INFLUENCE OF GLYCOPROTEINS B, C AND D ON THE CONVERSION OF VIRUS-TO-CELL ATTACHMENT FROM HEPARIN SENSITIVITY TO RESISTANCE

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Summary. – Glycoprotein C-negative (gC^-) mutants of herpes simplex virus type 1 (HSV-1) derived from strains KOS and ANGpath were used to analyse the influence of soluble heparin on the phase of adsorption/attachment of HSV-1 to cells. A dose of 200 μg/ml heparin given 20 mins after infection of cells with the gC-positive (gC^+) strains KOS and ANGpath at 4°C reduced the adsorption of infective particles to 20 – 30% of the controls. A weaker heparin effect was observed with gC^- mutants. However, also the gC^- mutants exhibited a short heparin-sensitive phase. Mutations in amino acids of gB or gD at positions 854 or 25 and 27, respectively, did not alter the attachment capacities of these HSV mutants in the presence of heparin despite their peculiar fusion properties and resistance to soluble gD. We conclude that HSV-1 strains exhibit a heparin-resistant phase of attachment, which is determined by gC. Lack of gC delays the heparin-resistant attachment phase of HSV-1 to cells.

Key words: herpes simplex virus; glycoproteins B, C, D; virus-to-cell attachment; heparin

Introduction

Glycosaminoglycans, especially heparin (heparan sulphate), represent the cellular receptor for HSV (WuDunn and Spear, 1989; Lycke *et al.*, 1991). Treatment of cells with heparinase causes inhibition of their capacity to adsorb virus (WuDun and Spear, 1989; Walev *et al.*, 1991). gC is the most important heparin-binding structure (Langeland *et al.*, 1990; Herold *et al.*, 1991; Svennerholm *et al.*, 1991), moreover, also gB and gD are involved in the attachment (Herold *et al.*, 1994). Strains of HSV treated with monoclonal antibodies (MoAbs) against gC are able to infect cells, however, at a strongly reduced rate only (Svennerholm *et al.*, 1991). Herold *et al.* (1995) found by means of various derivatives of heparin that gC and gB interact with different parts of heparin. The existence of a second, non-heparin-like cellular receptor which probably binds through gD has been reported (Lee and Fuller, 1993; Johnson *et al.*, 1990; Johnson and Ligas, 1988). Brunnetti *et al.* (1994, 1995) raised evidence for binding of gD to mannose phosphate-receptors. Ten percent of adsorbing HSV could not be inhibited with heparin, but could be with chondroitin sulphate, and soluble gD-1 reduced the HSV infection rate in a significant manner (Banfield *et al.*, 1995). Zsak *et al.* (1991) and Karger and Mettenleitner (1993) for pseudorabies virus (PsRV), and McClain and Fuller (1994) for HSV provided evidence for two distinct phases of adsorption of these viruses. In the

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Abbreviations: aa = amino acid; BHK = baby hamster kidney; CPE = cytopathic effect; EDTA = ethylenediamine tetraacetate; FFWI = fusion from within; FFWO = fusion from without; gB, gC, gD = glycoproteins B, C, D; gC^+, gC^- = gC-positive, gC-negative; HSV = herpes simplex virus; HSV-1 = HSV type 1; MoAb = monoclonal antibody; MOI = multiplicity of infection; PBS = phosphate buffered saline; PsRV = pseudorabies virus; SDS = sodium dodecyl sulphate

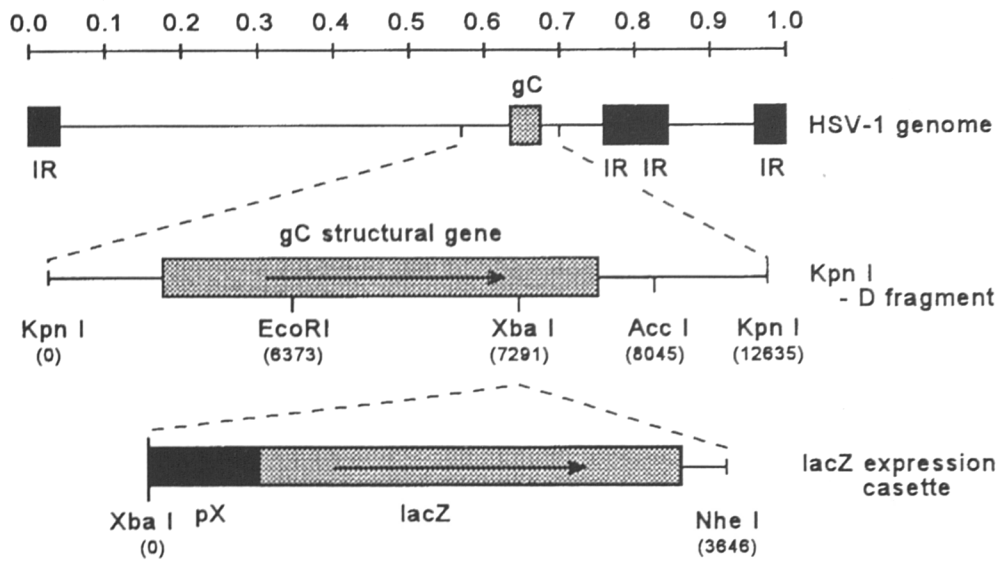

Fig. 1
Location of the structural gene of gC in the HSV-1 genome and position of the lacZ cassette

first, heparin-sensitive phase, virus particles are adsorbed, but heparin washing causes their release. In the second, heparin-resistant phase, bound virus particles cannot be released by heparin washing. Herold *et al.* (1994) used a pair of gC^+ and gC^- HSV mutants and performed dose-response experiments using heparin, but they did no time course-experiment, whereas McClain and Fuller (1994) used only laboratory HSV gC^+ strains. Therefore, we used different mutants/strains of HSV to study the kinetics of the heparin-resistance of the virus-to-cell attachment at different temperatures. A gC^- mutant of HSV strain KOS was constructed by cloning a lacZ cassette into the transmembrane part of gC. Besides, we used the gC^- strain ANGpath. The used strains KOS and ANGpath differ in their effects on cells: whereas KOS induces the wild type cytopathic effect (CPE), ANGpath induces cell fusion from within (FFWI). Especially, we tried to correlate in time the heparin-resistant attachment phase and the penetration phase by using "fusion from without"-positive ($FFWO^+$) strains with certain mutations in gB and gD.

Materials and Methods

Cells and viruses. African green monkey kidney (Vero) cells and baby hamster kidney (BHK) cells were used. The following HSV-1 strains were used: KOS 321 (Holland *et al.*, 1983), ANG, ANGpath, ANGpath gC18 (gC^-) and ANGpath gC^-gE^- (obtained from Dr. H.C. Kaerner, Heidelberg, Germany) (Schranz *et al.*, 1989), KOS 854Q, 27/III, 17 syn^+, 17 syn3 (Seck *et al.*, 1994), IES, F (Podlech *et al.*, 1996) and KOS 804Q (obtained from Dr. P. Schaffer, Boston, USA). In order to obtain high virus titers, Vero cells in roller bottles were infected at a multiplicity (MOI) of 0.03 – 0.1 PFU per cell and incubated for 48 hrs at 37°C. Stock virus was aliquoted, titrated and tested for bacterial contamination (Seck *et al.*, 1994).

Construction of vector pCXL9. We constructed a gC^- HSV by insertion of a lacZ expression cassette into the gC of strain KOS 321. Plasmid pTT-80 (Mettenleiter and Rauh, 1990), contained the promotor of glycoprotein X (pX) of PSRV and the bacterial lacZ gene.

We inserted the lacZ expression cassette into the hydrophobic transmembrane sequence of gC, so that gC was unable to anchor in the cell membrane. No gC was expressed intracellularly as tested by immunofluorescence with mono- and polyclonal gC-antibodies (data not shown).

Briefly, vector pTT80 was digested with restriction endonucleases *Sal*I and *Bam*I. The resulting fragment containing the lacZ expression cassette was subcloned into the polylinker region of the pUC18 vector. The new vector was called pLAC 8B. In parallel, the 3'-moiety of the gC structural gene was excised from vector pGX123 with rectriction endonucleases *Acc*I and *Eco*RI. pGX123 contained the HSV-1 KpnI - D restriction fragment, encompassing the whole gC gene. The resulting vector was called pGC18-7. The lacZ expression cassette was excised from pLAC 8B with endonucleases *Nhe*I and *Xba*I, and cloned into the

unique *Xba*I restriction site of pGC18-7, located in the gC structural gene. The resulting vector was called pCXL9. Transcription directions of the gC gene and lacZ expression cassette were identical (Fig. 1).

Recombination of HSV-1 strain KCL (gC⁻). Viral DNA from HSV-1 strain KOS 321 was prepared by infecting confluent monolayers of Vero cells at a MOI of 1-3. Cells were incubated at 37°C in 5% CO_2 for 24 hrs. Cells from a 60 mm-plate were harvested with trypsin/ ethylenediamine tetraacetate (EDTA) solution, washed twice with PBS and carefully lysed in 50 µl lysis buffer (0.5% sodium dodecyl sulphate (SDS), 10 mmol/l Tris-HCl pH 8.0, 100 mmol/l NaCl, 1 mmol/l EDTA). The resulting lysate was incubated with 0.1 mg/ml proteinase K at 50°C for 12 hrs and dialysed twice against TE buffer (10 mmol/l Tris-HCl pH 8.0, 1 mmol/l EDTA). Cotransfection experiments were carried out using the standard calcium phosphate method with 9 µg of viral DNA and 3 µg of uncut vector pCXL9 and Vero cells as target. After plaque formation, supernatants were taken and aliquots were plated on Vero cells and incubated until plaque formation. Cells were fixed with 0.05% glutaraldehyde and stained with X-Gal (300 µg/ml X-Gal (Sigma), 20 mmol/l $K_3Fe(CN_6)$, 20 mmol/l $K_4Fe(CN_6)$, 2 mmol/l $MgCl_2$ in PBS). $lacZ^+$ virus was plaque purified and tested for the $lacZ^+/gC^-$ phenotype.

Analysis of conversion of heparin-sensitive to heparin-resistant attachment. 2 x 10^5 Vero or BHK cells were seeded into 24-well plates (Nunclon) and kept overnight at 37°C in 5% CO_2. For analysis, the cells were incubated in ice-water for 15 mins. The cells were then infected with 1000 PFU per well. In the following three minutes virus particles were allowed to bind to cells. The wells were then washed carefully twice with ice-cold Hanks' solution.

After 3, 5, 10, and 40 mins, they were washed again; the "heparin"-wells twice with 200 µg/ml heparin (Thrombophob-25,000, Nordmark Arzneimittel GmbH, Germany) in Hanks' solution and once only with Hanks' solution to remove heparin. The "control"-wells were washed three times with Hanks' solution only. Then a medium containing 10% human serum with anti-HSV antibodies of ELISA titer of 2400 was added in order to neutralize remaining adsorbed HSV and the plates were incubated at 37°C in 5% CO_2 for 1-3 hrs. Plaques were counted 36 – 48 hrs after infection. Each experiment was repeated at least 3 times.

Results

Differentiation of two phases of virus attachment to cells and kinetics of the attachment

In order to assess the influence of heparin on the adsorption process, we performed first a dose-response experiment to determine the optimal concentration of heparin for this purpose. Fig. 2 shows that 200 µg/ml heparin in the culture fluid reduced the percentage of adsorbed virus strain KOS to about 20 – 30% of the control. This concentration was used in all further experiments. As shown elsewhere, adsorption rates at 4°C were identical for all strains tested (Lingen *et al.*, 1995). But, interestingly, there was 20 – 30% of the adsorbing virus which could not be washed off after 20 mins of incubation with heparin at 4°C. The same results were obtained also with strains ANGpath and KOS 854 Q.

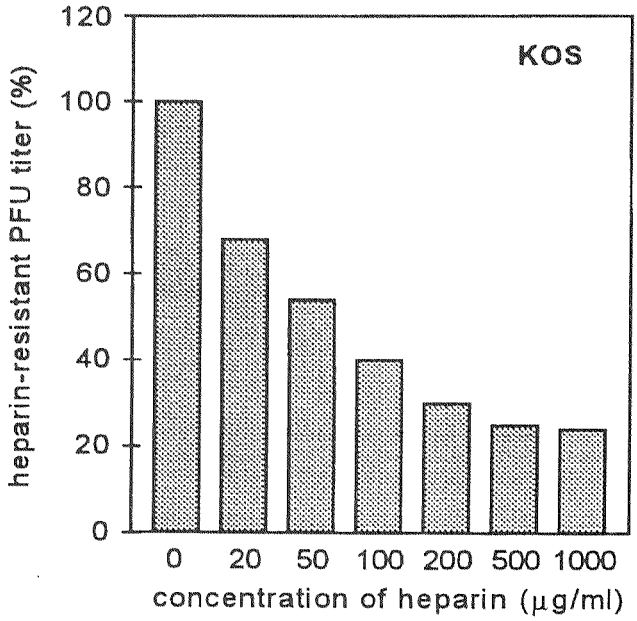

Fig. 2
Dose-response experiment with heparin washing after infection with strain KOS
Virus was inoculated at zero time at 4°C, heparin washing was done 20 mins later, fresh medium was added and the incubation continued at 37°C for 2 hrs. Then the antibodies were added. Reading of the test was done 24 – 36 hrs after infection.

We decided next to analyse the kinetics of the heparin-sensitive/resistant phase conversion with strains KOS and ANGpath. The heparin washing was done at different times after infection. Fig. 3 clearly shows that the heparin washing reduced the attachment of both HSV strains.

Influence of gC on the heparin sensitivity of the virus-to-cell attachment

gC is the most important glycoprotein of HSV which determines the attachment process. We therefore constructed a gC^- recombinant of strain KOS (KCl) and used the ANGpath gC^- mutant (gC18) to see whether the lack of gC influences the heparin resistance of the adsorption. Fig. 4

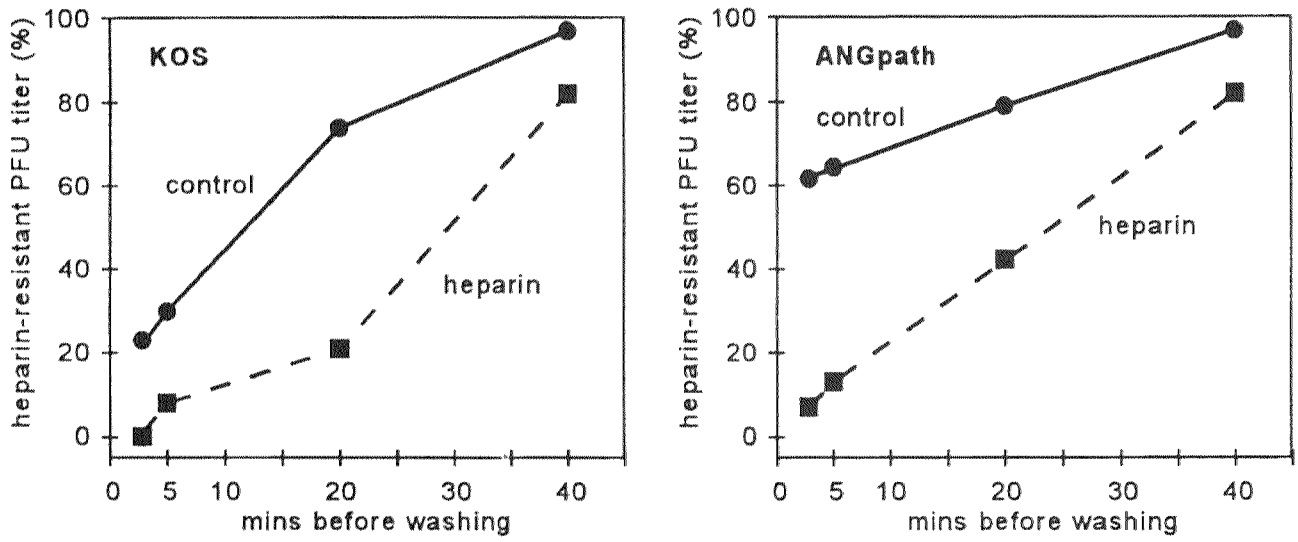

Fig. 3

Influence of heparin washing at different times on the attachment of strains KOS and ANGpath

After infection at 4°C, the washing was done at different times without or with 200 μg/ml heparin and fresh medium was added. Sixty mins after infection, the antibodies were added and the incubation was continued at 37°C. The 100% value was calculated as the number of PFU obtained in the untreated control.

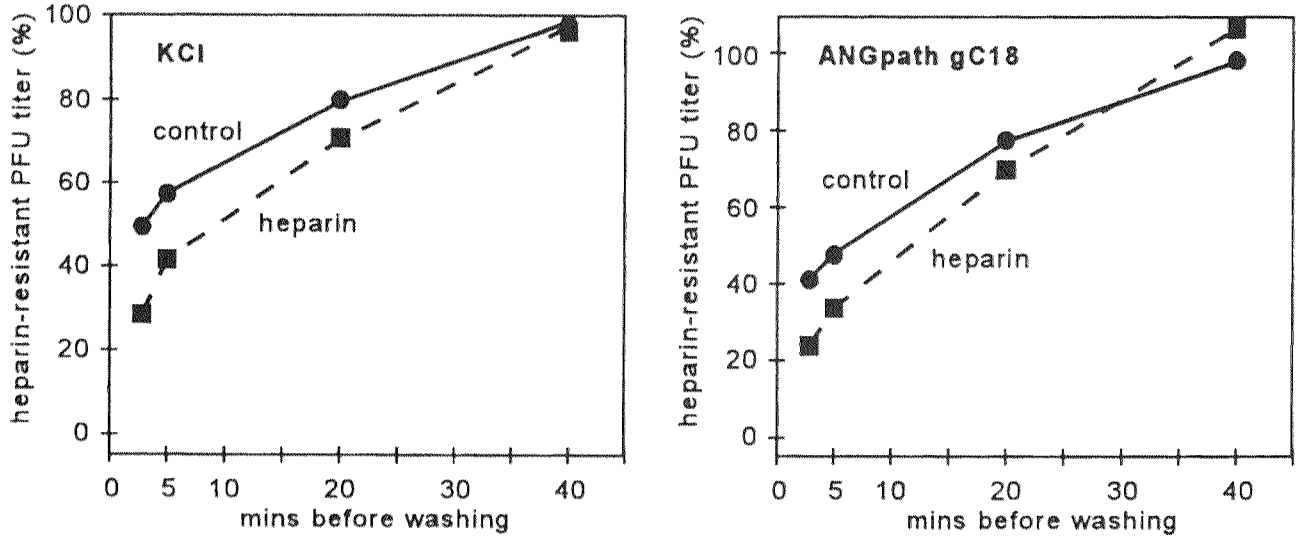

Fig. 4

Influence of heparin washing at different times on the attachment of mutants KCL and ANGpath gC18

demonstrates that there was apparently no influence of the heparin washing at the indicated times on the rate of virus adsorption. Thus, gC represents the most effective glycoprotein reactive in the attachment process and responsible for its heparin sensitivity.

Next, the wild type strain KOS and its gC⁻ recombinant (KCl) were used to test the influence of 200 μg/ml heparin on the virus adsorption after different times of incubation at 4°C.

Fig. 5 shows a strong influence of heparin on the adsorption. In the case of strain KOS, but not gC⁻ recombinant KCl, heparin enabled a washing off of a considerable proportion of the adsorbed infective particles of HSV for up to 20 mins. The parental strain ANGpath and its gC⁻ mutant ANGpath gC18 showed identical results.

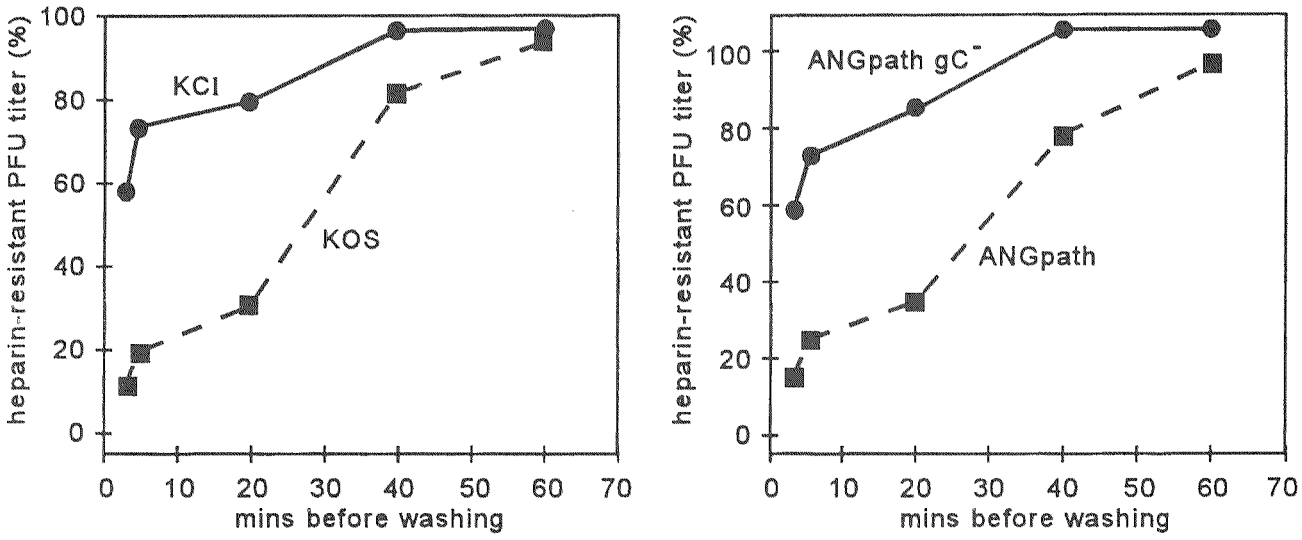

Fig. 5
Comparison of strain KOS and its gC⁻ mutant KCL, and strain ANGpath and its gC⁻ counterpart with regard to their sensitivity to heparin washing

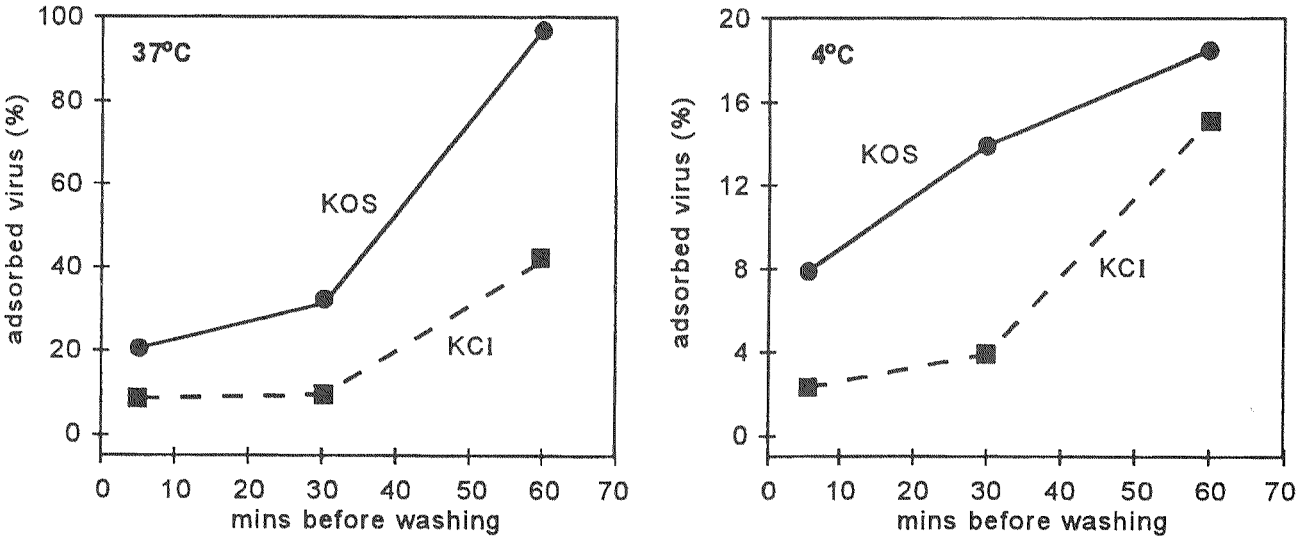

Fig. 6
Comparison of attachment rates of strains KOS and KCl at 37°C and 4°C
Heparin washing was done 5, 30, and 60 mins after infection at 4°C. The 100% value was that of KOS 321 for 60 mins at 37°C.

Comparison of the virus-to-cell attachment of strain KOS and the gC⁻ recombinant KCl at 4°C and 37°C

After having shown the influence of gC of HSV on the emergence of the heparin-resistant phase of the attachment, we wanted to see whether gC determines the actual rate of binding of HSV to cells. The temperatures 37°C and 4°C were compared. Cells were infected with strain KOS or mutant KCl at either temperature and washed with Hanks' solution (without heparin) at different times thereafter. The results obtained show (Fig. 6) that in the case of the gC⁻ recombinant KCl the attachment phase was delayed; the adsorption rates were higher at 37°C than at 4°C. Identical results were obtained with the strain pair ANGpath and ANGpath gC⁻ (gc18) (data not shown).

Influence of mutations in the amino- terminus of gD and the carboxy-terminus of gB on the heparin sensitivity of virus-to-cell attachment

Next, we tested the influence of gD and gB of HSV on the heparin sensitivity of the attachment by using HSV

Table 1. Influence of mutations in gD and gB of HSV on the heparin sensitivity of virus-to-cell attachment

HSV strain/ recombinant	Mutations in gD*	Mutations in gB**	Properties of strains
17 syn$^+$	–	–	wild type
KOS	–	–	wild type
F	–	–	wild type
ANG	25,27	854	laboratory strain
ANGpath	25,27	854	laboratory strain
27/III	25,27	–	gB from strain KOS
ANGpath (gD-gDI KOS)	–	854	gD-gI from strain KOS
ANGpath (gD-gDI ANGpath)	25,27	854	Re-recombinant
KOS 854 Q	–	854	C-t (gB) into KOS
17 syn 3	–	854	C-t (gB) into 17 syn$^+$
ANGpath gC$^-$	25,27	854	gC$^-$ by point mutation

*Mutations in the amino-terminus of gD at positions 25 and 27 (Lingen *et al.*, 1996).
**Mutations in the carboxy-terminus of gB at position 854 (Lingen *et al.*, 1995).

strains with mutations in these glycoproteins. Table 1 lists a number of such strains and recombinants. These mutations are assumed to be of peculiar importance for penetration and cell/cell fusion processes (Lingen *et al.*, 1995, 1996).

The mutation in amino acid (aa) 854 from Ala to Val in the carboxy-terminus of gB endows the strains with the ability of FFWO in BHK cells (Lingen *et al.*, 1995). Additional mutations in gD supply a capacity of penetration at 4°C, FFWO in BHK and Vero cells, and a resistance to washing with soluble gD (Lingen *et al.*, 1996). All mutant and recombinant strains were identical regarding the heparin washing described above; they exhibited the same behaviour as the reference strains KOS, 17 syn$^+$ and F. Further experimentation showed that the lack of gC in strains ANGpath gC18 and ANGpath gC$^-$gE$^-$ did not influence the heparin sensitivity of FFWI if given 3 hrs after infection (Seck *et al.*, 1994) (Table 2).

Table 2. Effect of heparin on FFWI

Treatment	Type of CPE		
	ANG	ANGpath gC18	ANGpath gC$^-$gE$^-$
Control	Cell fusion	Cell fusion	Cell fusion
Heparin	Cell rounding	Cell rounding	Cell rounding

Heparin (1 μg/ml) washing performed 3 hrs after infection.

Discussion

We analysed thoroughly the adsorption/attachment phase of the HSV growth cycle by using HSV-1 strains lacking gC (gC$^-$) in comparison to the parental strains (gC$^+$) and the washing with heparin. The gC$^-$lacZ$^+$ recombinant of strain KOS 321 (KCl) and the gC$^-$ mutant of strain ANGpath gC18, both functionally inactive, yielded identical results. In our washing experiments, heparin in concentration of 200 μg/ml was used as delineated by the dose-response study. In this way, about 75% of the adsorbed infective particles could be washed off, if the adsorption was allowed to proceed at 4°C for 20 mins. Heparin-sensitive and a heparin-resistant phases were indeed detectable after infection with various HSV strains.

In order to study the kinetics of the heparin sensitivity/resistance conversion, we used strains KOS 321 and ANG, and the heparin washing at different times of the virus adsorption period. The attachment of HSV particles to cells and its heparin resistance increased with time, but still a considerable amount of the adsorbed virus was sensitive to this washing procedure. By using the gC$^-$ mutants of HSV-1 in similar experiments, however, there were clearly smaller effects of the heparin washing. We conclude that gC of HSV-1 is the main determinant of the heparin resistance of the virus-to-cell attachment. In addition, gC inhibits the conversion of the heparin resistance. gC is one of the largest glycoproteins of HSV, measuring 24 nm in length. Possibly, this fact is responsible for inhibition of virus-cell interactions mediating the heparin-resistant attachment phase. This was shown to be true both at 4°C and 37°C, and the attachment rate was higher at 37°C than at 4°C. Another interesting point is the observation of a short heparin-sensitive attachment phase also with the both gC$^-$ mutants.

Taken together, HSV-1 exhibits a heparin-sensitive and heparin-resistant phase of attachment to cell comparable to PsRV (Karger and Mettenleitner, 1993), depending on the presence or absence of gC. Moreover, gC seems to delay the heparin-resistant attachment to cells. In the absence of heparin, the presence of gC appears to accelerate the attachment.

FFWI induced by mutations in the syn 3 locus – but not by mutations in other syn loci – can be inhibited by addition of 1 mg/ml heparin 3 hrs after infection (Seck *et al.*, 1994). Also in the case of the strains ANGpath gC18 and ANGpath gC$^-$gE$^-$ the same inhibition was observed. This observation can be interpreted that gC is not involved in the process of the cell-cell fusion leading to FFWI depending on the syn 3 locus. Especially the amino-terminus of gC is not involved in the cell-cell fusion as shown by our experiments with addition of neomycin (data not shown).

Herold *et al.* (1994) concluded from their experiments that the attachment to cells of HSV-1 strains lacking gC

depends on gB. We therefore tested some well defined mutants of HSV with mutations in gD and gB. These mutants exerted special activities: FFWO, cell-type specificity of FFWO and resistance to washing with soluble gD-containing medium (Lingen *et al.*, 1995). It could be shown that neither the FFWO-inducing mutation in position 854 of the carboxy-terminus of gB nor the mutations in positions 25 and 27 in the region of the amino-terminus of gD altered the attachment phase or its heparin resistance. In conclusion, gC of HSV-1 is the main determinant of control of virus-to-cell attachment. Its effect seems to be very specific and selective as is the cooperative function of gB and gD during this phase of the replication cycle.

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