior chamber of 10^8 PFU of NDV or 10^8 EID_{50} of influenza virus respectively (Fig. 8). The corneal opacity was the result of stromal edema (Fig. 10). In this experiment, attempts were made to suppress the virus-induced corneal opacity by

the intravenous injection of varying amounts of bacterial endotoxin into groups of rabbits; this was followed by NDV injection into the anterior chamber at various time intervals. A new group of 8 rabbits was used for each time interval. The corneal opacity was examined 24 hours after virus injection.

With 100 μg of endotoxin, a resistant (clear corneas) state was produced in all rabbits which received endotoxin 3 hours, 1 day or 2 days before an NDV challenge (Text-fig. 1). Protection was observed in about 80 to 90 per cent of the rabbits receiving endotoxin 0 hours, 4 or



TEXT-FIG. 1. Effect of typhoid endotoxin on the production of corneal opacity by NDV.

8 days before virus inoculation. With 10 μg of endotoxin, 100 per cent suppression was evident only in those animals which received the endotoxin 1 day prior to the virus. Suppression was manifested in 25 to 75 per cent of the rabbits which received endotoxin at 0, 3, 12 hours, 2 or 4 days before virus injection. The injection of 1 μg of endotoxin showed a slight suppressive effect; saline had no effect.

The injection of *E. coli* and *S. enteritidis* endotoxins also produced resistance to NDV. The minimal suppressive dosage was 1 μg for *E. coli* endotoxin and 0.1 μg for *S. enteritidis* endotoxin (Table I). The intravenous injection of endotoxins of *S. typhosa* (10 to 100 μg), *E. coli* (1 to 10 μg), or *S. enteritidis* (0.1 to 10 μg), failed to suppress influenza virus-induced corneal opacity (Tables I and II).

Microscopic Lesions Induced by NDV and Influenza Virus. The preceding experiments showed that endotoxins had little or no effect upon corneal opacity induced by influenza virus although NDV-induced opacity was suppressed markedly. The development of opacity was shown to be related directly to the appearance of microscopic lesions in the endothelium following virus injection.[1,2] Thus it is to be expected that differing endotoxin effects upon the corneal opacity would be associated with difference in the microscopic lesions produced.

Wilcox, Wood, Oh, Everett and Evans[1] reported that in endothelium the morphologic changes induced by influenza virus were identical to those induced by NDV; the observation was made on endothelium which represented but a small portion of the whole. More recently, a technique has been developed by which the entire endothelium may be obtained for microscopic examination.[2] Therefore, the virus-induced lesions were re-examined in both normal and endotoxin-induced resistant corneas.

When normal corneal endothelium was stained with silver nitrate and hematoxylin, the cell borders were outlined with silver; the nuclei, with hematoxylin (Fig. 1). The cells were uniformly polygonal in shape and were arranged in a regular mosaic pattern. The majority of the cells contained single nuclei; a few contained 2 or 3.

Within one hour after inoculation of 10^8 PFU of NDV, a loss of endothelial boundaries (fusion) and well isolated clusters of radially arranged endothelial cells (rosettes) began to appear (Fig. 2). During the next several hours, widespread fusions appeared and endothelial rosettes steadily increased in number. Many leukocytes, mainly polymorphonuclears, were present. After 6 hours, fusions were the most predominant feature (Fig. 3). Any cells which were still outlined with silver maintained their original size and shape fairly well. After 12 hours the endothelial lines disappeared almost completely but many irregularly distributed nuclei were still present (Fig. 4). Distinctive corneal opacity was not found until 12 hours after inoculation of the virus.

After inoculation of 10^8 EID_{50} of influenza virus, the first recognizable microscopic changes of corneal endothelium were observed at 12 hours. The cells were distorted in size and shape. There were very few rosettes. These changes became more apparent 24 hours after injection (Fig. 5). Many leukocytes were seen on the endothelial cells. Forty-eight and 72 hours after the virus injection, the endothelium consisted mostly of large irregularly shaped cells outlined by rather fine wavy lines of silver which sometimes crossed over nuclei (Fig. 6). Throughout the entire period of observation, fusions of endothelial cells, such as were seen in the endothelium inoculated with NDV, were not observed.

Definite opacity began to appear 24 hours after inoculation, reaching a maximum at 72 hours.

Endotoxin-induced resistant corneas showed intact endothelium following injection of toxic doses of NDV into the anterior chamber (Fig. 7) and did not become opaque. In contrast (see Table II) endotoxins

TABLE II

LACK OF EFFECT OF IV INJECTION OF TYPHOID ENDOTOXIN ON THE PRODUCTION OF CORNEAL OPACITY BY INFLUENZA VIRUS

Time of virus injection		Dose of endotoxin		
Relation to endotoxin injection	Hour	100 μg	10 μg	Normal saline *
Before	3	4/4 †	ND	8/8
	1	3/4	4/4	8/8
Same time		4/6	4/4	8/8
After	2	6/6	8/8	8/8
	6	4/4	4/4	6/6
	24	3/4	4/4	7/8

* Non-pyrogenic normal saline was used as a diluent for the endotoxin.
† Number of opaque corneas per number of eyes inoculated with NDV.
ND, Not done.

failed to protect corneas from the production of both microscopic lesions of endothelium and opacification by influenza virus.

Relationship between Increased Permeability of Ciliary Processes and the Development of Corneal Resistance to NDV. The membrane separating blood from aqueous humor exhibits a high degree of selectivity; thus the aqueous humor from a normal rabbit eye is almost completely protein-free (0.5 mg per ml). Similarly, a large number of dyes, for example, Evans blue, do not appear in the aqueous humor if they are injected into the blood stream. However, the intravenous injection of endotoxin affects the ciliary processes, and leukocytes and intravenously injected Evans blue appear in the aqueous humor.

Since changes in the permeability of the ciliary system might play some role in the development of corneal resistance, experiments were conducted to examine the relationship between the appearance of both leukocytes and intravenously injected Evans blue in aqueous humor and the development of resistance to NDV and influenza virus following various treatments.

Twenty-four hours prior to the virus injection into the anterior chamber, the animals were treated as outlined in Table I. Evans blue, an indicator of ciliary process permeability, was injected intravenously immediately after each treatment. The presence of dye in the aqueous humor was determined macroscopically at hourly intervals for 12 hours.

In all cases in which Evans blue was detected the dye disappeared completely from aqueous humor within 12 hours after treatment. For the detection of leukocytes separate groups of rabbits were used. The rabbits were pretreated as outlined in Table I without Evans blue injection. At 3, 6 and 12-hour intervals, aqueous humor was collected and the presence or absence of leukocytes was determined. Two rabbits were used for each interval.

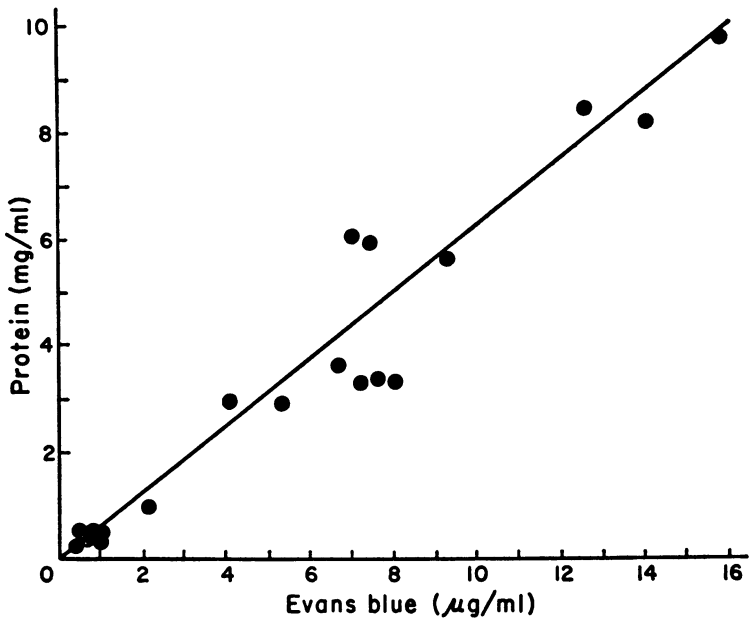
There was close agreement between the development of corneal resistance to NDV and the appearance of dye in aqueous humor (Table I). Intravenous injections of 10 μg of typhoid endotoxin, 1 μg of *E. coli* endotoxin, or 0.1 μg of *S. enteritidis* endotoxin caused the appearance of Evans blue in the aqueous humor and produced resistance in the eye. Similar results were produced by the instillation into conjunctival sacs of 400 μg histamine acid phosphate (Eli Lilly Co., Toronto, Canada), 2 intravenous injections of nitrogen mustard (Merck, Sharp & Dohme, Montreal, Canada) or paracentesis of the anterior chamber. Even the intra-ocular injection of non-pyrogenic normal saline resulted in appearance of Evans blue in the aqueous humor and protected the cornea.

Treatments which did not cause the appearance of Evans blue in the aqueous humor failed to produce resistance to NDV. It is interesting to note that rabbits immunized against NDV failed to acquire corneal resistance even though a high titer of specific virus antibody was found in their sera. This indicates that circulating antibody plays no major role in the production of corneal resistance. No correlation was found between the appearance of leukocytes in aqueous humor and the development of resistance against NDV. Corneal resistance to NDV was produced following each of the procedures described above carried out without Evans blue. Therefore Evans blue was not a factor in this resistance.

All treatments listed in Table I failed to produce a corneal resistance to influenza virus. These results are contrary to those observed with NDV and suggest a difference in the toxic mechanisms of the two viruses. NDV was used exclusively in subsequent experiments aimed at explaining the mechanism of corneal resistance induced by endotoxin.

Evans Blue as an Indicator for Presence of Protein in Aqueous Humor. Intravenously injected Evans blue is reported to combine with plasma proteins primarily albumin.[8] Therefore, the appearance of dye in the aqueous humor after treatments presumably indicates the presence of plasma proteins in the anterior chamber. To test the validity of this assumption, measurement of both Evans blue and protein in aqueous humor was carried out following the intravenous injection of 10 μg typhoid endotoxin. Aqueous humor free of contamination with blood

was collected from both eyes and pooled. A new group of 6 rabbits was used each time. A plot of the concentration of Evans blue in each specimen against the amount of protein in the same aqueous humor suggests a nearly linear relationship between the two (correlation coefficient



TEXT-FIG. 2. Quantitative relationship between Evans blue and protein in aqueous humor of rabbits following intravenous injection of 10 μg typhoid endotoxin. Each dot represents pooled aqueous humor from both eyes of a rabbit. (Correlation coefficient $r = 0.97$).

$r = 0.97$) (Text-fig. 2). This appears to justify the use of Evans blue as an indicator for the presence of protein in aqueous humor, and suggests that protein may be the active factor conferring corneal resistance.

Virus-induced Toxic Reaction in Eyes of Endotoxin-tolerant Rabbits. Endotoxin-tolerant rabbits, which had previously received repeated intravenous injections of endotoxin, failed to undergo changes in the permeability of the ciliary processes following injection of a challenge dose of endotoxin. The appearance of Evans blue in aqueous humor was used as the indicator. If the presence of protein in aqueous humor before virus injection is directly related to the occurrence of corneal resistance to virus, it is reasonable to expect that the eyes of such tolerant rabbits would be as susceptible to virus as those of normal rabbits. To study this possibility, 8 rabbits were each given 5 intravenous injections of 10 μg typhoid endotoxin at intervals of 3 days. One ml of 1 per cent Evans blue solution was injected intravenously immediately after the

last endotoxin injection. Twenty-four hours later a toxic dose of NDV was inoculated into the anterior chamber. Evans blue appeared in some eyes within 3 hours after the last endotoxin injection, but the dye disappeared completely from these eyes between 6 and 12 hours.

As expected, the eyes which were free of Evans blue in the anterior chamber showed corneal opacity as marked as in normal eyes (Table III). Partly tolerant eyes (left eyes of rabbits no. 5 and 6), which showed slight intensity of dye in the anterior chamber, produced partial refractoriness to the virus.

The second experiment was conducted to determine whether paracentesis of the anterior chamber could induce resistance of the cornea of endotoxin-tolerant rabbits. Two-tenths ml of aqueous humor was withdrawn from the right eye of endotoxin-tolerant rabbits, and Evans blue was injected intravenously. The left eye was untouched and served as a control. Normal rabbits were included in this experiment to compare the degree of corneal opacity and were treated in the same manner as tolerant rabbits. Within 1 hour the anterior chamber of the right eye of both tolerant and normal rabbits was dark blue due to presence of the dye; clarity returned within 12 hours. During this period, no Evans blue appeared in the anterior chamber of the left eye of either group although ciliary processes were slightly blue-tinged. NDV

TABLE III

NDV-INDUCED TOXIC CORNEAL REACTION IN ENDOTOXIN-TOLERANT EYES

Endotoxin-tolerant rabbit	Right eye		Left eye	
	Evans blue in anterior chamber *	Corneal opacity †	Evans blue in anterior chamber *	Corneal opacity †
1	−	++	−	++
2	−	+++	−	++
3	−	++	−	+++
4	−	++	−	++
5	−	++	±	+
6	−	++	+	+
7	−	+++	−	++
8	−	++	−	++

* Intensity of Evans blue in aqueous humour was scored in the same manner as in Table I.

† Corneal opacity was scored as follows: −, none; ±, doubtful; +, definite but weak, outline of pupil can be seen easily through cornea; ++, intermediate between + and +++; +++, strong, outline of pupil can not be seen through cornea.

was injected into the anterior chambers of both eyes 24 hours after the treatment, and corneal opacity was examined 1 day later. The cornea of both tolerant and normal rabbits which exhibited Evans blue were

completely resistant, while eyes not exhibiting the dye were fully susceptible to the viral toxic effect (Table IV).

TABLE IV

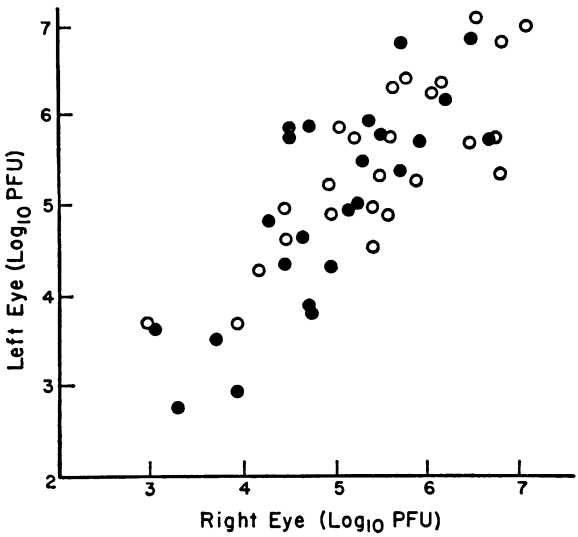
EFFECT OF PARACENTESIS OF THE ANTERIOR CHAMBER OF ENDOTOXIN-TOLERANT EYES ON THE PRODUCTION OF CORNEAL TOXIC REACTION BY NDV

Group	Rabbit Number	Right eye (paracentesis)		Left eye (not treated)	
		Evans blue in anterior chamber *	Corneal opacity †	Evans blue in anterior chamber *	Corneal opacity †
Endotoxin-tolerant	1	+++	−	−	++
	2	++	−	−	+++
	3	++	−	−	++
	4	+++	−	−	++
	5	+++	−	−	+++
Normal	1	+++	−	−	+++
	2	+++	−	−	+++
	3	++	−	−	++
	4	++	−	−	+++

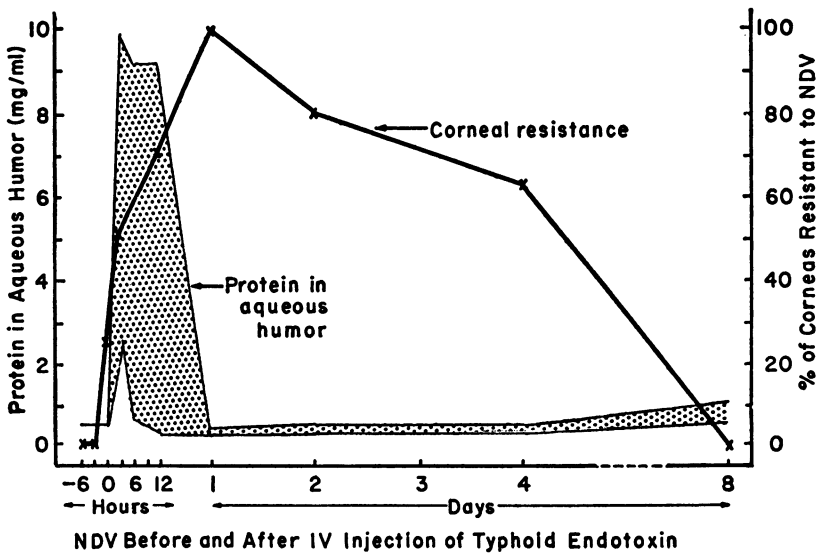
* Same as in Table I
† Same as in Table III

Titer of Infectious Virus in Aqueous Humor of Resistant and Susceptible Corneas. Rabbit serum contains non-specific inactivating substances for NDV.[9,10] Therefore, corneal resistance produced by endotoxin, paracentesis of anterior chamber, etc., could be due to the rapid inactivation of virus in the anterior chamber through the action of inhibitors brought into that site following a permeability change of the ciliary processes. To study this possibility, one eye was subjected to anterior chamber paracentesis so that a high concentration of viral inactivating substances from serum could be introduced into the chamber[1]; the other eye was left untreated. Twenty-four hours later the same number of PFU of NDV was inoculated into the anterior chambers of both eyes. As a control, normal rabbits were inoculated with the same amount of NDV into both eyes. At varying intervals, the aqueous humor of each eye was collected separately from new rabbits in both test and control groups for virus titration.

The virus titer of the right eye was plotted on log scale against that of the left eye (Text-fig. 3). The differences in titer between the two eyes of rabbits in the test group showed the same pattern as those of normal rabbits. Yet, complete resistance was observed in the right eye, but no resistance in the left eye of animals subjected to paracentesis of the right eye. Both eyes of normal rabbits were equally susceptible to NDV. It would appear that resistance is not brought about by rapid viral inactivation by inhibitory substances in aqueous humor.



TEXT-FIG. 3. Titers of NDV in aqueous humors of resistant and susceptible corneas: the relation of virus titer in the right to that in the left eye in individual rabbits. Control rabbits (•) received no treatment and both corneas were equally susceptible to NDV. Right eyes of test rabbits (o) were subjected to paracentesis of the anterior chamber 24 hours before injection of virus and were resistant to virus. Left eyes in the same rabbits were untreated and were fully susceptible to NDV.



TEXT-FIG. 4. Relation between the appearance of protein in the aqueous humor and the development of corneal resistance. The curve indicating corneal resistance is identical to that of 10 μg in Text-figure 1 since two experiments were done on the same rabbits simultaneously. The dotted area of the curve for proteins indicates ranges between the highest and the lowest value of protein in the aqueous humor within a group of 6 rabbits at each time interval.

Relationship between the Appearance of Protein in Aqueous Humor and the Development of Corneal Resistance. Typhoid endotoxin (10 μg) was injected intravenously into groups of 6 rabbits. At varying intervals, aqueous humor was collected from both eyes of 1 group of 6 rabbits for protein assay, and 0.2 ml of a toxic dose of NDV was injected into the anterior chamber. Extreme care was taken not to contaminate the aqueous humor with blood during the collection. Corneal opacity was recorded 24 hours later. Corneal resistance developed gradually after a sharp increase of protein in aqueous humor (Text-fig. 4). Significantly, substantial corneal resistance was observed for several days after the return of the protein concentration to normal. These results indicated that virus inhibitory substances in the aqueous humor played no direct significant role in corneal resistance.

Effect of Typhoid Endotoxin on NDV Infectivity. Each of 2 tubes containing 1 ml of 10 μg typhoid endotoxin in 1 ml solution and 1 ml of non-pyrogenic normal saline respectively was inoculated with approximately 8.0×10^6 PFU of virus. Saline was used as a diluent for the endotoxin. The tubes were incubated at 37° C. Aliquots of each were collected at various intervals and placed on a monolayer of chick embryo cells for infectivity titration. The results are presented in Table V. There were no substantial differences between the virus titer in the endotoxin solution and that in saline at any given time.

TABLE V

EFFECT OF TYPHOID ENDOTOXIN ON INFECTIVITY OF NDV

Hours at 37° C	Titer of NDV (PFU/ml) *	
	Endotoxin	Normal saline †
0	5.0×10^6	8.3×10^6
1½	3.9×10^6	6.0×10^6
3	8.3×10^5	2.6×10^6
6	1.5×10^4	3.9×10^4

* Mean titer of 4 plates.

† Non-pyrogenic normal saline was used as a diluent for the endotoxin.

DISCUSSION

The present studies have shown that, although corneal opacities produced by NDV and influenza virus were identical grossly, the microscopic lesions of corneal endothelium produced by the viruses were strikingly different. This would indicate that corneal opacity and microscopic lesions of the endothelium produced by these viruses were mediated by different mechanisms. This conclusion is also supported by the fact that bacterial endotoxins that produced a cornea completely resistant to NDV failed to suppress the toxic effect of influenza virus.

It is significant that not only endotoxin but also procedures such as paracentesis of the anterior chamber of the eye, instillation of histamine into the cornea, intravenous injections of nitrogen mustard, or even intra-ocular injection of non-pyrogenic normal saline, could induce complete resistance of the cornea to NDV. The corneal resistance induced by either intravenous endotoxin injection, paracentesis of the anterior chamber or intra-ocular injection of non-pyrogenic normal saline, lasted almost 1 week. The characteristic common to these procedures and to endotoxin is the ability to increase the permeability of ciliary processes as indicated by the passage of intravenously injected Evans blue into the anterior chamber. Procedures which did not accomplish this failed to induce resistance. It was particularly interesting that paracentesis of the anterior chamber could, in endotoxin-tolerant rabbits, render virus-susceptible corneas completely resistant to NDV. It seems clear that permeability changes in the blood-aqueous barrier of ciliary processes are essential for the induction of corneal resistance by the methods employed in this study.

Relatively little has been learned about the factor(s) immediately responsible for resistance. Leukocytes played no significant role. This is consistent with a previous finding that leukocytes are not essential in suppression of NDV-induced corneal toxicity in rabbits pretreated with nitrogen mustard.[11] The protective factor appears to be of a humoral nature and to be derived from blood. Evans blue has been reported capable of inducing a protective effect in chick embryos against NDV.[12] However, corneal resistance to NDV was also observed following the protective procedures listed in Table I without Evans blue injection. Therefore, the dye appears to have no significance in the corneal system.

Evans blue has been reported to be bound with the plasma proteins, primarily albumin,[8] and a splitting of dye from proteins does not occur during the permeation from the blood through the blood-aqueous barrier.[13] The present study shows that the concentration of Evans blue in the aqueous humor varies directly as the protein concentration. Therefore, it was considered that the plasma proteins or protein-bound substance could be important factors in the resistance to NDV. It is also possible that resistance could be induced by some other compound which can be introduced into anterior chamber as readily as Evans blue following permeability changes of ciliary processes.

Whatever the active factor(s) may be, endotoxin injection induces a resistant state in cornea through a modification of the endothelial cells. This is supported by the following observations: (a) endotoxin did not directly inactivate virus, (b) serum virus inhibitors did not play any

major direct role in the induction of resistant cornea, (c) circulating virus specific antibody failed to suppress corneal toxic reaction to NDV, (d) a resistant state persisted after the complete disappearance of Evans blue or protein from aqueous humor.

Whether increased permeability of the vascular system also plays any important role in endotoxin-induced resistance to viral toxic reactions in other organs is uncertain. In view of the evidence obtained in the corneal system, it may be worthwhile to explore the role of permeability changes of the vascular system in other tissues by using NDV as an indicator of altered reactivity.

Summary

A state of marked resistance to the toxic effects of NDV was observed in the rabbit eye following the intravenous injection of 10 μg of *S. typhosa* endotoxin, 1 μg of *E. coli* endotoxin and 0.1 μg of *S. enteritidis* endotoxin. These endotoxins were found to have little or no suppressive effect on influenza-induced toxic corneal reactions. The results indicated that the mechanisms by which these two viruses induced corneal opacity were different. This was confirmed by the findings that the microscopic lesions of corneal endothelium produced by NDV were strikingly different from those produced by influenza virus.

The development of the corneal resistance to NDV following injection of endotoxin was preceded by an increase in the protein content of aqueous humor resulting from increased permeability of the "blood-aqueous barrier" in ciliary processes of the iris. Treatments other than injection of the endotoxin causing similar changes in permeability of the iris also produced a cornea markedly refractory to NDV. Treatments without such an effect on the iris failed to produce resistance in the cornea against the virus.

Endotoxin alone, in the concentration used in this study, did not inactivate NDV. Non-specific inactivating substances of serum, circulating specific antibody for NDV or leukocytes appeared to play no direct significant role in this refractory state. The present studies emphasize the importance of changes of ciliary permeability in determining the protective effect of various treatments, including endotoxins, against toxic corneal reaction induced by NDV.

References

1. Wilcox, W. C.; Wood, E. M.; Oh, J. O.; Everett, N. B., and Evans, C. A. Morphological and functional changes in corneal endothelium caused by the toxic effects of influenza and Newcastle disease viruses. *Brit. J. Exper. Path.*, 1958, **39**, 601–609.
2. Oh, J. O., and Evans, C. A. Suppressive effects of pyrilamine maleate and

d-lysergic acid diethylamide (LSD-25) on early corneal lesions produced *in vitro* by Newcastle disease virus (NDV) and compound 48/80. *Virology*, 1960, **10**, 127–143.

3. OH, J. O. Modification of virus-induced corneal toxic reaction in rabbits by typhoid endotoxin. *Abstracts, 8th Internatl. Congress Microbiol., Montreal, Canada*, 1962, p. 116.
4. PAPPAS, G. D.; SMELSER, G. K., and BRANDT, P. W. Studies on the ciliary epithelium and the zonule. II. Electron and fluorescence microscope observations on the function of membrane elaborations. *Arch. Ophth.*, 1959, **62**: 959–965.
5. AYO, C., and MEYER, K. Protein content of rabbits' aqueous humor following intravenous injection of *E. coli* toxin. *Proc. Soc. Exper. Biol. & Med.*, 1942, **51**, 130–132.
6. DULBECCO, R. Production of plaques in monolayer tissue cultures by single particles of an animal virus. *Proc. Natl. Acad. Sc.* 1952, **38**, 747–752.
7. LOWRY, O. H.; ROSEBROUGH, N. J.; FARR, A. L., and RANDALL, R. J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, 1951, **193**, 265–275.
8. RAWSON, R. A. The binding of T-1824 and structurally related diazo dyes by the plasma proteins. *Am. J. Physiol.*, 1943, **138**, 708–717.
9. GINSBERG, H. S., and WEDGWOOD, R. J. Properdin and the inactivation of viruses. *Ann. New York Acad. Sc.*, 1960, **83**, 528–532.
10. KARZON, D. T., and BUSSELL, R. H. Studies of the nonspecific inactivation of Newcastle disease virus by mammalian sera. *Ann. New York Acad. Sc.*, 1960, **83**, 539–550.
11. OH, J. O., and YANG, Y. T. Suppression of virus-induced corneal toxicity in rabbits by pre-treatment with nitrogen mustard. *Proc. Soc. Exper. Biol. & Med.*, 1961, **106**, 413–420.
12. FINKELSTEIN, R. A. Protection of chick embryos against Newcastle disease virus by trypan blue and other compounds. *J. Immun.*, 1961, **87**, 707–713.
13. VON SALLMANN, L., and DILLON, B. The effect of di-isopropyl fluorophosphate on the capillaries of the anterior segment of the eye in rabbits. *Am. J. Ophth.*, 1947, **30**, 1244–1262.

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[*Illustrations follow*]

Legends for Figures

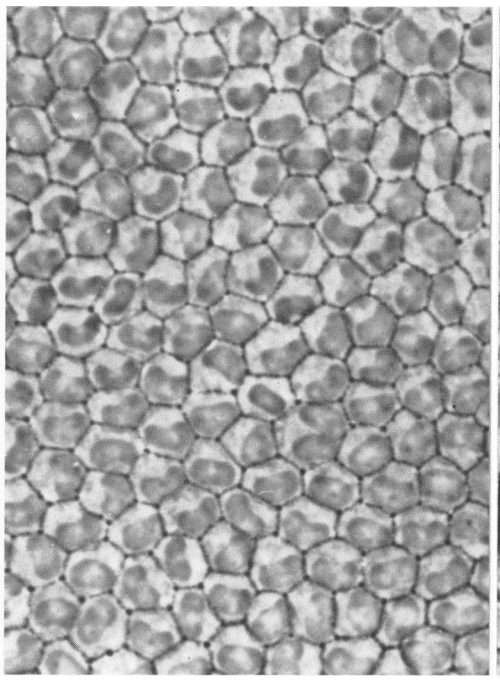
Figures 1 to 7 represent flat preparations of rabbit corneal endothelium stained with silver nitrate and hematoxylin. Both hematoxylin and eosin were used to stain sections of corneas shown in Figures 9 and 10.

Fig. 1. Normal corneal endothelium. × 400.

Fig. 2. Endothelium 3 hours after injection of a toxic dose of NDV into anterior chamber. Two rosettes (clusters of radially arranged endothelial cells) appear at the upper left and center of the photograph. Fusions (loss of endothelial cell boundaries) are seen in the right half. Nuclei of endothelial cells were less stainable with hematoxylin than in normal cells. Polymorphonuclear leukocytes (arrows) are present. × 400.

Fig. 3. Endothelium 6 hours after the injection of NDV. Extensive fusions of endothelial cells and leukocytes (arrows) are evident. Slight degree of corneal opacity was seen in this cornea. × 400.

Fig. 4. Endothelium 12 hours after NDV-injection. Endothelial outlines have almost completely disappeared. Corneal opacity was distinct at this stage. × 400.



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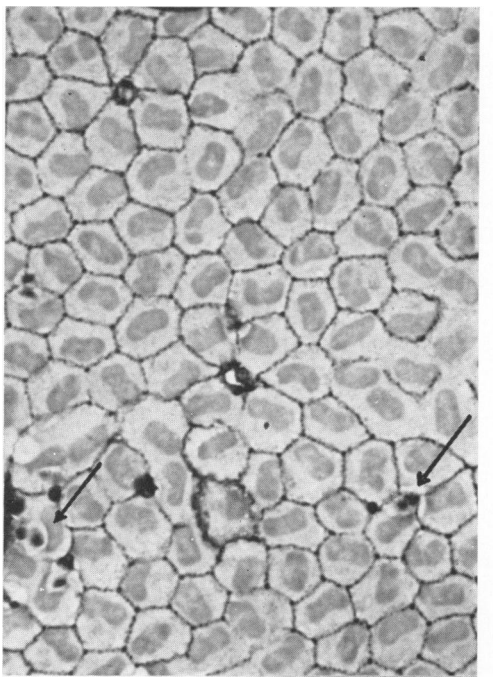

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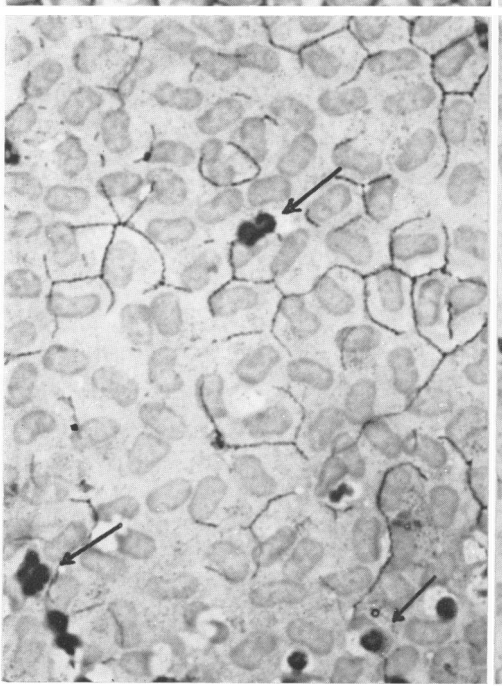

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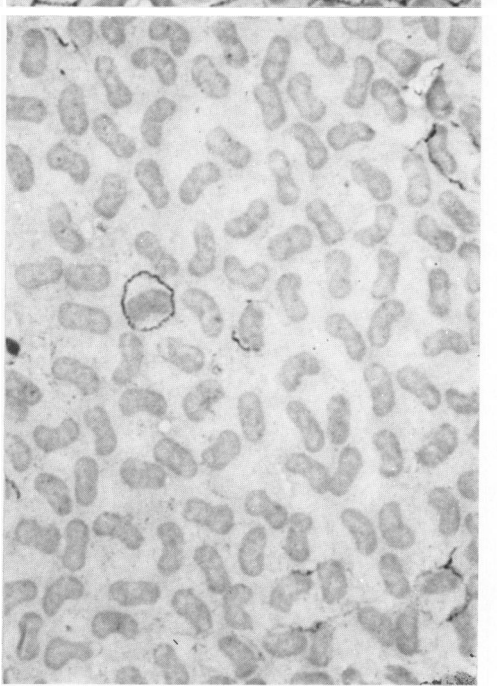

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FIG. 5. Endothelium 24 hours after injection of a toxic dose of influenza virus into the anterior chamber. The general pattern of cell arrangement is distorted and cells are irregular in shape and size. Doubtful corneal opacity was seen at this stage. × 400.

FIG. 6. Endothelium 48 hours after the injection of influenza virus. Cells are markedly irregular in shape and size. No fusions or rosettes are seen. Opacity was prominent in this cornea. × 400.

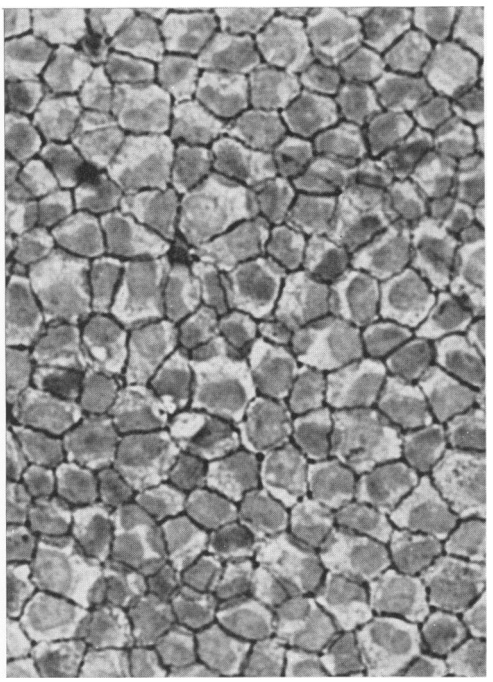
FIG. 7. Corneal endothelium in a rabbit treated with an intravenous injection of 10 μg typhoid endotoxin 24 hours prior to intra-ocular injection of toxic dose of NDV. Neither rosettes nor fusions are seen. Endothelium is normal in appearance; corneal opacity was not observed in this rabbit. × 400.

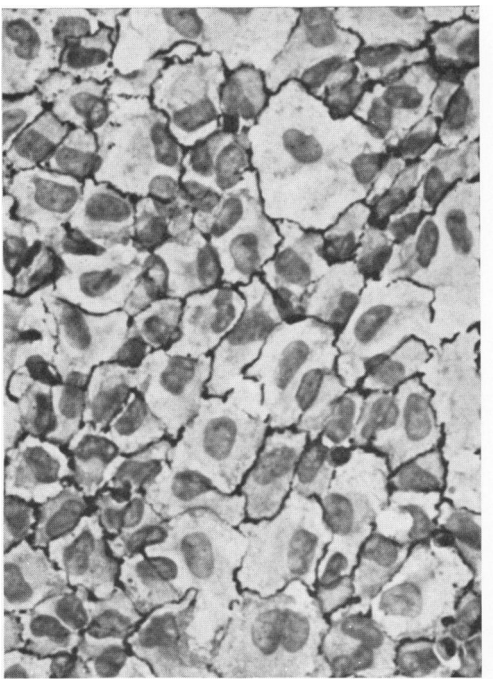
FIG. 8. Opaque cornea produced by NDV. Influenza virus produced an identical corneal opacity to this.

FIG. 9. Section of an NDV-resistant cornea induced by typhoid endotoxin. No stromal edema is present. Endothelium of this cornea is shown in Figure 7. × 5.

FIG. 10. Section of the opaque cornea shown in Figure 12. The cornea is markedly thickened due to extensive stromal edema. × 5.

5





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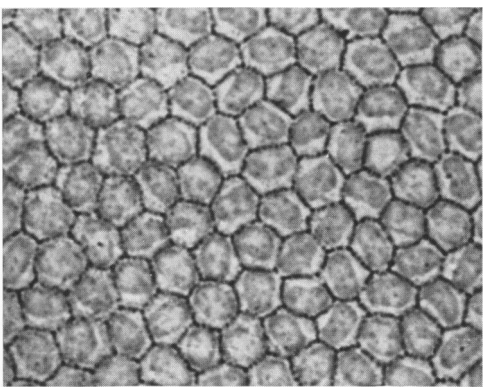

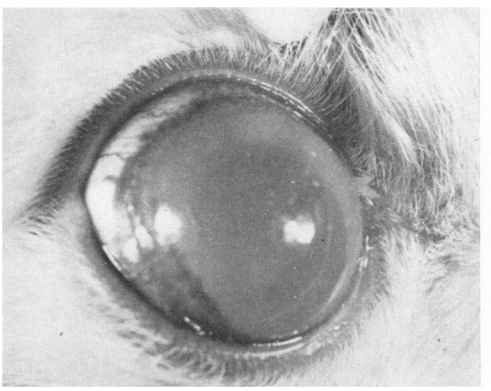

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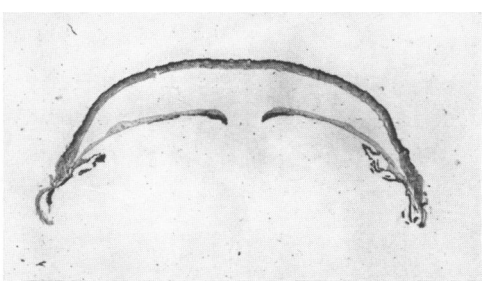

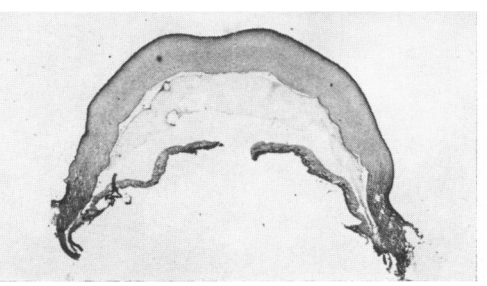

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