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MOLECULAR BASIS OF HERPES SIMPLEX VIRUS ENTRY INTO THE CELL AND RETARGETING OF THE VIRAL TROPISM FOR THE DESIGN OF ONCOLYTIC HERPESVIRUSES

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Chapter I: introduction

1.1 Herpesvirus

1.1.1 HISTORICAL PERSPECTIVE ON HERPESVIRUSES

Herpes simplex virus is one of the most fascinating biological organism known to man and in the 25-plus centuries that have passed since investigations into HSV first began, the focus of inquiry has undergone drastic changes.

Hippocrates coined the phrase “herpes” to describe lesions that appear to creep or crawl along the skin, but such lesions could be attributed to other kind of infection.

Celsus was the first to describe an actual herpetic lesion, in fact he noted that they were round initially but then diffused like a serpent to form a belt. Then Herodotus found an association between cutaneous eruption and fever and his descriptions were elaborated on by Galen, who recognized that HSV recurrences developed at the same anatomical site.

During the centuries, the terminology and meaning used by early investigators continued to change.

In the late 19th and early 20th Centuries, transmission of infectious agents to human volunteers was in vogue and in the 1930s Andrews and Carmichael made the observation that recurrent infections occurred only in adults who carried neutralizing antibodies.

In the following years two important articles were published: Doerr stated that HSV infections in man resulted from the endogenous production of a virus-like agent by the cell, under the influence of certain stimuli, and were not caused by exogenous infection. Secondly, Burnet and Williams stated that HSV infection, once contracted, seem to persist for life.

The next step was the discovery of aetiological agents with the application of tissues culture and, consequently, new classification criteria were introduced.

For mucocutaneous and visceral HSV infections, the real therapeutic advance has been the discovery of acyclovir by the pioneering work of Gertrude Elion.

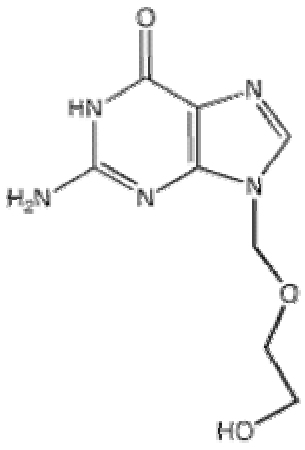


FIG 1: Acyclovir [1]

The golden age of HSV research was in the 1960s and 1970s with the discovery of the structure of the herpesvirus particle, the size and complexity of its DNA and the large number of its proteins thanks to new technologies.
In the past 20 years the approach to the study of Herpesviruses changed: there is a growing awareness that they have the potential to be used as an instrument against specific deseases [1].

1.1.2 TAXONOMY OF HERPESVIRIDAE

Identification of the new and apparently related virus led to a scientific desire for classification, but the criteria applied were necessary confined to what is technically possible, and thus taxonomy has an important historical component.
However, herpesvirus taxonomy has been addressed since 1971 by the International Committee on Taxonomy of Viruses: first formal names were given to viruses, secondly viruses were divided into subfamilies on the basis of biological criteria, then they are divided into genera on the basis of molecular data. In the latest report of ICTV Herpesviridae Group, the family Herpesviridae consists of three subfamilies: Alphaherpesvirinae (Simplexvirus, Varicellovirus, Mardivirus and Iltovirus genera), Betaherpesvirinae (Cytomegalovirus, Muromegalovirus and Roseolovirus genera) and Gammaherpesvirinae (Lymphocrytovirus and Rhadinovirus genera).
Nine herpesvirus have been isolated from humans: herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), human cytomegalogirus (HCMV), varicella-zoster virus (VSV), Epsein-Barr virus (EBV), human herpesvirus 6A and 6B (HHV-6A, HHV-6B) human herpesvirus 7 (HHV-7) and human herpesvirus 8 (HHV-8).
HSV-1, HSV-2 and VZV are members of alphaherpesvirinae. They share variable host range, short reproductive cycle, rapid spread in tissue culture, efficient destruction of infected cells, and the ability to establish latent infections in sensory ganglia.
Human members of betaherpesvirinae are HCMV, HHV-6 AND HHV-7. They are characterized by a limited host range, long reproductive cycle, and slow infection progression in tissue culture. Cells that are infected often become enlarged (cytomegalia) and the viruses can maintain latency in secretory glands, lynphoreticular cells kidneys and other tissues.
The members of gammaherpesvirinae show a very narrow host range, they are usually specific for T or B cells and establish a latent infection in lymphoid tissues. Members of this subfamily are EBV and HHV-8.

Herpes simplex viruses were the first of the human herpesviruses to be discovered and they are intensively investigated because their biological properties and their ability to cause a variety of infection, to remain latent in their host for life and to be reactivated at or near the site of initial infection [2].

Subfamily	Designation	Vernacular Name
Alphaherpesvirinae	***Human herpesvirus 1 (HHV-1)***	**Herpes simplex virus type 1 (HSV-1)**
	Human herpesvirus 2 (HHV-2)	**Herpes simplex virus type 2 (HSV-2)**
	Human herpesvirus 3 (HHV-3)	**Varicella-zoster virus (VZV)**
	Cercopithecine herpesvirus 1 (CeHV-1)	Herpesvirus B, Simian Herpesvirus
	Gallid herpesvirus 1 (GaHV-1)	Infectious laryngotracheitis virus
	Gallid herpesvirus 2 (GaHV-2)	Marek's disease herpesvirus 2
	Suid herpesvirus 1 (SuHV-1)	Pseudorabies virus, Aujesky's disease
	Felid herpesvirus 1 (FeHV-1)	Feline herpesvirus 1, Feline rhinotracheitis herpesvirus
	Ictalurid herpesvirus 1 (IcHV-1)	Channel catfish herpesvirus
Betaherpesvirinae	***Human herpesvirus 5 (HHV-5)***	**Cytomegalovirus (CMV)**
	Cercopithecine herpesvirus 8 (CeHV-8)	Rhesus monkey cytomegalovirus
	Murid herpesvirus 1 (MuHV-1)	Mouse cytomegalovirus
	Murid herpesvirus 2 (MuHV-2)	Rat cytomegalovirus
	Suid herpesvirus 2 (SuHV-1)	Pig cytomegalovirus
	Felid herpesvirus 2 (FeHV-1)	Cat cytomegalovirus
	Human herpesvirus 6A (HHV-6A)	
	Human herpesvirus 6B (HHV-6B)	**Roseolovirus**
	Human herpesvirus 7 (HHV-7)	
Gammaherpesvirinae	***Human herpesvirus 4 (HHV-4)***	**Epstein-Barr virus (EBV)**
	Human herpesvirus 8 (HHV-8)	**Karposi's sarcoma-associated herpesvirus (KSHV)**

TAB 1: Herpesvirus taxonomy by International Committee on Taxonomy of viruses.

1.1.3 CLINICAL SIGNIFICANCE OF HSV

HSV-1 and HSV-2 infections occur worldwide, have no seasonal variation and naturally only infect human beings: HSV must contact mucosal surfaces or abraded skin to initiate infection and then is transported by retrograde flow along axons that connect the point of entry into the body to the nuclei of sensory neurons. Viral multiplication occurs in a small number of sensory neurons; the viral genome then remains in a latent state for the life of the host. Many events cause viral reactivation, such as physical or emotional stress, fever, ultraviolet light, or tissue damage.

HSV-1 is normally associated with orofacial infections and encephalitis: recurrent orolabial lesions are preceded by other symptoms and it may cause fever, sore throat, vescicular or ulcerative lesions.

HSV-2 usually causes genital infections and can be sexually transmitted, transmitted from infected mother to neonates and during pregnancy. Its most severe clinical symptoms are encountered with primary infection, characterized by the appearance of macules and papules followed by vesicles, pustules, and ulcers.

HSV can infect also eye and cause herpetic keratocojunctivitis which most often involve only a single eye. Repeated attacks can last for weeks or months and progressive disease can result in vision loss.

Immunocompromised patients, especially organtransplant recipients, are at risk of severe HSV infection. These patients can develop progressive disease involving respiratory tract, oesophagus, or gastrointestinal tract.

Other consequences of HSV infection are encephalitis, meningitis, myelitis, radiculitis and respiratory diseases [3].

Chapter I: introduction

1.2 HSV

1.2.1 ARCHITECTURE OF HERPESVIRION

Virions of herpes virus can vary in size from 120 nm to 300 nm and consist of multiple stratified structures: an electron-dense core, an icosahedral capsid around the core, an amorphous tegument around the capsid and an outer envelope containing glycoprotein spikes.

The core contains viral DNA with a toroidal structure that appears to be suspended by proteinaceous spindle to the capsid.

The capsid is approximately 125 nm in diameter and is composed of 162 capsomers that can be pentons or hexons, consisting of 5 or 6 copies of the major capsid protein.

The proteinaceous layer that surrounds the capsid is the tegument. It appears fibrous and can be distributed asymmetrically, in fact its thickness can vary depending on the location of the virion particle within the infected cell.

The envelope, the outer covering, has a typical trilaminar structure. It appears to be made up of altered cellular membranes and contains viral glycoproteins that are responsible for viral attachment and entry to host cells [4].

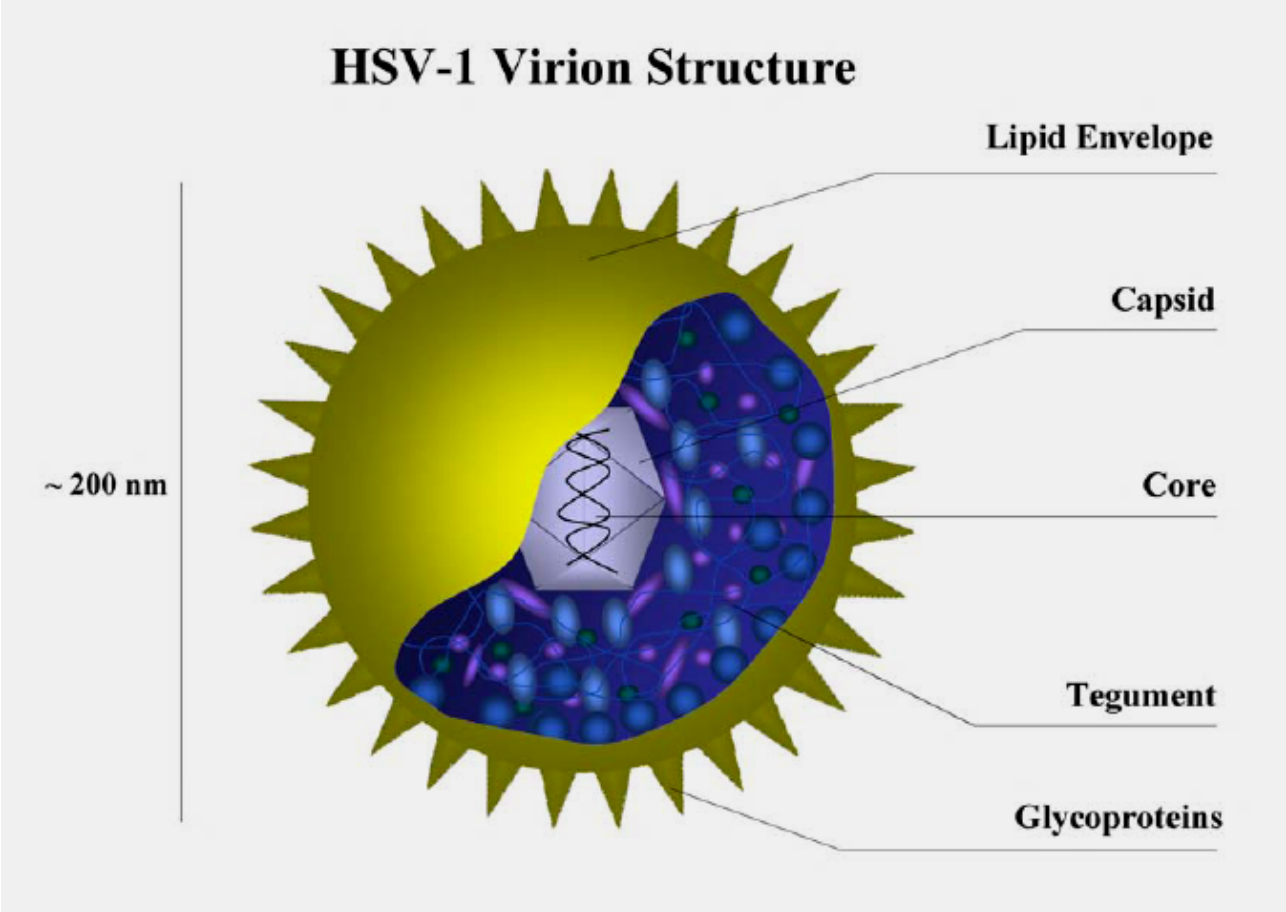


FIG 2: Schematic representation of HSV-1 virion structure.

1.2.2 VIRUS REPLICATION CYCLE

The main steps of herpesvirus infection are: attachment to cell surface, entry into the cell, replication and transcription of the viral genome, control of cell gene expression, egress and estabilishment of latency. I will describe in details attachment, entry and viral glycoprotein required for these steps in next paragraphs (1.2.7, 1.3.1, 1.3.2, 1.3.3, 1.3.4, 1.3.5) because they are pertinent to the background of this thesis.

1.2.3 ORGANIZATION, TRANSCRIPTION AND REPLICATION OF VIRAL GENOME

Herpesvirus genomes consist of linear, double-stranded DNA molecules that range in size from about 125 to 240 Kbp and in nucleotide composition from 32 to 75% G+C, depending on the virus species. Genomes are not simple length of unique DNA but contain direct or inverted repeats. The HSV-1 genome is 152 Kbp in length with a base composition of 68 % GC and encodes for 90 transcription units, and for 84 different gene products (fig. 3).

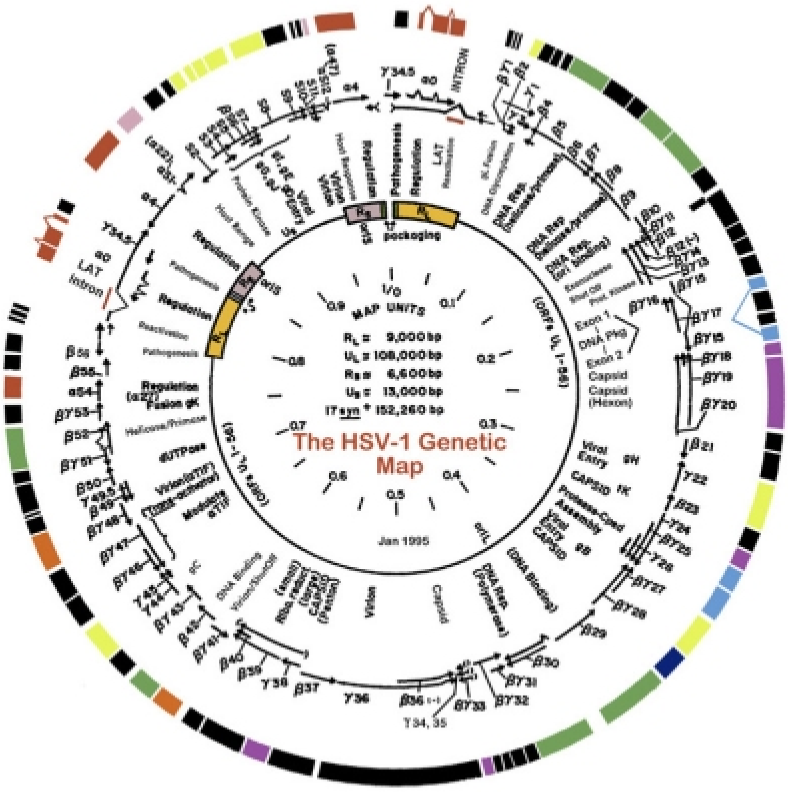


FIG 3: Functional organization of the HSV-1 genome. From inside out, circles are: 1. Maps units and kilobase pairs. 2. Sequence arrangement of HSV genome. 3. The transcriptional map of the HSV-1 genome; arrows indicate the direction of transcription. The designation between the second and the third ring identifies known functions encoded by the open reading frames. The designation outside the third ring identifies the number and the kinetic class (α, β or γ) to which the corresponding open reading frame belogs.

It could be divided into six important regions:

1- **a-sequences** are the end of the linear molecules and are important in both circularization of viral DNA, and in packaging the DNA in the virion.

2- $\mathbf{R_L}$ is the 9000bp long repeat which encodes both an important immediate early regulatory protein ($\alpha 0$) and the promoter of the most of the gene for the latency associated transcript (LAT).

3- $\mathbf{U_L}$ is the 108000 bp long unique sequence which encodes at least 60 distinct proteins. It contains genes for DNA replication enzymes and the capsid proteins, as well as many other proteins.

4- $\mathbf{R_S}$ are the 6600 bp short repeat which encode the very important an immediate early protein. This is a very powerful transcriptional activator. It acts along with $\alpha 0$ and $\alpha 27$ (in the U_L) to stimulate the infected cell for all viral gene expression that leads to viral DNA replication.

5- The origins of replication. The oriL is in the middle of the U_L region. The oriS is in R_S and thus is present in two copies.

6- $\mathbf{U_S}$ is the 13000 bp short unique region which encodes 14 ORFs, a number of which are glycoproteins important in viral host range and response to host defence.

By the inversion of U_L and U_S, four different isomers are originated: P (prototype), I_L (inversion if L component), I_S (inversion of S component) or I_{SL} (inversion of both two) (fig. 4). These isomers are present in equimolar amount in virus stocks.

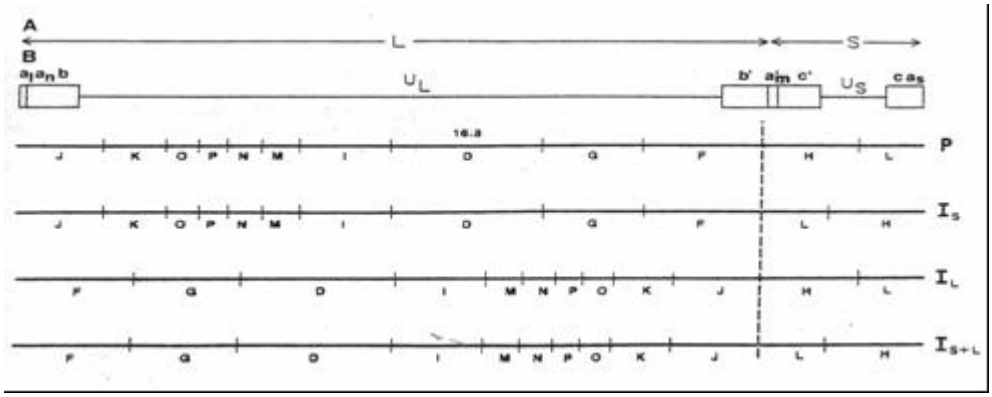


FIG 4: (A) Schematic representation of the sequence arrangement of the HSV genome. Thin line represents the unique sequences U_L and U_S, while boxes represent the inverted repeats (*n* and *m* represent a variable number of additional sequences). (B) Hind III restriction endonuclease map of HSV-1 (F) strain and representation of the fourth different isomers of viral DNA generating from inversion of the unique sequences relative to each other.

The majority of HSV genes are not spliced, so each viral gene encodes for a single protein, with few exceptions. Many mRNAs initiate in the middle of an expressed ORF and encode only the C-terminal part of the protein. Some ORFs are antisense to other ORFs (γ34.5 and ORF P, UL27.5 and UL27).

Gene products frequently have more than one function, not necessarily related one to the other.

Few transcripts do not appear to encode for expressed ORFs and their function in productive infection is not known; an example is the latency-associated transcripts (LATs) which are expressed in latent infection.

The HSV genome is transported to the nucleus of infected cells in a capsid-tegument complex which uses the cell's microtubule network. After being deposited into the nucleus of the infected cell, HSV-1 viral genome localizes to nuclear ND10 structures where the transcription of viral genes takes place. The host RNA polymerase II is responsible for the transcription of all viral genes during infection.

The genes of HSV-1 are divided into three classes: immediate early genes (α genes), early genes (β genes) and late genes (γ genes) (fig. 5).

HSV encodes a function responsible for transactivation of α genes immediately after infection, and this was termed α gene transactivating factor (α-TIF). At 2 to 4 h post infection α genes are expressed at peak levels. There are six viral α genes: ICP0, ICP4, ICP22, ICP27, ICP47, and Us1.5. Five of the six α genes stimulate viral β genes expression.

The β genes, which are produced at peak levels between 4 and 8 h post infection, product proteins involved in viral DNA replication (i.e. the single strand DNA binding protein ICP8) nucleotide metabolism (i.e. the large subunit of ribonucleotide reductase ICP6) and stimulate γ gene transcription. The β genes can be divided into two general groups: β_1 genes, which are expressed shortly after the synthesis of α proteins; and β_2 genes, which are expressed with more of delay after α genes expression.

γ gene products, which are produced at peak levels only after viral DNA replication, include structural proteins of mature virions and tegument components required to prepare newly infected cells for an efficient infection. The γ genes have been subdivided into two groups based on timing expression and their dependence on viral DNA replication: γ_1 genes, which are expressed relatively early in infection and γ_2 genes, which do not accumulate in appreciable amounts until after DNA replication and are not expressed in the presence of inhibitors of viral DNA synthesis [5]

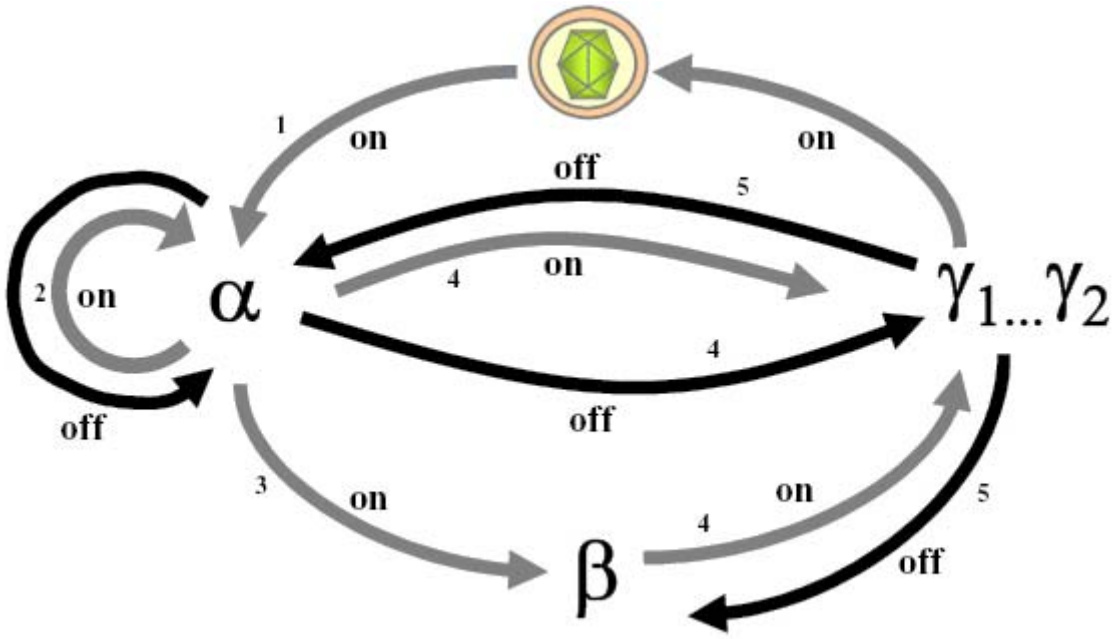


FIG 5: Schematic representation of the regulation of HSV gene expression. Grey arrows: events that turn gene expression "on"; black arrows: events that turn gene expression off. (1) α gene expression is stimulated by αTIF, a γ protein packaged in the virion. (2) α protein turn off transcription of α gene. (3) α proteins stimulate transcription of β gene. (4) α and β proteins transactivate γ genes. (5) Late infection, γ proteins turn off α and β gene expression.

The basic model for the replication of HSV DNA proceeds as follows. First parental viral DNA is circularized upon entering the nucleus of infected cell. After α and β gene expression, UL9 binds to specific elements in either oriL or oriS and begins to unwind the viral DNA. UL9 then recruits the ssDNA binding protein ICP8 to unwound portion of viral DNA. At this point, UL9 and ICP8 recruit the remaining five proteins to replication forks. The helicase-primase and viral DNA polymerase complexes assemble at each replication fork and initiate theta form replication. Through an unknown mechanism, replication switches from theta form to the rolling circle form for replication and UL9 is not required. The rolling circle replication forms long head-tail concatamers of viral DNA, which become cleaved into individual units during packaging of viral DNA into capsid (fig. 6)[6].

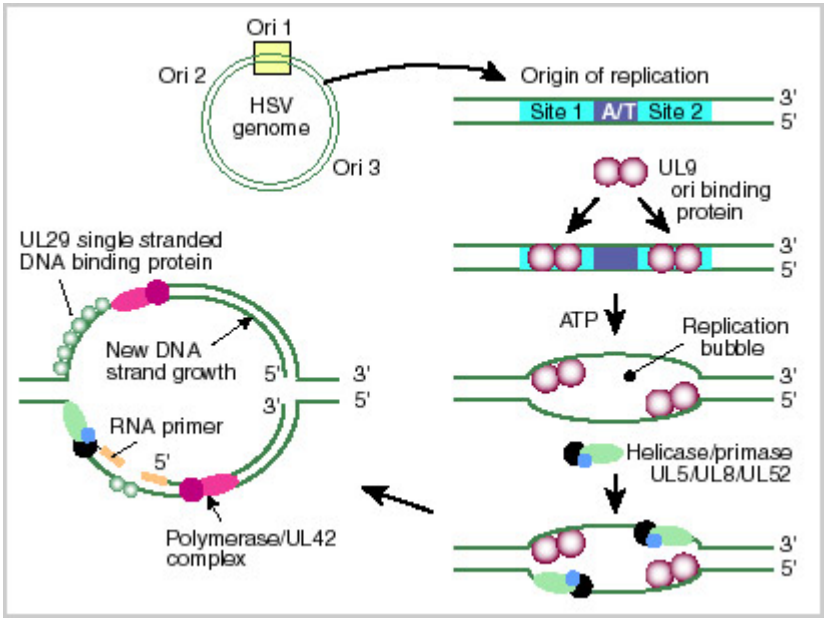


FIG 6: The steps of viral DNA replication in which many proteins take part: DNA polymerase (U_L30), DNA binding proteins (U_L42 and U_L29 or ICP8), ORI binding protein (U_L9), and the helicase/primase complex (U_L5, 8 and 52).

1.2.4 ASSEMBLY AND EGRESS

After DNA replication has started, the γ genes are transcribed, including those encoding HSV-1 capsid protein, then mature capsid is able to proceed along the viral egress pathway. It acquires a primary envelope by budding through the inner nuclear membrane into the perinuclear space. [7]. Two pathways of virus exit are proposed:

-the single envelopment model: virions leave the perinuclear space by becoming encased in vesicles-vacuoles formed by the outer nuclear membrane (fig. 7) [8]

- the de-envelopment-re-envelopment pathway: the envelope of virus present in the perinuclear space fuses with the outer nuclear membrane (de-envelopment), thus releasing the nucleocapsid into the cytoplasm. The de-envelopment nucleocapsids acquire a tegument in the cytoplasm and undergo a secondary envelopment (re-envelopment) by nucleocapsid budding into a trans-Golgi compartment, or trans-Golgi network, or into an endosomal compartment (fig. 7) [9,10].

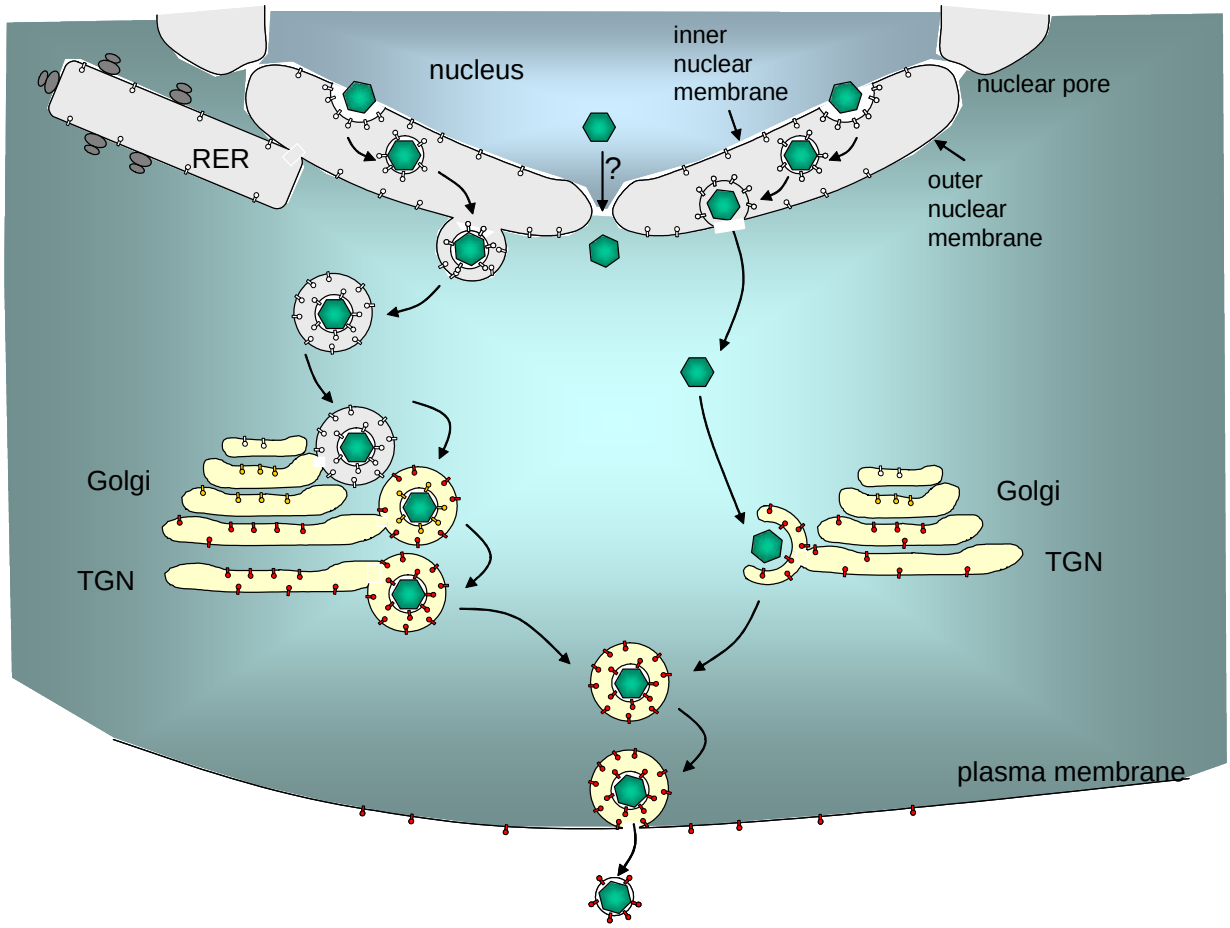


FIG 7: Schematic drawing showing the two alternative pathways of alphaherpesvirus egress from infected cells. The single envelopment pathway is despicted to the left, and the double envelopment, or de-envelopment-re-envelopment is despicted to the right of the illustration.

1.2.5 LATENCY

HSV-1 has developed a particularly successful form of accommodation with the host. After the initial infection in epithelial cells, the virus enters neuronal cell axons and migrates to the ganglia where it estabilishes a latent infection. (fig .8)

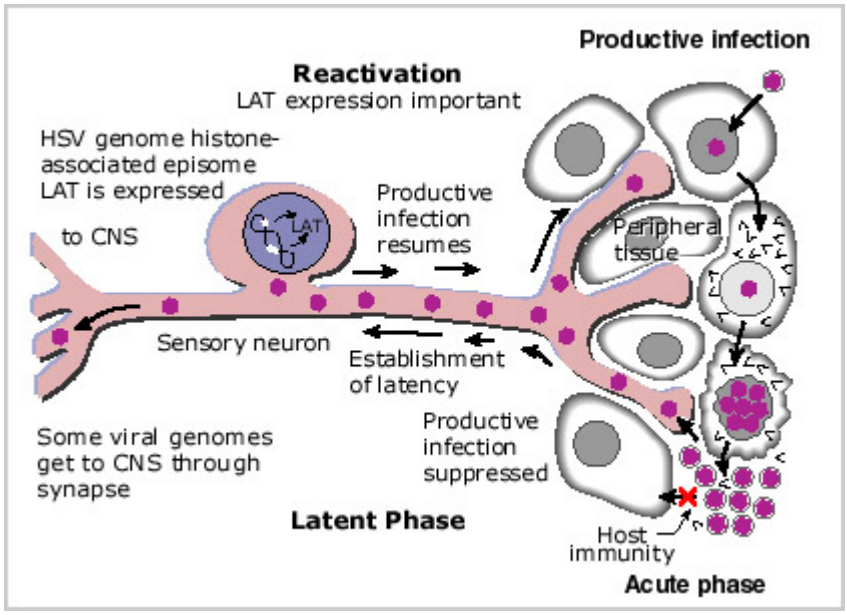


FIG 8: HSV establishment of latency. In productive infection, HSV enters at mucosal surfaces, replicates in epithelial cells at the site of entry and spreads through the tissue. Virus enters nerve endings of sensory neurons and is transported to neuron cell body in the ganglion where virus can either replicate productively or establish a latent infection: viral DNA circularizes into neuronal cell nucleus and latency-associated transcripts are expressed (LATs genes). Upon a variety of stimuli, the virus reactivates: viral DNA replicates, viral proteins are made, capsids are assembled and transported in anterograde direction to mucosal surfaces where virions are released and cause recurrent lesions.

Latency is characterized by lack of expression of all of the viral gene products that are required for productive, lytic infection. Instead, the virus resides in a quiescent state, producing only a family of latency-associated transcripts (LATs) that are retained in the nucleus and not polyadenylated. The LAT gene is located within the inverted repeat sequence that brackets the unique long segment, therefore, there are two copies of LAT gene in each HSV-1 genome. This gene encodes an 8.5 Kb transcript that is present at low abundance (minor LAT). Splicing of this trancript produces a series of highly stable introns (major LATs) that accumulate at high levels within the nuclei of lantently infected neurons. [41]

No protein product has been attributed to the LAT gene and the machanism by which LAT protects cells from apoptosis is not yet perfectly known.

Recently, it has been shown [59] that a microRNA (miRNA) encoded by the HSV-1 LAT gene confers resistence to apoptosis. In fact Gupta et al. [59] proved that miR-LAT exerts its anti-apoptotic effect by downregulation of trasforming growth factor (TGF)-β1 and SMAD3 expression, both of which are functionally linked in the TGF-β pathway.

This suggests that the miRNA encoded by the HSV-1 LAT gene regulates the inactivation of apoptosis in infected cells by modulation of TGF-β signalling and thus contributes to the persistence of HSV in a latent form in sensory neurons.

Periodically, HSV reactivates and causes episodes of lytic infection. The molecular basis of reactivation is not completely understood. External or endogenous stimuli, such as UV-light, stress and fever, have been known for dacades to induce HSV reactivation.

1.2.6 ALTERATION IN INFECTED CELL

HSV infection dramatically modifies the infected cell: the nucleolus becomes enlarged, disaggregates or fragments, host cromosomes become marginated, the nucleus becomes distorted and multilobed. There are also changes in intracellular membranes, fragmentation of Golgi vesicles, insertion of viral protein into cellular membranes, rearrangement of microtubular network and the formation of intranuclear inclusion bodies, globular nuclear structures where viral DNA replication proteins accumulate.

Furthermore, there is evidence that HSV uses multivesicular bodies (MVBs) as platforms for its envelopment/egress, thus cellular distribution and components of these structures change during HSV infection: it has been proved that modified MVBs membranes constitute a platform for HSV cytoplasmic envelopment, and that MVBs components are recruited to the site(s) of envelopment [61]. In uninfected cells, in fact, MVBs are dispersed throughout the cytoplasm, by contrast, in infected cells 1 (12 h and 24 h post infection) they are concentrated in a perinuclear region, and appear to be overall increased, possibly reflecting an augmentation of the compartment itself. These morphological changes are consistent with an involvement of the MVBs pathway in HSV replication [61].

Host macromolecular metabolism is altered in infected cells in at least four different ways: mRNA present in infected cells at the time of infection is degradated, host cell transcription appears to be turned off, cellular proteins are selectively degraded or stabilized and cellular proteins are redirected to perform novel tasks [4].

Moreover, HSV encodes other functions that block host defense against infection: inhibition of apoptosis, inhibition of MHC class I peptide presentation on infected cells and blocking maturation of , and antigen presentation by dentritic cells [41].

1.2.7 VIRUS ATTACHMENT AND ENTRY

Enveloped viruses enter cells by fusion with plasma or endocytic membranes: fusion at the plasma membrane is known to occur in Vero cells in a pH indipendent fashion [12]. In most cell types virion entry is by endocytosis, but this is also variable: in some cells fusion with endosomal membrane requires a low pH environment in other cases it does not require acid pH.

The entry process consists of three basic steps: recognition of cellular receptors by a viral glycoprotein, triggering of fusion and fusion execution. [13] These steps are carried out by virion glycoproteins, in concert with their cognate receptors, as only proteins have sufficient complexity and information content to organize and regulate membranes.

HSV, the paradigm of herpesviruses with respect to virus entry into the cell, encodes 11-12 glycoproteins and a number of additional membrane proteins.

The current model of HSV entry envisions that, first, the virus attaches to cell membranes by interaction of gC and possibly gB to glycosaminoglycans (GAGs) that decorate heparan sulphate (HS) [14] Although binding lacks specificity and is not absolutely required, likely creates multiple points of adhesion, is reversible, and the detached virus maintains its infectivity, indicating that fusion has yet to take place.

This step is followed by gD binding to one of three alternative entry receptors; nectin 1, herpes virus entry mediator (HVEM, also named HveA for herpes simplex mediator A) and specific o-sulphates (3O-S) moieties in HS. The different use of these receptors is important, and may help to account foe entry of HSV into such wide range of different cell types.

After binding of gD to a HSV-1 virus entry receptor, the last step in virus entry is fusion of virion envelope with the plasma membrana of target cell. gB, gH, gL appear to be the executors of fusion and constitute the conserved fusion machinery across the herpesvirus family. Critical properties of gH and gB have been elucidated recently, and provide an intriguing scenario. On one hand, molecular and biochemical analysis of gH highlighted properties of class 1 fusion proteins, but its structure has not been solved. On the other hand, the crystal structure of gB has yet solved, it exhibits a remarkable similarity to that of vesicula stomatitis G protein, and to viral fusion glycoprotein in general. [15]. How the two glycoproteins cooperate to execute fusion, and why two, and not one fusion executors are required in herpesvirus family is unclear. It is worthwhile to note that entry by fusion at plasma membrane, and entry by fusion in endocytic vesicle required all four glycoproteins (gD, gB, gH and gL). [16,17]. These requirements rule out the possibility that gB serves as

fusion executor in one cell compartment, and gH-gL serves as fusion executor in another cell compartment.

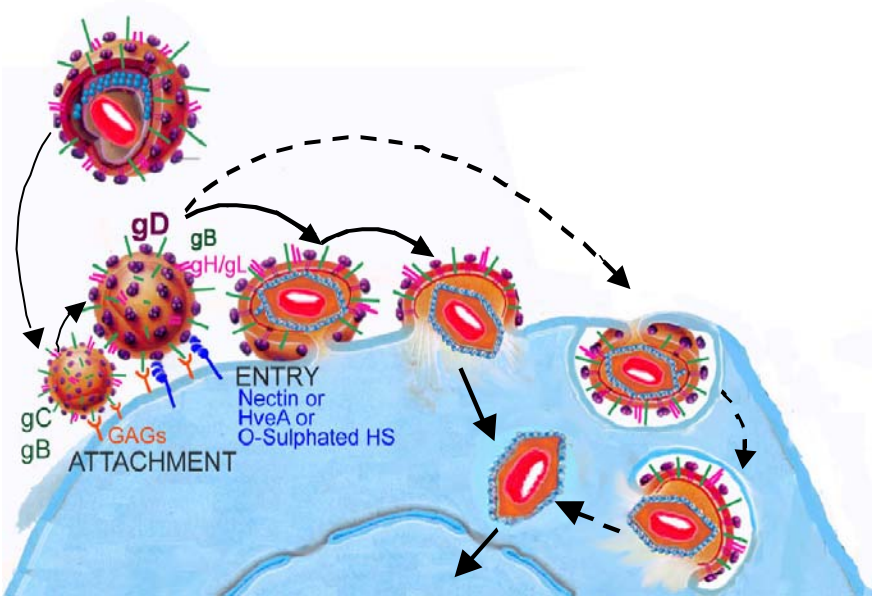


FIG 9: Attachment and entry of herpes simplex virus into cells. The first step is virion attachment to glycosaminoglycans of cell surface (heparin sulphate). The second step involves the interaction of glycoprotein D to one of the number receptors. This, with the participation of the other glycoprotein gB and gH/gL, leads to fusion of the viral envelope with the plasma membrane or endocytic compartments (in HeLa and CHO cells), followed by uncoating of virions, transport of capsid to the nuclear pores and release of viral DNA into nucleous.

Chapter I: introduction

1.3 Viral glycoprotein and cell receptors

1.3.1 GLYCOPROTEIN C

Glycoprotein C is the major actor during attachment and is a non essential glycoprotein encoded by the UL44 gene (1536 bp).

gC contains a 25 aa signal sequence at the N-terminus, a long 453 aa extracellular domain, a 23 aa transmembrane anchoring domain, and a short 10 aa C-terminal cytoplasmatic tail (Homa et al 1986). Its ectodomain structure is provided in part by cysteines, and harbors two physically separate antigenic regions, antigenic I and II, which map at the C- and N-termini of the molecule respectively. It is a mucin-type glycoprotein because of its high content in N-linked and O-linked oligosaccharides [18].

gC can mediate the initial binding of HSV-1 to cell surface GAG, Heparan sulphate (HS). The heparan sulphate binding site of gC has been localized to the N-terminal 120 aa. [19]. The main function of gC binding to GAG seems to be concentration of the virus on cell surface, enabling the more stable interaction of gD with an entry receptor.

Another function of gC is its ability to inactivate complement to facilitate immune evasion of HSV-1, in fact it is able to bind C3b [20].

1.3.2 GLYCOPROTEIN D

HSV-1 glycoprotein D is a type I membrane glycoprotein consisting of 369 aa, after cleavage of the signal sequence (25 aa), with an N-terminal ectodomain of 316 aa and three N-linked oligosaccharide attachment sites.

The protein has six cysteines that form three disulfide bonds (Cys66-Cys189, Cys106-Cys202, Cys118-Cys127).

The most N-terminal portion of the glycoprotein is unstructured in gD alone and forms an harpin in the HVEM-bound gD. It packs against the core of the molecule, to which it is connected through a short flexible proline rich region, spanning aa 45-54. The core consists of two prominent structures, an Immunoglobulin Variable (IgV) folded region (residues 56-184), and a 17 long helix (a-helix3) that ends at aa 240 and packs between the IgV and the most N-terminal portion.

Downstream of a-helix3 is a long flexible proline rich region, spanning aa 244-312. The C-terminus of gD ectodomain, past residue 260, does not participate in direct interaction with receptors, but biochemical and structural studies concordantly indicate that prominent properties of the ectodomain C-terminal region in the unliganded gD are the ability to be displaced from its binding site when gD interacts with HVEM and possibly with nectin1.

Moreover the C-terminal membrane region (aa 250/260-310) carries a domain required for the triggering of fusion, named the pro-fusion domain (PFD) [21].

The mutagenesis of gD at the C-terminus has shown that residue 294 (Trp294) is the necessary anchor in bringing the C-terminus in close proximity to the N-terminus, hence interfering with receptor binding.

Altogether, it has been proposed that gD exhibits conformational flexibility and the C-terminal region may be in an equilibrium between a transiently bound and a partially displaced state, that would permit the binding of the receptor. Receptor binding may well change this equilibrium and stabilize or induce the opened gD conformation.

At the structural level, much more is known of the interaction of gD with HVEM than with nectin1 and it has been shown that a number of mutations that hamper the interaction with HVEM also hamper the interaction with 3-O-S HS, suggesting that the binding surfaces for the two receptors overlap, at least in part. In contrast, the binding surfaces to HVEM and nectin1 do not exhibit significant overlap. Thus, deletion of the first 32 aa of gD and a number of mutations (aa 25, aa 27…) abrogate the interaction with HVEM, but not with nectin1, indicating that the binding sites of the two receptors on gD are partially non overlapping and independent one of the other, and that the primary interface with nectin1 is downstream of

residue 32. Critical aa residues for nectin1 binding include V34, Y38 and the cluster D215, R222, F223, all of which occupy a same surface of the molecule. The surface appears to be partially occluded by the N-terminal hairpin, in the HVEM bound gD. The gD surfaces binding to HVEM and nectin1 can not be ascribed to physically separated segment; cumulatively they map to aa 1-250/260.

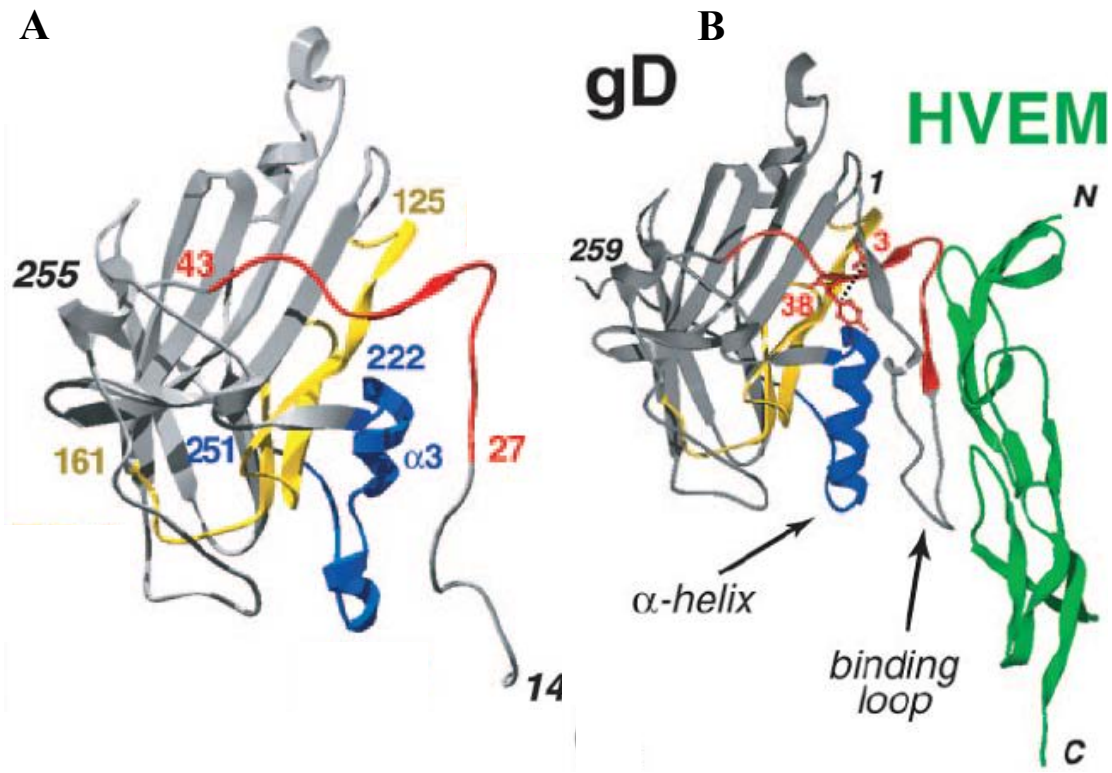


FIG 10: (A)Ribbon diagram of the crystal structure of unligated gD. The N- and the C-terminal residues observed in the crystal structure are indicated in black. (B) Ribbon diagram of the HVEM-bound gD (gD is red and HVEM is green). [22]

1.3.3 HSV-1 RECEPTORS

Binding of HSV glycoprotein D to an entry receptor sets in motion an irreversible chain of events leading to the fusion of the virion envelope with the plasma membrane of the target cell. The three natural gD receptors belong to structurally unrelated molecular families (fig.11).

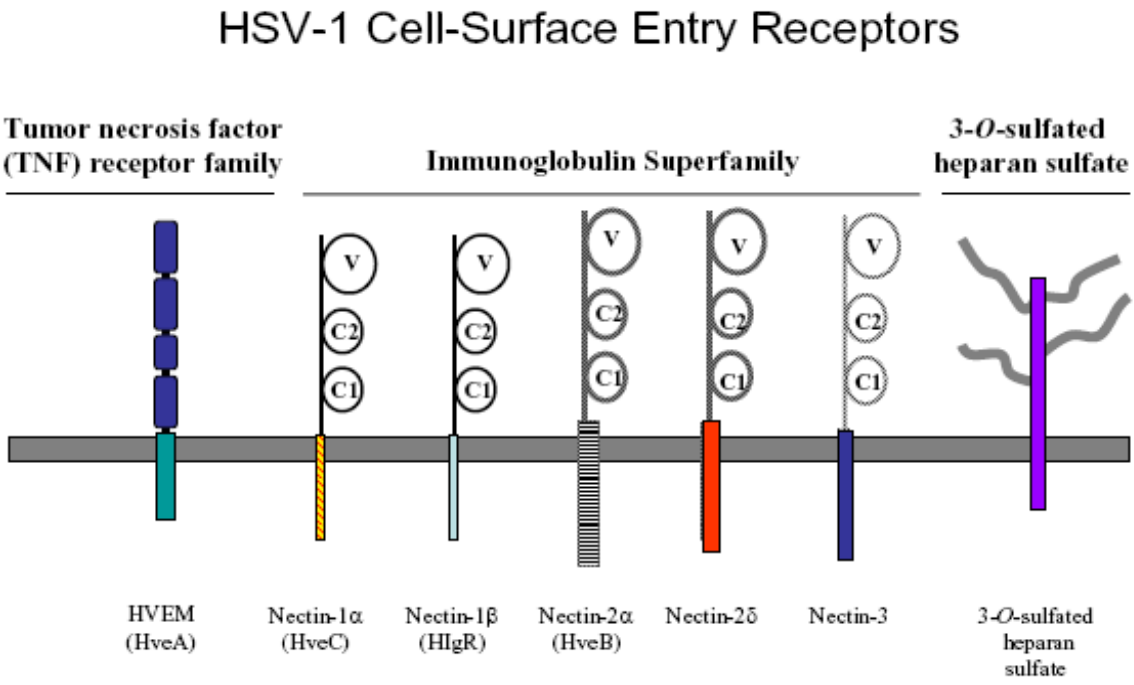


FIG 11: The three classes of cell surface receptors for HSV entry are: the tumour necrosis factor (TNF) receptor family consisting of HVEM, the immunoglobulin superfamily consisting of the nectins, the 3-O-sulfated heparin sulphate. Only viral attachment can occur in the absence of HSV entry receptor.

HVEM

HVEM/Herpes Virus Entry Mediator A (HveA) was first identified as a HSV receptor and was classified as a novel member of the TNFR family based on structural motifs. This family includes signal transduction molecules involved in regulation of cell proliferation, differentiation and apoptotic death. Structurally their ectodomain is composed of four typical cystein-rich domains.

HVEM belongs to the second group of TNF receptors, in fact it lacks a death domain and instead its cytoplasmatic tail interacts with several members of the TRAF family, leading to the activation of target like NF-kB, Jun N-terminal Kinase, and AP-1, and the consequent induction of T cell activation, proliferation, cytokine release, and expression of cell surface activation markers.

HVEM was found to be expressed mainly in cell of the immune system, and in a number of non-hematopoietic tissues and organs. Expression was not observed in brain or skeletal muscle. [23,24].

The gD contact site on HVEM involves CRD1 and CRD2, with the majority of contact lying in CRD1. Residues 35-37 form the intermolecular antiparallel β-sheet [25].

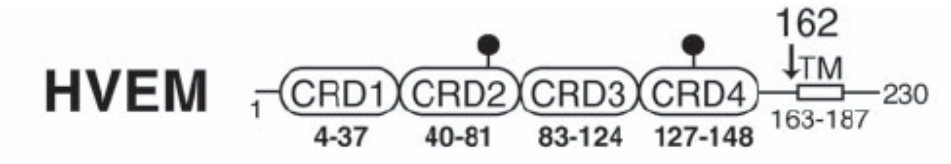


FIG 12: Diagram of HVEM: the HVEM aminoacid comprising each of the four cysteine-rich domain (CRD) are labelled. The position of N-glycosilation sites (lollipops) and the transmembrane regions (TM) are indicated.

NECTINS

Nectins are another family of HSV entry receptors, belong to immunoglobulin superfamily and include several isoforms present in both human and nonhuman cells. Nectins form homo cis-dimers on the plasma membranes and trans-dimers with nectins present on adjacent cell. Their main attribute is the formation, together with cadherins, of the adherens junction of epithelial cells, and in cooperation or not with cadherins the organization of claudin-based tight junctions. They are involved in the formation of synapses in neurons and the organization of heterotypic junctions between Sertoli cells and spermatids in the testis [26].

Most nectins carry a C-terminal conserved motif that binds afadin; this domain is absent in nectin1b.

Nectin-mediated signalling activity leads to activation of a variety of extracellular and intracellular molecules, such as scatter factor/hepatocyte growth factor, Ras, Cdc42 and Rac small G proteins [26].

Nectin1 interacts physically with gD [27]. The interaction requires the first 250 residues of gD and the V domain of nectin1.

Nectin1α (Herpes Virus Entry Mediator C) and Nectin1β (Herpesvirus Immunoglobulin-like receptor, (HIgR) are two mRNA spicing variants containing the same ectodomain. They are expressed on epithelial, fibroblastic, neural and hematopoietic cells, in kerationocyte, and in human tissues that are target of HSV infection including skin, brain, and spinal ganglia [28].

Nectin1γ is a natural soluble form which has a narrow distribution in human tissues. It has the capacity to bind to virions and block infectivity.

Nectin2 (nectin2α or Herpes Virus Entry Mediator B, HveB and nectin2δ) mediate the entry of HSV-2, PrV and certain viable mutants of HSV-1 but not wild type HSV-1 [29].

MODIFIED HEPARAN SULFATE

Modification of heparan sulphate by D-glucosaminyl 3-O- sulfotransferase creates 3-O-sulfated Heparan Sulfate (3-OS HS), generating a gD binding site that allows heparan sulphate proteoglycans to function as entry receptor for HSV-1. 3-O-sulfated Heparan Sulfates are broadly distributed on human cells and tissues and mediate HSV-1 but not HSV-2 entry [30].

B5

A type II glycoprotein, designed B5 was identified by its ability to render otherwise resistant cells more susceptible to HSV entry [31]. The same cells were rescued by HVEM, and it was thus proposed that B5 serves as HSV receptor. However, the ability to bind virions or a specific glycoprotein was not documented. As of now, it remains to be determined whether B5 exhibits properties of a HSV receptor.

1.3.4 GLYCOPROTEIN B

The HSV-1 UL27 gene encodes the 904 aa glycoprotein B (gB), which is a type I virion glycoprotein, high conserved across all subfamilies of herpesvirus: its crystal structure, a trimer with a coiled coil core, resemble closely that of vescicular stomatis virus G protein [32, 33].

gB is a trimeric spike, each of the three promoters appear to be composed of five distinct domains (named I-V), displaing multiple contact sites (fig. 13). Domain I, the "base", is a continous chain with a fold typical of pleckstrin homology domains. Domain II, the "middle", is made of two discontinuous segments, forming a structure reminiscent of a plekstrin homology superfold. Domain III, the "core", comprises three discontinuous segments: its prominent feature is a 44-residue α-helix that forms the central coiled coil with its trimeric counterpart. Domain IV, the "crow", adopts a novel structure, and is fully exposed on top of trimeric spike. Domain V, "the arm", is a long extension spanning the full length of the promoter [32].

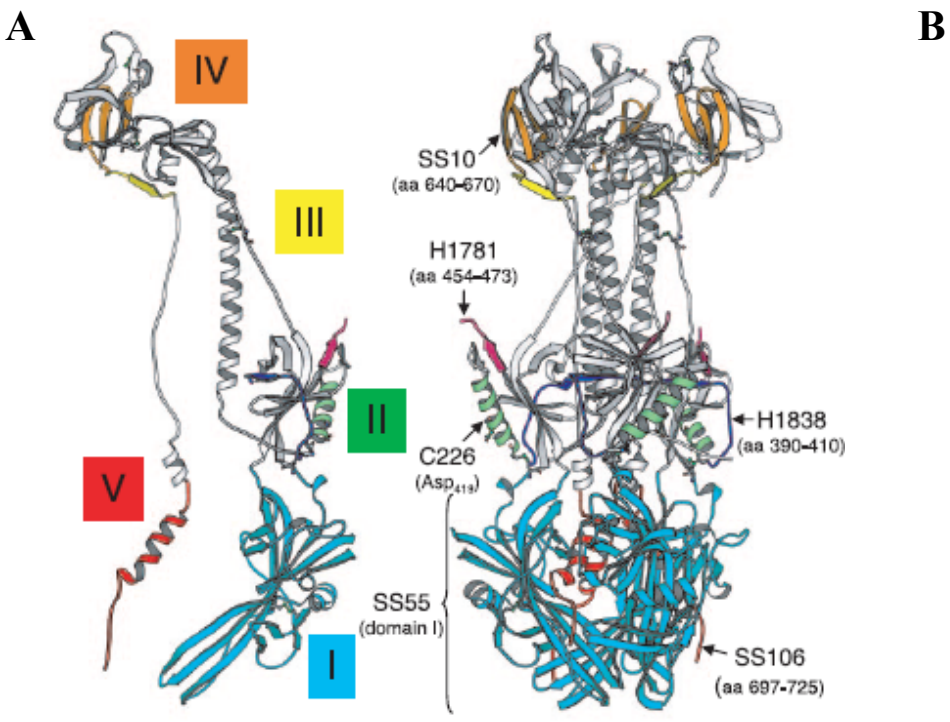


FIG 13: (A) Ribbon diagram of a gB protomer showing in color the epitopes of representative neutralizing MAbs. The epitopes of SS10 and SS67 (amino acids 640 to 670) include β–strands 33 to 35 from domain IV (orange) and β-strand 36 from domain III (yellow). The H1838 (blue) and H1781 (magenta) epitopes (residues 391 to 410 and residues 454 to 473, respectively) are linear stretches within domain II. Part of the H1781 epitope is not visible since it is located in a disordered portion of gB (residues 459 to 473). The C226 epitope includes Asp419 located in α-helix B (green) also of domain II. The conformational epitope for SS55 includes

unidentified amino acids within domain I (cyan). Finally, the epitope of MAb SS106 (amino acids 697 to 725) is located at the C terminus of domain V (red). Disulfide bonds are shown in ball-and-stick representation. (B) Ribbon diagram of a gB trimer. Color code is as in panel A.

Beyond its role in the attachment, where it interacts with GAGs that decorate heparan sulphate (HS) using a polylysine motif between aa 68 and 76, gB plays two opposite roles in fusion, its ectodomain participates in fusion execution, and its cytoplasmatic tail exerts anti-fusion activity.

In a recent study [60], it has been identified two loops in gB, aa 173 to 179 and 258 to 265, structurally homologous to the fusion loops of VSV G protein. The results of a stucture-based mutagenesis show that three of the five tested residues, W174, Y179, A261, are essential for gB's function in cell-cell fusion. So they proposed that gB has internal fusion loops, functionally homologous to those of VSV G class II fusion proteins, that are an important functional domain and possibly interact with the target cellular membrane.

1.3.5 GLYCOPROTEINS H AND L

The 2517 bp UL22 gene encodes the 838 aa glycoprotein H (gH), a type I membrane glycoprotein containing an 18 aa signal peptide, a long 785 aa ectodomain, a single 21 aa transmembrane hydrophobic domain close to the C-terminus, and a 14 aa C-terminal cytoplasmatic tail.

In its organization gH resembles viral fusion glycoproteins: in general, the fusion peptide is a hydrophobic α-helix able to penetrate the membrane of target cell; it may be located either at the N-terminus or in a fusion loop contained in the ectodomain, depending on whether the fusion glycoprotein undergoes a maturational cleavage, or not. The fusion glycoprotein form a bridge between the virion envelope and the cell membrane, an event that initiates pore formation and fusion of the viral and cellular membranes. The ectodomain of gH contains a possible α-helix at residues 377-397 (α-H1) with the characteristic of an internal fusion peptide. The partial or entire removal of the α-helix, or mutagenesis of critical residues abrogate HSV infectivity and cell fusion activity. Its replacement with well characterized fusion peptide from human immunodeficiency virus (HIV) gp42 or from vescicula stomatitis virus (VSV) glycoprotein (G), but not with their antisense sequences, rescued the infectivity and the fusion activity of the deleted form of gH [34]. gH has an other α-helix at residues 513-531 (α-H2).

Recently in our laboratory two regions downstream of the predicted fusion peptide α-H1, localized between residues 443-471 and 556-585, have been identified as heptad repeats (HR-N and HR-C): they could potentially interact and adopt a coiled coil conformation. The formation of coiled coil bundles is a key conformational change in the transition of a fusion glycoprotein from the fusion-inactive to the fusion-active state, and contributes to bring the viral and the cellular membranes in close juxtaposition, so to initiate the fusion pore formation.

The N-terminal heptad repeat was particurly interesting (HR-N), as it exhibited a high probability score to form a coiled coil. A double amino acid substitution which abrogated the predicted capacity to form the coiled coil also abrogated infectivity and cell-cell fusion activity, indicating that the predicted coiled coil motif is critical in HSV gH [35].

Concerning the ability of HR-N and HR-C peptides to interact with each other, three lines of evidence support this conclusion. First, a complex between HR-N and HR-C synthetic peptides was revealed in nondenaturing PAGE. Second, the mixture of HR-N and HR-C peptides exhibited an α-helical content higher than that of the two single peptides. Third, a mixture of HR-N and HR-C mimetic peptides reversed the inhibition of infection exerted by

the single peptides. These results clearly show that HR-N and HR-C peptides interact with each other. Interestingly, complex formation is an intrinsic property of the HR-N and HR-C peptides, independent of the presence of adjacent gH sequences and of the additional viral glycoproteins required for fusion [56].

In addiction, synthetic peptides mimicking α-H1 and α-H2 interact with nude lipid vesicles in the absence of adjacent gH sequences and in the absence of membrane proteins in the vesicles. They induce fusion of the lipid vesicles, and, more importantly, increase virus infection and cell-cell fusion. Taken together, these effects suggest that the interaction of α-H1 and α-H2 with the lipids possibly leads to lipid destabilization in the membranes and thus increases fusion and virus entry. The lipid destabilization may be critical for inducing the curvature of the membrane at the site of fusion and/or for decreasing the energy barrier. Interaction of gH with lipids may be critical also in guiding the glycoprotein refolding.

The synthetic peptides mimicking α-H1 and α-H2 differ from peptides mimicking HR-C. All induce fusion of the lipid vesicles, denoting a certain ability to interact with lipids. However, the first two enhance infection and fusion, whereas the latter inhibits these activities. It is well known that HR-C mimetic peptides block virus infection by forming a complex with the HR-N located in the glycoprotein and thus hinder the glycoprotein refolding. Therefore, the different behavior of the two groups of mimetic peptides reflects the different molecules they target: i.e., the membrane lipids in the case of α-H1 and α-H2 mimetic peptides and gH itself in the case of the HR-C mimetic peptide.

α-H2 and α-H1 differ in a number of important properties that reinforce the view that α-H1, but not α-H2, exhibits properties typical of fusion peptides. Collectively, the results reported here and elsewhere highlight gH as a structurally complex glycoprotein. It carries sequences able to interact with lipids, namely α-H1, with properties of a candidate fusion peptide, in addition to α-H2 and the pretransmembrane sequence with a tendency to partition at the membrane interface. It also carries two functional HRs. These elements are typical of class I fusion glycoproteins [54].

Of note, gH's function is dependent on forming an heterodimer complex with gL, which acts as gH chaperone for proper processing and gH trafficking to viral envelope.

The 675 bp UL1 gene encode the 224 aa glycoprotein L (gL) which contains a 25 aa signal peptide but not a transmembrane domain. It is not an integral membrane protein and its presence in the virion envelope is ensured by complex formation with gH. In absence of gH, gL is secreted from cells [36]: the first 323 aa of gH and the first 161 aa of gL can form a stable secreted hetero-oligomer. The exact role of gL in fusion remains to be elucidated: HSV

virions lacking gL also lack gH, and although they bind to cell surface, they do not penetrate cellular membranes [37, 38].

Chapter I: introduction

1.4 *Herpes as therapeutic agent*

1.4.1 ONCOLYTIC HSV

A number of features makes HSV-1 an attractive vector for cancer gene therapy: its ability to infect a wide range of host cells, to trasduce nondividing as well as dividing cells and express transgene products with excellent efficiency. Moreover, as much as 30 Kbp of its genome can be deleted and replaced by transgenes in replication defectives HSV-1 mutants, allowing for simultaneous delivery of multiple transgenes and use of heterologous promoters. Furthermore, HSV-1 genome does not integrate into the cellular genome, eliminating the concern of insertional mutagenesis; lastly, recombinant HSV-1 can be readily constructed, and purified.

There are two types of HSV-1 vectors, both have been used in clinical studies for cancer treatment: *Replication defective vector*, in which transgene expression cassettes are inserted in a viral genome with one or a few essential viral gene deleted. This viral gene therapy (fig. 14B) utilizes the virus as a means for bringing the gene of interest into the tumour cells where it is transcribed and translated into proteins. These proteins directly or indirectly interfere with tumour cell replication and survival. The second types of vectors are *Conditionally replicating vectors*, in which deletion of some nonessential viral genes results in a virus that prefentially infects, replicates in and lyses tumour cells. This oncolytic viral therapy (fig. 14A) is based on many strategies which could be grouped into three general approaches. The first approach is to delete gene functions that are critical for efficient viral replication in normal cells but are dispensable in tumour cells. Because HSV-1

carrying deletion of a single gene often retains residual replication, and toxicity in normal cells, additional deletions or alterations have to be made to further increase the safety profile of the vectors as well as to decrease the likelihood of reversion to wild type. The second approach is to limit the expression of a critical viral gene to tumour tissues through the use of tumour and /or tissue-specific promoters. The last approach is to alter viral tropism through modification of surface proteins (vector re-targeting) [41].

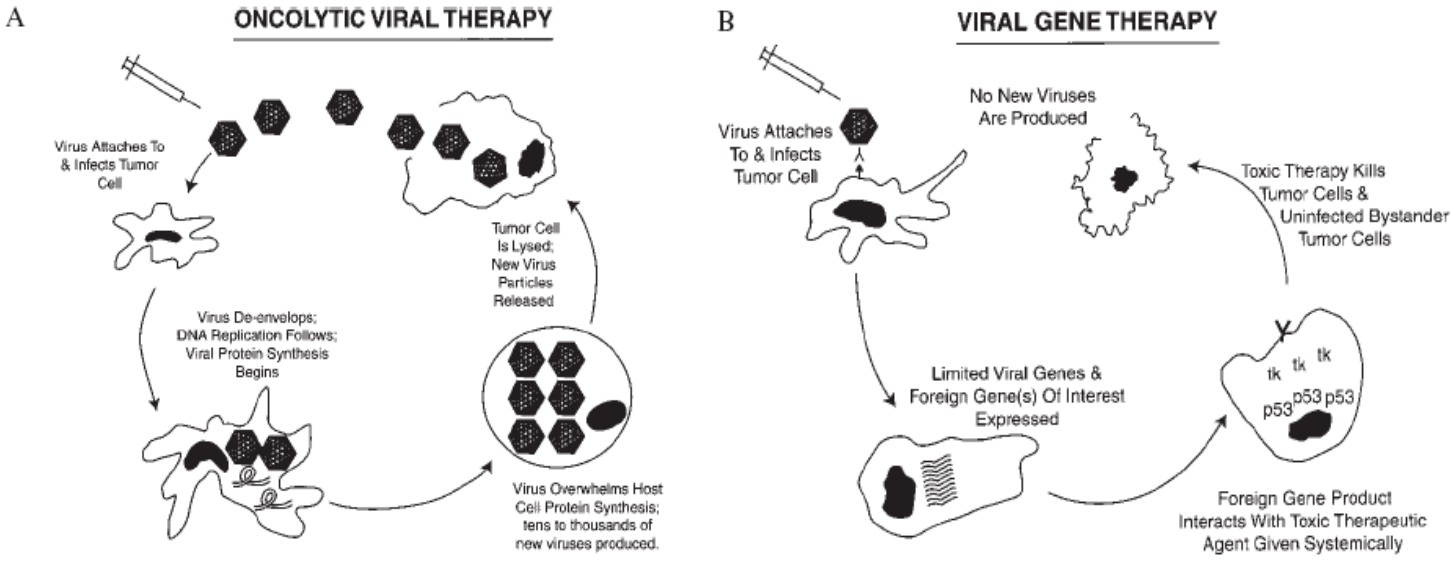


FIG 14: The two predominant therapeutic approaches to the treatment of malignant glioma: oncolytic viral therapy (A) and viral gene therapy (B).

Because infection and lysis of 100% of tumour cells is hard to achieve in vivo using oncolytic viruses alone, they are often "armed" with genes that can augment their cytolytic capacities. However, combination of oncolytic HSV strains with chemotherapy or radiotherapy generally displays enhancement in antitumour activity [41]. For example there were preclinical data which demonstrate that a single fraction of radiation, when administred approximately 24 h after G207 (a double HSV-1 mutant which produce antiglioma effects in multiple preclinical models (tab. 2), increases the replication and spread of viral infection within brain tumour as well as many other neoplasm [57].

Use of HSV as an oncolytic virus has been explored primarily for diseases of central and peripheral nervous system, due to its ability to both infect a variety of neurons and persist in latent state. Moreover the unequivocal lack of therapeutic success and associated grim prognosis has encouraged exploration of a variety of novel molecular therapies for the treatment of brain tumours, like malignant glioma. Genetically engineered HSVs studied for glioma therapy have included mutations in one or more of the viral genes thymidine kinase, DNA polymerase, uracil DNA glycosilase, ribonucleotide reductase, and $\gamma_1$34.5. These mutations all act to decrease the toxicity of HSV infection on the normal central nervous system. They are all enzymes necessary for successful nucleotide synthesis and replication. As a rule, non dividing cells, such as the postmitotic neurons of the adult CNS, do not support

the replication of viruses containing these mutations. Replicating cells, however, can supply cellular homologues to this enzyme in trans and allow viral replication to take place. Thus, these viruses can replicate only in dividing tumour cells [42].

To increase the antiglioma effects of genetically engineered HSV, a foreign gene (e.g. cytochine or interleukin genes) can be engineered into HSV in order to enhance the killing of the tumour cells. [43].

While the concept of genetically engineered oncolytic HSV-1 was originally developed for the treatment of malignant glioma and other CNS tumours, it was soon explored in preclinical models of other difficult to treat tumour, that developed outside the CNS, including neurofibrosarcomas, melanoma, and non-small cell lung carcinoma, as well as cancer of the breast, liver, pancreas, ovary, head and neck, prostate. For example, to explore the possible use of HSV in the treatment of metastatic melanoma, five patients with stage 4 melanoma underwent inoculation of HSV1716 (tab. 2) into subcutaneous melanoma nodules. Flattering of injected nodules was seen in patient receving a single injection, while tumour necrosis was seen in patient receiving multiple injections; no necrosis was seen in control nodules treated with saline. [57]

Another oncolytic HSV, NV1020 (tab. 2) has been studied in patients with colorectal metastases to the liver and a phase I/II trial is under way which, aimed at examining safety and tolerability of NV1020, as well as possible synergies with chemotherapy. [57]

Although extensive studies using HSV-1 vectors for cancer treatment have demonstrated remarkable safety and encouraging antitumour efficacy data, there are also several limitations. For example, multigene deleted HSV-1 vectors are in general more difficult to produce and less stable in aqueous solution or in lyophilized form than wild type virus. Another problem is caused by the induction of antiviral or antitransgene product immune response which may reduce the effectiveness of HSV-1-derived vectors, especially when multiple injections of the vectors are necessary. Moreover, the impact of pre-existing immunity on HSV-1 cancer therapy still needs to be carefully evaluated by further studies.

Extensive studies on the safety of HSV-1 vector are also necessary, in fact efficient replication of HSV-1 in a wide spectrum of human cells raises the possibility of serious damage to untargeted normal tissues. Another significant risk lies with the possibility of recombination: homologous recombination occurs when a replication defective or conditionally replicating HSV-1 vector infects a cell which already harbours a wild type latent virus. Homologous recombination between the incoming vector and the resident wild type

virus might occur and could result in the generation of a fully virulent virus that carries e biological active gene.

HSV-gene therapy heralds a new era of HSV biology and the conversion of hazardous foe into a user-friendly surgical tool.

VIRUS	Tumour-selective virus-derivative	Engineered mutation(s) to target tumours	Clinical trials	Tumour target
Herpes simplex virus-1	G207	1.Deletion of both $\gamma_1$34.5 copies. 2.LaZ insertion in UL39 (ICP6 inactivation	Phase I, IB completed Phase I XRT trial approved	Glioma
	HSV1716	1.Deletion of both $g_1$34.5 copies.	3 Phase I studies, pilot completed	Glioma, melanoma
	NV1020	1.Deletion, 15kbp region (UL56 gene to internal ILR) 2.Insertion of HSV-2gG, gD, gI, partial gE 3.Insertion of HSV-1 tk under alpha-4 4.700 kbp deletion of UL23-UL24	PhaseI completed PhaseI/II underway	Colorectal metastases

TAB 2: HSV for oncolytic viral treatment of tumours.

1.4.2 RE-TARGETING OF HSV-1

One way to use genetically engineered HSV-1 as oncolytic agents to treat malignant glioma is to modify the surface of the virus particles so as to target the virus to specific receptor present solely or predominantly on the surface of the tumour cell.

G. Zhou et al [52] engineered a recombinant HSV-1 (R5111) re-targeted to IL13Rα2 receptor present in malignant glioma cells. Unlike the more prevalent IL13αR1 receptor, IL13Rα2 receptor has a shorter cytoplasmic domain, it is monomeric, does not interact with IL-4, of which IL-13 is a close relative, and in nature it is present in high grade malignant gliomas or astrocytomas but also in human testes. First of all they mutagenize gB and gC to preclude their interaction with heparan sulfate and then they inserted IL-13 into gD at amino acid 24 to distrupt the binding site of HVEM. So they reported that IL-13 gD chimeric virus can use IL13Rα2 for entry into cells carrying only that receptor and its entry does not depend on endocytosis as its primary mechanism of entry into cells. Moreover, before using this recombinant virus in cancer therapy, the ability of gD to interact with nectin has been ablated without affecting the fusogenic and antiapoptotic function of gD with the mutation V34S. Furthermore this retargeted HSV could be used for both oncolytic activity and visualization of tumour cells.

The same group [53] targeted HSV-1 to malignant glioma cells by inserting into gD, between amino acid 24 and 25, an uPA peptide of 135 residues for the human uPAR reported to be highly expressed in malignant gliomas. uPAR is a 335 residue protein attached to cell surface via a glycosylphosphatidylinositol anchor. It lacks transmembrane and cytosolic domains. uPAR binds and localizes the urokinase plasmilogen activation (uPA) at the cell surface. Increased uPA activity has been reported in malignant astrocytomas in vitro and in malignant brain tumour in vivo. In malignant brain tumours uPA activity was correlated with poor prognosis. Increased cell surface UPA activity facilitates cellular movement via extracellular matrix (ECM) degradation, which is necessary for tumour cell invasion, chemotaxis, and cellular adhesion.

In a next work [55], in order to enhance the yield of a virus designed to target uPAR, they obtained an infectious virus with two frameshifts, one after codon 60 and the second after codon 201 of gD. The frameshifts replaced codons 61–201 with a sequence that contained four stop codons. The salient feature of the recombinant virus that emerged from these studies is that gD consisting of polypeptide A containing the N-terminal domain of uPA fused to residues 33–60 of gD and polypeptide B containing the C-terminal domain of gD was infectious. Attempts to construct recombinant viruses in which the A polypeptides consisted

solely of gD residues 1–60 or of the 132 residues encoding IL-13 fused to gD residues 33–60 yielded viruses that were not infectious. The significance of these findings stems from three conclusions:

(*i*) Residues 61–218, which cincide almost entirely with the Ig-like core of gD (located between residues 56 and 184) do not execute a function required for HSV-1 entry into cells, but they appear to serve as linkers between the N-terminal domain and the C-terminal profusion domains of gD.

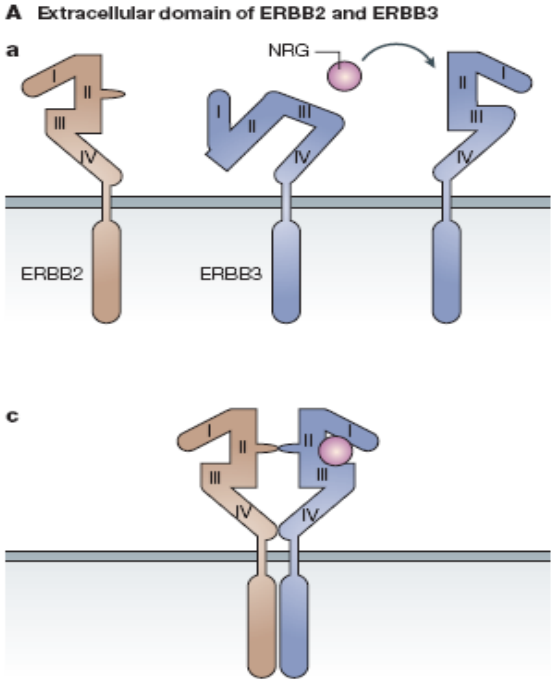
(*ii*) The fundamental difference between the constructs that yielded an infectious virus and those that failed is that polypeptide A consisting of $uPA_{33–60}$ interacted with the C-terminal domain of gD, whereas those containing $gD_{1–60}$ or IL-13– $gD_{33–60}$ did not. Although it may be tempting to suggest that the N-terminal domain of uPA conferred a conformation to $gD_{33–60}$ that enabled it to interact with the C-terminal domain of gD, the evidence supports the conclusion that uPA itself can interact with $gD_{219–314}$. The results support the conclusion that physical interaction of the domain capable of binding a cell surface receptor with the C-terminal domain of gD may lead to successful virus entry into cells, whereas lack of physical interaction results in failure.

(*iii*) It is unlikely that each of the three ligands (HVEM, uPA, and IL-13) selected by chance alters the conformation of the remaining portions of gD in an identical manner to enable virus entry.

The fact that the uPA ligand might be replaced by any of a number of alternative ligands in trans, provided that they can associate with the profusogenic domain to extend the host range of recombinant viruses in useful ways.

1.4.3 HER2 RECEPTOR

The human epidermal growth factor receptor 2 (HER2 also known as erbB2 or neu) encodes a 185 KDa transmembrane tyrosine kinase growth factor receptor with extensive homology to other members of epidermal growth factor receptor family (EGFR or HER). All members of EGFR family (EGFR/erbB1/HER1, erbB2/HER2, erbB3/HER3 and erbB4/HER4) have a cytoplasmatic tyrosine-kinase domain, a single membrane spanning region and an extracellular ligand binding region, which consists of four domains: domains I and III are important in peptide binding, domain II contains dimerization arm involved in receptor-receptor interaction; in the inactive state the domain II dimerization interface is blocked by intramolecular interactions between domain II and IV. The structure of HER2's extracellular region is radically different from the others. HER2 has a fixed conformation that resembles the ligand activated state: the domain II-IV interaction is absent and the dimerization loop in domain II is exposed. This structure is consistent with the data that indicate that HER2 is the preferred partner for the other activated HERs, as it is permanently poises for interaction with another ligand bound receptor. Furthermore, this structure explains why no soluble EGFR-related ligand has been found. It predicts that HER2 possesses a unique subdomain I-III interaction that makes ligand binding impossible because the site is buried and not accessible for interaction.



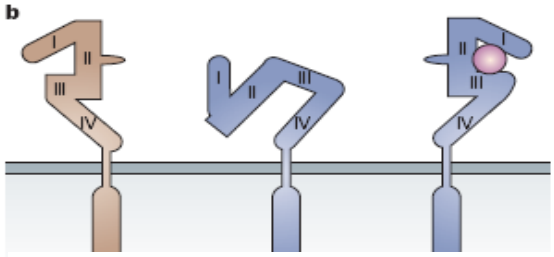


FIG 15: HER receptor ectodomain structure.

The extracellular region of each HER receptor consists of four domains (I-IV). Domain I and III are involved in neuregulin (NRG) binding and following this, the dimerization arm in domain II is exposed and promotes receptor-receptor interaction. HER2 has a fixed conformation that resembles the ligand activated state of the other HER receptors [44].

Homo- or etero-dimerization results in receptor activation through many intracellular signalling pathways and, despite extensive overlap in molecules that are recruited to the different active receptors, different HERs prefentially modulate certain signalling pathways, owing to the ability of individual HERs to bind specific effector proteins. This process of signal transduction culminates in the nucleus, where gene control and protein transcription are modified, producing effects on key cellular regulatory processes, such as differentiation, adhesion, growth, migration and apoptosis.

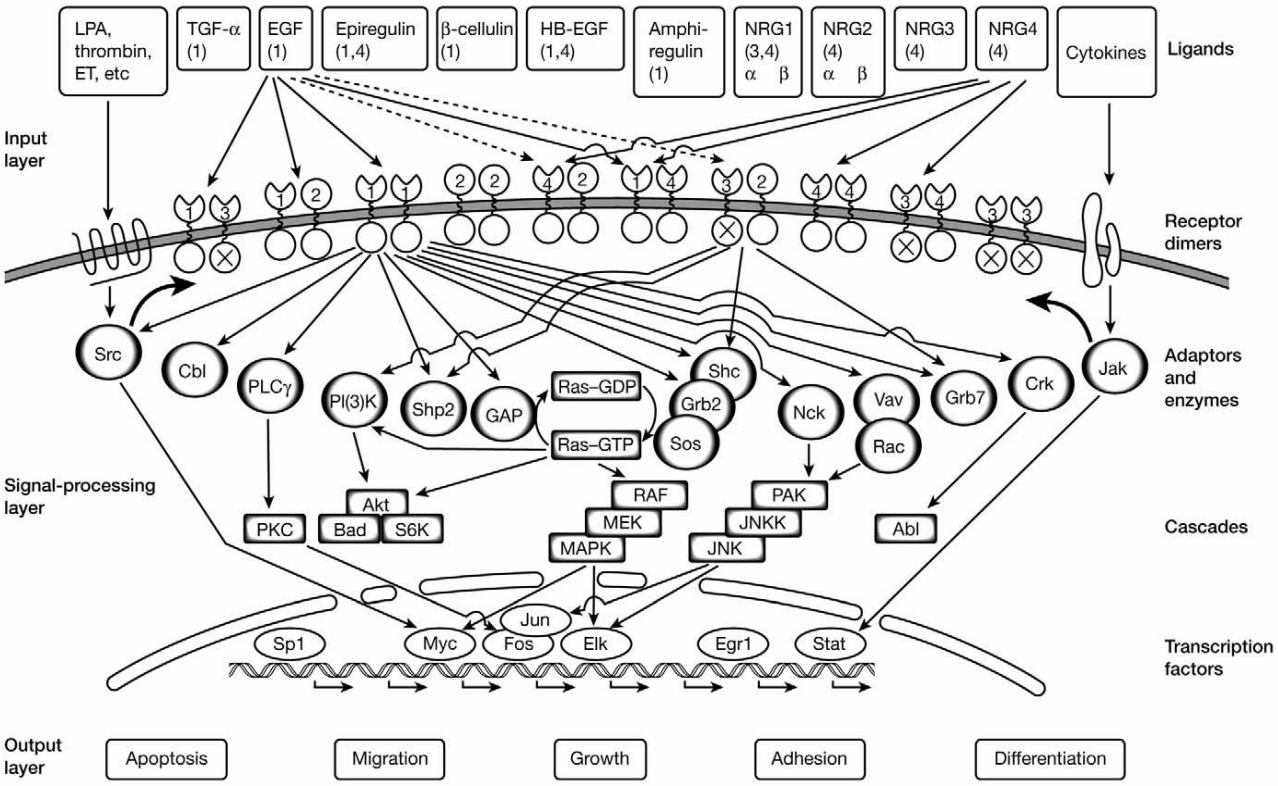


FIG 16: The HER signalling network. [45]

The HER receptors are expressed in various tissues of epithelial, mesenchymal and neuronal origin and under normal physiological conditions, their activation is controlled by the spatial and temporal expression of their ligands.

The most widely studied and the best understood HER receptors are EGFR and HER2. Both display abnormal or enhanced expression in many types of cancer, suggesting their involvement in tumourigenesis.

In human, HER2 expression is barely detectable in a variety of epithelial cells; by contrast, the HER2 protein is overexpressed (as much as 100-fold) in approximately 30% of human carcinomas, especially in breast and ovarian tumours, as a consequence of gene amplification and/or transcriptional alterations. In mammary tumours, HER2 expression correlates with particular invasiveness, metastatic ability, overall aggressiveness of the tumour, and a poor prognosis.

Because of these properties, HER2 is a target for therapy. Therefore, a range of therapeutic approaches has evolved: inhibition of receptor activity by the humanized anti-HER2 antibody called Herceptin or Trastuzumab. They applied in conjunction with standard chemotherapy, has led to an increase in the response rate time to disease progression, and overall survival of patients with HER2-positive metastatic breast cancer. Despite these proven benefits, however, treatment with herceptin fails to eradicate the tumour or its metastases, and a more effective treatment is needed.

Another strategy is based on interfering with receptor expression by gene therapy: the adenovirus type 5 E1A gene known to downregulate HER2 expression can inhibit human ovarian and breast cancer cells in vitro and suppress the production of HER2 protein in mice bearing human tumour xenografts, greatly improving survival. Studies using this approach are continuing [45].

In the past few years, a new strategy has been developed: the generation of viruses retargeted to the HER2 receptor. In one instance, retroviral vector particles derived from spleen necrosis virus were pseudotyped with the antigen binding site of antibody to HER2 [46].

The second instance concerns a vesicular stomatitis virus recombinant exhibiting a Sindbis virus glycoprotein modified with the scFv to HER2 [47].

The objectives of my thesis were twofold. They were described separately in chapter II and chapter III, respectively.

Chapter II: ENGINEERING OF RECOMBINANT HSV-1 RETARGETED TO HER2 RECEPTOR

2.1 Objective I

2.1 OBJECTIVE I

One objective was to genetically engineer an oncolytic HSV-1 capable of infecting cells through the human epidermal growth factor receptor 2 (HER2), which, as described above, is overexpressed in human breast and ovarian cancer. Because HER2 has no specific natural ligand, in order to generate a recombinant HSV specifically redirected to HER2, we engineered into gD a single chain antibody (scFv) derived from monoclonal antibody (MAb) 4D5 against HER2.

Furthermore, we had to be secure that this recombinant virus could not infect through its natural receptors (HVEM and Nectin1): the HVEM binding site has been abrogated by the insertion of the scFv into gD at amino acid 24 as already seen in the virus R5111 engineerizated by G. Zhou [52]. The nectin1 binding site was modified either through mutations or deletions in gD as I will describe into the next sections.

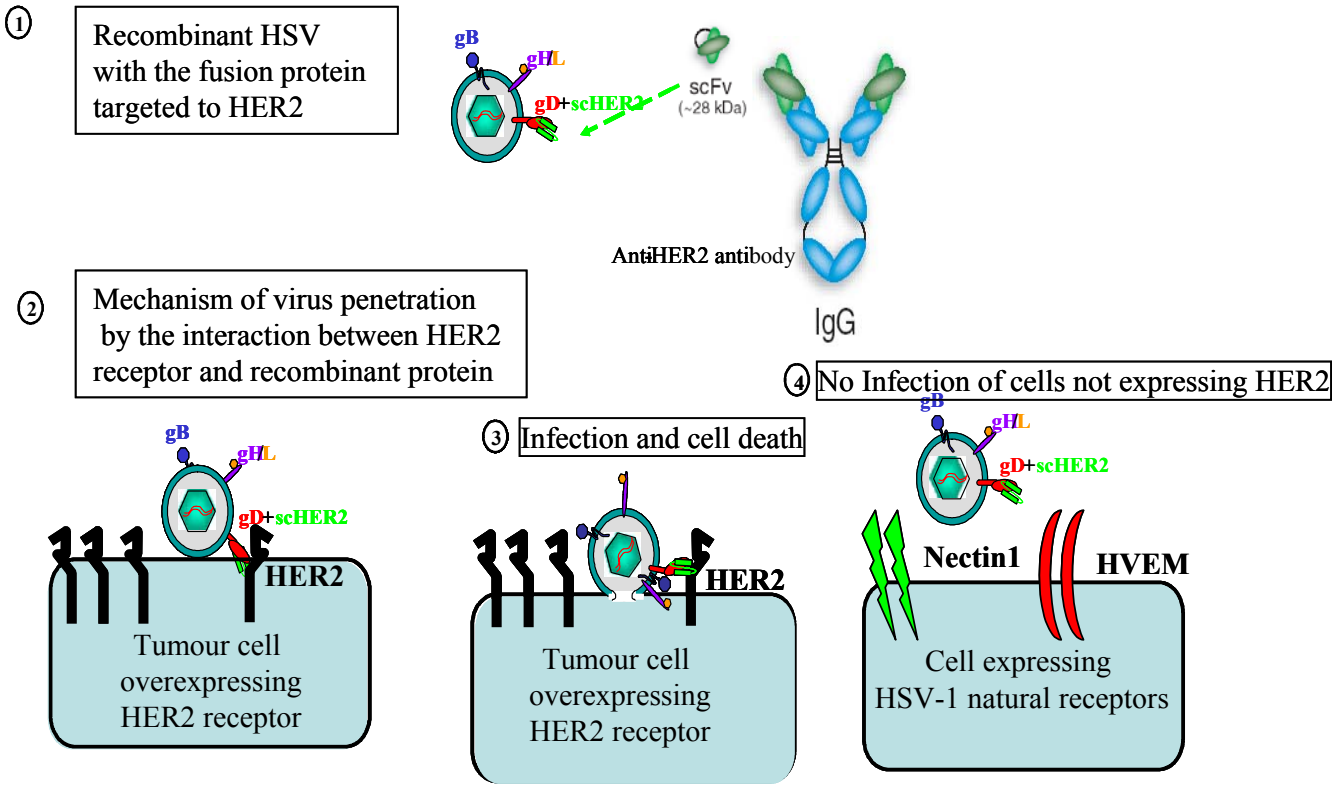


FIG 17: This figure describes the aim of work: the construction of a recombinant virus that infects and causes death of tumour cells overexpressing HER2 and is not able to infect via HSV-1 natural receptors, HVEM and nectin1.

Chapter II: ENGINEERING OF RECOMBINANT HSV-1 RETARGETED TO HER2 RECEPTOR

2.2 Materials and Methods

2.2.1 CELLS AND VIRUSES

Rabbit skin (**RS**), **Vero** green monkey kidney cells (obtained from John McLaren and American Type Culture Collection, respectively) and African green monkey kidney fibroblast-like cell line (**COS**) were maintained in Dulbecco's modified Eagle medium supplemented with 5% newborn calf serum (NCS).

Cells were grown in Dulbecco's modified Eagle medium supplemented with 5% Fetal Bovin Serum (FBS), except when is otherwise specified.

RGDP6 or **R6** cell line, a derivative of rabbit skin, expresses glycoprotein D under the control of HSV late promoter $\gamma U_L 26.5$.

J cell line is a derivative of **BHK-tk$^-$** cells (Baby Hamster Kidney) that lacks gD recetors.

J-HVEM, **J-hNectin1** and **J-mNectin1** are derivatives of J cells that express HVEM, human Nectin1 and mouse Nectin1 respectively.

The receptor negative Chinese hamster ovary (**CHO**) cells were cultured in F-12 nutrient mixture medium supplemented with 5% fetal calf serum.

Human cell lines utilized for infection assays are:

293T: a highly transfectable derivative of human epithelial cell line 293.

SKOV-3: a derivative of ovarian adenocarcinoma maintained in RPM plus glutaMAX^{TM} medium (Gibco)

MCF7: a derivative of adenocarcinoma of mammary gland.

RH4: a derivative of alveolar rabdosarcoma

I143tk-: fibroblast

Hep-2: epithelial cell line

Mouse cell lines utilized for infection assays are:

NIH/3T3:fibroblasts

TT12E2: fibroblasts expressing murin HER2

L-mouse: fibroblasts

Wild type HSV-1(F) and HSV-1(KOS)tk12, which express β-galattosidase from an insert in the viral thymidine kinase gene were grown in rabbit skin or baby hamster kidney BHK cells and routinely titrated by plaque assay in Vero cells

The gD$^-$ virus HSV-1(KOS)tk12/FRT-GFP, carrying green fluorescence protein (GFP) cDNA in place of gD gene, was grown in R6 cells, the complementing rabbit skin cell line expressing inducible gD, described above.

2.2.2 CONSTRUCTION OF J-HER2 AND CHO-HER2 CELL LINES

J and CHO cells were tranfected with the pcDNA-HER2 plasmid, selected with neomycin G418 at a concentration of 400 to 800 μg/ml for 5 days. Individual clones were obtained by limiting dilution and were checked for HER2 expression by indirect immunofluorescence (IFA).

2.2.3 ANTIBODIES

MAb9G6 (Santa Cruz) is a monoclonal antibody directed to the ectodomain of HER2.

4D5 (Santa Cruz) is monoclonal antibody directed to HER2.

Herceptin (Genetech) is the humanized monoclonal antibody derived from 4D5.

MAbH170 is a monoclonal antibody directed to N-terminus of gD (Goodwin Institute, Plantation, FL); Although in some recombinants this region was deleted, we were able to use MabH170 because the epitope was present into the linker between the heavy and light chains of scHER2. HD1 and MAb30 are monoclonal antibodies directed to conformational epitopes of gD (Goodwin Institute, Plantation, FL).

H1380.1 is a monoclonal antibody directed to gD (Goodwin Institute, Plantation, FL)

BD80 is directed to a membrane proximal domain of gD

H1380.1 and BD80 are antibodies type specific for HSV-1, while H170 is a type common antibody because it recognizes gD of HSV-1 and HSV-2.

Anti-mouse IgG-FITC (fluorescin-isothiocyanate) conjugated antibodies were from Jackson Immunoresearch.

Peroxidase-conjugated anti mouse IgG horseradish peroxidase linked whole antibody (from sheep) were from GE Healthcare

2.2.4 INDIRECT IMMUNOFLUORESCENCE

J-HER2 and CHO-HER2 were grown on glass coverslips for 24 h and then fixed with paraformaldehyde for 10 min at room temperature and incubated for 1 h with MAb 9G6, diluted 1:50 in 20% new born calf serum in phosphate-buffered saline (PBS), and for 45 minutes with fluorescein isothiocyanate (FITC)-conjugated anti mouse IgG (Jacksonm Immunoresearch).

Moreover all the gD constructs were sequenced for accuracy and checked for expression of chimeric gD by indirect immunofluorescence: BHK and RS cells transfected with plasmid carrying gD under the control of the immediate-early CMV promoter were fixed with -20°C cold methanol for 10 min at 30 h after transfection, and reacted with monoclonal antibodies to

gD: MAbs H170 and HD1 or MAb30. Furthermore, cells transfected with plasmids carrying gD under the control of the natural promoter were superinfected with HSV-2(G) (3 PFU/cell) 6 h after transfection in order to induce gD gene expression, fixed with -20°C cold methanol for 10 min at 16 h after infection, and reacted with the type 1-specific MAb30 and H1380.1, diluted 1:400, 1:300 respectively. In all cases, primary antibodies to gD were followed by FITC-conjugated anti-mouse IgG (Jackson Immunoresearch).

2.2.5 PLASMIDS AND CONSTRUCTS

For all gD constructs, the starting plasmid was pEA99, which contains the wt gD coding sequence in pcDNA3.1(-) (Invitrogen).

To allow cloning of the scHER2 sequence between aa 24 and 25 of gD, two unique EcoRI and BamHI restriction sites were inserted at nucleotides 137 to 142 and nucleotides 162 to 167 of the gD coding sequence by site-directed mutagenesis with primer gD_21EcoRI_30BamHI (GCT TTC GCG GCA AAG GAA TTC CGG TCC TGG ACC AGC TGA CGG ATC CTC CGG GGG TCC). The EcoRI site insertion introduces the two D21G and L22I substitutions in mature gD. The BamHI site insertion is silent.

pS2019a served as a template to PCR amplify the sequence for scHER2 (previously derived from MAb 4D5). Briefly, scHER2 was amplified with primers scFv_EcoRI_f (GCA AAG GAA TTC CGG TCT CCG ATA TCC AGA TGA CCC AGT CCC CG) and scFv_BamHI_r (CGG AGG ATC CGT CAG CTG GTC CAG GGA GAC GGT GAC TAG TGT TCC TTG ACC); similarly, scHER2L (where scHER2 is followed by a 9-residue serine-glycine linker) was amplified with primers scFv_EcoRI_f (GCA AAG GAA TTC CGG TCT CCG ATA TCC AGA TGA CCC AGT CCC CG) and scFv_SGlink_BamHI_r (CGG AGG ATC CGT CAG CTG GTC CAG ACC GGA ACC AGA GCC ACC GCC ACT CGA GG).

For the construction of both gD+scHER2 and gD+scHER2linker, the primer sequences for gD amplification, included the sequences encoding aa 21 to 24 and aa 25 to 30 of mature gD, such that in the final constructs gD lacked no amino acid residue and only contained the insert at aa residue 24.

For the generation of recombinant viruses by homologous recombination, gD+scHER2 and gD+scHER2linker chimeras were subcloned into recombination plasmids named **pLM11** and **pLM11L**, which contained about 500 bp of the natural upstream and downstream gD-flanking sequences. Moreover the gD+scHER2 and gD+scHER2linker chimeras were

subcloned into vectors for constitutive expression under the control of the immediate-early cytomegalovirus (CMV) promoter, generating plasmids pLM10 and pLM10L, respectively.
Primers used to screen by PCR recombinant viruses derived from plasmid pLM11 and pLM11L are: gD_96_f (GCG GCA AAT ATG CCT TGG CGG ATG CC) and gD_200_r (GGG GCT GGA ACG GGT CCG GTA GGC CCG), flanking the site of scHER2 insertion in gD, and primers scFv_EcoRI_f and scFv_BamHI_r or scFv_SGlink_BamHI_r, also used to amplify scHER2.
When we decided to use BAC Mutagenesis-Procedure to engineer new recombinant viruses, we used a BAC previously built in our laboratory which derived from pYbac102 [65] and between UL3 and UL4 pBeloBAC11 sequences were inserted. In this BAC, named *"pYEbac102gD-flipped"*,gD sequence was deleted.
First of all we constructed two shuttle vectors, one containing LacZ gene and one containing EGFP (enhanced green fluorescent protein) gene downstream the HSV α-27 promoter. The α27-EGFP cassette or the α27- LacZ cassette was inserted between two 700 bp sequences PCR amplified from the plasmid pBeloBac11, designated as pBeloBac11-up (primers Sal_pBelo_1209_f: TTG CCA GTC GAC ATT CCG GAT GAG CAT TCA TCA GGC GGG CA and pBelo_1897_Xho_r: GCA AAA ACT CGA GTG TAG ACT TCC GTT GAA CTG ATG GAC) and pBeloBac11-down (primers Mun_pBelo_1898_f: GGA AGT CAA TTG GAA GGT TTT TGC GCT GGA TGT GGC TGC CC and pBelo_2586_Eco_r: CAC ACT GAA TTC GCA ATT TGT CAC AAC ACC TTC TCT AGA AC). In the resulting constructs, designated the α27-EGFP cassette or the α27-LacZ cassette resulted inserted between nt 1897 and 1898 (original coordinates) of pBeloBac11. The cassettes plus the pBeloBac11 flanking sequences were subcloned in the shuttle vector pST76KSR [85] to generate **pSBG** and **pSBZ** containing α27-EGFP and α27LacZ respectively, and to proceed with BAC mutagenesis: Bacs obtained (gD-BZ and gD-BG) were used as recipient for the generation of recombinant Bac containing engineered gD.

In **pS31** shuttle vector, gD-scHER2L was inserted between aa 24 an 25 of gD which contains mutation V34S: first of all we introduced the mutation into gD sequence of pLM11L using primer gD34S_StuI (TCC TCC GGG GAG CCG GCG CGT GTA CCA CAT CCA GGC AGG CCT ACC GG) and we obtain pLM31L. Then we transferred recombinant gD mutated at aa 34 into shuttle plasmid pST76KSR to obtain pS31 for homologous recombination in E.coli containing Bac gD-BZ.

In **pS39** shuttle vector, gD-scHER2L was inserted between aa 24 an 25 of gD which contains mutation V34S, D215G, R22N and F223I: first of all we introduced mutations into gD sequence of pLM31L and obtain pLM39L. To this end we used the following primers: gD_rev_215G-222N-223I_PvuI (GGT TCT CGG GGA TGA TGT TGG GCA GCA TCC CGA TCG AGC CCA CCG TCA CCC CCT) and gD _215G-222N-223I_PvuI (AGG GGG TGA CGG TGG GCT CGA TCG GGA TGC TGC CCA ACA TCA TCC CCG AGA ACC). Then recombinant gD with mutations was subcloned to shuttle plasmid pST76KSR to obtain pS39 for homologous recombination in E.coli containing Bac gD-BG.

In the **pS113** shuttle vector, scHER2 was inserted between aa 6 and 38 of gD. Mutagenesis and cloning was performed on pLM5, a plasmid containing gD ORF cloned in pcDNA3.1(-), flanked by two 500-bp upstream and downstream genomic flanking sequences. Firstly we introduce EcoRI and BamHI restriction sites at the nucleotide 832 and 923 of gD respectively using the following couples of primers: gD_6/8_EcoRI_f (CAA ATA TGC CTT GGC GGA GAA TTC TCT CAA GAT GGC CG) and gD_6/8_EcoRI_r (CGG CCA TCT TGA GAG AAT TCT CCG CCA AGG CAT ATT TG) and gD_37/38_BamHI_f (CGG GGG TCC GGC GCG GAT CCC ACA TCC AGG CGG G) and gD_37/38_BamHI_r (CCC GCC TGG ATG TGG GAT CCG CGC CGG ACC CCC G). The restrictions sites EcoRI and BamHI are placed at 6/8 and 37/38 aa respectively and their insertions caused the following substitutions: D6E, A7N, R37G and Y38S. Then the scHER2 was amplified from pS2019a [86] with primers scFv_EcoRI_f (GCA AAG GAA TTC CGA TAT CCA GAT GAC CCA GTC CCC G) and scFv_SGlink_BamHI_r (CGG AGG ATC CAC CGG AAC CAG AGC CAC CGC CAC TCG AGG) and inserted into EcoRI BamHI digested plasmid. Finally the cassette containing the engineered gDΔ6-38+scHER2 plus gD genomic upstream and downstream flanking sequences was subcloned to pST76KSR shuttle vector to obtain pS113 for homologous recombination in E.coli containing BacgD-BG.

In the **pS249** shuttle vector, gD is engineered with scHER2 flanked by serine-glycine linkers (upstream 8 aa: HSSGGGSG; downstream 12 aa: SSGGGSGSGGSG) in place of gD aa residues 61 to 218. Mutagenesis and cloning was performed on pLM5, as for pS113. First, two NdeI sites were inserted at the coding sequence for aa residues 61-62 and 218-219 of mature gD with mutagenic primers gD_61/62_NdeI_f (ACG GTT TAC TAC GCC CAT ATG GAG CGC GCC TGC C) and gD_218/219_NdeI_f (GAC GGT GGA CAG CAT CCA

TAT GCT GCC CCG CTT C). Next, a 9 aa serine-glycine linker was inserted by annealing and ligating into the NdeI site the two phosphorylated oligos P-SG9Bam7/Nde_f (TAG TAG TGG CGG TGG CTC TGG ATC CGG) and P-SG9Bam7/Nde_r (TAC CGG ATC CAG AGC CAC CGC CAC TAC), containing a silent BamHI site. The scHER2 was amplified from pS2019a [86] with primers scFv_Bam_f (GGC TTA TGG ATC CGA TAT CCA GAT GAC CCA GTC CCC) and scFv_SG_x37_BamH_r (CGG AGG ATC CAC CGG AAC CAG AGC CAC CGC CAC TCG AGG) and inserted into the BamHI site of the serine-glycine linker. Finally the cassette containing the engineered gDΔ61-218+scHER2 plus gD genomic upstream and downstream flanking sequences was subcloned to pST76KSR shuttle vector to obtain pS249 for homologous recombination in E.coli containing BacgD-BG.

2.2.6 GENERATION OF RECOMBINANT VIRUSES.

We used two different methods to generate recombinant viruses: homologous recombination in mammalian cells and homologous recombination in bacterial cells with the aid of BAC-HSV system.

1. Recombination in mammalian cells: BHK cells were transfected by means of Fugene 6 (Roche, Milan, Italy) with pLM11 or pLM11L and were superinfected 6 h later with the gD⁻ virus HSV-1(KOS)tk12/FRT GFP at 3 PFU/cell. At 24 h after infection, the cell lysate was plated in RS cells. Plaques were harvested and screened by PCR. The recombinant viruses **R-LM11** and **R-LM11L** are derived from pLM11 and pLM11L, respectively.

R-LM5, a recombinant carrying wt gD in a EGFP reporter-carrying HSV-BAC, was generated by homologous recombination. RS cells were cotransfected with purified DNA of BAC gD⁻BG.53 and pLM5, a plasmid carrying the coding sequences for wt gD flanked by 500 bp of genomic upstream and downstream flanking sequences. **R-LM13** was generated in similar fashion, using plasmid pLM13, carrying gD and scHER2 and a 9 aa serine-glycine linker between aa 24 and 25 of mature gD.

2. Recombination in bacterial cells (BAC Mutagenesis-Procedure or two-step replacement in E.coli DH10B) was performed essentilly as described with slight modifications [87,88,89]. Briefly, electrocompetent DH10B E.coli harbouring the HSV-BAC were electroporated with the shuttle vector in 0.2 cm electroporation cuvettes (Bio-Rad) at 200 Ω, 25 μF, 2.5 kV, plated on LB agar containing 25 μg/ml kanamycin (the shuttle vector's marker) and 20 μg/ml chloramphenicol (the BAC's

marker), and incubated at 30°C o/n to allow the expression of RecA from the shuttle vector. The clones were re-plated onto LB+kanamycin+chloramphenicol at 43 °C to allow the identification of those harbouring the cointegrates (visible as large colonies, as compared to the temperature sensitive small colony phenotype determined by non-integrated shuttle vectors). Subsequently, the cointegrates were allowed to resolve by plating the clones onto LB+ chloramphenicol at 30°C, and clones containing the resolved HSV-BAC were selected on LB+chloramphenicol plates supplemented with 10% sucrose. Finally, the clones were checked for loss of kanamycin resistance, and for the presence of the desired insert by colony PCR.

Recombination between the HSV-BAC 102gD^{-}flip and the shuttle vectors pSBZ or pSBG generated the gD^{-}BZ and gD^{-}BG recombinants respectively, which contains the α27promoter-LacZ cassette or α27promoter-EGFP cassette inserted into the BAC sequences. The viruses were reconstituted by transfection in R6 gD^{-} complementing cells [90].

The gD^{-}BZ BAC and gD^{-}BG BAC were used as recipients for the generation of recombinants containing engineered gD: recombination between gD^{-}BZ BAC and pS31 generated BAC LM31, instead BAC LM39, BAC LM113 and BAC LM249 were generated by recombination between gD-BG BAC and pS39, pS113 and pS249 respectively. The recombinant genomes were checked by PCR and sequencing, and the viruses R-LM31, R-LM39, R-LM113 and R-LM249 were reconstituted by transfection into R6 cells, followed by one passage into BHK cells, and amplified in HER2 expressing cells.

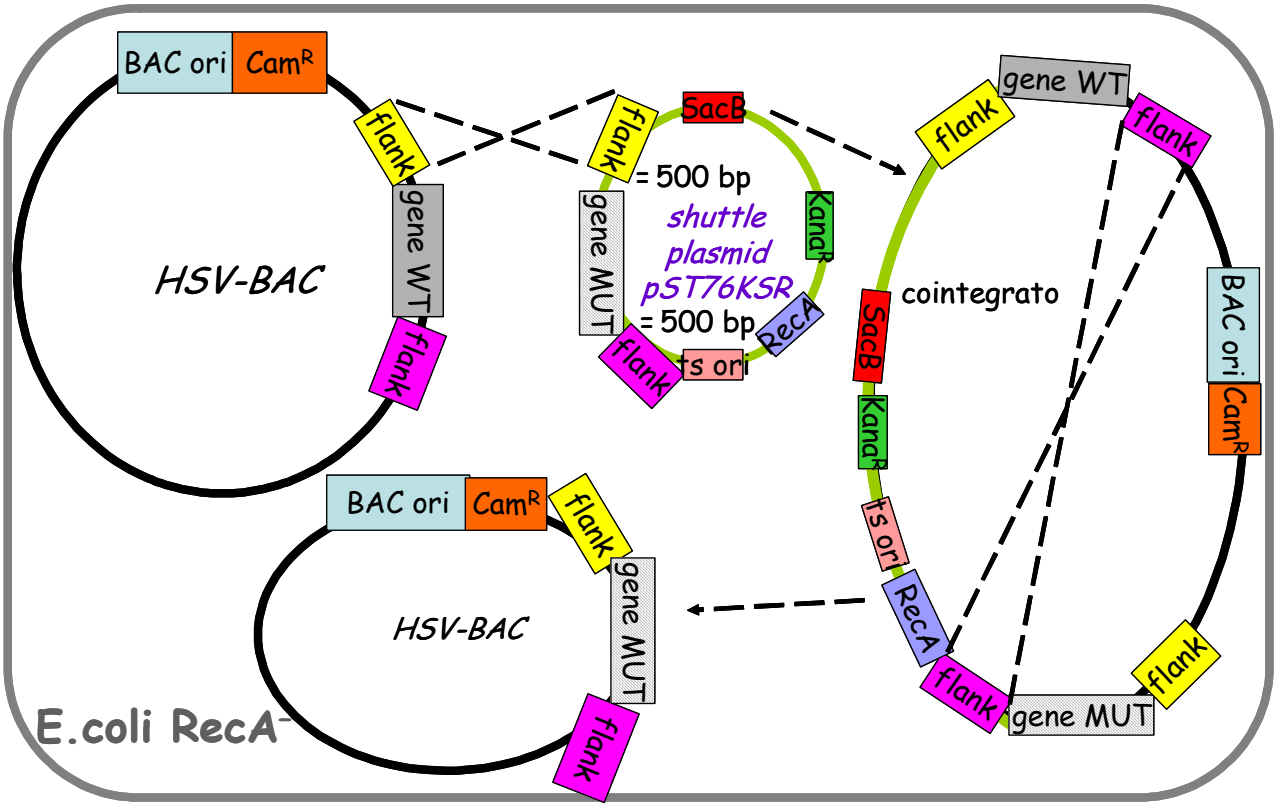


FIG 18: Schematic representation of the steps of BAC Mutagenesis-Procedure in E.coli DH10B described in this paragraph.

The choice of R6 was based on the fact that this cell line is permessive, expresses gD ectopically and enables virus lacking gD to replicate and spread. So transfected recombinant HSV-BACs could produce virions carrying wild type gD in their envelope and recombinant gD sequence in their genome. After a variable numbers of passages in this cell line, BHK cells were infected, in order to generate a totally (not only genotipically, but also fenotipically) recombinant virus stock ready for the following passages in cell line expressing HER2 as the sole receptor. The passage in BHK is necessary to eliminate wild type protein present in the envelope of recombinant virus. In fact, recombinant viruses loose wild type gD following BHK entry and the progeny produced in this cell line is genotipically and fenotipically recombinant.

2.2.7 WESTERN BLOTTING

We determined the relative electrophoretic mobility of chimeric gD and WT gD: the cells infected with WT or mutant viruses were lysed directly in SS (solubilizing solution) containing SDS and β-mercaptoethanol and boiled for 3 minutes. The proteins were separated by denaturing polyacrylamide electrophoresis (PAGE) and transferred onto Hybond-ECL nitrocellulose-membrane (Amersham Biosciences). The membranes were blocked with 5% non-fat dry milk in PBS for 30 minutes at 37°C, washed and reacted with MAbH170 or MAbBD80, followed by peroxidase-conjugated anti mouse IgG horseradish peroxidase linked whole antibody (from sheep) (GE Healthcare) and enhanced chemioluminescence (ECL;Western blotting detection reagents; GE Healthcare).

2.2.8 CELL-CELL FUSION ASSAY

The cell-cell fusion assay was performed as follows: effector COS cells were seeded in 24-well dishes ($5 \cdot 10^5$ cells/well) and transfected with plasmid pCAGT7pol, plasmids encoding gB, gH, and gL, and either wt gD (pEA99) or one of the gD-scHER2 chimeras (pLM10 and pLM10L). Target cells, namely, J-HVEM, J-Nectin1, or J-HER2 cells, seeded in T25 flasks, were transfected with the pEMCVLuc reporter construct. At 24 h after transfection, the target cells were seeded in a 1:1 ratio with COS effector cells, and cells were lysed after 24 h of cocultivation. The extent of fusion was measured by means of the luciferase assay system by Promega (Florence, Italy) in a TD20/20 luminometer (Turner Designs). All samples were run in triplicate.

2.2.9 PLATING EFFICIENCY

J, J-nectin1, J-HVEM, J-HER2, CHO, CHO-nectin1, CHOHER2, and Vero cells were infected with serial dilutions of R-LM11, R-LM11L, or HSV-1(KOS)tk12. At 24 or 48 h after infection, cells were fixed with 0,2% glutaraldehyde and 0,2% formaldehyde in PBS and plaques were visualized by in situ X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining The samples were run in triplicate.

2.2.10 VIRUS REPLICATION ASSAYS

J, J-nectin1, J-HVEM, and J-HER2 cells grown in 12-well plates were infected with R-LM11, R-LM11L, or HSV-1(KOS)tk12 at 10 PFU/cell for 90 min at 37°C. Extracellular virus was inactivated by means of an acid wash (40 mM citric acid, 10 mM KCl, 135 mM NaCl [pH 3]) [58]. Replicate cultures were frozen at 3, 24, or 48 h after infection, and the viral progeny (intracellular plus extracellular) was titrated on Vero cells.

2.2.11 INHIBITION OF VIRUS INFECTION

To measure the effects of anti-HER2 antibodies on infection with the R-LM11 and R-LM11L recombinant viruses, cells grown in 96-well plates were preincubated with increasing concentrations of purified IgGs of MAb 4D5 or 9G6 (Santa Cruz Biotechnology), directed to HER2 conformational epitopes, or herceptin, or of irrelevant mouse IgGs, or of R1.302 directed to nectin1 in 30 μl for 1 h at 37°C. The recombinant viruses were added in 3μl and allowed to absorb to cells for 90 min at 37°C. To measure the effect of a recombinant soluble form of HER2, aliquots of R-LM11 or R-LM11L were mixed with increasing concentrations of a HER2-Fc chimera (ErbB2/Fc; R&D Systems) and CTLA4-Fc as a negative control for 1 h at 37°C and were allowed to absorb to J-HER2 and CHO-HER2 cells for 90 min at 37°C. In both types of experiments, the viral inoculum was removed at the end of the absorption interval, and the cells were rinsed twice, overlaid with medium containing the same concentration of IgGs or proteins as was present during virus absorption, and incubated for 16 h at 37°C. Expression of β-galactosidase was a direct measure of the extent of virus infection. The optical density was read in a Bio-Rad enzyme-linked immunosorbent assay (ELISA) reader. For each antibody or protein concentration, triplicate samples were run. A value of 100% represents data obtained with infected cells not exposed to antibodies or to a recombinant receptor.

To determine which receptor, HER2 or nectin1, mediated infection of R-LM39, R-LM113 or R-LM249, SKOV3 cells grown in 96-well plates were incubated for 2 h on ice with increasing concentrations of purified antibodies (R1.302 to nectin1, Herceptin to HER2, mouse immunoglobulins, or a mixture of R1.302 and Herceptin) diluted in DMEM without serum, and then with the viral inoculum at the multiplicity of infection of 2 pfu/cell (as titered in SKOV3 cells) for further 90 min on ice. Following virus adsorption, the non-attached virus was removed and cells were washed twice with ice cold RPMI+Glutamax supplemented with 2.5% FBS. Cells were overlaid with the same concentration of antibodies or IgGs, rapidly shifetd at 37°C and incubated for 16 h. Infection was quantified as EGFP fluorescence

intensity by means of a Victor plate reader (Perkin Elmer). Alternatively, digital pictures were taken and infection quantified as described above. A 100% value represents data obtained with cells infected with virus, without antibody treatment.

2.2.12 INFECTION ASSAY

A number of cell lines grown in 96-well plates were infected with R-LM39, R-LM113 or R-LM249 stocks grown in cell line expressing HER2 as the sole receptor (J-HER2 or SKOV3). Infection was monitored as EGFP expression 24 or 48 h later. Digital pictures were taken with a Kodak camera connected to a Zeiss Axioplan fluorescence microscope, and EGFP expression was quantified with Photoshop Histogram tool.

Chapter II: ENGINEERING OF RECOMBINANT HSV-1 RETARGETED TO HER2 RECEPTOR

2.3 Results

2.3.1 GENETIC ENGINEERING OF HSV RECOMBINANT WHOSE gD CARRIES A scFv TO HER2 INSERTED BETWEEN RESIDUES 24 AND 25: R-LM11 AND R-LM11L

The overall objective was to insert a ligand to HER2 in gD. Because HER2 has no known natural ligand, the selected ligand was a scFv derived from MAb 4D5 [75], herein designated scHER2. scHER2 was inserted at aa 24, a site previously reported to tolerate the IL-13 and uPA insertions. The site of insertion is very close to the binding site for HVEM receptor, which was mapped to a continuous region that includes residues 27 to 29 [25,67]. The experimental design consisted first in the insertion of two restriction sites, EcoRI and BamHI, at aa residues 21 and 30 of mature gD, respectively, and subsequently in the insertion of a fragment encoding scHER2. Downstream of it, one construct contained a 9-aa serine-glycine flexible linker and another construct contained no linker (Fig.19). In the final constructs, named gD-scHER2 and gD-scHER2L, scHER2 was inserted between gD aa residues 24 and 25, and gD lacked no amino acid residue but carried two substitutions, D21G and L22I, as a consequence of the EcoRI restriction site insertion. gD-scHER2 and gD-scHER2L were cloned into pcDNA3.1 under the control of the CMV promoter for constitutive expression. The expression and proper folding of the chimeric forms of gD in transfected BHK or RS cells were essentially similar to those of wt gD, as detected by IFA (data not shown). Of note, the two substitutions introduced with the EcoRI restriction site did not alter the expression of gD or its ability to bind receptors and mediate cell-cell fusion.

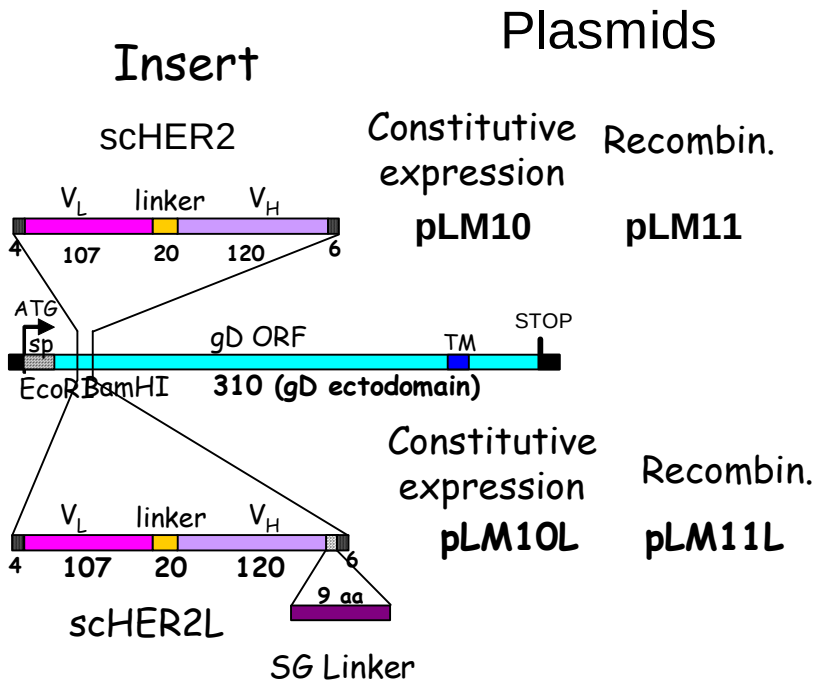


FIG 19: Schematic representation of the gD gene carrying the sequence encoding scHER2. The cDNA encoding scHER2 was inserted into the gD gene, previously modified to carry EcoRI and BamHI restriction sites. The insert was flanked by sequences encoding 4 and 6 aa residues at the 5' and 3' ends, respectively, to restore the complete gD sequence. pLM10L and pLM11L carried a 9-aa Ser-Gly linker (L) downstream of the insert. In pLM10 and pLM10L, the chimeric gD gene was cloned into pcDNA3.1 for constitutive expression. pLM11 and pLM11L carry the chimeric gD gene bracketed by upstream and downstream gD sequences and were employed to generate recombinant (Recombin.) viruses. Numbers indicate the length in amino acid residues of each fragment. The insertion of the EcoRI site in gD caused the D21G and L22I substitutions in mature gD. VH and VL, heavy- and light-chain variable domains of the anti-HER2 antibody 4D5. sp, signal peptide. Bars are drawn to scale.

2.3.2 CONSTRUCTION OF CELL LINES STABLY EXPRESSING HER2

In order to construct cell lines expressing HER2 in the absence of any other HSV-1 entry receptors, an expression plasmid encoding HER2 was transfected into HSV-resistant J or CHO cells. Both cell lines lack the receptors necessary for entry of HSV. The transfected cells were cloned by limiting dilution, and individual clones were scored for HER2 surface expression by IFA on nonpermeabilized cells. Figure 20 shows the cell surface localization of the receptor in overexpressing stable clonal cell lines J-HER2 (fig. 20A) or CHO-HER2 (fig. 20B).

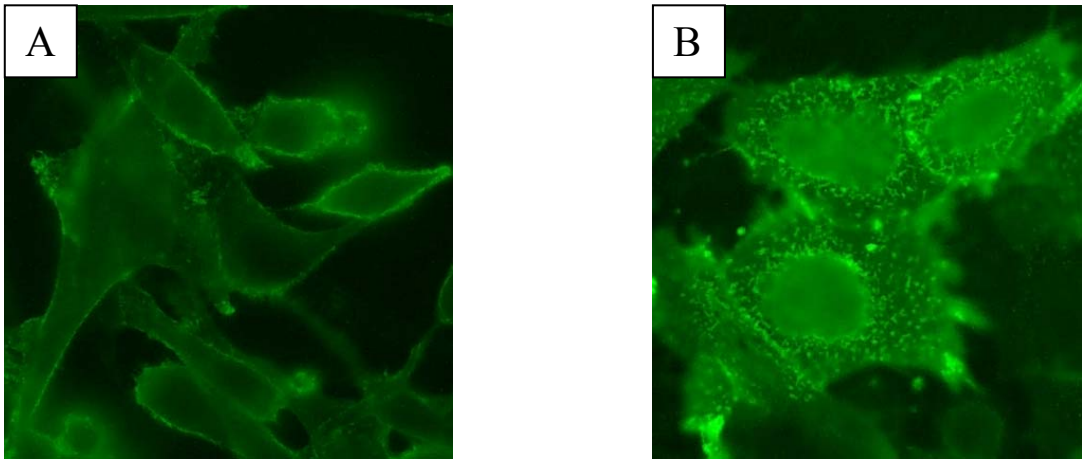


FIG 20: Expression of HER2 in the stable cell lines J-HER2 (A) and CHO-HER2 (B). Cells were fixed with paraformaldehyde and reacted with MAb 9G6 to HER2 ectodomain, followed by FITC-conjugated secondary antibody. Fluorescence localized at the plasma membrane.

2.3.3 CHIMERIC gD-scHER2 OF R-LMM11 AND R-LM11L MEDIATES CELL-CELL FUSION THROUGH THE HER2 RECEPTOR

As a first assay to check whether the chimeric forms of gD-scHER2 were able to interact with the HER2 receptor and thus trigger fusion, we performed a cell-cell fusion assay. In this assay, the effector COS cells, cotransfected with plasmids encoding wt or chimeric gD under the control of the CMV promoter, plus gB, gH, gL, and a T7 polymerase (pCAGT7pol), fuse with receptor-positive target cells transfected with a T7 promoter-driven luciferase reporter gene. The luciferase activity is a direct measure of the fusion capacity of the transfected cells. J-HER2 cells were used as target cells, and J-nectin1 and J-HVEM cells were used as positive controls. The results in fig. 21 show that gD-scHER2 and gD-scHER2L mediated fusion with J-HER2 cells to an extent comparable to, or slightly higher than, that exhibited by wt gD with J-HVEM cells, providing a first line of evidence that the insertion of scHER2 resulted in a functional gD exhibiting redirected tropism. The chimeric forms of gD mediated fusion with J-nectin1 cells to an extent indistinguishable from that of wt gD, providing evidence that the scHER2 insertion did not affect the binding to nectin1 or the pro-fusion activity of gD. By contrast, fusion with J-HVEM cells was almost abolished. Of note, the fusion activity of wt gD-expressing cells with J-HVEM cells was much lower than that withJ-nectin1 cells.

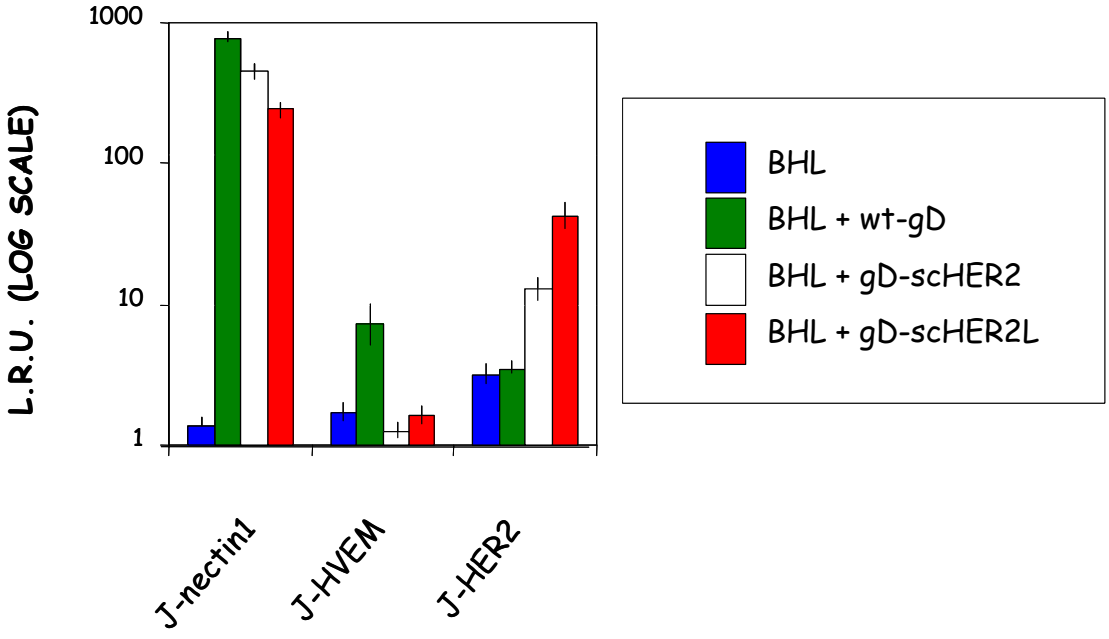


FIG 21: Cell-cell fusion mediated by chimeric scHER2-gD. The effector COS cells, cotransfected with plasmids encoding gB, gH, gL (BHL), and pCAGT7pol plus chimeric or wt gD, were cocultivated with J-nectin1, J-HVEM or J-HER2 terget cells transfected with pEMCVLuc. Luciferase activity was expressed as relative light units (RLU) on log scale. Each experiment was performed at least three times, and samples were run in triplicate; mean values are shown. Vertical bars, standard deviation.

2.3.4 CONSTRUCTION OF HSV RECOMBINANTS CARRYING gD-scHER2: R-LM11 AND R-LM11L

We next generated recombinant viruses carrying gD-scHER2 or gD-scHER2L by homologous recombination between the gD deletion virus HSV-1(KOS)tk12/FRT-GFP [76] and the recombination plasmids containing gD-scHER2 and gD-scHER2L, designated pLM11 and pLM11L. To aid recombination, the plasmids carried the chimeric gD genes bracketed by the upstream and downstream sequences that flank the gD coding sequence. The recipient virus encodes the *lacZ* gene under the control of the α4 promoter in place of the thymidine kinase gene, and therefore, the recombinants can be traced and quantified as β-galactosidase activity.

The recombinants, named R-LM11 and R-LM11L, were verified genotypically and for production and properties of the chimeric gD. First, the scHER2 sequences were amplified with primers annealing to the gD sequences flanking the insertion (fig. 22A) or with primers specific for the scHER2 insert (f ig. 22B). In either case, the amplimers exhibited the expected size.

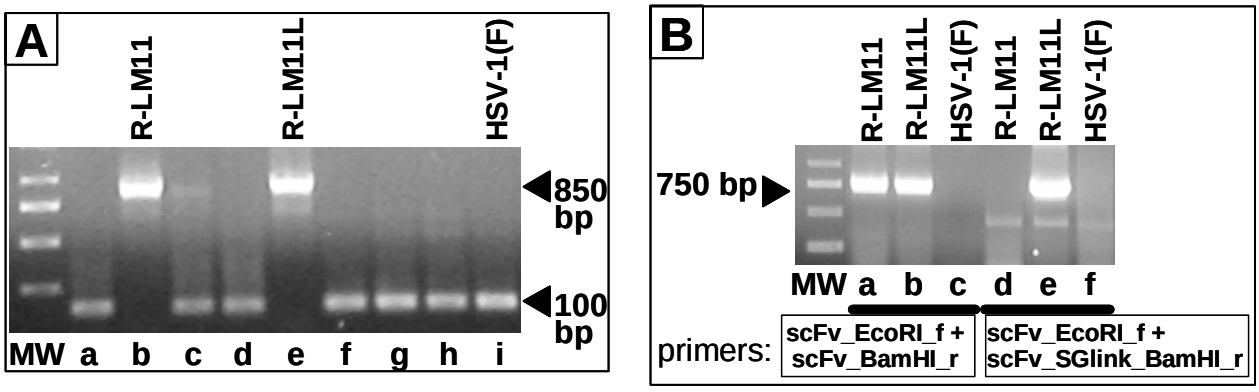


FIG 22: Amplification of the sequences encoding scHER2 from lysates of R-LM11 and R-LM11L-infected cells.

(A) PCR was performed with primers annealing to the gD sequences that flank the site for scHER2 insertion. The presence of the insert causes an increase in the size of the amplification product from 100 bp (non recombinant plaques (lanes a, c, d, f, g, and h) to 850 bp (lanes b and e). Lane I, HSV-1(F), used as a control. MW, 1kp DNA ladder.

(B) PCR was performed with primers annealing to the scHER2 insert. Lanes a to c, PCR with primers used for cloning the scHER2 insert. Lanes d to f, PCR with the same forward primer as in lane a to c and a reverse primer annealing to ser-gly linker, thus amplifying R-LM11L rather then R-LM11. The amplification products from the recombinant exhibit the expected length (about 750 bp). HSV-1(F) was used as negative control and did not give rise to any amplification product (lane c and f)

Second, lysates of cells infected with the recombinants were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. As shown in figure 23, gD from R-LM11 and R-LM11L exhibited a decrease in electrophoretic mobility and an apparent *M*r consistent with the insertion. Both wt gD and the slower-migrating bands from the recombinants reacted in Western blots with MAb H170 against gD.

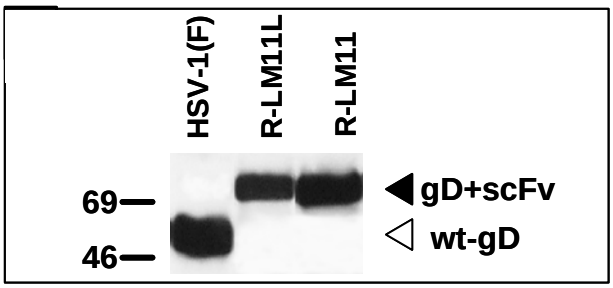


FIG 23: Electrophoretic mobility of chimeric scHER2-gD. Lysates of cells infected with R-LM11, R-LM11L, HSV-1(F) were subjectd to SDS-PAGE, transferred to nitrocellulose membranes, and visualized by western blotting with MAb H170 against gD, followed by peroxidase-conjugated anti-mouse IgG and ECL. In recombinant the presence of scHER2 results in a slower-migrating band (black arrowhead) than that with wtgD (white arrowhead). Numbers to the left represent migration position of molecular mass markers (in kilodaltons).

Next, we analysed the reactivity of the chimeric gD-scHER2 to antibodies directed to conformation-dependent epitopes by cell ELISA. Cells infected with R-LM11, or with HSV-1(KOS)tk12 as a control, were reacted with MAbs HD1 and DL11, two potent neutralizing antibodies that react with a region involved in virus entry, and with MAb AP7, which reacts to a discontinuous epitope localized in part at the N terminus and in part at the C terminus (residues 290 to 300) [72,74].

The results in figure 24 show that reactivity to MAb H170, which recognizes aa 1 to 23, was not modified in chimeric gD relative to wt gD, as expected. Reactivity to the neutralizing MAbs was somewhat decreased but still present, in accordance with the ability of the virus to carry out infection. The reactivity of the chimeric gD-scHER2 to MAb AP7 was lost. The results indicate that (i) critical epitopes for infection are maintained and (ii) in the receptor-unbound gD-scHER2, the ectodomain N and C termini do not interact with each other anymore. Of note, the insertion did not confer instability on the viruses; the recombinants were passaged for several months, and their properties were stable.

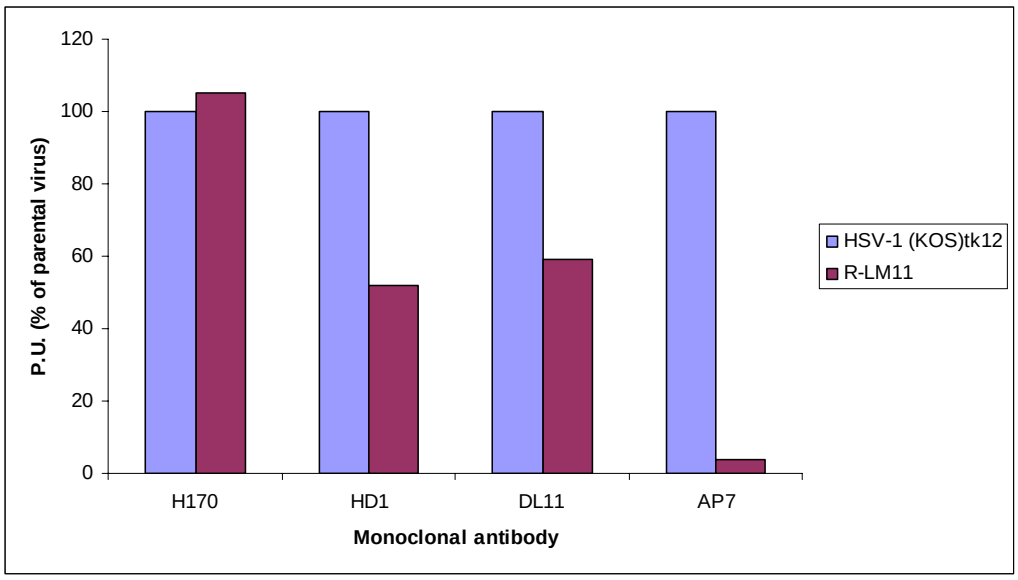


FIG 24: Reactivities of wt gD and the gD-HER2 chimera to a panel of monoclonal antibodies, measured by cell ELISA. Vero cells were infected with the indicated viruses. At 16 h after infection, they were reacted with the indicated antibodies, followed by a peroxidase-conjugated anti-mouse antibody and o-phenylenediamine. Binding of antibodies was quantified as peroxidase units (P.U.), and expressed as a percentage relative to the cells infected with parental HSV-1 (KOS)tk12 virus. Each assay was performed in quadruplicate.

Given that the *M*r of gD was almost doubled by the insertion, we also asked whether recombinant virions were able to incorporate gD in amounts similar to those present in wt virions. Extracellular virions were pelleted and analyzed for the content of gD and for that of gB as a reference.

The results in figure 25 show that amounts of gD were very similar in RLM11 and wt virions.

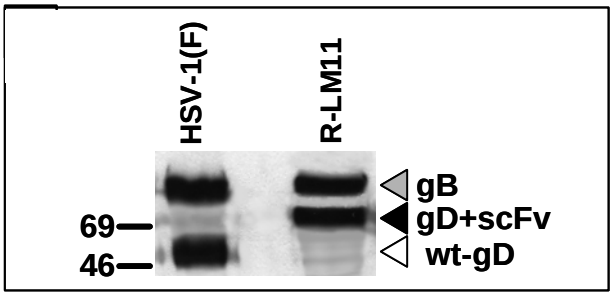


FIG 25: Quantification of gD and gB present in virions. Virions were pelleted from the extracellular medium of infected Vero cells after growth for 24 h. Equal amounts of virions, measured as PFU, were loaded for SDS-PAGE separation. Amounts of gB and gD were detected by Western blotting with MAbs H1817 and H170, respectively. Arrowhead indicate migration position.

2.3.5 R-LM11 AND R-LM11L INFECT CELLS THAT EXPRESS HER2 AS THE SOLE RECEPTOR.

Replicate cultures of J, J-nectin1, J-HER2, J-HVEM, CHO, CHO-nectin1, CHO-HER2, or CHO-HVEM cells were exposed to R-LM11 and R-LM11L viruses, or to the parental virus HSV1(KOS)tk12 as a control. After 24 h, infection was detected as β-galactosidase activity. Figure 26 shows that R-LM11 and R-LM11L, but not the parental HSV-1(KOS)tk12, were able to infect J-HER2 and CHO-HER2 cells (fig. 26 g, h, i, s, t, and u). The recombinants were still able to infect J-nectin1 and CHO-nectin1 cells (fig. 26 d, e, p, and q) but lost the ability to infect J-HVEM and CHO-HVEM cells (fig. 26 j, k, v, and w).

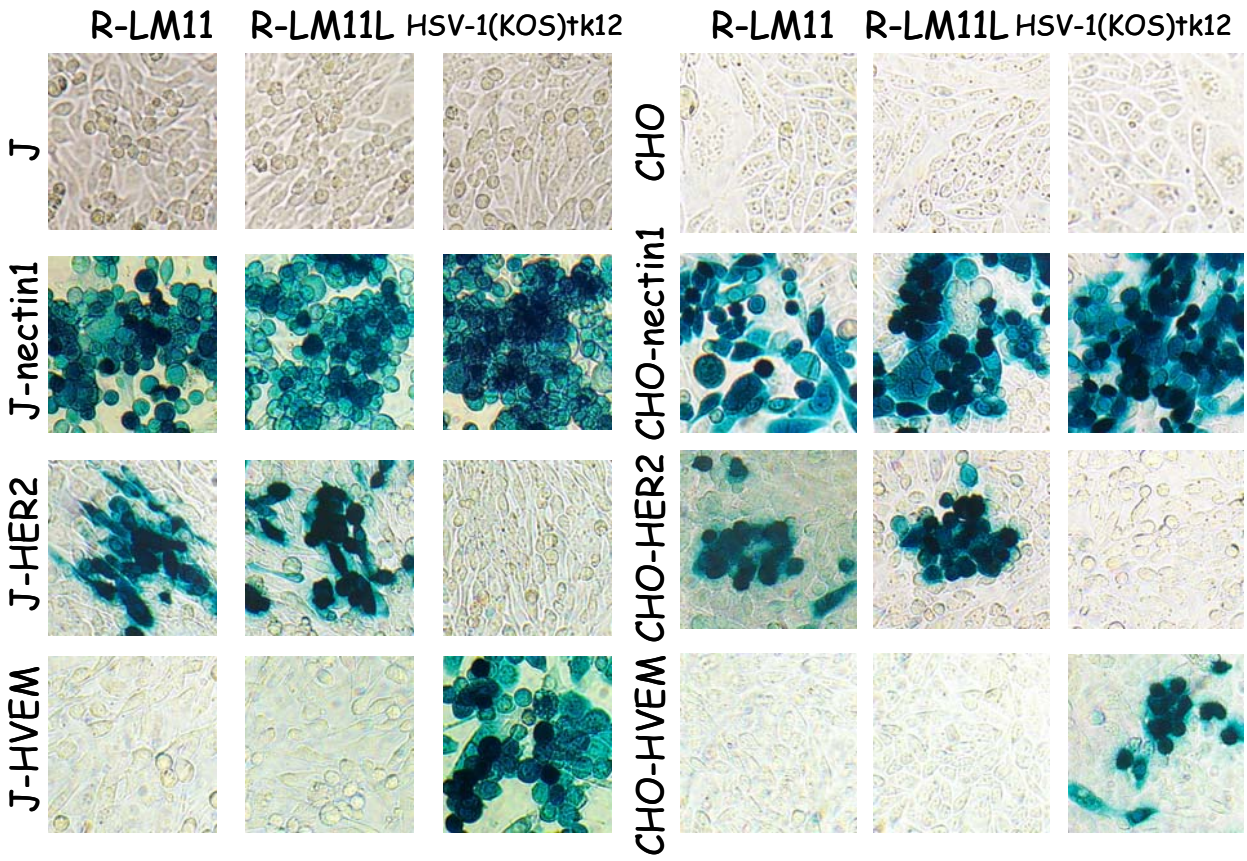


FIG 26: The recombinant viruses R-LM11 and R-LM11L infect cells via the HER2 receptor. Micrographs show J, J-nectin1, J-HER2, J-HVEM, CHO-nectin1, CHO-HER2, and CHO-HVEM cells exposed to the recombinant viruses R.LM11 and R-LM11L or to the parental virus HSV-1(KOS)tk12 at 10 pfu/cell. Infection was monitored as β-galactosidase activity by X-Gal staining 16 h following infection.

2.3.6 R-LM11 AND R-LM11L GROW AND SPREAD IN CELLS EXPRESSING HER2 AS THE SOLE RECEPTOR

Replicate cultures of J-HER2, J-nectin1, J-HVEM, and J cells were exposed to R-LM11 and R-LM11L (10 PFU/cell). After 24 and 48 h, the cells were harvested and progeny viruses titrated on Vero cells. The results in figure 27A to D show that R-LM11 and R-LM11L grew in J-HER2 cells. The titer was about 20-fold lower than that in J-nectin1 cells. Both recombinants were unable to grow in J-HVEM cells, suggesting that the HVEM binding site on gD was altered by the insertion of scHER2. As expected, the parental HSV-1(KOS)tk12 did not replicate in J-HER2 cells.

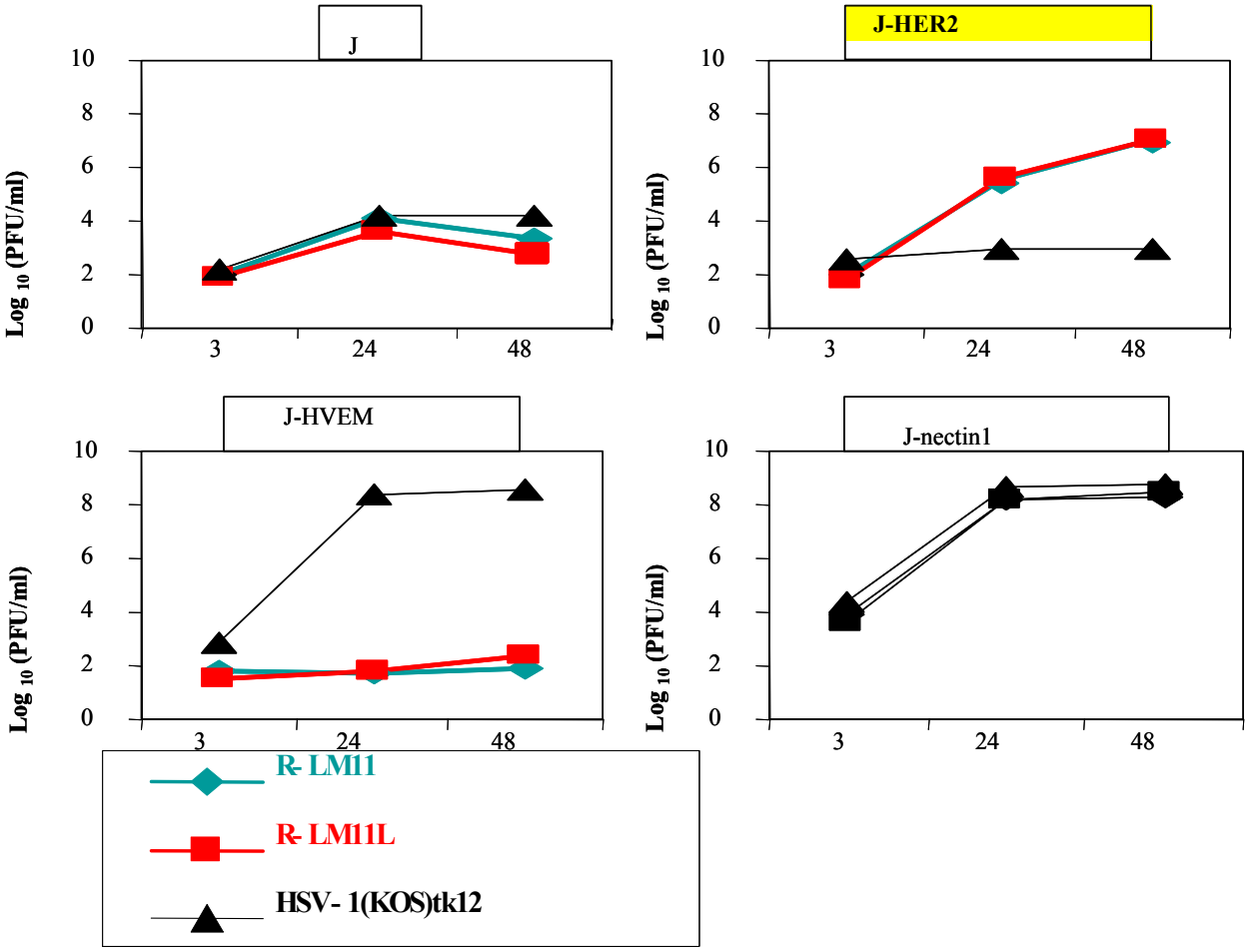


FIG 27: Growth curves of R-LM11 and R-LM11L. Replicate cultures of J (A), J-HER2 (B), J-HVEM (C), or J-nectin1 (D) cells were infected with R-LM11, R-LM11L, or HSV-1(KOS)tk12 at 10 pfu/cell. Progeni virus was harvested at 3, 24 and 48 h after infection and titred on Vero cells.

In the next series of experiments, R-LM11 and R-LM11L were assayed for the ability to spread from cell to cell in J-HER2 and CHO-HER2 cells, and in J-nectin1 and CHO-nectin1 cells as controls (fig. 28). The plaques formed by the recombinants in J-HER2 cells were fewer in number (700-fold and 250-fold reductions) than those in J-nectin1 cells. The reduction observed in plaque numbers in CHO-HER2 versus CHO-nectin1 cells was only 10-fold. HSV-1(KOS)tk12 did not form plaques in J-HER2 and CHO-HER2 cells. The ratio between the number of plaques in Vero cells and that in J-nectin1 cells, or between that in Vero cells and that in CHO-nectin1 cells, was practically the same for the two recombinants and the wt virus. The recombinants did not form plaques in J-HVEM cells, in agreement with the lack of growth observed in figure 28.

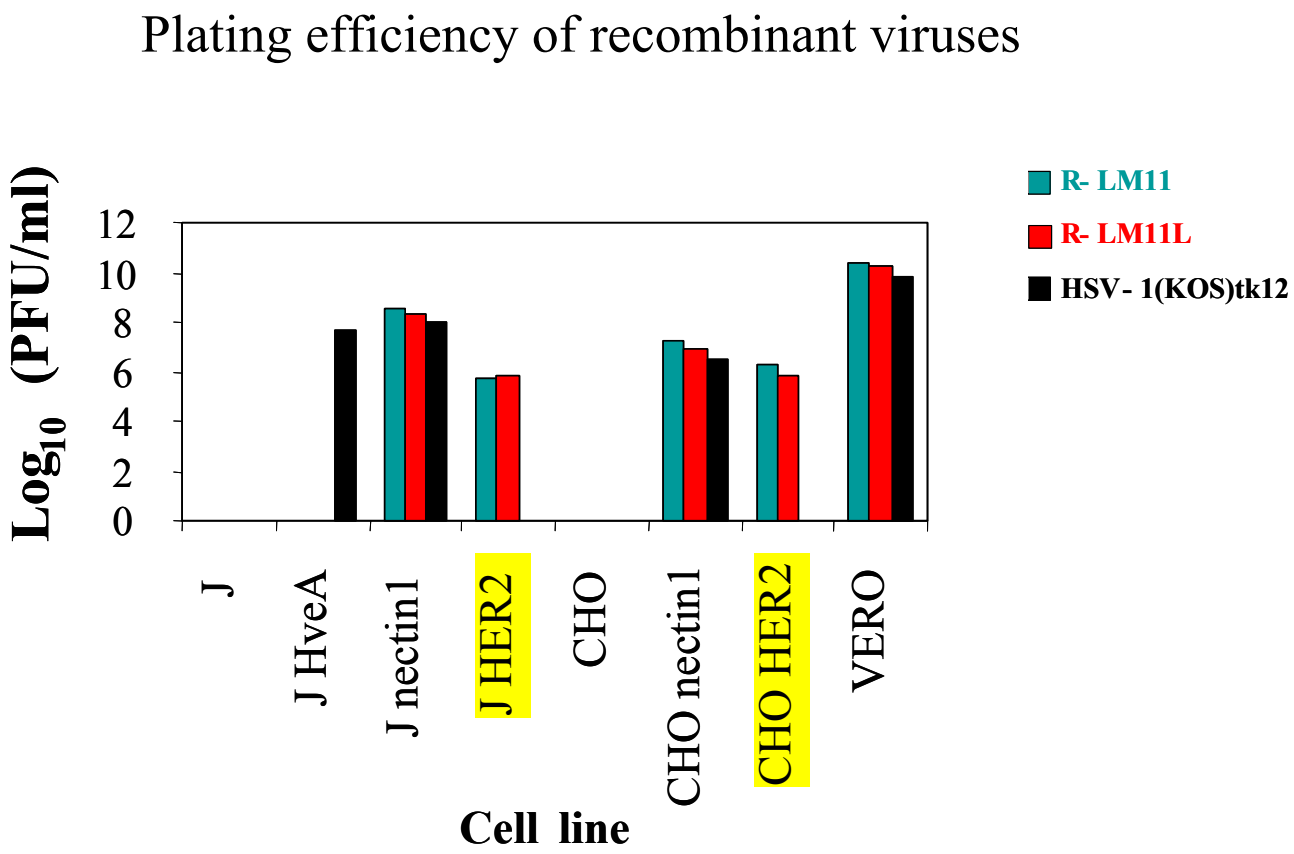


FIG 28: Plating efficiency of R-LM11 and R-LM11L. R-LM11, R-LM11L and HSV-1(KOS)tk12 in the indicated cell lines. Infected monolayers were fixed 24 or 48 h after infection, and plaque were visualized by X-Gal or Giemsa staining.

As a measure of the abilities of R-LM11 and R-LM11L recombinants to spread from cell to cell, we also determined their plaque sizes. As shown in figure 29, the plaque sizes of wt viruses and R-LM11L did not differ significantly from each other, whereas the plaque size of R-LM11 was 50% reduced.

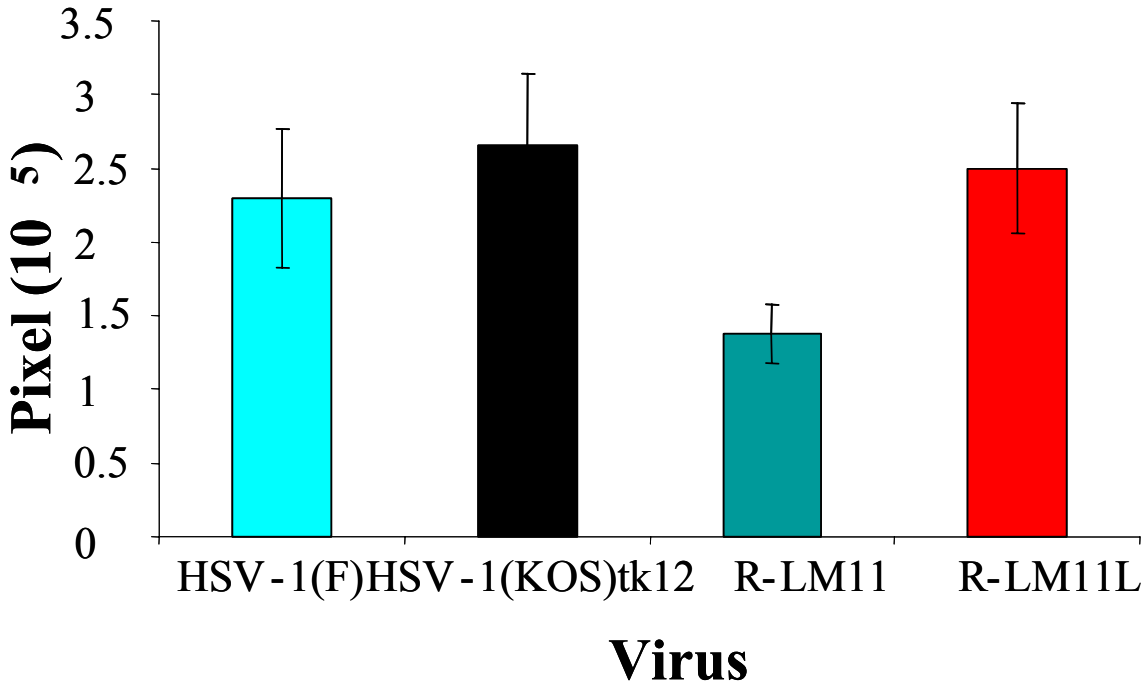


FIG 29: Plaques formed as shown in fig were photographed, and the plaque areas were measured by means of the histogram program and expressed as pixel. For each virus, the area of at least 20 plaques were measured. Histogram represent averages; errors bars, standard deviation.

Cumulatively, these results provide evidence for the following conclusions.

1. R-LM11 and R-LM11L have acquired the ability to grow and spread in J-HER2 and CHO-HER2 cells, although at reduced efficiency relative to that in J-nectin1 and CHO-nectin1 cells. The reduction is more evident for the R-LM11 virus, which lacks a linker between scHER2 and gD, at least as far as cell-to-cell spread is concerned. This indicates that the recombinants exhibited a modified tropism and were effectively redirected to the HER2 receptor.

2. The recombinants maintained the ability to infect through nectin1 but not through HVEM.

2.3.7 ENTRY OF R-LM11 AND R-LM11L INTO J-HER2 OR CHO-HER2 CELLS IS DEPENDENT ON HER2

To provide evidence that entry of R-LM11 and R-LM11L recombinants into JHER2 or CHO-HER2 cells was dependent on the HER2 receptor, we measured whether infection was inhibited by MAbs to HER2 or by a soluble form of HER2. In the first series of experiments, we used two MAbs, addressed to different epitopes of HER2. MAb 4D5 is addressed to domain IV and is the MAb from which the scFv was derived. MAb 9G6 is addressed to an unmapped conformational epitope. Figure 30 shows that both MAbs inhibited R-LM11L infection of J-HER2 cells in a dose-dependent manner. Mouse IgG had no effect. In the second series of experiments, we tested the effect of a recombinant form of HER2 in which the ectodomain is fused to the Fc portion of IgG (HER2-Fc). As shown in figure 30B, HER2-Fc inhibited entry of R-LM11L into CHO-HER2 cells in a dosedependent manner, whereas an unrelated receptor, CTLA4-Fc, did not.

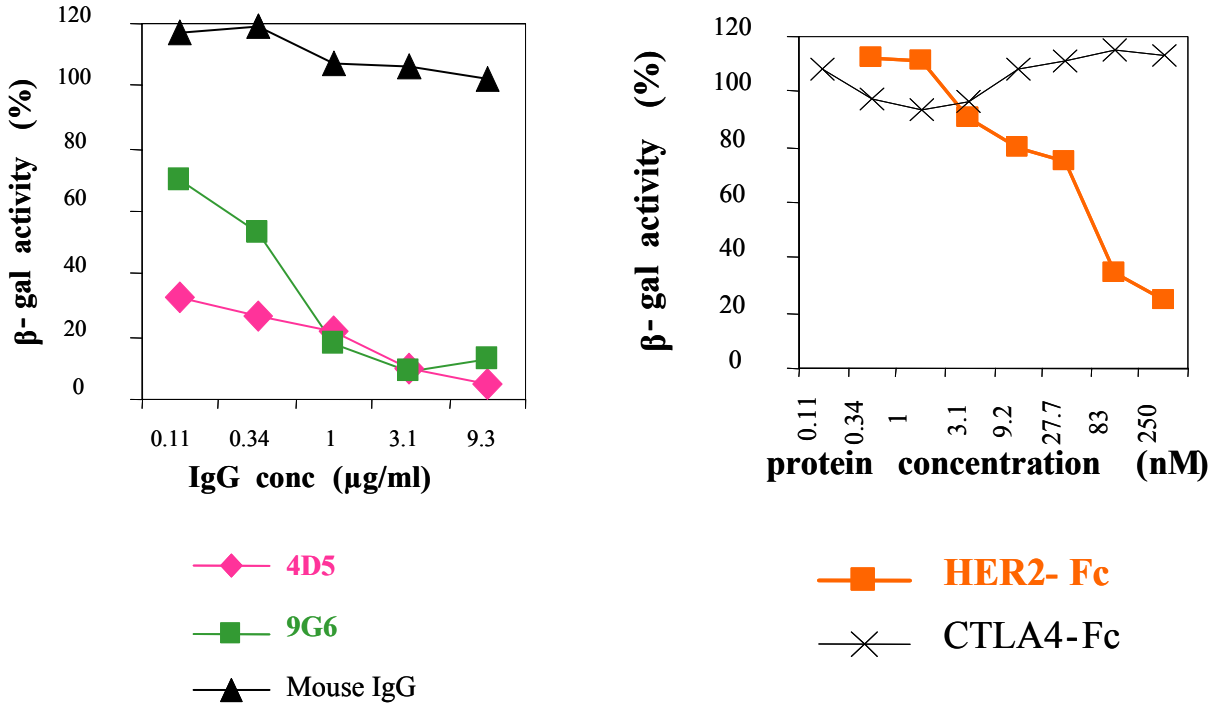


FIG 30 : Infection of R-LM11 and R-LM11L recombinants in HER2-expressing cells is blocked by antibodies to HER2 and by a soluble form of HER2, HER-Fc. (A) J-HER2 cells were preincubated with the indicated concentration of purified IgG of monoclonal antibody 4D5, or 9G6 against HER2 or of irrelevant mouse IgGs for 1 h at 37°C. Virus was added to the antibody-containing medium and allowed to infect the cells for 90 min at 37°C. Infection was monitored 16 h later as β-galactosidase activity. (B)Replicate aliquots of R-LM11L were preincubated with the indicated concentrations of purified soluble recombinant HER2-Fc or CTLA4-Fc for 1 h at 37°C and allowed to absorb to CHO-HER2 cells for 90 min at 37°C. Infection was quantified 16 h later as β-galactosidase activity. Each point represents the average of triplicate assays. The standard error ranged from 0,6

to 1,9% of means values. One hundred percent indicates the optical density measured in untreated, virus-infected cultures.

2.3.8 DETARGETING FROM NATURAL RECEPTORS BY MEANS OF POINT MUTATIONS

The objective of the following experiments was to mutagenize gD in order to ablate the virus ability to infect through nectin1 (detargeting). This was done by insertion of a single (V34S) or multiple (V34S-D215G-R222N-F223I) mutations.

V34S MUTATION

The first mutation we introduced into recombinant gD of R-LM11L genome to try to destroy nectin1 binding site was V34S, a mutation reported to abrogate the capacity of R5141 and R5321, herpes simplex visuses retargeted to cells expressing IL13Rα2 by means of IL-13 ligand fused to residue 33 of gD, to use nectin1 as receptor. [64]. Recombinant was generated by the "BAC Mutagenesis-Procedure".

First, we engineered a HSV-1gD$^-$LacZ virus: the starting construct was a HSV-1 genome deleted in gD with the bacterial artificial chromosome (BAC) inserted between UL3 and UL4 (pYeBac102gD$^-$ flipped). We introduced the LacZ reporter gene into BAC sequence between two LoxP sites, so it would have been possible to remove LacZ and BAC sequence using Cre recombinase and to obtain a recombinant virus different from wild type HSV-1 only for the introduced mutations.

The virus, named **R-LM31,** was still able to infect J-HER2 cells, thanks to scHER2, as expected. However, it retained also the ability to infect cells via nectin1 (fig 31).

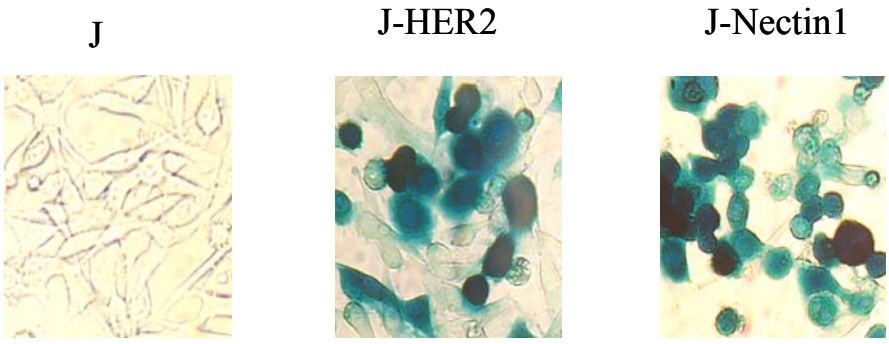


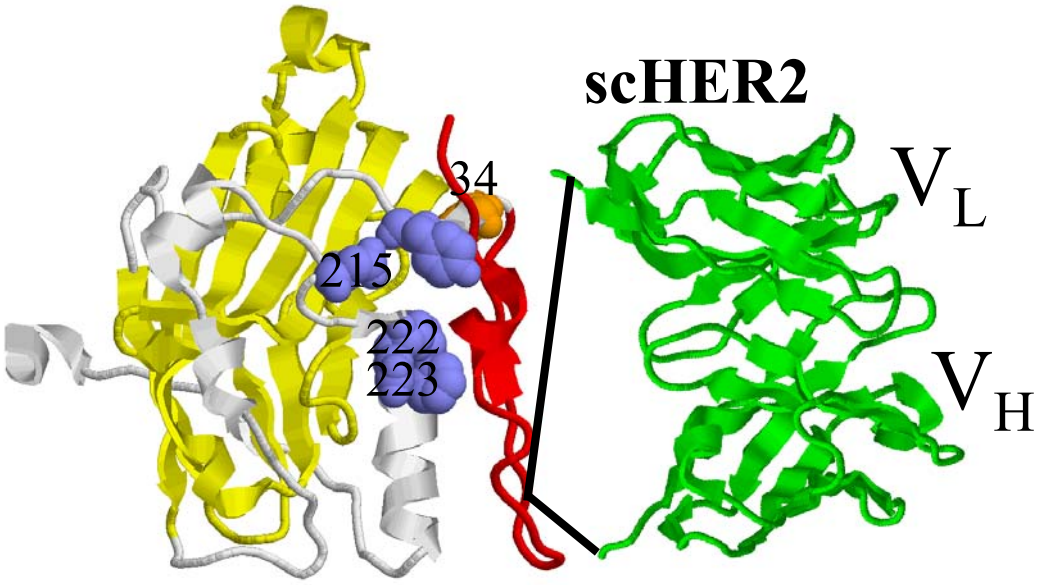
FIG 31: The recombinant R-LM31 was able to infect J-nectin1 cells too. Micrographs show J, J-HER2 and J-nectin1 cells exposed to the recombinant virus R-LM31 at 10 pfu/cell. Infection was monitored as β-galactosidase activity by X-Gal staining 16h following infection.

V34S-D215G-R222N-F223I MUTATIONS

It was reported that amino acids 215-222-223 of gD are located in a region involved in nectin1 binding. Thus Manoj et al [62] showed that mutations of these residues debilitated gD ability to use nectin1 for infection. In that study, infection was only measured in complemented viruses; recombinant viruses were not generated.

By BAC Mutagenesis-Procedure, we engineered a recombinant virus, named **R-LM39, carrying** V34S-D215G-R222N-F223I mutations, scHER2 sequence inserted between aa 24 and 25 and EGFP as reporter gene. In this recombinant LacZ gene was repleaced with EGFP gene to monitor virus growth and infectivity in live cultures and not in fixed-stained cultures. In addition, EGFP gene is smaller than LacZ gene (EGFP sequence is 700bp long, while LacZ sequence is 3300bp long), so viruses with EGFP gene are genotipically more stable than those carrying LacZ gene.

First, we verified that gD sequence was as expected: recombinant gD was amplified by PCR and sequenced. Then we tested the ability of recombinant virus to infect cells of different types and origin: the results (Fig. 32) show a complete detargeting in mouse cells, only a partial detargeting in rodent cells (hamster and rabbit), and lack of detargeting for primate cells (human and monkey).



A

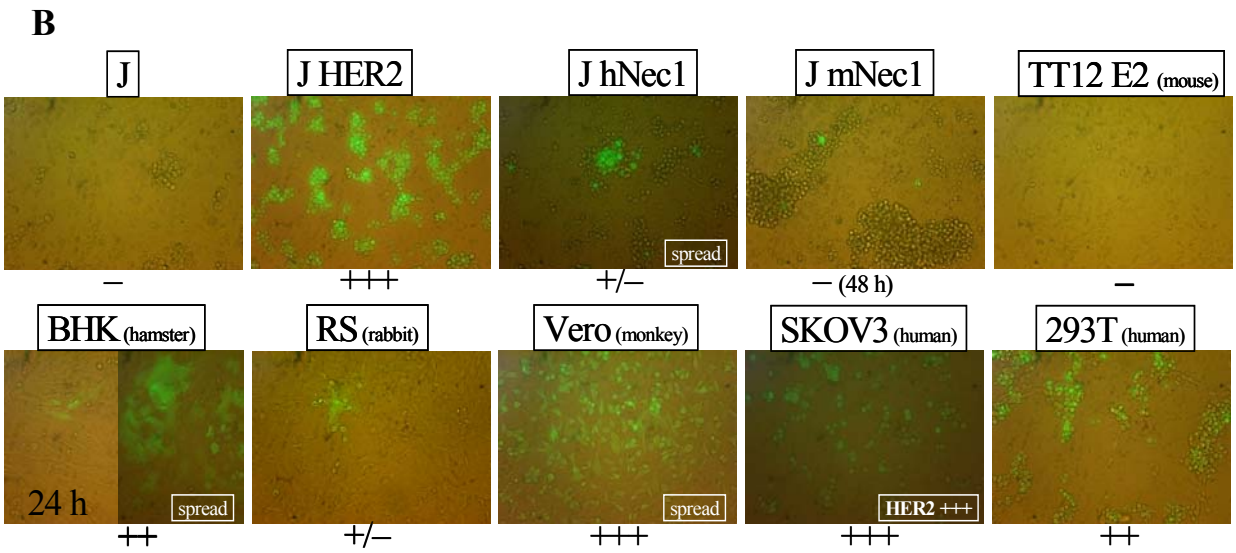


FIG 32 (A) Hypotetical model of chimeric gD structure obtained by combining the 3D structure of scHER2 and gD: scHER2 is green and bubbles indicate mutated residues.
(B) Micrographs show the indicated cell lines exposed to R-LM39 and visualized with fluorescence microscope. Infection was monitored at 72 h post infection.

Next, we performed a neutralizzation assay in SKOV3 cells, which express both HER2 and human nectin1. The results (Fig. 33) show that infection was not blocked by MAb to HER2 (herceptin) or to nectin1 (R1.302) used separately, but only by the mixture of the two antibodies. The results indicate that R-LM39 entry is mediated by nectin1 and HER2; thus, in absence of HER2 receptor the recombinant could use nectin1. Viceverse, in the absence of nectin1, it can use HER2.

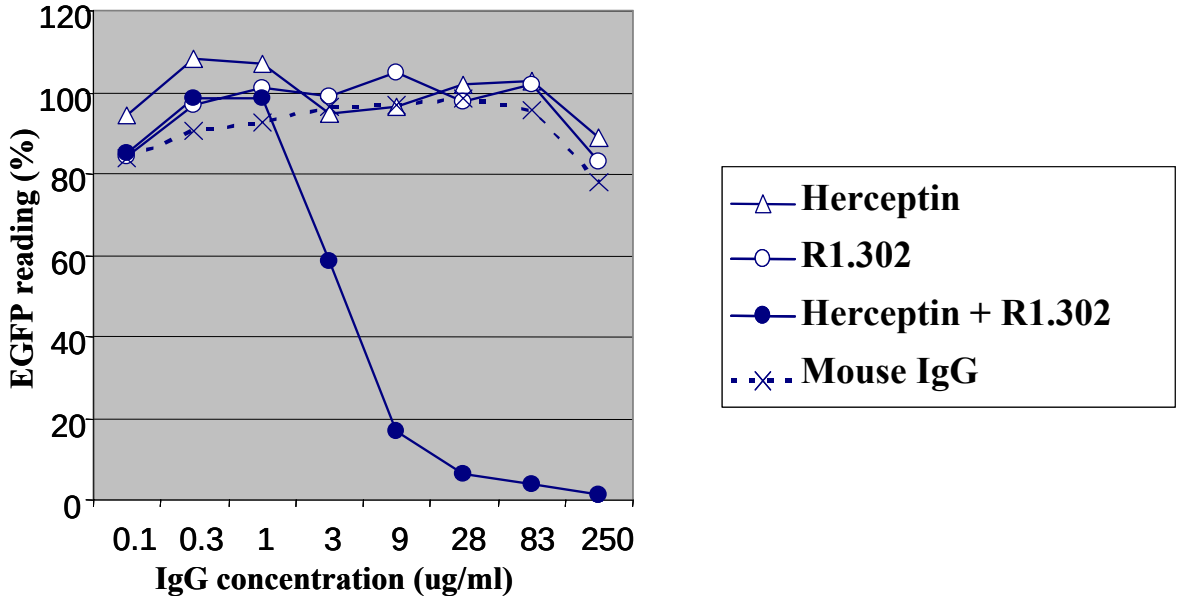


FIG 33:Infection of R-LM39 in SKOV3 cells is blocked only by a mixture of antibodies to HER2 (Herceptin) and to nectin1 (R1.302), but is not blocked by each antibody used separately.

SKOV3 cells grown in 96-well plates were incubated for 2 h on ice with increasing concentrations of purified antibodies (R1.302 to nectin1, Herceptin to HER2, mouse immunoglobulins, or a mixture of R1.302 and Herceptin) and then with the viral inoculum at the multiplicity of infection of 2 pfu/cell for further 90 min on ice. Following virus adsorption, the non-attached virus was removed and cells were overlaid with the same concentration of antibodies or IgGs, rapidly shifted to 37°C and incubated for 16 h. Infection was quantified as EGFP fluorescence intensity by means of a Victor plate reader (Perkin Elmer). Each point represents the average of assays.

2.3.9 DETARGETING FROM NATURAL RECEPTORS BY DELETION OF N-TERMINUS OR Ig DOMAIN OF gD

The results of the preceding paragraph show that a complete detargeting from nectin1 cannot be achieved by single or multiple mutations. Here, in order to abrogate virus ablility to use natural receptors, we undertook a novel strategy, based on deletion of gD portions. Two viruses were generated, named R-LM113 and R-LM249 respectively.

The rationale for the design of **R-LM113** was to insert the scHER2 in a different gD position relative to that of R-LM39. The novel position was chosen to mask the nectin1 binding site, as follows. By means of gD 3D structure observation, we decided to remove a large portion at the N-terminus of gD (from aa 6 to aa 38), insert scHER2 sequence plus 9-aa serine-glycine flexible linker at position 39. The scHER2 insertion was in a different position respect to previous recombinant gDs (in R-LM11-11L-31 and 39 scHER2 was between aa 24 and 25). The linear map of R-LM113 gD and its predicted structure are shown in figure 34. The virus was generated as described in Materials and Methods. To verify the presence of scHER2, the recombinant gD was amplified by colony PCR and sequenced, before virus reconstitution steps.

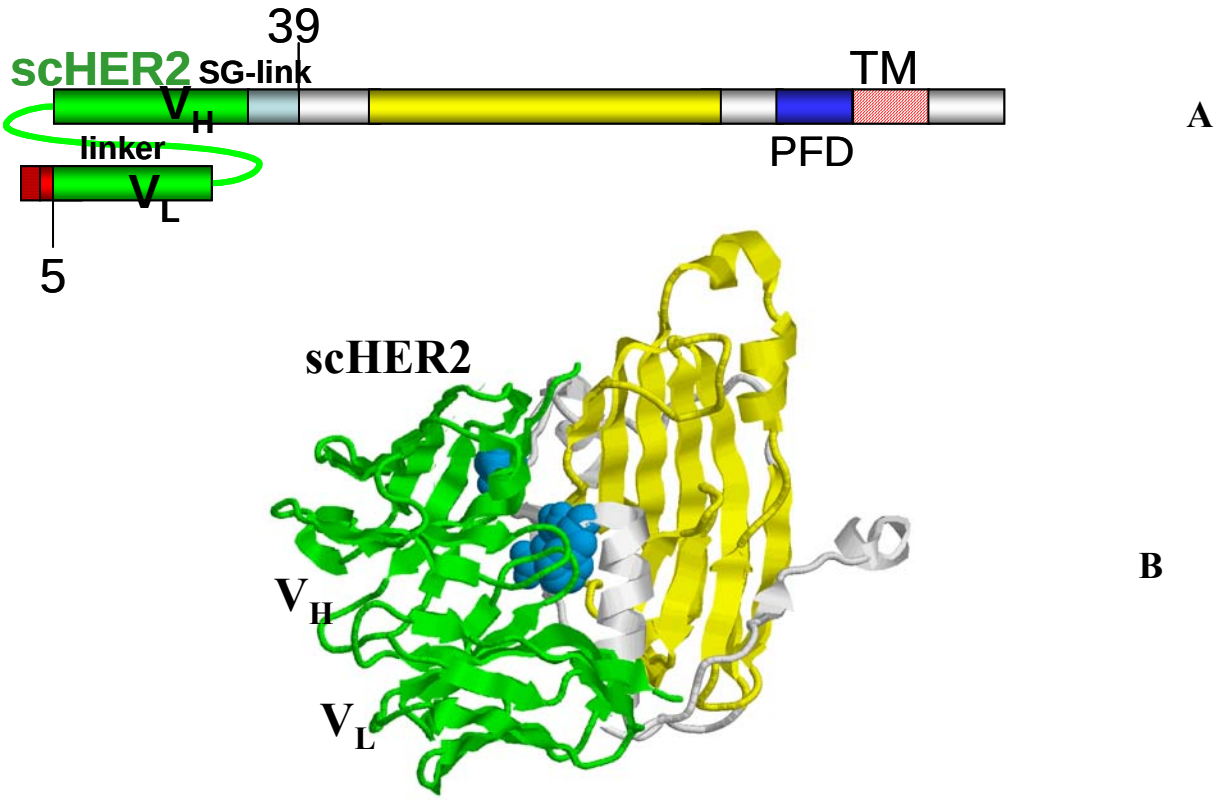


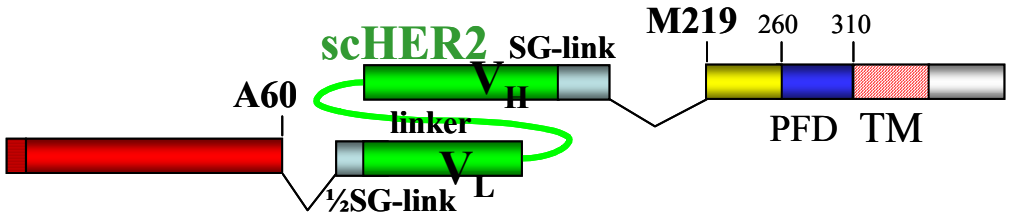
FIG 34: (A) Schematic representation of gD gene carrying the sequence encoding scHER2 with a little linker Ser-Gly inserted between aa 5 and 39 of gD (B) Hypotetical model of chimeric gD structure obtained by combining the 3D structure of scHER2 and gD: scHER2 is green, gD is yellow and the bobbles represent aa 34-215-222-223.

The rationale for the design of R-LM249 stems from the finding of Zhou and Roizman [55], who reported on a HSV-1 recombinant (R5322) whose gD was spit into two distinct peptides: polypeptide A containing the gD signal peptide fused to N-terminal domain (155 residues) of urokinase plasmilogen activator (uPA) linked to residues 33-60 of gD, with mutation V34S, and polypeptide B containing the C-terminal domain of gD (residues 219-369). By immunoprecipitation assay they showed that the two peptides interact through the kringle domain (residues 50-132) of the N-terminal domain of uPA. Although R5322 virus was infectious and produced vital progeny, it retained the ability to use its natural receptors. Nonetheless, the results clearly indicate that residues 61-218 of gD do not execute a function required for HSV-1 entry into cells. It is interest to note that residues 61-218 coincide almost entirely with Ig core of gD located between residues 56-184. Based on these finding, we replaced the Ig-folded core of gD with scHER2: scHER2 sequence was flanked by a short Ser-Gly linker (8 aa) at the N-terminus and a longer Ser-Gly linker (16 aa) at the C-terminus, gD had no mutations at N-terminus sequence and had only about 40 aa (from 219 to 260) before the profusion domain.

We next asked whether the recombinant virus

- was infectious and capable to produce vital progeny
- was able to infect HER2 expressing cells
- was detargeted from HSV-1 natural receptors, HVEM and nectin1.

A



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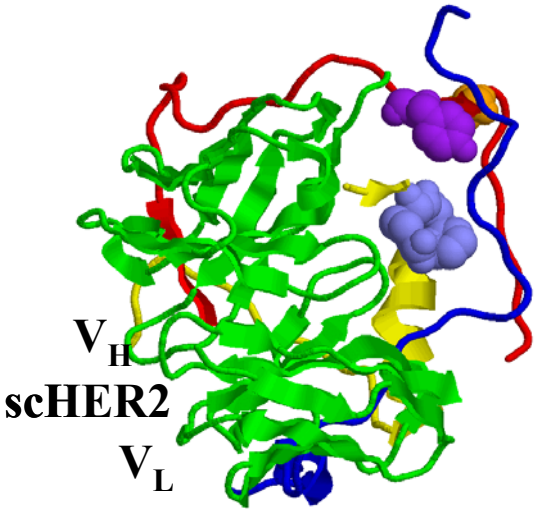

FIG 35: (A) Schematic representation of gD gene carrying the sequence encoding scHER2 flanked by two Ser-Gly linker in place of gD residues from aa 60 to aa 219.

(B) Hypotetical model of chimeric gD structure obtained by combining the 3D structure of scHER2 and gD: scHER2 is green,

R-LM113 and R-LM249 were generated by "BAC Mutagenesis-Procedure". Prior to transfection of recombinant Bacs into mammalial cells the sequences of recombinant gDs were determined following colony PCR amplification.

For virus reconstitution recombinant Bacs were transfected into R6 cells that express glycoprotein D under the control of HSV late promoter $\gamma U_L 26.5$.

The viruses obtained in R6 cells were fenotypically wild type; genotypically they carried recombinant gDs. After several passages in R6 cells, the viruses were passaged in BHK cells and subsequentes in J-HER2 or SKOV3. The viruses grown in cells expressing HER2 were employed for subsequent studies. The results of figure 36 show the ability of R-LM113 and R-LM249 to infect a variety of cells. Cumulatively, R-LM113 and R-LM249 infected only cells expressing HER2 and lost the ability to infect via nectin1.

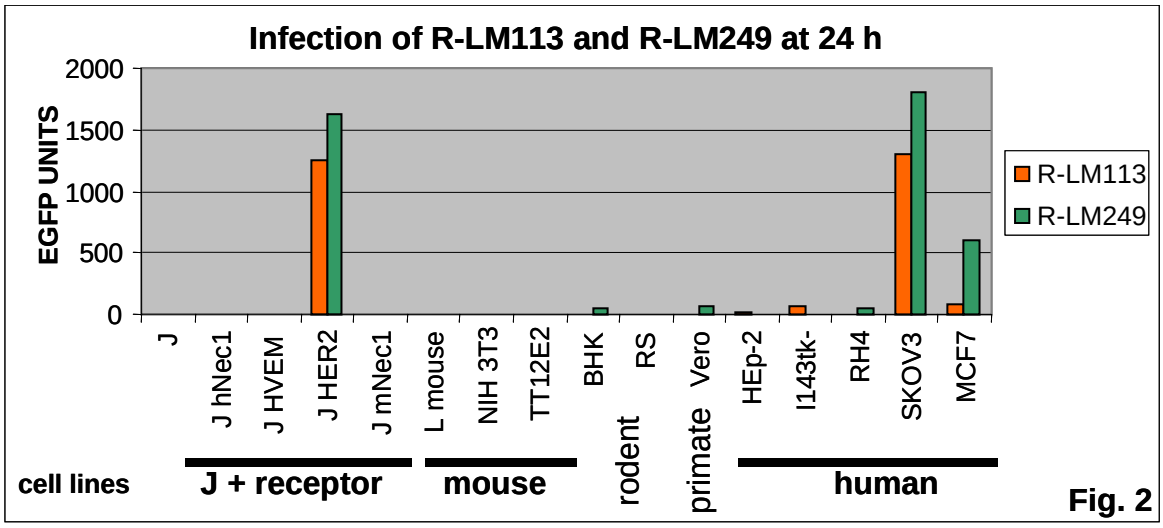

FIG 36: R-LM113 and R-LM249 infection assay of the indicated cell lines. Quantification of infection was made with photos made 24 h post infection analized by Photoshop (Adobe).

To confirm that entry of R-LM113 and R-LM249 recombinants into SKOV3 cells was mediated only by HER2, and not by nectin1, we measured whether infection was inhibited by MAbs to HER2 (herceptin) or by MAb to nectin1 (R1.302). The results in figure 37 clearly show that both R-LM113 and R-LM249 infection was blocked by MAb to HER2 or by a mixture of herceptin and MAb to nectin1, but not by R1.302 alone.

Hence, R-LM113 and R-LM249 entry was not via nectin1, in agreement with the lack of infection of cells expressing only nectin1, like JhNec1 and JmNec1.

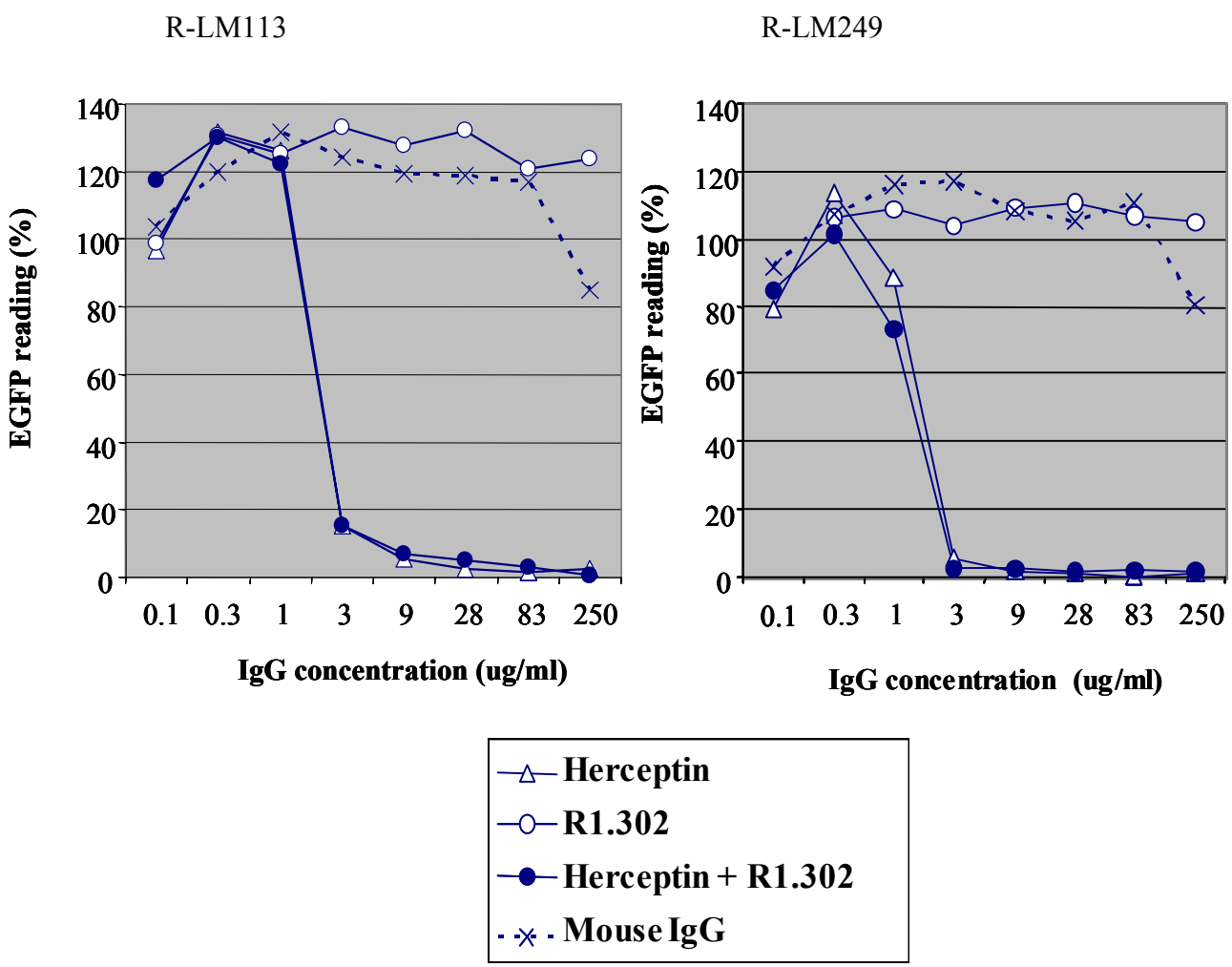

FIG 37: Infection of R-LM113 and R-LM249 in SKOV3 cells is blocked by antibodies to HER2 (Herceptin) and by a mix of Herceptin and antibody to nectin1 (R1.302), but is not blocked by R1.302.
SKOV3 cells grown in 96-well plates were incubated for 2 h on ice with increasing concentrations of purified antibodies (R1.302 to nectin1, Herceptin to HER2, mouse immunoglobulins, or a mixture of R1.302 and Herceptin) and then with the viral inoculum at the multiplicity of infection of 2 pfu/cell for further 90 min on ice. Following virus adsorption, the non-attached virus was removed and cells were overlaid with the same concentration of antibodies or IgGs, rapidly shifetd at 37°C and incubated for 16 h. Infection was quantified as EGFP fluorescence intensity by means of a Victor plate reader (Perkin Elmer). Each point represents the average of assays.

We further checked the electroforetic mobility of chimeric gDs: lysates of SKOV3 cells infected with recombinants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. As control we used lysates of SKOV3 infected by R-LM5, which had gD wild type and EGFP as reporter gene, and by R-LM13, which had gD plus scHER2 between aa 24 and 25 and EGFP as reporter gene. As shown in figure 38, all recombinant gDs had the expected molecular weigh.

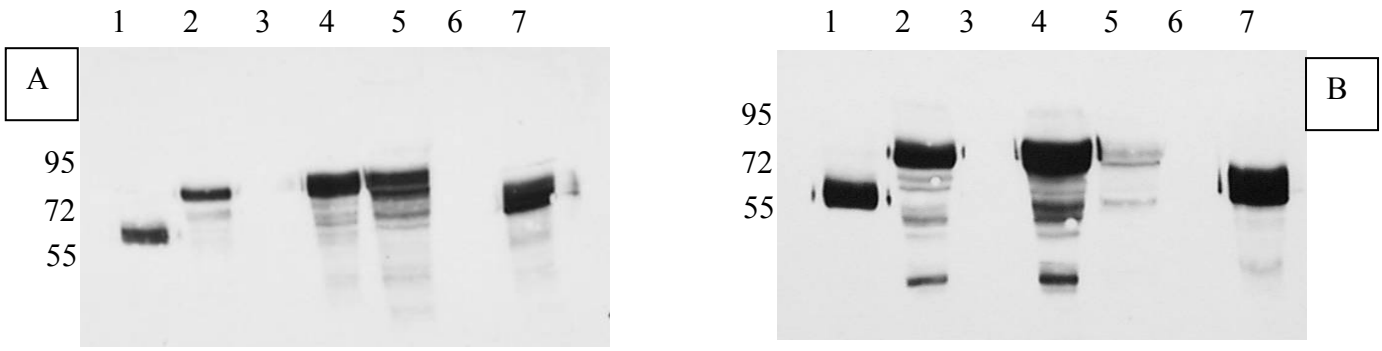

FIG 38: Electroforetic mobility of chimeric gDs visualized by western blotting with MAb BD80 (A) and H170 (B). Lysates from SKOV3 infected by R-LM5 (lane 1), by R-LM13 (lane 2), by R-LM39 (lane 4), by R-LM113 (lane 5), by R-LM249 (lane 7). Lanes 3 and 6 are empty. Numbers to the left and to the right represent migration position of molecular mass markers (in kilodaltons)

In summary, these results provide evidence that both gD sequences, aa 6-38 and aa 61-218:

- could be removed and gD retains its functionality, and recombinant viruses are detergeted from HSV-1 natural receptors, HVEM and nectin1
- could be replaced by an heterologous ligand, like scHER2.

Chapter II: ENGINEERING OF RECOMBINANT HSV-1 RETARGETED TO HER2 RECEPTOR

2.4 Discussion

2.4.1 DISCUSSION

We report on the construction and properties of the following HSV recombinants: R-LM11, R-LM11L, R-LM31, R-LM39, R-LM113, R-LM249 carrying the insertion of a scFv to HER2 and mutations or deletions of gD sequences to modify virus tropism. The aim of the work was the engineering of a HSV-1 retargeted to tumour cells overexpressing HER2 receptor and de-targeted to normal cells expressing HSV-1 natural receptors (HVEM and Nectin1). The key finding is that all these viruses were able to infect cells that express HER2 as the sole receptor (J-HER2), but not in the parental receptor-negative J cells and therefore were redirected to HER2 receptor.

First of all we engineered two recombinant viruses, R-LM11 and R-LM11L, in which scHER2 was inserted between aa 24 and 25 of gD and that differed only for a Gly-Ser linker present in R-LM11L downstream of scFv sequence. We demonstrated that these viruses entered into J-HER2 cells using HER2, were able to form plaques in J-HER2 or CHO-HER2 cells, although at an efficiency several hundredfold lower than that in J-nectin1 or CHO-nectin1 cells, and that their replication in J-HER2 cells was 20-fold lower than that in J-nectin1 cells.

As for the detargeting from natural receptors is conceive, the scHER2 insertion in gD between aa 24 and 25 abolished the ability of HSV to enter cells via the HVEM receptor. The result was somehow unexpected, given that the IL-13 insertion in the same position in gD failed to disrupt the HVEM binding site [66]. In either case, the site of insertion is adjacent to the binding site for HVEM receptor, which was mapped to a continuous region (1-32) that includes the interacting residues 27 to 29 [25,67]. A major difference between the current and previous results was the size of the insert, which was 247 or 256 aa in our constructs and 134 aa in the IL-13–gD chimera. We speculate that the larger size of the scHER2 insert may hinder the flexibility of the gD N terminus such that it prevents the formation of the N-terminal hairpin and therefore the HVEM binding site. By contrast, the scHER2 insertion did not modify the capacity of the recombinants to enter cells via the nectin1 receptor. The nectin1 binding site on gD is not fully characterized yet. A number of substitutions or insertions, at residues 34, 38, 126, 151, 187, 215, 222, 223, 243, 246, etc. [68,62,63], were reported to affect entry through nectin1, as measured by an infectivity complementation assay. However, recombinant viruses specifically defective in entry through nectin1 and still capable of pro-fusion activity remained to be constructed.

Knocking down the recombinant virus ability to infect through nectin1 was our next goal in order to obtain a fully retargeted oncolytic virus with a high safety profile.

To this end, we introduced point mutations at residues reported in the literature as involved in nectin1 binding. The first was V34S mutation into gD of R-LM11L, as this mutation abrogated the capacity of R5141, a recombinant HSV-1 with IL-13 ligand fused to residue 33 of gD to use nectin1 as receptor [64]. However, our recombinant virus designated R-LM31 mantained the ability to infect J-nectin1 cells. We tried to improve the virus by adding to this mutation other point mutations in a different portion of gD and generated R-LM39 recombinant carrying V34S, D215G, R222N and F223I mutations. Moreover R-LM39 had EGFP gene as reporter to monitor cell infection in vivo instead of LacZ gene. For the construction of R-LM31, R-LM39 and the following two recombinat viruses we used the BAC Mutagenesis-Procedure (described in paragraph 2.2.5).

R-LM39 resulted completely detargeted to mouse nectin1, only partially detargeted to rodent nectin1, but it was still able to use primate nectin1. In fact, entry in SKOV3 cells expressing both nectin1 and HER2 could be inhibited only by the exposure of cells to antibodies to both receptors simultaneously.

By an alternative strategy, involving deletions and substitutions of portions of gD, we obtained two viruses completely detargeted to human nectin1: the first virus (R-LM113) is a recombinant HSV-1 in which scHER2 was inserted in place of gD sequence from aa 6 to aa 38. We decided to remove this gD portion on the basis of gD 3D structure. The rationale was that we would remove residues involved in HVEM binding and hinder residues involved in nectin1 binding (215-222-223).

Results confirmed these predictions and R-LM113 resulted retargeted to HER2 and detargeted from both HVEM and nectin1.

R-LM249 was designed following the observation by Zhou and Roizman that the Ig-fold of gD can be deleted, provided that the N- and C-ter portions interact via a heterologous kringle domain. In R-LM249 scHER2 is inserted in place of Ig-like domain of gD. R-LM249 and R-LM113 were able to infect through HER2 as the sole receptor and did not retain the ability to enter via nectin1 and HVEM: in fact, neutralization assays showed that MAb to HER2 was sufficient to block R-LM113 and R-LM249 infection of SKOV3 cells.

The first remarkable aspect of our results is that gD can tolerate an insert almost as big as gD itself, its *M*r can be doubled and its Ig-like domain can be removed without loss of key functions (e.g. profusion activity). In particular, in view of the current model of gD action [69,21,22], results presented here and elsewhere imply that the ability of gD to modify its conformation and trigger fusion takes place independently of whether gD binds to its natural receptors or to foreign receptors for which it carries a ligand.

In the past few years, several efforts to construct viruses retargeted to a number of different cellular receptors have been described. To our knowledge, only two reports illustrated the generation of viruses retargeted to the HER2 receptor.

In one instance, retroviral vector particles derived from spleen necrosis virus were pseudotyped with the antigen binding site of an antibody to HER2 [70]. Compared to the pseudotyped retrovirus, our recombinant viruses are genetically engineered to express the chimeric gD, and therefore any progeny virus at any replication cycle will carry the retargeted envelope. This ensures that the redirected tropism and, consequently, the possible oncolytic activity are maintained for as many viral replication cycles as needed. The second instance concerns a vesicular stomatitis virus recombinant exhibiting a Sindbis virus glycoprotein modified with the scFv to HER2 [47] Compared to small RNA viruses, such as vesicular stomatitis viruses, oncolytic viruses based on HSV have the advantage of a large genome capacity and genetic stability and therefore provide the possibility to deliver to the tumour cells additional heterologous therapeutic or immunomodulatory gene products (e.g., IL-12 or granulocyte-macrophage colonystimulating factor) [42,71].

A remarkable result we achieved is the loss of virus ability to use its natural receptors, human and murine, thus the R-LM113 and R-LM249 recombinants are ready to be assayed in animal models of mammary and ovary tumours, like transgenic mice expressing human HER2 receptor or mammary tumour xenographted nude mice.

This work provides proof of principle that retargeting HSV tropism by fusion of a scFv to gD is feasible, that the insertion can be performed in different positions of gD, and that it is possible to remove and substitute a big portion of gD mantaining its funtionality. In recent years, a high number of single-chain antibodies have been generated, some of which have entered clinical practice. Our finding and the availability of a large number of scFvs greatly increases the collection of potential receptors to which HSV can be redirected. It has been proposed that HSV recombinants that target specific surface markers can be used to visualize the distribution of tumour cells and their metastases in tissues by at least two methods [52]. The first involves the viral thymidine kinase-dependent incorporation of a radioactive precursor. A more attractive approach is to fuse a nonessential tegument protein present in high numbers per virion to GFP or similar molecules capable of being visualized in vivo. The studies presented in this report make the specific targeting of HSV for both oncolytic activity and visualization of tumour cells possible for a large variety of cell surface molecules to which antibodies and scFvs are available.

Chapter III: PRODUCTION, PURIFICATION AND FUNCTIONAL ASSAYS OF RECOMBINANT gHgL HETERODIMER

3.1 Objective II

3.1 OBJECTIVE II

The second project of my thesis centered on production and purification of a recombinant truncated form of the heterodimer gHgL, named $gH_{t5E1\text{-}strep}$gLV5His. To this end, we cloned a stable insect cell line expressing a soluble form of gH in complex with gL, under the control of a metalloprotein inducible promoter and we purified the heterodimer using affinity chromatography.

Next, we asked whether $gH_{t5E1\text{-}strep}$gLV5His exhibits some functional activities: it was capable to bind cell surface.

Chapter III: PRODUCTION, PURIFICATION AND FUNCTIONAL ASSAYS OF RECOMBINANT gHgL HETERODIMER

3.2 Materials and Methods

3.2.1 CELLS

The **S_2** cell line was derived from a primary culture of late stage (20-24 h old) *Drosophila melanogaster* embryos. Many characteristics of S_2 cell line suggest that it is derived from a macrophage-like lineage. S_2 cells grow at 28°C or room temperature without CO_2 as a loose, semi-adherent monolayer in tissue culture flasks and in suspension in spinners flasks using *"The Stovall Low Profile Roller"*. (Invitrogen, Drosophila expression system). The complete medium for S_2 cells is Schneider's Drosophila Medium containing 5-10% heat-inactivated fetal bovine serum. S_2 cells have to be passed always in new flasks when the culture density reaches 6 to $20 \cdot 10^6$ viable cells/ml and they have to be seeded not below a density of $5 \cdot 10^5$ cells/ml.

HeLa is an epithelial cell line derived from cervix adenocarcinoma.

SK-N-SH is a human neuroblastoma cell line.

293T, J, CHOK1, Fcl13 (J-nectin1), RS, COS, I143, Hep-2 have been described previously (par.2.2.1).

3.2.2 PLASMIDS AND CONSTRUCTS

S_2 cell were co-transfected with truncated form of gH and full length gL, cloned into pMT/BiP/V5-His (invitrogen) (fig. 39) and pCoBlast plasmid (invitrogen), which carries blasticidin resistence gene, as selective marker.

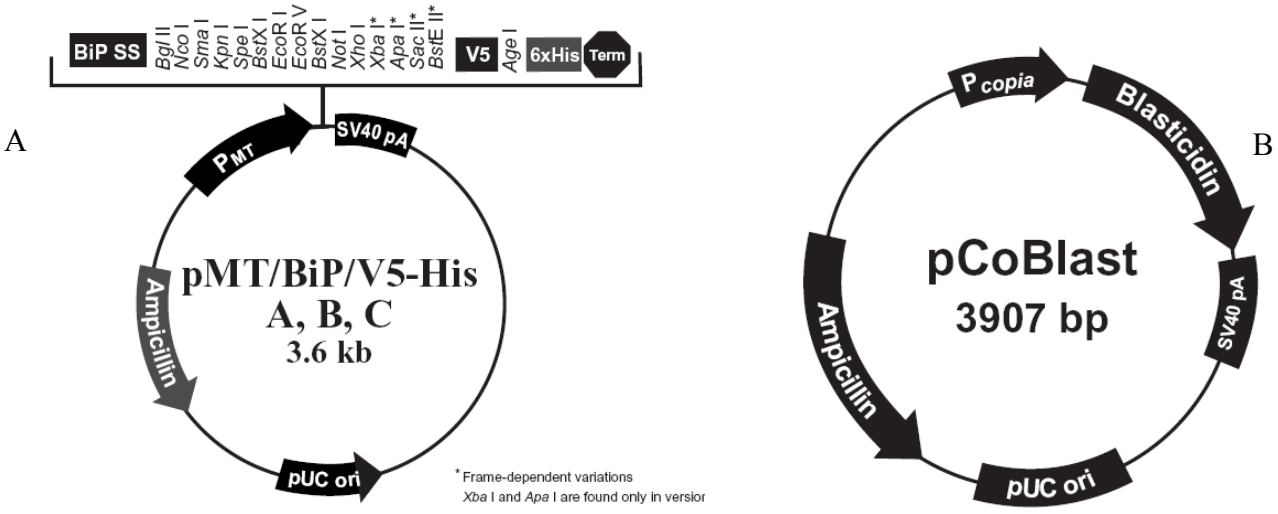

FIG 39: Schematic map of insect expression plasmid pMT/Bip/V5-His (invitrogen) (A) and insect selection plasmid pCoBlast (invitrogen) (B).

To enable gH detection we inserted a heterologous epitope recognized by MAb 5E1 (5E1 epitope), preceded by the consensus for factor Xa protease. Downstream of these sequences, we inserted the ONE-STrEP-tag sequence for purification of recombinant gHgL (herein named $gH_{t5E1\text{-}strep}$ gLV5His) by chromatography to STrEP-tactin resin (IBA).

The linear map of $gH_{t5E1\text{-}strep}$ is shown in figure 40.

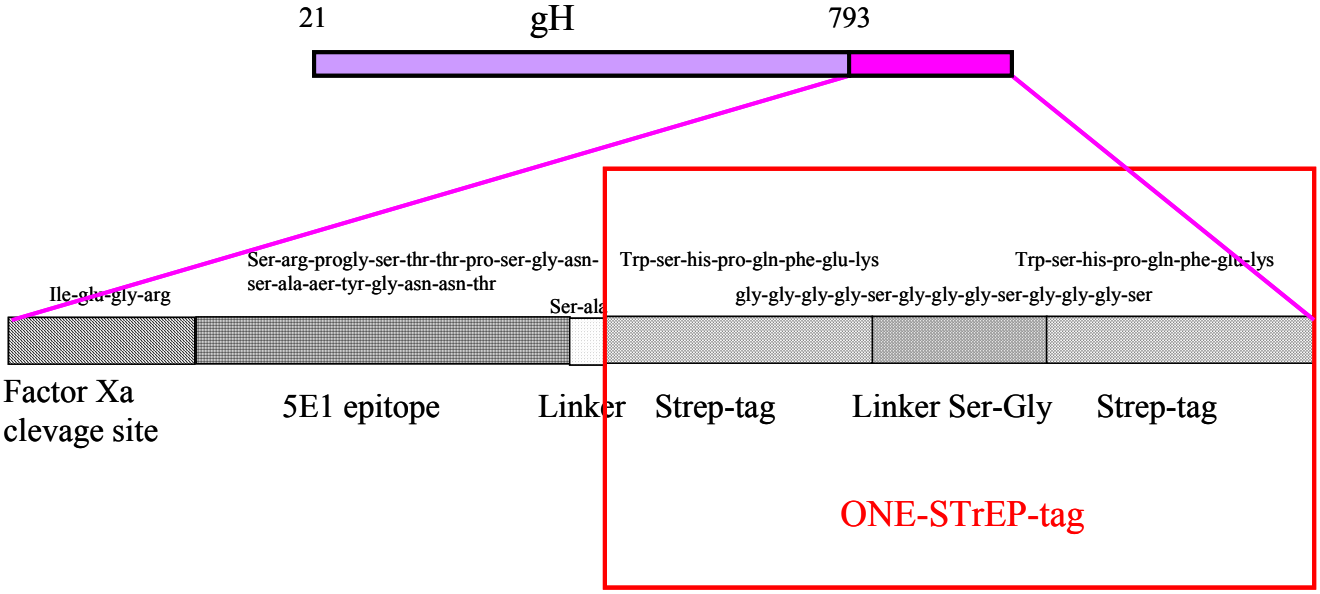

FIG 40: Linear map of recombinant gH

To generate $gH_{t5E1\text{-}strep}$ protein, the appropriate gH sequences (corresponding to aa 21-793 without signal sequence) were cloned in the pMT/BiP/V5His vector (invitrogen), under the drosophila metallothionein promoter and drosophila BiP secretion signal. The $gH_{t5E1\text{-}strep}$ construct was generated by PCR amplification of the gH sequence from viral DNA with the following primers:

gH NotI_forw: GCG TGG GGC GGC CGC CAC GAC TGG ACT GAG C containing the restriction site NotI and *gH XhoI_rev*: CCG TCA TTC ATT TGC TAG CCC TCG AGC ACG CAG CCC containing the restriction site XhoI. The amplimer was digested with NotI and XhoI and cloned in pMT/BiP/V5His vector.

In a second step, the epitope that encodes sequentially the aa consensus for *factor Xa protease-epitope recognized by MAb5E1 and epitope-ONE STrEP tag*, was generated with the following primers:

*ONE-STREPNheI*_forw (containing the restriction site NheI for cloning into gH and the silent site Sal I for screening):

GCC GCG CTA GCC ATC GAA GGG CGA AGT CGA CCA GGA AGC ACT ACA CCC TCT GGG AAC TCT GCA AGG TAT GGG AAT AAC ACA AGC GCT TGG AGC CAC CCG CAG TTC G

ONE-STREPXhoI_rev (containing the restriction site XhoI for cloning into gH) GCC GGC TCG AGT CAT TTT TCG AAC TGC GGG TGG CTC CAC GAT CCA CCT CCC GAT CCA CCT CCG GAA CCT CCA CCT TTC TCG AAC TGC GGG TGG CTC CAA GC.

The amplimer was digested with NheI and XhoI and cloned in pMT/BiP/V5-His containing gH.

The gL-expressing construct (gLV5His) was derived by cloning the appropriate sequence (aa 20- 224 without signal sequence), amplified from HSV-1 DNA by PCR by means of the primers *gL EcoRI_forw*: GTG TGT GAA TTC GGG CTT GCC TTC AAC CG containing the restriction site EcoRI and *gL NotI_rev*: CGG CGC CTC TTG CGG CCG CCT CGA CGG AAA CCC G containing the restriction site NotI which causes gL stop codon mutation (introduction of phe) to allow V5-His expression downstream of gL protein. The amplimer was digested with EcoRI and NotI and cloned in pMT/BiP/V5His vector. The constructs were sequenced for accurancy.

3.2.3 ANTIBODIES

Antibodies to gH we used are: MAb52S (1:200), which recognizes a gL-indipendent discontinuous epitope with critical residues at position 536-537, MAb53S (1:400), which recognizes a discontinuous epitope and strickly requires gL for reactivity, and MAb5E1 (1:1000 in IFA and 1:5000 in western blotting) which recognizes the epitope 5E1 cloned downstream of gH sequence.

Antibodies to gL we used are: MAbL4 (1:400), which recognizes residues 168-178 [84], MAb antiV5 (invitrogen) (1:400 in IFA and 1:5000 in western blottig) which recognizes the epitope present at the C-terminus of gL.

Anti-mouse IgG-FITC (fluorescin-isothiocyanate) and anti-mouse IgG-TRITC conjugated antibodies were from Jackson Immunoresearch.

Peroxidase-conjugated anti mouse IgG horseradish peroxidase linked whole antibody (from sheep) was from GE Healthcare.

3.2.4 SELECTION OF A STABLE CELL LINE

S_2 cells were tranfected, using 150µl of Arrest-in transfection agent (invitrogen) with the following plasmids: 13 µg of $gH_{t5E1\text{-}strep}$, 6 µg of gLV5His and 1 µg of pCoBlast; five days

after transfection S_2 were selected with blasticidin (invitrogen) at a concentration of 5 to 50 μg/ml. Selected cell line was checked for gH-gL expression after induction by western blotting.

3.2.5 INDUCTION OF $gH_{t5E1\text{-}strep}$gLV5His EXPRESSION

To induce expression of $gH_{t5E1\text{-}strep}$gLV5His, we seeded S_2 $gH_{t5E1\text{-}strep}$gLV5His cells at a density of $1x10^7$ cells/ml and added copper sulphate ($CuSO_4$) to the serum-free medium at the final concentration of 500μM. Cells were harvested 7-10 days after induction; the presence of $gH_{t5E1\text{-}strep}$gLV5His in the cell supernatant was assayed by western blotting and dot blotting, after removing cells by centrifugation at 1000xg for 10 min.

3.2.6 WESTERN BLOTTING

To test the production of $gH_{t5E1\text{-}strep}$gLV5His we determined its electrophoretic mobility: the supernatant was boiled in SS (solubilizing solution) containing SDS and β-mercaptoethanol and boiled for 3 minutes. The proteins were separated by denaturing polyacrylamide electrophoresis (PAGE) and transferred onto Hybond-ECL nitrocellulose-membrane (Amersham Biosciences). The membranes were blocked with 5% non-fat dry milk in PBS for 30 minutes at 37°C, washed and reacted with MAb5E1 and with MAbantiV5, followed by peroxidase-conjugated anti mouse IgG horseradish peroxidase linked whole antibody (from sheep) (GE Healthcare) and enhanced chemioluminescence (ECL;Western blotting detection reagents; GE Healthcare).

3.2.7 DOT BLOTTING

To determine the amount of $gH_{t5E1\text{-}strep}$gLV5His produced or purified and to determine the reactivity to antibodies that recognize conformation-dependent epitopes, we performed dot blotting assay using Bio-Dot Microfiltration Apparatus (BIO-RAD): we spotted on a nitro cellulose membrane (Hybond-C Extra Amersham Biosciences) decreasing amounts of samples. The membranes were blocked with 5% non-fat dry milk in PBS for 30 minutes at 37°C, washed and reacted with MAb53S (1 h at 37°C), followed by peroxidase-conjugated anti mouse IgG horseradish peroxidase linked whole antibody (from sheep) (GE Healthcare) and enhanced chemioluminescence (ECL;Western blotting detection reagents; GE Healthcare).

3.2.8 AFFINITY CHROMATOGRAFY

For $gH_{t5E1\text{-}strep}$gLV5His complex purification we used ONE-STrEP-tag system (IBA BioTAGnology): we eluted by means of desthiobiotin, which allows regeneration of the resin. The principle of this system is illustrated in figure 41 and the protocol is detailed in paragraph 3.3.3.

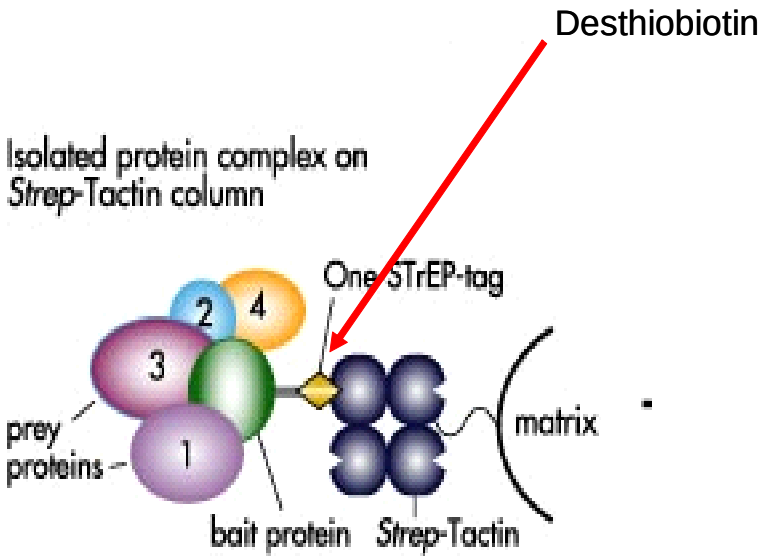

FIG 41: $gH_{t5E1\text{-}strep}$ in complex with gLV5His binds Strep-Tactin thanks to the One-STrEP-tag epitope at its C-term. After washing contaminants present in the medium with washing buffer, $gH_{t5E1\text{-}strep}$gLV5His complex was eluted with elution buffer containing desthiobiotin. Desthiobiotin competes with One STrEP-tag for strep-tactin binding (from IBA).

3.2.9 CELL BINDING ASSAY

The different cell lines described in paragraph 3.2.1 were grown on glass coverslips for 16 h, then they were fixed with ice cold methanol at -20°C for 10 min or with paraformaldeyde at room temperature (RT) for 10 min, permalized with 0,1% Triton X-100 in PBS for 10 min when indicated. Cells were incubated for 1 h at 37°C with 1% BSA in PBS, followed by 1 µM purified $gH_{t5E1\text{-}strep}$gLV5His for 1 h at RT. or with negative control (data not shown). Binding of $gH_{t5E1\text{-}strep}$gLV5His was deteched by indirect immnofluorescence, as described in paragraph 2.2.4.

Chapter III: PRODUCTION, PURIFICATION AND FUNCTIONAL ASSAYS OF RECOMBINANT gHgL HETERODIMER

3.3 Results

3.3.1 GENETIC ENGINEERING OF A SOLUBLE FORM OF $gH_{t5E1\text{-}strep}$gLV5His FOR THE INDUCIBLE EXPRESSION IN INSECT CELLS

To generate an insect cell line expressing $gH_{t5E1\text{-}strep}$ gLV5His, two plasmids were engineered, encoding gH and gL respectively.

The first plasmid ($gH_{t5E1\text{-}strep}$) contained gH (aa 21-793), cloned between the pMT/BiP/V5 His promoter and the C-terminal epitope "ONE-STrEP", essential for the subsequent purification step. The experimental design consisted in insertion of soluble gH sequence into the multiple cloning site of the expression plasmid, then, insertion of "*multiple epitope*" sequence downstream of protein sequence. As described in paragraph 3.2.2, this multiple epitope contains Factor Xa cleavage site to remove all the epitopes, if necessary, 5E1 epitope to detect gH in western blotting and indirect immunofluorescence, and ONE-STrEP-tag for purification step.

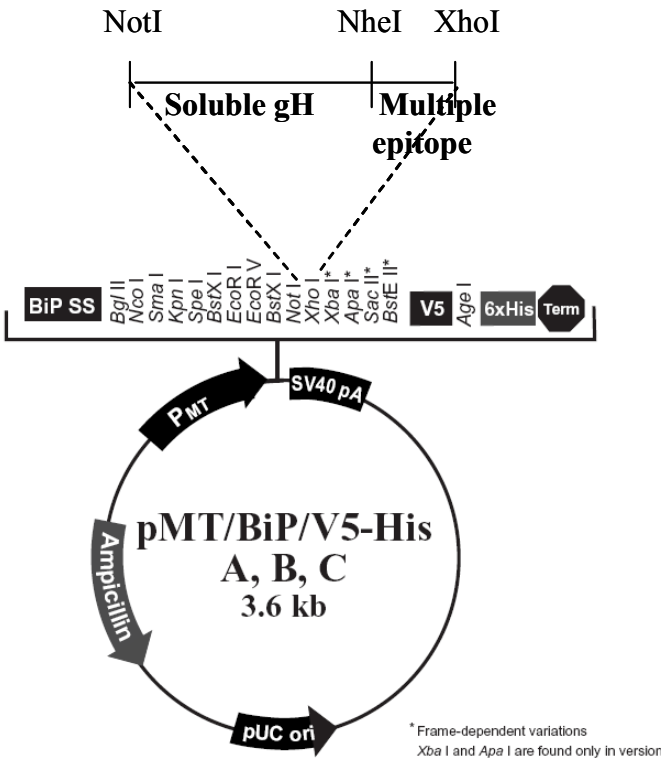

FIG 42: Schematic representation of the construct for gH expression in S_2 cells: soluble gH (aa 21-793) and the multiple epitope (Xa cleavage site-5E1-ONE-STrEP-tag) were cloned into the multiple cloning site under the control of metalloprotein promoter (P_{MT}).

The second plasmid contains gL sequence downstream of the pMT/BiP/V5 His promoter. Thus, gL contains the V5 epitope at its C-terminus, whose sequence was present in the plasmid.

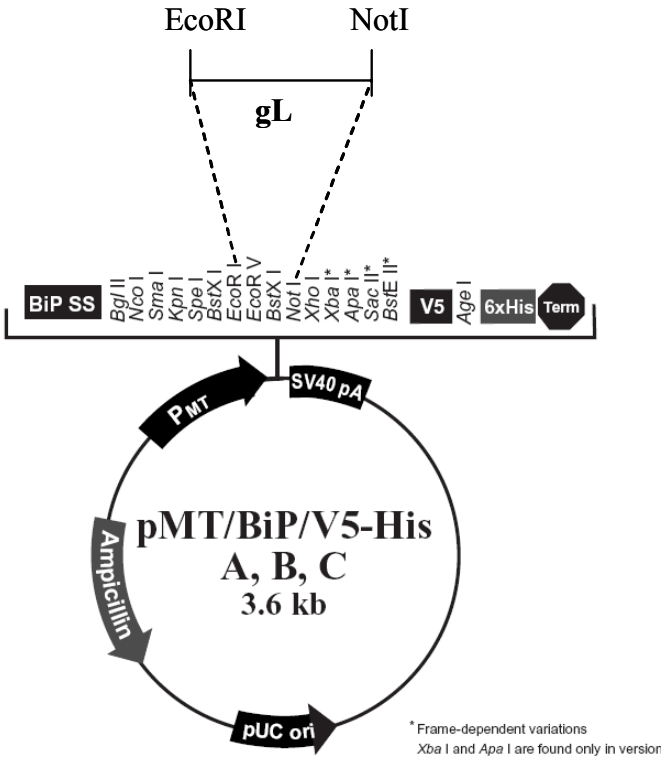

FIG 43: Schematic representation of the construct for gL expression in S_2 cells. gL was cloned into the multiple cloning site under the control of the metallo protein promoter (P_{MT})

3.3.2 CONSTRUCTION OF S_2 INSECT CELL LINE EXPRESSING $gH_{t5E1\text{-}strep}gLV5His$

In order to construct the S_2 insect cell line expressing $gH_{t5E1\text{-}strep}gLV5His$, the two expression plasmids described in paragraph 3.3.1, $gH_{t5E1\text{-}strep}$ and gL-V5His and one selection plasmid (pCoBlast) were co-transfected in S_2 cells by means of arrest-in transfection agent (invitrogen). The transfected cells were selected with 50 µg/ml blasticidin.

As first assay to check whether this S_2 cell line, named S_2 $gH_{t5E1\text{-}strep}$, was able to produce $gH_{t5E1\text{-}strep}gLV5His$, we induced the expression with cupper sulphate ions and the supernatant was assayed for $gH_{t5E1\text{-}strep}gLV5His$ presence by western blotting (fig. 44). We also determined the time of highest expression.

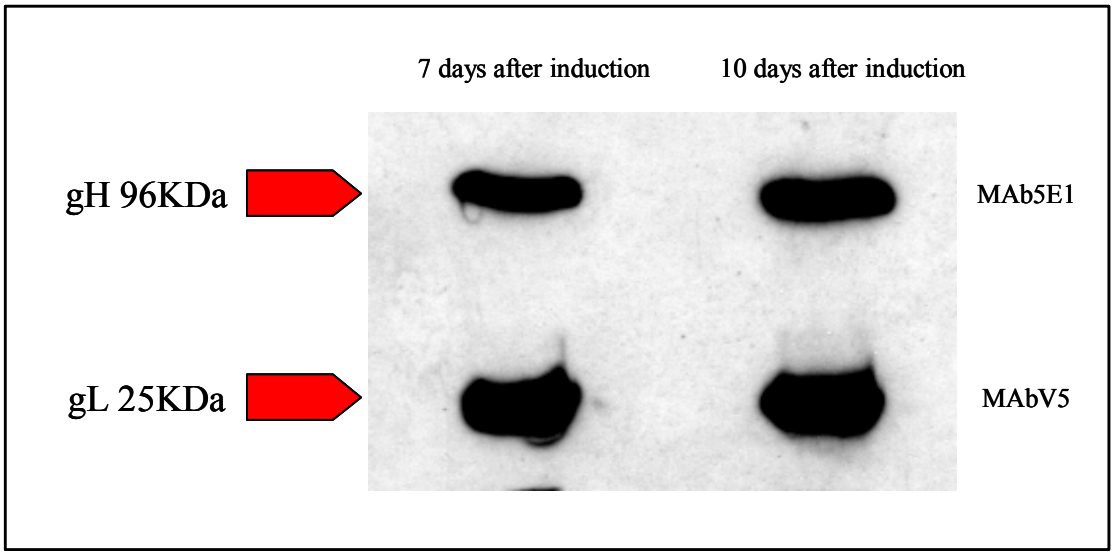

FIG 44: Analysis of supernatant of S_2 $gH_{t5E1\text{-}strep}$ cells: seven and ten days after induction 40 µl of cells were spinned and the supernatant was tested. Amount of $gH_{t5E1\text{-}strep}$ and gLV5His was detected by western blotting with MAbs 5E1 and V5, respectively. Arrowheads indicate migration position.

As shown in figure 44, $gH_{t5E1\text{-}strep}gLV5His$ did not appear degradated and migrated at expected positions.

$gH_{t5E1\text{-}strep}gLV5His$ was also detected by indirect immunofluorescence (data not shown) with antibodies MAb 53S and MAb 52S directed to gH discontinuous epitopes.

These results indicate that S_2 insect cells are the proper instrument to produce $gH_{t5E1\text{-}strep}gLV5His$, and that the secreted protein adopts a correct conformation.

3.3.3 PURIFICATION OF $gH_{t5E1\text{-}strep}$gLV5His BY MEANS OF ONE-STrEP-TAG CHROMATOGRAPHY

$gH_{t5E1\text{-}strep}$gLV5His was purified by means of STrEP Tactin resin. The S_2 $gH_{t5E1\text{-}strep}$ cells at a density of $1 \cdot 10^7$ cells/ml were induced with 500µM $CuSO_4$, according to Invitrogen protocol. Ten days after induction, the medium was collected, filtred with a 50mm Filter Unit (Nalgene), and concentrated 10 times with Centricon Plus-70 (amicon-Millipore). The concentrated medium containing $gH_{t5E1\text{-}strep}$gLV5His was centrifuged and filtred again and brough to pH 8.5.

Moreover to avoid that the biotin contained into the medium could interfere with the binding of $gH_{t5E1\text{-}strep}$ to the resin, we added avidin. $gH_{t5E1\text{-}strep}$gLV5His was absorbed to STrEP Tactin resin over night at 4°C .

The column was then washed with washing buffer (100mM Tris /HCl, 150mM NaCl, 1mM EDTA pH8) and eluted with elution buffer (IBA: 100mm Tris /HCl, 150mM NaCl, 1mM EDTA, 2mM desthiobiotin pH8). 0,5 ml fractions were collected.

The result of purification is shown in figure 45: $gH_{t5E1\text{-}strep}$gLV5His was present in eluted fractions 2-3-4 and 5 (lanes 6-9).

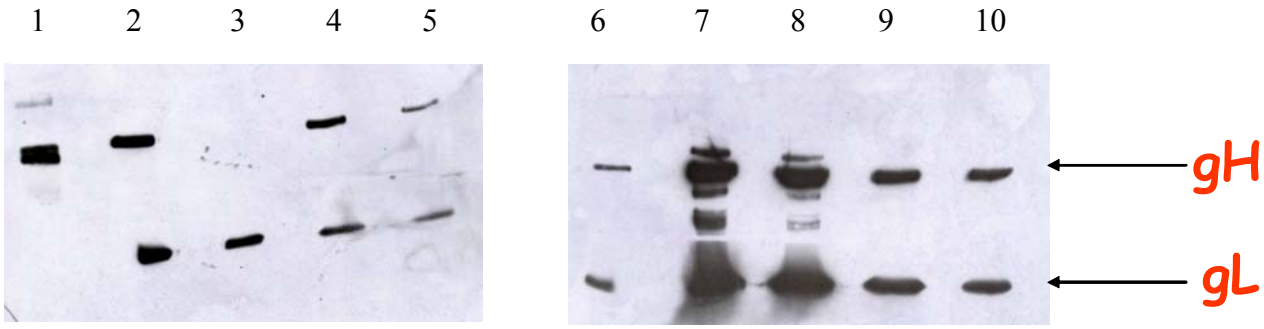

FIG 45: Electrophoretic mobility of $gH_{t5E1\text{-}strep}$gLV5His. Medium containing gHgL (lane 2), unbound fraction (lane 3), wash 2 (lane 4) and eluted fraction from I to VI (from lane 5 to 10) were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and visualized by western blotting with MAb 5E1 against the epitope 5E1 cloned at the C-term of $gH_{t5E1\text{-}strep}$, and with Mab-V5 against the epitope V5 cloned at the C-term of gLV5His, followed by peroxidase-conjugated anti-mouse IgG and ECL. Lane 1 contains gB-5E1 that is the positive control for reactivity of MAb 5E1.

We next analyzed the extent of purification and the yield of purified protein by silver-staining coloration. The results in figure 46 show that only the two bands of gH and gL were present.

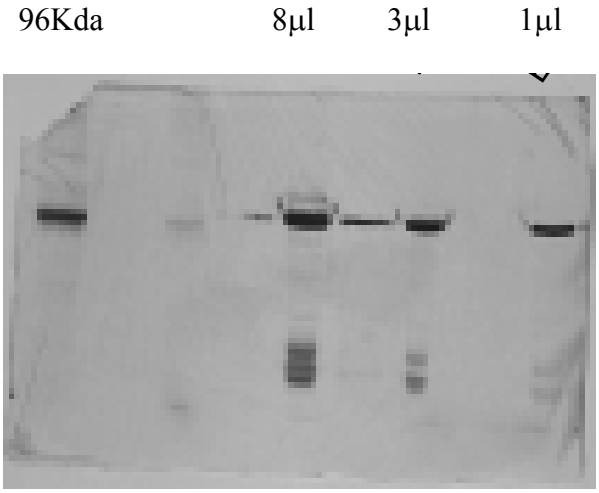

FIG 46: The indicated fractions were subjectd to SDS-PAGE and then developed by silver staining.
The eluted $gH_{t5E1\text{-}strep}$gLV5His contains the indicated volumn of the concentrated 10X mix of E2+E3+E4+E5. First lane contains Molecular mass marker.

We determined the concentration of purified $gH_{t5E1\text{-}strep}$gLV5His by dot blotting, comparing it to a known amount of gHgL (fig. 47) and by protein determination according to *Lowry* [11].

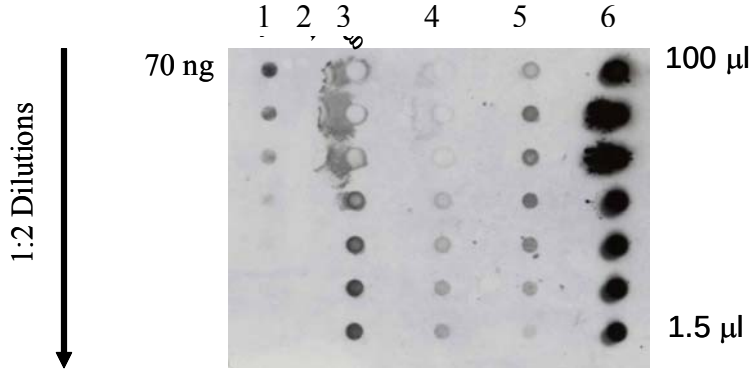

FIG 47: DOT-BLOTTING: Decreasing amounts of $gH_{t5E1\text{-}strep}$gLV5His were spotted to a nitrocellulose membrane (Hybond-C Extra Amersham Biosciences) and visualized with MAb 53S against a conformational dependent epitope of gH. Samples are: known amount of gHgL (lane 1), medium without $gH_{t5E1\text{-}strep}$gLV5His (lane 2), medium containing $gH_{t5E1\text{-}strep}$gLV5His (lane 3), unbound franction (lane 4), wash 2 fraction (lane 5), concentrated 10X mix of E2+E3+E4+E5.

The table 3 summarizes all the purification experiments performed the amount of medium produced, and the yield of purified $gH_{t5E1\text{-}strep}$gLV5His achieved

EXP.	starting medium	purificated protein	yield
1^	300 ml	40 ug	0.133 ug/ml
2^	800 ml	150 ug	0.187 ug/ml
3^	500 ml	290 ug	0.58 ug/ml
4^	2200 ml	400 ug	0.181 ug/ml
5^	800 ml	125 ug	0.156 ug/ml
6^	2400 ml	300 ug	0.125 ug/ml
7^	1000 ml	1200 ug	1.2 ug/ml

TAB 3: The table summarizes all the purification experiments we have done and the yield of production we have obtained.

In conclusion, we developed a protocol for production and purification of $gH_{t5E1\text{-}strep}$gLV5His.

3.3.4 BINDING OF $gH_{t5E1\text{-}strep}$gLV5His TO CELLS

It was of interest to determine whether $gH_{t5E1\text{-}strep}$gLV5His heterodimer purified above exhibited some biological properties. The dot blot assay, shown above, and indirect immunofluorescence assays (data not shown) proved that $gH_{t5E1\text{-}strep}$ was able to interact with conformation-dependent monoclonal antibody, like MAb53S. Here, we asked whether the complex is able to bind cells, in particular cell surface.

In preliminary experiments, we analysed complex binding to permeabilized cells. Thus, fixed and permeabilized cell lines of different types were incubated with $gH_{t5E1\text{-}strep}$gLV5His. The results (fig.48) show the binding of $gH_{t5E1\text{-}strep}$gLV5His occurred to all the cell type tested.

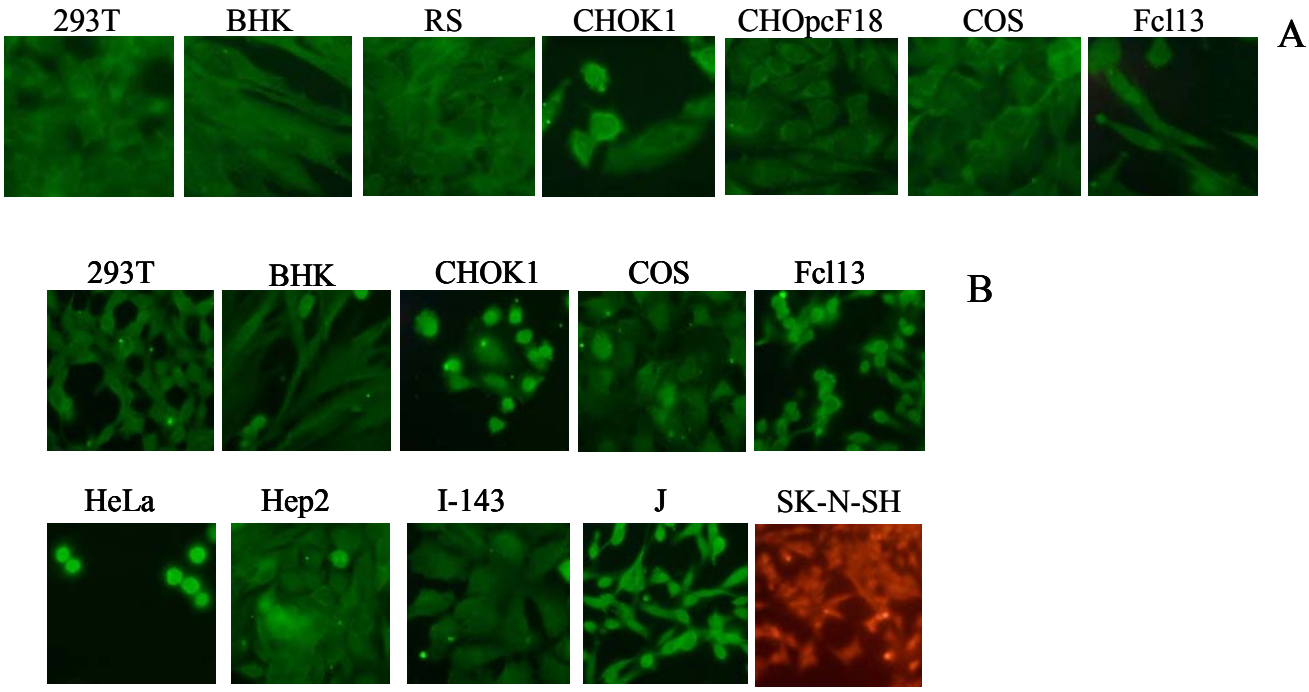

FIG 48: Binding of purified $gH_{t5E1\text{-}strep}$gLV5His on the indicated cell lines. (A) Cells were fixed with methanol incubated for 1h at room temperature with 1 μM $gH_{t5E1\text{-}strep}$gLV5His and reacted with MAb 5E1 to $gH_{t5E1\text{-}strep}$ 5E1 epitope, followed by FITC-conjugated secondary antibody. (B) Cells were fixed with paraformaldehyde, permebilized with Triton 0,1% and reacted with MAb L4 to gL, followed by FITC or TRITC-conjugated secondary antibody. Both in A and in B fluorescence localized in part at the plasma membrane, and in part at the cytoplasm.

Furthermore binding to non permeabilized cells indicated that $gH_{t5E1\text{-}strep}$gLV5His adopts a correct conformation and is capable of binding to cell surface (fig. 49).

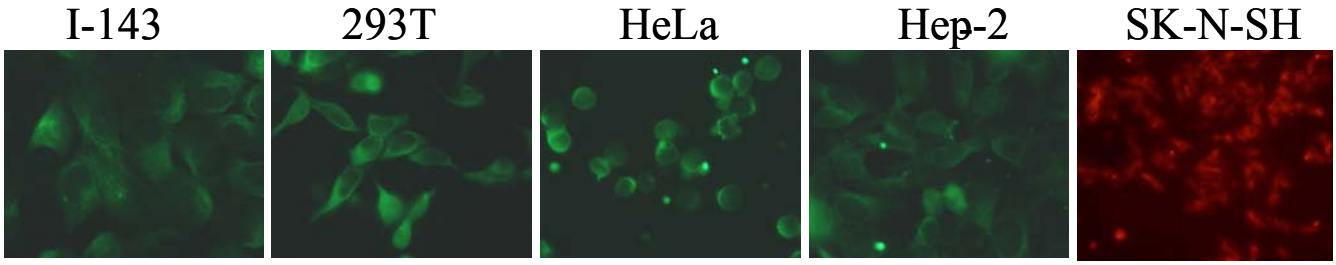

FIG 49: Binding of $gH_{t5E1\text{-}strep}$gLV5His purified at the plasma membrane of indicated cell lines. Cells were fixed with paraformaldehyde, incubated for 1 h at room temperature with 1 μM $gH_{t5E1\text{-}strep}$gLV5His and reacted with MAb L4 to gL, followed by FITC or TRITC-conjugated secondary antibody. Fluorescence localized at the plasma membrane.

Chapter III: PRODUCTION, PURIFICATION AND FUNCTIONAL ASSAYS OF RECOMBINANT gHgL HETERODIMER

3.4 Discussion

3.4.1 DISCUSSION

In the past there have been numerous attempts to produce and purify a soluble recombinant form of gHgL from HSV, as well as from other herpesviruses, in a number of laboratories, including our own. While other major HSV glycoproteins have been produced, purified and their structure solved, the production and purification of gHgL has been a demanding task. Our laboratory has been committed to this project for years, with no success.

The trials that were not successful included production of gHtgL in baculovirus, purification of gHtgL by affinity chromatography to a number of monoclonal antibodies to gH, 52S and 53S, or to the heterologous epitopes, 5E1 and His.

The successful approach described here couples

- the production in a permanent insect cell line,
- the avoidance of constitutive expression, and the choice of inducible expression through a melloprotein promoter,
- the purification by means of the One-STrEP-tag system by IBA.

The yield of produced $gH_{t5E1\text{-}strep}$gLV5His is in the range of about 1mg/lt of medium, e.i. relatively low. Our largest scale of production was 1-2 lt of medium, containing 1 x 10^{10} cells/lt. Production needs to be systematically monitored over the 7-10 days of induction. We have adapted cells to grow in suspension, rather than as monolayers.

The main aim of the work was to obtain a biologically functional form of gH, in order to (i) study biological properties of gH, in particular its role in HSV entry; (ii) determine the crystal structure (to be done in collaboration with Dr. Felix rey, institute Pasteur, France).

With respect to biological function we provide here two lines of evidence:

- $gH_{t5E1\text{-}strep}$gLV5His is capable of reacting to antibodies that recognize conformation dependent epitopes and that neutralize virion infectivity.
- $gH_{t5E1\text{-}strep}$gLV5His is capable of binding a variety cells at the cell surface, implying that cell surface bears a component able to interact with $gH_{t5E1\text{-}strep}$gLV5His. Indeed, the availability of soluble $gH_{t5E1\text{-}strep}$gLV5His makes it feasible to identify more clearly whether gHgL has a cellular partner, and what is the role of this interaction in the process of virus entry. Based on the fact that gH sequence presentes a potential integrin binding motif Arg-Gly-Asp (RGD), future experiments will address the issue whether integrins are involved in gH binding to cell surface. Integrins are indicated as a family of structurally, immunochemically and functionally related cell-surface heterodimeric receptors characterized by the ability of each members to recognize multiple ligands with various recognition sequences. [84]

Parry et al [73] have shown that mutated gH, in which RGD sequence was become Arg-Gly-Glu (RGE), was no more able to bind CHO cells expressing $\alpha_V\beta_3$. Moreover, integrin $\alpha_V\beta_3$ seems to have an importantant role in HHV-8 and HCMV infection. Galdiero et al [80] have demonstarted that a HSV-1 with gH-RGD mutated in gH-RGE is not inhibited with respect to virus entry. These results do not rule the possibility that, as for HCMV also for HSV-1, $\alpha_V\beta_3$-mediated infection depends on a ligand motif different from the typical tripeptide RGD motif [76]. If gH cellular partner was integrin indeed, it could be possible that to destroy this binding it is necessary to mutagenize other sequence of gH...

No doubt, the availability of biologically active purified $gH_{t5E1\text{-}strep}$gLV5His, in sufficient quantities, will speed up the efforts to solve its crystal structure.

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