JOURNAL OF VIROLOGY, Feb. 1989, p. 819–827
0022-538X/89/020819-09$02.00/0

Vol. 63, No. 2

Herpes Simplex Virus Glycoprotein D Mediates Interference with Herpes Simplex Virus Infection

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Received 12 August 1988/Accepted 2 November 1988

We showed that the expression of a single protein, glycoprotein D (gD-1), specified by herpes simplex virus type 1 (HSV-1) renders cells resistant to infection by HSV but not to infection by other viruses. Mouse (LMtk$^-$) and human (HEp-2) cell lines containing the gene for gD-1 under control of the human metallothionein promoter II expressed various levels of gD-1 constitutively and could be induced to express higher levels with heavy metal ions. Radiolabeled viruses bound equally well to gD-1-expressing and control cell lines. Adsorbed viruses were unable to penetrate cells expressing sufficient levels of gD-1, based on lack of any cytopathic effects of the challenge virus and on failure to detect either the induction of viral protein synthesis or the shutoff of host protein synthesis normally mediated by a virion-associated factor. The resistance to HSV infection conferred by gD-1 expression was not absolute and depended on several variables, including the amount of gD-1 expressed, the dosage of the challenge virus, the serotype of the challenge virus, and the properties of the cells themselves. The interference activity of gD-1 is discussed in relation to the role of gD-1 in virion infectivity and its possible role in permitting escape of progeny HSV from infected cells.

The last step of successful viral replication is the egress of progeny viruses from infected cells. A progeny virus must escape from the cell that produced it without superinfecting that cell. Superinfection would result in eclipse of progeny virions and a decrease in the infectious yield.

Different viruses may use different mechanisms to prevent eclipse of progeny virions by host cells. Members of the paramyxovirus and orthomyxovirus families express a receptor-destroying enzyme, neuraminidase. This enzyme removes sialic acid, the virus receptor, from viral and cell glycoproteins and glycolipids (35, 52). Similarly, some coronaviruses express a receptor-destroying esterase with a unique specificity (29). Cells producing retrovirus gene products are resistant to infection by closely related strains of retroviruses and presumably to superinfection by progeny virus released from the cells (49). The mechanism(s) responsible for this interference is not fully understood. It has been shown that the CD4 receptor for the human immunodeficiency virus is reduced in amount on the surfaces of infected cells, at least in part because of sequestering of the receptor within the cell by the *env* gene product (18, 47). Expression of the *env* gene product alone can reduce the levels of CD4 on the surfaces of transformed cells (46).

Superinfection exclusion or interference has not been documented for herpesviruses, in part because of difficulties associated with detecting superinfection of cells infected by a lytic virus. As shown here, expression of herpes simplex virus type 1 (HSV-1) glycoprotein D (gD-1) in the absence of lytic infection is sufficient to render permissive cells resistant to HSV infection, just as expression of a retroviral *env* gene product can make cells resistant to infection by closely related retroviruses. Our study follows on the observations of Campadelli-Fiume et al. (5), who found that a baby hamster kidney cell line transformed by the *Bam*HI J fragment of the HSV-1 genome and some subclones of the cell line were resistant to HSV infection. The cells were shown to express gD-1 but may also have expressed other HSV proteins, inasmuch as the DNA fragment used for transformation contains four complete open reading frames encoding membrane glycoproteins (gG, US5, gD, and gI) and truncated forms of the open reading frames for a protein kinase and gE (32). In the subclones of the transformed cell line analyzed by Campadelli-Fiume et al. (5), it was possible to detect expression of gD-1, but not gI-1 or gE-1, by immunoprecipitation; attempts to detect the other potentially expressed HSV proteins were not reported.

In the experiments reported here, HSV DNA fragments encoding gD-1 plus gI-1 (the gI-1 transcription unit is contained totally within the gD-1 transcription unit) or gD-1 alone were cloned into expression vectors and used to transform cells. In all plasmid constructs, the endogenous promoter of gD-1 was replaced by the human metallothionein promoter II. Because this promoter is inducible by heavy metal ions (22), we could assess the effects on interference of increased levels of gD-1 expression within a cell line.

gD is one of seven virion envelope glycoproteins known to be encoded by herpes simplex viruses; the others are gB, gC, gE, gG, gH, and gI (1, 9, 14, 28, 38, 40, 44). Genetic analyses have demonstrated that gD, gB, and gH are all required for virion infectivity (4, 6, 26, 41). The other four HSV glycoproteins appear to be dispensible for viral replication in cell culture (15, 28, 33, 51).

Monoclonal antibodies specific for gD can be strong neutralizers of HSV infectivity. These antibodies block the penetration step rather than adsorption of virus to cells (11, 12, 16, 36). In addition, some monoclonal antibodies specific for gD can, at high concentrations, inhibit stable attachment of the virus to cells (11). Anti-gD monoclonal antibodies can also block virus-induced cell-cell fusion (34). Finally, mutant virions devoid of gD can adsorb to cells but fail to penetrate the cells (26). These data suggest that gD plays an important role in viral entry at the fusion-penetration step.

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MATERIALS AND METHODS

Viruses and cells. HEp-2 cells and mouse LMtk$^-$ fibroblasts were grown in Dulbecco modified Eagle medium (DMEM; GIBCO Laboratories) supplemented with 10% fetal calf serum (FCS; Hyclone and GIBCO). African green monkey kidney cells (Vero cells) were grown in Hanks 199 supplemented with 5% FCS. The virus strains used for these experiments were HSV-1(KOS), HSV-1(F), HSV-2(G), HSV-2(333), vaccinia virus(WR), and vesicular stomatitis virus (VSV)(San Juan). Virus stocks were prepared by low-multiplicity passage in HEp-2 cells, and their titers were determined on Vero and HEp-2 cells.

Plasmid construction. The coding sequences for gD-1 and gI-1 were taken from a plasmid, pDW15, which contains the 3,641-base-pair (bp) *Sma*I subfragment of *Bam*HI fragment J of HSV-1(KOS) DNA inserted into the *Sma*I site of pUC8 (produced by Darrell WuDunn). The endogenous gD-1 promoter was removed by digestion of the plasmid with *Hin*dIII and *Sma*I. The *Hin*dIII site lies very close to the transcription initiation site for gD-1 mRNA (7, 19, 50). The 2,900-bp *Hin*dIII-*Sma*I fragment containing the gD-1 and gI-1 coding sequences was filled in with the Klenow fragment of DNA polymerase and inserted into the *Sma*I site of plasmid pHSI containing the human metallothionein promoter II (22; plasmid kindly provided by E. Kieff). The resulting plasmid, with the insert oriented such that gD-1 was immediately downstream of the metallothionein promoter, was designated pRJ20. An *Nru*I-*Nru*I 813-bp deletion within pRJ20 removed the translation initiation codon and the N-terminal coding sequences of gI down to its transmembrane domain. gD-1 was the only remaining intact HSV coding sequence within this plasmid, which was designated pRJ41.

A general purpose vector, pRJ40, containing both the human metallothionein II promoter and the neomycin selectable marker from Tn*5* was produced by inserting the *Bam*HI fragment (containing the resistance cartridge) from pBKneo (kindly provided by R. Manservigi) into the *Nar*I site of pHS1 after filling both fragments in with T4 DNA polymerase. The metallothionein-HSV transcription units from plasmids pRJ20 and pRJ41 were transferred into the pRJ40 vector by exchanging a *Hin*dIII-*Eco*RI fragment with a *Hin*dIII-*Eco*RI fragment in pRJ40. The new plasmids containing the gD-1 and gI-1 genes or only the gD-1 gene, along with the metallothionein promoter and the neomycin selectable marker, were designated pRJ35 and pRJ42, respectively. Restriction enzymes were from New England BioLabs.

Transfections. Transfections were done by the calcium phosphate precipitation procedure described by Spandidos and Wilkie (43). All vectors were linearized outside of the sequences of interest before transfection. Mouse LMtk$^-$ or HEp-2 cells were plated 24 h before transfection at densities that yielded monolayers about 75% confluent at the time of transfection. Calcium phosphate precipitates of plasmid DNA (0.5 to 1.0 μg) along with salmon sperm DNA as the carrier were added to the monolayers in 25-cm^2 tissue culture flasks or in 60-mm-diameter dishes containing 5 ml of DMEM, 10% FCS, and gentamicin (GIBCO) at 25 μg/ml. The medium containing DNA was replaced with fresh medium 17 to 20 h after transfection was initiated. After allowing 24 h more for expression of the selectable marker, the medium was changed to DMEM–10% FCS with geneticin (GIBCO) at 400 μg/ml. Selection medium was changed every 3 to 4 days, and individual colonies were picked 10 to 15 days after transfection. LMtk$^-$ cells transformed very efficiently and were limiting diluted to assure clonal populations. The cell line designated CA35gD10 was obtained by cotransfecting pRJ20 with pSV2neo at a 5 μg:1 μg ratio. The parent L cell line for CA35gD10 was CA14.11.35 (30).

Induction of gD-1 expression. Cell lines were induced to express higher levels of gD-1 by incubation with DMEM–10% FCS containing either 2 μM cadmium chloride or 100 μM zinc chloride for 6 to 18 h. The metal chloride stock solutions were acidified with HCl to dissolve the metal ions and then adjusted to pH 5.0.

Southern blotting and Western immunoblotting. For Southern blots, cells from two 25-cm^2 flasks were detached with EDTA and pelleted. DNA was isolated as described by Maniatis et al. (31). Proteinase K and RNase A were from Boehringer Mannheim Biochemicals. A 10-μg sample of DNA from each cell line was digested with *Pvu*II and separated on a 0.8% agarose gel. Before denaturation, the DNA in the gel was depurinated by two 15-min incubations in 0.25 M HCl. The DNA was denatured with NaOH and transferred to nitrocellulose (BA85; Schleicher & Schuell, Inc.) with a Vacublot apparatus (American Bionetics). Prehybridization and hybridization conditions were those outlined by Gatti et al. (13). The probe used was a purified *Bam*HI-*Nru*I fragment nick translated (Bethesda Research Laboratories, Inc.) with ^{32}P dCTP (Amersham Corp.). The probe encompasses the entire gD coding sequence. Washes were done at 60°C.

Samples for Western blots were prepared by lysing cell monolayers with 1% Nonidet P-40–10 mM Tris hydrochloride (pH 8)–0.15 M NaCl–2 mM phenylmethylsulfonyl fluoride–8 mM iodoacetamide at 4°C for 30 min. The lysates were collected and centrifuged for 5 min in a water-cooled microcentrifuge. The supernatant was collected, and 4× sodium dodecyl sulfate (SDS) sample buffer was added to yield 1% SDS–0.01 M phosphate buffer (pH 7.0)–0.1 M dithiothreitol–0.02% bromophenol blue–10% glycerol. Samples were loaded onto an 8.5% polyacrylamide gel crosslinked with *N*,*N*′-diallyltartardiamide (Bio-Rad Laboratories). At the completion of the separation, the proteins were transferred to nitrocellulose (BA85) in Tris-glycine-methanol buffer. Nitrocellulose was blocked with phosphate-buffered saline (PBS)–5% milk and incubated with diluted polyclonal antiserum (hyperimmune rabbit serum, Rb58/6, kindly provided by I. Halliburton) in PBS–1% bovine serum albumin–0.2% sodium azide for 2 to 4 h at room temperature or 4°C overnight. Detection was accomplished with [^{125}I]protein A (Amersham). Washes were performed with PBS–5% milk–0.2% Tween 20. Blots were wrapped and placed against Cronex film (Du Pont Co.) with an intensifying screen for 12 to 72 h.

Infection of cells and labeling with [^{35}S]methionine. Monolayers of HEp-2 and LMtk$^-$ cells grown in 24-well tissue culture dishes (Corning Glass Works) were infected with HSV in 0.1 ml of PBS–1% heat-inactivated FCS–0.1% (wt/vol) glucose for 2 h at 37°C with frequent shaking. After 2 h, the inoculum was removed and replaced with 1.5 ml of Hanks 199 with 1% heat-inactivated FCS. At 5 h after infection, the monolayers were washed three times with methionine-free medium 199–1% heat-inactivated FCS and labeled for 30 min in DMEM lacking cold methionine and containing 2% heat-inactivated FCS and [^{35}S]methionine (Amersham) at 15 μCi/ml. After removal of the radioactive medium, the monolayers were harvested in 1× SDS sample buffer and separated on an 8.5% polyacrylamide gel. Gels were fixed and treated with Amplify (Amersham), dried, and placed against Cronex film for 12 to 24 h.

Adsorption of radioactive virus to cells. Radiolabeled HSV-1(KOS) was prepared from HEp-2 cells infected at 4 PFU per cell and incubated with [^{3}H]thymidine at 0.02 mCi/ml from 4 to 36 h after infection. Virions were purified on dextran T10 (Pharmacia) gradients as previously described (45). Binding assays were done in 96- and 24-well tissue culture plates by exposing the cells to various concentrations of purified labeled virus in PBS containing 0.1% glucose, 1% heat-inactivated FCS, and bovine serum albumin at 1 mg/ml in a total volume of 30 μl for 96-well plates and 100 μl for 24-well plates. For assays done at 37°C, binding was allowed to proceed for 30 min. At 4°C, binding was allowed to proceed for 2 h. After the adsorption period, the cell monolayers were washed twice with PBS containing 0.1% glucose and 1% heat-inactivated FCS and once with PBS and then harvested in 50 μl of PBS containing 1% SDS–1% Triton X-100. The lysates were added to 4 ml of Econolume scintillation fluid (ICN Pharmaceuticals Inc.) and counted in a scintillation counter (LS3133T; Beckman Instruments, Inc.).

Flow cytometry. Cells were dislodged with EDTA and suspended in PBS containing 5% heat-inactivated FCS–0.1% sodium azide. The cells were incubated with 1:10 dilutions of ascites fluid containing III-114 (monoclonal antibody specific for gD) or purified monoclonal antibody II-125 (specific for gB) for 30 min on ice. These monoclonal antibodies were previously described (36). The second-step antibody was fluorescein isothiocyanate-coupled goat anti-mouse immunoglobulin G from Southern Biotechnology. Stained cells were fixed in PBS–1% paraformaldehyde and analyzed on an EPIX flow cytometer.

RESULTS

Isolation of transformed cell lines expressing gD-1. The plasmids used to transform cells either contained the neomycin phosphotransferase selectable marker (Fig. 1B) or were cotransfected with pSV2neo (Fig. 1A). The plasmids carried the gD-1 transcription unit from HSV-1(KOS) DNA, modified such that the endogenous viral promoter was replaced by the human metallothionein promoter II. Two open reading frames are contained in the gD-1 transcription unit; the downstream one encodes gI-1. To produce a plasmid capable of expressing gD-1 but unable to express gI-1, most of the gI-1 open reading frame, including the translation initiation codon, was deleted in pRJ42 (Fig. 1C). The control plasmid used, pRJ40, was essentially as shown in Fig. 1B except that no HSV DNA sequences were inserted.

Mouse LMtk$^-$ cells and HEp-2 cells were transfected with the plasmids described above, and stable transformants were selected for the ability to replicate in medium containing G418. Table 1 lists the cell clones characterized here, the plasmids used for the transfections, and shorthand designations for the clones. The mouse cell lines are prefixed with an L and human cell lines are prefixed with an H, followed by the HSV glycoprotein-coding sequences potentially expressed by the clone and an arbitrary number to identify different clones. Control cell lines were transfected with pRJ40, which contains no HSV DNA, and were selected in medium containing G418 in parallel with the other clones.

As expected, Southern blot analysis revealed the presence of appropriate plasmid DNA sequences in the transformed clones. Figure 2A shows the results of hybridization of a labeled probe specific for HSV DNA sequences (the *Bam*HI-*Nru*I fragment indicated in Fig. 1A) with *Pvu*II digests of

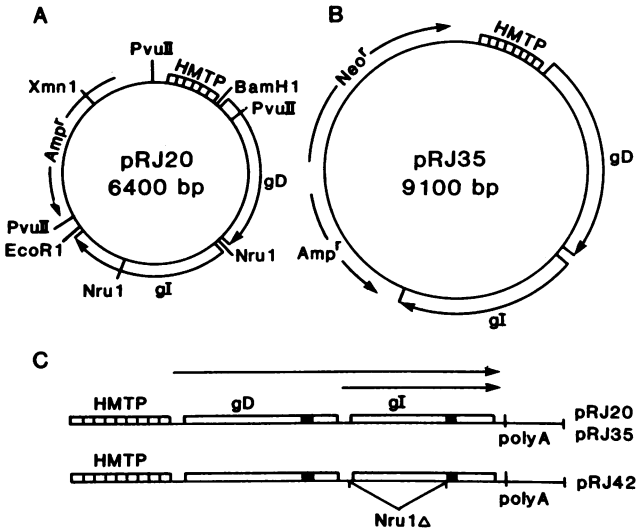

FIG. 1. Plasmids used for transfection to isolate mouse and human cell lines expressing HSV-1 gD. (A) Plasmid cotransfected with pSV2neo. (B) Plasmid incorporating the neomycin selectable marker. (C) Transcription units of the different vectors, showing the human metallothionein promoter II (HMTP), the glycoprotein-coding regions for gD and gI (open boxes with black shading to denote the membrane-spanning regions of each), and transcripts. The promoter for gD was replaced by HMTP in these vectors. Most of the coding region for gI was deleted from pRJ42.

DNA isolated from the transformed clones. For digests from all except the control clones, the probe detected HSV DNA sequences in fragments of sizes similar to those present in digests of the plasmid used for transfection. A couple of the transformed clones (L-gDgI-1 and L-gDgI-2) also contained extensively rearranged forms of these HSV DNA sequences. The clones transformed by the plasmid from which most of the gI-1 sequences were deleted (pRJ42) can be identified by loss of the 2,883-bp band and appearance of a new band at 2,070 bp.

Transformed clones carrying the gD-1 gene expressed different constitutive levels of gD-1, as determined by Western blot analysis with a polyclonal rabbit antiserum. Figure 2B shows the constitutive levels of gD-1 expression for some

TABLE 1. Transformed mouse and human cell lines

Cell line		Plasmid used for transformation	Presence of coding sequence:	
Source and name	Descriptive name used here		gD	gI
Mouse				
CA35gD10	L-gDgI-1	pRJ20, pSV2neo	+	+
351d-11	L-gDgI-2	pRJ35	+	+
351d-6	L-gDgI-3	pRJ35	+	+
421d-4	L-gD-1	pRJ42	+	−
421d-3	L-gD-2	pRJ42	+	−
401-1	L-control	pRJ40	−	−
Human				
35H7-3	H-gDgI-1	pRJ35	+	+
35H7-8	H-gDgI-2	pRJ35	+	+
42H9-9	H-gD-1	pRJ42	+	−
40H7-1	H-control	pRJ40	−	−

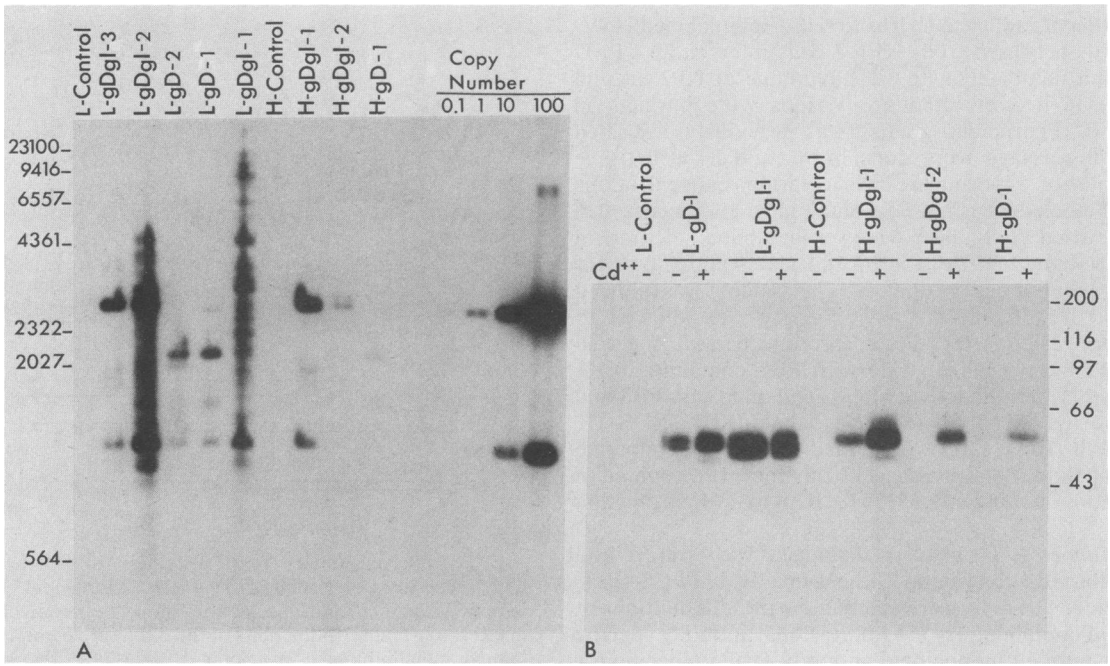

FIG. 2. (A) Southern blot showing HSV DNA sequences incorporated into the transformed cell lines and the approximate copy number per cell genome. DNAs extracted from the cell lines indicated were digested with *Pvu*II, and equivalent samples of the digests were fractionated by electrophoresis on an agarose gel and then blotted onto a nitrocellulose filter. The labeled probe used was a *Bam*HI-*Nru*I fragment of pRJ20 containing the gD-1-coding sequences (Fig. 1). The plasmid used for the copy number control was pRJ20 digested with *Pvu*II. Lambda *Hin*dIII digest markers are shown on the left margin. (B) Western blot of several mouse and human cell lines, showing basal levels of gD-1 expression and increased levels following induction with cadmium. An equivalent amount of protein was loaded in each lane. Molecular weight (in thousands) markers are indicated on the right margin.

of the clones, along with the induced levels detected 8 h after addition of 2 μM cadmium chloride. Zinc chloride could also be used to enhance gD-1 expression in these cell lines (data not shown). The heavy metal ion induction was more effective for the human clones than for the mouse clones. A possible explanation is that the promoter is a human promoter or the mouse cells are thymidine kinase negative (or both). Thymidine kinase is believed to be part of the metallothionein induction pathway (25).

For the human clones, the maximal inducible levels of gD-1 expression seem to correlate with the estimated numbers of gD-1 gene copies present. The same does not hold true for the mouse cell lines. It is uncertain whether the transformed clones carrying the gI-1 gene actually express this glycoprotein. All of the bands detected on the Western blots by the rabbit polyclonal antiserum were removed by immunoprecipitation of the Western blot samples with a monoclonal antibody specific for gD-1 (data not shown).

Plaque formation on transformed cell lines. Because plaque formation by HSV is poor on mouse L cells, only the human HEp-2 cell clones were tested for ability to support plaque formation after challenge with HSV-1(KOS), HSV-2(G), VSV, and vaccinia virus(WR) (Table 2). All of the gD-1-expressing HEp-2 cell transformants tested (including H-gDgI-2, in addition to those shown in Table 2) failed to support HSV-1 plaque formation. In fact, little if any cytopathic effect was observed when 10^6 PFU of HSV-1(KOS) were plated on 25-cm^2 monolayers (about 4×10^6 cells) of clone H-gDgI-1 or H-gD-1. By this test, cells expressing gD-1 were resistant to cytopathic effects of HSV-1 whether or not there was potential for gI-1 expression.

The results were not so straightforward with HSV-2. HSV-2(G) formed plaques as well or better on the H-gD-1 cell line than on the H-control cell line but produced considerably fewer plaques on the H-gDgI-1 cell line. Curiously, the HSV-2(G) plaques on the H-gD-1 cell line were much larger and had a different morphology than HSV-2(G) plaques on untransformed HEp-2 cells, the H-control cell line, or the H-gDgI-1 cell line. We have no explanation for this observation nor is it known whether presence of the gI-1-coding sequence, as well as expression of gD-1, contributes to the reduced plating efficiency of HSV-2(G) on H-gDgI-1 cells. These results and others described below show that the gD-1-expressing clones are less resistant to HSV-2 than to HSV-1. The H-control cell line and untransformed HEp-2 cell lines did not differ in the ability to support HSV plaque formation.

The gD-1-expressing clones that failed to support HSV-1 plaque formation were able to support plaque formation by

TABLE 2. Plaque formation on transformed human cells

Cell line	No. of plaques/plate (% of control)[a]			
	HSV-1	HSV-2	Vaccinia virus	VSV
H-control	54 (100)	69 (100)	192 (100)	350 (100)
H-gDgI-1	0 (<0.1)	0 (0.6)	300 (160)	337 (96)
H-gD-1	0 (<0.1)	82 (120)	370 (190)	70 (20)

[a] Serial dilutions of viruses were plated (1-ml samples) on monolayers in 25-cm^2 flasks. After incubation for 2 h to permit virus adsorption, the inocula were replaced with medium containing pooled human gamma globulin. After plaques had developed (1 to 3 days), the cells were stained with Giemsa. The numbers shown are the average of duplicate counts for a single dilution of each virus. Plaques of HSV-1 could not be detected on H-gDgI-1 and H-gD-1 cells, even at the next most concentrated serial dilution. Some of the percentages given are based on counts obtained at other dilutions.

TABLE 3. Binding of HSV-1 to transformed human cells

Cell line	Presence of heparin[a]	Adsorption at 4°C[b]		Adsorption at 37°C[c]	
		PFU/cell	cpm bound[d]	PFU/cell	cpm bound[d]
H-control	+	100	120 ± 40	150	32 ± 8
	−	100	2,700 ± 100	150	380 ± 80
	−	50	1,500 ± 30	100	280 ± 50
H-gDgI-1	+	100	110 ± 10	150	20 ± 10
	−	100	3,100 ± 200	150	350 ± 50
	−	50	1,700 ± 100	100	270 ± 30
H-gDgI-2	+	100	90 ± 10	ND[e]	ND
	−	100	2,970 ± 30	ND	ND
	−	50	1,800 ± 70	ND	ND
H-gD-1	+	100	100 ± 10	150	30 ± 10
	−	100	2,800 ± 100	150	390 ± 30
	−	50	1,640 ± 40	100	230 ± 20

[a] Heparin was present during adsorption at 50 μg/ml. Heparin inhibits HSV adsorption to cells (48, 53).
[b] The virus was added to cell monolayers in 24-well plates.
[c] The virus was added to cell monolayers in 96-well plates.
[d] Counts per minute per well adjusted to compensate for slight differences in cell density (micrograms of protein per well) for the different cell lines. The values represent averages of three (4°C) or five (37°C) wells and the standard deviations.
[e] ND, Not done.

vaccinia virus and VSV, except that VSV plated with somewhat reduced efficiency on H-gD-1 cells (Table 2). Vaccinia virus actually formed more and larger plaques, with altered morphology, on H-gD-1 and H-gDgI-1 clones than on the H-control cell line. Whether this relates to gD-1 expression remains to be determined.

Adsorption of HSV to transformed cells. Experiments were done to determine whether adsorption of HSV-1 to transformed human cells was less efficient than adsorption to control cells. Cells in 24- or 96-well plates were exposed to purified, labeled virions at 4°C for 2 h or at 37°C for 30 min, respectively. After the unbound virus was washed away, the cells were solubilized for quantitation of the bound virus. The results presented in Table 3 demonstrate that HSV-1 adsorbed as efficiently to cells expressing gD-1 as to control cells and that, as expected, virus adsorption was inhibitable by heparin (48, 53).

Ability of HSV to initiate viral protein synthesis in transformed cells. Within 1 h of addition of HSV to susceptible cells, cell protein synthesis is sharply inhibited because of the action of a virion-associated component brought into the cell along with the genome (8, 37, 42). This immediate inhibition of cell protein synthesis does not require viral gene expression. Induction of viral protein synthesis normally accompanies inhibition of cell protein synthesis.

Experiments were done to determine whether inhibition of cell protein synthesis and induction of viral protein synthesis could be detected after exposure of transformed cells to the virus. For the experiments shown in Fig. 3 and 4, the transformed cells were exposed to HSV-1(KOS) or HSV-2(G) at two different input multiplicities and then pulse-labeled for 30 min with [^{35}S]methionine at 5 h after addition of the virus. Because HSV PFUs are not readily quantifiable on mouse L cells, the multiplicities chosen for infection of the mouse cell transformants with HSV-1 and HSV-2 were those that induced comparable rates of viral protein synthesis at 5 h after infection of control cells. The lower multiplicity used (× in Fig. 3) corresponds to 1 PFU per cell for

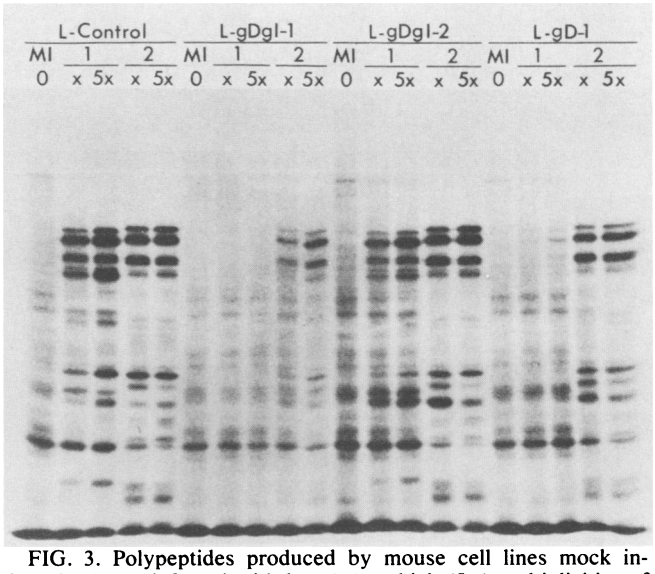

FIG. 3. Polypeptides produced by mouse cell lines mock infected (MI) or infected with low (×) or high (5×) multiplicities of HSV-1(KOS) and HSV-2(G). Cells were labeled for 30 min with [^{35}S]methionine at 5 h after infection, lysed in sample buffer, and electrophoresed through an SDS–8.5% polyacrylamide gel.

HSV-1(KOS) and 1.5 PFU per cell for HSV-2(G), on the basis of titers obtained on HEp-2 cells, or about one-third of the concentration of the virus to which the HEp-2 cells were exposed (Fig. 4). The same virus stocks had titers of 6 PFU per cell for HSV-1(KOS) and 3 PFU per cell for HSV-2(G) when assayed on Vero cells, which we use routinely for plaque assays.

The results (Fig. 3 and 4) demonstrate that all of the gD-1-expressing clones (except L-gDgI-2) failed to respond normally to challenge with HSV-1(KOS) in that viral protein synthesis was diminished and cell protein synthesis was not as efficiently shut off. In contrast, there was only partial, if

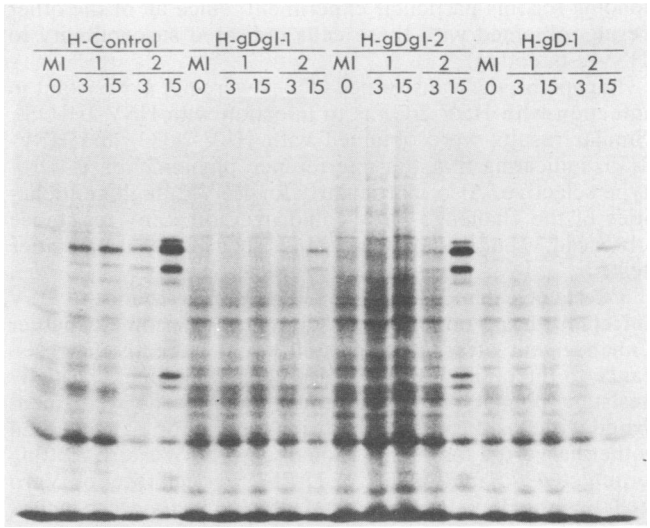

FIG. 4. Polypeptides produced by human cell lines mock infected (MI) or infected with low (3 PFU per cell) and high (15 PFU per cell) multiplicities of HSV-1(KOS) and HSV-2(G) viruses. Cells were labeled for 30 min with [^{35}S]methionine at 5 h after infection, lysed in sample buffer, and electrophoresed through an SDS–8.5% polyacrylamide gel.

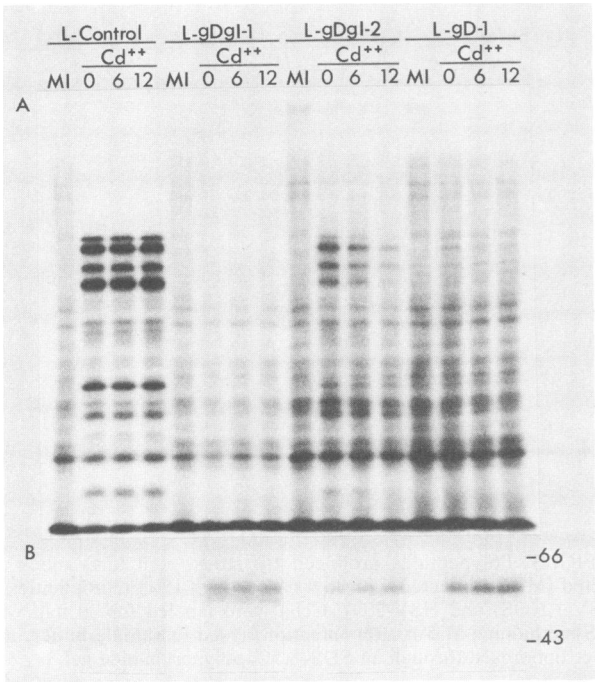

FIG. 5. Effect of enhancing gD-1 expression before infection of mouse cell lines. (A) Polypeptide profiles of cell lines induced with cadmium for 0, 6, or 12 h before infection with HSV-1(KOS). At 5 h after infection, cells were labeled for 30 min with [^{35}S]methionine, lysed in sample buffer, and electrophoresed through an SDS–8.5% polyacrylamide gel. MI, mock infected. (B) Western blot of parallel samples showing the levels of gD-1 expressed at the time of infection. Molecular weight (in thousands) markers are shown at the right.

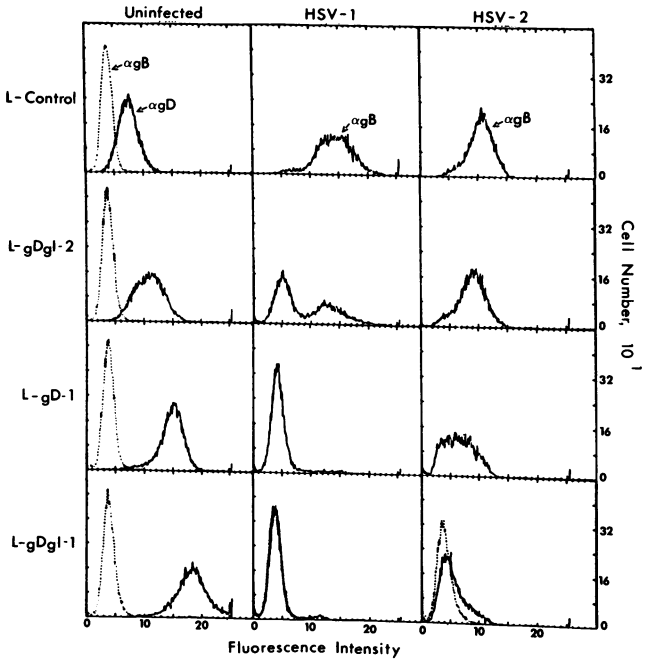

FIG. 6. Flow cytometric analysis of mouse cell lines before and after infection. Uninfected cells were stained with an anti-gD antibody (column 1) to show levels of surface expression of gD-1. Parallel cultures were infected with HSV-1(KOS) (column 2) or HSV-2(G) (column 3) and stained 9 h after infection with an antibody specific for gB. gB is expressed at relatively high levels early after infection. The dotted profiles in column 1 show the lack of reactivity of anti-gB with uninfected cells. The dotted profiles in the bottom panels of columns 2 and 3 show the background staining with the second antibody (fluorescein isothiocyanate-coupled goat anti-mouse IgG) alone.

any, interference with HSV-2(G) infection. Interference with HSV-2(G) infection was most evident for H-gDgI-1 (the apparent interference with HSV-2 infection of H-gD-1 cells was probably due to an error in sample preparation or loading for this particular experiment, since all of the other results obtained with these cells indicated susceptibility to HSV-2 infection).

In general, the transformed clones were not as resistant to infection with HSV-2(G) as to infection with HSV-1(KOS). Similar results were obtained with HSV-2(333) and HSV-1(F), indicating that the interference phenomenon is serotype selective. Also, particularly for HSV-2, high multiplicities of the challenge virus could overcome any resistance observed, as indicated by the trends seen at the multiplicities tested.

Effects of enhanced gD-1 expression on resistance to HSV infection. Experiments were done to determine whether enhancement of gD-1 expression could increase the resistance to infection of transformed cells exhibiting a partially resistant phenotype. Transformed mouse cells were incubated with 2 μM cadmium chloride for 0, 6, or 12 h and then either harvested for quantitation of gD-1 by Western blotting or infected at a multiplicity of 15 PFU per cell (titer on Vero cells) with HSV-1(KOS). The infected cells were pulse-labeled with [^{35}S]methionine at 5 h after infection.

For two of the mouse transformants (L-gDgI-2 and L-gD-1), cadmium induction increased the amount of accumulated gD-1 (Fig. 5B) and rendered the cells more resistant to HSV-1 infection, as judged by the rates of viral protein synthesis at 5 h (Fig. 5A). For the third mouse transformant (L-gDgI-1), full resistance to the dose of HSV-1 used was observed even without cadmium induction.

A comparable experiment could not be done with the HEp-2 cell transformants, because they are all highly resistant to HSV-1 infection.

We conclude that, for a single cloned cell line, the level of gD-1 expression correlates with the degree of resistance to HSV-1 infection, whereas in comparisons of different cell lines, the degree of resistance to HSV infection may be influenced by as yet unrecognized differences between the clones as well as by the level of gD-1 expression.

Basis for partial susceptibility of transformed cells to HSV infection. Experiments were done to investigate whether the transformed-cell populations were heterogeneous with respect to gD-1 expression and whether the intermediate phenotype, with respect to resistance to HSV infection, was due to a fraction of the cells becoming infected or to reduced rates of viral protein synthesis in each infected cell. Cells of four transformed mouse clones were divided into three samples. One sample of each was suspended, incubated with an anti-gD monoclonal antibody, stained with a fluorescein isothiocyanate-coupled second antibody, and then fixed. The other two samples were infected at a multiplicity of 5 PFU per cell (titer on Vero cells) with HSV-1(KOS) or HSV-2(G) and, at 9 h after infection, stained following reaction with an anti-gB monoclonal antibody. All samples were analyzed on a flow cytometer (EPIX).

The results (Fig. 6) show that the transformed clones constitutively expressed different amounts of gD-1 on cell surfaces and that the cells in each population were relatively

homogeneous with respect to the amount of gD-1 expressed. (The background nonspecific level of staining with the anti-gD antibody was higher than that with the anti-gB antibody [upper left panel of Fig. 6], possibly because the anti-gD antibody was not purified from the ascites fluid, whereas the anti-gB antibody was purified.)

After exposure to HSV-1 or HSV-2, all of the L control cells became infected. They all expressed gB, as shown by the shift to a higher fluorescence intensity of virtually all of the cells. The lower mean fluorescence of HSV-2-infected L control cells than that of HSV-1-infected cells indicated that the antibody bound less avidly to gB-2 than to gB-1 or that less gB-2 than gB-1 is expressed on infected cell surfaces.

Whereas the cells that expressed the highest constitutive levels of gD-1 uniformly failed to express gB-1 after exposure to HSV-1 and therefore were probably not infected, L-gDgI-2 cells formed two distinct populations after exposure to HSV-1. Some of the cells expressed near-normal levels of gB-1 and were obviously infected, whereas the others apparently expressed little or no gB-1.

Similar results were obtained after exposure of the cells to HSV-2, except that for each of the gD-1-expressing clones a larger proportion of cells expressed gB after HSV-2 infection than after HSV-1 infection. Also, the less intense staining of HSV-2-infected cells made it difficult to discern distinct subpopulations of cells that were either positive or negative for gB-2 expression.

These results indicate that the intermediate phenotype of L-gDgI-2 cells was due to the fact that a fraction of the cells was susceptible to HSV-1 infection, whereas the rest of the cells were resistant. Moreover, the results suggest that a certain threshold level of gD-1 expression is required for resistance to a given dose of a virus.

DISCUSSION

Hamster, mouse, or human cells expressing sufficient levels of gD-1 can be resistant to infection by HSV (this study; 5). We show here that expression of gD-1 alone among HSV gene products is sufficient to render cells resistant to HSV infection. Moreover, in a given transformed cell line, the degree of resistance to HSV-1 infection is proportional to the amount of gD-1 expressed. The resistance observed is not a generalized interference with viral infection but is specific for HSV among those viruses tested and is more pronounced for HSV-1 than for HSV-2. It remains to be determined whether cells expressing the HSV-2 form of the glycoprotein, gD-2, can also exhibit resistance to HSV infection.

Although other cell lines expressing gD-1 have been isolated, resistance of these cells to HSV infection has not been noted previously, in part because the studies done had other aims and resistance was not looked for. In the cases of an L cell line (3) and a Chinese hamster ovary cell line (2), both expressing gD-1, attempts to infect the cells were not reported; the latter cell line could not provide information about the role of gD-1 in interference in any event, because Chinese hamster ovary cells are naturally resistant to HSV infection. With an L-cell line expressing gD-2, infection of the cells with HSV-1 was successfully performed to determine whether the endogenous gD-2 was processed differently in infected and uninfected cells (20). These results are not necessarily contradictory to those reported here and elsewhere (5), because gD-2 may differ from gD-1 in interfering activity, gD-2 may exhibit only type-specific interfering activity, levels of gD-2 expressed by the particular transformed cells used may not have been sufficiently high to induce complete resistance (partial resistance of the cells was not ruled out by the experiments done), or the variable expression of gD-2 on cell surfaces documented by the researchers may have resulted in heterogeneity of the transformed cell population with respect to resistance.

The mechanism by which gD-1 renders cells resistant to HSV infection is not known, but it seems likely, as suggested also by Campadelli-Fiume et al. (5), that gD-1 interacts with some cell surface component required for viral penetration, thereby preventing its interaction with gD-1 in virions. Several observations are consistent with this hypothesis. (i) gD-1 is essential for virion infectivity; it is required for penetration, not adsorption (26). (ii) Penetration occurs by fusion of the virion envelope with the plasma membrane (12), and anti-gD monoclonal antibodies can neutralize infectivity by blocking this fusion without inhibiting adsorption (12, 16). (iii) Virions bind normally to resistant gD-1-expressing cells but are unable to penetrate (this study; 5).

Expression of gD-1 by cells could somehow tie up all of the unidentified cell surface component with which gD-1 in virions is proposed to interact. Alternatively, expression of gD-1 could result in sequestering inside the cell of the putative cell surface component, thereby rendering the cell surface devoid of a receptor needed for HSV penetration. A precedent for this latter hypothesis exists in part. There is a reduction in amount of CD4 present on the surfaces of cells producing HIV or expressing the HIV glycoprotein, as mentioned above.

It seems likely that an orderly cascade of interactions between virion and cell surface components is required for induction of fusion between the virion and the cell (P. G. Spear, M. Wittels, A. O. Fuller, D. WuDunn, and R. Johnson, *in* R. Compans, A. Helenius, and M. B. A. Oldstone, ed., *Cell Biology of Virus Entry, Replication, and Pathogenesis*, in press). The first interaction is binding to virions to cell surface heparan sulfate (53). We propose that this interaction is followed by other specific interactions, including one between gD and some other cell surface component.

Some important biological consequences of gD-mediated interference can be envisioned. The presence of gD in membranes of infected cells may ensure that progeny virions do not superinfect the cells that produced them. Because HSV acquires its envelope at the inner nuclear membrane and is transported out of the cell in membrane-bounded vesicles and cisternae of the Golgi (21), a mechanism to prevent superinfection may be especially important to permit escape of progeny virions from infected cells.

The consequences of this gD-mediated interference have probably already been seen in studies of HSV-induced cell-cell fusion. It is known that fusion occurs more readily at low multiplicities of infection, <1, than at higher multiplicities (17, 39). Mixing an infected cell population with a noninfected cell population gives much higher rates of fusion than mixing two infected cell populations (23, 24). To the extent that virus-induced cell-cell fusion resembles virion-cell fusion, the decreased ability of infected cells to fuse with one another may parallel the decreased ability of virions to fuse with gD-expressing cells.

While this report was under review, another kind of interference with HSV infection was reported (10). It was found that transformed L cells expressing a truncated form of the HSV regulatory protein VP16 (also known as α-TIF) did not support HSV replication as well as did control cells. After infection, the transformed cells produced about 1/12 of

the normal levels of an immediate-early viral mRNA, the synthesis of which is influenced by VP16. Whether resistance of cells to HSV infection or replication is caused by expression of truncated VP16 or wild-type gD, both phenomena belong to the general category of viral interference mediated by homologous viral gene products. The two phenomena may differ in that the interfering VP16 must be mutated, whereas the interfering gD may have to retain most or all wild-type functions. The truncated form of VP16 used, which itself lacks normal regulatory activity, is postulated to occupy sites that wild-type VP16 must interact with to initiate viral gene expression (10). As discussed above, wild-type gD is proposed to have different roles, depending on the membrane in which it is found—interference activity when present in cell membranes and an essential role in infectivity when found in the virion envelope.

It is tempting to speculate, as did Friedman et al. (10), that these observations will lead to the development of novel antiviral agents, particularly inasmuch as expression of truncated VP16 or gD is not toxic to cultured cells. It remains to be seen, however, what effects these viral proteins or their analogs might have in normal cells of developing and adult tissues in intact animals.

ACKNOWLEDGMENTS

We thank I. Halliburton for the rabbit antiserum used, E. Kieff and D. WuDunn for plasmids, N. Soltys for assistance with cell culture, and J. Hartley for assistance with cytofluorometric analysis.

R.M.J. is an MSTP trainee supported by Public Health Service training grant 5 T32 GM07281 from the National Institutes of Health. This work was supported by grants CA19264 and CA21776 from the National Institutes of Health and by a Marietta Klinman memorial grant for cancer research from the American Cancer Society.

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