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Received October 28, 1963. P.S.E.B.M., 1964, v116.

Inhibition of Herpes Virus by Natural and Synthetic Acid Polysaccharides. (29183)

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A synthetic sulfated polysaccharide, sodium dextran sulfate, has been reported by this laboratory to inhibit the multiplication of certain viruses (EMC, ECHO, Coxsackie A9, and herpes) while other viruses (type 1 poliovirus and variants from attenuated types 1 and 2 poliovirus) not only were resistant but their plaque-forming ability was enhanced by the presence of the acid polysaccharide in the medium(1). Later Nahmias and Kibrick(2) reported that heparin, a naturally occurring sulfated polysaccharide, inhibited several strains of herpes virus. Our original observations on the inhibition of herpes virus by dextran sulfate have been extended and the nature of the inhibition as well as its reversal by basic polymers are included here. The effects on herpes virus growth of heparin, chondroitin sulfate, and the sulfated polysaccharide from agar are also described.

Materials and methods. Cell cultures. HeLa cells were grown in Eagle's medium supplemented with 10% bovine serum. Procedures for plaque assays in these cells as well as the medium employed were the same as previously described(3).

Virus. Herpes virus was originally obtained from Dr. A. S. Kaplan who studied its growth in rabbit kidney cultures(4). Plaque counts of this virus were found to be increased 5- to 10-fold when DEAE-dextran(6) was added to the agar overlay, indicating that inhibitor-sensitive particles which were incapable of forming plaques in untreated overlay medium were present in this original virus stock. In addition, when this stock virus was plated in untreated agar overlay, plaques of 2 sizes were observed. These were plaque-purified for use in the experiments described below, and designated Lpf (large plaque former) and Mpf (minute plaque former). The Lpf virus induces polykaryocyte formation while the Mpf virus causes cell rounding and clumping; in this respect, they correspond to Roizman's Mp and mp strains(5). Since they were selected for their ability to form plaques

in the presence of agar inhibitor, both strains plated with equal efficiency in DEAE-dextran treated and the untreated agar overlays.

Acid polysaccharides. The following sulfated polysaccharides were tested for anti-herpes activity by methods described below: Sodium dextran sulfate 2000 (Pharmacia, Uppsala, Sweden), heparin and chondroitin sulfate (Calbiochem, Los Angeles, Calif.) and sulfated agar polysaccharide, prepared in this laboratory as previously described(3).

Inhibition tests. Anti-viral activity of the compounds was tested by 3 different methods:

1. *Plaque inhibition.* Monolayer cultures were inoculated with varying numbers of plaque forming units (pfu's) of virus and after a 2 hour adsorption period, plates were overlaid with agar medium containing the test compound at the desired concentrations. After staining, the plaques were counted and examined for inhibition of plaque development.

2. *Adsorption inhibition test.* Known numbers of pfu of virus were mixed with the desired concentration of test compound and inoculated into cell cultures. After 2 hours adsorption, the plates were washed free of unadsorbed virus and compound, and standard agar overlay medium added. In this type of test, the number of plaques formed was a direct measure of the number of virus particles adsorbed in the presence of the test compound.

3. *Inhibition of cytopathic effects (CPE).* Cultures were infected at low multiplicity (0.001) and after a 2 hour adsorption period, medium containing the test compound was added. In this test, inhibition of CPE together with a decrease of virus yield were the criteria of effectiveness of the test compound.

Results. Plaque inhibition tests. Cultures were infected with virus concentrations ranging from 50 to 50,000 pfu of virus and after 2 hours' adsorption, they were overlaid with medium containing 0.333 mg/ml of the test compound. After 4 days' incubation, a second overlay containing 1/40,000 neutral red was added and plaques counted on the 5th and 7th days. Of the 4 acid polysaccharides tested (Table I), only dextran sulfate inhibited plaque formation of both strains of herpes virus. Heparin, chondroitin sulfate, and excess agar polysaccharide (added in addition to that already present in the agar) had no effect on either plaque size or number. Fig. 1 shows the almost total inhibition of plaque formation by the Mpf strain caused by dextran sulfate. The minimal effective concentration of dextran sulfate was determined to be between 25 and 50 γ/ml.

TABLE I. Effect of Acid Polysaccharides in Overlay Medium on Plaque Formation of Lpf Herpes Virus.*

Polysaccharide†	pfu/ml‡
Dextran-SO_4	$<10^2$
Heparin	21.5×10^6
Agar	20×10^6
Chondroitin-SO_4	23.5×10^6
Control	25×10^6

* Similar results obtained with Mpf virus.
† 0.333 mg/ml in overlay.
‡ Plaque count at 7 days.

Inhibition of adsorption of herpes virus by acid polysaccharides. In previous studies, it was shown that acidic polymers interfered with the adsorption of EMC virus to susceptible cells. Similar experiments with both strains of herpes virus were performed as follows: equal volumes of virus dilutions in phosphate buffered saline, pH 7.0 (PBS) and acid polysaccharides (2 mg/ml) were mixed and 0.5 ml of the mixtures were immediately plated on HeLa cell monolayers. Controls consisted of virus mixed with PBS. After 2 hours incubation, cultures were washed free of unadsorbed virus and polysaccharide and normal overlay medium added. Plates were subsequently stained and counted as described. Table II summarizes the results obtained with the Lpf virus and shows that the only compound incapable of interfering with adsorption of herpes virus was chondroitin sulfate; similar results were found

TABLE II. Inhibition of Adsorption of Lpf Herpes Virus by Acid Polysaccharides.

Polysaccharide*	Virus titer (pfu)	% inhibition
Dextran-SO_4	40×10^2	99.8
Heparin	12×10^3	99.5
Agar	73×10^3	97.0
Chondroitin-SO_4	20×10^5	20.0
Control	25×10^5	0

* 0.5 mg/plate.

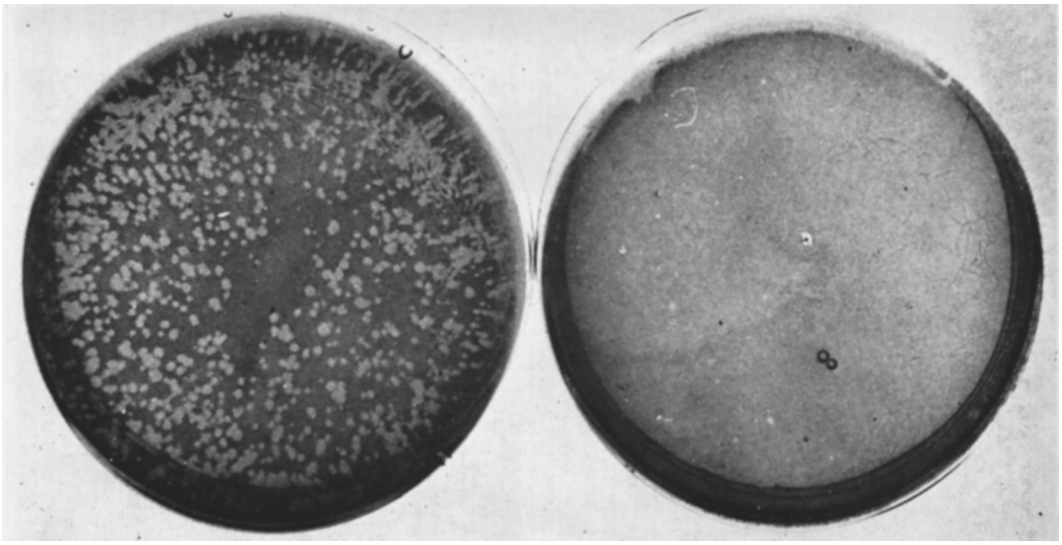

FIG. 1. Plaque inhibition of Lpf herpes virus by dextran sulfate. Control plate (left) with normal overlay and plate inoculated with same number of pfu's of virus but overlaid with medium containing 0.333 mg/ml dextran sulfate.

with the Mpf virus. It is of interest that the results of this experiment showed that while heparin and the agar polysaccharide inhibited adsorption of virus to cells, results of the previous experiment indicated that they have no effect on virus multiplication after adsorption. In addition, basic polymers (DEAE-dextran or protamine) were found to have no effect on adsorption, a finding similar to that reported by Allison and Valentine(7) with fowl plaque and vaccinia viruses.

Reversal of adsorption-inhibition by DEAE-dextran. To test whether virus was irreversibly prevented from adsorbing to cells, the following experiment was performed: A group of monolayer cultures of HeLa cells was inoculated with an average of 150 pfu of Lpf virus; another group of cultures was inoculated with the same number of pfu's of virus mixed with a concentration of dextran sulfate calculated to give complete inhibition (400 γ/ml). After 1 hour incubation at 37°C DEAE-dextran (400 γ/plate) was added to half of the cultures inoculated with the virus-dextran sulfate mixtures; the remainder served as inhibited controls. At varying time intervals 2 plates from each group were washed free of unadsorbed virus and normal overlay medium added. The results are shown in Fig. 2. In plates with dextran sulfate, virus adsorption was completely blocked over a 4 hour period. That this inhibition was reversible was shown by the resumption of viral

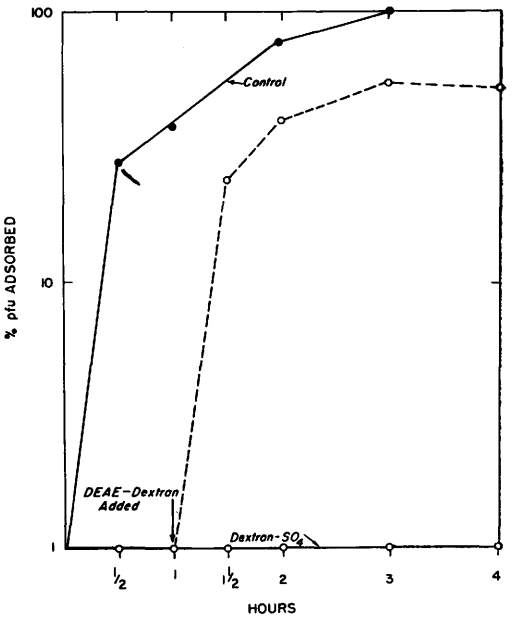

FIG. 2. Reversal of dextran sulfate inhibition of Lpf herpes virus adsorption by addition of DEAE-dextran. (●——●) = normal control adsorption curve, (○- - -○) = adsorption of virus after addition of 400 γ DEAE-dextran, and (○——○) = total inhibition of adsorption by dextran sulfate.

TABLE III. Effect of Various Acid Polysaccharides on Multiple Growth Cycles of Herpes Virus.*

Polysaccharide†	CPE‡	Log_{10} pfu/ml‡	Diff log_{10}
Dextran-SO_4	0	4.5	2.0
Heparin	2+	5.3	1.2
Agar	2+	5.8	0.7
Chondroitin-SO_4	4+	6.5	0
Control	4+	6.5	0

* Lpf virus at 0.001 multiplicity.
† 100 γ/ml in medium.
‡ CPE and virus assays at 48 hr after infection.

adsorption after addition of DEAE-dextran which formed an immediate insoluble complex with the inhibitor. Failure to recover 100% of the input virus may have been due to co-precipitation of the virus. This experiment also indicated that the interaction between inhibitor and virus did not result in an irreversible loss of infectivity.

Inhibition of CPE and viral growth in liquid medium. Monolayer cultures were infected with herpes virus at low multiplicity (0.001) and after 2 hours adsorption, medium containing 100 γ/ml of the various acid polysaccharides was added. Infected control cultures received medium only. The cultures were observed over a 48 hour period at which time they were frozen, thawed, and assayed for infectious virus. Results with both the Lpf and Mpf viruses were similar. The greatest inhibitory effect was noted in the culture containing dextran sulfate where no CPE was seen at 48 hours whereas the control culture was completely destroyed by virus. This inhibition was also reflected in the difference in final virus titers between the two cultures (Table III). Less marked effects were noted with heparin and the agar polysaccharide, while chondroitin sulfate was completely ineffective under these conditions.

Discussion. Several investigators have established that viral antibody does not affect the cell to cell spread of herpes virus when applied after adsorption and penetration of virus. In view of this fact, the marked suppression of plaque development observed with dextran sulfate suggests possible intracellular inhibition of viral growth with this compound. The effects of heparin and the agar polysaccharide, on the other hand, are similar to that of viral antibody; their activity appears to be only on the adsorption of extracellular virus and no effect was observed in the plaque inhibition tests. Unlike antibody, however, they do not possess neutralizing activity and their effects are reversible.

Vaheri and Cantell(8) have recently reported inhibition of herpes virus by heparin and from their experiments also concluded that heparin probably inhibited virus multiplication by preventing the attachment of virus to cells. Since the activity of heparin and the agar polysaccharide is only on free virus, it would be expected that in multicyclic viral growth in liquid medium they would prevent the subsequent adsorption of virus released after the initial growth cycle. The result would therefore be a delay in appearance of CPE with a concomitant reduction in virus yields. The lack of effect with chondroitin sulfate in any of the tests remains unexplained. Nahmias and Kibrick(2) have also reported that chondroitin sulfate (A, B, and C) had no anti-herpes activity.

Although spontaneous mutants which are resistant to dextran sulfate have been observed for poliovirus(1) and foot-and-mouth disease virus(9), such mutants have not been found in the strains of herpes virus used in this study. Repeated attempts to develop dextran sulfate mutants of herpes virus by growing the virus in the presence of the inhibitor have thus far failed.

The high degree of inhibition of herpes virus by dextran sulfate prompted a preliminary investigation on its possible antiviral activity in hosts other than cell cultures. In a few experiments in chick embryos, no effect was seen when virus was allowed to adsorb onto the chorioallantoic membrane for 2 hours, followed by the application of various concentrations of dextran sulfate at the same site. The reasons for the lack of inhibition in this system are unknown at present.

Summary. The effect of 4 acid polysaccharides on 2 variants of herpes virus was investigated by 3 different methods for measuring anti-viral activity. Dextran sulfate inhibited adsorption, plaque formation, and multicyclic viral growth in liquid medium. Heparin and agar polysaccharide inhibited

adsorption and virus multiplication in liquid medium, while chondroitin sulfate was completely ineffective as an inhibitor. No difference was noted in the behavior of the 2 variants with respect to their sensitivity toward the 4 sulfated polysaccharides.

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Received February 12, 1964. P.S.E.B.M., 1964, v116.

A Complement-Fixing Antigen for Varicella-Zoster Derived from Infected Cultures of Human Fetal Diploid Cells.* (29184)

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General application of the complement fixation technic for serologic diagnosis of infections caused by the varicella-zoster (V-Z) virus has been limited by difficulties in obtaining sufficiently potent antigens free from anticomplementary activity.

While fairly potent antigens can be prepared from vesicular fluids(1), the source is too undependable to meet the requirements of the diagnostic laboratory. In early studies on the V-Z virus, Weller and Witton(2) showed that infected tissue cultures contained specific complement-fixing antigen, but concentration of the culture fluids by ultrafiltration was necessary to obtain antigens with sufficient reactivity. Concentrates prepared in this manner were anticomplementary, and while heating abolished this activity, it also reduced the antigenicity. Caunt *et al*(3) prepared complement-fixing antigens to the V-Z virus in cultures of human amnion cells; fluids from infected cultures were concentrated by drying from the frozen state, and the resulting antigens were free from anticomplementary activity.

The procedures described to date for preparation of V-Z complement-fixing antigens from infected cell cultures are cumbersome, involving replacement of the culture medium at relatively frequent intervals after infection as well as concentration of the antigenic material from large volumes of culture fluid. More importantly, despite such concentration the resulting antigens have been of low potency. This report describes a simple method for preparation of relatively high-titered antigens from the cellular phase of infected cultures of human fetal diploid skin and muscle cells.

Materials and methods. Human fetal diploid cell strains. Initiation of cell strains: Cultures of human fetal diploid skin and muscle (HFDSM) cells were initiated from fetuses 2 to 5 months of age. Tissue from the limbs and torso of the fetus was minced and washed in 3 changes of Hanks' balanced salt solution (BSS). Cells were dispersed by overnight trypsinization at 4°C in 200 ml of a 0.25% trypsin solution, pH 7.5. The dispersed cells were centrifuged at 600 rpm for 20 minutes and the trypsin solution was removed. The cells were then resuspended in 40 ml of Hanks' BBS, transferred to a 50 ml graduated, conical contrifuge tube and centrifuged at 600 rpm for 20 minutes. The volume of the cell pack was noted and, after

* Supported by grant from Nat. Inst. of Allergy and Infect. Dis., U.S.P.H.S.