REPLACEMENT OF GLYCOPROTEIN B GENE IN THE HERPES SIMPLEX VIRUS TYPE 1 STRAIN ANGpath DNA BY THAT ORIGINATING FROM NONPATHOGENIC STRAIN KOS REDUCES THE PATHOGENICITY OF RECOMBINANT VIRUS

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Summary. – Herpes simplex virus type-1 (HSV-1) strain ANGpath and its recombinants, in which the 8.1 kbp *Bam*HI G restriction fragment (0.345 – 0.399) containing the glycoprotein B (gB^{path}) gene (UL27) or its subfragments–coding either for cytoplasmic or surface domains of gB-had been replaced with the corresponding fragments from nonpathogenic KOS virus DNA (gB^{KOS}), were tested for their pathogenicity for DBA/2 mice and rabbits. The recombinant ANGpath/$B6^{KOS}$ prepared by transferring the 2.7 kbp *Sst*I-*Sst*I subfragment (0.351 – 0.368) of the *Bam*HI G^{KOS} fragment still had the original sequence of ANGpath DNA coding for the syn^3 marker in the cytoplasmic domain of gB and was pathogenic for mice as well as for rabbits. Virological and immunohistological studies in DBA/2 mice infected with the latter pathogenic recombinant and with ANGpath showed the presence of infectious virus and viral antigen at inoculation site (epidermis, subcutaneous connective tissue and striated muscle in the area of right lip), in homolateral trigeminal nerve and ganglion, brain stem, midbrain, thalamic and hypothalamic nuclei. In contrast, nonpathogenic recombinants ANGpath/$syn^{+}B6^{KOS}$ (prepared by transferring the whole *Bam*HI G^{KOS} fragment) and ANGpath/syn^{+KOS} (prepared by transferring the 0.8 kbp *Bam*HI-*Sst*I subfragment of the *Bam*HI G^{KOS} fragment) showed limited haematogenous and neural spread, but no evidence of replication in CNS; thus, their behaviour resembled that of the wild type strain KOS.The recombinant ANGpath/syn^{+KOS}, which was not pathogenic for mice, still remained pathogenic for rabbits, a phenomenon indicating the presence of an additional locus in the gB molecule participating on virulence. Sequencing the 1478 bp *Sst*I-*Sst*I subfragment of the *Bam*HI G^{path} fragment (nt 53,348 – 54,826 of UL segment) showed the presence of at least 3 mutations as compared to the KOS sequence, from which the change of cytosine to thymine at nt 54,251 altered the codon for arginine to that for histidine (amino acid 515) in the gB polypeptide chain.

Key words: herpes simplex virus type 1; glycoprotein B; pathogenicity; recombinants; mutation

Introduction

The gB of HSV-1 is essential for its infectivity by inducing membrane fusion and facilitating penetration (Haffey and Spear, 1980; Spear, 1985). The gB polypeptide chain has a N-terminal 29 amino acid (aa) long cleavable signal sequence, a 696 aa long hydrophilic surface domain with 6 potential glycosylation sites, a 109 aa long C-terminal domain projecting into cytoplasm and a 69 aa hydrophobic transmembrane domain (Pellet *et al.*, 1985). A single aa change in the cytoplasmic domain at position 857 causes extensive fusion in tsB5 mutant-infected Vero cells (Bzik *et al.*, 1984). This locus called syn^3 (Ruyechan *et al.*, 1979) was mapped to coordinates 0.345 – 0.355 (deLuca *et al.*, 1982). The suggested role of gB in HSV envelopment was not confirmed (Cai *et al.*, 1987). Monoclonal antibodies (MoAbs) distinguished up to 18 epitopes clustered in 4 antigenic domains (Pereira *et al.*, 1989). The gB gene maps to the *Bam*HI G fragment (0.345 – 0.399) (Pereira *et al.*, 1982), more precisely to UL 27 (nt 53,083 – – 55,794 (McGeoch *et al.*, 1988).

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When *Bam*HI G fragment from KOS DNA (*Bam*HI G^{kos}) was cotransfected with the ANGpath DNA into BHK cells, the recombinant selected was syn^+ (nonsyn) and positive for staining by MoAbs B6 which detects a specific epitope for gB^{KOS} (Weise *et al.*, 1987). The B6 locus maps in the *Sst*I-*Sst*I (2.7 kb) subfragment of the *Bam*HI G fragment upstream from the *Sal*I site (Fig. 1). Additional recombinants were prepared after cotransfection of ANGpath DNA with smaller *Bam*HI G^{KOS} subfragments such as *Bam*HI-*Sst*I^{KOS} (0.8 kbp) and *Sst*I-*Sst*I^{KOS} (2.7 kbp), and selected according to the syn^+ (no giant cells) and/or the $B6^+$ phenotypes. Strain ANGpath virus derived from strain ANG (Kaerner *et al.*, 1983) is pathogenic for mice after peripheral inoculation (Kümel *et al.*, 1986). While ANG as well as KOS can be transported to the regional sensory ganglion by axonal route to establish latency (Schröder and Kümel, 1986; Rajčáni *et al.*, 1990*a*), only ANGpath would spread beyond the first neuron and reach CNS. Neural spread of ANGpath in the mouse model is more complex; it can be traced by immunofluorescene and peroxidase-anti-peroxidase (PAP) staining because of active replication and antigen production in Schwann cells of peripheral nerves and in glial cells at the entry site of cerebrospinal nerves (Rajčáni *et al.*, 1990*a*). Here we present evidence that recombination of KOS DNA fragment(s) coding for gB into ANGpath DNA reduces the ability of ANGpath to spread from the inoculation site both by haematogenous and neural routes. The latter remains restricted to silent axonal spread followed with establishment of latency in the absence of virus replication in CNS during the acute postinfection period.

Materials and Methods

Viruses. Strain ANGpath (Kaerner *et al.*, 1983), wild type strain KOS (Holland *et al.*,1983) and the recombinants (Table 1) were obtained from the German Cancer Research Center (DKFZ), Heidelberg. The recombinants were prepared as previously described (Weise *et al.*, 1987; Lange-Bay, 1990). Briefly, BHK-21 cells were cotransfected with either the *Bam*HI G fragment from KOS DNA or its subfragments *Bam*HI-*Sst*I (0.345 – 0.351) or *Sst*I-*Sst*I (0.351 – 0.368) and with ANGpath DNA by the calcium phosphate precipitation method (Graham *et al.*, 1973). The "black plaque assay" before selection of gB^{KOS} B6 marker was made according to Holland *et al.* (1983) using the anti-B6 MoAb (kindly provided by Dr.J.C.Glorioso, University of Pittsburgh, PA, USA), anti-mouse IgG/Px-labelled conjugate (Dianova, Hamburg, Germany) and 4-chloro-1-naphthol as substrate.

Animals. DBA/2 mice were simultaneously inoculated into the right lip subcutaneosly (sc) and into the right cornea with a total virus dose of 2×10^6 PFU. The lethality was registered and autopsies were performed on days 3, 6 and 9 post infection (p.i.). Following organs were sampled for virus titration and morphological examination: right lip, both trigeminal ganglia, brain stem, cerebellum, midbrain, brain hemispheres, spinal cord with spinal ganglia, adrenal glands with kidneys and retroperitoneal vegetative ganglia, liver and spleen. Albino rabbits (3 000 g) were inoculated with 2×10^6PFU of virus into the right scarified cornea in a volume of 50 µ l. The animals were observed for 2 – 4 months. At autopsy, both trigeminal ganglia, right brain stem (at the entrance of trigeminal nerve root) and right cornea were removed under sterile conditions, minced and cultured for 10 days as described below.

Virus infectivity titrations were made in Vero cells grown on 24-well microplates (Nunc) in Eagle' s Basal Medium supplemented with 10% foetal calf serum (FCS), 10 mmol/l Hepes buffer, 2 mmol/l L-glutamine and antibiotics. Plaques were counted within 2,3 and 4 days of incubation at 37 °C in 5% CO_2 atmosphere; titers were expressed in PFU either per total tissue sample used to prepare the 10% suspension (for details see Rajčáni *et al.*, 1990*a*) or per 0.1 g organ weight.

Morphological examinations. For indirect immunofluorescence (IF) staining tissues were quickly frozen in liquid nitrogen, cut in cryostat and fixed in acetone. The sections were stained with rabbit anti-HSV-1 (strain McIntyre) hyperimmune globulin (Da-

Table 1. Recombinants constructed by replacement of BamHI G fragment or its subfragments in ANGpath DNA by KOS DNA fragments

Designation	Recipient virus	Fragment transferred	Phenotype	Pathogenicity	Axonal spread
KOS[a]	KOS	none	syn^+, $B6^+$	low	frequent
ANGpath	ANGpath	none	syn, $B6^-$	high	frequent
ANGpath/syn^+B6^{KOS}	ANGpath	*Bam*HI G^{KOS} (8.1 kbp, 0.345 – 0.399)	syn^+, $B6^+$	low	present
ANGpath/syn^{+KOS}	ANGpath	*Bam*HI-*Sst*I^{KOS} (0.8 kbp, 0.345 – 0.351)	syn^+, $B6^-$	low	present
ANGpath/$B6^{KOS}$	ANGpath	SstI-SstI^{KOS} (2.7 kbp, 0.351 – 0.368)	syn, $B6^+$	high	frequent

[a]Wild type strain; syn = giant cell morphology; syn^+ = nonsyncytial morphology

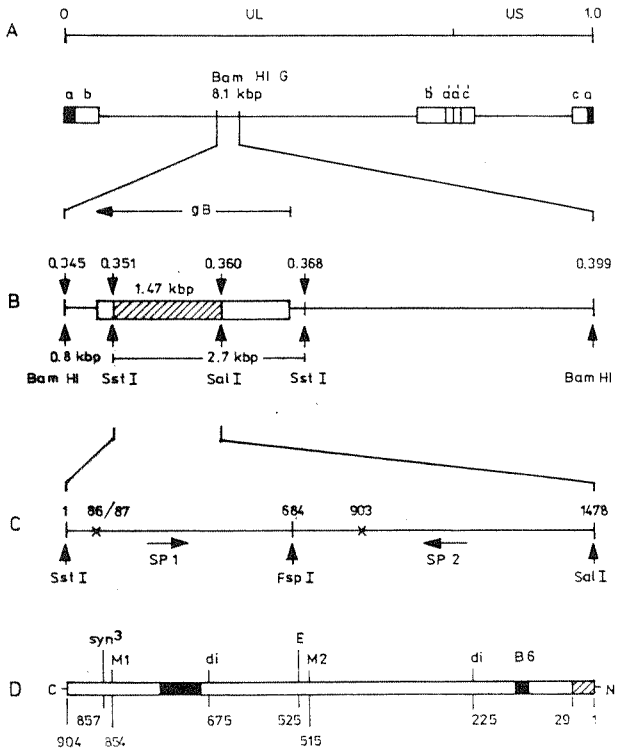

Fig. 1

Location of BamHI fragment and its subfragments in HSV-1 DNA

A: genomic DNA. Map coordinates 0–1.0, segments UL and US, inverted repeats a, b, c, and a', b', c'. B: *Bam*HI G fragment (8.1 kbp). Restriction sites and their map coordinates. gB gene (box), the sequenced *Sst*I-*Sal*I subfragment (hatched area), direction of transcription of gB gene (horizontal arrow). C: the sequenced *Sst*I-*Sal*I subfragment (nt 1–1478). Specific primers SP1 and SP2 are marked by horizontal arrows. D: gB polypeptide chain (aa 1–904). Hydrophobic transmembraneous regions (black areas), mutations M1 (aa 854) and M2 (aa 515), loci syn3 (aa 857), B6, disulfhydril bridges for dimer formation (aa 225 and 675), E (entry site, aa 525), signal sequence (aa 1–29). For M1 see Lange-Bay (1990), for syn^3 Bzik *et al.* (1984) and for E de Luca *et al.* (1982).

ko) and goat anti-rabbit conjugate (GAR/FITC) as described (Rajčáni *et al.*, 1990*a*). The sections were counterstained with thiazine red and mounted into Tris-bufferd glycerine (1 part of 0.1 mol/l Tris.HCl pH 7.5 and 9 parts of glycerine). For immunoperoxidase staining the tissues were fixed with acid formalin (73.5 % ethanol, 24.5% glacial acetic acid, 2% formalin) when removed from animals perfused through the left ventricle using the same fixative in deep Methophane anesthesia. After embedding into paraffin the sections were deparaffinized in xylol and alcohol series, and rinsed in PBS. The endogenous peroxidase was blocked by incubation with 3% H_2O_2, then the sections were washed in PBS, blocked with normal swine serum and subsequently incubated with hyperimmune rabbit serum at 37 °C for 40 mins. Following 3 washes in PBS the sections were treated with the bridging antibody (swine anti-rabbit IgG, Dako) and then with the rabbit PAP complex (Dako) according to manufacturer's instructions. After final washing the sections were rinsed in 0.1 mol/l acetate pH 5.2 and incubated for 30 mins with aminoethylcarbazol (Sigma) in acetate buffer as described (Culling *et al.*, 1985). The sections were counterstained with Mayer's haemalaun and mounted into glyceringelatine.

Latency reactivation. Latent HSV-1 infection was tested in Gasserian ganglia of DBA/2 mice after lip/cornea inoculation at 31 – 46 days p.i. and in rabbits after corneal inoculation at 40 – 60 days p.i. as previously described (Rajčáni *et al.*, 1990*b*). The minced ganglion fragments were cultured in plastic dishes for 10 days in medium RPMI-1640 supplemented with 10% FCS, 10 mmol/l Hepes, 2 mmol/l L-glutamine and 5 µmol/l 5-azacytide (Serva) as inducer. Medium was changed on days 3 and 7 (inducer-free) in culture. The medium samples from days 7 and 10 in culture as well as the collected ganglion fragments were tested for virus presence in Vero cells grown on 24-well microplates.

DNA sequencing. The 1.5 kbp *Sst*I (*Sac*I)-*Sal*I subfragment of the *Bam*HI G^{path} fragment (Fig. 1) from nt 53,348 to 54,826 of the UL segment in strain 17 DNA (McGeoch, 1988) was cleaved with *Fsp*I (cleavage site at nt 54,033 – 54,038). The resulting *Sst*I-*Fsp*I (684 nt long) and *Fsp*I-*Sal*I (794 nt long) subfragments were inserted into *Sac*I-*Sma*I and/or *Sma*I-*Sal*I polylinker sites of pUC19. The plasmid DNA was first cleaved with *Sma*I to create blunt ends and then digested either with *Sst*I or with *Sal*I. Then the *Sst*I-*Fsp*I and the *Fsp*I-*Sal*I subfragments were ligated at corresponding ends of the vector and transfected into *E. coli* WK6 cells. The cloned vector DNAs containing either of inserted subfragments were purified by CsCl-gradient centrifugation and sequenced by the chain-termination dideoxynucleoside triphosphosphate method (Sanger *et al.*, 1977) using the T7-Sequencing kit (Pharmacia LKB Biotechnology). Specific downstream (5'-GTGGATGACCGTGTCGA-3', nt 275 – 292) and upstream (5'-GACTGCATCGGCAAGGA-3', nt 1217 – 1201) primers were synthesized taking advantage of the published strain 17 sequence and used downstream from the *Sst*I site or upstream from *Sal*I site (Fig. 1). The universal sequencing primers were used in downstream direction from *Sst*I and/or *Fsp*I insertion sites and in upstream direction from *Sal*I and/or *Fsp*I sites. The labelling reaction was performed with [alpha-^{35}S]dATP and 3 units of T7 DNA polymerase (5 mins incubation at 37 °C). The denatured reaction mixture was electrophoresed on 6% acrylamide gel containing 8 mol/l urea in TBE buffer at 55 °C and 50 V/cm using the 2010 Macrophor apparatus (Pharmacia LKB Biotechnology). By the end of the run, the gel was washed in 10% acetic acid, air dried and exposed to Kodak X-AR film.

Results

Virus distribution in mice and rabbits during acute and latent infection

Following combined lip and corneal inoculations, the strains KOS, ANGpath as well as all the 3 recombinants replicated at the inoculation site. ANGpath strain and the

pathogenic recombinant ANGpath/B6^{KOS} spread to CNS and visceral organs (Table 2). Despite a limited replication of KOS was also found in the right trigeminal ganglion by day 6 p.i., both nonpathogenic recombinants (ANGpath/syn^{+}B6^{KOS} and ANGpath/syn^{+KOS}) remained restricted to the inoculation site (Table 3). Lethality and distribution of ANGpath/B6^{KOS} in DBA/2 mice closely resembled that of ANGpath indicating the presence of a dominant virulence-associated mutation in the *Bam*HI-*Sst*I subfragment of ANGpath coding for the cytoplasmic domain of gB. Nevertheless, the ANGpath/B6^{KOS} recombinant was still pathogenic for rabbits (Table 4), indicating that lethality of ANGpath for rabbits was reduced (to 13%) only when the whole gB gene had been replaced by the KOS sequence (ANGpath/syn^{+}B6^{KOS}). Table 4 also shows that both nonpathogenic viruses, KOS and ANGpath/syn^{+}B6^{KOS}, spread by axons to the right trigeminal ganglion establishing latency in about 33% of survivors. The presence of infectious virus was also tested in the cornea and brain of rabbits which succumbed infection on days 4 – 12 p.i. In lethal cases the virus was present in brain stem, occasionally also in brain cortex, spinal cord and spleen (data not shown). Unlike to mice, no virus was isolated from adrenal glands (compare Tables 2 and 3), which indicates that neural spread was the main route of virus transmission after corneal inoculation. Latency competence as an indicator of the ability of recombinants to spread by axonal route to the regional sensory ganglion (first neuron) is documented in Table 5. The spread of the nonpathogenic recombinants ANG-

Table 2. Distribution of infectious virus in mice infected with ANGpath strain and pathogenic recombinant ANGpath/B6

Organ (tissue)	ANGpath			ANGpath/B6^{KOS}		
	Virus titers at days p.i.					
	3	6	9[a]	3	6	9
Right lip	2×10^4	1×10^3	9×10^2	1×10^5	6×10^3	nd
RTG	1×10^2	1×10^2	2×10^1	1×10^3	1×10^2	nd
LTG	neg	neg	neg	neg	neg	nd
Brain stem	1×10^3	3×10^3	neg	2×10^1	1×10^3	nd
Cerebellum	neg	neg	neg	neg	1×10^2	nd
Midbrain	4×10^2	1×10^4	neg	neg	2×10^0	nd
Cortex	neg	neg	neg	neg	neg	neg
Spinal cord	neg	1×10^1	neg	5×10^1	neg	nd
SVG	neg	neg	neg	2×10^1	neg	nd
Adrenal gl.	neg	neg	neg	1×10^5	2×10^2	nd
Spleen	neg	neg	neg	neg	neg	neg
	Lethality			Lethality		
	0/16	7/13	11/13	0/15	3/12	11/12

Inoculation dose 2×10^6 PFU. Virus titers expressed in PFU per 0.1 g tissue (lip, different brain samples) or per whole organ sample (ganglia, spinal cord, adrenal gland, spleen). RTG – right trigeminal (Gasserian) ganglion; LTG – left trigeminal ganglion; SVG – spinal and vegetative ganglia; nd – not done; neg – $<1 \times 10^0$. [a] No virus was found in the CNS of survivor mouse.

Table 3. Distribution of infectious virus in mice infected with KOS strain and nonpathogenic recombinants ANGpath/syn^{+}B6^{KOS} and ANGpath/syn^{+KOS}

Organ (tissue)	KOS			ANGpath/syn^{+}B6^{KOS}			ANGpath/syn^{+KOS}		
	Virus titers at days p.i.								
	3	6	9	3	6	9	3	6	9
Right lip	1×10^5	4×10^1	neg	2×10^4	neg	neg	3×10^4	5×10^5	neg
RTG	neg	1×10^1	neg	neg	neg	neg	neg	neg	neg
LTG	neg	neg	neg	neg	neg	neg	neg	neg	neg
Brain stem	neg	neg	neg	neg	neg	neg	neg	neg	neg
Cerebellum	neg	neg	neg	neg	neg	neg	neg	neg	neg
Midbrain	neg	neg	neg	neg	neg	neg	neg	neg	neg
Cortex	neg	neg	neg	neg	neg	neg	neg	neg	neg
Spinal cord	neg	neg	neg	neg	neg	neg	neg	neg	neg
SVG	neg	neg	neg	neg	neg	neg	neg	neg	neg
Adrenal gl.	neg	neg	neg	neg	neg	neg	neg	neg	neg
Spleen	neg	neg	neg	neg	neg	neg	neg	neg	neg
	Lethality			Lethality			Lethality		
	1/15	1/12	1/9	0/15	0/12	0/9	0/15	1/12	1/9

Virus titers expressed in PFU per 0.1 g lip tissue or per whole ganlion sample. For details see Table 2.

Table 4. Lethality and latency competence of KOS strain and ANGpath/gB^{KOS} recombinants for rabbits

Virus	Lethality	Latency			
		RTG	LTG	RBS	RC
KOS	0/7	3/7 (42.8%)	0/7	1/7 (14.3%)	0/7
ANGpath/syn^{+}B6^{KOS}	2/15 (13%)	5/13 (38.4%)	1/13 (7.6%)	2/13 (15.3%)	1/13 (7.7%)
ANG/syn^{+KOS}	19/22 (86%)	0/3	0/3	0/13	0/13
ANGpath/B6^{KOS}	7/8 (87%)	0/1	0/1	0/1	0/1

For other abbreviations see Table 2.

Table 5. Latency competence of the ANGpath recombinants for mice

Virus	Virus dose (PFU)	Days p.i.	RTG	LTG	Brain stem
ANGpath[a]	1×10^5	30	6/8 (75%)	0.8	nd
	1×10^4	90	10/16 (62.5%)	0/16	nd
KOS	5×10^5	31	14/17 (82%)	3/17 (17.6%)	0/17
ANGpath/syn^{+}B6^{KOS}	5×10^5	46	6/18 (33%)	0/18	2/18 (11.1%)
ANGpath/B6^{KOS} [a]	5×10^4	32	10/17 (59%)	0/17	0/17
ANGpath/syn^{+KOS}	5×10^5	36	7/15 (47%)	0/15	0/15

[a]For lethality during acute infection see Table 2. For abbreviations see Table 2.

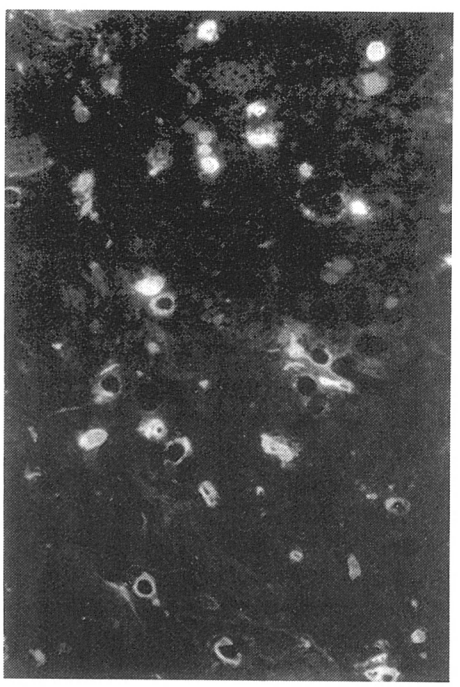

Fig. 2
Subcutaneous connective tissue at the site in the lip of DBA/2 mouse on day 3 p.i. with recombinant ANGpath/B6^{KOS}
HSV antigen is present in elongated fibrocytes, mononuclear cells and endothelium cells of postcapillary venules (magn. × 200).

path/syn^{+}B6^{KOS} and ANGpath/syn^{+KOS} was reduced to 33% and/or 47%, respectively. In comparison to KOS this was a significant reduction ($p = 0.05$); nevertheless, both nonpathogenic recombinants clearly spread by axonal route.

Morphological findings in mice during acute infection

Immunohistological localization of viral antigen in the tissues was in accord with the virus infectivity assays described above. The pathogenic strain ANGpath and the pathogenic recombinant ANGpath/B6^{KOS} showed clearcut positive staining at the inoculation site, within peripheral nerves, in the trigeminal ganglion, brain stem and in other parts of CNS (Table 6).In addition, there was no HSV-specific antigen in the CNS of mice infected with KOS w.t. and with the nonpathogenic recombinants ANGpath/syn^{+}B6^{KOS} and ANGpath/syn^{+KOS} (Table 7). The latter recombinant showed occasional positive staining in the right trigeminal ganglion on day 3 p.i. (compare Table 3).

In mice infected with the pathogenic strain ANGpath and pathogenic recombinant ANGpath/B6^{KOS} (i.e. those expressing syn phenotype), HSV-1 antigen was present in the following structures of inoculated lip area: epidermis, mononuclear cells and fibrocytes of subcutaneous connective tissue, endothelium cells of venules and capillaries (Fig. 2), and in the Schwann cells of nerve fibers. Extensive

Table 6. Distribution of viral antigen in mice infected with ANGpath strain and pathogenic recombinant ANGpath/B6^{KOS}

Organ (tissue)	ANGpath			ANGpath/B6^{KOS}		
	Antigen at days p.i.					
	3	6	9[a]	3	6	9
Right lip	+	+	+	+	+	+
RTG	+	+	–	+	+	+
LTG	–	–	–	–	–	–
NTNT	–	+	–	+	+	+
FR	–	+	–	–	+	+
Cerebellum	–	+	–	–	+	–
Midbrain	+	+	–	+	+	+
Hypothalamus	–	–	–	–	+	+
Thalamus	–	+	–	–	+	+
Cortex	–	–	–	–	+	–
Gyn. dentatus	–	–	–	–	–	–
Spinal cord	–	–	–	–	–	–
SVG	–	–	–	–	–	–
Adrenal gl.	–	–	–	–	–	–
Liver	–	–	–	–	–	–
Spleen	+	–	–	–	–	–

The presence of antigen was tested by IF and PAP staining. NTNT – nucleus terminalis nervi trigemini; FR – formatio reticularis; (++) – antigen present; (–) – antigen absent. For other abbreviations see Table 2.
[a]No viral antigen was found in CNS of the survivor mouse.

Table 7. Distribution of viral antigen in mice infected with KOS strain and nonpathogenic recombinants ANGpath/syn^{+}B6^{KOS} and ANGpath/syn^{+KOS}

Organ (tissue)	KOS			ANGpath/ syn^{+}B6^{KOS}			ANGpath /syn^{+KOS}		
	Antigen at days p.i.								
	3	6	9	3	6	9	3	6	9
Right lip	+	+	–	+	+	–	+	+	+
RTG	–	+	–	–	–	–	+	–	–
LTG	–	–	–	–	–	–	–	–	–
NTNT	–	–	–	–	–	–	–	–	–
FR	–	–	–	–	–	–	–	–	–
Cerebellum	–	–	–	–	–	–	–	–	–
Midbrain	–	–	–	–	–	–	–	–	–
Hypothalamus	–	–	–	–	–	–	–	–	–
Thalamus	–	–	–	–	–	–	–	–	–
Cortex	–	–	–	–	–	–	–	–	–
Gyr. dentatus	–	–	–	–	–	–	–	–	–
Spinal cord	–	–	–	–	–	–	–	–	–
SVG	–	–	–	–	–	–	–	–	–
Adrenal gl.	–	–	–	+	–	–	–	–	–
Liver	–	–	–	–	–	–	–	–	–
Spleen	–	–	–	–	–	–	–	–	–

For legend see Tables 2 and 6.

positive staining was found in the sarcoplasm of adjacent striated muscle cells with accumulation of the antigen below sarcolemma, in small nerve fibers, and in the walls of venules (Fig. 3). Sections of lips from mice infected with nonpathogenic recombinants ANGpath/syn^{+}B6^{KOS} and ANGpath/syn^{+KOS} showed HSV antigen in a few mononuclear macrophages in subcutaneous connective tissue (Fig. 4) and in a few striated muscle cells. In contrast to these nonpathogenic recombinants, the pathogenic ANGpath and ANGpath/B6^{KOS} recombinants showed abundant fluorescence in the neurons and satellite cells of right trigeminal ganglion (Fig. 5, 6), in Schwann cells of the homolateral trigeminal nerve and in CNS. Positive neurons and glial cells were seen most frequently in the brain stem, mainly in the area of nucleus terminalis nervi trigemini (Fig. 7), in the midbrain, thalamic nuclei and in the hypopthalamic area. Brain cortex and cerebellum remained spared. Dissemination of the virus via bloodstream to spleen or adrenal glands occurred rarely. In the spleen viral antigen was seen mainly in the red pulp and in the wall of sinuses within endothelium cells, reticulum cells and mononuclear leukocytes. Viral antigen was present in adrenal cortex of a mouse infected with the nonpathogenic recombinant ANGpath/syn^{+}B6^{KOS}.

Sequencing of SstI-SalI subfragment of ANGpath DNA

The gB (UL 27) gene spans from nt 53,079 to 55,794 of the UL segment. The *SstI-SalI* subfragment of ANGpath DNA corresponds to the portion of gB gene from nt 53,348 to 54,826. The 1478 nt long *SstI-SalI*^{path} subfragment differed from that of KOS in 3 nucleotides. At nt 54,251 (903 in Fig. 8) cytosine was changed to thymine, which altered the codon CGC (coding for arginine in the transcribed strand of KOS DNA) to CAC (coding for histidine in ANGpath DNA). This changed aa 515 of the gB polypeptide chain. An another change was found at nt 86/87 (Fig. 8), altering the codon GGC (glycine) in the transcribed strand of KOS to GCG (alanine) in that of ANGpath; the latter may cause

Fig. 3
Subcutaneous muscle tissue adjacent to the inoculation site on day 3 p.i. with strain ANGpath
Excessive positive fluorescence in the muscle fibers (mainly cross sections) with the antigen accumulated in the sarcoplasm in perinuclear areas and below the sarcolemma. Occasional positivity is also in elongated fibrocytes, nerve fibers and capillary walls (magn. × 500)

Fig. 4
Subcutaneous connective tissue on day 3 p.i. with nonpathogenic recombinant ANGpath/syn^{KOS}
Virus antigen in a few mononuclear cells (magn. × 250)

mutation of aa 787 of the gB polypeptide chain. The KOS and ANGpath DNA sequences of the *Sst*I-*Sal*I fragment differed from that of strain 17 in 10 nucleotides. From all these nucleotide exchanges only 2 caused codon alterations. The mutation at nt 789 (54,137) of the transcribed strand changed guanine in strain 17 to adenine in strains KOS and ANGpath, altering the codon for alanine to that for valine at aa 553. The mutation of adenine to thymine at nt 1119 (54,467) changed leucine to glutamine at aa 443. The sequence in question of strain 17 differed from that of strain F in 5 nucleotides. Finally, the corresponding sequences of strains KOS and ANGpath differed from that of strain F in 8 nucleotides.

Discussion

Based on different pathogenicity for Balb/c mice HSV strains were divided into 3 groups (Dix *et al.*, 1983). Class I strains were highly virulent after ic as well as peripheral inoculations, class II strains were virulent after ic inoculation only, while class III strains were avirulent by both inoculation routes. Strains KOS (wild type) and ANGpath are class II, and I strains, respectively. Our data present evidence that the nonpathogenic recombinants of ANGpath could be prepared when either the whole *Bam*HI G fragment or its *Bam*HI-*Sst*I subfragment are replaced by the corresponding KOS DNA sequences.

Many structural and nonstructural genes of HSV have been with respect to pathogenicity using deletion or insertion mutants (reviewed by Rajčáni, 1992). The most thoroughly investigated gene in this respect were those coding for thymidine kinase (Ben-Hur *et al.*, 1983), the DNA polymerase components (Day *et al.*, 1987), and the immediate early transactivation proteins ICP0, ICP4 and ICP27 (Leib *et al.*, 1989). The *Mlu*I restriction fragment 0.761– 0.832 (Rosen *et al.*, 1986) and the genome region 0.82 – 0.832 were also found to play an important role in HSV pathogenicity. Less attention, however, has been paid to glycoproteins. As shown previously, the deletion of gE gene converted ANGpath into a class II virus nonpathogenic after peripheral inoculation (Schranz *et al.*, 1989; Rajčáni *et al.*, 1990*a*). This paper presents results on the role of gB mutations for pathogenicity.

The essential glycoprotein B harbors up to 18 epitopes (Pereira *et al.*, 1989) and mar mutation sites (Highlander *et al.*, 1989) in its external domain, which provide important

Fig. 5
The right trigeminal ganglion on day 6 p.i. with strain ANGpath
Excessive positivity is in pseudounipolar neurons, perineural satellites and Schwann cells (magn. × 500).

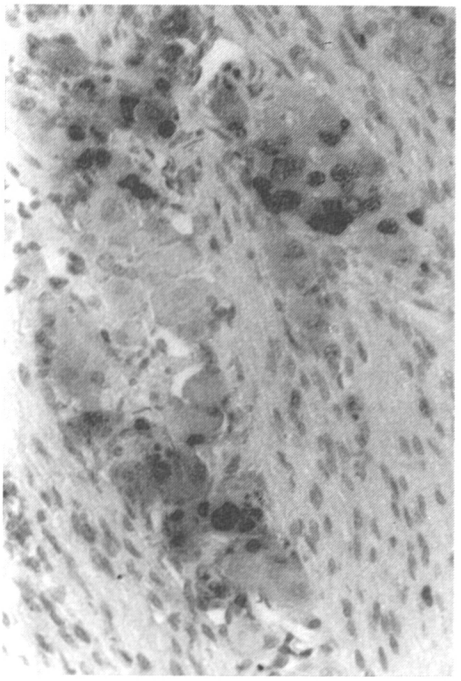

Fig. 6
The right trigeminal ganglion on day 6 p.i. with strain ANG
PAP staining. The same finding as in Fig. 5 (magn. × 600).

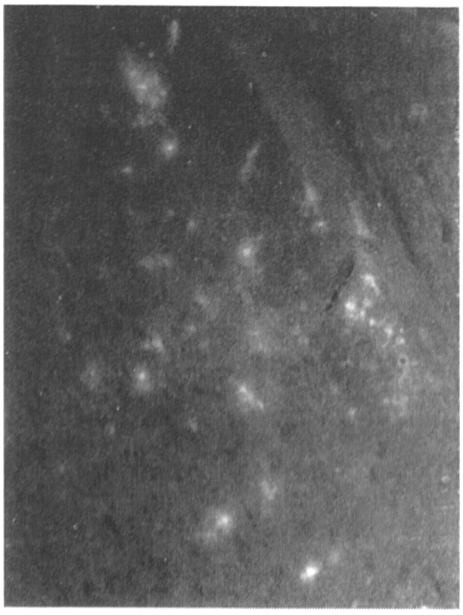

Fig. 7
Brain stem on day 6 p.i. with strain ANGpath
Virus antigen is present in neurons and glial cells of nucleus terminalis nervi trigemini (magn. × 200).

antigens including a neutralization site. As mentioned above, the syn^3 locus is located in the cytoplasmic part of the polypeptide chain confirming that gB is involved in virus penetration and membrane fusion (de Luca *et al.*, 1982; Bzik *et al.*,1984). The latter function has been repeatedly associated with pathogenicity, though loci coding for virulence-related functions and giant cell formation are not identical. The *Eco*RI F fragment (0.32 – 0.42) which includes the gB gene was designated inv-II, was associated with neuroinvasivity (Goodman *et al.*, 1989). In our hands ANGpath, a syncytial strain, was virulent in comparison to KOS nonsyn. In addition, the recombinant ANGpath/$B6^{KOS}$ containing the original *Bam*HI-*Sst*I fragment of ANGpath was as virulent as the ANGpath strain. The recombinants nonpathogenic for mice had either the whole gB^{KOS} gene or the *Bam*HI-*Sst*I subfragment of KOS DNA. Thus, at least one of several virulence-related loci resides in the cytoplasmic domain of gB. Lange-Bay (1990) has shown by sequencing of the 270 nt long *Bam*HI-*Sst*I subfragment of ANGpath DNA that it differs from the same sequence of KOS DNA in 5 nucleotides, from which the change of thymidine (in the coding sequence of KOS) to cytosine (in the coding sequence of ANGpath) at nt 53,230 of the UL segment (McGeoch *et al.*,1988) alters the codon

for valine (KOS) to that for alanine (ANGpath) at aa 854 of the gB polypeptide chain. This mutation is close to that reported aa 857 in tsB5 (Bzik *et al.*, 1984).

Because the recombinant nonpathogenic for mice ANGpath/syn^{+KOS} was still pathogenic for rabbits, and because other preliminary results indicated that the B6 region itself was not related to virulence (Kúdelová *et al.*, 1991) we have further sequenced the *Sst*I-*Sal*I subfragment of ANGpath DNA coding for the region close to the transmembraneous domain of the gB molecule. In comparison to KOS DNA we found an additional mutation at aa 515 close to the entry locus (aa 525) in the surface domain (Pereira *et al.*, 1989). This finding is in accord with observations on dissociation between syncytial phenotype and virulence (Wheeler, 1964; Yamada *et al.*, 1986) and indicates that gB has at least 2 infectivity-related loci, one for penetration and another for membrane fusion.

Highly pathogenic strains such as SC16 and ANGpath showed a combined neural spread because they replicate in Schwann cells and endoneural cells of peripheral nerves (Rajčáni and Conen, 1972; Rajčáni *et al.*,1990*a*), while the nonpathogenic recombinants can reach the first neuron in regional sensory ganglion by axonal transport only without subsequent multiplication in ganglion cells. At early postinfection intervals pathogenic viruses spread by haematogenous route invading the endothelium cells of postcapillary venules at the inoculation site. No involvement of remote CNS structures such as brain cortex and hippocampus was seen after entering the brain along the trigeminal nerve. The

```
          1             14                                                  59
KOS       CCTTGGTGGT TAGAGGGTAC AGGGCCTTCA TGGGGTTGCT CTGCAGCCGC ATGACGTAAC
F                       C                                                 G
17                      C                                                 G
ANGpath                 A                                                 A
         61                 78       86/7
KOS       GAAAGGCGAA GAAGGCCGCC GCCAGGCCGG CCAGGACCAA CAGACCCACG GCCAGCGCCC
F                           A         GC
17                          G         GC
ANGpath                     G         CG
        121
          CAAAGGGGTT GGACATGAAG GAGGACACGC CCGACACGGC CGATACCACG CCGCCCACGA

        181
          TGCCCATCAC CACCTTGCCG ACCGCGCGCC CCAGGTCGCC CATCCCCTCG AAGAACGCGC

        241        251
KOS       CCAGGCCCGC GAACATGGCG GCGTTGGCGT CGGCGTGGAT GACCGTGTCG ATGTCGGCGA
F                  G
17                 A
ANGpath            G

        301
          AGCGCAGGTC GTGCAGCTGG TTGCGGCGCT GGACCTCCGT GTAGTCCAGC AGGCCGCTGT

        361
          CCTTGATCTC GTGGCGGGTG TACACCTCCA GGGGGACAAA CTCGTGATCC TCCAGCATGG

        421
          TGATGTTGAG GTCGATGAAG GTGCTGACGG TGGTGATGTC GGCGCGGCTC AGCTGGTGGG

        481                                          520
KOS       AGTACGCGTA CTCCTCGAAG TACACGTAGC CCCCACCGAA GGTGAAGTAG CGCCGGTGTC
F                                                  G
17                                                 A
ANGpath                                            A

        541
          CCACGGTGCA CGGCTCGATC GCATCGCGCG TCAGCCGCAG CTCGTTGTTC TCCCCCAGCT

        601
          GCCCCTCGAC CAACGGGCCC TGGTCTTCGT ACCGAAAGCT GACCAGGGGG CGGCTGTAGC

        661
          AGGCCCCGGG CCGCGAGCTG ATGCGCATCG AGTTTTGGAC GATCACGTTG TCCGCGGCGA

        721
          CCGGCACGCA CGTGGAGACG GCCATCACGT CGCCGAGCAT CCGCGCGCTC ACCCGCCGGC
```

Fig. 8

Sequence of the SstI-SalI subfragment of gB gene strain ANGpath DNA nt 53,348 (numbered 1) – 54,826 (numbered 1478) as compared to that of strains KOS, F and 17

The crucial mutation by which KOS DNA differs from ANGpath DNA is at nt 903.

```
      781     789
KOS     CCACGGTGAC CGAGGCGATG GCGTTGGGGT TCAGCTTGCG GGCCTCGTTC CACAGGGTCA
F              G
17             G
ANGpath        A

      841          854
KOS     GCTCGTGATT CTGTAGCTCG CACCACGCGA TGGCAACGCG GCCCAACATA TCGTTGACAT
F                   C
17                  C
ANGpath             T

      901 3                                  934
KOS     GGCGCTGTAT GTGGTTGTAC GTAAACTGCA GCCGGGCGAA CTCGATGGAG GAGGTGGTCT
F         C                                  G
17        C                                  T
ANGpath   T                                  G

      961
        TGATGCGCTC CACGGACGCG TTGGCGCTGG CCCCGGGCGG CGGGGGCGTG GGGTTTGGGG

     1021            1037
KOS     GCTTGCGGCT CTGCTCTCGG AGGTGTTCCC GCACGTACAG CTCCGCGAGC GTGTTGCTGA
F                     T
17                    G
ANGpath               T

     1081                                      1119
KOS     GAAGGGGCTG GTACGCGATC AGAAAGCCCC CATTGGCCTG GTAGTACTGC GGCTGGCCCA
F                                               A
17                                              A
ANGpath                                         T

     1141
        CCTTGATGTG CGTCGCGTTG TACCTGCGGG CGAAGATGCG GTCCATGGCG TCGCGGGCGT

     1201
        CCTTGCCGAT GCAGTCCCCC AGGTCCACGC GCGAGAGCGG GTACTCGGTC AGGTTGGTGG

     1261                    1286
KOS     TGAAGGTGGT GGATATGGCG TCGGAGGAGA ATCGGAAGGA GCCGCCGTAC TCGGAGCGCA
F                               G
17                              A
ANGpath                         G


     1321          1334
KOS     GCATCTCGTC CACTTCCTGC CACTTGGTCA TGGTGCAGAC CGACGGGCGC TTTGGCACCC
F                   C
17                  C
ANGpath             T

     1381
        AGTCCCAGGC CACGGTGAAC TTGGGGGTCG TGAGCAGGTT CCGGGTGGTC GGCGCCGTGG

     1441
        CCCGGGCCTT GGTGGTGAGG TCGCGCGCGT AGAAGCCG
```

Fig. 8 (continued)

latter rather occurs after intranasal inoculation (Webb *et al.*, 1989). Because HSV is known to spread by transneuronal transport (Norgen and Lehman, 1989) and its distribution within CNS depends on the inoculation site, we assume that haematogenous dissemination may not be essential for invasion of nervous system.

The gB seems to contribute to virulence, acting in accord with other genes such as the DNA polymerase gene complex, thymidine kinase and the immediate early transactivation proteins. To obtain further evidence for this hypothesis, the *Bam*HI G fragment of ANGpath DNA was recombined into KOS DNA. However, the recombinant KOS/gB^{path} contained only the ANGpath sequence corresponding to the *Bam*HI-*Sst*I and *Sst*I-*Sal*I subfragments and was of B6^{+} phenotype. This latter recombinant was only moderately pathogenic showing properties intermediate between strain

KOS and ANGpath (data not shown). This results is not contradictory to our hypothesis. It shows that gB is one of many genes possibly involved in HSV-1 virulence.

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