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LATENCY COMPETENCE OF HERPES SIMPLEX VIRUS STRAINS ANG, ANGpath AND ITS gC AND gE MINUS MUTANS

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Summary. — The latency competence of herpes simplex virus type 1 (HSV-1) strains SC16, KOS, ANG, ANGpath and its mutants ANGpathgC18 (gC minus, spontaneous point mutation), KOSgC39 (gC minus deletion), ANGpathI2-4 (gE minus deletion), and ANGpathgCI-8 (gE and gC minus double mutant) was compared and DBA/2 mice. While the latent SC16 and KOS reactivated spontaneously in explanted homolateral trigeminal ganglion fragments coming from Velaz DBA/2 mice, methylation inhibitor 5-azacytidine (5-AzaC) was required to achieve reactivation of SC16 in the ganglion explants from Hannover DBA/2 mice. Reactivation of ANGpath in the cultured trigeminal ganglia from both lines of DBA/2 mice occurred only in the presence of the drug. The compound also enhanced the reactivation incidence in the ganglion explants from ANG-infected Hannover DBA/2 mice but not from Velaz DBA/2 mice: in the latter it remained low even in the presence of the inducer. Both gE^- mutants failed to establish latency as judged by the failure of reactivation either in the presence or the absence of 5-AzaC. This seemed in accordance with the absence of neural (quick axonal) spread of these mutans in mice (Rajčáni *et al.*, 1990). In contrast, both gC^- mutans established latency: ANGpathgC18 at an unchanged rate and KOSgC39 at a lower frequency than the parent strain.

Key words: *latent infection*; *herpes simplex virus*; *mutants*; *DBA mice*

Introduction

It is known that herpes simplex virus (HSV) mutants not expressing glycoprotein C (gC) grow well in cell culture (Holland *et al.*, 1983; 1984; Draper *et al.*, 1984). Also the gE minus mutans were shown to grow well *in vitro* (Neidhardt *et al.*, 1987; Schranz *et al.*, 1989). The gC^- mutant of HSV-1 strain MP established latency in the lumbosacral spinal ganglia when inoculated into the hind footpad (Mannini-Palenzona *et al.*, 1988). Not only

intracerebral but also intravaginal inoculation of Balb/c mice with a HVS-1 gC minus mutant leads to the involvement of nervous system (Johnson *et al.*, 1986). In contrast, the gE minus mutant was nonpathogenic after peripheral inoculation (Rajčáni *et al.*, 1990) although it caused encephalitis after intracerebral administration. Here we describe the latency competence of two gC minus mutans (a spontaneous and a deletion mutant), of a gE minus deletion mutant and of the gC/gE minus double mutant.

Materials and Methods

Virus strains. The strain SC16 of HSV type 1 (Hill *et al.*, 1975) was kindly provided by dr. T. E. Hill, University of Bristol, U. K. The strain ANG (Kaerner *et al.*, 1981) and its pathogenic variant ANGpath (Kaerner *et al.*, 1983) were coming from the same stock as in the previous study (Rajčáni *et al.*, 1990). The strain KOS provided by dr. V. Vonka (Sevac, Prague) originated from Prof. J. L. Melnick s collection (WHO Collaborating Centre for Virus Reference and Research, Houston, Texas, U.S.A.). The deletion mutant KOS gC39 was previously characterized (Holland *et al.*, 1986). ANGpathgC18, a spontaneous gC minus mutant, was described by Weise (1987). The construction of the gE minus deletion mutant ANGpathI2-4 was reported by Neidhardt *et al.* (1987) and the construction of the gC negative gE negative double mutant has been published recently (Schranz *et al.*, 1989). The ANGpath K5/I mutant was derived from ANGpath gC18/gB^{KOS} (Weise *et al.*, 1987) by deleting a part of the ICP4 gene (Schröder *et al.*, 1985) and displayed the following phenotype: gC negative, ICP4 negative, and an exchange of the gB6 sequences from ANGpath for the gB6 sequences of KOS; the latter was defined with the monoclonal antibody B6 which reacted with gB KOS but not with gB ANGpath.

Latency in DBA/2 mice (Table 1). In independent trials using different virus doses and two mouse lines of different susceptibility to HSV-1, the latent infection was established by inoculation of 5 μl virus suspension to the right scarified cornea; simultaneously 10 μl of the virus suspension was administered intra- and/or subcutaneously into the right lip using an air-tight microsyringe. The blister formation in the latter case was checked under the microscope. The animals were housed under pathogen-free conditions on a standard diet. At intervals ranging from 1 month to 6 months post-infection, both trigeminal ganglia (from some also the brain stem) were removed, immersed into sterile phosphate buffered saline (PBS pH 7.2) containing 5% foetal calf serum (FCS), minced and explanted in plastic Petri dishes (tissue culture grade) in medium RPMI-1640 supplemented with 10% FCS and antibiotics. The cultures were kept for 10 days; medium was exchanged on days 4 and 7. In explants cultured in the presence of 10 μmol/l 5-aza-cytidine (Merck), the last exchange (day 7) was made with drug-free medium. On day 10 in culture, the tissue fragments coming from the same sample were washed in PBS and homogenized (20% suspension in BEM). Occasionally some collected fragments were quickly frozen, cut in cryostat and stained by indirect immunofluorescence (IF) method to check the occurrence of HSV antigen-containing cells.

Virus infectivity assay of the medium fluids and tissue homogeneates was made in BHK cells grown in 24-well plastic dishes or in Vero cells grown in Leighton glass tubes. When grown to confluency in BEM (supplemented with 10% calf serum) (CS), the infected cells were replenished with the same medium containing 2% CS. The plaques were counted at highest positive dilution by daily intervals for 3–4 days.

Pathogenicity during acute infection. Deaths were registered for the first 21 days in each animal group. Signs of sickness developed in SC16- and ANGpath-infected Hannover DBA/2 and in SC16-infected Velaz DBA/2 mice. On days 6 and 7 post-infection (p.i.) the sick mice were sacrified, their trigeminal ganglia, brain stem, the rest of brain, spleen, adrenals, and kidneys were removed and examined either for virus presence or HSV-specific antigens using indirect IF and PAP staining. For IF the tissues were quickly frozen in liquid nitrogen and cut in cryostat. For the peroxidase-antiperoxidase staining the tissues were fixed in neutral formalin, embedded into paraffin and stained according to the instructions of the kit manufacturer (CRL Universal Immunoperoxidase staining kit, Cambridge, U.K.). For more details see Rajčáni *et al.* (1990).

Table 1. Latency competence of three HSV-1 strains in DBA/2 mice

Virus strain	Inoculation dose (PFU)	Inoculation route	Mouse strain	Autopsy day p.i.	Reactivation in culture		Iducer*
					RTG	LTG	
SC16	1×10^5	cornea	DBA/2**	92	7/8	2/8	none
	2×10^4	cornea	DBA/2**	71	11/15	3/15	none
	7.5×10^4	cornea, lip	DBA/2***	40	1/3	0/3	none
	8×10^3	cornea, lip	DBA/2***	60	2/7	0/7	none
	1×18^4	cornea, lip	DBA/2***	32	8/8	2/6	yes
ANGpath	6×10^5	cornea, lip	DBA/2**	91	6/32	1/32	none
	2×10^5	cornea, lip	DBA/2**	81–88	19/23	3/23	yes
	1.2×10^4	cornea, lip	DBA/2***	54–90	10/16	0/16	yes
	7.5×10^4	cornea, lip	DBA/2***	60	0/8	0/8	none
	1.5×10^5	cornea, lip	DBA/2***	40	2/11	0/11	none
	7×10^5	cornea, lip	DBA/2***	32	0/6	0/6	none
	1.2×10^5	cornea, lip	DBA/2***	30	6/8	0/8	yes
ANG	1.5×10^6	cornea, lip	DBA/2***	30–42	2/24	0/24	none
	1×10^6	cornea, lip	DBA/2***	54	7/12	2/12	yes
	2×10^6	cornea, lip	DBA/2**	80	3/20	0/20	none
	2×10^6	cornea, lip	DBA/2**	79	2/24	0/24	yes
	2×10^6	cornea, lip	DBA/2**	173	4/17	0/17	yes

RTG, LTG = right (left) trigeminal ganglion
* 5-azacytidine 10 μ mol/l for 7 days in culture
** from breed Velaz, ČSFR
*** from Deutsche Versuchstieranstalt, Hannover

Results

Variations in the latency competence of the wild type virus strains

As shown in Table 1, in DBA/2 mice strain SC16 has established latency at a high rate in the homolateral (73 and 88%, respectively) and at a lower rate (20 and 25%, respectively) in the contralateral trigeminal ganglion. The dose of 1×10^5 PFU caused the death of more than 50% of mice in the acute phase of infection. Alternatively, 12 out of 15 (80%) of DBA/2 mice died within 14 days after inoculation of 3×10^4 PFU SC16 (Table 3). In DBA/2 mice the virus spread to neural tissues (brain stem) as well as by bloodstream (adrenal gland, kidneys, spleen; Table 2). Using IF and PAP staining the HSV antigen was found in pseudounipolar neurons and satellite cells of the homolateral trigeminal ganglion (Fig. 1), in many neurons and glial cells of the brain stem as well as in the higher parts of CNS including thalamic nuclei and temporal cortex (Table 3). When the survivors were examined for latency, the incidence of spontaneous virus reactivation was surprisingly low (3 out 10, 30%). Therefore, 5-AzaC was added to the medium fluid of ganglion explants. Already since day 4 in culture a part of ganglion explants had released infectious virus. The total latency incidence

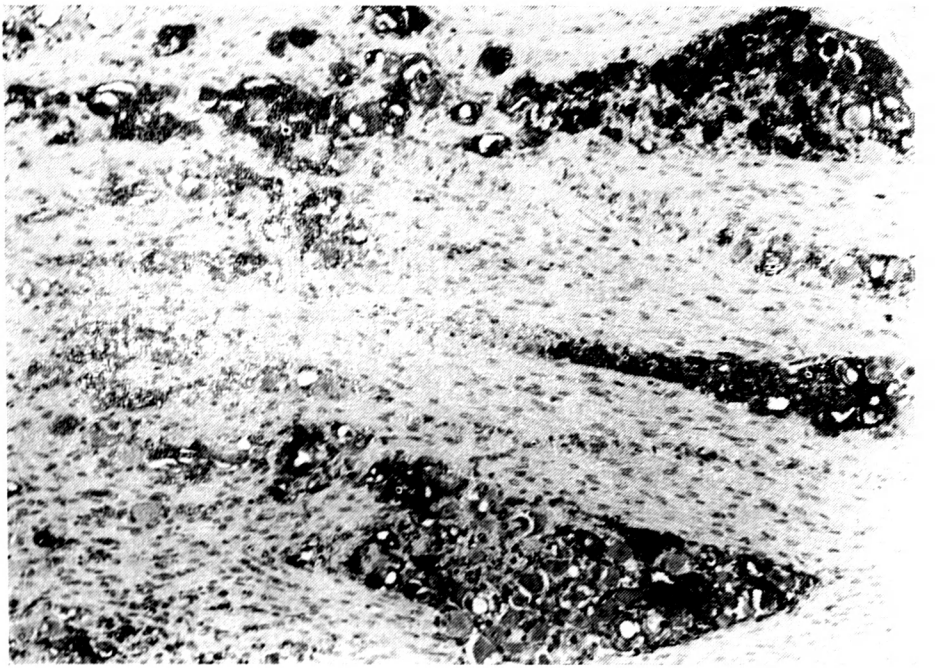

Fig. 1

Detection of HSV-1 antigen in pseudounipolar neurons and satellite cells of the right trigeminal ganglion on day 6 after inoculation of SC16 (3×10^4 PFU) into the scarified cornea of DBA/2 mice (PAP strain and HE, $\times 120$)

was as high as 100% (Table 1) even when the inoculation dose per mouse was as low as 8×10^3 PFU SC16 (less than 1 LD_{50}).

In ANGpath-infected DBA/2 mice the virus spread to the trigeminal ganglion, brain stem, and adrenal gland. The titre in the rest of the brain was lower then in SC16-infected DBA/2 mice (Table 2). HSV antigen was seen predominantly in the brain stem, namely in *nc. terminalis n. trigemini*, in *formatio reticularis*, in the vestibular nuclei in the bottom of the fourth ventricle (Table 3). The latter Table also shows that in DBA/2 mice the dose of 7.5×10^4 ANGpath corresponded to 8×10^3 of SC16.

Explantation of the right trigeminal ganglion revealed latency only in 2 out of 25 mice (8%) when the explants were kept in the absence of 5-AzaC. When the inducer was added, the latency increased to 66.6% (16/24). The importance of the inducer for latency reactivation in the ganglion explants coming from ANGpath-infected DBA/2 mice becomes evident from the fact that the incidence increased from 18.8% in the absence of the drug to 82.6% in its presence (Table 1).

In ANG-infected Hannover DBA/2 mice the addition of the inducer increased he latency rate as detected by explantation from 8.3% to

Table 2. Distribution of HSV in the organs of DBA/2 mice after corneal and lip inoculations

Organ	Day 6 post-infection	
	ANGpath[1]	SC16[2]
Trigeminal ganglia (both)	1×10^4*	2×10^3
Brain stem	(3×10^4)	$> 1 \times 10^3$
Spinal cord	—	2×10^3
Brain	2×10^2	3×10^3
Kidneys	—	6×10^3
Adrenal glands	3×10^4	6×10^3
Spleen	—	—

* PFU per organ; [1] inoculation dose of 7×10^5 PFU/mouse; [2] inoculation dose of 7.7×10^4 PFU/mouse

58.3%. However, this was not the case in Velaz DBA/2 mice, in which the rate of latency remained low, regardless whether the inducer was present or not (14.5% and 15%, respectively). When KOS was used to establish latency in Velaz DBA/2 mice, on the other hand, spontaneous reactivation of the latter virus occurred in 78.9% of cultured homolateral ganglia, by a frequency as high as the spontaneous reactivation incidence with SC16-infected Velaz DBA/2 mice (73.3%). It should be mentioned in this context that the ANGpath K5/I mutant established latency in DBA/2 mice at a slightly lower rate than w.t. ANGpath when tested in the presence of the inducer (50% as compared to 66.6%). On the other hand, the latter mutant reactivated spontaneously at a frequency similar to that in the presence of 5-AzaC (Table 4).

Latency competence of the gC and gE negative ANGpath mutants

Using the KOSgC39 deletion mutant the reactivation incidence in the right trigeminal ganglion explants from DBA mice was significantly reduced (from 78.9% to 33.3%, $p = 0.025$). Using the spontaneous ANGpathgC18 (gC negative) mutant in Velaz DBA/2 mice, the reactivation frequency was similar regardless the ganglion explants were kept in the presence or absence of the inducer (82.6% and 71.4%, respectively).

No reactivation of infectious virus could be achieved from the explants at culturing the ganglia from DBA/2 mice inoculated with the gE negative ANGpathI2-4 mutant and/or the gE/gC negative ANGpathgCI-8 double mutant. Results were the same regardless whether the explants were kept in the presence or absence of the inducer. In addition, no latency of ANGpathgCI-8 was found in DBA/2 mice either by spontaneous or drug mediated reactivation (Table 4). It seems, therefore, that the gE minus mutants were unable to establish latent infection, at least as judged by the results of the explantation procedure.

Table 3. Distribution of SC16 and ANGpath strains after oral (lip) inoculation into DBA/2 mice

Organ	SC16[1] Days post-infection				ANGpath[2] Days post-infection			
	6 (IF)	6 (PAP)	7 (IF)	7 (PAP)	6 (IF)	6 (PAP)	7 (IF)	7 (PAP)
Right trig. ganglion	+	+	+	+	+	+	+	+
Left trig. ganglion	−	−	−	−	−	−	−	−
Medulla oblongata (brain stem)								
Nc. terminalis n. trigemini	+	+	+	+	+	+	+	+
Formatio reticularis	+	+	+	+	+	+	+	+
other structures	+	+	+	+	+	+	+	+
Cerebellum	+	+	+	+	−	−	−	−
Midbrain	+	+	+	+	−	−	−	−
Hypothalamus	+	+	+	+	−	−	−	−
Thalamus	+	+	+	+	−	−	−	−
Brain cortex	+	+	+	+	−	−	−	−
Spinal cord	−	+	−	+	−	−	−	−
Vegetative nerves	−	+	−	−	−	−	−	−
Adrenal gland	−	−	−	−	−	−	−	−

Lethality (at given dose)		day 5	day 6	day 7	day 8	day 9	day 10	day 12	Total	
8×10^3	SC16		6	2	2			2	12/27	44%
1×10^4			5	4	3				12/25	48%
3×10^4			8	2			2		12/15	80%
7.5×10^4			4	7					11/11	100%
7.5×10^4	ANGpath		3				3		6/14	43%
1.2×10^5		3	8	3			2		16/24	66%
7.5×10^5			2	4	6				12/18	66%

[1] inoculation dose of 7.5×10^4 PFU/mouse; [2] inoculation dose of 7×10^5 PFU/mouse

Table 4. Latency competence of two HSV strains as compared to their mutants

Virus strain	Inoculation dose (PFU)	Inoculation route	Mouse strain	Autopsy day p.i.	Reactivation in culture		Inducer*
					RTG	LTG	
ANGpath	2×10^5	cornea, lip	DBA/2**	81–88	19/23	3/23	yes
	1.5×10^5	cornea, lip	DBA/2***	30	6/8	0/8	yes
	1.2×10^4	cornea, lip	DBA/2***	54–90	10/16	0/16	yes
ANGpathI2-4 (gE minus)	2×10^5	cornea, lip	DBA/2**	172	0/13	0/13	yes
ANGpathgCI-8 (gE, gC minus)	6×10^5	cornea, lip	DBA/2***	56–70	0/28	0/28	yes
	6×10^5	cornea, lip	DBA/2***	70	0/12	0/12	none
	2×10^5	cornea, lip	DBA/2**	182	0/14	0/14	yes
ANGpathgC 18 (gC minus)	2×10^6	cornea, lip	DBA/2**	122	15/21	1/21	yes
ANGpathK5/I (gC^-, $ICP4^-$, $gB6^{KOS}$)	2×10^5	cornea, lip	DBA/2***	83	4/8	1/8	none
	2×10^5	cornea, lip	DBA/2***	79	4/8	0/8	yes
KOS	1×10^5	cornea, lip	DBA/2**	81	15/19[a]	1/19	none
KOSgC39 (gC minus)	1×10^5	cornea, lip	DBA/2**	81	5/15[a]	0/15	none

[a] reduction significant at $p < 0.5$
* 5-azacytidine 10 μmol in culture
** from breeding farm Velaz, ČSFR
*** from Deutsche Versuchstieranstalt, Hannover

Discussion

It is evident from presented results that strain ANGpath reactivates in the explanted trigeminal ganglion fragments in the presence of 5-AzaC only. The spontaneous reactivation incidence was low in the explants from both lines of DBA/2 mice. When the ganglion explants from SC16-infected DBA/2 mice were cultured, there was no reactivation till day 10 in culture as detected by virus release to medium fluid. Only 3 out of 10 explants contained HSV antigen-positive neurons (Table 1). On the other hand, when inducer had been added to the medium, several explants released virus already by day 4 in culture. The effect of hypomethylating agents such as 5-AzaC, L-ethionine, and dimethylsulphoxide (DMSO) has been described by Whitby *et al.* (1987) who found enhanced latency incidence in SC16-infected outbred (Bristol/2) mice in the presence of 5-AzaC or L-ethionine and earlier virus detection in culture in the presence of 5-AzaC and DMSO. The inducer effect of methylation inhibitors occurs despite of the fact that the latent HSV DNA was not found extensively methylated *in vivo* using methylation-sensitive restriction endonucleases (Dressler *et al.*, 1987). Probably methylation is restricted to a few GC nucleotides in the promoter region of early and/or immediate early genes. This was demonstrated with the thymidine kinase gene promoter. The affinity of cellular transcription factors Sp1 and CTF to their binding site, a 33 bp promoter segment upstream from the TATA box, was 20 times reduced when a single CpG dinucleotide in that segment became methylated (Ben-Hattar and Jiricny, 1988). This may explain while on one hand, the *Sma*I/*Xma*I and *Sal*I/*Sae*II enzyme pairs failed to detect DNA methylation, but, on the other hand, hypomethylating drugs display an inducer activity in HSV latency.

We suggest that at least 2 categories of latent genomes exist: those which easily reactivate in culture and those which reactivate in the presence of the onducer only. Most recently the abovementioned findings have found further support in enhanced reactivation of *Herpesvirus saimiri* by 5-AzaC (Mossmann *et al.*, 1989). Some light has been shed on the possible role of latency associated transcripts (LAT) in regulation of latency. A mutant not expressing LAT was found to reactivate with a considerable delay as compared to its parent strain expressing LAT (Steiner *et al.*, 1989). Therefore, it is tempting to assume that dual regulation may be at work also in HSV latency: a down regulation by methylation and a positive regulation linked to LAT. Kudelová and Rajčáni (1990) comparing the DNA/DNA hybridization results with those of virus reactivation in culture pointed at the possible existence of spontaneously nonreactivable HSV genome. This seems in accord with the older complementation experiments which rescued the nonreactivable genome in culture (Brown *et al.* 1979; Lewis *et al.*, 1984). Recently Tenser *at al.* (1989) found that their thymidinkinase-negative HSV mutant could not been reisolated from the cultured ganglion fragments, but LAT could be detected. Further studies are necessary to determine whether the spontaneously nonreactivating HSV genome is always inducible, or whether truly noninducible genomes may also exist.

The most important point to be discussed here is the absence of any reactivation in both mouse lines despite of the presence of the inducer with the two gE minus mutants. Based on our report that the negative ANGpathI2-4 did not spread by quick axonal transport, we assume that this was due to the highly restricted or absent spread of these mutants to the ganglion. In contrast, the gC negative mutants spread to the ganglion, althought the establishment of latency was less frequent with the gC negative KOSgC39 mutant. The gC negative spontaneous mutant spread as well as did the parent strain, a finding similar to the results of others (Mannini-Palenzona, 1988; Johnson *et al.*, 1986).

Finally, the behaviour of the ANCpathK5/I mutant (gC negative, ICP4 deletion gB6 exchange) was of considerable interest. In this mutant, in addition to the gC minus spontaneous mutation, the B6 region of ANGpath gB, not reacting with the B6 monoclonal antibody, was exchanged for the same region of KOS gB recognized by this antibody (Weise *et al.*, 1988). The latency rate was slightly decreased, but on the other hand, 5-AzaC had no effect on the reactivation incidence. Whether this was due to the ICP4 deletion or due to the gB6 region exchange should be further investigated using the ANGpath Y1 (ICP4del) mutant. ANGpath K5/I and ANGpath Y1 were pathogenic for rabbits and spread to their trigeminal ganglia (results not shown). In rabbits the gE negative mutants were the only ones which did not kill the animals after intracorneal inoculation. Because KOS did not kill the rabbits when given in the same dose to the scarified cornea, the B6 region of gB seems not to be responsible for the higher virulence of ANGpath in rabbits (manuscript in preparation).

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