Molecular and Biological Characterization of a Herpes Simplex Virus Type 1 (HSV-1) Neuroinvasiveness Gene

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Summary

Pathogenetic studies of herpes simplex virus type 1 (HSV-1) strains ANG and its mouse brain-passaged descendant ANG path revealed no difference in neurovirulence but a significant difference in neuroinvasiveness. Thus, both viruses induced a fatal encephalitis in mice after direct injection into the brain, but only ANG path induced lethal neurologic disease after inoculation on rear footpads. The difference in neuroinvasiveness is not related to the capacity to replicate in mouse neural tissues or mouse cells in general, but is specifically related to virus entry into the peripheral nervous system in the footpad. Marker rescue experiments in which ANG path genes were used to confer neuroinvasiveness on ANG indicated that the gene that codes for glycoprotein D (gD) is responsible for the phenotypic difference. Analyses of the gD genes by dideoxy-sequencing techniques identified a base difference in the coding sequences and predicted that the ANG gD gene codes for alanine (GCC codon) at amino acid position 84 in the open reading frame and the ANG path gD gene codes for glycine (GGC codon) at this site. Using these data, an oligonucleotide probe predicted to be specific for the ANG path gD gene was prepared, and in Southern blot analyses, this probe revealed that neuroinvasiveness-rescued agents had incorporated the base change seen in the ANG path gD gene. We conclude that HSV-1 glycoprotein D functions to effect neuroinvasiveness and we discuss potential mechanisms that may be involved.

A definition of the specific interactions that take place between viruses and target organs is a critical goal in understanding viral pathogenesis. Powerful approaches employed in this area are those designed to identify and functionally characterize viral genes and gene products that are required for infection and replication in unique differentiated cell types in these organs (1, 2). A target organ that has received considerable attention from virologists is the brain, and HSV is an agent that has well-appreciated but incompletely understood interactions with both the brain and the peripheral nervous system (3). To understand the pathogenesis of HSV infections as they relate to the nervous system, three phases of the process must be understood. Thus, the mechanisms by which HSV (*a*) gains access to the nervous system (neuroinvasiveness), (*b*) causes disease in the central nervous system (neurovirulence), and (*c*) is maintained for extended periods in a nonreplicating form in neurons (latency) require definition. The focus of this investigation is identification and characterization of HSV genes that specifically effect neuroinvasiveness.

We show that HSV-1 strain ANG (4, 5) demonstrates a phenotype specifically reduced for neuroinvasiveness. Thus, in contrast to most HSV strains, ANG is completely nonlethal for mice when inoculated on rear footpads, but it is fully neurovirulent when injected directly into the brain. By comparison, a brain-passaged derivative of ANG named ANG path (4, 5) causes lethal neurologic disease after both footpad inoculation (it is now neuroinvasive) and direct injection into the brain. The basis of this biologic difference is not related to the capacity to replicate in mouse neural tissues or mouse cells in general. Rather, tracing studies of virus infection in the mouse indicate that the difference is specifically related to the ability of the neuroinvasive ANG path agent to enter the peripheral nervous system in the footpad. Marker rescue experiments employing ANG path genes to confer neuroinvasiveness on ANG indicate that the gene that codes for glycoprotein D (gD)[1] is responsible for this difference. To establish unequivocally that gD is involved, the gD genes from both agents were sequenced, and a single base difference was identified. Analysis of the gD gene sequences predicts that the ANG gD gene codes for an alanine at position 84 in the mature protein, and the ANG path gD gene codes for a glycine at this position. Using these data, an oligonucleotide

[1] *Abbreviations used in this paper:* gD, glycoprotein D; MEF, mouse embryo fibroblasts.

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Volume 172 August 1990 487–496

probe was prepared and employed in Southern blot procedures to show that neuroinvasive agents derived by marker rescue encode the same base change seen in the ANG path gD gene.

Materials and Methods

Virus Strains and Tissue Culture. HSV-1 strains ANG and ANG path have been described previously (4, 5). The procedures for tissue culture of rabbit skin cells, preparation of primary mouse embryo fibroblasts, and infection, propagation, titration and plaque purification of viruses were described previously (6, 7).

Mouse Inoculations and Virulence Testing. Outbred, 4-week old male Swiss-Webster mice (Simonsen Laboratories, Gilroy, CA and Harlan Sprague Dawley, Indianapolis, IN) were used, and equivalent results were obtained with mice from the two suppliers. Standard methods for intracranial inoculation (6, 7), scarified rear footpad inoculation with and without saline pre-treatment (8), and quantitation of virulence were used (9).

DNA Cloning and Analysis. Methods for DNA cloning, transformation, propagation and storage of bacteria, and mini-preparations of plasmid DNA have been described (10). Restriction enzymes BamHI, BglII, ClaI, EcoRI, HindIII, HpaI, NarI, NcoI, NruI, PstI, PvuII, SalI, SmaI, and XbaI (Bethesda Research Laboratories, Gaithersburg, MD); Asp718 and NaeI (Boehringer Mannheim Biochemicals, Indianapolis, IN); AatII, AccI, and KpnI (New England Biolabs, Beverly, MA); and DdeI, SacI, and SacII (Stratagene, La Jolla, CA) were used according to manufacturer's directions.

Transfection and In Vivo Selection of Recombinants. The method for isolating virion and cloned DNA for transfection by isopycnic centrifugation has been described (11). ANG was marker rescued by calcium phosphate transfection of rabbit skin cells with ANG DNA and a cloned DNA fragment as described previously, except that neuroinvasive recombinants were selected by inoculating virus onto saline-pretreated rear feet of mice and recovering virus from the brains of killed mice (6, 7, 12, 13).

DNA Sequencing. Sanger dideoxy DNA sequencing was performed using Sequenase v2.0 (U.S. Biochemical Corp., Cleveland, OH) according to the manufacturer's directions with [^{35}S]dATP (Amersham Corp., Arlington Heights, IL). Gel electrophoresis and autoradiography have been described previously (10).

Oligonucleotide Primers and End-labeling. Oligonucleotide primers were prepared by Dr. Dohn Glitz, Department of Biological Chemistry, UCLA. Primers were purified on a 20% acrylamide sequencing gel using published methods. Standard procedures for 5′ end-labeling resulted in specific activities greater than 5×10^8 cpm/μg (10).

Southern Blot Analyses. Preparation of viral DNA for analytical purposes has been described (12), and standard methods for agarose gel electrophoresis were used. Procedures for Southern blotting, hybridization and autoradiography have been described previously (10).

Results

HSV-1 ANG and ANG Path Differ Specifically in Neuroinvasiveness. Comparative neuroinvasiveness of the two agents was established by inoculating virus on scarified rear footpads of mice and scoring for death resulting from encephalomyelitis. As shown in Table 1, ANG path is lethal and demonstrates a median lethal dosage (LD_{50}) of $\sim 10^5$ PFU. In contrast, ANG is completely non-lethal for mice even when inoculated with as much as 10^8 PFU, the highest dosage of virus we could obtain, so we were unable to calculate an LD_{50}. Following virus inoculation directly into the brain, ANG and ANG path are similarly neurovirulent and demonstrate LD_{50}s of about 1 PFU. This result indicates that ANG's inability to kill mice after footpad inoculation cannot

Table 1. *Median Lethal Dosages (PFU/LD_{50}) of HSV-1 ANG and ANG Path after Intracranial or Rear Footpad Inoculation*

Virus	Intracranial inoculation	Footpad inoculation
ANG	0.2	$>1.9 \times 10^8$
ANG path	0.1	7.2×10^5

Mice were inoculated with serial decimal dilutions of virus stock by the route indicated, and deaths from encephalomyelitis were scored over a period of 21 d after inoculation. PFU/LD_{50} ratios were calculated as described in Materials and Methods.

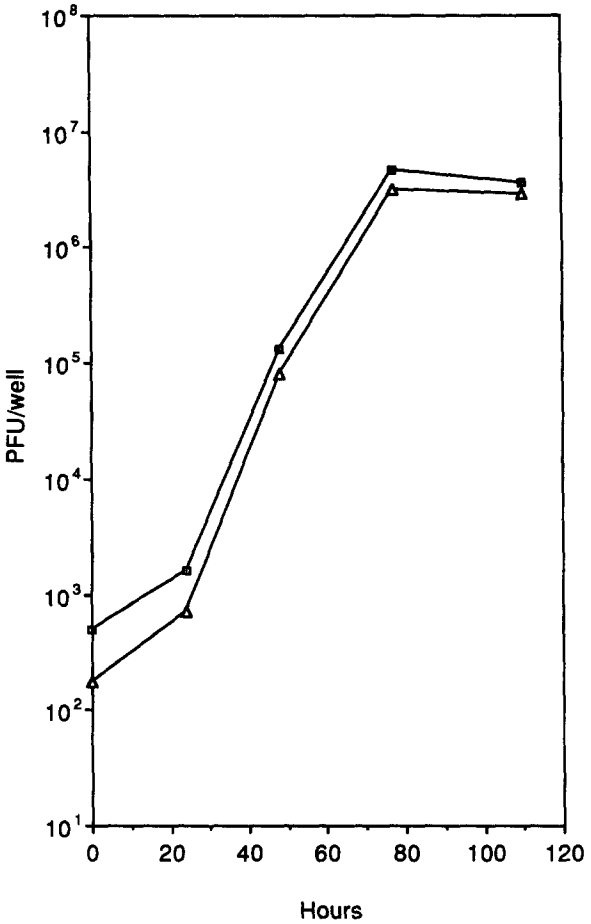


Figure 1. Replication cycles of HSV-1 strains ANG and ANG path in primary mouse embryo fibroblasts maintained at 38.5°C. 10^6 cells were infected at a multiplicity of 0.001 PFU/cell. At the times indicated, three samples were frozen at −70°C. When all samples were accumulated, samples were thawed and frozen twice before they were titrated for virus. ANG (□), ANG path (△).

be attributed to a generalized lack of virulence for the nervous system.

To further establish that the biologic difference is specific for neuroinvasiveness, virus replication was studied in mouse embryo fibroblasts (MEF) maintained in tissue culture at 38.5°C, the normal body temperature for mice. A low multiplicity of infection (0.001 PFU/cell) was used to approximate more closely infection in vivo. As seen in Fig. 1, ANG and ANG path demonstrate similar replication profiles. As an extension of this experiment, virus replication in vivo following direct injection of 100 PFU into mouse brains was also studied. As shown in Fig. 2, ANG and ANG path replicate to similar levels in the mouse brain through 84 h. After 84 h, all mice are dead. These results indicate that ANG is not restricted for replication in mouse neural tissues in vivo and, when taken with those obtained in vitro, show that ANG's inability to kill after footpad inoculation is not attributable to a general defect for replication in mouse cells.

Anatomic Site of Restriction of ANG Neuroinvasiveness. To determine where in the neural route from the footpad to brain the ANG invasion is restricted, we inoculated mice with 10^6 PFU on scarified rear footpads. Feet, sciatic nerves, lumbosacral spinal ganglia, dorsal roots, spinal cords, and brains were removed at various intervals after inoculation, and these tissues were titrated for virus content. In the footpad (Fig. 3 *A*), ANG is somewhat restricted for replication compared with ANG path. In the nervous tissues that were examined (Fig. 3, *B–F*), ANG demonstrates restricted replication compared with ANG path, the difference between the replication of ANG and ANG path is more pronounced in more central nervous tissues, and no ANG is detected in the brain. These

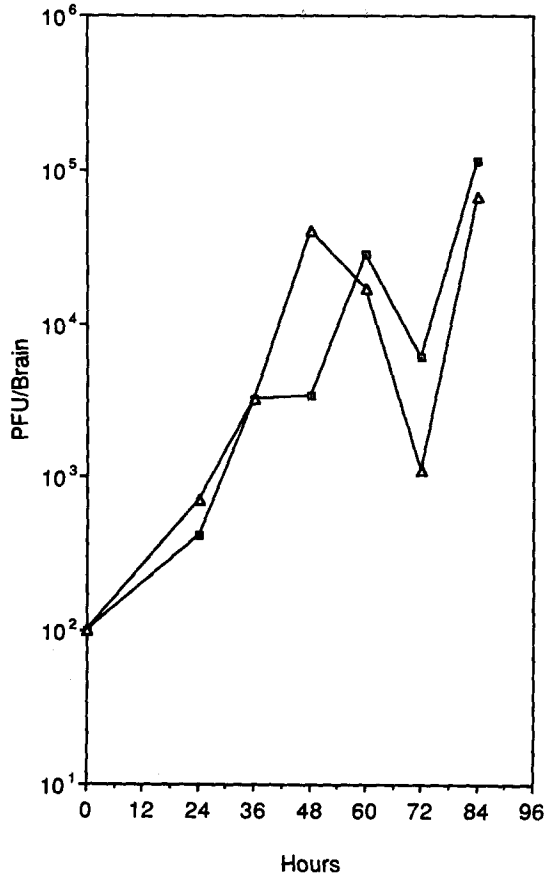


Figure 2. Replication cycles of HSV-1 strains ANG and ANG path in mouse brains in vivo. 100 PFU in 0.03 ml were injected into the left cerebral hemisphere. At the times indicated, three mice per virus were killed, and their brains were removed and frozen at −70°C. When all samples were collected, brains were homogenized as 10% (wt/vol) suspensions and centrifuged 5 min at 3,000 *g*. The resulting supernatant was titrated for virus. ANG (□), ANG path (Δ).

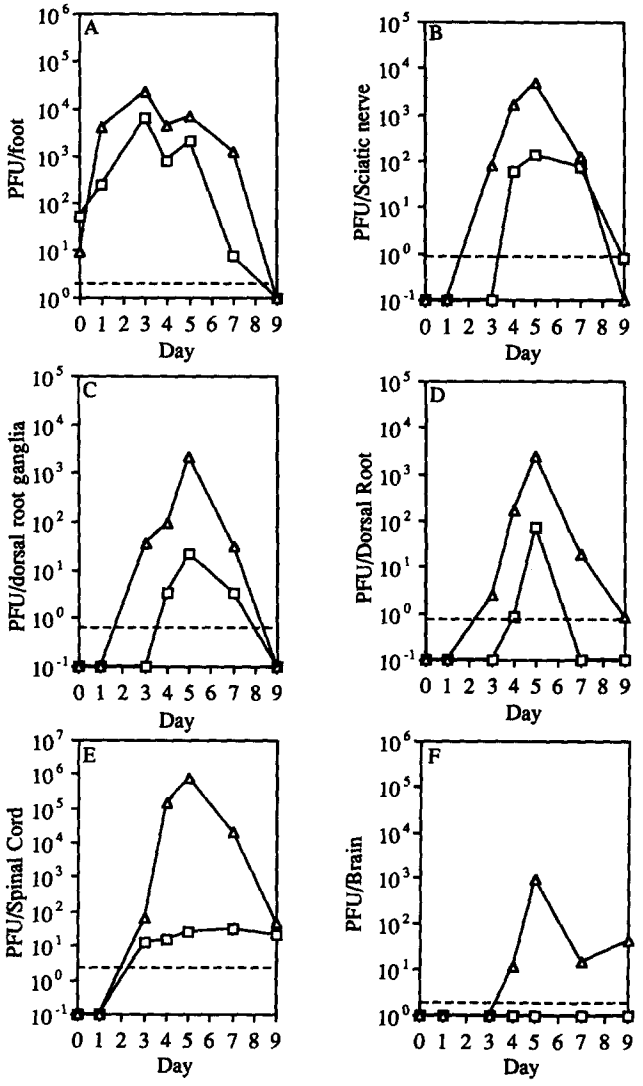


Figure 3. HSV in tissues along the neural route of infection from the footpad to the brain. Mice were inoculated on scarified rear footpads with 10^6 PFU of virus. On the days indicated, three mice were killed, feet (*A*), sciatic nerves (*B*), lumbosacral spinal ganglia (*C*), dorsal roots (*D*), spinal cords (*E*), and brains (*F*) were removed, and all tissues were frozen at −70°C. Once all samples were accumulated, tissues were homogenized as 10% (wt/vol) suspensions and centrifuged 5 min at 3,000 *g*. The resulting supernatant was titrated for virus. ANG (□), ANG path (Δ). Dashed line indicates limit of assay sensitivity.

results indicate that the ANG invasion is restricted in the footpad, and this restriction may lead to reduced amounts of ANG in neural tissues.

Although the results just presented indicated that the ANG invasion is restricted in the footpad, the exact nature of the neuroinvasiveness defect might be related to restricted replication in some cell type unique to the footpad or it could be due to reduced entry into the nervous system in the footpad. To investigate this further, we inoculated mice on rear footpads with 10^8 PFU of ANG and 10^6 PFU of ANG path and assessed virus invasion into the nervous system. In the footpad (Fig. 4 *A*), there is more ANG than ANG path until day 3, at which time both agents reach 4×10^4 PFU/foot. On days 4 and 5, there is more ANG path than ANG, and by day 9 neither virus is recovered in the footpad. Clearly, the cumulative amounts of ANG and ANG path in the footpad are similar. And there is more ANG in the footpad during the first 3 d of infection. Nevertheless, in the nervous tissues that were examined (Fig. 4, *B–E*), ANG demonstrates restricted replication compared with ANG path, the difference between the replication of ANG and ANG path is more pronounced in more central nervous tissues, and no ANG is detected in the brain. From these results, we conclude that a defect in ANG leads to reduced virus entry into the nervous system in the footpad. In addition, there may be a restriction related to entry into the central nervous system.

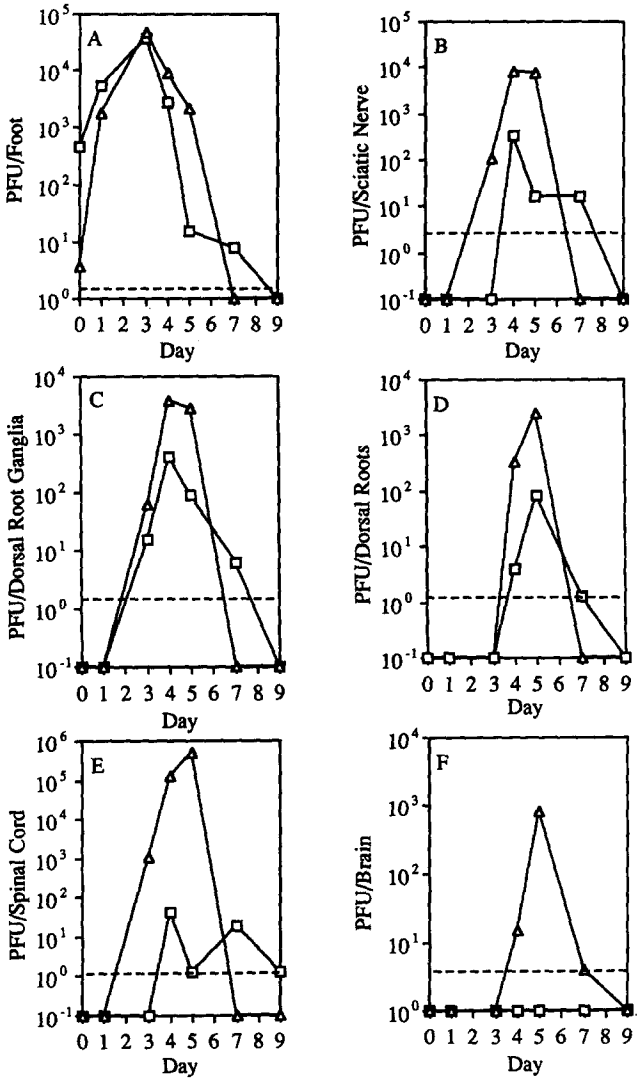


Figure 4. HSV in tissues along the neural route of infection from the footpad to the brain after rear footpad inoculation with 10^8 PFU of ANG and 10^6 PFU of ANG path. On the days indicated, three mice were killed, feet (*A*), sciatic nerves (*B*), lumbosacral spinal ganglia (*C*), dorsal roots (*D*), spinal cords (*E*), and brains (*F*) were removed, and all tissues were frozen at −70°C. Once all samples were accumulated, tissues were homogenized as 10% (wt/vol) suspensions and centrifuged 5 min at 3,000 g. The resulting supernatant was titrated for virus. ANG (□), ANG path (△). Dashed line indicates limit of assay sensitivity.

Identification of the Genetic Defect in ANG. The general strategy of marker rescue has been described previously (7, 13). In brief, ANG DNA is transfected into cells with cloned genes from ANG path. Virus recombinants are generated and analyzed by inoculation on mouse rear footpads. If at least one mouse out of a group of four is killed, the co-transfection culture is scored positive and is presumed to contain neuroinvasive recombinant agents. Initially, cloned XbaI and EcoRI fragments spanning the entire HSV genome were used, and the results (data not shown) indicated that the ANG path EcoRI h fragment (0.867–0.963 map units) conferred neuroinvasiveness on ANG. Virus from cell cultures transfected with ANG DNA and its own EcoRI h fragment never killed mice.

Viruses recovered from the brains of mice killed by infection with ANG path EcoRI h and ANG DNA co-transfection cultures were plaque-purified and analyzed for neuroinvasiveness. As shown in Table 2, these agents (5AP130 3.1, 6 12 6.1, 8 12 C4, and 9 8 A2) demonstrate LD_{50}s of $\sim 10^6$ PFU, ANG path was ~10 times more lethal (LD_{50} of $\sim 10^5$ PFU), and ANG was at least 100 times less lethal ($LD_{50} > 10^8$ PFU). To demonstrate that these results were not attributable

Table 2. *Median Lethal Dosages (PFU/LD_{50}) of HSV-1 ANG, ANG Path, and ANG Path EcoRI h Rescued Viruses after Rear Footpad Inoculation*

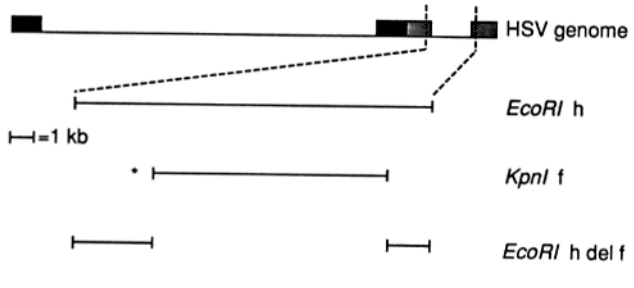
Virus	Exp. 1	Exp. 2
ANG	$>6.1 \times 10^7$	$>6.5 \times 10^7$
ANG path	2.4×10^5	1.1×10^5
5AP130 3.1		1.8×10^6
6 12 6.1	1.6×10^8	7.1×10^6
8 12 C4	4.4×10^6	5.6×10^6
9 8 A2	1.5×10^6	1.5×10^6

Mice were inoculated with serial decimal dilutions of virus stock on scarified rear footpads, and deaths from encephalomyelitis were scored over a period of 21 d after inoculation. PFU/LD_{50} ratios were calculated as described in Materials and Methods.

to transfection-induced artifact (14), we carefully tested the genetic stability of ANG. Mouse footpads were injected subcutaneously with 10% (wt/vol) sodium chloride, a procedure that enhances susceptibility to infection (15), before they were inoculated with virus generated by ANG DNA co-transfection with its own EcoRI h fragment. On day 6, the approximate day mice inoculated with virus generated by ANG path EcoRI h DNA and ANG DNA co-transfections had died, mice were killed. 16 separate viruses were recovered from brains, stocks were made in cell culture, and viruses were analyzed by inoculating 10^8 PFU on rear footpads. None of these virus isolates induced a lethal infection. These results indicate that the neuroinvasiveness defect in ANG is not altered by transfection per se but by the genes included in the transfection.

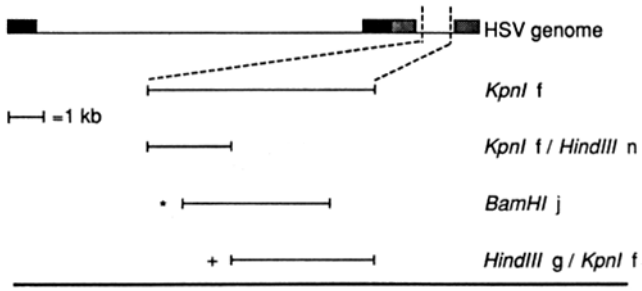
We next attempted to precisely localize the defect in ANG with subclones of the ANG path EcoRI h fragment. EcoRI h DNA was subcloned into three pieces, KpnI f (the middle fragment) and EcoRI h del f (the end fragments), and these were used in marker rescue experiments. The results (Fig. 5) indicate that the KpnI f fragment rescues ANG for neuroinvasiveness. Although the EcoRI h del f fragment gave a positive result in one experiment, we were unable to repeat this, and we attribute this one individual result to a group of mice that was uniquely susceptible to infection (the KpnI f fragment was positive in seven of nine of the cultures tested in this experiment).

The ANG path KpnI f fragment was then divided into smaller fragments, KpnI f/HindIII n, BamHI j, and KpnI f/HindIII g. In three experiments (Fig. 6), the BamHI j fragment and the KpnI f/HindIII g fragment conferred neuroinvasiveness on ANG, so attention was focused on the three open reading frames (ORFs) that are encoded in the region



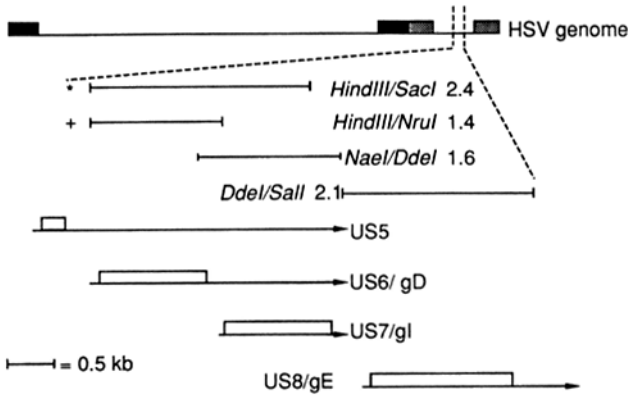
Cloned fragment	Exp A	Exp B	Exp C
ANG path *KpnI* f	5/9	7/9	3/9
ANG path *EcoRI* h del f	0/9	2/9	0/9

Figure 5. Rescue of HSV-1 ANG neuroinvasiveness using subclones of the ANG path EcoRI h DNA fragment. Cloned ANG path genes were transfected into cells with ANG DNA. The progeny virus was tested by inoculation on rear footpads of mice. Fractions indicate the number of co-transfection cultures that killed mice divided by the total number of cultures tested. As shown, ANG path KpnI f (*) consistently conferred neuroinvasiveness on ANG. The top line represents the HSV genome. (▬) Long segment inverted repeats; (▩) short segment inverted repeats. Beneath this, the EcoRI h, the KpnI f, and the EcoRI h del f fragments are expanded from their physical location in the genome.



Cloned fragment	Exp A	Exp B	Exp C
ANG path *KpnI* f/*HindIII* n	0/7	0/6	0/6
ANG path *BamHI* j	0/7	1/6	2/6
ANG path *HindIII* g/*KpnI* f	4/7	2/6	1/6

Figure 6. Rescue of HSV-1 ANG neuroinvasiveness using subclones of the ANG path KpnI f DNA fragment. Cloned ANG path genes were transfected into cells with ANG DNA. The progeny virus was tested by inoculation on rear footpads of mice. Fractions indicate the number of co-transfection cultures that killed mice divided by the total number of cultures tested. As shown, ANG path BamHI j (*) and HindIII g/KpnI f (+) conferred neuroinvasiveness on ANG. The top line represents the HSV genome. (▬) Long segment inverted repeats; (▩) short segment inverted repeats. Beneath this, the KpnI f, the KpnI f/HindIII n, the BamHI j, and the HindIII g/KpnI f fragments are expanded from their physical locations in the genomes.

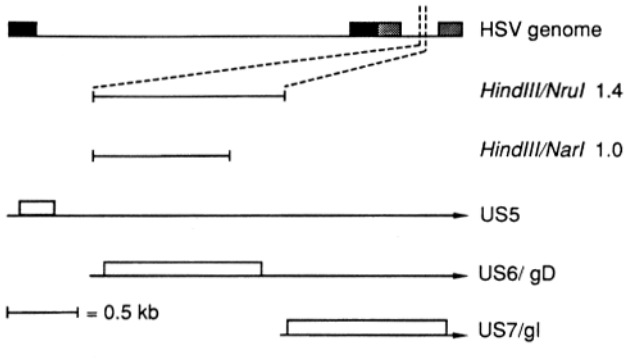


Cloned fragment	Exp A	Exp B	Exp C	Exp D	Exp E
ANG path *HindIII/SacI* 2.4	1/6	1/6			
ANG path *HindIII/NruI* 1.4			0/3	2/6	1/6
ANG path *NaeI/DdeI* 1.6	0/6	0/6	0/3	0/6	0/6
ANG path *DdeI/SalI* 2.1	0/6	1/6	0/3	0/6	0/6

Figure 7. Rescue of HSV-1 ANG neuroinvasiveness using clones of the ANG path genes that comprise gD, gI, and gE. Cloned ANG path restriction fragments were transfected into cells with ANG DNA. The progeny virus was tested by inoculation on rear footpads of mice. Fractions indicate the number of co-transfection cultures that killed mice divided by the total number of cultures tested. As shown, ANG path HindIII/SacI 2.4 (*) and HindIII/NruI 1.4 (+) conferred neuroinvasiveness on ANG. The top line represents the HSV genome. (▬) Long segment inverted repeats, (▩) short segment inverted repeats. Beneath this, the HindIII/SacI 2.4, the HindIII/NruI 1.4, NaeI/DdeI 1.6, and the DdeI/SalI 2.1 fragments are expanded from their physical locations in the genome. Also shown in relation to these fragments are the US5, US6/gD, US7/gI and the US8/gE transcripts (→) and open reading frames (▭).

shared by the BamHI j and KpnI f/HindIII g fragments. Those three ORFs code for glycoprotein D or US6, glycoprotein I or US7, and glycoprotein E or US8. A 2.4-kb HindIII-SacI fragment comprising the ORFs for glycoprotein D (gD) and glycoprotein I (gI), a 1.6-kb NaeI-DdeI clone encompassing the gI ORF, and a 2.1-kb DdeI-SalI fragment spanning the ORF for glycoprotein E (gE) were used in marker rescue experiments. As shown in Fig. 7, the HindIII-SacI fragment comprising the gD and gI ORFs and the DdeI-SalI fragment encoding the gE ORF conferred neuroinvasiveness on ANG. Since the fragment that spans the ORFs for both gD and gI but not the 1.6-kb *NaeI-DdeI* clone encompassing the gI ORF was positive, the gD ORF was implicated as a gene responsible for the neuroinvasiveness defect in ANG. To examine this, marker rescue experiments using a 1.4-kb HindIII-NruI fragment that encompasses the gD ORF were performed. As shown in Fig. 7, the ANG path HindIII-NruI fragment rescued ANG for neuroinvasiveness. Here, we were not able to repeat the positive result obtained earlier with the 2.1-kb DdeI-SalI fragment that spans the ORF for gE and have not pursued this aspect further.

The ANG path 1.4-kb HindIII-NruI fragment was compared with the homologous ANG fragment and, as summarized in Fig. 8, the ANG path but not the ANG clone conferred neuroinvasiveness on ANG. These data implicated the gD gene with the neuroinvasiveness defect in ANG, but it was still possible that sequences that encode the promoter



Cloned fragment	Exp A	Exp B	Exp C	Exp D	Exp E
ANG path *HindIII/NruI* 1.4	1/4	1/5			
ANG *HindIII/NruI* 1.4	0/6	0/6			
ANG path *HindIII/NarI* 1.0			1/6	1/6	1/4
ANG *HindIII/NarI* 1.0			0/6	0/6	0/6

Figure 8. Rescue of HSV-1 ANG neuroinvasiveness using clones of the ANG and ANG path genes that comprise gD. Cloned restriction fragments were transfected into cells with ANG DNA. The progeny virus was tested by inoculation on rear footpads of mice. Fractions indicate the number of co-transfection cultures that killed mice divided by the total number of cultures tested. ANG path HindIII/NruI 1.4 and ANG path HindIII/NarI 1.0 conferred neuroinvasiveness on ANG. The top line represents the HSV genome. (▬) Long segment inverted repeats, (▩) short segment inverted repeats. Beneath this, the HindIII/NruI 1.4 and the HindIII/NarI 1.0 fragments are expanded from their physical location in the genome. Also shown in relation to these fragments are the US5, US6/gD, and US7/gI transcripts (→) and open reading frames (▭).

Table 3. *Rescue of HSV-1 ANG Neuroinvasiveness using DNA Clones of the ANG Path and the ANG gD Gene Attached to the ANG HindIII n DNA Fragment*

Cloned fragment	Exp. A	Exp. B
ANG path gD + ANG HindIII n	3/6	2/6
ANG gD + ANG HindIII n	0/6	0/6

The ANG and the ANG path HindIII-NarI fragments (0.914–0.921 map units) which code for most of gD were ligated to the ANG HindIII n DNA fragment (0.878–0.914 map units) to generate DNA clones that span the region from 0.878 to 0.921 map units. Cloned DNA constructs were transfected into cells with ANG DNA. The progeny virus was tested by inoculation on rear footpads of mice. Fractions indicate the number of co-transfection cultures that killed mice divided by the total number of cultures tested. As shown, the ANG path gD + ANG HindIII n fragment conferred neuroinvasiveness on ANG.

of transcription for the gI gene were responsible. To examine this possibility, ~400 bp were removed from one end of the 1.4-kb HindIII-NruI fragments by cleavage with NarI. The resulting 1.0-kb HindIII-NarI fragments span ~80% of the gD open reading frame and stop ~340 bp upstream of the 5′ end of the gI transcript. As seen in Fig. 8, the ANG path HindIII-NarI fragment but not the ANG clone rescued ANG for neuroinvasiveness. As the cumulative data indicate, decreasing the length of DNA fragments employed decreases the relative ratio of positive transfections. A likely reason for this result is the decreased efficiency of recombination which is known to take place for markers present in shorter DNA fragments. To determine whether this was the case, we attempted to enhance rescue of the neuroinvasiveness defect in ANG by ligating a 5.6-kb ANG HindIII n fragment (0.878–0.914 map units) to the 1.0-kb ANG path HindIII-NarI fragment (0.914–0.921 map units). As shown in Table 3, three of six and two of six cultures generated by co-transfecting the expanded ANG path gD clone with ANG DNA rescued ANG for neuroinvasiveness. As Table 3 also indicates, a similarly expanded ANG HindIII n plus ANG HindIII-NarI fragment did not kill mice (zero of six and zero of six cultures were positive). Clearly, this maneuver restores the efficiency of rescue of ANG neuroinvasiveness to a level (42%) comparable to the 37% obtained with the similarly sized ANG path HindIII g/KpnI f fragment described earlier (Fig. 6). Based upon this and the other marker resuce analyses described, we conclude that a significant defect in the virus ANG which restricts neuroinvasiveness maps within the 1.0 kb HindIII-NarI fragment which codes for most of the open reading frame of glycoprotein D.

Site of Defect in the ANG gD Gene. To determine where in the gene which codes for gD the alteration occurred, two approaches were taken. First, the ANG and ANG path gD genes were sequenced. Second, viral isolates recovered from the brains of mice that were killed by infection in marker rescue experiments were analyzed both phenotypically and

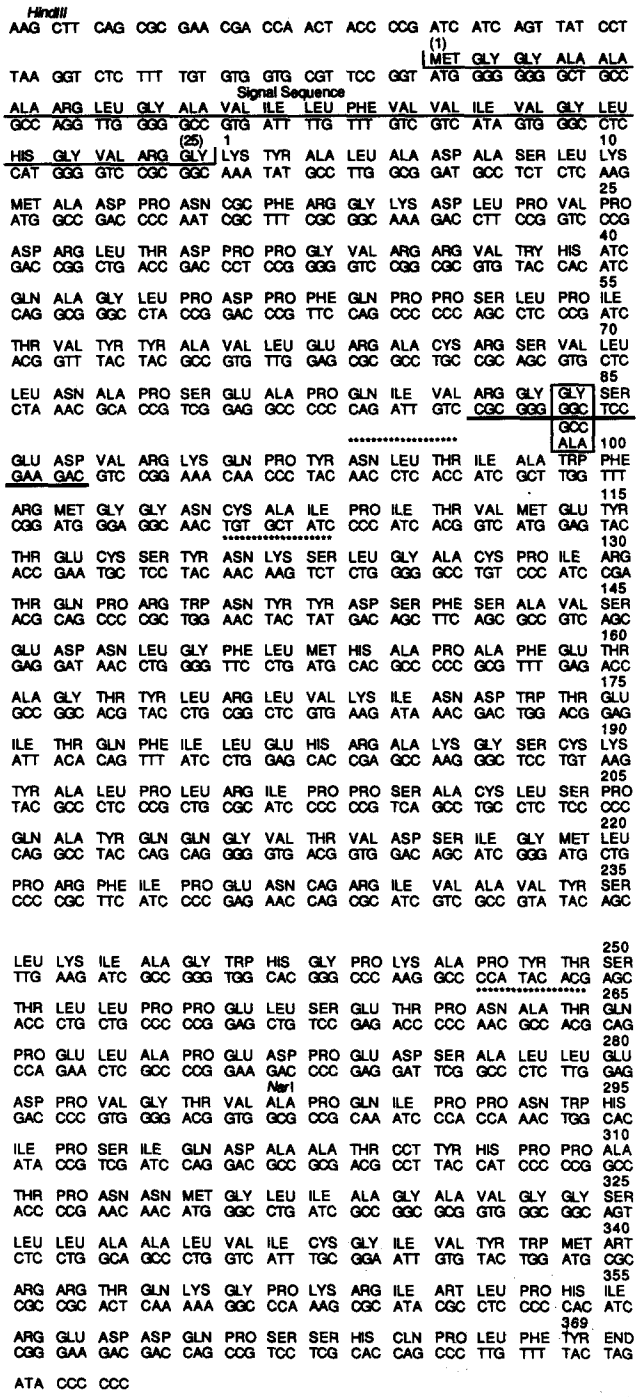


Figure 9. Nucleotide and predicted amino acid sequence of the HSV-1 ANG and ANG path regions encoding glycoprotein D. Relevant restriction sites are labeled and three potential N-linked glycosylation sites are marked by asterisks. The putative signal sequence is marked with a line, the sequence difference between ANG and ANG path is boxed, and the oligonucleotide primer used in Southern blot analysis is indicated by the dark underline.

genotypically. DNA sequencing analysis of the ANG and ANG path 1.4-kb HindIII/NruI fragments reveals a single base pair difference. Analysis of this difference predicts that ANG encodes alanine (GCC codon) at amino acid position

Table 4. *Median Lethal Dosages (PFU/LD_{50}) of HSV-1 ANG, ANG Path, and ANG Path 1.0 kb HindIII-NarI Fragment Rescued Viruses after Rear Footpad Inoculation*

Virus	Exp. 1	Exp. 2	Exp. 3
ANG	$>1.15 \times 10^8$	$>1.10 \times 10^8$	$>1.09 \times 10^8$
ANG path	5.03×10^4	1.01×10^4	4.51×10^5
33LB1	7.08×10^5	2.49×10^6	
33LC2	1.95×10^6	3.06×10^6	
39O1D		2.53×10^8	8.27×10^7

Mice were inoculated with serial decimal dilutions of virus stock on scarified rear footpads, and deaths from encephalomyelitis were scored over a period of 21 d after inoculation. PFU/LC_{50} ratios were calculated as described in Materials and Methods.

number 84 in the mature protein and ANG path encodes glycine (GGC codon) at this same site (Fig. 9).

Phenotypic analysis (Table 4) of plaque-purified viruses recovered from the brains of mice killed by infection with virus generated by ANG DNA co-transfection with the ANG path HindIII-NarI fragment indicates these agents are lethal for mice following footpad inoculation. These agents (33LB1,

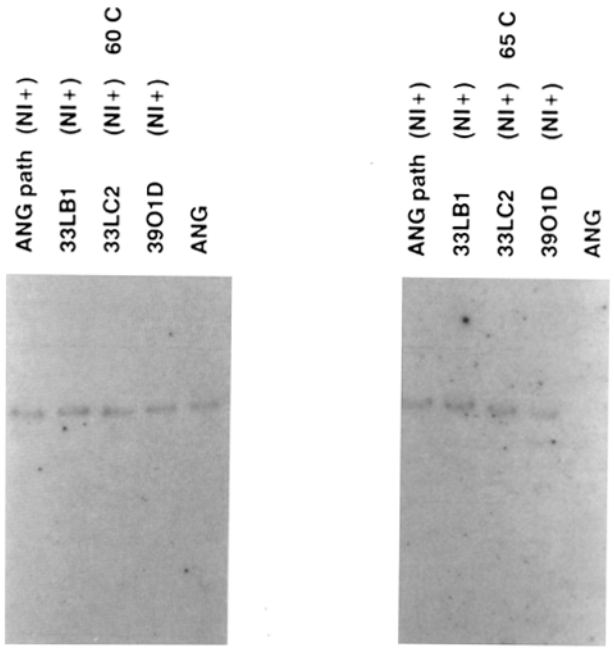


Figure 10. Duplicate Southern blots of BamHI generated fragments of ANG path, ANG, and three neuroinvasive viruses 33LB1, 33LC2, and 39O1D derived from co-transfections of ANG DNA with the 1.0-kb ANG path HindIII/NarI fragment that comprises gD. These blots were hybridized with ^{32}P end-labeled oligonucleotide primer 5'CGCGGGGGCTCCGAAGAC3' which is predicted to be specific for ANG path gD, and washed at 60°C or 65°C. Note that the probe remained hybridized to ANG BamHI j fragment at 60°C but is removed at 65°C. The probe remained hybridized to the BamHI j fragment derived from ANG path and viruses 33LB1, 33LC2, and 39O1D at both temperatures. Neuroinvasive viruses are shown (NI+).

33LC2, and 39O1D) demonstrate LD_{50}s that range from 10^6 to 10^8 PFU. The genotypes of these agents were established by stringent Southern hybridization. An oligodeoxynucleotide probe predicted to be specific for the ANG path gD gene (see legend to Fig. 10) was synthesized and then hybridized to Southern blotted DNA derived from the various isolates. Under appropriate hybridization stringency (Fig. 10, 65°C), this probe can distinguish the gD-encoding ANG path BamHI j fragment from ANG BamHI j fragment. By comparison, the probe reveals no difference under reduced hybridization stringency (Fig. 10, 60°C). Neuroinvasive agents recovered from the brains of mice killed by infection with virus generated by ANG DNA co-transfection with the ANG path HindIII/NarI fragment (33LB1, 33LC2, and 39O1D) hybridize to the probe similar to ANG path. The simplest interpretation of these results is that these neuroinvasive agents possess the same coding sequence as the ANG path gD gene. Taken together, these results indicate that the defect in ANG maps to nucleotide number 138,744 in the HSV-1 sequence (18, 19) and amino acid position 84 in glycoprotein D. This predicts that an alanine in the ANG gD protein becomes glycine in the ANG path gD protein.

Discussion

We have employed HSV-1 ANG (4, 5), a strain that does not cause lethal neurologic disease in mice following infection on rear footpads, and ANG path, a fully lethal brain-passaged derivative of ANG, to show that this phenotypic difference is related to a gene that specifically effects virus entrance into the nervous system. Marker rescue experiments utilizing cloned ANG path genes to confer neuroinvasiveness on ANG revealed that the gene that codes for gD is responsible for this phenotypic difference. When ANG and ANG path gD genes were sequenced, a single nucleotide difference predicted to change an alanine to glycine at amino acid number 84 in the mature protein was noted. Using these data, an oligodeoxynucleotide probe was prepared and employed in Southern blot analysis to demonstrate that neuroinvasive agents derived by marker rescue encode that base change seen in the ANG path gD gene. From these results, we conclude that HSV-1 gD protein functions in vivo to effect neuroinvasiveness, and that gD amino acid 84 is critical to this process.

Before speculating on the potential mechanisms by which a change in the primary amino acid sequence of gD could alter neuroinvasiveness, it is important to summarize potentially relevant properties of this protein. gD lies on the surface of the virion and is anchored to the envelope at the COOH terminus. The mature protein is 369 amino acids long, it has three potential N-linked glycosylation sites, it is synthesized both early and late during the viral replication cycle, and it is translated between short unique segment nucleotides 138,419 to 139,601 from an unspliced 2.8-kb polyadenylated transcript (16–19). The predicted amino acid difference between the ANG gD (position 84 alanine) and the ANG path gD (position 84 glycine) lies in a region that may be α-helical. This secondary structure is further predicted to lie in an exposed reverse turn domain that contains an N-linked glycosylated amino acid (20, 21).

The predicted alteration in the primary amino acid sequence of ANG gD could potentially affect neuroinvasiveness through two mechanisms. Since the ANG invasion is restricted in the footpad because of reduced virus entry into the nervous system and since gD protein performs an essential function in virus replication to facilitate virion penetration into cells (22, 23), it could be hypothesized that the alteration in the ANG gD induces a structural change in the protein that may lead to less efficient virion penetration into nerve terminals in the footpad. Such a phenomenon could also be a property of nerve terminals between the peripheral and central nervous systems. This change may be specific for nerve terminals as opposed to neuron somas since ANG replicates efficiently in the mouse brain where neuron somas are accessible to infection. The anatomic separation of neuron soma and nerve terminals in vivo, which is required to examine this hypothesis, can be reproduced in vitro using Campenot chambers to culture dissociated spinal dorsal root ganglia (24). Such a system may help to address the question of altered viral penetration of nerve terminals that may be caused by the predicted change in the ANG gD protein.

The second mechanism that may be responsible for the reduced entry of ANG into the nervous system relates to immunologic phenomena. Many investigations have shown that gD protein stimulates production of protective antibody and possibly cytotoxic T cells and is a major neutralizable herpes antigen (25–30). Therefore, the ANG gD protein could be more immunogenic or render the virion more susceptible to restriction by adaptive immunity than the ANG path gD protein. Mechanisms involving immune restriction of viral invasion would predict that ANG and ANG path viruses demonstrate similar capacity to induce lethal neurologic disease in immune-suppressed mice following rear footpad inoculation.

Finally, since replacement with ANG path gD gene sequences did not fully rescue ANG to the PFU/LD_{50} ratio demonstrated by ANG path, it seems likely that another gene in addition to the gD gene is involved in the reduced neuroinvasiveness phenotype of ANG. In this regard, since the recombinants generated with the ANG path gD gene are at least 10 times less neuroinvasive than ANG path, a second gene probably would function to a minor degree and might function only in concert with ANG path gD gene. It is possible that the ANG path gD × ANG recombinant viruses produced here (strains 33LB1, 33LC2, or 39O1D) could be used in marker rescue experiments to identify other genes involved with the reduced neuroinvasiveness of ANG. As one possibility, since HSV glycoproteins function together (31) and since it is likely that gD protein interacts with other glycoproteins in the envelope, marker rescue with genes encoding an ANG path glycoprotein and ANG path gD gene might reveal the full potential neuroinvasiveness.

In summary, we have used biological and genetic methods to show that HSV-1 neuroinvasiveness is, in part, a function

of gD. Since this protein is well characterized, continued investigation of this system is likely to lead to additional insights concerning the mechanism by which HSV-1 enters the nervous system.

This work was supported by grants from the National Institutes of Health (AI-06246), the National Multiple Sclerosis Society (RG-1647-A-1), and the W.M. Keck Foundation. K.M. Izumi is a predoctoral trainee supported by by U.S. Public Health Service National Research Service Award GM-07104 and the Ursula Mandel Scholarship, University of California, Los Angeles.

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Received for publication 8 February 1990 and in revised form 24 April 1990.

References

1. Mims, C.A., and D.O. White. 1984. Viral pathogenesis and immunity. Blackwall Scientific Publications, Oxford. 158–199.
2. Notkins, A.L., and M.B.A. Oldstone. 1984. Concepts in viral pathogenesis. Springer-Verlag, New York. 97–116.
3. Goodpasture, E. 1929. Herpetic infection with especial reference to involvement of the nervous system. *Medicine (Baltimore).* 8:223.
4. Kaerner, H.C., C.H. Shröder, A. Ott-Hartman, G. Kümel, and H. Kaerner. 1983. Genetic variability of herpes simplex virus: development of a pathogenic variant during passaging of a nonpathogenic herpes simplex virus type 1 in mouse brain. *J. Virol.* 46:83.
5. Kümel, G., H. Kirchner, R. Zawatsky, H. Engler, C.H. Schroder, and H.C. Kaerner. 1982. Experimental infection of inbred mice with herpes simplex virus V. Investigation with a virus stain non-lethal after peripheral infection. *J. Gen. Virol.* 63:315.
6. Thompson, R.L., and J.G. Stevens. 1983. Biological characterization of a herpes simplex virus intertypic recombinant which is completely and specifically non-neurovirulent. *Virology.* 131:171.
7. Thompson, R.L., E.K. Wagner, and J.G. Stevens. 1983. Physical location of a herpes simplex virus type 1 gene function(s) specifically associated with a 10 million-fold increase in HSV neurovirulence. *Virology.* 131:180.
8. Cook, M.L., and J.G. Stevens. 1973. Pathogenesis of herpetic neuritis and ganglionitis in mice: evidence for intra-axonal transport of infection. *Infect. Immun.* 7:272.
9. Reed, L.J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* 27:493.
10. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor. 545 pp.
11. Walboomers, J.M.M., and J. Ter Schegget. 1976. A new method for the isolation of herpes simplex virus type 2 DNA. *Virology.* 74:256.
12. Javier, R.T., R.L. Thompson, and J.G. Stevens. 1987. Genetic and biological analyses of a herpes simplex virus intertypic recombinant reduced specifically for neurovirulence. *J. Virol.* 61:1978.
13. Thompson, R.L., M.L. Cook, G.B. Devi-Rao, E.K. Wagner, and J.G. Stevens. 1986. Functional and molecular analyses of the avirulent wild-type herpes simplex virus type 1 strain KOS. *J. Virol.* 58:203.
14. Calos, M.P., J.S. Lebkosky, and M.R. Botchan. 1983. High mutation frequency in DNA transfected into mammalian cells. *Proc. Natl. Acad. Sci. USA.* 80:3015.
15. Olitsky, P.K., and R.W. Schlesinger. 1941. Effect of local edema and inflammation in the skin of the mouse on the progression of herpes virus. *Science (Wash. DC).* 93:574.
16. Cohen, G.H., D. Long, and R.J. Eisenberg. 1980. Synthesis and processing of glycoproteins gD and gC of herpes simplex virus type 1. *J. Virol.* 36:429.
17. Matthews, J.T., G.H. Cohen, and R.J. Eisenberg. 1983. Synthesis and processing of glycoprotein D of herpes simplex virus types 1 and 2 in an in vitro system. *J. Virol.* 48:521.
18. McGeoch, D.J., A. Dolan, S. Donald, and F.J. Rixon. 1985. Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. *J. Mol. Biol.* 181:1.
19. McGeoch, D.J., M.A. Dalrymple, A.J. Davison, A. Dolan, M.C. Frame, D. McNab, L.J. Perry, J.E. Scott, and P. Taylor. 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *J. Gen. Virol.* 69:1531.
20. Strynadka, N.C., M.J. Redmond, J.M. Parker, D.G. Scriba, and R.S. Hodges. 1988. Use of synthetic peptides to map the antigenic determinants of glycoprotein D of herpes simplex virus. *J. Virol.* 62:3474.
21. Isola, V.J., R.J. Eisenberg, G.R. Siebert, C.J. Heilman, W.C. Wilcox, and G.H. Cohen. 1989. Fine mapping antigenic site II of herpes simplex virus glycoprotein D. *J. Virol.* 63:2325.
22. Johnson, D.C., and M.W. Ligas. 1988. Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus-specific cell surface receptors. *J. Virol.* 62:4605.
23. WuDunn, D., and P.G. Spear. 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *J. Virol.* 63:52.
24. Ziegler, R.J., and R.E. Herman. 1980. Peripheral infection in culture of rat sensory neurons by herpes simplex virus. *Infect. Immun.* 28:620.
25. Berman, P.W., T. Gregory, D. Crase, and L.A. Lasky. 1985. Protection from genital herpes simplex virus type 2 infection by vaccination with cloned type 1 glycoprotein D. *Science (Wash. DC).* 227:149.

26. Cremer, K.J., M. Mackett, C. Wohlenberg, A.L. Notkins, and B. Moss. 1985. Vaccinia virus recombinant expressing HSV-1 type 1 glycoprotein D prevents latent herpes in mice. *Science.* 228:737–740.
27. Eisenberg, R.J., C.P. Cerini, C.J. Heilman, A.D. Joseph, B. Dietzschold, E. Golub, D. Long, M. Ponce de Leon, and G.H. Cohen. 1985. Synthetic glycoprotein D related peptides protect mice against herpes simplex virus challenge. *J. Virol.* 56:1014.
28. Zarling, M.J., P.A. Moran, R.L. Burke, C. Pachl, P.W. Berman, and L.A. Lasky. 1986. Human cytotoxic T cell clones directed against herpes simplex virus infected cells IV. Recognition and activation by cloned glycoproteins gB and gD. *J. Immunol.* 136:4669.
29. Blacklaws, B.A., A.A. Nash, and G. Darby. 1987. Specificity of the immune response of mice to herpes simplex virus glycoproteins B and D constitutively expressed on L cell lines. *J. Gen. Virol.* 68:1103.
30. Martin, S., B. Moss, P.W. Berman, L.A. Lasky, and B.T. Rouse. 1987. Mechanisms of antiviral immunity induced by a vaccinia recombinant expressing herpes simplex virus type 1 glycoprotein D: cytotoxic T cells. *J. Virol.* 61:726.
31. Johnson, D.C., M.C. Frame, M.W. Ligas, A.M. Cross, and N.D. Stow. 1988. Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins gE and gI. *J. Virol.* 62:1347.