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Increased susceptibility to the pathogenic effects of wild-type and recombinant herpesviruses in MPS VII mice compared to normal siblings

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In previous studies, we have shown that a herpesvirus vector can transfer a therapeutic cellular gene (β-glucuronidase) from peripheral sites of inoculation into the central nervous system in mice with a model neurodegenerative disease caused by a deficiency of this enzyme (mucopolysaccharidosis type VII, Sly disease). The vector corrects the enzymatic deficiency in transduced cells but the number of cells corrected is too low to alter the pathology of the disease. The recombinant vector virus, which has the foreign gene substituted into the viral LAT locus, had reduced pathogenicity after corneal inoculation compared to the wild-type virus from which it was derived (HSV-1 strain 17+). We therefore attempted to increase the number of corrected cells in the MPS VII brain by increasing the inoculating dose of the vector. However, the vector was acutely pathogenic in the diseased mice at doses that were non-pathogenic in normal littermates. The pathogenic effect of the vector virus in the mutants could be blocked by passive immunization with human gamma-globulin containing anti-HSV-1 antibodies on the day of infection but not when given at the peak of viral replication (day 4). However, effective protection also blocked transduction by the vector, thereby abrogating the effects of increased vector dosage. The effect was virus specific because inoculation of a high dose of a non-pathogenic variant of strain 17+ virus (1716) directly into the brains of MPS VII mice was not lethal. We found no apparent differences in the acute inflammatory response in mutant versus normal animals. These data suggest that the increased susceptibility to vector virulence was related to the overall compromised state of health of the diseased animals, which is further supported by the observations that the mutant mice are more sensitive to stress and to anesthetics than normal littermates. These findings indicate that adverse effects of gene transfer vectors for genetic diseases may not be fully apparent when tested in normal animals.

Keywords: gene therapy; lysosomal storage disease; MPS VII; β-glucuronidase; herpesvirus vector; pathogenicity; anesthesia

Introduction

A deficiency of the lysosomal acid hydrolase β-glucuronidase (GUSB) causes mucopolysaccharidosis (MPS) VII (Sly disease) (Sly *et al*, 1973), a defect in glycosaminoglycan metabolism. Undegraded glycosaminoglycans accumulate in the lysosomes of a number of cell types including neurons and glia in the brain, producing mental retardation in human patients (Neufeld and Meunzer, 1995). True homologues of human MPS VII have been described in dogs (Haskins *et al*, 1984) and mice (Birkenmeier *et al*, 1989).

A promising approach to the treatment of MPS VII and other lysosomal storage diseases affecting the brain is somatic gene transfer (Wolfe and Sands, 1996). We have previously reported the development of a herpes simplex virus (HSV)-1 -based vector to correct GUSB deficiency in cells of the MPS VII mouse brain (Wolfe *et al*, 1992a). A GUSB cDNA was inserted into the HSV-1 LAT locus to drive transcription after latency was established (Wolfe *et al*, 1992a, Deshmane *et al*, 1995). A replication competent vector was used to target the vector to the central nervous system (CNS) by

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viral spread after peripheral inoculation. However, the number of cells transduced for long-term expression of the normal GUSB was very low. Although less than 5% of normal levels of GUSB expression can reverse pathology after somatic gene transfer in some organs (Wolfe *et al*, 1992b), the number of cells transduced in the brain by the HSV vector was below the level needed for a therapeutic effect. The low level of transduction is a major impediment to using herpesvirus vectors for gene therapy for widely disseminated lesions in the brain such as occur in MPS VII and most inherited metabolic disorders (Snyder and Wolfe, 1996).

The recombinant vector virus had reduced pathogenicity relative to the wild-type virus when tested in Balb/c mice, therefore, one method of increasing the number of cells transduced might be to increase the dosage of vector virus inoculation. However, a 10-fold higher inoculum in the MPS VII mice resulted in an increased number of animals that died from the acute infection. Inbred strains of mice vary in their susceptibility to HSV-1 infection (Lopez, 1975; Kastrukoff *et al*, 1986) and the MPS VII mutation occurred in C57B1/6-*H-2K*^{bm1} strain, a congenic of C57BL/6 with a unique *H-2K* class I MHC gene (Birkenmeier *et al*, 1989). The C57BL/6 and related strains are generally highly resistant to HSV-1 and Balb/c mice display intermediate resistance patterns (Lopez 1975; Kastrukoff *et al*, 1986). However, the normal littermates of the MPS VII were completely resistant to the pathogenic affects of the recombinant virus. Thus the susceptibility of the MPS VII mice to the vector virus appears to be a function of their compromised state-of-health.

Results

The recombinant virus showed reduced pathogenicity in a corneal inoculation assay in Balb/c mice compared to the 17+ parental strain (Table 1). The infected animals exhibited typical signs of acute infection between 6–10 days post-inoculation and some mortality occurred (Table 1). In contrast, when normal littermates of the MPS VII mice were inoculated with either the vector or the more pathogenic wild-type virus, all of the mice survived the acute disease even at the highest dose we could administer by corneal inoculation (Table 1). The differences in susceptibility between MPS VII mice and their normal littermates was significant (Fisher's exact test) at doses of 2×10^5 PFU/eye for 17+ virus (P=0.014) and at 2×10^6 PFU/eye for the vector virus (P=0.009).

There was clinical evidence of acute infection following inoculation, such as exudate around the eyes but it appeared to be less severe than in the mutants. Histologic examination of the trigeminal ganglia during the acute infectious phase (4 days post-inoculation) showed similar inflammatory responses in the MPS VII and normal mice (not shown). To demonstrate that infection had occurred, the trigeminal ganglia of latently infected normal mice were explanted and co-cultured with CV-1 cells to reactivate the virus (Table 2) (Deshmane *et al*, 1995). Most of the normal mice were positive, showing that they had been infected and latency had been established.

After infection, frozen sections of the trigeminal ganglia and the brainstems from MPS VII mice were assayed for the presence of GUSB enzymatic activity *in situ*, as described previously (Wolfe *et al*, 1992a). No positive cells were ever seen in untreated MPS VII mice, as described previously (Wolfe *et al*, 1992a; Snyder *et al*, 1995), since they are null mutants for GUSB (Sands and Birkenmeier, 1993). Nor were any positive cells seen in control MPS VII mice infected with the wild-type virus (strain 17+) from which the vector was derived (not shown), also as seen previously (Wolfe *et al*, 1992a). The dose of 2×10^5 PFU per eye may have been near the threshold dose to form a latent infection because only a few positive cells were seen in each animal, and because positive cells were only detected in one ganglion in some animals even though all were inoculated bilaterally. We therefore increased the inoculating dose ten-fold, which resulted in much greater mortality from the acute disease (Table 1), but did not substantially increase the number of corrected cells.

We attempted to block the pathogenic affect of the high dose of virus in MPS VII mice by passive immunization with human serum containing anti-HSV antibodies (Shimeld *et al*, 1990). Immuniza-

Table 1 Acute mortality after corneal inoculation of HSV vector virus

Virus	Dose (PFU/eye)	Deaths per total (%) Balb/c	B6.C-H-2K^{bm1}/ByBir-gus^{mps} Normal	MPSVII
17+	2×10^5	9/20 (45)	0/9 (0)	3/4 (75)
	2×10^6	(>90)[a]	0/10 (0)	ND
ΔLAT-GUSB	2×10^5	7/40 (18)	0/11 (0)	2/10 (20)
	2×10^6	ND	0/11 (0)	3/4 (75)

[a] average observation over many experiments. ND=not done

Table 2 Reactivation of HSV from latently infected normal littermates of MPS VII mice

Dose	# positive ganglia (%) 17+	ΔLAT-GUSB
2×10^5	8/9 (88)	8/10 (80)
2×10^6	6/6 (100)	5/8 (62)

Table 3 Effect of passive immunization on acute susceptibility to vector infection and expression of GUSB

Day	*Balb/c* 2×10^5 17+ mortality	*MPS VII* 2×10^6 ΔLAT-RGUSB mortality	GUSB+[b]
none	9/20	3/4	2/2
−2		0/4	0/4
0	0/30	0/4	1/3
0 (0.1)[a]		0/4	0/4
+2		1/4	2/3
+4		4/4	2/2

[a](1/10 dose of γ glob). [b]All mice with tissues that could be examined were assayed, but tissue necrosis prevented analysis of some mice which died overnight

tion at the time of infection, with a dose that inhibits viral replication *in vitro*, protected all of the animals (Table 3). However, this also blocked infectivity and the establishment of latent infection since positive cells were seen in only one out of seven animals immunized before or at the time of vector infection (Table 3), negating the effect of increasing the viral dose. Using a 10-fold lower dose of antiserum produced similar results (Table 3). We therefore attempted to passively immunize mice 4 days after viral inoculation, a time which is after the viral genome reaches neuronal cells but before the onset of clinical illness. However, this regimen failed to protect the animals (Table 3). Immunization at 2 days post-infection improved the resistance of the animals but the number of corrected cells was still very low even though two of three had evidence of transduction, and there was no net improvement in the total number of cells transduced and corrected.

To determine if the effects were virus specific, we inoculated MPS VII mice with a very high dose of HSV strain 1716 which has greatly reduced neurovirulence (MacLean *et al*, 1991) as a result of a deletion mutation in the ICP 34.5 gene near the LAT locus. Since this virus does not move from trigeminal ganglion to the brain stem (Spivak *et al*, 1995), we injected it directly into the brains of MPS VII mice. This is a much more rigorous test of pathogenicity than peripheral inoculation because the wild-type virus (17+) was lethal (five out of five acute deaths) in the intermediate-resistant Balb/c mice at dose of only 10 PFU (not shown). In contrast, a dose 5×10^5 PFU of 1716 was non-pathogenic (0 out of three acute deaths) when directly injected into the brains of MPS VII mice (not shown). *In situ* hybridization detected expression of the LAT transcript in all three MPS VII mice injected with 1716 virus to demonstrate that the brains had become infected and latency had been established by this virus (Figure 1). Antibody staining in serial sections for products of the viral replicative cycle were negative (not shown).

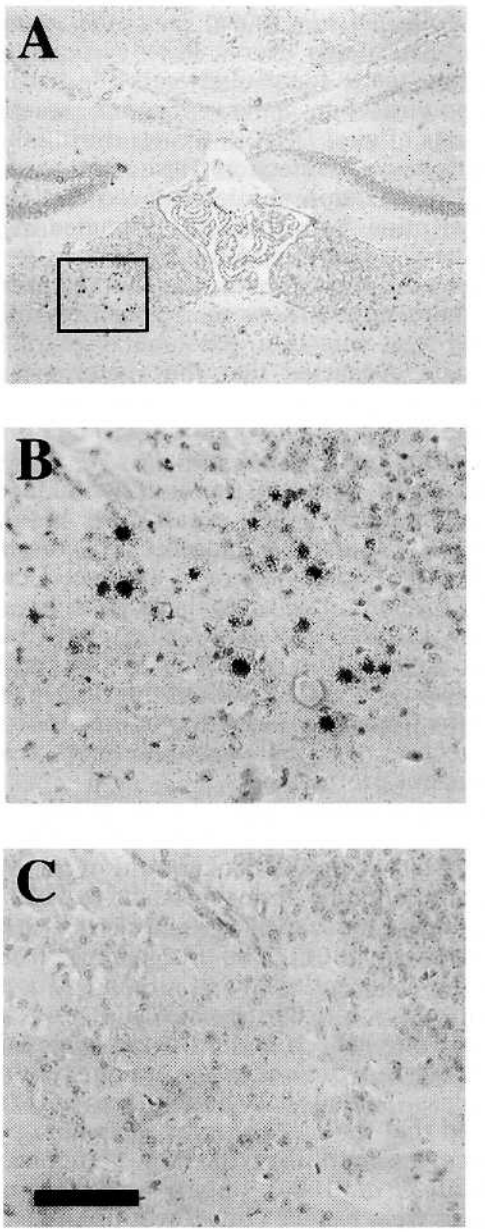

Figure 1 Expression of HSV-1 specific LAT RNA in a latently infected MPS VII mouse brain after injection of 5×10^5 PFU of 1716. (A) 1716 virus infected MPS VII brain tissue showing positive signal; (B) Area boxed in panel A from the thalamus showing typical LAT-specific hybridization of HSV-1 strain 1716 in neurons; (C) Uninfected thalamus tissue (Balb/c). Scale bar represents 300 microns in A and 71 microns in B and C.

Discussion

Insertion of the GUSB cDNA into the LAT locus reduced the pathogenicity of the vector virus compared to wild-type parental strain virus in Balb/c mice. Previous studies showed that the vector can express GUSB enzyme activity stably in the trigeminal ganglia and brainstem of MPS VII

mice (Wolfe *et al*, 1992a), but the number of positive cells was very low. No appreciable differences were found between the vector and wild-type viruses in acute replication, reactivation, or amounts of viral DNA in tissues during the acute or latent phases of infection (Deshmane *et al*, 1995). *In situ* hybridization studies showed that the level of transcription from the LAT promoter in individual latently infected cells is similar from the vector or wild-type viruses, but the vector-infected ganglia have only 20% as many positive cells as in the wild-type infection (Deshmane *et al*, 1995). These data suggested that the vector established latency similar to wild-type virus but the LAT promoter-driven gene expression was compromised in the recombinant virus.

Attempts to increase the number of corrected cells by increasing the viral dose resulted in significantly increased mortality in the mutant mice. The pathogenic effect of the vector could be blocked in the mutant animals by passive immunization with anti-HSV antiserum, but this also blocked transduction of cells by the vector. This is likely to have been the result of interfering with the acute infection since passive immunization 4 days after infection, the time of maximum viral replication, was not protective.

The mutant mice exhibited susceptibility similar to the Balb/c strain. Previous studies had shown that C57B1/6 mice, the background of the MPS VII strain, are generally more resistant to HSV-1 than Balb/c mice (Lopez, 1975; Kastrukoff *et al*, 1986; Thomas *et al*, 1991). The *H-2* complex of genes influences susceptibility to some viruses (e.g. leukemogenic retroviruses, Wolfe and Hardy, 1989) and the MPS mutation arose on the C57B1/6-*H-2*^{bm1} strain which carries a unique allele at the *H-2K* class I locus. Although previous studies indicated that the *H-2* complex does not appear to influence susceptibility to HSV-1 (Lopez, 1975; Kastrukoff *et al*, 1986), only a limited number of congenics were tested. Thus it was possible that the allele at the *H-2K* locus was responsible for the change in susceptibility. However, the vector was completely non-pathogenic in the normal littermates of the mutants. This finding, and the observation that the inflammatory infiltrate during the acute infection is similar in mutant and normal mice, suggest that the host immune response to the virus is not involved in the differences in susceptibility.

The increased susceptibility of the MPS VII mice compared to their healthy littermates is probably the result of the overall compromised state of health of the diseased animals, rather than a specific effect of GUSB activity on viral pathogenesis. The disease is characterized by progressive degeneration leading to early death (on average between 5–6 months of age, Birkenmeier *et al*, 1989). We and others have observed that the diseased mice can die simply from the stress of handling (unpublished). The mutants also are more susceptible to the effects of the anesthetic tribromoethanol (Avertin) than normal mice, in which it is usually very safe. The diseased mice require only half the dose of a normal littermate of the same weight to become fully anesthetized and to achieve a surgically acceptable level of analgesia (Wolfe and Sands, 1996). Although it is unclear whether the increased sensitivity is related to the pathology of the central nervous system versus a more general affect of the disease, the observation that there were differences between both the anesthetic and analgesic effects suggest that they are more likely to be related to the central nervous system pathology.

Since the vector virus exhibited reduced pathogenicity when tested in one strain of normal mice, it is apparent that some adverse effects of gene transfer vectors may not become known until they are used in diseased animals. The non-pathogenicity of the 1716 virus in the diseased animals suggests that it may be an excellent vector backbone and we are constructing a GUSB-expressing vector based on it. The *in situ* hybridization for LAT expression from latent 1716 also suggests that it may be possible to transduce an increased number of cells with this virus in the MPS VII mouse brain. However, expression may be altered when a foreign gene is inserted into the LAT region as occurred with the 17/LAT-GUSB vector (Deshmane *et al*, 1995). Reducing pathogenicity of gene transfer vectors will be especially important when attempting to treat animals or human patients debilitated by genetic disease.

Materials and methods

Cell culture and HSV-1 growth titration

Subconfluent monolayers of CV-1 cells grown in Eagle's minimal essential medium supplemented with 5% fetal calf serum at 37°C with 5% CO_2 were infected with HSV-1 strain 17+ or with 17/LAT-RGUSB virus (Wolfe *et al*, 1992a; Deshmane *et al*, 1995) at 1 PFU per cell, and virus was concentrated from the medium as previously described (Deatly *et al*, 1987). As described earlier (Wolfe *et al*, 1992a) the 17/LAT-RGUSB virus vector contains a deletion spanning two *BstEII* sites located at nucleotide numbers 6260–7157 in one of the copies of LAT gene in the terminal repeat long region, and at nucleotide numbers 119198–120095 in the other copy of LAT gene in the internal repeat long region of the HSV-1 genome. These deletions have been replaced by a 2.4 kb *EcoRI* fragment of rat cDNA for the β-glucuronidase gene, such that the LAT promoter controls transcription of the inserted glucuronidase gene. The titers of virus stock were determined on CV-1 cells. The ability of virus to replicate in infected trigeminal ganglia was judged by titering virus in tissue homogenates. Individual

ganglia were homogenized in serum free medium and ten fold dilutions were plated onto CV-1 cells to score PFUs.

Animals and infection procedure
The MPS VII mice and normal littermates were bred from the carrier strain B6.C-*H-2*^{bm1}/ByBir-*gus*$^{mps/+}$ (Birkenmeier *et al*, 1989). Although this is an autosomal recessive trait, only about 10% of pups that survive to adulthood are MPS VII (Wolfe and Sands, 1996), which limits the number of animals available for testing. The mutants were identified both by gross appearance and lack of GUSB activity (Wolfe *et al*, 1992a). Balb/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were anesthetized with avertin to effect, and inoculated by corneal scarification and adsorption of 5 μl medium containing virus as described (Wolfe *et al*, 1992a).

Explant reactivation
At a minimum of 4 weeks after infection, latently infected mice were sacrificed and the trigeminal ganglia were removed and incubated with monolayers of CV-1 cells. The wells were inspected daily for signs of cytopathic effect. Ganglia were transferred every 4 to 5 days to new cells and observed until reactivation occurred.

In situ *hybridization*
In situ hybridization were performed essentially as described (Deshmane *et al*, 1995). Briefly, brain sections were perfused and fixed for 24 h in fresh paraformaldehyde-lysine-periodate fixative at 4°C, dehydrated in ethanol, and embedded in paraffin. Sections 5 to 6 mm thick were mounted on poly-L-lysine treated slides deparaffinized with xylene and treated with proteinase K. ^{35}S-labeled nick-translated probes were diluted in hybridization mixture to contain 1 ng of DNA probe per 5 ml and approximately 10^5 c.p.m. per tissue section. Heat-denatured hybridization mixture (5 ml) was placed on each tissue section and covered with baked siliconized coverslip and paraffin oil. After hybridization at 50°C for 48 h, the paraffin oil was removed with a chloroform wash and the slides were washed, dehydrated in ethanol, dipped in NTB-2 nuclear track emulsion (Eastman Kodak Co, Rochester, NY) and exposed for 4 days at 4°C. After processing with D19 developer (Kodak) and fixer (Kodak), the sections were stained with hematoxylin and eosin.

In situ *GUSB assay*
The brainstem and trigeminal ganglia were examined for GUSB expression as described (Wolfe *et al*, 1992a). The tissues were removed by dissection, immediately frozen in liquid nitrogen-cooled isopentane for 10 s then transferred to liquid nitrogen, and stored at −70°C. The blocks of frozen tissue were cut into 10 micron sections, attached to glass slides, and stored at −70°C. The frozen sections were reacted for GUSB activity (Wolfe *et al*, 1992a) and examined using differential interference contrast (Nomarski) microscopy.

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