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Differences in the Susceptibility of Herpes Simplex Virus Types 1 and 2 to Modified Heparin Compounds Suggest Serotype Differences in Viral Entry

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Although heparan sulfate (HS) serves as an initial receptor for the binding of both herpes simplex virus type 1 (HSV-1) and HSV-2 to cell surfaces, the two serotypes differ in epidemiology, cell tropism, and ability to compete for viral receptors in vitro. These observations are not necessarily contradictory and can be explained if the two serotypes recognize different structural features of HS. To compare the specific features of HS important for the binding and infection of HSV-1 and HSV-2, we took advantage of structural similarities between heparin and cell surface HS and compared the abilities of chemically modified heparin compounds to inhibit plaque formation. We found that the antiviral activity of heparin for both serotypes was independent of anticoagulant activity. Moreover, specific negatively charged regions of the polysaccharide, including N sulfations and the carboxyl groups, are key structural features for interactions of both HSV-1 and HSV-2 with cell surfaces since N desulfation or carboxyl reduction abolished heparin's antiviral activity. In contrast, 6-O sulfations and 2-,3-O sulfations are important determinants primarily for HSV-1 infection. The O-desulfated heparins had little or no inhibitory effect on HSV-1 infection but inhibited HSV-2 infection. Using a series of intertypic recombinant mutant viruses, we found that susceptibility to O-desulfated heparins can be transferred to HSV-1 by the gene for glycoprotein C of HSV-2 (gC-2). This supports the notion that the envelope glycoproteins of HSV-1 and HSV-2 interact with different affinities for different structural features of heparin. To determine if the modified heparin compounds inhibited plaque formation by competing with cell surface HS for viral attachment, binding studies were also performed. As anticipated, most compounds inhibited binding and plaque formation in parallel. However, several compounds inhibited the binding of HSV-1 to cells during the initial attachment period at 4°C; this inhibitory effect was reversed when the cells and inoculum were shifted to 37°C. This temperature-dependent differential response to modified heparin compounds was evident primarily when glycoprotein C of HSV-1 (gC-1) was present in the virion envelope. Minimal temperature-dependent differences were seen for HSV-1 with gC-1 deleted and for HSV-2. These results suggest differences in the interactions of HSV-1 and HSV-2 with cell surface HS that may influence cell tropism.

The entry of alphaherpesviruses into cells requires the binding of virus to receptors on the cell surface, which is followed by fusion of the virion envelope with the cell plasma membrane. This process is complex and may involve multiple interactions between viral glycoproteins and cell surface components, including both low- and high-affinity interactions (16, 50). Recent studies have begun to elucidate the viral and cellular requirements for herpes simplex virus (HSV) entry. For HSV type 1 (HSV-1) and HSV-2 (as well as related herpesviruses, pseudorabies virus [PrV], bovine herpes virus type 1, and cytomegalovirus), the initial interaction of virus with cells is the binding of virus to cell surface heparan sulfate (HS) (5, 27, 28, 35, 38, 40–42, 44, 45, 48, 58, 61). The evidence for HSV includes the following: (i) cells devoid of HS (but not other glycosaminoglycans [GAGs]) because of either enzymatic treatment or genetic mutation have greatly reduced numbers of receptors for viral binding (14, 19, 48, 58), and (ii) heparin, which is structurally similar to HS, inhibits the binding of viruses to cells (58). However, although both HSV-1 and HSV-2 bind HS, the two serotypes exhibit differences in epidemiology, cell tropism, and susceptibility to inhibitors of viral binding that may reflect differences in viral entry. For example, HSV-1 is more likely to cause oral lesions and sporadic encephalitis, whereas HSV-2 commonly causes genital lesions. This epidemiological observation is supported by in vitro studies which showed that HSV-1 binds to human synaptosomes and glial cells more efficiently than does HSV-2, whereas HSV-2 binds HeLa cells (a cervical cell line) more efficiently than does HSV-1 (56, 57). In addition, neomycin, poly-L-lysine, and platelet factor 4 inhibit the binding of HSV-1, not HSV-2 (3, 32–34, 43). The observations that HS is the initial receptor for both HSV-1 and HSV-2, that different cells preferentially bind one serotype more than the other, and that select inhibitors of viral binding show serotype specificity are not necessarily contradictory. Rather, these results can be explained if the relative contributions of individual glycoproteins to viral binding are different and/or individual glycoproteins recognize different structural features of HS. We recently showed that there are serotype differences in the role of glycoprotein C (gC) in viral attachment (13). Although both gC of HSV-1 (gC-1) and gC of HSV-2 (gC-2) are heparin-binding glycoproteins, deletion of gC-1, not gC-2, results in mutants that are impaired in the ability to bind to cells and have reduced specific infectivities (13, 20, 46).

The studies presented here were designed to test the hypothesis that HSV-1 and HSV-2 preferentially bind different structural features of HS. The notion that HSV-1 and HSV-2

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TABLE 1. Effects of heparin and modified heparin compounds on HSV plaque formation on Vero cells

Heparin compound	IC_{50} (μg/ml)[a]			aPTT (% heparin)[b]
	HSV-1(KOS)	HSV-2(G)	HSV-1(KOS)gC$^-$	
Heparin	6	3	10	100
N-desulfated heparin	>1,000	600	>1,000	3
N-desulfated, N-reacetylated heparin	100	15	1,000	3
N-DA heparin	4	1	20	65–85
N-DA, N-resulfated heparin	8	3	20	55–59
2-,3-O-DS heparin	350	15	400	11
N-DA, N-resulfated, 2-,3-O-DS heparin	200	10	200	ND[c]
N-desulfated, N-reacetylated, 2-,3-O-DS heparin	>1,000	300	>1,000	<0.5
6-O-DS heparin	>1,000	50	>1,000	11
CR heparin	700	300	>1,000	5
AMS heparin	9	4	60	40
NAC-heparin	4	1	4	5–10

[a] The concentrations of heparin compound that inhibited 50% of viral plaques formed (IC_{50}) compared with plaques formed in the absence of heparin compound were determined from the dose-response curves.
[b] aPTT, activated partial thromboplastin time as a measure of anticoagulant activity. Data were provided by Glycomed, Inc.
[c] ND, not determined.

might recognize different structural features of HS seems likely when one considers the complexity and structural diversity of mammalian proteoglycans. Proteoglycans are ubiquitous molecules that are integral membrane proteins of cells and components of the extracellular matrix (31). The core proteins are modified by the addition of long unbranched sulfated polysaccharides called GAGs and by N-linked and O-linked oligosaccharides. The structures of proteoglycans are cell type and possibly differentiation specific (7, 23). The three most abundant GAGs on plasma membrane proteoglycans are HS, chondroitin sulfate, and dermatan sulfate. HS proteoglycans serve as important regulators of cellular signaling by modulating, for example, the stability and biologic activity of heparin-binding growth factors (23). HS chains are heterogeneous, varying in sites of N acetylation, N sulfation, O sulfation, and epimerization of glucuronic acid (GlcA) to iduronic acid (IdoA). Thus, it is plausible that this heterogeneity could specify distinct receptors for various ligands, including HSV-1 and HSV-2. For example, heparin binds antithrombin III via a specific pentasaccharide structure which occurs infrequently within heparin chains near the nonreducing ends (37).

HS is distinguished from the closely related GAG heparin by its lower degree of sulfation, higher degree of N acetylation compared with the N sulfation of glucosamine residues, and the predominance of glucuronic acid rather than iduronic acid (36). Taking advantage of the similarities between heparin and HS, we previously examined the abilities of a series of modified heparin compounds to inhibit HSV-1 binding at 4°C (17). In these studies we extend those observations and compare the abilities of modified heparin compounds to inhibit HSV-1 and HSV-2 binding and plaque formation both at 4 and 37°C. The results obtained suggest differences in the initial interactions of HSV-1 and HSV-2 with cell surface HS.

MATERIALS AND METHODS

Cells and viruses. HEp-2 cells and Vero cells were obtained from the American Type Culture Collection. HEp-2 cells were passaged in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum; Vero cells were passaged in medium 199 supplemented with 5% bovine serum. The wild-type viral strains used were HSV-2(G) (30), HSV-2(333) (53), HSV-1(F) (8), HSV-1(KOS) (49), and HSV-1(17) (2). The mutants HSV-1(KOS)ΔgC2-3 (provided by C. Brandt, University of Wisconsin) and HSV-2(G)gC$^-$ are gC-1 and gC-2 deletion viruses, respectively (13, 19). Intertypic recombinant viruses RHIG13, RSIG25, and R7015 (provided by B. Roizman, University of Chicago) have previously been described (3, 6). These viruses are summarized in the legend to Fig. 4. All viruses were propagated on HEp-2 cells. Viral stocks had titers of approximately 10^9 PFU/ml on Vero cells.

Virion binding and infectivity assays. Two types of binding assays were performed. First, the number of radiolabeled viral particles bound to cells in the presence or absence of heparin or modified heparin at 4°C was determined by methods previously described (17, 20). Second, the effects of modified heparin compounds during the initial binding period on subsequent plaque formation were determined as follows. HEp-2 or Vero cells in 25-cm^2 flasks were precooled at 4°C for 30 min, and then duplicate flasks were inoculated with 200 to 500 PFU of virus per flask in phosphate-buffered saline (PBS) in the presence or absence of a modified heparin compound at 4°C. After a 2-h adsorption period, the viral inoculum and modified heparin compound were removed, and cells were washed three times with PBS, overlaid with fresh PBS with or without a modified heparin compound, and then transferred to 37°C for 1 h to allow penetration of the bound virus to occur. Cells were subsequently overlaid with medium 199 supplemented with 1% fetal bovine serum and 0.1% pooled human gamma globulin (199O). Plaques were counted after 2 days, as described below.

Viral plaque assays in the presence or absence of a modified heparin compound were also performed in duplicate at 37°C. HEp-2 or Vero cells in 25-cm^2 flasks were inoculated with virus in PBS in the presence or absence of a modified heparin compound. After a 2-h adsorption period at 37°C, the viral inoculum was removed and cells were overlaid with medium 199O. Plaques were counted after 2 days of incubation. Vero cells were fixed and stained with Giemsa to visualize plaques. To visualize plaques on HEp-2 cells, an immunoassay was performed, as previously described, with monoclonal antibody II-105 (anti-gB), III-114 (anti-gD), III-174 (anti-gD), or III-188 (anti-gC-2) (gifts of P. Spear) (13, 20, 21).

Assay for rate of virus penetration. The rate of virus penetration was assessed by determining the rate at which adsorbed virus became resistant to inactivation by a low-pH citrate buffer (20). Confluent Vero cells in 25-cm^2 flasks were inoculated with virus (500 to 1,000 PFU per dish) in the presence or absence of a modified heparin compound for an adsorption period of 2 h at 4°C. Flasks were then shifted to 37°C to allow virus penetration to proceed. At selected times after the temperature shift, experimental flasks were treated with 1 ml of citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0) and control flasks were treated with 1 ml of complete PBS for 1 min. Monolayers were then washed three times with PBS and overlaid with 199O. Plaques were counted as described above. Experiments were performed in duplicate.

Preparation of modified heparin compounds. The syntheses and structural characterizations of all the modified heparin compounds (obtained from Glycomed, Alameda, Calif.) have previously been described (17). The anticoagulant activity was measured by activated partial thromboplastin time. Lyophilized compounds were resuspended in PBS, aliquoted, and stored at −20°C.

Statistical analysis. The data presented are means with standard deviations. Student unpaired, two-tailed *t* tests were performed by the Bonferroni method for multiple groups. The significance level was adjusted to 0.005.

RESULTS

Antiviral activity of heparin for both HSV-1 and HSV-2 is independent of anticoagulant activity. A series of oxidation and reduction reactions yields a modified heparin compound, termed nonanticoagulant heparin (NAC-heparin or GM1077), that has 5 to 10% of heparin's anticoagulant activity, as mea-

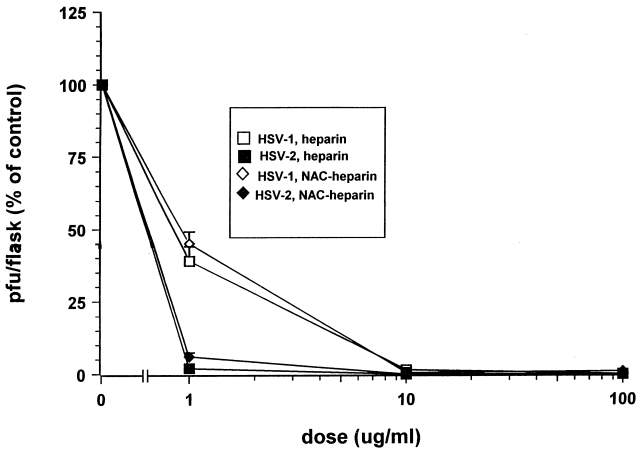

FIG. 1. Effects of heparin and NAC-heparin on viral plaque formation by HSV-1(KOS) and HSV-2(G). Cultures of HEp-2 cells in 25-cm^2 flasks were inoculated with HSV-1(KOS) or HSV-2(G) virus (200 to 500 PFU per flask) in the absence or presence of heparin and NAC-heparin. After a 2-h adsorption period at 37°C, the inoculum was removed and cells were overlaid with medium 199O. Plaques were counted by immunoassay after 2 or 3 days. The results presented are the PFU formed in the presence of heparin or NAC-heparin expressed as percentages of the PFU detected in the absence of heparin (control). Two independent experiments were performed in duplicate. Each point is the mean of values obtained, and error bars indicate the ranges of values obtained.

sured by activated partial thromboplastin time (Table 1). We examined the ability of this compound to inhibit HSV-1 and HSV-2 plaque formation and found that NAC-heparin exhibited antiviral activity almost identical to that of heparin on both Vero cells (Table 1) and HEp-2 cells (Fig. 1). For example, on HEp-2 cells, both heparin and NAC-heparin inhibited >90% of viral plaque formation at a dose of 1 μg/ml for HSV-2 and at a dose of 10 μg/ml for HSV-1. Similar results were obtained for several different strains of each serotype on several cell types, including epithelial, neuroblastoma (SK-N-SH), and cervical (Ca Ski) cell lines (data not shown). Thus, the structural features of HS required for HSV binding must differ from the features required for antithrombin III binding.

N sulfations and a negative charge at the carboxyl site are important for the antiviral effects of heparin. HS and heparin are characterized by their high negative charge density due to the presence of sulfate and carboxyl groups. In general, N-sulfated saccharide sequences tend to contain more IdoA than GlcA units and carry *O*-sulfate groups, whereas N-acetylated regions retain their GlcA units and essentially lack *O*-sulfate residues (31, 36). Carboxyl groups also contribute to the negative charge characteristic of both heparin and HS and presumably to the conformation of the polysaccharides. We compared the effects of unmodified, N-desulfated, N-deacetylated (N-DA), N-reacetylated, carboxyl-reduced (CR), and aminomethylsulfonate (AMS) (replacement of the carboxyl group with an AMS group) heparins on HSV-1 and HSV-2 viral plaque formation (Table 1). Virus was mixed with heparin or modified heparin at various concentrations immediately prior to inoculating Vero or HEp-2 cells for plaque assays at 37°C. After a 2-h adsorption period, the inoculum was removed and cells were overlaid with medium. Plaques were counted after 2 days. The results for Vero cells are summarized in Table 1; comparable results were obtained for HEp-2 cells. For each compound, the concentration of modified heparin compound required to inhibit 50% of plaque formation was determined from the dose-response curve. N desulfation or carboxyl reduction of heparin, not N deacetylation of heparin, markedly decreased the antiviral activity for both HSV-1 and HSV-2. This observation supports the notions that negative charges at these sites contribute to the antiviral activity of heparin and that it is the N-sulfated regions, not the N-acetylated regions, which are involved in virus-cell interactions. Interestingly, some antiviral activity, particularly for HSV-2, is regained by reacetylation of heparin at the N-desulfated sites. This suggests that presence of a negatively charged sulfate group may not be required for antiviral activity against HSV-2; rather, the absence of a positive charge at the N-desulfated site may be required. Similarly, substitution for the carboxyl group by a different negatively charged side chain, an AMS group, restores the antiviral effect for both HSV-1 and HSV-2.

Contribution of O-sulfations of heparin to antiviral activity. The regions of heparin or HS that are N sulfated are also rich in 6-O and 2-O sulfations. To determine the relative contributions of these sulfation sites on viral infection, we compared the effects of selective 6-O desulfation or 2-,3-O desulfation of heparin on the inhibition of viral plaque formation. The results of plaque assays for HSV-1(KOS) and HSV-2(G) on HEp-2 cells are shown in Fig. 2. Both 6-O-desulfated (6-O-DS) heparin and 2-,3-O-DS heparin inhibited HSV-2 infection, albeit less efficiently than did unmodified heparin, but had little or no inhibitory effect on HSV-1 infection. Comparable results were obtained on Vero cells (Table 1). To determine whether this serotype differential effect was limited to these two strains, we also compared the abilities of O-DS heparins to inhibit other commonly used wild-type strains of HSV-1 and HSV-2. The results for 6-O-DS heparin on HEp-2 cells are shown in Fig. 3 (comparable results were obtained on Vero cells). 6-O-DS heparin had little or no inhibitory effect on PFU for HSV-1(KOS), HSV-1(F), or HSV-1(17) but inhibited approximately 75% of viral PFU for HSV-2(G) and HSV-2(333).

Susceptibility to O-DS heparin can be transferred to HSV-1 by gC-2. For both HSV-1 and HSV-2, gC and gB bind heparin-

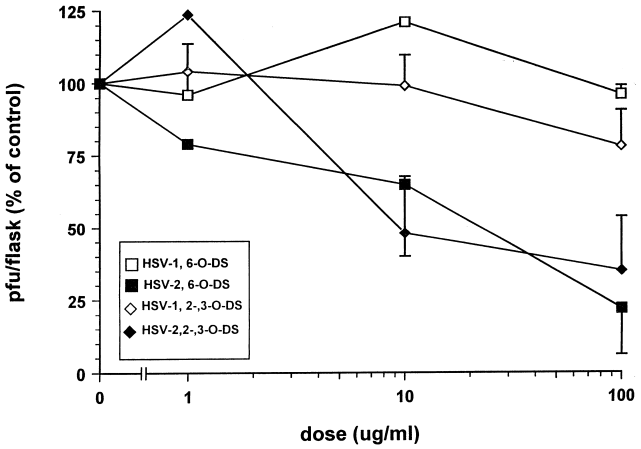

FIG. 2. Effect of O desulfations on plaque formation by HSV-1(KOS) and HSV-2(G). Cultures of HEp-2 cells in 25-cm^2 flasks were inoculated with virus (200 to 500 PFU per flask) in the absence or presence of various concentrations of 6-O-DS heparin or 2-,3-O-DS heparin. After a 2-h adsorption period at 37°C, the inoculum was removed, cells were overlaid with medium 199O, and plaques were counted by immunoassay after 2 or 3 days. The results presented are the PFU formed in the presence of O-DS heparin expressed as percentages of the PFU detected in the absence of O-DS heparin (control). Each point is the mean of values obtained from three or four independent experiments performed in duplicate, and error bars indicate standard deviations. At a dose of 100 μg/ml, the abilities of 6-O-DS heparin and 2-,3-O-DS heparin to inhibit HSV-2(G) compared with inhibition of HSV-1(KOS) are significantly different ($P = 0.0015$ for 6-O-DS heparin and $P = 0.0002$ for 2-,3-O-DS heparin).

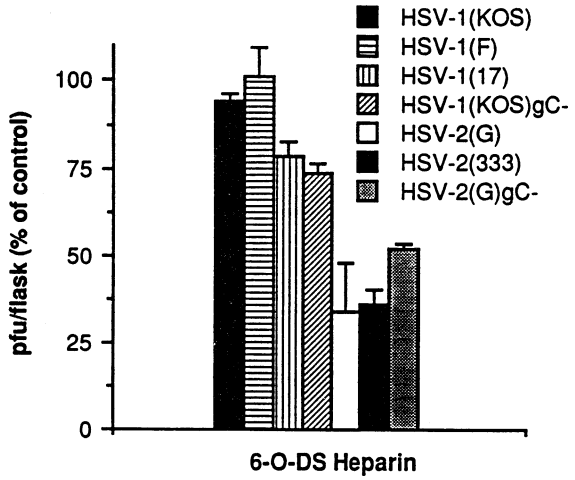

FIG. 3. Effects of 6-O-DS heparin on plaque formation for several strains of HSV-1 and HSV-2. Flasks (25 cm^2) were inoculated with virus in the absence or presence of 100 μg of 6-O-DS heparin per ml. After a 2-h adsorption period at 37°C, the inoculum was removed and cells were overlaid with medium 199O. Plaques were counted by immunoassay after 2 or 3 days. The results presented are the PFU formed in the presence of 6-O-DS heparin expressed as percentages of the PFU detected in the absence of 6-O-DS heparin (control). For each virus, at least two experiments were performed in duplicate. Each point is the mean of values obtained, and error bars indicate the ranges of values obtained. The inhibitory effect of 6-O-DS heparin on HSV-2(G) and HSV-2(333) was significantly different than the effect on wild-type HSV-1 strains ($P < 0.001$).

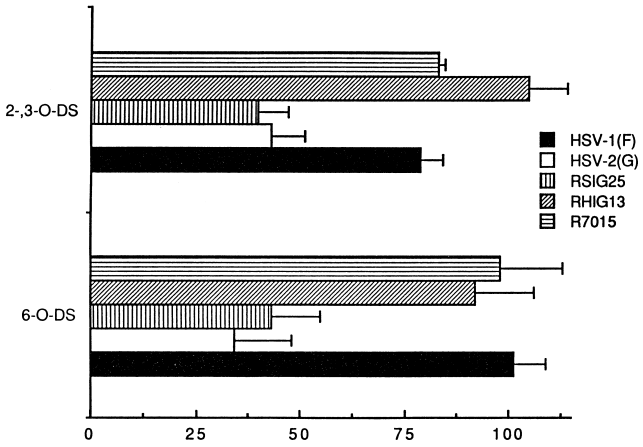

FIG. 4. Effects of 2-,3-O-DS heparin and 6-O-DS heparin on plaque formation by intertypic strains of HSV-1(F) and HSV-2(G). Cultures of HEp-2 cells in 25-cm^2 flasks were inoculated with wild-type or intertypic recombinant virus in the absence or presence of 100 μg of 2-,3-O-DS heparin or 6-O-DS heparin per ml. RSIG25 contains HSV-2 sequences in place of the corresponding HSV-1 sequences from map positions 0.575 to 0.725 and encodes the *gC-2* gene (3). RHIG13 contains HSV-2 sequences in place of the corresponding HSV-1 sequences from map positions 0.325 to 0.425 and encodes the *gB-2* gene (6). In R7015, the HSV-1 S component sequence between map positions 0.823 and 0.985 was replaced with the corresponding HSV-2 DNA sequence. The resulting intertypic virus encodes HSV-2 gD, gG, gE, and gI (3). After a 2-h adsorption period at 37°C, the inoculum was removed and cells were overlaid with medium 199O. Plaques were counted by immunoassay after 2 or 3 days. The results presented are the PFU formed in the presence of O-DS heparin expressed as percentages of the PFU detected in the absence of O-DS heparin. For each virus, at least two experiments were performed in duplicate. Each point is the mean of values obtained, and error bars indicate the ranges of values obtained. The inhibitory effects of 6-O-DS heparin and 2-,3-O-DS heparin on HSV-2(G) and RSIG25 were significantly different than the effects on HSV-1(F) and other intertypic strains ($P < 0.001$).

affinity columns under physiologic conditions (13, 20). However, the relative contributions of each of these glycoproteins to viral binding and infection may differ. For HSV-1, deletion of gC-1 decreases the specific binding activity (number of viral particles bound per cell at 4°C), deletion of gB-1 has little effect, and deletion of both gC-1 and gB-1 abolishes viral binding (19, 20). For HSV-2, deletion of gC-2 has little or no effect on specific binding activity; studies of viruses with gB-2 deleted have not yet been reported (13). To determine whether the differences in susceptibility to 6-O-DS heparin mapped to either of the heparin-binding glycoproteins, we first compared the effects of 6-O-DS heparin on plaque formation by wild-type HSV-1 and HSV-2 and HSV-1 and HSV-2 with gC deleted on HEp-2 cells (plaque assays with gB-deleted viruses cannot be conducted because the virus is not infectious). The results are shown in Fig. 3. 6-O-DS heparin had little or no inhibitory effect on plaque formation for HSV-1(KOS) independent of the presence of gC-1 in the virion envelope. In contrast, 6-O-DS heparin inhibited wild-type HSV-2, but deletion of gC-2 decreased the inhibitory activity. At a dose of 100 μg/ml, 6-O-DS heparin inhibited approximately 75% of PFU for HSV-2(G) but only 50% of PFU for HSV-2(G)gC^-. Taken together, these results suggest that for HSV-1, resistance to 6-O-DS heparin is independent of gC-1; for HSV-2, the presence of gC-2 may contribute to susceptibility to 6-O-DS heparin.

To further explore the serotype differences in susceptibility to O-DS heparins, we took advantage of a series of intertypic recombinant viruses between HSV-1(F) and HSV-2(G) (generous gifts of B. Roizman). These viruses have previously been described (3, 6). The results of studies on HEp-2 cells using 100 μg of 6-O-DS or 2-,3-O-DS heparin per ml are summarized in Fig. 4. Comparable results were obtained with 50 μg of O-DS heparins per ml. As shown, RSIG25 was the only recombinant virus inhibited by 6-O-DS and 2-,3-O-DS heparin. This recombinant, in which gC-2 is transferred to HSV-1(F), could not be differentiated from HSV-2 with respect to susceptibility to O-DS heparins. The transfer of gB-2 (RHIG13) or gD-2, gG-2, gE-2, and gI-2 (R7015) resulted in intertypic recombinant viruses that behaved like HSV-1(F) and were resistant to inhibition by O-DS heparins. Taken together, these results suggest that gC-2 confers susceptibility to O-DS heparins on HSV-1.

Effects of modified heparin compounds on viral binding. To determine if the effects of modified heparin compounds on viral plaque formation could be explained by their effects on viral binding, binding studies were performed. We previously showed that for HSV-1, the effects of modified heparin compounds on the number of viral particles bound per cell (binding of radiolabeled virus to cells at 4°C) paralleled the effects of modified heparin compounds present during the initial binding period at 4°C on subsequent plaque formation (17). In pilot studies, parallel results for these two binding assays were also obtained for HSV-2. Subsequently, all studies conducted used the latter assay. As predicted, for most modified heparins, the results for binding were similar to those obtained for infection (standard plaque assays conducted at 37°C) (Table 2), suggesting that the ability of modified heparin compounds to inhibit plaque formation reflected the ability to competitively inhibit viral binding to cell surface HS. However, CR heparin, 6-O-DS heparin, and 2-,3-O-DS heparin inhibited HSV-1 binding at 4°C but had little or no effect at 37°C. No such differences were observed for HSV-2. For HSV-2, there was a tendency for O-DS heparins to inhibit HSV-2 infection at 37°C better than at 4°C. The observation that select modified heparins inhibited HSV-1 at 4°C better than at 37°C suggests differences in the interaction of HSV with the cell surface such that for HSV-1,

TABLE 2. Effects of modified heparin compounds at 4 and 37°C on viral plaque formation[a]

Modified heparin	Dose (μg/ml)	PFU/flask (% of control)			
		HSV-1(KOS)		HSV-2(G)	
		4°C	37°C	4°C	37°C
Heparin	10	14 ± 7	17 ± 3	8 ± 0.7	8 ± 1.4
	100	8 ± 7	6 ± 3	2 ± 0.7	5 ± 0.7
NAC-heparin	10	9 ± 1	20 ± 5	9 ± 1.4	7 ± 0
	100	4 ± 1	5 ± 2	2 ± 0	1 ± 1
N-desulfated heparin	10	70 ± 14	67 ± 1	98 ± 13	88 ± 4
	100	87 ± 11	71 ± 8	105 ± 1.4	90 ± 13
N-DA heparin	10	4 ± 0	30 ± 2	8 ± 3	14 ± 1
	100	3 ± 1	18 ± 1	4 ± 4	11 ± 3
CR heparin	10	52 ± 10	96 ± 20	95 ± 14	99 ± 0
	100	32 ± 7	97 ± 1	74 ± 11	84 ± 11
AMS heparin	10	17 ± 8	45 ± 2	11 ± 7	18 ± 8
	100	6 ± 0	17 ± 2	4 ± 2	8 ± 2
6-O-DS heparin	10	54 ± 5	98 ± 5	68 ± 11	82 ± 14
	100	32 ± 8	90 ± 13	64 ± 1	43 ± 8
2-,3-O-DS heparin	10	90 ± 5	103 ± 7	82 ± 4	45 ± 8
	100	42 ± 16	71 ± 17	32 ± 13	23 ± 7

[a] Vero cells were precooled to 4°C or maintained at 37°C and then exposed to virus (200 to 500 PFU per flask) in the presence or absence of heparin or modified heparin at the concentrations shown for 2 h at the respective temperatures. For the 4°C flasks, after the 2-h adsorption period, the inoculum was removed and cells were washed with PBS three times prior to transfer to 37°C to allow penetration to occur. For the 37°C flasks, after the 2-h adsorption period, the inoculum was removed and cells were overlaid with medium (199O). Plaques were counted after 2 days, and data are the PFU per flask remaining expressed as percentages of the control (absence of heparin compound). Two to four independent experiments were performed in duplicate, and data are the means of the values obtained ± standard deviations.

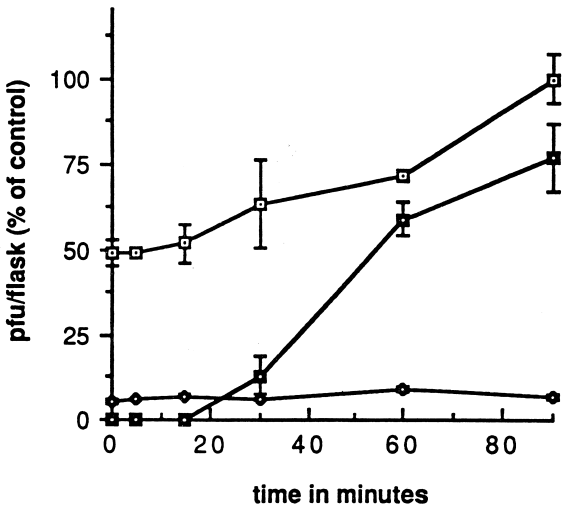

FIG. 5. Comparison of the rate of transition from susceptibility to resistance to 6-O-DS heparin and the rate of HSV-1(KOS) penetration. Confluent monolayers of Vero cells in 25-cm^2 flasks were inoculated with ~1,000 PFU of virus for a 2-h adsorption period at 4°C in the presence or absence of 6-O-DS heparin or N-DA heparin (50 μg/ml). Flasks were shifted to 37°C (without wash) to allow penetration to proceed. At selected times after the temperature shift (t = 0), duplicate monolayers were treated for 1 min with 1 ml of citrate buffer or with PBS. Monolayers were then washed three times and overlaid with 199O. Plaques were counted after 2 days. The number of plaques on the control cultures inoculated in the absence of any modified heparin compound and treated with PBS was essentially the same for all time points, and the average value was taken as 100%. The results presented are the PFU formed in the presence of 6-O-DS heparin (open boxes) or N-DA heparin (open diamond) for the indicated length of time after the temperature shift or the PFU formed in the presence of 6-O-DS heparin and surviving citrate treatment for the indicated length of time after the temperature shift (closed boxes) expressed as percentages of the PBS control value. Each point is the average of duplicate determinations, and error bars indicate the ranges of values obtained.

interactions at 4°C are easier to inhibit than those at 37°C, whereas for HSV-2, there is a tendency for interactions at 37°C to be inhibited better than those at 4°C. This latter observation, although not statistically significant, may reflect an additional inhibitory effect of modified heparin compounds on virus penetration.

To determine if the inhibitory effect evident only at 4°C for HSV-1 might be reversed at physiologic temperatures, assays were modified as follows. Vero or HEP-2 cells were inoculated with virus in the presence or absence of select inhibitors at 4°C. After a 2-h adsorption period, flasks were either directly transferred to 37°C (no wash) or the inoculum was removed, and cells were washed three times, overlaid with fresh PBS with or without modified heparin, and then transferred to 37°C to allow penetration to occur (wash). Plaques were counted after 2 days. For HSV-1, not HSV-2, there were differences in the effects of CR heparin, 6-O-DS heparin, and 2-,3-O-DS heparin on subsequent plaque formation, depending on whether or not cells were washed after the initial adsorption at 4°C (data not shown). For example, if unbound virus was not removed at the end of the initial adsorption at 4°C but cells were transferred to 37°C (no wash), the PFU per flask remaining, as a percentage of the control, was 97% ± 8% for CR heparin at 100 μg/ml. This result is similar to results obtained when the entire study were conducted at 37°C (Table 2). In contrast, if unbound virus was removed after the 4°C adsorption period, HSV-1 plaque formation was inhibited (34% ± 7%). No additional inhibition of HSV-1 plaque formation was observed when, after washing unbound virus, cells were overlaid with PBS containing a modified heparin compound for the 1-h penetration period at 37°C. Hence, no additional HSV-1 virions were eluted after the temperature shift.

These results are consistent with a model in which there is more than one type of interaction between HSV and HS. We hypothesize that at 4°C, a low-affinity interaction occurs between HSV-1 and HS; this interaction can be reversibly inhibited by CR heparin, 6-O-DS heparin, and 2-,3-O-DS heparin. As cells and inoculum are warmed to physiologic temperatures, a higher-affinity interaction occurs. This latter interaction is resistant to inhibition by CR heparin and O-DS heparin but is still susceptible to N-DA heparin, suggesting that it is still mediated by an interaction of virus with HS molecules. To begin to test this model, we next correlated the rate of transformation from susceptibility to resistance to select modified heparins with rates of virus penetration. For these experiments, Vero cells were inoculated with HSV-1(KOS) in the presence or absence of 50 μg of 6-O-DS heparin (or N-DA heparin as a control) per ml at 4°C. At the end of the initial adsorption period (defined as time [t] = 0), dishes were directly transferred to 37°C to allow penetration to proceed. At the times indicated, the inoculum was removed and duplicate monolayers were exposed for 1 min to a low-pH citrate buffer (or to PBS as a control) to inactivate any bound virus that had not yet penetrated cells. The results in Fig. 5 show that the rates of transformation from susceptibility to resistance to 6-O-DS heparin paralleled the rate of virus penetration. 6-O-DS heparin inhibited just over 50% of viral PFU at 4°C (t = 0). However, the inhibitory effect of 6-O-DS heparin on HSV-1(KOS) was reversed with increased time at a physiologic tem-

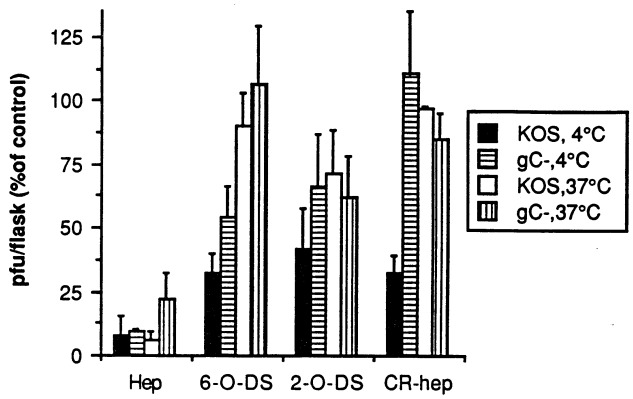

FIG. 6. Effects of unmodified heparin (Hep), CR heparin (CR-hep), 6-O-DS heparin, and 2-,3-O-DS heparin (2-O-DS) on HSV-1(KOS) (KOS) and HSV-1(KOS)ΔgC2-3 (gC−) at 4 and 37°C. Cultures of Vero cells in 25-cm^2 flasks were precooled to 4°C or maintained at 37°C and inoculated with HSV-1(KOS) or virus with gC-1 deleted, HSV-1(KOS)ΔgC2-3, in the absence or presence of 100 μg of heparin, CR heparin, 2-,3-O-DS heparin, or 6-O-DS heparin per ml. For dishes at 4°C, after a 2-h adsorption period at 4°C, the inoculum was removed and cells were washed three times in PBS, transferred to 37°C for 1 h to allow penetration to occur, and subsequently overlaid with medium 199O. For flasks at 37°C, after a 2-h adsorption period at 37°C, the inoculum was removed and cells were washed three times in PBS and subsequently overlaid with medium 199O. Plaques were counted after 2 or 3 days. The results presented are the PFU formed in the presence of the indicated heparin compound expressed as percentages of the PFU detected in the absence of any heparin compound (control). For each virus, two or three experiments were performed in duplicate. Each point is the mean of values obtained, and error bars indicate the ranges of values.

perature. The percentage of PFU that penetrated cells also increased in parallel with increased time at 37°C. Within 60 min, approximately half of the virus that had been inhibited by 6-O-DS heparin became resistant to the inhibitory effect of 6-O-DS heparin and, in parallel, about half of the bound virus had penetrated. These results suggest that the transition from susceptibility to resistance to 6-O-DS heparin occurs immediately before or concomitant with penetration and is consistent with a model of primary binding at 4°C followed by secondary binding at 37°C, as has been described for several other viruses (9–11, 16). N-DA heparin inhibited viral plaque formation throughout the time course. The observation that the inhibition of viral PFU by N-DA heparin at 4°C is not reversed when cells are transferred to 37°C indicates that part of the secondary binding evident at 37°C may also be mediated by interactions with HS moieties.

It was previously shown that the binding of HSV-1 to cells is mediated independently by interactions between gC-1 and/or gB-1 and cell surface HS (19, 20) and that select modified heparin compounds differentiate the binding of gC-1 and gB-1 with cell surface HS (17). We hypothesized that for HSV-1, the initial adsorption at 4°C might consist predominantly of interactions between gC-1 and HS, whereas the secondary binding evident at 37°C might consist predominantly of interactions between gB-1 and HS. To explore this possibility, we compared the effects of temperature-dependent modified heparin compounds on infection by wild-type HSV-1 or HSV-1 with gC-1 deleted. The results are summarized in Fig. 6. Similar to the results obtained for HSV-2, there was little or no temperature-dependent differential effect on viral PFU for the virus with gC-1 deleted. CR heparin and 2-,3-O-DS heparin had little or no inhibitory effect at either 4 or 37°C. 6-O-DS heparin did inhibit about 50% of PFU at 4°C but had little or no effect at 37°C. These results support the hypothesis that most of the initial binding evident at 4°C for wild-type HSV-1 reflects interactions between gC-1 and HS. Interestingly, at 37°C, the effects of all the modified heparin compounds were similar for both wild-type HSV-1 and HSV-1 with gC-1 deleted (Table 1). Taken together, these results suggest that the effects of modified heparin compounds on wild-type HSV-1 at 37°C reflect the interactions between gB-1 and HS, whereas the effects at 4°C primarily reflect interactions between gC-1 and HS. For HSV-2, minimal differences in the effects of modified heparins at 4 and 37°C were observed, suggesting that the interactions of HSV-2 virus with cell surface HS at both temperatures are similar and perhaps mediated by only one heparin-binding glycoprotein.

DISCUSSION

Although HS serves as a receptor for the binding of both HSV-1 and HSV-2, the results reported here suggest that the specific features of HS recognized differs for each serotype. This notion is supported by the observation that 6-O-DS and 2-,3-O-DS heparins inhibit plaque formation for HSV-2 but have little or no inhibitory effect for HSV-1. This difference was observed for several strains and on several human cell lines, including epithelial cells (HEp-2), cervical cells (Ca Ski), and neuroblastoma cells (SK-N-SH) (data not shown). Thus, 6-O and 2-,3-O sulfation sites on iduronic acids are important determinants for the interactions of HSV-1, not HSV-2, with heparin and cell surface HS. One would anticipate that cells which express HS structures rich in O-sulfated iduronic acids might be more permissive for HSV-1 infection than are cells expressing few O-sulfated HS structures.

Interestingly, the transfer of gC-2 to an HSV-1 genomic background results in virions that are susceptible to 6-O-DS and 2-,3-O-DS heparins. The transfer of gC-2 to HSV-1 has also previously been shown to alter the phenotype of HSV-1 with respect to neomycin susceptibility (3). In contrast to O-DS heparins to which HSV-2 is more susceptible than HSV-1, neomycin inhibits HSV-1 infection more effectively than it does HSV-2 infection (3, 32–34, 43). The effects of neomycin on HSV-1 infection, however, are complex. We previously reported that neomycin has two distinct effects on HSV-1 infection. First, it inhibits the binding of gC-1-positive, not gC-1-negative, virions to cells because, as a polycation, it competes with gC-1, not gB-1, for HS receptors (18). Second, neomycin inhibits postbinding events independent of the presence of gC in the virion envelope by an unknown mechanism (18). Neomycin fails to inhibit HSV-2 binding but does inhibit postbinding events for HSV-2 (16a). The results with intertypic recombinants suggest that when gC-2 is transferred to an HSV-1 background, it influences viral binding so that binding is resistant to neomycin (presumably because gC-1 is absent and neomycin does not compete with gC-2 or gB-1 for HS receptors) but susceptible to 6-O-DS and 2-,3-O-DS heparins (presumably because gC-2 binds O-DS heparins).

Not only do the glycoproteins of HSV-1 and HSV-2 appear to preferentially bind different structural features of HS, but the relative contributions of gB and gC to viral binding for the two serotypes differ. We and others have previously shown that the binding of HSV-1 to Vero or HEp-2 cells is mediated principally by gC-1 (19, 20, 52, 54). HSV-1 mutants with gC-1 deleted show decreased specific binding activities (particles bound per cell at 4°C), a marked lag in penetration, and reduced specific infectivities (PFU per particle) (20). In contrast, an HSV-2(G) mutant with gC-2 deleted shows no impairment in specific binding activity, rate of penetration, or specific infectivity on Vero or HEp-2 cells (13), although differences in susceptibility to neomycin have been reported (3, 32–34, 43).

These results suggest that gB-2, the other major heparin-binding glycoprotein, plays the key role in HSV-2 attachment to these cells. At first glance, the observations that gC-2 does not play the key role in mediating the binding of HSV-2 to cell surface HS on Vero or HEp-2 cells and that the transfer of gC-2 to HSV-1 alters the phenotype of HSV-1 with respect to susceptibility to inhibitors of viral adsorption might seem contradictory. However, these results are consistent with the notion that in an HSV-1 background, the presence of either gC-1 or gC-2 in the viral envelope influences viral binding and relative susceptibilities to inhibitors of viral adsorption. In an HSV-2 background, the presence or absence of gC-2 does not affect specific binding activity but, because gC-2 is a heparin-binding protein, influences susceptibilities to inhibitors of viral adsorption.

Most viruses bind at low temperatures (0 to 4°C) but penetrate cells only at physiologic temperatures. For some viruses, a secondary binding step follows the initial binding at 4°C (16). This secondary binding generally occurs only at temperatures permissive for penetration and may be required for penetration. In these studies, we explored differences in HSV-1 and HSV-2 binding at 4 and 37°C by comparing the effects of modified heparin compounds present during the adsorption period at these two temperatures. These studies focused on different types of interactions with HS and differ from those reported by McClain and Fuller (39), which focused on defining multiple attachment steps for wild-type HSV-1 by the ability of bound virus to be eluted by washes with PBS, heparin, or a low-pH citrate buffer. For wild-type HSV-1, we found that several modified heparin compounds could competitively inhibit viral binding at 4°C, but this inhibitory effect was reversed when the inoculum and cells were warmed to physiologic temperatures. Such a differential effect was less evident for viruses with gC-1 deleted and for HSV-2. These results suggest that the binding of HSV-1 to HS is a multistep process. We postulate that the initial step in HSV-1 adsorption is a low-affinity interaction between gC-1 and HS structures. This interaction is relatively easy to competitively inhibit both by polycations such as neomycin (18) and by modified heparin compounds (17). The only modified heparin compound that failed to inhibit the binding of HSV-1 at 4°C was N-desulfated heparin. This initial binding is observed at 4°C and promotes efficient adsorption but is not absolutely required. This interaction may serve to concentrate virions at the cell surface, and, at physiologic temperatures, may promote a conformational change in the virus so that a second, higher-affinity interaction with HS occurs. This second interaction is mediated principally by gB-1 binding either to a different subset of HS structures or with higher affinity to similar HS moieties (17). This interaction cannot be inhibited by N-desulfated heparin, O-DS heparin, CR heparin, or neomycin but can be inhibited, for example, by N-DA heparin. Studies conducted at physiologic temperatures primarily reflect this second interaction, as evidenced by the observation that there are no significant differences between wild-type viruses (containing both heparin-binding glycoproteins, gC and gB) and viruses with gC-1 deleted (containing only gB) with respect to susceptibility to inhibitors of viral adsorption at 37°C; differences are detected only at 4°C. This second interaction may be essential and presumably triggers subsequent heparin-independent interactions between the virus and cell surface that culminate in virus-cell fusion. Subsequent interactions probably include interactions between gD and/or gH-gL and other cell surface components (12, 24–26, 39). In support of this model of different interactions between HSV-1 and cell surface HS at 4 and 37°C is the recent finding that soluble gC inhibits HSV-1 attachment at 4°C but has little or no inhibitory effect on plaque formation at 37°C (54).

Although we cannot exclude dual interaction between HSV-2 and HS moieties, it is not evident from the data obtained thus far. Minimal temperature-dependent differences were observed for HSV-2, although there was a tendency for O-DS heparins to inhibit HSV-2 plaque formation better at physiologic temperatures. Serotype differences in the abilities of modified heparins to inhibit viral infection at 4 and 37°C may reflect serotype differences in the roles of heparin-binding envelope glycoproteins in viral infection (13). For example, for HSV-2, binding at both 4 and 37°C may be mediated principally by only one glycoprotein, presumably gB-2.

Three distinct stages in PrV adsorption, including biphasic interaction with HS, have also been proposed (26, 60, 61). The first step in PrV attachment is presumed to be via a low-affinity binding of gC (gIII) with an HS receptor molecule. After this primary adsorption, virus cannot be eluted by PBS but can be eluted by a heparin wash (26). The second step in PrV attachment is presumed to be mediated by the binding of the central portion of gC with higher affinity to HS. Deletion of the middle segment of PrV gC results in mutant virions that are impaired in this secondary stable binding interaction (60). Evidence suggests that the third stage in PrV stable adsorption is mediated by interactions between gD and an unknown cell surface. Both the second and third steps in PrV adsorption are resistant to elution by heparin wash. In support of the notion of biphasic interaction between PrV and HS, in preliminary studies, we found that O-DS heparins inhibit PrV infection when present at 4°C but have little or no inhibitory effect at 37°C (16a).

Low- and high-affinity interactions with HS that promote conformational changes in the heparin-binding protein have also been described for several other ligands. For example, the binding of antithrombin III to a specific heparin pentasaccharide sequence leads to a conformational change in antithrombin III and is responsible for the catalytic activity of heparin in the coagulation cascade (1, 37). The binding of basic fibroblast growth factor (FGF) to heparin may change the conformation of basic FGF to allow for higher-affinity interaction between basic FGF and its receptor (15, 22, 29, 55, 59). An alternative mechanism has been proposed for acidic FGF (51). Soluble heparin induces oligomerization of acidic FGF, resulting in subsequent FGF receptor dimerization and activation.

The binding of HSV to cell surfaces is clearly a complex, multistep process. The studies reported here show serotype differences in susceptibility to modified heparin compounds. These differences vary, depending on the experimental conditions (4 versus 37°C) and the presence or absence of gC in the virion envelope. These results suggest that there may be differences in the structural sequences of HS with which HSV-1 and HSV-2 preferentially bind and differences in initial (evident at 4°C) and secondary (evident at 37°C) interactions with cell surface HS. These differences, coupled with serotype differences in the roles of gC and gB in viral binding, may contribute to the observed differences in epidemiology and cell tropism. Additional serotype differences may be uncovered as the subsequent steps in the cascade of virus-cell interactions that occur to promote viral entry are identified.

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