

INHIBITORY EFFECT OF HEPARIN ON HERPES SIMPLEX VIRUS¹

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ABSTRACT

NAHMIA, ANDRÉ J. (Boston University School of Medicine, Boston, Mass.), AND SIDNEY KIBRICK. Inhibitory effect of heparin on herpes simplex virus. *J. Bacteriol.* 87:1060-1066. 1964.—A substance inhibitory to herpes simplex virus was observed during experiments with leukocyte cultures. The component in the cultures responsible for this inhibition was identified as heparin. The minimal inhibitory concentration required to inhibit 30 to 300 TCD₅₀ of the virus in human amnion tissue culture was found to be 1 to 2 units per ml (10 to 20 µg/ml). This effect was confirmed with other strains of herpes simplex virus, other tissue-culture systems, and other media. The inhibitory activity of the heparin was found to be related to the sulfate groupings on the molecule. The effect of heparin appears to be on the virus, rather than on the cell. The virus is not inactivated, however, and the heparin-virus "complex" is readily dissociable on dilution. Heparin was shown to affect viral infection in its earliest phase, probably at the primary electrostatic attachment of virus to cell. The import of these and related observations on common virological laboratory procedures and the possible biological significance of our findings are discussed.

During studies demonstrating the multiplication of herpes simplex virus (HSV) in human leukocytes (Nahmias, Kibrick, and Rosan, *J. Immunol.* *in press*), it was noted that a constituent of the leukocyte cultures had an inhibitory effect on the virus. Experiments leading to the identification of this inhibitory substance as heparin will be presented. Also reported are studies which attempt to elucidate the active grouping on the heparin molecule responsible for its inhibitory property, and observations on the nature of the heparin-virus-cell interaction.

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MATERIALS AND METHODS

Leukocyte and control cultures. Leukocyte cultures were prepared from heparinized blood (50 units of heparin per ml) by the method of Moorhead et al. (1960). This blood was obtained from donors lacking neutralizing antibodies to HSV. Control plasma cultures were prepared with the same constituents, except that 1 ml of the donor's plasma was used instead of the leukocyte-plasma suspension.

Viruses tested. Most of the experimental work was done with a laboratory strain of HSV (Rodanus) which had undergone multiple passages in eggs and primary human amnion tissue cultures. Two other laboratory strains with similar passage histories (Zaccardi and Virtue), the proliferating and giant cell strains of HSV (Scott, McLeod, and Tokumaru, 1961), and four recent isolates from human herpetic infections of skin and cornea were also tested.

Tissue culture and media. Primary human amnion cell cultures were prepared and maintained with bovine amniotic fluid media (Milovanovic, Enders, and Mitus, 1957). For certain experiments, minimal essential medium (MEM) (Eagle, 1959) with 10% calf serum was used. The latter medium was also employed for hamster and rabbit kidney tissue cultures and for a human embryonic kidney diploid strain (MA 10) supplied by Microbiological Associates, Inc., Bethesda, Md.

Titration of virus in leukocyte cultures. Duplicate leukocyte and control plasma cultures were inoculated with 0.2 ml of virus suspension (titer: 10⁵ per 0.1 ml) and incubated at 36 C. At regular intervals, titrations for virus were performed with two or more human amnion cultures per dilution and an inoculum of 0.2 ml per tube.

Estimation of heparin inhibitory effect. For all experiments with heparin, the following preparation was employed unless otherwise stated: Panheprin (no. 2945; Abbott Laboratories, North Chicago, Ill.), 20,000 USP units per ml (100 units

= 1 mg; no preservatives). A virus suspension, prepared with Hanks balanced salt solution (BSS) to contain 30 to 300 TC₅₀ per 0.1 ml, was added to tubes containing an equivalent volume of heparin in various dilutions. The virus-heparin mixtures were incubated for 1 hr at 4 C, and 0.2 ml was used as an inoculum in each of two human amnion tissue culture tubes. Heparin-free controls containing similar amounts of virus were included. The amnion cultures were examined for cytopathic effect (CPE) every 2 to 3 days for 3 weeks. During this period no medium changes were made.

Protamine neutralization test. To each of ten tubes containing 2 mg of heparin was added protamine sulfate (Eli Lilly & Co., Indianapolis, Ind.) in concentrations of 2, 1.8, 1.6, 1.4, 1.2, 1.0, 0.8, 0.6, 0.4, and 0.2 mg. Tubes showing precipitation were centrifuged at 5,000 rev/min for 60 min at 4 C, and the individual supernatant fluids were then tested for both heparin-inhibitory effect and their effect on blood-clotting time. For the latter test, 0.1 ml of the supernatant fluid was added to each of ten tubes; 2 ml of blood were obtained from a donor, immediately placed in the tubes containing the supernatant fluids, and clotting times were determined. As controls, tubes containing 2 ml of blood and 0.1 ml of saline were used.

Recording of heparin concentration needed to inhibit virus. Of the several heparin preparations tested, some were available as solutions (e.g., Panheprin), and others as powders (Table 3). Because the potency of these preparations (i.e., equivalences of international units per mg) was variable, the heparin concentrations required for viral inhibition are recorded both in units and in micrograms. In addition, because it was not at first known whether heparin affected the virus or the cell, heparin concentrations are expressed in two ways: initial, heparin concentration per 1 ml

in the initial virus-heparin mixture; and final, heparin concentration per 1 ml in the final tissue-culture medium. The final heparin concentration represents one-fifth that of the virus-heparin mixture, because the tests were done by adding a 0.2-ml virus-heparin inoculum to tissue cultures containing 0.8 ml of medium.

RESULTS

Discovery of a virus-inhibitory substance in leukocyte and plasma preparations. The initial observations which revealed an inhibitory effect of some culture constituent on HSV are presented in Table 1. It was first noted that, on titration of the leukocyte cultures, there was an absence of viral CPE in amnion tubes inoculated with undiluted specimens (Table 1-A). In addition, a delay of CPE in the 10⁻¹ dilution, as compared with the 10⁻² dilution, was observed on the second titration day. Because it had previously been reported (Gresser, 1961) that leukocytes exposed to certain viruses could produce interferon, the possibility that interferon was responsible for this effect was first considered. The presence of a similar inhibitory effect in the undiluted specimens of the plasma control series, however, refuted this hypothesis (Table 1-B).

The possibility was then investigated that some plasma component not present in serum (e.g., fibrinogen) was responsible for this inhibition. The initial experiments on this point appeared to support this view, because they revealed that the heparinized plasma, but not serum, was inhibitory to HSV. When plasma prepared from the same donor by defibrination or by use of ethylenediaminetetraacetate (EDTA) or citrate as anticoagulants was tested, it was found to lack the inhibitory effect. These data suggested that heparin was the inhibitory substance.

Titration of heparin inhibitory effect. With the procedure described under Materials and Meth-

TABLE 1. Inhibitory effect on herpes simplex virus of leukocyte and control plasma cultures

Culture	Day culture titrated	Day cytopathogenic effect (CPE) first observed in human amnion		
		Dilution of culture		
		Undiluted	10 ⁻¹	10 ⁻²
(A) Leukocyte culture inoculated with herpes simplex virus	1st	No CPE	3rd	No CPE
	2nd	No CPE	14th	6th
(B) Plasma control culture inoculated with herpes simplex virus	1st	12th	4th	8th
	2nd	No CPE	4th	No CPE

TABLE 2. Titration of heparin against herpes simplex virus (100 TC₅₀)

Heparin concn				CPE
Amt in initial heparin-virus mixture		Amt in final medium		
units/ml	μg/ml	units/ml	μg/ml	
100	1,000	20	200	0
50	500	10	100	0
25	250	5	50	0
10	100	2	20	0
5	50	1	10	0 to +
2.5	25	0.5	5	+
1	10	0.2	2	+
0	0	0	0	+

ods and a constant virus inoculum (100 TC₅₀), it was found (Table 2) that the minimal concentration of heparin required for inhibition of viral CPE in cultures of human amnion cells was 5 to 10 units per ml or 50 to 100 μg/ml (initial concentration). This represents 1 to 2 units per ml or 10 to 20 μg/ml (final concentration).

Consistency of heparin inhibitory effect on various strains of HSV and with different tissue-culture systems and media. Four recent isolates from human infections with HSV, when tested at a dose of 100 TC₅₀ with varying heparin concentrations, were also found to be inhibited by 5 to 10 units per ml of heparin (initial concentration). In addition, three laboratory strains and the proliferative and giant cell variants of HSV were inhibited by similar concentrations. Testing of the Rodanus strain in hamster kidney, rabbit kidney, and diploid (MA 10) cultures revealed inhibition of virus by heparin at similar concentrations in these tissue-culture systems. This was also the case when Eagle's MEM with 10% calf serum was substituted for the bovine amniotic fluid medium.

Verification that the inhibitory effect is due to heparin. Because heparin is a biological rather than a synthetic product, the possibility was examined that impurities in the preparation, rather than heparin, were responsible for the inhibitory effect.

Table 3 presents results of tests evaluating the inhibitory activity of six biological heparin preparations. These materials, supplied by three different companies, had been prepared from three different body sources of pork and beef. The activity of these preparations in units per

milligram, as reported by the manufacturer, is recorded in Table 3, as is the minimal inhibitory concentration of the preparation in the initial heparin-virus mixture. It is apparent that all six products had very similar end points. Although this evidence did not completely rule out the possible existence of a common impurity in the six preparations of varied origin, this possibility was held as unlikely.

Correlation of inhibitory activity with sulfate groupings on heparin molecule. Because heparin is a sulfated mucopolysaccharide whose sulfate groupings are believed to be closely associated with its anticoagulating property, it was considered of interest to determine whether neutralization of sulfate groups with protamine would affect the virus-inhibition capacity. The effect of protamine neutralization on blood-clotting time was also determined.

As the concentration of protamine was decreased, thus increasing the number of available sulfate groups on the heparin molecules, there was a close parallel between the curves for viral CPE and blood-clotting inhibition (Fig. 1). When 1.0 mg or less of protamine was added to 2 mg of heparin, enough sulfate groups were left unneutralized to inhibit completely further viral CPE and blood clotting.

Results of tests for virus-inhibitory activity with synthetic polymers of varying degrees of sulfation (to be reported) afforded further support for the importance of the sulfate group in this inhibitory activity.

Observations on the nature of the heparin-virus-cell interaction: effect on cell of pretreatment with heparin. Human amnion tissue cultures were

TABLE 3. Effect of various biological heparins on herpes simplex virus

Animal source	Amt	Minimal inhibitory concn in initial mixture	
		units/ml	μg/ml
Pork intestine*	~100	7.5	75
Pork liver*	123	7.5	60
Beef intestine*	115	10	85
Beef lung*	130	7.5	60
Beef lung†	~100	7.5	75
Pork intestine‡	163	10	60

* Abbott Laboratories, North Chicago, Ill.

† Organon Inc., West Orange, N.J.

‡ Riker Laboratory, Northridge, Calif.

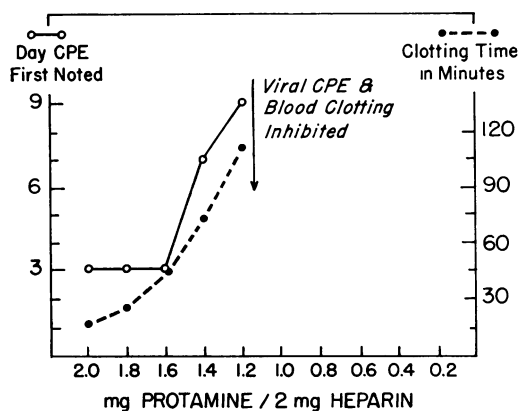


FIG. 1. Protamine neutralization test.

exposed for 2 days at 36 C to 1 ml of medium containing 20 units of heparin. The tubes were washed three times with Hank's BSS; 100 TC_{50} of HSV in 1 ml of medium were added both to these and to heparin-free control tubes. The time of appearance and extent of viral CPE were similar in test and control tubes. These results suggest that the inhibitory action of heparin is not mediated by an irreversible effect on the cells.

Effect of various concentrations of heparin on virus of varying inoculum size. To obtain some indication of the possible mode of action of heparin, a box titration was performed, whereby different doses of heparin (from 5 to 10,000 units per ml) were added to varying dilutions of virus (from 10 to 10^7 TC_{50} per ml). Duplicate amnion tubes inoculated with the different virus-heparin mixtures were then observed for the appearance of CPE, as well as for the rapidity of spread of virus throughout the monolayer. It was noted that increasing concentrations of heparin were needed to inhibit increasing concentrations of virus. Thus, the minimal inhibitory dose of heparin for viral inocula of up to 10^4 TC_{50} was 100 units per ml (initial concentration) or less. With higher concentrations of virus, doses of heparin up to 10,000 units per ml could not inhibit viral CPE, although its appearance was delayed for several days. When CPE did appear, moreover, it was focal with very slow progression, as contrasted with virus spread in heparin-free cultures which was nonfocal and rapidly progressing. This finding of focal spread would suggest that heparin, like antibody, prevents

cell-to-medium-to-cell, rather than cell-to-cell, spread. The slow progression of CPE, once the first focus is noted, suggests also that virus demonstrable at these higher heparin concentrations is not a heparin-resistant variant. These results also indicate that heparin does not have a direct inactivating effect on the virus.

Lack of viral inactivation by heparin. Absence of viral inactivation by heparin was further confirmed as follows. Varying concentrations of heparin and 1,000 TC_{50} of virus were incubated for 19 hr at 4 C. The virus titers of these mixtures were then determined in cultures of human amnion cells. As controls, tubes containing saline instead of heparin were employed. The results reveal that virus was demonstrable in the higher dilutions of the inoculum, even with high heparin concentrations (Table 4). CPE was absent in the undiluted mixtures containing 25 and 100 units of heparin, and at a 10^{-1} dilution of the 100 units per ml mixture. This is consistent with the presence of residual heparin inhibitory concentration in these tubes. The data also suggest that, if a heparin-virus complex is formed, it is readily dissociated on dilution.

Effect of heparin on the viral infectious cycle. Table 5 presents results obtained when amnion tubes inoculated with 1,000 TC_{50} of virus were then exposed to 20 units per ml of heparin at various time intervals ranging up to 10 hr after viral inoculation. Complete inhibition of CPE was found to occur only when heparin was added at 0 hr. Up to 1 hr, there was some delay in the appearance of CPE. Subsequently, CPE appeared at the same time in tissue-culture tubes both with and without heparin. These results suggest that the heparin effect occurs very early in the viral infectious cycle, probably at the first phase of adsorption during the initial ionic at-

TABLE 4. Lack of inactivation of herpes simplex virus by heparin

Dilution of virus-heparin or virus-saline mixture	Saline control	Heparin concn in heparin-virus mixture		
		5*	25	100
Undiluted	+	+†	0	0
10^{-1}	+	+	+	0
10^{-2}	+	+	+	+
10^{-3}	+	+	+	+

* Indicates units per ml.

† Symbols: 0 = no CPE; + = CPE.

TABLE 5. *Effect of heparin on viral infectious cycle*

Time heparin added	Time of appearance of 50% CPE
<i>min</i>	<i>day</i>
0.....	No CPE
15.....	8
30.....	4
45.....	4
60.....	2
90 and over.....	1
Control (no heparin).....	1

tachment of virus to cell. This is corroborated by the observation that when human amnion tissue-culture tubes are exposed for 2 hr to a heparin-virus mixture, followed by five washes of the cultures with Hank's BSS and addition of heparin-free medium, viral CPE is not evidenced.

DISCUSSION

Although other workers (Cohen, 1942; Heyman et al., 1958; Young and Mora, 1960; Vaheri and Penttinen, 1962; Feltz and Regelson, 1962) reported on the effect of various polyanions on certain bacterial, plant, animal, and human viruses, heparin presents certain features of particular biological import as regards viruses affecting man. Heparin is present in various animal and human tissues (Riley, 1963), and it is an agent that can be administered safely to man when certain precautions are taken. If heparin were found to be effective *in vivo*, its potential use as an antiviral agent, unlike the other polyanions with *in vitro* inhibitory properties, would thereby be clinically facilitated. Preliminary results (to be reported) on its use for prevention and treatment of herpetic keratitis in the rabbit, however, have been disappointing.

Higginbotham (1963) recently suggested that heparin is a preformed type of protective substance in the body, as compared with antibody which is an induced protective substance. It could be postulated that, as a result of the inflammatory reaction produced by certain viral infections, heparin, which is released from mast cells (Riley, 1963), forms a complex with susceptible viruses, localizing them to their site of infection and thus limiting their spread. Our *in vitro* data demonstrating that high viral concentrations can escape the heparin effect would provide a possible *in vivo* limitation to this protective mechanism, were it to be actually

operative. These results with heparin and HSV are analogous to those obtained by Roizman and Roane (1963) with antiserum and HSV, whereby, with the use of large viral inocula, a small fraction of virus was found to resist neutralization by all dilutions of antiserum.

Many explanations have been offered for the ability of herpes simplex virus to remain latent and produce recurrent lesions in spite of the presence of circulating antibodies (Glasgow and Habel, 1963). Since *in vitro* experiments revealed that heparin and other polyanions exert an inhibitory but reversible effect on the infectivity of the virus, the possibility may be considered that such an effect may also occur *in vivo*. Under certain stimuli (e.g., fever and stress), infective virus may become dissociated from a polyanion complex, thus permitting its local proliferation, with further spread prevented by the presence of circulating antibodies.

The inhibitory action of heparin and other polyanions has practical significance in several procedures commonly employed by virologists. Because herpes simplex and certain other viruses are inhibited by these compounds, attempts to recover such agents from heparinized blood (e.g., Geller, Coleman, and Jawetz, 1953) would probably fail. We have, in fact, found no record of isolation of HSV from heparinized blood. On the other hand, two successful recoveries of this agent from the blood of patients were made from specimens of clotted blood (Ruchman and Dodd, 1950; McKenzie, 1961, *personal communication*). In this regard, it should be noted that plasma prepared with EDTA or citrate had no inhibitory effect on HSV.

Although the amount of heparin employed for preparation of plasma clot tissue cultures is usually very small, it might still interfere with the growth of particularly susceptible viruses. Indeed, the "other factor" which Scott et al. (1953) found to cause nonspecific adsorption of HSV to plasma clot cultures might well have been heparin.

An inhibitory effect of acid mucopolysaccharides present in agar used as overlay in the plaquing of a number of viruses was reported recently by several workers (Takemoto and Liebhaber, 1961, 1962; Liebhaber and Takemoto, 1961; Schulze and Schlesinger, 1963; Colón, Idoine, and Brand, 1963). With regard to HSV, Tytell and Neuman (1963) observed that the plaquing of this virus could be greatly improved

by use of protamine to neutralize sulfate groups in the agar mucopolysaccharide, or, better still, by use of a methyl cellulose overlay. We were unable to find any difference in the susceptibility to heparin of the proliferative and giant cell strains of Scott et al. (1961), but have not tested the microplaque and macroplaque variants (Roizman and Roane, 1963) which also have differing cytopathic manifestations in tissue culture.

The observations obtained to date on the nature of the heparin-virus-cell interaction suggest that heparin and other sulfated polyanions tested inhibit susceptible viruses by the strongly anionic charge provided by their sulfate groups. Heparin seems to affect the virus at its earliest infective stage, and the heparin-virus "complex" formed is readily reversible, at least on dilution. It would appear from these findings that we are dealing with an effect at the primary attachment level (Tolmach, 1957). Corroboration for the effect being at this level comes from the kinetic studies of Mandel (1957) with a mouse intestine acid mucopolysaccharide inhibitor and Theiler's encephalomyelitis virus, and those of Liebhaber and Takemoto (1963) with sodium dextran sulfate and encephalomyocarditis virus. In addition, an inhibitory effect on hemagglutination of certain viruses (Colón et al., 1963) would also suggest that the site of action of these polyanionic compounds is at this level.

Based on the assumption that we are dealing with an ionic effect on cell and virus surface, the relative susceptibility of viruses to heparin may offer a clue as to the surface charge on virus particles. Such information is now lacking for most mammalian viruses (Brinton and Lauffer, 1959).

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ADDENDUM

We received recently a communication from A. Vaheri and K. Cantell from the Virus Depart-

ment, State Serum Institute, Helsinki, Finland, who, after reading our abstract (*Bacteriol. Proc.*, 1963, p. 145), informed us of their very similar findings. These workers independently discovered that heparin inhibited herpes simplex virus, and arrived at analogous conclusions regarding its probable mode of action.

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