Role of Interferon-Like Viral Inhibitor in Endotoxin-Induced Corneal Resistance to Newcastle Disease Virus

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ABSTRACT

OH, J. O. (University of British Columbia, Vancouver, B.C., Canada), AND E. J. GILL. Role of interferon-like viral inhibitor in endotoxin-induced corneal resistance to Newcastle disease virus. J. Bacteriol. 91:251–256. 1966.—A state of marked resistance to the toxic corneal effects of Newcastle disease virus (NDV) was observed in rabbit eyes after intravenous injection of 10 or 100 μg of typhoid endotoxin. By use of tissue cultures of rabbit corneal endothelial cells for assay, high titers of an interferon-like viral inhibitor were detected in serum and in ocular aqueous humor of these animals. The pretreatment of eyes with aqueous humor or serum containing the inhibitor markedly suppressed the production of corneal toxicity by NDV. The intravenous injection of 1 μg of the endotoxin had a negligible effect on the corneal reaction, and little or no inhibitor was found in either serum or aqueous humor. Normal aqueous humor or serum contained no inhibitor and had no suppressive effect on the NDV-induced reactions. The results indicated that the inhibitor played an important role in the induction of corneal resistance to NDV in vivo. The inhibitor in aqueous humor of endotoxin-injected rabbits was found to be derived from blood after an increase in the permeability of "blood-aqueous barrier" of iris due to the endotoxin. Therefore, intravenously administered typhoid endotoxin induced corneal resistance to NDV in rabbits through its dual action on host: (i) release of an interferon-like viral inhibitor into the blood stream and (ii) disruption of the blood-aqueous barrier of the iris, thus allowing the passage of the viral inhibitor from blood into the anterior chamber, where it modified the corneal endothelial cells to render them resistant to NDV.

When a high concentration of influenza, western equine encephalitis (WEE), mumps, or Newcastle disease virus (NDV) is inoculated into the anterior chamber of the rabbit eye, there results a toxic reaction characterized by corneal opacity (1, 4, 5, 17, 20) and characteristic microscopic lesions of corneal endothelial cells (16, 20). No evidence of increase in infective virus is associated with the above reaction. In a recent study, gram-negative bacterial endotoxins were found to suppress the corneal reaction induced by NDV (16). The study also suggested that the corneal resistance might be induced through a modification of the corneal endothelial cells by an active factor of protein nature in aqueous humor.

The objectives of the present study were to gain more definite evidence for the presence of an active factor in aqueous humor of rabbits injected with typhoid endotoxin and to define further the mechanisms by which the intravenous injection of the endotoxin induced corneal resistance to the toxic effect of NDV in rabbits.

MATERIALS AND METHODS

Rabbits. White New Zealand rabbits of both sexes, weighing 2 to 2.5 kg, were used for all experiments except in the tissue culture of corneal endothelial cells. Here, 3- to 4-kg rabbits of mixed sex were the source of cells.

Typhoid endotoxin. The same batch of typhoid endotoxin as used in a previous investigation (16) was utilized in the present studies. This was an alcohol-precipitated, ether-extracted endotoxin of Salmonella typhosa (TO-901), and was kindly supplied by E. Neter, The Children's Hospital, Buffalo, N.Y. A stock solution was made by dissolving endo-
toxin in nonpyrogenic sterile saline (Baxter, Alliston, Ontario, Canada) and filtering through a Swinnin filter. It was stored at −20°C. Prior to use, dilutions to the desired concentration were made with nonpyrogenic sterile saline.

**Viruses.** The sources of the L-Kan 1948 strain of NDV and the PR8 strain of influenza A virus were described in a previous paper (16). They were grown in the allantoic cavity of 10-day-old embryonated chick eggs. The Indiana strain of vesicular stomatitis virus (VSV) was furnished by A. F. Howatson, University of Toronto, and was grown in mouse fibroblast cells (L strain). Western equine encephalitis (WEE) virus and a recent isolate of herpes simplex virus were obtained from D. M. McLean, Hospital for Sick Children, Toronto, Canada, and from W. L. Parker, University of Manitoba, respectively. Herpes simplex virus was passaged in primary cultures of rabbit kidney cells, and WEE virus was passed in the brains of newborn mice. Vaccinia virus was recovered from glycerinated vaccinia lymph (Connaught Laboratory, Toronto, Canada) through inoculation on the chorioallantoic membrane of 10-day-old chick embryos.

**Cultivation of rabbit corneal endothelial cells.** The technique for cultivation of rabbit corneal endothelial cells has been described elsewhere (15). Under the dissecting microscope, Descemet's membrane with the endothelial cells was peeled from a piece of the cornea. This was transferred to a cover slip (6 by 22 mm) which had been previously coated with rabbit plasma. The plasma was clotted by the addition of 1 to 2 drops of chick embryo extract. These preparations were then added to cultures containing 1 ml of culture medium consisting of medium 199 (Difco) with 10% heat-inactivated calf serum (Microbiological Associates, Inc., Bethesda, Md.), 100 units of penicillin, 100 μg of streptomycin, and 20 μg of nystatin. The tubes were plugged with silicone rubber stoppers and were incubated at 37°C. The endothelial cells commenced migration and proliferation in 1 to 2 days. A large confluent sheet of cells was noted after 6 to 10 days and had an average diameter of 0.5 cm. Cultures 6 to 10 days old were used in the experiments.

**Collection of serum and aqueous humor from rabbits.** Blood was obtained aseptically by cardiac puncture without anesthesia. After incubation at 37°C for 1 hr, serum was collected immediately for titration of the inhibitor. Both intravenous injection of Nembutal (Abbott Laboratories, Montreal, Que., Canada) and topical application of Pontocaine Hydrochloride (Winthrop Laboratories, Aurora, Ont., Canada) to cornea were used to anesthetize rabbits from which aqueous humor was collected. A 26-gauge needle attached to an empty 0.5-ml disposable syringe (Beckton, Dickenson & Co., Clarkson, Ont., Canada) was introduced into the anterior chamber through the cornea, and 0.2 ml of blood-free aqueous humor was aspirated. Aseptic technique was observed during the procedure. For titration of the inhibitor, the aqueous humor from both eyes of each rabbit was pooled and diluted to a desired concentration with the culture medium immediately after aspiration.

**Intraocular inoculation of virus.** The techniques for injection of the inoculum into the anterior chamber of the eye were those described previously (17).

**RESULTS**

**Protection of tissue cultures of corneal endothelial cells against NDV by pretreatment with serum of endotoxin-injected rabbits.** The results of a previous in vivo study (16) indicated the presence of an active factor in blood and aqueous humor of endotoxin-injected rabbits. The factor was thought to induce a resistance in rabbit corneas against the toxic effects of NDV by modifying the corneal endothelial cells. In this experiment, attempts were made to detect such a factor in the serum of endotoxin-injected rabbits by use of tissue cultures of corneal endothelial cells. In each experiment, 100 μg of typhoid endotoxin was injected into the ear veins of each of two rabbits, and blood was obtained 3 hr later. Amounts of 1 ml of serial twofold dilutions of pooled serum in the culture medium were overlaid on sets of rabbit corneal endothelial cell cultures in Leighton tubes and incubated at 37°C for various lengths of time. At the end of each incubation period, the culture medium was decanted, the cultures were washed once with 2 ml of phosphate-buffered saline (PBS) at pH 7.2 and were inoculated with 1 ml of the medium containing 10 TCID50 of NDV (10 plaque-forming units). After 24 hr of stationary incubation at 37°C, the cytopathic effects (CPE) of NDV, as described elsewhere (15), were recorded. As controls, sets of corneal endothelial cell cultures were inoculated with serial twofold dilutions of normal rabbit serum and challenged with NDV in the same manner as in the test. Inhibitory titers, expressed as units per milliliter, were determined as reciprocals of the highest dilutions of sera which inhibited CPE completely.

Although treatment of cells with normal rabbit serum induced no protective effect, suppression was observed as early as 3 hr after exposure of the cells to serum of endotoxin-injected rabbits (Fig. 1). A maximal degree of suppression (320 units per ml) was obtained after 24 hr incubation of the cells with the serum. The effect declined gradually as the incubation period was prolonged, and 40 units of inhibitory activity was detected in the cells incubated with serum for 8 days prior to NDV inoculation. Therefore, 24-hr incubation was adopted in the succeeding experiments. The majority of the resistant cells were found to be viable by a neutral red staining technique. Simultaneous inoculation of both virus and diluted serum of endotoxin-injected rabbits showed no suppression of CPE in the endothelial cell cultures. Pretreatment of cells with either 100 μg of the endotoxin or a mixture of normal rabbit
Fig. 1. Effect of time of virus challenge on the inhibitory activity of endotoxin-injected rabbit serum.

serum and the endotoxin failed to suppress the virus effect.

The results confirmed the presence in serum of endotoxin-injected rabbits of an inhibitor for NDV which rendered corneal endothelial cells resistant after an adequate incubation period.

Presence of inhibitor for NDV in aqueous humor of endotoxin-induced NDV-resistant corneas. A previous in vivo study (16) showed that corneal resistance to NDV was observed in all rabbits as early as 3 hr after a single intravenous injection of 100 μg of typhoid endotoxin and 24 hr after injection with 10 μg. Only a slight suppressive effect was observed in rabbits which had received 1 μg. Since the aqueous humor of NDV-resistant corneas might contain an inhibitor some time after endotoxin injection, the correlation between the presence of the inhibitor in aqueous humor as well as serum and the induction of corneal resistance to NDV was studied.

Groups of three or four rabbits were injected intravenously with 100, 10, or 1 μg of typhoid endotoxin. At various times the aqueous humor and blood were collected separately from each animal, and titers of inhibitor were determined as described in the preceding section.

As shown in Fig. 2, a viral inhibitor was present in both aqueous humor and serum in all rabbits 1.5, 3, and 6 hr after intravenous injection of 100 μg of endotoxin. Inhibition appeared to be greatest 1.5 and 3 hr after the injection and almost completely disappeared from both aqueous humor and serum in 12 hr. With 10 μg, the inhibitor was demonstrated in both aqueous humor and serum at 1.5 and 3 hr. In no case was the titer of the inhibitor in aqueous humor higher than that in the serum. Corneal resistance to NDV was observed in all rabbits at 1.5, 3, 6, and 12 hr after injection of 100 μg of endotoxin and 12 hr after injection of 10 μg. Although aqueous humor of the 12-hr groups showed no inhibiting effect in vitro, corneal resistance to NDV was demonstrated in these rabbits. In rabbits injected with 1 μg of endotoxin, neither a significant degree of corneal resistance nor the presence of an inhibitor was detected in aqueous humor, although two of four rabbits developed a low titer of inhibitor in serum 1.5 hr after the endotoxin injection.

Induction of corneal resistance in vivo by viral inhibitor.

Endotoxin serum was obtained from the rabbits 1.5 hr after intravenous injection of 100 μg of
typhoid endotoxin. A 0.2-ml amount of endotoxin serum was inoculated into the anterior chamber of the right eye, and 0.2 ml of normal rabbit serum was injected into the left eye of the same rabbit after the withdrawal of the same volume of aqueous humor from each eye. At various intervals, a toxic dose of NDV (10^6 plaque-forming units) was then inoculated into the anterior chamber, and corneal opacity was recorded 20 to 24 hr later. The viral inhibitor in both sera was also titrated in tissue cultures of rabbit corneal endothelial cells in the manner described previously.

As shown in Table 1 (group 1), the pretreatment of eyes with endotoxin serum containing 32 to 128 units of the inhibitor suppressed the NDV-induced corneal opacity in most animals, whereas normal rabbit serum which lacked the inhibitor failed to induce resistance. The resistance appeared in half of the eyes as early as 3 hr after the treatment with endotoxin serum and in almost all eyes after 6 hr.

Although intravenously injected endotoxin is rapidly removed from circulating blood, a trace amount of endotoxin may remain in the blood for some time. Also, injection of 1 μg of typhoid endotoxin into the anterior chamber was shown to induce a marked resistance to NDV-induced corneal opacity in rabbits (16). Therefore, the suppressive effect of endotoxin serum on the NDV-induced corneal opacity, observed in the preceding experiment, might be due to the local action of residual endotoxin present in the serum, rather than being caused by the inhibitor. To test this possibility, the following experiment was carried out. The endotoxin-induced viral inhibitor was found to be completely inactivated by heatt at 70 C for 1 hr, whereas the antiviral activity of endotoxin resisted this temperature. If residual endotoxin in serum was responsible for the induction of the resistance in the eyes pretreated with unheated endotoxin serum, the eyes treated with heated endotoxin serum would be as resistant to NDV as the eyes treated with unheated endotoxin serum. Endotoxin serum was obtained from endotoxin-injected rabbits in the same manner as in the preceding experiment. The left eyes of all rabbits were treated with unheated endotoxin serum containing inhibitor and the right eyes with heated endotoxin serum. At various intervals, both eyes were inoculated with 10^6 plaque-forming units of NDV. The results (Table 1, group 2) showed that the eyes pretreated with heated endotoxin serum failed to resist NDV-corneal reaction, whereas the majority of the eyes pretreated with unheated serum were completely protected from the viral effects, indicating that residual endotoxin in the serum, if any, played no part in the induction of resistance.

The eyes pretreated with endotoxin aqueous humor containing the inhibitor were also resistant to the virus (Table 1, group 3). The endotoxin aqueous humor was obtained from rabbits 3 hr after intravenous injection of 100 μg of typhoid endotoxin. Aqueous humor of normal rabbits contained no inhibitor and had no suppressive effect.

Properties of the viral inhibitor. The properties of the endotoxin-induced viral inhibitor were studied with pooled rabbit serum (titer of 320) and pooled aqueous humor (titer of 80) obtained 3 hr after intravenous injection of 100 μg of typhoid endotoxin. The properties of the inhibitor in both serum and aqueous humor were identical. The protective effect against NDV was partially abolished by heating at 56 C for 1 hr and completely abolished by heating at 70 C for 1 hr. Its activity was greatly reduced by incubation with crystalline trypsin at a concentration of 0.01%, for 1 hr at 37 C. The titer was reduced to one-eighth of the original titer after the serum or aqueous humor was kept at pH 2.0 for 1 hr. All inhibitory activity remained in the superna-

<table>
<thead>
<tr>
<th>Group</th>
<th>Pretreatment of cornea with</th>
<th>In vitro inhibitory titer (NDV)</th>
<th>Length of pretreatment (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Endotoxin serum</td>
<td>32–128</td>
<td>3/6*</td>
</tr>
<tr>
<td></td>
<td>Normal serum</td>
<td>&lt;10</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7/8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7/8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5/7</td>
</tr>
<tr>
<td>2</td>
<td>Endotoxin serum</td>
<td>16–64</td>
<td>3/7</td>
</tr>
<tr>
<td></td>
<td>Heated endotoxin serum (70 C, 1 hr)</td>
<td>&lt;10</td>
<td>7/7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7/7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8/8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ND†</td>
</tr>
<tr>
<td>3</td>
<td>Endotoxin aqueous humor</td>
<td>16–32</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>Normal aqueous humor</td>
<td>&lt;10</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5/7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4/6</td>
</tr>
</tbody>
</table>

* Number of toxic corneas per number of corneas inoculated with NDV.
† Not done.
Among the viruses tested, the inhibitor is most effective in suppressing CPE produced by NDV and less effective against CPE caused by influenza, VSV, and WEE viruses. This may explain our previous in vivo findings that endotoxins produced a cornea completely resistant to NDV but failed to suppress the toxic corneal effect of influenza virus (16). This also suggests that NDV is the virus of choice for detecting the endotoxin-induced inhibitor in rabbits.

The possible role of nonspecific serum-inactivating substances for viruses (8, 13) in the induction of resistance in corneal endothelial cell cultures can be ruled out by the following observations: (i) cultures pretreated with serum were washed with PBS prior to virus inoculation; (ii) simultaneous inoculation of both NDV and either endotoxin-injected rabbit serum or normal rabbit serum produced as much extensive CPE as that in cultures inoculated with NDV alone; and (iii) cultures pretreated with normal rabbit serum failed to resist NDV. Role of nonspecific serum-inactivating substance for viruses in endotoxin-induced corneal resistance in vivo was already ruled out in a previous study (16).

The study has shown a good correlation between the presence of a viral inhibitor in aqueous humor and the induction of corneal resistance to NDV in endotoxin-injected rabbits. It suggests that the inhibitor in aqueous humor plays an important role in the induction of corneal resistance to viruses in such animals. This view is strengthened by the finding that pretreatment of eyes with serum or aqueous humor containing the inhibitor suppressed the production of corneal opacity by NDV.

The inhibitor in aqueous humor appears to be derived from the blood, since properties of the inhibitor in both serum and aqueous humor are identical and the titer of the inhibitor in aqueous humor is never higher than that in serum of the same rabbit at any time. The results of a previous study (16) also suggested the hematic origin of the viral inhibitor in aqueous humor.

The aqueous humor originates from the ciliary processes of the iris. Normally, the membrane in the ciliary processes separating the blood from the aqueous humor exhibits a high degree of selectivity. The intravenous injection of either 100 or 10 μg of typhoid endotoxin was shown to break this "blood-aqueous barrier" and to permit the passage of rather large molecules, such as protein, from the blood into aqueous humor; 1 μg of the same endotoxin failed to do so (16). This is in complete agreement with the results of the present study that, although the viral inhibitor was induced in sera by intravenous injection of 100, 10, or 1 μg of typhoid endotoxin, the inhibitor was demonstrated in the aqueous

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**Table 2. Comparative inhibitory effect of pooled serum and aqueous humor of typhoid endotoxin-injected rabbit on the production of CPE by various viruses**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Comparative inhibitory titer Serum</th>
<th>Aqueous humor</th>
</tr>
</thead>
<tbody>
<tr>
<td>NVD</td>
<td>100*</td>
<td>100*</td>
</tr>
<tr>
<td>Influenza A</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>VSV</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>WEE</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Herpes simplex</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*The pooled serum and aqueous humor were obtained 3 hr after intravenous injection of 100 μg of typhoid endotoxin. The serum or aqueous humor was serially diluted and incubated for 24 hr with the corneal endothelial cell tissue cultures before inoculation of 10 τCD₉₀ of each virus. The resulting titers were expressed in comparative values to illustrate the sensitivity of the various viruses to the inhibitor.

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**Discussion**

In the present study, a viral inhibitor was demonstrated in both serum and aqueous humor of rabbits inoculated intravenously with 100 or 10 μg of typhoid endotoxin. The characteristics observed are similar to those of interferon (12) with the exception of acid sensitivity. The presence of "sparing factor" in the serum of mice inoculated with endotoxin was reported by Gledhill (9, 10). An interferon-like inhibitor was recently demonstrated by Ho (11) in the serum of rabbits which were inoculated with *Escherichia coli* endotoxin or live *E. coli*, and by Stinebring and Youngner (18) in the plasma of mice with *E. coli* endotoxin or live bacteria. The properties of these inhibitors are identical to those of the inhibitor induced in rabbits by typhoid endotoxin.
humor only of those injected with 100 or 10 μg. Therefore, the inhibitor in the blood is apparently brought into the anterior chamber after the breakdown of the barrier by the endotoxin.

The evidence indicates that the mechanisms by which typhoid endotoxin induces corneal resistance to virus involve at least two steps: (i) the intravenous injection of endotoxin induces the interferon-like viral inhibitor in blood; and (ii), by disrupting the blood-aqueous barrier of the iris, it allows the escape of the inhibitor of the blood into the anterior chamber to act on and modify the target cells for the virus, the corneal endothelial cells.

The present investigation emphasizes the importance of changes of permeability in determining the protective effect of endotoxin against the toxic corneal reaction induced by NDV. It also provides evidence for the passage of the viral inhibitor from the blood stream to the extraocular site. Since endotoxins increase the vascular permeability, particularly in lungs and gastrointestinal tracts (7), and also disrupt the “blood-brain barrier” (3), the inhibitor may be present in the perivascular tissues of these organs as well as in the blood of endotoxin-injected animals and may render the tissues resistant to viral infection. Therefore, it will be of interest to study whether the suppressive effect of endotoxins on certain viral infections (2, 6, 10, 14, 19) can be explained by the above mechanisms.

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LITERATURE CITED