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MODE OF ACTION
OF AN INHIBITOR FROM AGAR
ON GROWTH AND HEMAGGLUTINATION
OF GROUP A ARBOVIRUSES

APRIL 1963

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK
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ABSTRACT

A polysaccharide obtained from agar inhibits growth or plaque formation by the equine encephalitis viruses in liquid or solid medium. When free EEE virus is mixed with the inhibitor and incubated at 37°C, its capacity to form plaques and to agglutinate goose red blood cells is reduced.

The evidence presented suggests that the mode of action of inhibition by the polysaccharide is through direct combination with the free virus particles. Evidence is also presented that shows an inhibition of virus growth that occurs as a result of interaction between the polysaccharide and chick fibroblast cells.

Takemori and Nomura showed that minute plaque mutants of poliovirus were inhibited by agar extracts and that the inhibitory factor was responsible for the failure of these mutants to develop normal-sized plaques. Takemoto and Liebhaber showed with encephalomyocarditis virus that a sulfated polysaccharide present in agar inhibited the growth of minute plaque-forming strains but had no effect on strains that form large plaques. Schulze and Schlesinger also showed inhibition of growth of Dengue-2 virus by a similar polysaccharide. This report is concerned with similar findings with the equine encephalitis viruses and with the probable mode of action of the inhibitor in this group of viruses. Viral activity, determined by growth in chick fibroblast monolayers and by hemagglutination with goose red blood cells, is markedly suppressed by an agar extract prepared by the method of Takemoto and Liebhaber. The experiments to be described here suggest that the inhibitor, besides acting by direct combination with the virus particles, might also have another mode of action that involves the host cells.

Chick embryo fibroblast monolayers grown in lactalbumin hydrolysate medium containing 10 per cent calf serum were used throughout this study. Three members of the group-A arboviruses were used: eastern, western, and Venezuelan equine encephalitis viruses. The virus assays were performed by the plaque method, employing chick embryo fibroblast monolayers grown in 60-millimeter plastic Petri dishes that were seeded with an initial cell concentration of 20 x 10^6 cells per plate. Cultures were grown at 37°C in a humidified incubator containing 95 per cent air and 5 per cent carbon dioxide. The virus was diluted in beef heart infusion broth, and each plate was inoculated with 0.1 milliliter of diluted virus suspension. After an adsorption period of 20 minutes, the inoculum was removed and each plate received 5 milliliters of agar overlay consisting of Hanks' balanced salt solution, 0.1 per cent yeast extract, 0.5 per cent
gelatin, 0.5 per cent lactalbumin hydrolyzate, 1.1 per cent Noble agar, 0.14 per cent sodium bicarbonate, and 500 micrograms of DEAE-dextran. After 48 hours the plates were stained with neutral red (2 ml of a 1:8000 solution) and the plaques were counted. The aqueous agar extract was prepared by the method described by Takemoto and Liebhaber and will be referred to hereafter as inhibitor or polysaccharide.

The data in Figure 1 show the effect of inhibitor on the growth of western equine encephalitis (WEE) virus. Plates were inoculated with WEE virus at a multiplicity of 0.0001. After adsorption of virus, the monolayers were washed with saline A, and 5 milliliters of lactalbumin hydrolyzate medium containing 1.2 milligrams of inhibitor per milliliter were added. As a control, another group of cultures received 5 milliliters of lactalbumin hydrolyzate medium. At the indicated intervals the supernatant was removed from plates in both groups and assayed. Only a very small difference was observed in the amount of virus released during the first cycle of growth in the two groups of plates, but a marked inhibition of further virus production was observed in the monolayers grown in medium containing the inhibitor. This inhibition, as it will be shown later, was the result of direct combination of the inhibitor to the free virus, rendering the virus unable to penetrate the cells. It was possible to recover at least part of the inhibitor-coupled virus in an infectious form by incubating it with DEAE-dextran. Intracellular virus content of control and experimental cultures has been shown to have the same infectivity quantitatively. The suppression of viral infectivity was clearly evident in the tissue culture fluid. Since only a slight inhibition was observed during the first virus production, it could be concluded that the inhibitor does not influence replication of the virus once it has been adsorbed.

The effect of the inhibitor on suppression of growth of eastern equine encephalitis (EEE) virus and its reversal by DEAE-dextran is shown in Figure 2. The virus was adsorbed for 20 minutes and the plates were washed with saline A. Medium containing inhibitor, or inhibitor plus DEAE-dextran, was added to the monolayers. Appropriate controls without inhibitor were included in the experiment. The amount of DEAE-dextran used completely reversed the inhibition by the agar extract. The curves for both controls were identical, but only one is shown. In parallel experiments with Venezuelan equine encephalitis virus (VEE), which forms large plaques on monolayers overlayed with agar, the inhibitor at the concentration used did not influence virus multiplication as measured by virus release. A mutant of VEE virus (10t) that forms only small plaques without DEAE-dextran was inhibited by the polysaccharide. The difference in plaque size is therefore due to the presence of inhibitor in agar. These data also indicate that inhibitor combines with the small-plaque formers and not with large-plaque formers. Evidence to support this conclusion will be shown later.

The effect of the inhibitor on free EEE and VEE viruses was tested by two assay methods, plaque titration and hemagglutination. Stock virus preparations were diluted to a concentration of approximately 10⁷ plaque-forming units per milliliter. Various concentrations of inhibitor were
Figure 1. Effect of Inhibitor on Growth of VEE Virus Adsorbed Prior to Addition of Inhibitor.
Figure 2. Effect of Inhibitor on Growth of Adsorbed EEE Virus, and Reversal of Inhibition by DEAE-Dextran.
added and samples for titration were taken at zero time and after one hour at 37°C. The virus samples were diluted in heart infusion broth to eliminate the effect of the inhibitor and then were assayed in chick fibroblast monolayers. For control purposes, virus samples without polysaccharide were similarly treated and titrated at the times indicated in Table I. Incubation of the virus with the polysaccharide brought about a two-log drop in titer at the end of one hour with the lowest concentration tested. At higher concentrations (1.0 to 2.5 mg per ml) 99.99 per cent reduction of plaque-forming units was observed. There was no reduction in titer at zero time with inhibitor prepared by either the Takemoto⁹ or Schulze and Schlesinger³ method. These results are not in agreement with those obtained by Schulze and Schlesinger with Dengue-2 virus. Those authors obtained an immediate reduction in titer in the presence of inhibitor and the rate of later inhibition paralleled that of the untreated virus. Similar experiments with VEE virus, which forms large plaques under agar and is resistant to the inhibitor in liquid medium, revealed that a significantly higher concentration (fourfold) of inhibitor was required to obtain any reduction in titer after a one-hour incubation.

The hemagglutination reaction between the equine encephalitis viruses and goose red blood cells involves specific attachment of the virus to the red blood cells. It was of interest to test the effect of the inhibitor on the hemagglutination reaction of this group of viruses. Goose red blood cells were collected in Alsevers solution and were washed five times with dextrose-gelatin-Veronal buffer, pH 7.2. A stock solution of 12.5 per cent by volume was prepared that was equal to 17,500 cells per cubic milliliter. The cells were diluted (1:95) in phosphate-buffered saline for the micro-assay. The procedure of Clarke and Casals⁴ was followed. The final pH of the reaction was 6.35. In the experiment shown in Figure 3, the goose red blood cells were suspended in different concentrations of inhibitor and used in the test. The data shown that the hemagglutination by VEE virus was least affected, followed by WEE virus and EEE in increasing order, similar to the growth and plaque formation inhibition pattern. An increase in the inhibition of hemagglutination by the polysaccharide was observed when the antigen was incubated with the inhibitor at 37°C for 1½ hours prior to the addition of the goose red blood cells. The interpretation of the reversal of the hemagglutination inhibition by the polysaccharide with DEAE-dextran was difficult because DEAE-dextran mixed with the inhibitor agglutinated the goose red blood cells in the absence of antigen.

Groups of chick fibroblast monolayer cultures were treated with inhibitor (1.2 mg per ml) for one hour. One group of monolayers was washed three times with saline A; another, two times with DEAE-dextran (100 µg per ml) and once with saline A; and a third group two times with protamine sulfate (salmine, 100 µgs per ml) and once with saline A. Controls not treated with inhibitor received three washes with saline A. Plates were then inoculated with EEE virus, washed one time with saline A after adsorption for 20 minutes, and then were incubated at 37°C after adding five ml of lact-calf medium. As may be seen in Table II, suppression of growth was clearly evident at six and at ten hours.
### TABLE I. REDUCTION IN PLAQUE-FORMING TITER OF EEE VIRUS TREATED WITH AGAR EXTRACT AT 37°C

<table>
<thead>
<tr>
<th>Concentration of Inhibitor, mg per ml</th>
<th>PFU b/ per ml at 37°C</th>
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<tbody>
<tr>
<td></td>
<td>0 min.</td>
</tr>
<tr>
<td>0</td>
<td>$10^6.7$</td>
</tr>
<tr>
<td>0.5</td>
<td>$10^6.6$</td>
</tr>
<tr>
<td>1.0</td>
<td>$10^6.5$</td>
</tr>
<tr>
<td>2.5</td>
<td>$10^6.4$</td>
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a. Tissue culture EEE virus diluted $10^{-3}$ in beef heart infusion broth (BHIB) was mixed with the desired concentration of inhibitor incubated at 37°C and diluted further in BHIB at the indicated time.

b. PFU—plaque-forming units.

### TABLE II. ADSORPTION OF THE INHIBITOR TO CF CELLS IN MONOLAYERS AND ITS EFFECT ON EEE VIRUS GROWTH

<table>
<thead>
<tr>
<th>MONOLAYERS</th>
<th>VIRUS TITER OF SUPERNATANT FLUID, Plaque-forming units per ml</th>
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<tbody>
<tr>
<td>Control (untreated)</td>
<td>$1.1x10^6$</td>
</tr>
<tr>
<td>Inhibitor-Treated b/</td>
<td>$1.3x10^6$</td>
</tr>
<tr>
<td>Washed with Saline A</td>
<td>$6.3x10^6$</td>
</tr>
<tr>
<td>Washed with DEAE-Dextran</td>
<td>$2.8x10^6$</td>
</tr>
</tbody>
</table>

a. Monolayers were incubated with lact-calf medium containing 0.6 mg inhibitor per ml at 37°C for one hour.

Inoculum $10^3$ pfu EEE virus per monolayer

At 24 hours titer was the same in control and experimental samples.
Figure 3. Effect of Inhibitor on Hemagglutination by the Equine Encephalitis Viruses. Goose red blood cells were suspended in the indicated dilutions of the inhibitor and used in the test.
At 24 hours after virus infection, no inhibition was detected. These results could be interpreted in two ways: (a) the inhibitor, which undoubtedly has firmly attached to the cells, binds the virus and thus inhibits its penetration, or (b) the inhibitor destroys or blocks cell receptor sites essential for virus penetration. Titrating of the intracellular virus revealed that there was less infectivity in intracellular virus from samples containing inhibitor than in the controls. This and other experiments failed to eliminate the possibility that cell receptors are blocked by the inhibitor. There is no question that the inhibitor does bind to the virus as shown here for EEE virus and elsewhere by Schulze and Schlesinger for Dengue-2 virus.

It has been shown here that the polysaccharide from agar interacts with free virus particles and inhibits the infectivity and hemagglutinating property of the virus. It was also shown that interaction between the inhibitor and chick fibroblast cells was manifested by suppression or delay of virus multiplication. It is not known whether the action of the inhibitor in this case is on the free virus, on the cell, or possibly on both. It is suggested that the inhibitor attaches to specific sites of the virus envelope, since viruses resistant to the inhibitor do not interact with it.

