REGIONS US6 AND US7 OF HERPES SIMPLEX VIRUS TYPE 1 DNA ENCODING GLYCOPROTEINS D AND I MAY INFLUENCE NEUROINVASIVITY

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Summary. – Recombinants were prepared by replacing a 1931 bp region of the *Bam*HI J fragment (0.906 – 0.920) of the pathogenic ANGpath DNA – coding for glycoprotein D (gD) and a part of glycoprotein I (gI) – by the corresponding sequence of nonpathogenic KOS DNA (Kaerner *et al.*, 1991) and tested in DBA/2 mice. The strain ANGpath and the control recombinant ANGpath/gD-gI^{path}, prepared by back transfer of the given ANGpath DNA fragment into ANGpath/gD-gI^{del}lacZ^{+} DNA, were pathogenic after intraperitoneal inoculation. In contrast, mice infected with the strain KOS and the low-pathogenic recombinant ANGpath/gD-gI^{KOS} survived peripheral virus administration. Both the strain KOS and the low-pathogenic recombinant ANDpath/gD-gI^{KOS} spread by bloodstream to spleen, liver and adrenal glands but did not multiply in spinal cord. Nevertheless, the antigen of low-pathogenic recombinant ANGpath/gD-gI^{KOS} was found in retroperitoneal vegetative nerves and ganglia. On the other hand, the strain ANGpath and the pathogenic recombinant ANGpath/gD-gI^{path} multiplied in cerebrospinal nerves and spinal cord causing typical hind leg paralysis.

Key words: herpes simplex virus type 1; glycoproteins D and I; pathogenicity for mice; neural spread

Introduction

Several genes of herpes simplex virus (HSV), but mainly those coding for nonstructural proteins such as thymidine kinase, DNA polymerase, the immediate early transcription proteins (ICP0, ICP4) and latency-associated transcripts have been considered to be connected with the ability of this virus to spread from inoculation site after peripheral administration (reviewed by Rajčáni, 1992). A mutant deleted in the alpha/transcription initiation factor was also nonpathogenic for mice (Steiner *et al.*, 1990). The nonpathogenic strain HFEM as compared to strain F has a deletion at DNA region 0.762 – 0.789 (Rosen *et al.*, 1986; Rosen-Wolff *et al.*, 1988, 1989). A little less attention in this respect was payed to structural glycoproteins. We have previously shown that the gE deletion mutant (Neidhardt *et al.*, 1987) and the gC defective gE deletion mutant (Schranz *et al.*, 1989) failed to spread along nerves in mice (Rajčáni *et al.*, 1990*a*) and rabbits (Kúdelová *et al.*, 1991). The ANGpath recombinant in which the *Bam*HI G fragment–carrying the gene for gB – was replaced by the corresponding fragment from KOS DNA was nonpathogenic for mice (Košťál *et al.*, 1994) and rabbits (Kúdelová *et al.*, 1991). Here we bring evidence that replacement of ANGpath gD gene and a part of adjacent gI gene by coresponding KOS DNA sequences yields a recombinant with abolished neuroinvasivity after peripheral administration. Preparation of the ANGpath/gD-gI^{KOS} construct in which the gD^{path} sequence and a part of gI^{path} sequence were replaced by the corresponding KOS DNA sequences was reported elsewhere (Kaerner *et al.*, 1991).

Materials and Methods

Viruses. Strain ANGpath was the pathogenic variant of wilde type strain ANG prepared as described (Kaerner *et al.*, 1983). Wild type strain KOS originated from WHO Collaborating Center for Virus Research, Houston, TX. Recombinants ANGpath/gD-gI^{KOS} (full designation ANG/gD-gI^{del}lacZ^{+}/gD-gI^{KOS}) and ANG/gD-gI^{KOS} (full designation ANG/gD-gI^{del}lacZ^{+}/gD-gI^{KOS}) were prepared as

Table 1. Virus recombinants with exchanged gD or gB gene

Designation	Recipient virus DNA	DNA fragment transferred	Phenotype	Pathogenicity
ANGpath/gD-gI^{KOS}	ANGpath/gD^{del}lacZ^{+}	BamHI J^{KOS}	gD^{KOS}, gI$^{KOS/path}$	low
ANGpath/gD-gI^{path}	ANGpath/gD^{del}lacZ^{+}	*Bam*HI J^{path}	gD^{path}, gI^{path}	medium high
ANGpath/gB^{KOS}	ANGpath	*Bam*HI G^{KOS}	gB^{KOS}	low
ANG/gD-gI^{path}	ANG/gD^{del}lacZ^{+}	BamHI J^{path}	gD^{path}, gI^{path}	high

Table 2. Lethality for mice of HSV-1 strains and their gD-gI and gB recombinants inoculated by ip route

Virus	Lethality at days p.i.							Total (%)
	2	3	4	5	6	8	9	
ANGpath	0	0	1	3	4	2	0	10/10 (100)
ANGpath/gD-gI^{path}	0	0	2	2	2	4	3	13/26 (50)
ANGpath/gD-gI^{KOS}	0	0	0	0	0	0	0	0/26
KOS	0	0	0	0	0	0	0	0/15
ANGpath/gB^{KOS}	0	0	0	0	0	1	0	1/10 (10%)
ANG/gD$^{path(a)}$	0	0	1	3	2	2	0	8/10 (80%)

[a]Not used in further pathogenetic studies.

described (Kaerner *et al.*, 1991). A control recombinant ANGpath/gD-gI^{path} was also prepared by recombination of the *HindIII-BalI* subfragment of the *Bam*HI J^{path} fragment with ANGpath/gD-gI^{del}lacZ^{+} DNA and grown in BSC-1 cells expressing gD. Another control recombinant ANGpath/gB^{KOS} was prepared by recombination of the *Bam*HI G^{KOS} fragment and ANGpath DNA (Weise *et al.*, 1987) and selected by means of monoclonal antibody (MoAb) B6, which reacts with gB^{KOS} but not with gB^{ANGpath}, and according to the syn^{+} phenotype (Holland *et al.*, 1983). Virus strains their recombinants are listed in Table 1.

Animals. Six week-old DBA/2 mice were kept on a standard diet. They were infected by intraperitoneal (ip) route with 2×10^6 PFU of each virus or recombinant in 0.1 ml. On days 2,4,6 and 9 p.i., the organs (tissues) listed below were removed from at least 3 or more mice and tested by infectivity titration or examined morphologically. Following organs (tissues) were taken: intestines and mesenterium, liver, spleen, retroperitoneal connective tissue with aorta and large vessels, kidneys and suprarenal glands, lumbar cord, lumbar segment of spinal column, and brain stem.

Virus infectivity titrations. Organ suspensions (10%) were prepared in Eagle's Minimal Essential Medium supplemented with 2 mmol/l L-glutamine and antibiotics. The suspensios were sonicated and titrated as described elsewhere (Košťál *et al.*, 1994).

Immunofluorescence (IF) and immunoperoxidase (IP) stainings were performed as described by Košťál *et al.* (1994).

Results

Pathogenicity of individual recombinants for mice

At least 10 mice were inoculated by ip route with each virus or recombinant and observed for 10 days. It can be seen in Table 2 that the transfer of the fragment *Bam*HI J^{K} (carrying the gD-gI gene) into ANGpath DNA abrogated t pathogenicity of the ANGpath strain to nil, while the transf of the fragment *Bam*HI G^{KOS} (carrying the gB gene) c creased the pathogenicity by 90%. The "control" recomb nant ANGpath/gD-gI^{path} was pathogenic in 50% of anima indicating problems at obtaining this recombinant in a h mogeneous state.

Distribution of infectious virus at different intervals p

ANGpath strain and the pathogenic recombinant ANG path/gD-gI^{path} showed a similar distribution. Between da 2 – 3 p.i., they spread by bloodstream to spleen, liver a adrenal glands; later on, by days 4 – 9 p.i., they eviden multiplied in CNS (Table 3). In contrast, KOS strain and t low-pathogenic recombinants ANGpath/gD-gI^{KOS} a ANGpath/gB^{KOS} spread only via bloodstream to adrena liver, and spleen, but showed no invasion of CNS (Table Thus, the ability of ANGpath strain to spread to and replica in spinal cord after ip inoculation was abolished or signi cantly reduced when DNA fragments from KOS stra carrying either the gB or the gD gene replaced the origi sequence in ANGpath DNA.

Distribution of HSV antigen in mice after ip inoculati

The presence of HSV antigen was followed by IF and staining of semiserial sections (at least 8 per block) intervals from day 2 to 6 p.i., in survivors also on day 9 p With each virus recombinant local spread occured in t

Table 3. Distribution of ANGpath strain and its pathogenic recombinat ANGpath/gD-gI^{path} in mice after ip inoculation

Virus Organ (tissue)	Virus titers at days p.i.				
	2	3	4	6	9
ANGpath					
Mesenterium	9×10^3	nd	neg	neg	ns
Spleen	7×10^3	1×10^2	neg	neg	ns
Liver	2×10^2	3×10^3	neg	neg	ns
Kidneys	7×10^3	1×10^2	neg	neg	ns
Adrenal gland	2×10^4	2×10^5	2×10^4	5×10^4	ns
Spinal cord	nd	nd	2×10^1	5×10^2	ns
Brain stem	nd	nd	neg	2×10^2	ns
Rest of brain	nd	nd	neg	neg	ns
ANGpath/gd-gI^{path}					
Mesenterium	2×10^2	neg	neg	neg	neg
Spleen	1×10^3	2×10^3	neg	neg	neg
Liver	2×10^3	neg	neg	neg	neg
Kidneys	2×10^1	neg	neg	neg	neg
Adrenal gland	2×10^5	5×10^2	5×10^3	5×10^1	neg
Spinal cord	neg	5×10^1	5×10^2	5×10^2	neg
Brain stem	neg	neg	5×10^1	neg	neg
Rest of brain	neg	neg	neg	neg	neg

Virus titers expressed in PFU per whole organ sample (adrenals, spinal cord) or per 0.1 g tissue. No virus found in dead mouse (compare with Table 6). nd – not done; ns – no survivors; neg = $<1 \times 10^0$.

Table 4. Distribution of KOS strain and of low-pathogenic recombinants ANGpath/gD-gI^{KOS} and ANGpath/gB^{KOS} in mice after ip inoculation

Virus Organ (tissue)	Virus titers at days p.i.				
	2	3	4	6	9
KOS					
Mesenterium	neg	neg	neg	neg	neg
Spleen	5×10^3	neg	neg	neg	neg
Liver	2×10^3	neg	neg	neg	neg
Kidneys	neg	neg	neg	neg	neg
Adrenal gland	5×10^4	3×10^2	5×10^2	neg	neg
Spinal cord	neg	neg	neg	neg	neg
Brain stem	neg	neg	neg	neg	neg
Rest of brain	neg	neg	neg	neg	neg
ANGpath/gD-gI^{KOS}					
Mesenterium	1×10^2	5×10^2	neg	neg	neg
Spleen	1×10^3	1×10^2	neg	neg	neg
Liver	5×10^2	neg	neg	neg	neg
Kidneys	1×10^1	neg	neg	neg	neg
Adrenal gland	5×10^3	3×10^5	5×10^4	neg	neg
Spinal cord	neg	neg	neg	neg	neg
Brain stem	neg	neg	neg	neg	neg
Rest of brain	neg	neg	neg	neg	neg
ANGpath/gB^{KOS}					
Mesenterium	6×10^3	nd	neg	neg	
Spleen	3×10^3	nd	neg	neg	
Liver	3×10^3	nd	neg	neg	
Kidneys	8×10^3	nd	3×10^3	neg	
Adrenal gland	3×10^5	nd	2×10^3	neg	
Spinal cord	neg	nd	neg	neg	
Brain stem	neg	nd	neg	neg	
Rest of brain	neg	nd	neg	neg	

For legend see Table 3.

mesenterial tissue to the outer layers of intestinal wall. The nonpathogenic strain KOS and the low-pathogenic recombinants ANGpath/gD-gI^{KOS} and ANGpath/gB^{KOS} spread via bloodstream to adrenal glands, spleen and, less regularly, to liver (Table 5). In addition, the antigen of latter recombinants was also seen in retroperitoneal vegetative nerves and ganglia but not in dorsal root ganglia and spinal cord. ANGpath strain and the "control" pathogenic recombinant ANGpath/gD-gI^{path} showed the same distribution between days 2 – 3 p.i., later on (between days 4 – 9 p.i.), they also appeared in cerebrospinal nerves and ganglia, in spinal cord and occasionally in brain stem (Table 6).

Morphological findings in organs of infected mice

HSV-1 antigen was seen in mesenterial connective and adipose tissue (fibrocytes, mononuclear cells, adipose cells), and in outer layers of intestinal wall (Fig. 1). In the latter case it was present in subserosa, smooth muscle cells, and occasionally in neurons of the myenteric plexus. In the spleen positive mononuclear cells were found in red pulp and reticulum cells of sinuses. Antigen – positive cells in the liver were mainly hepatocytes; positive elongated Kupffer cells were seen in the wall of sinuses, positive fibrocytes in interstitial connective tissue surrounding the branches of portal vein and scattered positive endothelium cells were also found. Abundant antigen was present in adrenals: foci of parenchymal cells were regularly positive mainly in *zona fasciculata*, but later on all three cortical layers became involved. In adrenal medulla trabecular cells as well as endothelium cells were positive (Fig. 2). Retrop-

Table 5. Distribution of HSV-1 antigen in mice infected with KOS strain and low-pathogenic recombinants ANGpath/gD-gI^{KOS} and ANGpath/gB^{KOS}

Virus Organ (tissue)	Antigen at days p.i.				
	2	3	4	6	9
KOS					
Mesenterium	+	+	+	–	–
Intestinal wall	+	+	+	–	–
Spleen	+	+	–	–	–
Liver	–	–	–	–	–
Adrenal gland	+	+	+	–	–
Vegetative ganglia[a]	–	–	–	–	–
Spinal ganglia	–	–	–	–	–
Brain stem	–	–	–	–	–
ANGpath/gD-gI^{KOS}					
Mesenterium	+	+	–	–	–
Intestinal wall	+	+	–	–	–
Spleen	+	+	–	–	–
Liver	+	+	–	–	–
Adrenal gland	+	+	+	–	–
Vegetative ganglia[a]	–	–	+	–	–
Spinal ganglia	–	–	–	–	–
Spinal cord	–	–	–	–	–
Brain stem	–	–	–	–	–
ANGpath/gB^{KOS}					
Mesenterium	+	nd	–	–	nd
Intestinal wall	+	nd	–	–	nd
Spleen	+	nd	–	–	nd
Adrenal gland	+	nd	+	–	nd
Vegetative ganglia[a]	–	nd	+	–	nd
Spinal ganglia	–	nd	–	–	nd
Spinal cord	–	nd	–	–	nd
Brain stem	–	nd	–	–	nd

[a]Paraaortal and retroperitoneal ganglia, occasionally also paravertebral sympathetic ganglia; (+) = antigen present; (-) = antigen absent. For other abbreviations see Table 3.

Table 6. Distribution of HSV-1 antigen in mice infected with ANGpath strain and the pathogenic recombinant ANGpath/gD-gI^{path}

Virus Organ (tissue)	Antigen at days p.i.				
	2	3	4	6	9
ANGpath					
Mesenterium	+	+	+	–	ns
Intestinal wall	+	+	+	–	ns
Spleen	+	+	–	–	ns
Liver	+	+	–	–	ns
Adrenal gland	+	+	+	–	ns
Vegetative ganglia[a]	–	–	+	++	ns
Spinal ganglia	–	–	+	+	ns
Spinal cord	–	–	+	+	ns
Brain stem	–	–	–	+	ns
ANGpath/gD-gI^{path}					
Mesenterium	+	+	+	–	–
Intestinal wall	+	+	+	+	+
Spleen	+	+	–	–	–
Liver	+	+	–	–	–
Vegetative ganglia[a]	–	–	+	+	+
Spinal ganglia	–	–	+	+	+
Spinal cord	–	–	+	–[b]	+
Brain stem	–	–	+	–[b]	+

[a]Paraaortal and retroperitoneal ganglia, occasionally also paravertebral sympathetic ganglia. [b]Possibly, this finding reflects the lower lethality of this recombinant. For abbreviations see Tables 3 and 5.

eritoneal vegetative nerves and ganglia accompanying large arteries showed positive staining of Schwann cells, neurons and satellite cells from day 4 p.i. in mice infected with ANGpath strain or either recombinant but not in mice inoculated with KOS strain. The HSV-1 antigen could be clearly distinguished in nuclei as well as in cytoplasm of large vegetative neurons (Fig. 3).

Spinal nerves, spinal ganglia and roots were involved in lower thoracic and lumbal levels in mice infected with ANGpath strain and the pathogenic "control" recombinant ANGpath/gD-gI^{path}. Spinal cord at lumbal segments was also positive from day 4 p.i., namely a few neurons and foci of glial cells in lateral and posterior horns, and glial cells in lateral and posterior columns (Fig. 4). In brain stem the antigen-positive cells were neurons and glial elements located near to the bottom of IVth ventricle and below meninges indicating probable spread of virus within the cerebrospinal space.

Discussion

Glycoprotein D is an essential component of HSV envelope which stimulates synthesis of neutralizing antibody and promotes virus adsorption and penetration into susceptible cells (Noble *et al.*, 1983). Up to 8 epitopes were recognized with MoAbs (Eisenberg *et al.*, 1982), from which the group VII antibody neutralized virus infectivity when reacting with a type common epitope between aminoacids (aa) 8 – 23

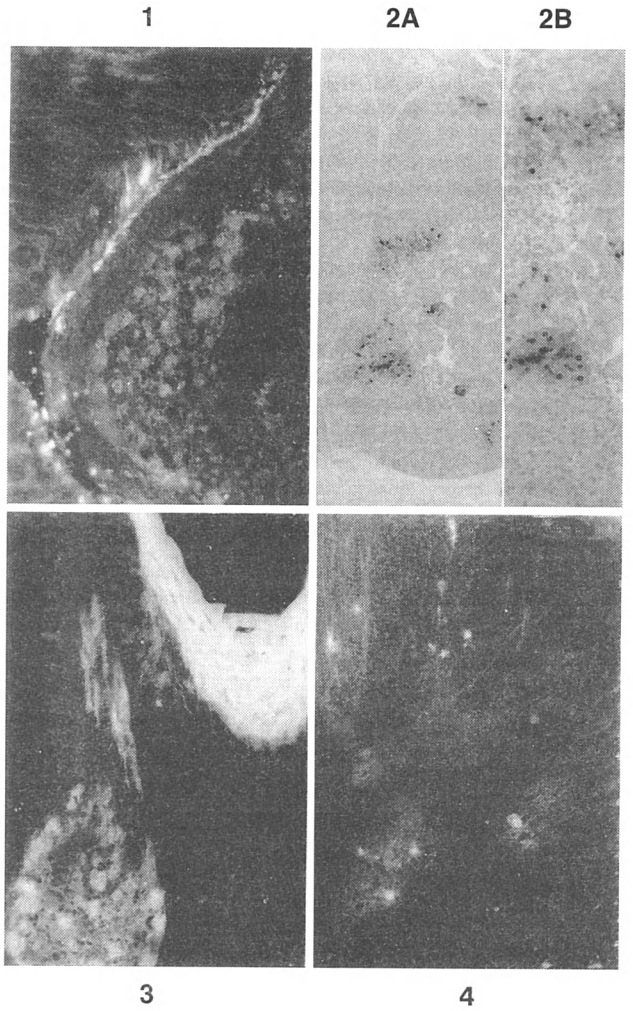

Fig. 1
KOS strain-infected mouse by day 2 p.i.
Viral antigen in subserosa and in the outer smooth muscle layer of intestinal wall. Indirect IF test. (Magn. × 240).

Fig. 2
ANGpath strain-infected mouse by day 4 p.i. IP staining
Viral antigen in parenchymal cells of adrenal cortex and in trabecular cells of adrenal medulla (A, magn. × 240). Enlarged area shows the presence of antigen in nuclei and cytoplasm of parenchymal cells in *zona fasciculata* and *reticularis* as well as in a few trabecular cells of adrenal medulla (B, magn. × 600).

Fig. 3
ANGpath/gD-gI^{KOS} recombinant virus-infected mouse by day 4 p.i.
Positive IF of viral antigen in a paraaortal vegetative ganglion (neurons, perineural satellites, Schwann cells of the cross-cut nerve). Nonspecific fluorescence of the counterstained large artery (upper right corner,magn. × 600).

Fig. 4
ANGpath/gD-gI^{path} control recombinant-infected mouse by day 9 p.i.
Positive IF of viral antigen in glial cells of white matter (dorsal columns) and in neurons of gray matter (dorsal and lateral horns) of spinal cord at lumbal level. (Magn. × 200).

(Cohen *et al.*, 1984). Sera from mice immunized with HSV-1 reacted with 12 epitopes from which peptides at aa 10 – 24, 260 – 284 and 340 – 354 corresponded to continuous epitopes of groups VII, II and II, respectively as defined by MoAbs (Geerlings *et al.*, 1990). Epitopes of groups I, III, IV and VI are discontinuous and cannot be recognized within denatured gD (Cohen *et al.*, 1986). The gD gene was mapped at US6 (McGeoch *et al.*, 1985).

When neutralized with anti-gD antibody, HSV-1 virions would attach to but would not penetrate into susceptible cells (Fuller and Spear, 1987). Mouse and human cells transfected with the gD DNA express gD on their surface and are resistant to challenge with HSV-1 (Johnson and Spear, 1989). Soluble forms of gD bind to the limited number of cell surface receptors and inhibit entry to cells (Johnson *et al.*, 1990). However, the adsorption of HSV-1 to susceptible cells involves more numerous attachment sites in addition to gD – dependent binding. A recombinant virus was prepared in which gD and a part of gI genes were replaced by beta-galactosidase sequences. The gD – defective particles underwent envelopment and release from conventional Vero cells, but they were unable to penetrate other cells and initiate there a replication cycle (Ligas and Johnson, 1989). In the presence of polyethylene glycol they induced plaque production suggesting that gD may be essential for penetration. The critical region was mapped to the group VII epitope where mutation of aa 25 altered the properties of gD (Campadelli-Fiume *et al.*, 1990).

Probably gD as well as gB cooperate at adsorption and entry of virions into susceptible cells. Other glycoproteins such as gH (Buckmaster *et al.*, 1984) as well as gE and gI (Johnson *et al.*, 1988*a*) may be also involved. It seems reasonable to assume that HSV gB and gC interact with different kinds of receptor molecules at cell surface and that gD is more involved in promoting endocytosis and membrane fusion (Johnson *et al.*, 1988*b*). The nature of cell receptors is unclear, though it was described that HSV interacts with the fibroblast growth factor (Kaerner *et al.*, 1990). Much less is known about neural receptors, but dopamine receptors might be considered as possible candidates because drugs like haloperidol inhibit the binding of HSV to brain cells (Shaskan *et al.*, 1984; 1987).

We investigated the spread in mice of ANGpath recombinants in which either gB or gD were replaced by the corresponding KOS sequences. Following ip administration, recombinants ANGpath/gB^{KOS} and ANGpath/gD-gI^{KOS} replicated in vegetative retroperitoneal ganglia but not in spinal ganglia and spinal cord. This difference does not concern the ability of ANGpath strain to spread via axons, because KOS strain as well as recombinant ANGpath/gB^{KOS} were able to establish latency in mice (Rajčáni *et al.*, 1990; Košťál *et al.*, 1994). Neurovirulent strains not only spread along axons, they also replicate in nonneural cells such as

Schwann cells of peripheral nerves (Rajčáni and Conen, 1972), or in astrocytes of nerve roots at the transition of peripheral nerve structure to the structure of CNS (Townsend and Baringer, 1978; Openshaw and Ellis, 1983). As demonstrated here, the low-pathogenic recombinants in which either gB or gD sequences in ANGpath DNA were replaced by the corresponding KOS sequences have lost the ability to multiply in neurons and supportive cells of CNS, cerebrospinal nerves and ganglia. Interestingly, their ability to multiply in nonneural cells and neurons of autonomous (vegetative) nervous system remained unaltered. This discrepancy cannot be explained by more excessive supply of HSV-1 virions for autonomous nerve endings in the intestinal wall, mesenterium and adrenal medulla (Hill *et al.*, 1986), as these structures revealed the presence of viral antigen in KOS strain–infected animals which showed no involvement of vegetative ganglia. In addition, the dramatic increase of lethality of the recombinant ANG/gD-gI^{path} for mice deserves further attention. Izumi and Stevens (1990) found that gD sequence of ANG strain differs from that of ANGpath strain by a mutation in codon GCC (gD^{ANG}) to GGC (gD^{path}), which changes alanine to glycine at aa 84 of the polypeptide chain. Keeping in mind that neuroinvasivity of different HSV strains is a complex phenomenon, there is reasonable to assume that it can be influenced by essential glycoproteins such as gB (Bergstrom, 1991; Košťál *et al.*, 1994), gE (Rajčáni *et al.*, 1990; Kúdelová *et al.*, 1991) and possibly gD in cooperation with other envelope components known to be associated with virus adsorption and penetration to susceptible cells (gI, gH).

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