Differentiation of Types 1 and 2 Herpes Simplex Virus by Plaque Inhibition with Sulfated Polyanions (36950)

ROBERT D. HUTTON, DONALD L. EWERT, AND GEORGE R. FRENCH
(Introduced by A. J. Nahmias)

Department of Dermatology, University of California San Francisco Medical Center, San Francisco, California 94122; and Department of Microbiology, Sixth US Army Medical Laboratory, Fort Baker, California 94965

Within the past decade, herpes simplex virus (HSV) isolates have been shown to have distinct antigenic and biologic properties that allow differentiation of almost all strains into 2 groups, designated Type 1 and Type 2. This recognition has given rise to the important finding that there are also clinicoepidemiologic differences between infections with the 2 types, *i.e.*, Type 1 has been found to be primarily associated with nongenital infection and transmission, and Type 2 with genital infection and transmission, including those that occur neonatally (1). The antigenic difference between the two types is subtle, and typing is usually accomplished by complex serologic or physiochemical methods (2–5). In addition, several distinct biologic properties are known for the 2 types, although none are very practical for type identification. One interesting biologic property of HSV, known for almost 10 yr, is a sensitivity to inhibition by certain sulfated polyanions (6–8). Most strains used in past inhibition studies, however, were apparently type unknown. Our study therefore undertook to compare sensitivities to inhibition by polyanions for several strains of HSV, specifically to see if the two antigenic types would show different reactivities.

Materials and Methods. Cell cultures. Vero monkey kidney cells (VMK) were grown in L-15 medium supplemented with 10% fetal calf serum. VMK cell monolayers for plaque assay experiments were seeded in 75 cm^2 T-flasks 48 hr prior to use.

Virus. All HSV strains tested were serologically typed by Dr. Andre Nahmias at Emory University. Virus stocks for plaque reduction experiments were prepared in L-15 media supplemented with 0.1% bovine serum albumin fraction V (BSA) at titers of approximately 2000 plaque forming units/0.1 ml (PFU/0.1 ml).

Polyanions. Dextran sulfate (32,000–48,000 MW), heparin (100 units/mg) and hyaluronic acid were obtained from Nutritional Biochemical Corp. Chondroitin sulfates A, B, C, and D were obtained from Miles Laboratories, Inc. Agar inhibitor substance was prepared by saline extraction of Bacto agar (Difco) by the method of Takemoto and Nomura [cited in (9)]. Stock solutions of polyanions were prepared in L-15 medium without antibiotics and stored at —70° until use.

Plaque inhibition studies. Plaque forming unit (PFU) determinations were done by the method of Dulbecco (10) utilizing a double agarose overlay assay. Studies of inhibition were done by mixing about 200 PFU of virus/0.1 ml with equal volumes of either polyanion solution in L-15, or L-15 without polyanion as the control. One-tenth milliliter of these mixtures were inoculated at room temperature, without preliminary incubation, onto each of triplicate VMK 75 cm^2 T-flasks. Ninety minutes adsorption preceded the first overlay of 0.5% agarose in L-15 medium with 2% FCS. The second overlay was on Day 4 with the same materials plus 1:15,000 neutral red. Plaques were counted 24 hr later. Control counts were about 100 PFU/flask. Percentage plaque inhibition was calculated by comparison of the polyanion–virus mixture counts to the L-15 diluent control counts. The 50% reduction concentrations of polyanion were determined by plotting percentage inhibition versus polyanion concentration on

TABLE I. Polyanion Screening for Inhibition of HSV Type 1 and Type 2 Plaque Formation.

	Reduction (%) of control plaque count			
	Type 1 Strain VET		Type 2 Strain KER	
	1000[a]	10	1000	10
Dextran sulfate	>90	51	>90	>90
Heparin	>90	10	>90	60
Agar inhibitor	>90	31	>90	78
Hyaluronic acid	<10	<10	<10	<10
Chondroitin sulfate A	<10	<10	<10	<10
B	<10	<10	68	15
C	<10	<10	36	14
D	<10	<10	<10	<10

[a] Concn (μg/ml) of polyanion tested.

probability paper, in the manner described by Lindenmann and Gifford (11).

Results. Polyanion sensitivity screening study. Table I shows the percentage inhibition of a known Type 1 and Type 2 HSV by 8 polyanions at concentrations of 1000 and 10 μg/ml. Dextran sulfate, heparin, and agar inhibitor substance showed inhibitory activity at both concentrations and against both types. Inhibition was virtually complete at 1000 for both types, but at 10 was much more pronounced for Type 2 than Type 1. Hyaluronic acid and chondroitin sulfate A and D showed no significant inhibition. Chondroitin sulfate B showed marked inhibition of the Type 2 strain, but only at the high concentration of 1000 μg/ml.

Fifty percent plaque reduction determinations. Because of the high degree of sensitivity noted at 10 μg/ml for dextran sulfate, heparin, and agar inhibitor, we sought to determine the concentrations of these polyanions that would result in 50% plaque reductions of the two types of HSV. Table II shows that the 50% plaque reduction concentration of dextran sulfate against Type 1 was 16 μg/ml, whereas it was 0.8 μg/ml against Type 2. The concentration ratio was thus 20-fold, *i.e.*, the Type 2 was 20 times more sensitive to inhibition by dextran sulfate than the Type 1. The levels for heparin were found to be 33 and 8 μg/ml, which gives a ratio of about 4:1. The Type 2 HSV was also more sensitive to inhibition by agar inhibitor than was the Type 1 virus, but the results were quite variable and the 50% plaque reduction concentration could not be determined with certainty for Type 2.

Inhibition of other strains. Two other Type 1 and two other Type 2 strains of HSV were then studied with dextran sulfate. Concentrations of dextran sulfate were used that might predictably cause plaque reduction above and below 50%. Results are shown in Table III. The percentage inhibition closely matched that predicted from experience with the initially tested Types 1 and 2. Again, it was observed that the Type 2 strains were distinctly more sensitive than the Type 1 strains.

Discussion. In a comparative study of transport media for HSV, Nahmias and coworkers (12) demonstrated that the frequency of recovery of Type 2 HSV was lower than that of Type 1 in agar-base transport

TABLE II. Fifty Percent Plaque Reduction Concentrations for Selected Polyanions.

	HSV Type 1 Strain VET	HSV Type 2 Strain KER	Ratio Type 1/Type 2
Dextran sulfate	16[a]	0.8	20
Heparin	33	8	4
Agar inhibitor	55	1–3[b]	≧18

[a] Concn (μg/ml).

[b] See text for explanation.

TABLE III. Predicted Versus Observed Plaque Inhibition by Dextran Sulfate.[a]

	Reduction (%) of control plaque count				
	Type 1			Type 2	
	33[b]	10		1.0	0.33
Predicted[c]	67	43	Predicted	62	32
Strain MAC	69	41	Strain MEN	54	32
Strain GER	63	48	Strain OLD	53	12

[a] Two additional strains each of Type 1 and Type 2 HSV.

[b] Concn (μg/ml).

[c] Predicted based on observed inhibition with Type 1 strain VET and Type 2 strain KER.

media, which contains sulfated polysaccharides. Agarose-base transport media, however, tended to increase and equalize the recovery rates for Type 1 and Type 2 HSV. The authors therefore suggested that HSV Type 2 may be more sensitive to sulfated mucopolysaccharides than Type 1. The results of our study provide confirmatory evidence for their assertion.

It is thought that one mechanism of sulfated polyanion inhibition of viral infectivity is the formation of electrostatic bonds between the negatively charged polyanion and oppositely charged sites on the virion surface, which results in the formation of reversible noninfective complexes (6, 7). Our findings would therefore imply that there are different net surface charges or distributions of surface charges on the two types of HSV. Plummer *et al.* (5) have already shown the two types to have subtle but distinct differences in nucleic acid density.

An expanding awareness of the wide spectrum of disease resulting from HSV infection and the clinicoepidemiologic differences between the two types of HSV infection has increased the need for a rapid, reliable method of type identification available to other than research laboratories. Determination of nucleic acid density is one alternative to serologic differentiation, but also requires techniques not normally utilized in the diagnostic laboratory. The possibility exists that a simple method of typing, based on relative sulfated polyanion sensitivities, may be developed.

Summary. The relative inhibition of Type 1 and Type 2 herpes simplex virus plaque formation by natural and synthetic polyanions has been studied. Type 1 and Type 2 HSV were found to have different sensitivities to several naturally occurring sulfated acid mucopolysaccharides and to the synthetic polyanion dextran sulfate. In all instances where sensitivity to a polyanion was observed, it was greater for Type 2 than for Type 1 HSV. These differences were especially marked for dextran sulfate, heparin and agar inhibitor. Chondroitin sulfate B inhibited Type 2 HSV at high concentration, but did not inhibit plaque formation of Type 1.

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