ELSEVIER

Antiviral Research 33 (1997) 187–200

Antiviral Research

The synthesis and immunogenicity of varicella-zoster virus glycoprotein E and immediate-early protein (IE62) expressed in recombinant herpes simplex virus-1

Philip W. Lowry[a], Celine M. Koropchak[a], Clara Y.H. Choi[a], Edward S. Mocarski[b], Earl R. Kern[c], Paul R. Kinchington[d], Ann M. Arvin[a,b,*]

[a]*Department of Pediatrics, Stanford University School of Medicine, Stanford, CA 94305-5119, USA*
[b]*Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305-5119, USA*
[c]*Department of Pediatrics, University of Alabama at Birmingham, Birmingham, AL 35294-0019, USA*
[d]*Department of Ophthalmology, University of Pittsburgh, Pittsburgh, Pennsylvania 15213, USA*

Received 27 June 1996; accepted 28 September 1996

Abstract

In order to evaluate the conditions for optimal expression and immunogenicity of varicella-zoster virus (VZV) proteins in a herpes simplex virus-1 (HSV-1) vector, we selected the VZV glycoprotein E (gE), encoded by ORF 68 and the VZV product of ORF 62, an immediate-early major tegument protein (IE62). Three HSV/VZV recombinants were generated: (1) VZV gE protein coding sequences along with the promoter region were inserted into the thymidine kinase (TK) gene of HSV-1 strain KOS; (2) VZV gE expressed from the HSV-1 ICP4 promoter was inserted into the glycoprotein C (gC) gene of HSV-1 strain F; and (3) VZV IE62 protein coding sequences under the control of the HSV-1 ICP4 promoter were inserted into the gC gene of HSV-1 strain F. Immunoblot analysis and immunoperoxidase staining of infected cell monolayers demonstrated vector expression of VZV proteins. Following intracranial inoculation in mice, both VZV gE-HSV (TK) and VZV IE62-HSV (gC) induced an IgG response against VZV gE or VZV IE62. When tested in cytotoxicity assays using T-lymphocytes from VZV immune human donors, the range of precursor frequencies for T-lymphocytes that recognized VZV gE or VZV IE62 was similar whether these proteins were expressed by HSV-1 or a vaccinia vector. These experiments demonstrate that HSV-1 is a competent vector for expression of these VZV proteins and support the feasibility of engineering a combined vaccine for these closely related α-herpesviruses.

Keywords: Herpes simplex virus-1; Varicella-zoster virus; Viral vaccines; Vaccines; Attenuated

* Corresponding author.

PII S0166-3542(96)01014-5

1. Introduction

Varicella-zoster virus (VZV) and herpes simplex virus (HSV) are the two α-herpesviruses responsible for human disease. Since these viruses are highly transmissible in the human population, the morbidity caused by primary infections with VZV and the two types of HSV, HSV-1 and HSV-2, is significant. The capacity to establish latency leads to the additional morbidity associated with recurrent disease in many individuals. A live attenuated VZV vaccine strain (LAVV) has recently been approved for use in the United States (Arbeter et al., 1986; Gershon, 1992), the high level of safety and efficacy of LAVV supports the concept that attenuated infectious virus preparations are likely to be a worthwhile strategy for herpes viral vaccine design. The rationale for investigating the effects of the expression of major VZV proteins in HSV was to assess the feasibility of engineering a combined vaccine for these closely related human herpesviruses, HSV was selected as the vector because of the formidable technical problems posed by engineering stable VZV recombinants.

Two VZV proteins, the VZV gE and the immediate-early protein (IE62), were selected for expression in HSV. IE62 and gE proteins are both important targets in natural and vaccine-induced humoral and cell-mediated immunity to VZV (Arvin et al., 1986; Giller et al., 1989; Watson et al., 1990; Bergen et al., 1991). Memory $CD4^+$ and $CD8^+$ T-cells that recognize these proteins persist in humans up to 20 years after natural infection (Diaz et al., 1989; Arvin et al., 1991; Huang et al., 1992; Sharp et al., 1992). VZV IE62 and gE proteins are also effective immunogens in the guinea pig model of VZV infection, when introduced as purified antigens or expressed by vaccinia recombinants (Arvin et al., 1987; Lowry et al., 1992a,b; Sabella et al., 1993).

Because of their ability to stably accommodate a wide range of foreign DNA, HSV-1 strains have been increasingly studied as potential candidates for vectors of human gene therapy (Glorioso et al., 1995) and have been used to express a variety of DNA and RNA viral proteins (Shih et al., 1984; Sawyer and Wu, 1990; Dormitzer et al., 1992) as well as bacterial and human proteins (Ho and Mocarski, 1988; Dolter et al., 1993). The potential for using HSV-1 as a vaccine vector has been demonstrated, a live recombinant HSV-1/HSV-2 strain (R7020) with reduced neurovirulence was constructed by insertion of the HSV-2 genes encoding gD, gG, gI and part of gE into the thymidine kinase (TK) region of HSV-1 (F strain) (Meignier et al., 1990); this vaccine strain elicited HSV antibodies in seronegative human volunteers (Cadoz et al., 1992).

Two strains of HSV-1, F and KOS strain, were evaluated as vectors for VZV gene expression. The F strain was selected because it was used as the backbone for the HSV-1/HSV-2 recombinant, R7020 (Meignier et al., 1990), the KOS strain was chosen because the virulence of the parent strain has been well characterized in the mouse model (Dix et al., 1983; Thompson et al., 1986). The HSV-1 glycoprotein C (gC) gene and the TK gene were selected as alternative loci for insertion of VZV genes. The HSV gC is a nonessential glycoprotein for HSV replication but has an important role in apical viral attachment and infectivity of epithelial cells (Herold et al., 1991; Sears et al., 1991). Insertion into gC has the advantage of allowing full synthesis of early as well as late proteins and preserves the acyclovir sensitivity of the HSV-VZV recombinant. The effects of gC deletion on HSV virulence in animals are variable, but HSV strains lacking TK activity are less virulent in the mouse model (Field and Wildy, 1978; Tenser et al., 1979; Tenser, 1983; McDermott et al., 1984).

2. Materials and methods

2.1. Cells and viruses

Vero cells (American Type Culture Collection, Rockville, MD) were maintained in Eagle's minimum essential medium (MEM) supplemented with 2 mM glutamine, 50 U/ml of penicillin, 50 μg/ml of streptomycin, 0.5 μg/ml of fungizone and 5% fetal bovine serum (FBS). The F strain (Ejercito et al., 1968) and KOS strain of HSV-1 (obtained from Dr Jack Stevens, UCLA) were propagated in these cells.

2.2. Subcloning of VZV genes into HSV plasmids

The plasmid pBR322, containing the VZV strain 80-2 *Hin*dIII C fragment, which includes the gE coding region (ORF 68), was cloned by R. Hyman (Milton S. Hershey Medical Center, Pennsylvania State University). The plasmid pGEcoE, containing the VZV strain Ellen *Eco*RI-E fragment which includes the IE62 coding region, was provided by J. Ostrove (National Institute of Allergy and Infectious Diseases) (Reinhold et al., 1988). Two plasmids were provided by E.M. including pRB 103-pl, containing the HSV-1 *Bam*HI Q fragment, which includes the HSV-1 TK region with a PUC19 polylinker inserted into the *Sac*I site and pRB3 181 which contains the promoter-regulatory domain of the α-ICP4 gene (Shih et al., 1984). The plasmid pCD14 (Seidel-Dugan et al., 1988), which contains the complete gC-1 coding region of HSV-1, was provided by 0. Cohen (Department of Microbiology, School of Dental Medicine, University of Pennsylvania).

Restriction enzymes, DNA polylinkers, DNA ligase, T4 DNA polymerase, the KIenow fragment of *E. coli* DNA polymerase I and intestinal alkaline phosphatase, were obtained from New England Biolabs, Beverly, MA, and used according to the manufacturer's recommendations. Following restriction digestion, DNA fragments were separated by electrophoresis in 1% agarose and purified by electroelution. Transformation was conducted using *E. coli* DH5 α-cells (Bethesda Research Laboratories) (Maniatis et al., 1989).

2.3. Plasmid construction

(1) The plasmid pHk103 for VZV gE-HSV (TK), was constructed in the following way. The plasmid pBR322 containing the VZV *Hind*III C fragment was cut with restriction enzymes *Kpn*I and *bam*HI to generate a 2.9 kb fragment. This fragment contains the coding region for the VZV gE promoter and gene. The fragment was eluted on a 1% agarose gel and ethanol precipitated. The vector was prepared by performing a double digest on plasmid prb 103-pl with restriction enzymes *Kpn*I and *Bgl*II. A cohesive end ligation was then performed and the DNA was used to transform *E. coli* Dh5 α-cells. The plasmids isolated from the resultant colonies were screened for the presence and orientation of VZV gE by doing restriction enzyme analysis, the resultant plasmid was designated pHK103 (Fig. 1A).

(2) The plasmid pHF12 for VZV gE-HSV (gC) was constructed in the following manner. PRB3181 containing the promoter-regulatory domain of the α-ICP4 gene was linearized by digestion at a unique *Xba*I site and served as the initial vector. PBR322 containing the VZV *Hin*dIII C fragment was digested with *Bst*1107 (*Xca*I) and *Sma*I to yield a 2.1 kb insert containing VZV gE without its promoter-regulatory domain. *Xba*I linkers were ligated to the insert, and the insert was cloned into pBR3181. The resultant plasmid was digested with *Sal*I and *Bam*HI to yield a 4.0 kb insert containing the ICP4 promoter and VZV gE, this insert was blunt-ended by treatment with the KIenow fragment of *E. coli* DNA polymerase I, and *Sal*I linkers were attached.

PCD14 was linearized by digestion at a unique *Nar*I site 668 bp downstream from the start of the gC genome, blunt-ended by treatment with the Klenow fragment of *E. coli* DNA polymerase I and *Sal*I linkers were attached. The fragment containing the α-ICP4 promoter and the VZV gE insert were cloned into pCDl4 and the DNA was used to transform *E. coli* DH5 α-cells. The resultant plasmid was screened using restriction analysis and was designated pHF12 (Fig. 1B).

(3) The plasmid pHF30 for VZV IE62-HSV (gC) construction involved the following series of steps. PRB3181 was linearized by digestion at a unique *Xba*I site and served as the initial vector. PGEcoE was digested with *Bst*I 107 (*Xca*I) and *Sac*I to generate a 4.5 kb insert containing VZV IE62 without its promoter-regulatory domain. *Xba*I linkers were attached to the insert. This insert was cloned into PRB3181. The resultant plasmid was digested with *Pst*I and *Sac*I to generate a 6.4 kb insert containing the α-ICP4 promoter and IE62, this insert was blunt-ended by treatment with T4 DNA polymerase and ligated to *Sac*I linkers. PCD14 was linearized by digestion at a unique *Nar*I site, blunt-ended by treatment with the Klenow fragment of *E. coli* DNA

polymerase I and *Sac*I linkers were attached. The above α-ICP4 promoter/IE62 insert was cloned into the *Sac*I site. The DNA was used to transform *E. coli* DH5 α-cells as described above. The resultant plasmid was screened using restriction analysis and was designated pHF3O (Fig. 1C).

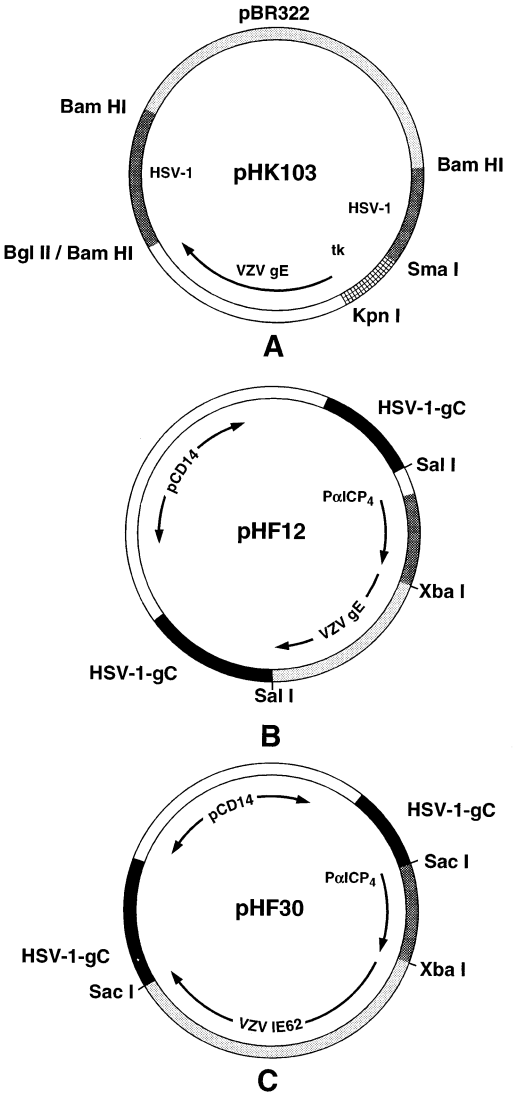

Fig. 1. Three plasmid constructs were made for recombining VZV gE or IE62 into the TK or gC regions of HSV-1. (A) The gE gene sequence including its promoter region was inserted into HSV-1 TK generating plasmid pHK103. (B) The gE gene sequence driven by the HSV-1 ICP4 promoter, was inserted into HSV-1 gC generating plasmid pHF12. (C) The IE62 gene sequence driven by the HSV-1 ICP4 promoter, was inserted into HSV-1 gC, generating pHF30.

2.4. Generation and selection of HSV-VSV recombinants

Vero cells were co-transfected with 5 μg of HSV-1 DNA and varying amounts (0.25–1.25 μg) of plasmid DNA using described techniques (Mocarski et al., 1980; Spaete and Mocarski, 1987; Ho and Mocarski, 1988). The flasks were harvested when 100% cytopathic effect was seen; ^{32}P-labelled gE and IE62 probes were used to map plaques containing recombinant virus by hybridization of nitrocellular discs (Villarreal and Berg, 1977). A minimum of three cycles of plaque purification was carried out to generate each HSV-VZV recombinant strain.

2.5. Southern blot analysis

Vero cells were infected with VZV gE-HSV (gC) or VZV gE-HSV (TK), as well as HSV-1 strains F and KOS or VZV Oka strain. Cellular DNA was isolated from one T-150 flask of infected Vero cells using a modification of a method previously described (Blin and Stafford, 1976). Genomic DNA (15 μg) was digested with *Sal*I or *Bam*HI which leaves VZV gE intact. Digested DNA was electrophoresed on a 0.7% agarose gel and transferred to a nylon membrane by the method described by Southern (Southern, 1975). Hybridization was conducted overnight using a 2.8 kb *Kpn*I/*Bam*HI fragment of VZV gE radiolabeled with [^{32}P] by random priming.

2.6. Preparation of immunoaffinity-purified VZV proteins

Vero cells were infected with HSV-VZV recombinants, HSV-1 KOS or F strains, vaccinia strains expressing VZV gE or IE62 protein or vaccinia control strains (Arvin et al., 1991, Kinchington et al., 1992). When generalized cytopathic effect was visible, HSV-1 KOS strain, HSV-1 F strain, vaccinia and uninfected cell control preparations were made in parallel using methods described previously (Arvin et al., 1986). Monoclonal anti-

bodies (MAbs) to VZV gE (90/58 kDa Mr complex)(Arvin et al., 1986) or to the IE62 protein (170 kDa) (Arvin et al., 1986) were coupled to cyanogen bromide-activated Sepharose 4B and incubated with the solubilized infected Vero cell extracts. Bound proteins were eluted with 3 M potassium thiocyanate pH 7.5, followed by centrifugation and osmotic dialysis membrane concentration using Aquacide (Calbiochem-Novabiochem).

2.7. Preparation of HSV-VZV cellular extracts

Vero cells were infected with HSV-VZV recombinants or HSV-1 F or KOS strains. After full cytopathic effect was observed, 2 ml of lysis buffer (2% SDS, 0.05 M Tris pH 7, 3% sucrose, 5% beta-mercaptoethanol and bromphenol blue) were added to each TlSO flask; the contents were sonicated for 30 s and boiled for 3–5 min.

2.8. Immunoblot assay

Immunoaffinity-purified VZV gE or VZV IE62 protein or infected cell extracts were separated and transferred by electrophoresis as described previously (Lowry et al., 1992). The strips were incubated with the polyclonal test sera at a dilution of 1:10 for 1.5–2.0 h. Bound human or mouse IgG was detected by immunoperoxidase staining using biotinylated goat antibody to human or mouse IgG (Vectastain ABC kit, Vector Labs). The M_r of visible bands was estimated using reference standards.

2.9. Immunoperoxidase staining

Vero cells were infected with VZV gE-HSV (TK), VZV gE-HSV (gC), or VZV IE62-HSV (gC), as well as VZV (Chase strain), vaccinia strains expressing either VZV gE or VZV IE62 and HSV-1 (KOS and F strains). After full cytopathic effect was observed, the cells were washed, fixed and washed again as previously described (Dormitzer et al., 1992). The cells were incubated for 1 h with a 1:500 dilution of hyperimmune serum from a patient with recent zoster, the serum was diluted in PBS containing 1% BSA and 0.2% Tween 20. Following three more washes, bound IgG was detected by immunoperoxidase staining using biotinylated goat antibody to human IgG (Vectastain ABC kit, Vector Laboratories).

2.10. Sensitivity of VZV gE-HSV (TK) to acylovir

The acyclovir sensitivity of VZV gE-HSV (TK) was tested by inoculating Vero cells in a 24-well microtiter plate with 50–100 pfu/well of virus. After adsorbing for 1 h, the monolayer was overlayed with agarose/2 × MEM containing 0, 2, 4, 6, 8 and 10 μM, acyclovir. A duplicate assay was set up with equivalent titers of parent HSV-1 KOS strain. Plaques were counted 3 days after inoculation.

2.11. Animal inoculation studies

2.11.1. Guinea pigs

Fifteen adults Hartley guinea pigs were purchased from Simonsen Laboratories, CA. Five animals were challenged with VZV gE-HSV (gC), five with VZV IE62-HSV (gC) and five with HSV-1 (F strain). The animals were inoculated intravaginally with a volume of 0.1–0.25 ml using a 1.0 ml syringe with a small plastic catheter attached. The day following inoculation, swab samples of vaginal secretions were collected for viral titer and stored at −70°C. During the acute infection, (days 2–13) animals were evaluated daily for the presence and number of genital lesions.

2.11.2. Mice intracranial challenge

Three-week-old Balb/c mice were purchased from Charles River Breeding Laboratories, NC. The mice were challenged with VZV gE-HSV (TK), VZV gE-HSV (gC), VZV IE62-HSV (gC), HSV-1 KOS or F strain. Stock viruses were serially diluted; ten mice were tested per dilution. The virus was administered in a volume of 0.03 ml and injected directly into the right cerebral hemisphere. All the viruses were tested in a coded manner. Following an observation period of 21 days, surviving mice were euthanized and blood was collected.

2.12. VZV-specific human T-lymphocyte cytotoxicity assays

Autologous targets infected with HSV-VZV recombinants were prepared using B-lymphocytes from VZV-immune, HSV-1 and HSV-2 nonimmune donors transformed with Epstein-Barr virus (EBV). Three donors were tested for each HSV-VZV recombinant autologous lymphoblastoid cell (LCL). Targets (2×10^6) were infected with VZV gE-HSV (TK), VZV gE-HSV (gC), VZV IE62-HSV (gC) or vaccinia recombinants expressing VZV gE or IE62 (5 pfu/cell) and incubated for 14 h at 37°C (Arvin et al., 1991). Following incubation, each LCL preparation was incubated with 300 μCi of [^{51}Cr] for 2 h, prepared as previously described (Arvin et al., 1991) and added to V-bottom microtiter plates at 3×10^3 cells/well in 0.1 ml of medium. Control targets included LCL infected with HSV-1 (KOS or F strains) and EBV-transformed uninfected LCL.

In order to prepare VZV specific cytotoxic effector cells, T-lymphocytes were recovered from PBMC, incubated with VZV antigen, and aliquoted to wells in concentrations of 0, 10^3, 5×10^3, 10^4, 5×10^4 and 10^5 cells/well as previously described (Arvin et al., 1991); 24 replicate wells were prepared for each T-cell concentration. Following incubation for 14–18 days, effector T-cells were transferred to V-bottom plates containing infected LCL target cells as described above. The plates were incubated at 37°C for 4 h and 100 μl of supernatant was removed from each well and counted in a gamma counter. The 24 replicate wells prepared at each of the effector cell concentrations were scored as positive if counts per minute were greater than 3.0 S.D. above the mean counts per minute for the control wells in the same assay.

2.13. Statistical analysis

In the animal studies, LD_{50}s were calculated using the dose effect analysis computer software (Elsevier, UK). Cytotoxicity assay data were evaluated by applying the maximum likelihood method to standard limiting dilution plots using a computerized analysis (Fazekas de St. Groth, 1982).

3. Results

3.1. Isolation of recombinant viruses

The plasmids pHF12 and pHF30 were co-transfected with HSV-1 F DNA and pHK1O3 was co-transfected with HSV-1 KOS DNA, following the appearance of 100% cytopathic effect, the flasks were harvested. Transfection progeny were subjected to a minimum of three cycles of plaque purification on Vero cells using plaque hybridization with a VZV IE62 or VZV gE ^{32}P-labelled probe to identify recombinant viruses. Following purification, plaque hybridization showed 100% recombinant virus for viruses designated VZV gE-HSV (TK), VZV gE-HSV (gC) and VZV IE62-HSV (gC).

3.2. Southern blot analysis

Following *Sal*I digestion of DNA from Vero cells infected with VZV gE-HSV (gC), and hybridization with a ^{32}P-radiolabelled DNA probe consisting of a 2.8 kb *Kpn*I/*Bam*HI fragment of VZV gE, a single band of approximately 5.5 kb was noted (Fig. 2, lane 1). This band was not detected when DNA from Vero cells infected with HSV-1 F strain DNA was digested with *Sal*I and hybridized with the same probe. The presence of the VZV gE insert was also confirmed in Southern blotting of DNA from Vero cells infected with VZV gE-HSV (TK) using a *Bam*HI digest. Hybridization with the 32P radiolabelled 2.8 kb *Kpn*I/*Bam*HI DNA probe revealed a 6.4 kb band (Fig. 2, lane 3) which was not present in *Bam*HI-digested DNA of Vero cells infected with HSV-1 KOS strain DNA.

3.3. Expression of VZV proteins in cells infected with HSV-VZV recombinants

Immunoperoxidase staining was performed on Vero cells infected with the recombinant viruses VZV gE-HSV (TK), VZV gE-HSV (gC) and VZV IE62-HSV (gC) to determine protein expression in these cells. Wells infected with VZV gE-HSV (TK), VZV gE-HSV (gC) and VZV IE62-HSV (gC) showed intense dark staining of mature

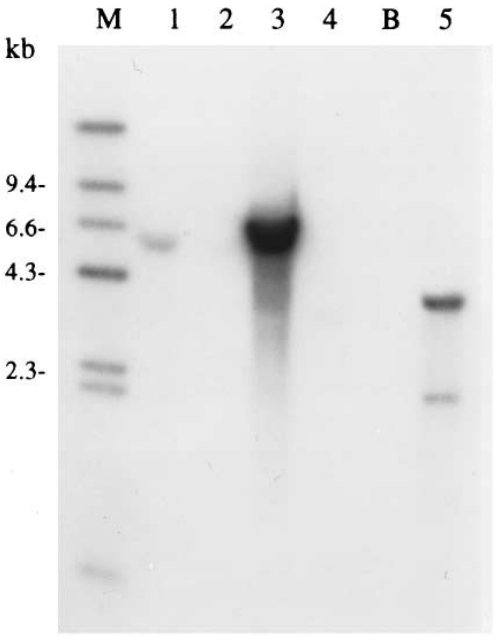

Fig. 2. Southern blot analysis showing that *Sal*I or *Bam*HI digests of DNA from Vero cells infected with recombinant virus contained fragments that hybridized with a gE specific probe, while digests of DNA from Vero cells infected with wild type HSV-1 did not. Genomic DNA (15 μg) was digested, electrophoresed, blotted and probed with a 2.8 kb *Kpn*I/*Bam*HI fragment of VZV gE. (1) DNA obtained from Vero cells infected with VZV gE-HSV (gC), and digested with *Sal*I. (2) DNA obtained from Vero cells infected with HSV-1 F strain, digested with *Sal*I. (3) DNA obtained from Vero cells infected with VZV gE-HSV (TK), and digested with *Bam*HI. (4) DNA obtained from Vero cells infected with HSV-1 KOS strain, and digested with *Bam*HI. (5) DNA obtained from Vero cells infected with VZV Oka strain, and digested with *Bam*HI. (M) Marker DNA fragments produced by *Hin*dIII digestion of lambda DNA. (B) Blank lane.

plaques, indicating abundant expression of VZV gE or VZV IE62, as expected from the insert carried by the different viruses. Vero cells infected by VZV strain Chase (wild type) showed comparable staining of plaques. However, monolayers infected with the parental strains, HSV-1 F or KOS, showed little or no staining of foci even where CPE was extensive (data not shown).

3.4. Evaluation of VZV protein synthesis by immunoblot

Solubilized extracts of Vero cells infected with VZV gE-HSV (TK) or VZV gE-HSV (gC) were separated by SDS-PAGE, transferred to nitrocellulose and tested against VZV hyperimmune human serum, obtained from a zoster patient who was HSV seronegative. For VZV gE-HSV (TK), gE expression was demonstrated by the presence of a band at 90 kDa (Fig. 3, lane B); this band is evident in an extract from a vaccinia/gE strain (Fig. 3, lane A) and is distinct from a co-migrating band at 96 kDa present in an HSV-1 KOS strain extract (Fig. 3, lane C). For VZV gE-HSV (gC), gE expression is demonstrated by the presence of a broad band at approximately 90 kDa (Fig. 4, lane C) which is present in an extract from cells infected with a vaccinia/gE strain but not in an extract from cells infected with HSV-1 F strain (Fig. 4, lane B).

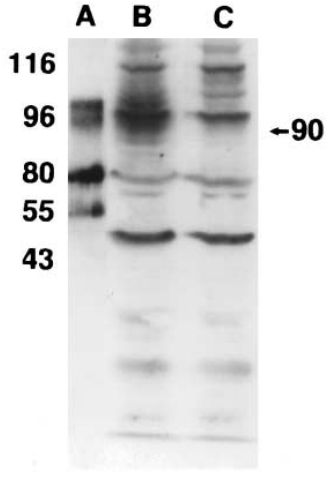

Fig. 3. Expression of VZV gE by VZV gE-HSV (TK). Immunoaffinity-purified VZV proteins or solubilized infected cell extracts were electrophoresed, transferred to nitrocellulose, and tested against polyclonal sera at a dilution of 1:10. A 90 kDa species (VZV gE) is present in lanes (A) immunoaffinity-purified VZV gE control produced by a vaccinia vector and (B) cellular extract of Vero cells infected with VZV gE-HSV (TK). (C) Cellular extract of Vero cells infected with HSV-1 KOS strain.

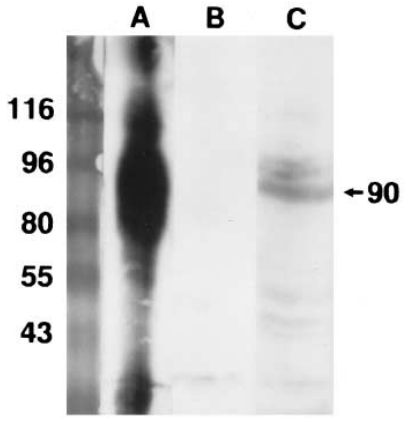

Fig. 4. Expression of VZV gE by VZV gE-HSV (gC). Immunoaffinity-purified VZV proteins or solubilized infected cell extracts were electrophoresed, transferred to nitrocellulose, and tested against polyclonal sera at a dilution of 1:10. A 90 kDa species (VZV gE) is present in lanes A and C; A, immunoaffinity-purified VZV gE control produced by a vaccinia vector; and C, cellular extract of Vero cells infected with VZV gE-HSV (gC). (B) Cellular extract of Vero cells infected with HSV-1 F strain.

Synthesis of the IE62 protein by the VZV IE62-HSV (gC) recombinant strain yielded a protein product that resembled the protein expressed by cells infected with a VZV IE62-vaccinia strain (Fig. 5, lanes C and A). As is evident in these blots, the proteolytic digestion of infected cells and the procedures required for immunoaffinity separation and immunoblot analysis break down the large VZV IE62 protein which is 175 kDa, to a number of smaller subspecies; these lower bands retain antigenic reactivity (Kinchington et al., 1992). Comparable bands were not visible in immunoaffinity-purified extracts from cells infected with the parental HSV-1 F strain (Fig. 5, lane D).

3.5. Acyclovir sensitivity testing

In order to evaluate TK production in VZV gE-HSV (TK), acyclovir sensitivity testing was performed. When acyclovir was added to infected cells, parental HSV-1 KOS strain showed a continuous decrease in the number and size of plaques, ranging from a 12% reduction with 2 μM acyclovir to an 88% reduction with 10 μM acyclovir. In contrast, acyclovir had no effect on the growth of VZV gE-HSV (TK) when tested in a concentration of 2–10 μM, indicating that insertion of the gE gene inhibited HSV TK synthesis, as was expected.

3.6. Animal inoculation studies

3.6.1. Guinea pig inoculation

VZV gE-HSV (gC), VZV IE62-HSV (gC) and parental HSV-1 F strain, were administered to guinea pigs by vaginal inoculation. Vaginal swabs were done 1 day later in order to assure that virus replication occurred at the site of inoculation, the

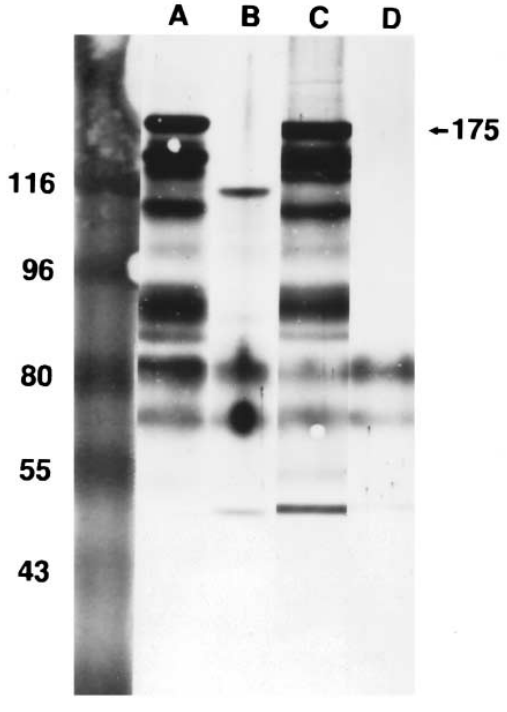

Fig. 5. Expression of VZV IE62 protein by VZV IE62-HSV (gC). All protein products in this immunoblot were immunoaffinity-purified, electrophoresed, transferred to nitrocellulose, and tested against polyclonal sera at a dilution of 1:10. (A) VZV IE62 protein produced in vaccinia vector. (B) Vaccinia infected cell control. (C) Vero cells infected with VZV 1E62-HSV (gC). (D) Vero cells infected with HSV-1 F strain. IE62 protein appears as multiple bands because this 175 kDa undergoes proteolytic degradation in infected cells before harvesting (Kinchington et al., 1992).

Table 1
Guinea pig genital challenge using recombinant HSV-1 F strain expressing VZV gE or VZV IE62

Virus	No. of animals	Titer (pfu/ml)	No. with lesions	No. of lesions (average)
gE-HSV (gC)	5	45×10^6	1 (20%)	1
IE62-HSV (gC)	5	20×10^7	2 (40%)	1
HSV-1 (F)	5	15×10^7	3 (60%)	1

virus was recovered in specimens from 13 of 15 (87%) animals. HSV-1 F strain has limited virulence in the guinea pig following vaginal inoculation. Three of five animals developed vaginal lesions but the mean number of lesions was one per animal. The HSV-VZV recombinants exhibited no clear alteration in this level of virulence in guinea pigs. Among animals inoculated with VZV gE-HSV (gC), only one of five developed lesions; similarly, two of five animals inoculated with VZV IE62-HSV (gC) developed lesions (Table 1). The mean number of lesions for animals inoculated with VZV gE-HSV (gC) or VZV IE62-HSV (gC) was one per animal. All lesions spontaneously resolved and no animals had signs of systemic infection.

3.6.2. Intracranial inoculation of mice

Mice were inoculated intracranially with multiple dilutions of the HSV-VZV recombinants as well as parental HSV-1 F or KOS strains in order to estimate the pfu/LD_{50} for each strain (Table 2). VZV gE-HSV (gC) and VZV IE62-HSV (gC), both constructed using HSV-1 F strain as the expression vector, had pfu/LD_{50}s of 8.6×10^1 and 5.4×10^2, similar to the parental HSV-1 F strain (1.6×10^3). However, the VZV gE-HSV (TK) strain, which is TK deficient, was three orders of magnitude less virulent than the parental HSV-1 KOS strain (Table 2).

Sera from mice surviving the intracranial challenge were tested for antibody responses to the VZV proteins by immunoblot. Five survivors from the group of animals receiving the highest titer of each HSV-VZV recombinant were tested. Among mice infected with VZV gE-HSV (TK), four of five (80%) had evidence of IgG antibody against VZV gE on immunoblot testing (data not shown). No IgG antibody was detectable from mice inoculated with VZV gE-HSV (gC). A sample of pooled serum from five mice infected with VZV IE62-HSV (gC) showed IgG reactivity against immunoaffinity-purified VZV IE62 (Fig. 6, lane C); no reactivity was evident against an uninfected cell preparation (Fig. 6, lane D).

3.7. Recognition of VZV gE and IE62 protein by human cytotoxic T-cells

Similar cytotoxic responder cell frequencies were evident in experiments comparing killing of targets infected with a VZV/HSV recombinant strain versus killing of targets infected with a vaccinia strain expressing the same VZV protein

Table 2
Mouse intracranial (IC) challenge using recombinant HSV-1 F strain expressing VZV gE or VZV IE62 or HSV-1 KOS strain expressing VZV gE

Virus	pfu/mouse	Dead/total	pfu/LD_{50}
gE-HSV (gC)	6.0×10^2	10/10	8.6×10^1
	6.0×10^1	3/10	
	6.0	3/10	
	0.6	4/10	
IE62-HSV (gC)	4.5×10^3	10/10	5.4×10^2
	4.5×10^2	4/10	
	4.5×10^1	3/10	
	4.5	4/10	
gE-HSV (TK)	1.0×10^4	4/10	1.2×10^4
	1.0×10^3	0/10	
	1.0×10^2	0/10	
F strain	4.5×10^4	10/10	1.6×10^3
	4.5×10^3	6/10	
	4.5×10^2	3/10	
	4.5×10^1	0/10	
KOS strain	1.8×10^5	10/10	3.8×10^1
	1.8×10^4	10/10	
	1.8×10^3	10/10	
	1.8×10^2	10/10	
	1.8×10^1	1/10	

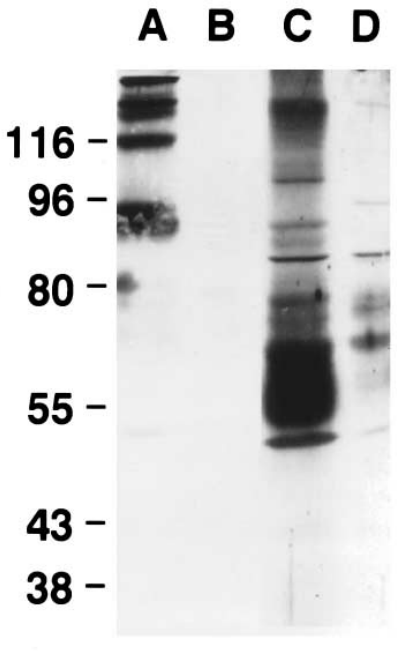

Fig. 6. Representative immunoblot of pooled sera from five mice showing reactivity after intracranial inoculation with VZV IE62-HSV (gC). Sera were run against test antigen immunoaffinity-purified IE62 (lanes A and C) as well as an uninfected cell preparation in parallel (lanes B and D). Lanes A and B were run against hyperimmune anti-IE62 serum (pooled sera of rabbits immunized with a synthetic peptide of the predicted C terminus of ORF 62 (Kinchington et al., 1992). Lanes C and D were run against a pooled sample of five mice intracranially inoculated with VZV IE62-HSV (gC) at a dosage of 4.5×10^2 pfu/mouse.

(Fig. 7). In the first experiment (Fig. 7A), the T-cell responder frequencies for autologous targets infected with vaccinia expressing VZV gE ranged from 1:70 000 to 1:203 000. In the same assays, T-cell responder frequencies for targets infected with VZV gE-HSV (TK) ranged from 1:49 000 to 1:118 000. In the next experiment, for targets infected with vaccinia expressing VZV gE, T-cell responder frequencies ranged from 1:18 000 to 1:168 000; frequencies for targets infected with VZV gE-HSV (gC) ranged from 1:24 000 to 1:287 000 (Fig. 7B). Finally, T-cell responder frequencies for T-lymphocytes that recognized the VZV IE62 protein expressed by vaccinia ranged from 1:17 000 to 1:94 000; in the same assays, targets infected with VZV IE62-HSV (gC) ranged from 1:22 000 to 1:33 000 (Fig. 7C).

4. Discussion

The approach of using a live vector to express viral proteins has a number of advantages over immunization with purified proteins: antigen presentation to the host is more sustained as a result of viral replication and synthesis of viral proteins by infected cells more closely resembles the stimulus to the immune system that is generated by infection with the wild virus. Towards the goal of engineering a combined α-herpesvirus vaccine, HSV can be more readily used as a live vector than VZV, HSV can be inexpensively produced in large quantities, maintains its potency in storage and most importantly, specific genetic manipulations of the virus can be readily performed. Our studies show that two VZV proteins of widely varying function and structure, the VZV gE and the IE62 protein can be expressed in sufficient quantities by HSV-1 to induce humoral immunity in mice and to serve as T-cell targets in human cytotoxicity assays.

Based on immunoperoxidase staining and immunoblotting, VZV gE and IE62 proteins were produced by these HSV-VZV recombinants, regardless of whether HSV-1 F strain or KOS strain was used. Two different promoters, the early HSV ICP4 promoter as well as the native VZV gE promoter, were both effective in regulating VZV gE expression in the HSV-1 vector. A VZV promoter regulating TK expression was also reported to be effective in a HSV-1 vector (Sawyer and Wu, 1990). None of the three HSV–VZV strains showed any marked changes in growth characteristics, although the VZV gE-HSV (TK) recombinant consistently displayed syncytium formation differing from that of the parental KOS strain, a characteristic noted of other strains altered in the UL24 region (Jacobson et al., 1989). The VZV gE or VZV IE62 inserts both maintained their stability in the HSV-1 genome in multiple tissue culture passages.

VZV gE-HSV (gC) and VZV IE62-HSV (gC) had similar virulence compared to wild type HSV F strain when inoculated vaginally in guinea pigs,

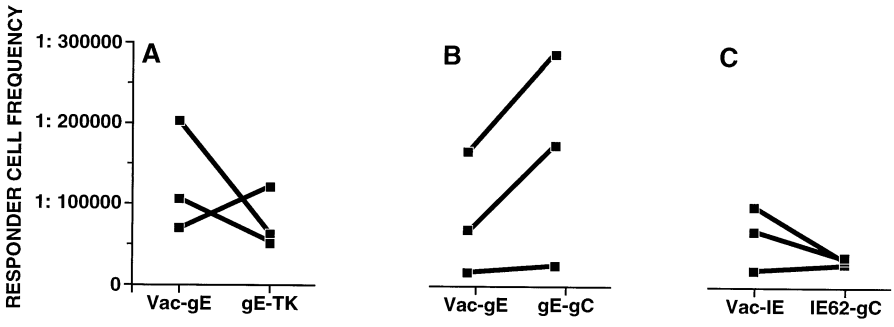

Fig. 7. Precursor frequencies of cytotoxic T-lymphocytes specific for IE62 and gE of VZV. T-lymphocytes from VZV immune, HSV nonimmune donors were incubated with inactivated VZV Ag in limiting dilution cultures, with initial T-cell concentrations ranging from 0, 1×10^3, 1×10^4, 5×10^4 and 1×10^5 and tested for lysis of autologous LCL infected with a vaccinia construct expressing gE (Vac-gE) and a HSVIVZV recombinant expressing gE (gE-TK or gE-gC), or a vaccinia construct expressing IE62 (Vac-IE) and the HSVIVZV recombinant expressing IE62 (IE62-gC). For each comparison assay, three donors were tested in parallel, the points connected by lines indicate the precursor CTL frequencies from these assays.

but had somewhat higher virulence than the parent strain when used in mouse intracranial challenge experiments. Alteration of gC would be expected to have little effect on neurovirulence, since gC is one of the few genes that can be deleted without affecting viral growth in the central nervous system (Kumel et al., 1985; Johnson et al., 1986; Sunstrom et al., 1988). Our finding that the 50% lethal doses of VZV gE-HSV (gC) and VZV IE62-HSV (gC) were somewhat lower than that of the HSV F strain was unexpected and the pathogenicity of these strains will need to be further studied using immunohistopathologic techniques in a larger number of animals.

Our HSV VZV-gE (TK) construct appeared to have markedly altered or diminished production of TK based on its resistance to acyclovir compared to the parental HSV-1 (KOS strain). When tested in intracranial challenge studies, the VZV gE-HSV (TK) construct was approximately 3 logs less virulent than the wild-type KOS strain. This loss of neurovirulence has been consistently observed in TK^- altered or deficient strains and TK^- strains have been noted to be 10-fold to 10^5-fold less virulent than wild-type HSV in intracranial challenges (Field and Wildy, 1978; Field and Darby, 1980). The deficiency of TK appears to impair viral replication in neuronal cells (Price and Khan, 1981), in part because neurons are postmitotic and undergo little deoxyribonucleic acid synthesis. Without viral TK, the rate of synthesis is even lower, neurons may resemble serum-starved cells and provide a poor environment for viral replication (Jamieson et al., 1974).

Interestingly, mice who survived intracranial challenge with VZV gE-HSV (TK) or VZV IE62-HSV (gC) mounted an IgG response to VZV gE and IE62 protein, respectively. These experiments demonstrate that these HSV-VZV recombinants synthesized sufficient VZV gE or IE62 protein to stimulate a primary immune response. Among surviving mice, either sufficient viral replication occurred in the CNS to stimulate a systemic humoral response or limited systemic viral replication may have occurred following CNS inoculation. Based on our failure to detect an IgG response in inoculated mice, it appears that gE expression by the VZV gE-HSV (gC) may have been relatively low. Alternately, since VZV gE-HSV (gC) was the most virulent construct, a shorter period of replication may have occurred with less activation of the humoral immune response.

Since cell-mediated immunity is critical in the human host response to VZV, we performed CTL assays to determine whether VZV gE or VZV IE62 in an HSV-1 vector was recognized effectively by T-cells with cytolytic function. Primary infection with VZV induces T-cells that recognize VZV gE, gB, gH, gC and IE62 (Arvin et al., 1986;

Giller et al., 1989; Bergen et al., 1991; Huang et al., 1992); cell-mediated immunity to these proteins persists for more than 20 years in the normal human host (Arvin et al., 1991). A specific cytotoxic T-cell response to both VZV gE and IE62 can be detected in limiting dilution cultures of T-lymphocytes from VZV immune donors tested against vaccinia recombinants expressing these proteins (Arvin et al., 1991). In our experiments, the T-cell responder frequencies for targets infected with two different HSV-1 vectors expressing VZV gE or targets infected with VZV IE62-HSV (gC) were similar to those for targets infected with vaccinia expressing VZV gE or IE62.

Our cumulative CTL results demonstrate that HSV-1 and vaccinia are equally competent vectors in the processing and presentation of VZV gE and IE62, and indicate that immunity elicited by their expression in HSV-1 will mimic naturally induced cell-mediated immunity. It has recently been shown that expression of ICP47 by HSV-1 infected fibroblasts blocks transport of class I proteins and renders them resistant to lysis by HSV-specific $CD8^+$ CTLs (York et al., 1994); however, LCL targets express both class I and class II molecules and VZV specific CTL are present in $CD4^+$ as well as $CD8^+$ T-cell subsets (Arvin et al., 1991). A disadvantage of using vaccinia as an expression vector is the inevitable development of immunity to vaccinia with suppression of replication and no advantage to the host (Cooney et al., 1991). If HSV-1 was used as the vector for a combined α-herpesvirus vaccine, induction of host immunity against the vector as well as the foreign expressed proteins would be a desirable feature.

Previous studies have shown that immunization of guinea pigs with a vaccinia recombinant expressing a single VZV protein (IE62) reduced viremia following VZV challenge, however, protection was not complete (Sabella et al., 1993). It is likely that expression of more than one VZV antigen by a viral vector would induce a higher level of immunity, thus, VZV gE and IE62 could be cloned into a single HSV-1 vector. Once immunogenicity was proved, further genetic manipulations such as elimination of the ICP34.5 gene product affecting neurovirulence (Chou et al., 1990), restoration of the TK gene to ensure sensitivity to acyclovir (Meignier et al., 1990) or ablation of the gH gene to promote production of non-infectious progeny (Farrell et al., 1994) could be performed in order to increase the safety of HSV as a vaccine vector.

Acknowledgements

P.W.L. was supported by an individual NRSA (IF32-AI108501) as well as a Developmental and Neonatal Biology training grant (NIH HD07249). The work was also supported by Public Health Service grants AI22280 and AI20459 to A.M.A, EY09397 and EY08098 to P.R.K, and AI24009 to E.R.K. We gratefully acknowledge the contributions of Gina M. Abbruzzi, Linda Lew, Taesun Park and Camille Sabella, Department of Pediatrics, Stanford University School of Medicine, Stanford, CA, William T. Ruyechan and John Hay, Department of Microbiology, State University of New York at Buffalo, Buffalo, NY, Peggy Vogt, University of Alabama at Birmingham, Birmingham, AL, Lenore Pereira, University of California San Francisco, School of Dentistry, San Francisco, CA, Rana Orangi, Northview Pacific Laboratories, Berkeley, CA and Philip Ng, Chiron Corporation, Emeryville, CA. The general care of the experimental animals used for this study was done in accordance with NIH guidelines for laboratory animals and in compliance with the Animal Welfare Act, PL 94–279. The specific project was reviewed and approved by the Stanford University Administrative Panel on Laboratory Animal Care.

References

Arvin, A.M., Kinney-Thomas, E., Shriver, K., Grose, C., Koropchak, C.M., Scranton, E., Wittek, A.E. and Diaz, P.S. (1986) Immunity to varicella-zoster viral glycoproteins, gp I (90/58) and gp III (gp 118) and to a nonglycosylated protein, p170. J. Immunol. 137, 1346–1351.

Arvin, A.M., Solem, S.M., Koropchak, C.M., Kinney-Thomas, E. and Paryani, S.G. (1987) Humoral and cellular

immunity to varicella-zoster virus glycoprotein, gpl, and to a non-glycosylated protein, p 170, in the strain 2 guinea pig. J. Gen. Virol. 68, 2449–2454.

Arvin, A.M., Sharp, M., Smith, S., Koropchak, C.M., Diaz, P.S., Kinchington, P., Ruyechan, W. and Hay, J. (1991) Equivalent recognition of a varicella-zoster virus immediate early protein (IE62) and glycoprotein I by cytotoxic T-lymphocytes of either $CD4^+$ or $CD8^+$ phenotype. J. Immunol. 146, 257–264.

Arbeter, A.M., Starr, S.E., Weibel, R. E. and Plotkin, S.A. (1986) Varicella vaccine studies in healthy children and adults. Pediatrics 78(Suppl.), 748–756.

Bergen, R.E., Sharp, M., Sanchez, A., Judd, A.K. and Arvin, A.M. (1991) Human T-cells recognize multiple epitopes of a major tegumentlimmediate early protein (IE62) and glycoprotein I of varicella-zoster virus. Viral Immunol. 4, 151–166.

Blin, N. and Stafford, D.W. (1976) A general method for isolation of high molecular weight DNA from eukaryotes. Nucleic Acids Res. 3, 2303.

Cadoz, M., Micoud, M., Seignneurin, J.M., Mallaaret, M.R., Baccard, Ch., Morand, P., Meignier, B., Whitley, R. and Roizman, B. (1992) Phase I trial of R7020: a live attenuated recombinant herpes simplex virus (HSV) candidate vaccine. Program Abstr. 32nd Intersci. Conf. Antimicrob. Agents Chemother., 341.

Chou, J., Kern, E.R., Whitley, R.J. and Roizman, B. (1990) Mapping of herpes simplex virus-1 neurovirulence to gamma 1 34.5, a gene nonessential for growth in culture. Science 250, 1262–1266.

Cooney, E.L., Collier, A.C., Greenberg, P.D., Coombs, R.W., Zarling, J., Arditti, D.E., Hoffman, M.C., Hu, S-L and Corey, L. (1991) Safety of and immunological response to a recombinant vaccirua virus vaccine expressing HW envelope glycoprotein. Lancet 337, 567–572.

Diaz, P.S., Hunter, E.L., Smith, S.M. and Arvin, A.M. (1989) T-lymphocyte cytotoxicity with natural varicella-zoster virus infection and after immunization with live attenuated varicella vaccine. J. Immunol. 142, 636–641.

Dix, R.B., McKendall, R.R. and Baringer, J.R. (1983) Comparative neurovirulence of herpes simplex virus type 1 strains after peripheral or intracerebral inoculation of Balb/c mice. Infect. Immun. 40, 103–112.

Dolter, K.E., King, S.R. and Holland, T.C. (1993) Incorporation of CD4 into virlons by a recombinant herpes simplex virus. J. Virol. 67, 189–195.

Dormitzer, P.R., Ho, D.Y., Mackow, E.R., Mocarski, E.S. and Greenberg, H.B. (1992) Neutralizing epitopes on herpes simplex virus-1-expressed rotavirus VP7 are dependent on coexpression of other rotavirus proteins. Virology 187, 18–32.

Ejercito, P.M., Kieff, E.D. and Roizman, B. (1968) Characterization of herpes simplex virus strains differing in their effect on social behavior of infected cells. J. Gen. Virol. 2, 357–364.

Farrell, H.E., McLean, C.S., Harley, C., Efstathiou, S., Inglis, S. and Minson, A.C. (1994) Vaccine potential of a herpes simplex virus type 1 mutant with an essential glycoprotein deleted. J. Virol. 68, 927–932.

Fazekas de St. Groth, S. (1982) The evaluation of limiting dilution assays. J. Immunol. Methods 49, R11–R23.

Field, H.J. and Darby, G. (1980) Pathogenicity in mice of strains of herpes simplex virus which are resistant to acyclovir in vitro and in vivo. Antimicrob. Agents Chemother. 17, 209–216.

Field, H.J. and Wildy, P. (1978) The pathogenicity of thymidine kinase-deficient mutants of herpes simplex virus in mice. J. Hyg. 81, 267–277.

Gershon, A.A. (1992) Varicella vaccine: the American experience. J. Infect. Dis. 166(S1), 63–68.

Giller, R.H., Winistorfer, S. and Grose, C. (1989) Cellular and humoral immunity to varicella zoster virus glycoproteins in immune and susceptible human subjects. J. Infect. Dis. 160, 919–922.

Glorioso, J.C., DeLuca, N.A. and Fink, D.J. (1995) Development and application of herpes simplex virus vectors for human gene therapy. Annu. Rev. Microbiol. 49, 675–710.

Herold, B.C., WuDunn, D., Soltys, N. and Spear, P.G. (1991) Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. J. Virol. 65, 1090–1098.

Ho, D.Y. and Mocarski, E.S. (1988) Beta galactosidase as a marker in the peripheral and neural tissues of the herpes simplex virus-infected mouse. Virology 167, 279–283.

Huang, Z., Vafai, A. and Hayward, A.R. (1992) Specific lysis of targets expressing VZV gpI or gpIV by $CD4^+$ clones. J. Virol. 66, 2664–2669.

Jacobson, J.G., Martin, S.L. and Coen, D.M. (1989) A conserved open reading frame that overlaps the herpes simplex virus thymidine kinase gene is important for viral growth in cell culture. J. Virol. 63, 1839–1843.

Jamieson, A.T., Gentry, J.H. and Subak-Sharpe, J.H. (1974) Induction of both thymidine and deoxycytidine kinase activity by herpes viruses. J. Gen. Virol. 24, 465–480.

Johnson, D.C., McDermott, M.R., Chrisp, C. and Glorioso, J.C. (1986) Pathogenicity in mice of herpes simplex virus type 2 mutants unable to express glycoprotein C. J. Virol. 58, 36–42.

Kinchington, P.R., Hougland, J.K., Arvin, A.M., Ruyechan, W.T. and Hay, J. (1992) The Varicella-zoster virus immediate-early protein IE62 protein is a major component of virus particles. J. Virol. 66, 359–366.

Kumel, G., Kaerner, H.C., Levine, M., Schroder, C.H. and Glorioso, J.C. (1985) Passive immune protection by herpes simplex virus-specific monoclonal antibodies and monoclonal antibody-resistant mutants altered in pathogenicity. J. Virol. 56, 930–937.

Lowry, P.W., Sabella, C., Koropchak, C.M., Watson, B., Thackray, H.M., Abbruzzi, G.M. and Arvin, A.M. (1992a) Investigation of the pathogenesis of varicella- zoster virus infection in guinea pigs using polymerase chain reaction. J. Infect. Dis. 167, 78–83.

Lowry, P.W., Solem, S., Watson, B.N., Koropchak, C.M., Thackray, H.M., Kinchington, P.R., Ruyechan, W.,

Zing, P., Hay, J. and Arvin, A.M. (1992b) Immunity in strain 2 guinea-pigs inoculated with vaccinia virus recombinants expressing varicella-zoster virus glycoproteins I, IV, V or the protein product of the immediate early gene 62. J. Gen. Virol. 73, 811–819.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, New York, NY.

McDermott, M.R., Smiley, J.R., Brais, L.J., Rudzroga, H.E. and Bienenstock, J. (1984) Immunity in the female genital tract after intravaginal vaccination of mice with an attenuated strain of herpes simplex virus type 2. J. Virol. 51, 747–753.

Meignier, B., Martin, B., Whitley, R. J. and Roizman, B. (1990) In vivo behavior of genetically engineered herpes simplex viruses R7017 and R7020. II. Studies in immunocompetent and immunosuppressed owl monkeys (Aotus trivirgatus). J. Infect. Dis. 162, 313–321.

Mocarski, E.S., Post, L.E. and Roizman, B. (1980) Molecular engineering of the herpes simplex virus genome: insertion of a second L-S junction into the genome causes additional genome inversions. Cell 22, 243–255.

Price, R.W. and Khan, A. (1981) Resistance of peripheral autonomic neurons to in vivo productive infection by herpes simplex virus mutants deficient in thymidine kinase activity. Infect. Immunol. 34, 571–580.

Reinhold, W.C., Straus, S.E. and Ostrove, J.M. (1988) Directionality and further mapping of varicella zoster virus transcripts. Virus Res. 9, 249–261.

Sabella, C., Lowry, P.W., Abbruzzi, G.M., Koropchak, C.M., Kinchington, P.R., Sadegh-Zadeh, M., Hay, J., Ruyechan, W.T. and Arvin, A.M. (1993) Immunization with the immediate-early tegument protein (open reading frame 62) of varicella-zoster virus protects guinea pigs against virus challenge. J. Virol. 67, 7673–7676.

Sawyer, M.H. and Wu, Y. (1990) Recombinant HSV 1 strains expressing the VZV thymidine kinase. Program Abstr. 15th Int. Herpesvirus Workshop, 166.

Sears, A.E., McGwire, B.S. and Roizman, B. (1991) Infection of polarized MDCK cells with herpes simplex virus 1: two asymmetrically distributed cell receptors interact with different viral proteins. Proc. Natl. Acad. Sci. USA 88, 5087–5091.

Seidel-Dugan, C., Ponce de Leon, M., Friedman, H.M., Fries, L.F., Frank, M.M., Cohen, G.H. and Eisenberg, R.J. (1988) C3b receptor activity on transfected cells expressing glycoprotein C of herpes simplex virus types 1 and 2. J. Virol. 62, 4027–4036.

Sharp, M., Terada, K., Wilson, A., Nader, S., Kinchington, P.E., Ruyechan, W., Hay, J. and Arvin, A.M. (1992) Kinetics and viral protein specificity of the cytotoxic T-lymphocyte response in healthy adults immunized with live attenuated varicella vaccine. J. Infect. Dis. 165, 852–858.

Shih, M.F., Arsenakis, M., Tiollais, P. and Roizman, B. (1984) Expression of hepatitis B virus S gene by herpes simplex virus type 1 vectors carrying alpha and beta regulated gene chimeras. Proc. Natl. Acad. Sci. USA 81, 5867–5870.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503–517.

Spaete, R.R. and Mocarski, E.S. (1987) Insertion and deleiton mutagenesis of the human cytomegalovirus genome. Proc. Natl. Acad. Sci. USA 84, 7213–7217.

Sunstrom, J.C., Chrisp, C.E., Levine, M. and Glorioso, J.C. (1988) Pathogenicity of glycoprotein C negative mutants of herpes simplex virus type I for mouse central nervous system. Virus Res. 11, 17–32.

Tenser, R.B. (1983) Intracerebral inoculation of newborn and adult mice with thymidine kinase-deficient mutants of herpes simplex virus type 1. J. Infect. Dis. 147, 956–957.

Tenser, R.B., Miller, R.L. and Rapp, F. (1979) Trigeminal ganglion infection of thymidine kinase-negative mutants of herpes simplex virus. Science 205, 915–918.

Thompson, R.L., Cook, M.L., Devi-Rao, G.B., Wagner, E.K. and Stevens, J.G. (1986) Functional and molecular analyses of the avirulent wild-type herpes simplex virus type 1 strain KOS. J. Virol. 58, 203–211.

Villarreal, L.P. and Berg, P. (1977) Hybridization in situ of SV40 plaques: detection of recombinant SV40 virus carrying specific sequences of nonviral DNA. Science 196, 183–185.

Watson, B., Keller, P.M., Ellis, R.W. and Starr, S.E. (1990) Cell-mediated immune responses after immunization of healthy seronegative children with varicella vaccine: kinetics and specificity. J. Infect. Dis. 162, 794–799.

York, I.A., Roop C., Andrews D.W., Riddell, S.R., Graham, F.L. and Johnson, D.C. (1994) A cytosolic herpes simplex virus protein inhibits antigen presentation to $CD8^+$ lymphocytes. Cell 77, 525–535.