



VIRAL TRANSFER TECHNOLOGY

BRIEF COMMUNICATION

Central nervous system toxicity of two adenoviral vectors encoding variants of the herpes simplex virus type 1 thymidine kinase: reduced cytotoxicity of a truncated HSV1-TK

C Cowsill¹, TD Southgate^{1,*}, G Morrissey^{1,*}, RA Dewey¹, AE Morelli¹, TC Maleniak¹, Z Forrest¹, D Klatzmann², GWG Wilkinson³, PR Löwenstein¹ and MG Castro¹

¹Molecular Medicine and Gene Therapy Unit, School of Medicine, University of Manchester, Manchester, UK; ²Laboratoire de Biologie et Thérapeutique des Pathologies Immunitaires, Université Pierre et Marie Curie, CNRS, Hôpital de la Pitié-Salpêtrière, Paris, France; and ³Department of Medicine, University of Wales College of Medicine, Heath Hospital, Heath Park, Cardiff, UK

Herpes simplex virus type 1-thymidine kinase (HSV1-TK) in combination with ganciclovir is an efficient and widely used strategy in brain tumour gene therapy. Recently, we have shown effective inhibition of glioma growth in a syngeneic rat model using recombinant adenoviruses expressing the full-length HSV1-TK and an N-terminus truncated variant, HSV1-ΔTK in the presence of ganciclovir. We also showed active chronic brain inflammation in the long-term survivors (3 months) treated with HSV1-TK plus GCV. Furthermore, our results indicated loss of myelinated fibres, oedema and indices of ongoing axonal degeneration. In this study, we assessed the cytotoxicity of both HSV1-TK variants in the presence or absence of ganciclovir, in primary cultures of neurones and glia, and in the rat brain *in vivo*. Our results

indicate that, at viral doses where tumour cells are sensitive to the enzyme/prodrug system, (1) there is no major cytotoxicity for either neurones or glial cells grown in primary cultures, (2) on its own the full-length HSV1-TK is more cytotoxic than its truncated version HSV1-ΔTK for a population of non-neuronal and non-glial cells within neocortical primary cultures, and (3) *in vivo*, when delivered into the striatum, RAdS encoding HSV1-TK are more cytotoxic than RAdS encoding HSV1-ΔTK, after administration of ganciclovir. The effectiveness of HSV1-ΔTK in preventing brain tumour growth *in vivo*, combined with its reduced cytotoxicity, both *in vivo* and in primary cultures of CNS cells, could represent an advantage for treatment of brain tumours using gene therapy. Gene Therapy (2000) 7, 679–685.

Keywords: thymidine kinase; brain tumours; flow cytometry; neurological; cytotoxicity

Retroviral, adenoviral, and herpes simplex virus type 1-mediated transfer of the HSV1-TK gene to mammalian brain tumour cells in combination with the peripheral administration of the nucleoside analogue ganciclovir (GCV) has been shown to be efficient as an antitumoral strategy in several preclinical studies.^{1–8} Using this approach, several clinical trials for the treatment of different kinds of tumours have been initiated,⁹ including clinical trials for brain tumours using retroviruses and adenoviruses encoding HSV1-TK in combination with GCV.¹⁰

When recombinant adenoviruses (RAdS) encoding HSV1-TK are administered into the brain, they will also infect (and might be cytotoxic for) peritumoral normal neurones and other non-tumoral cells in the CNS (eg glia, endothelium, microglia, meningeal cells). High-dose adenovirus-mediated HSV1-TK gene transfer to baboon (1.5×10^9 total plaque forming units (p.f.u.)) and rhesus monkey brain (1.5×10^{11} total p.f.u.) led to gliosis,

necrosis, meningitis and even death upon GCV administration.^{11,12} *In vitro*, Maron and co-workers¹³ recently described that RAd-TK plus GCV is cytotoxic for astrocytes grown in primary culture. Work from our laboratory has recently shown that both HSV1-TK and an N-terminus truncated version HSV1-ΔTK, encoded by recombinant adenoviruses are equally effective in inhibiting the growth of CNS-1 glioma cells in Lewis rats.¹ We also showed active brain inflammation, demyelination and indices of ongoing axonal degeneration, 3 months after successful inhibition of glioma growth after treatment with the recombinant adenovirus expressing the full-length HSV1-TK.¹

We therefore considered it of critical importance to study the cytotoxicity of HSV1-TK and HSV1-ΔTK within infected cells from the rat central nervous system *in vitro*, and in the rat brain *in vivo*. Our results show that neither neurones nor glial cells in primary cultures were killed by HSV1-ΔTK or HSV1-TK alone, or by GCV on its own. However, HSV1-TK was more toxic, both on its own and in the presence of GCV, to non-neuronal/non-glial cells present in primary CNS cell cultures, when compared with HSV1-ΔTK. Similarly, the nonspecific toxicity, as well as the activation of astroglia and infiltration of

Correspondence: MG Castro or PR Löwenstein, Molecular Medicine and Gene Therapy Unit, Room 1.302 Stopford Building, School of Medicine, University of Manchester, Oxford Road, Manchester M13 9PT, UK

*These authors contributed equally to the work

Received 17 June 1998; accepted 8 December 1999

inflammatory cells was much higher in animals injected with RAD expressing HSV1-TK when compared with HSV1- Δ TK. Thus, the *in vitro* and *in vivo* results presented in this paper shed light on the inherent direct cytotoxicity of HSV-TK towards neurones, glial and other brain cells in the absence of inflammatory and immune cells (*in vitro*), and in their presence (*in vivo*).

RAd127 (HSV1- Δ TK) and RAd128 (HSV1-TK) were generated by homologous recombination between the left-hand side of the adenoviral genome in pJM17 and the plasmid pAL119, containing the sMIEhCMV promoter¹⁴ upstream of HSV1-TK or HSV1- Δ TK, followed by the SV40 polyadenylation sequence as described previously.¹

Cell suspensions harvested from rat primary neocortical neuronal cultures infected with RAd127 or RAd128, were double immunostained to detect simultaneously the expression of cell type-specific neuronal (MAP-2) or glial (GFAP) markers, in combination with either transgene protein levels or apoptosis, using flow cytometry. The percentage of MAP-2-positive cells was 98% at 2 days after plating, and 69% at 6 days after plating. The percentage of GFAP expressing astrocytes increased from 1% to 10%, indicating ongoing cell division of non-neuronal cells (results not shown). Due to their low concentration in primary neuronal cultures, the population of astrocytes was electronically enriched to allow their analysis by flow cytometry. Enrichment was done by taking advantage of the astrocytes' higher cytoplasmic granularity (side scatter signal), and bigger size (forward scatter signal), compared with neurones. However, cells were classed as neurones by their MAP-2 immunoreactivity, or as glial cells through their GFAP immunoreactivity. Cells negative for both MAP-2 and GFAP (likely to represent fibroblasts, endothelial cells and/or microglia) were also analysed. Our cultures did not contain cells immunoreactive for β -galactocerebrosidase (a marker for oligodendrocytes). Both neurones and astrocytes expressed HSV1-TK and HSV1- Δ TK driven by the sMIEhCMV promoter (results not shown).

To study the conditional toxicity of adenovirally encoded HSV1-TK or HSV1- Δ TK plus GCV for astrocytes and neurones, we analysed the percentage of apoptotic neurones and astrocytes by flow cytometry.¹⁵ Neuronal cultures were infected 24 h after plating with RADs with 5×10^7 IU added per well (approximate MOI 30) for 48 h, followed by GCV (10 μ M) treatment for 3 days (Figure 1). RAd127 and RAd128 were not cytotoxic to either neurones or astrocytes in the presence or absence of GCV ($P \geq 0.05$; RAd127 + GCV, and RAd128 + GCV *versus* any other treatment group) (Figure 1a). However, we detected significant levels of apoptosis when we analysed MAP-2 and GFAP double negative cells. In the presence of GCV, RAd128 caused 43% apoptosis ($P < 0.001$; RAd128 + GCV *versus* all other treatment groups); in the absence of GCV, RAd128 caused 38% apoptosis in this cell population ($P < 0.001$; RAd128 *versus* all other treatment groups). The increased apoptosis after adding GCV was small but statistically significant ($P < 0.001$; RAd128 *versus* RAd128 + GCV). RAd127, however, was less toxic for non-neurones/non-glial cells showing 7% apoptosis in the presence of GCV ($P < 0.001$; RAd127 + GCV *versus* RAd127, and also *versus* all other treatment groups), and only basal levels of apoptosis (1%) were detected in the absence of GCV (not significant; RAd127 *versus* all other treatment groups).

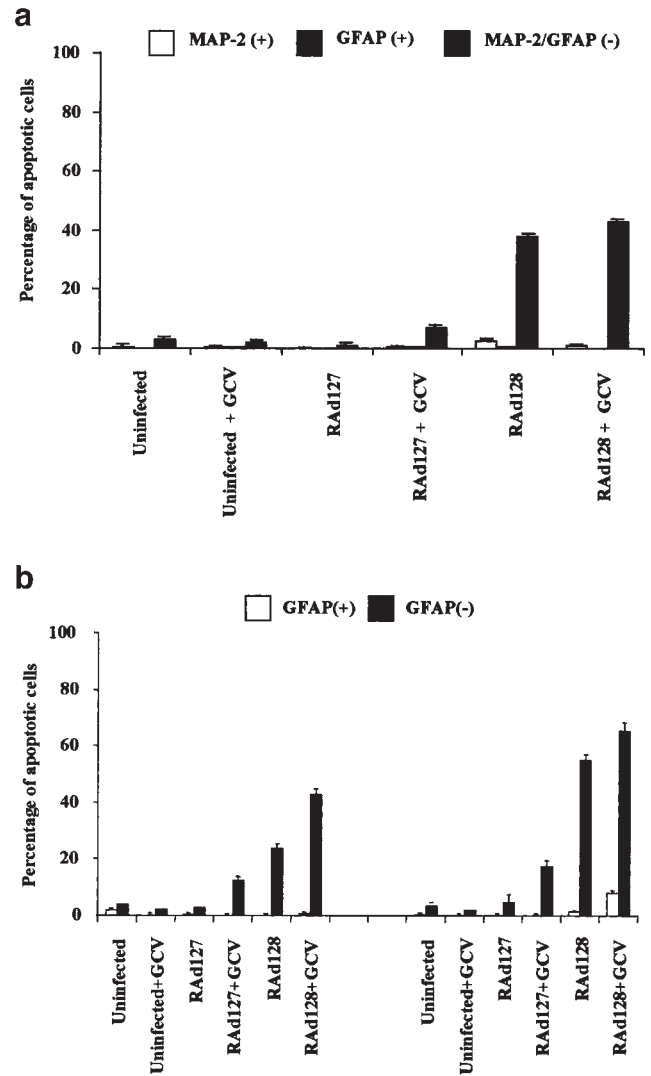
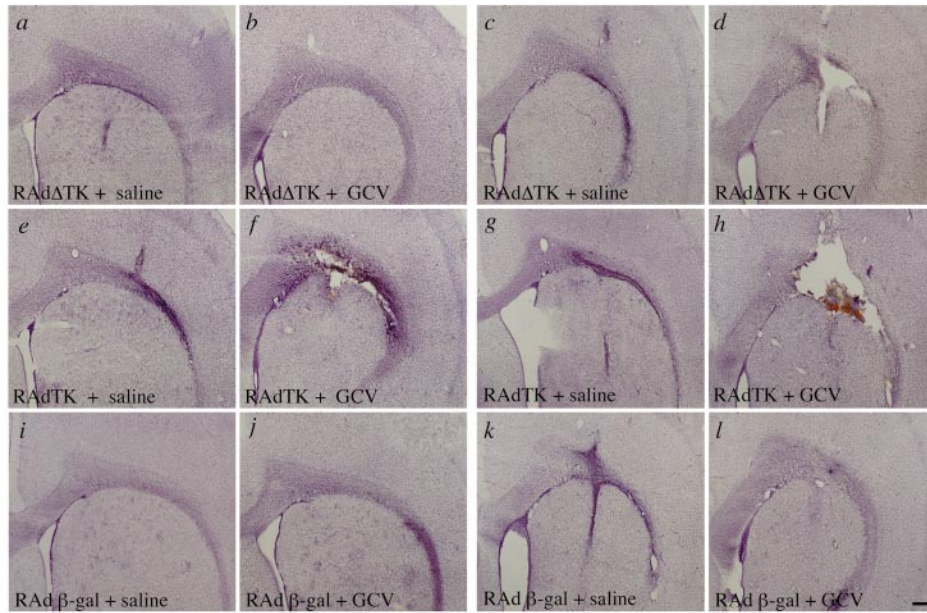


Figure 1 (a) Rat neurones in primary cultures: cytotoxicity of RAds expressing HSV1- Δ TK and HSV1-TK. Cultures were characterised using specific antibodies by flow cytometry: MAP-2-positive cells (neurones), GFAP-positive cells (astrocytes), and MAP-2/GFAP-negative cells (possibly endothelial, fibroblast or microglial cells) were studied. Cells were infected with RAd127 and RAd128 (MOI 30) in the absence or presence of GCV (10 μ M). Uninfected cells incubated in the absence or presence of GCV were used as controls. (b) Rat glia in primary cultures: cytotoxicity of HSV1- Δ TK and HSV1-TK expressed from RAds in rat primary glial cultures. Cells were plated at two different cell densities (low 3×10^5 cells per well (left panel) and high 1.25×10^6 cells per well (right panel), and infected with RAd127 and RAd128 (MOI 30) in the absence or presence of GCV (10 μ M). GFAP-positive cells (astrocytes) and GFAP-negative cells (possibly endothelial, fibroblast, microglial cells) were identified using an antibody specific for GFAP, and fluorescence was assessed by flow cytometry. Uninfected cells incubated in the absence or presence of GCV (10 μ M) were used as controls. The percentages of apoptotic cells were measured by flow cytometry.

However, due to the low percentage of astrocytes (<10%) present in primary neuronal cultures, we also studied the toxicity of our system in primary glial cultures, in which 40–50% of the population is GFAP immunoreactive. Thus, to determine if the toxicity of HSV1-TK and HSV1- Δ TK plus GCV is dependent on whether the cells are replicating or not we plated the cells at two den-

(I) Nissl staining



(II) TK immunoreactivity

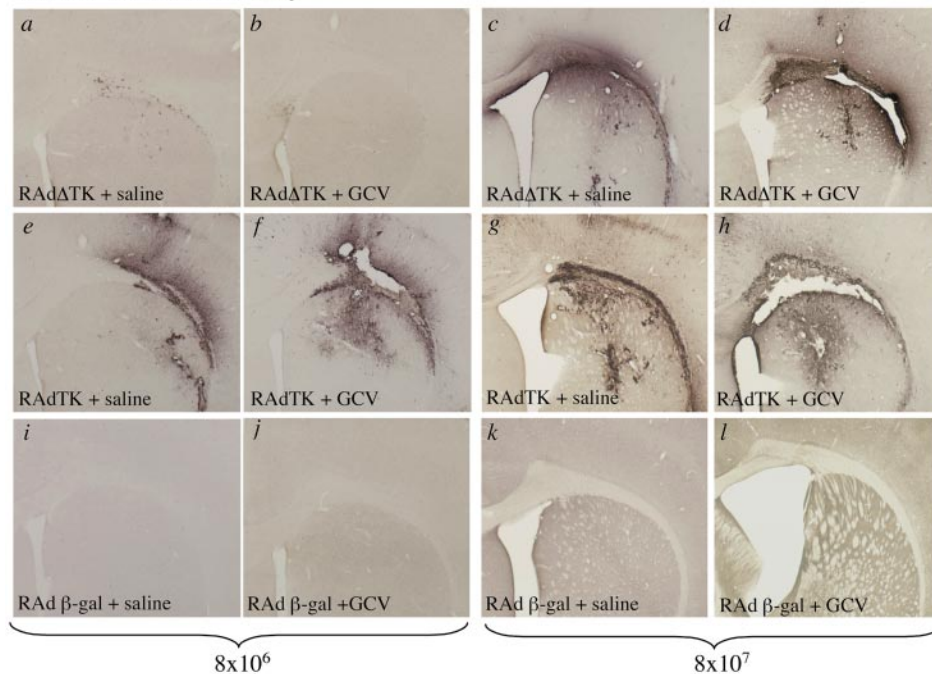


Figure 2 Determination of brain lesions by Nissl staining (I) and HSV1-TK or HSV1-ΔTK immunoreactivity (II) *in vivo*. Animals were injected with RAd128, (HSV-TK), RAd127, (HSV-ΔTK), or RAdβ-gal at a dose of 8×10^6 IU or 8×10^7 IU, then treated for 7 days with systemic GCV or saline injections twice daily. Nissl staining indicated no evidence of brain lesions in animals treated with RAdβ-gal (li-l). Animals injected with the higher dose of RAd127 or RAd128 followed by GCV, however, demonstrated a morphologically detectable lesion (ld and h) but not with saline (lc and g). Only animals treated with the lower dose of RAd128 plus GCV showed evidence of brain lesion (la, b, e, f). No HSV1-TK immunoreactivity was found in animals treated with RAdβ-gal (Iii-l). More intense HSV1-TK immunoreactivity was found at the higher dose of viral vector and in animals injected with RAd128 (Iia-h).

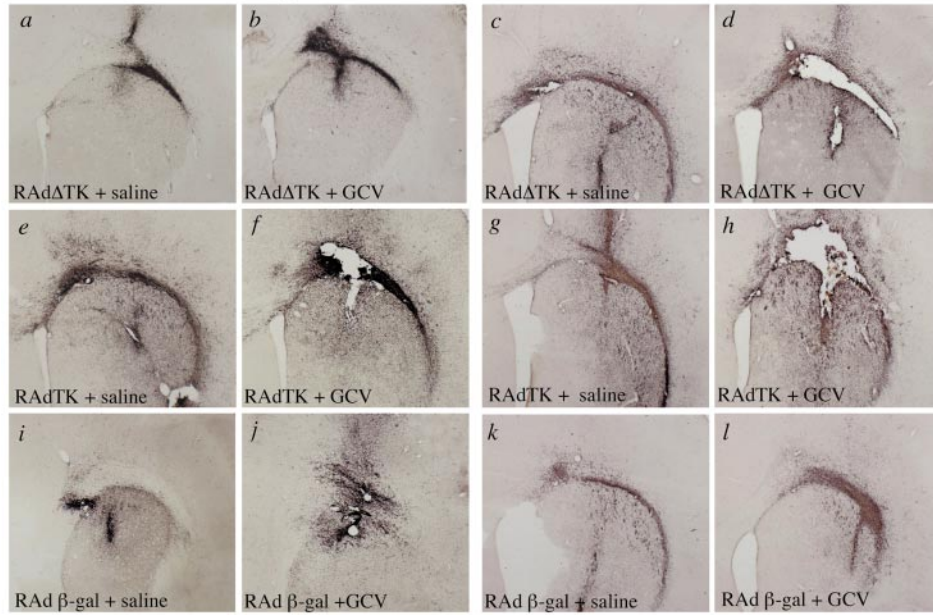
sities: high, confluent (non-dividing conditions) and low, non-confluent (dividing conditions).

Analysis of GFAP immunoreactive cells indicated that, in low-density culture (Figure 1b, left panels), neither RAd127 nor RAd128 (MOI 30) *per se* were toxic, either in the absence or presence of GCV ($P \geq 0.05$; RAd127 + GCV *versus* all other treatments, or RAd128 + GCV *versus* all

other treatments). In high-density cultures (Figure 1b, right panels), neither virus was cytotoxic in the absence of GCV ($10 \mu\text{M}$) ($P \geq 0.05$; RAd127 and RAd128 *versus* uninfected, and uninfected + GCV). In the presence of GCV, however, RAd128 killed 8% of the astrocytes ($P < 0.001$; RAd128 + GCV *versus* RAd128).

Analysis of GFAP-negative cells in low-density culture

(I) ED1 immunoreactivity



(II) GFAP immunoreactivity

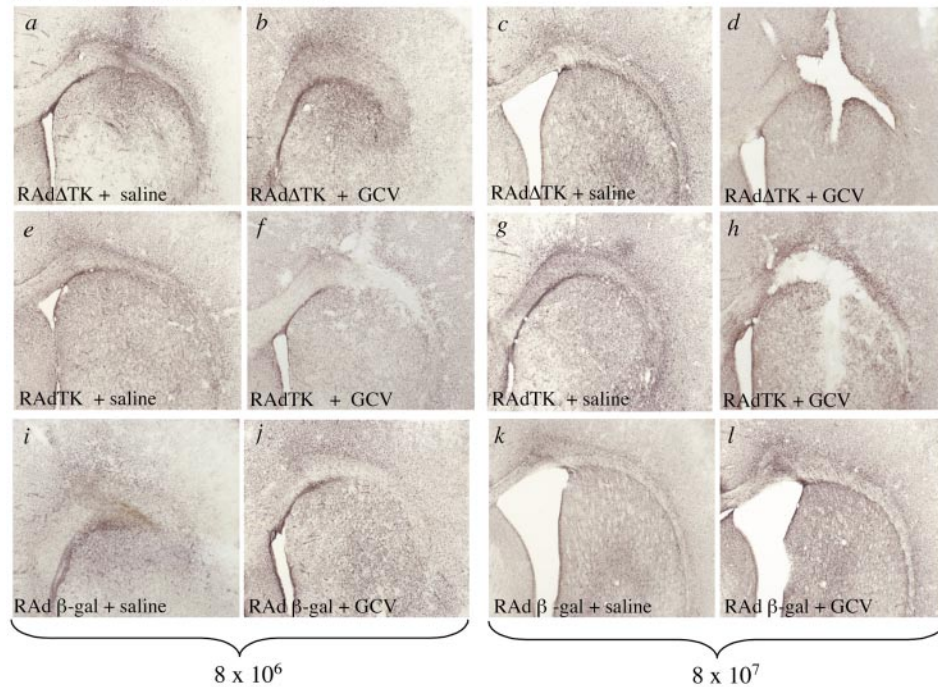


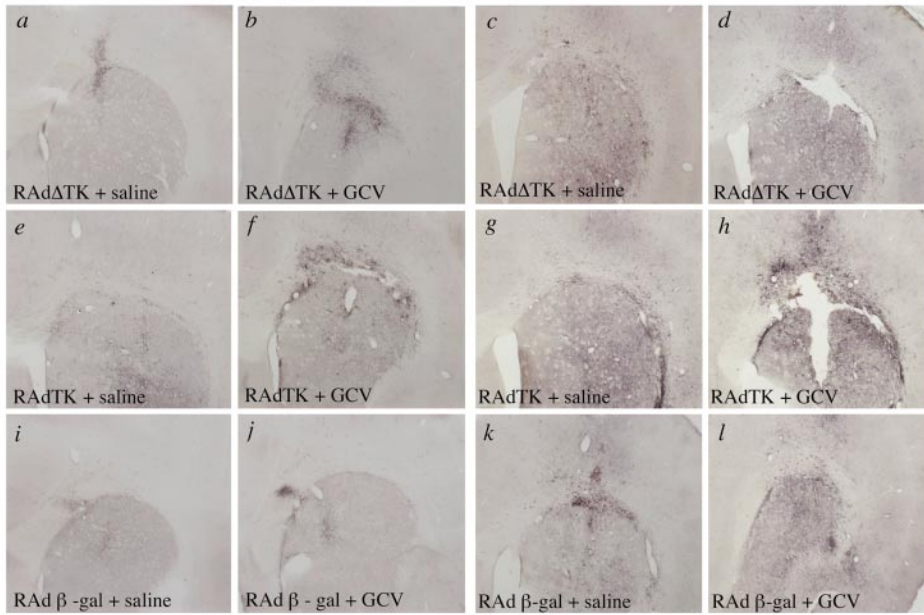
Figure 3 Infiltration of macrophages/microglia (ED1 immunoreactive I) and astrocytes (GFAP immunoreactive II) cells *in vivo* following administration of RAd127 and RAd128 into the brain. Up-regulation of ED1 and GFAP immunoreactivity was present in all animals. ED1 and GFAP immunoreactivity in animals treated with RAd β -gal did not increase following GCV treatment but did increase with a higher viral dose (Ii–I; IIi–I). Animals injected with RAd128 or RAd127 showed similar increases in ED1 and GFAP immunoreactivity with higher viral doses (Ic, d, g, h; IIc, d, g, h). Further increased ED1 and GFAP immunoreactivity was observed in animals treated with ganciclovir (Ib, d, f, h; IIb, d, f, h).

(Figure 1b, left panels) indicated that HSV1- Δ TK was non-cytotoxic *per se* ($P \geq 0.05$; RAd127 *versus* uninfected, and uninfected + GCV). In the presence of GCV, RAd 127 was cytotoxic ($P < 0.001$; RAd127 + GCV *versus* RAd127). HSV1-TK however, was cytotoxic in the absence of GCV (25% apoptosis) ($P < 0.001$; RAd128 *versus* uninfected, uninfected + GCV, RAd127, RAd127 + GCV). In the presence of GCV, the toxicity of RAd128 was significantly

increased (40% apoptosis) ($P < 0.001$; RAd128 + GCV *versus* RAd128).

Analysis of GFAP-negative cells in high-density culture (Figure 1b, right panels) indicated that HSV1- Δ TK was non-cytotoxic *per se* ($P \geq 0.05$; RAd127 *versus* uninfected, and uninfected + GCV). In the presence of GCV, RAd 127 was cytotoxic ($P < 0.0001$; RAd127 + GCV *versus* RAd127). HSV1-TK however, was cytotoxic in the

(I) CD4 immunoreactivity



(II) CD8 immunoreactivity

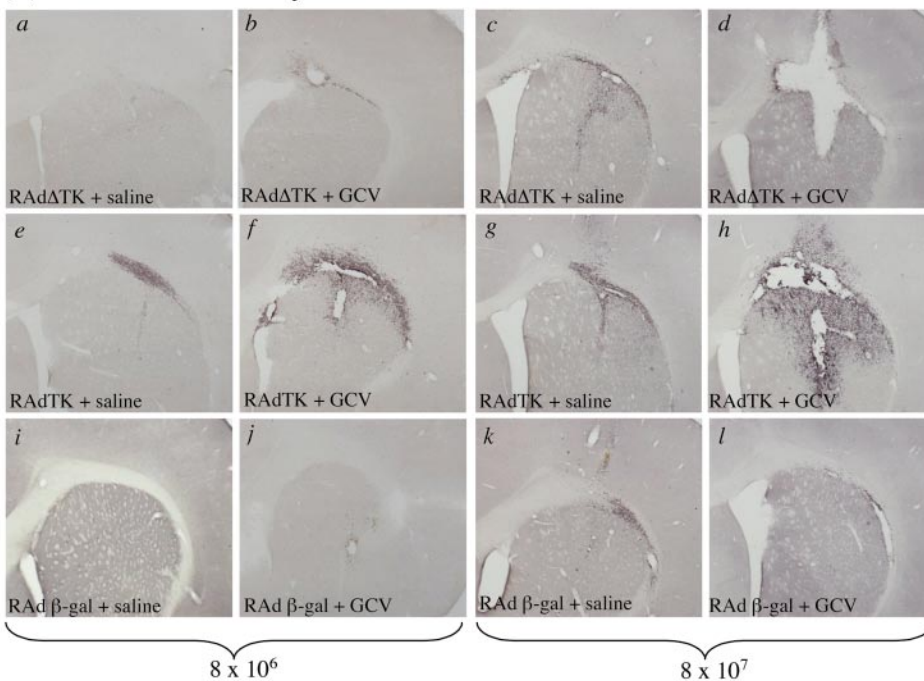


Figure 4 Infiltration of CD4⁺ immunoreactive and CD8⁺ immunoreactive lymphocytes following administration of RAd127 and RAd128 into the brain *in vivo*. There was a small but significant infiltration of CD4⁺ and CD8⁺ lymphocytes at the higher viral dose of RAdβ-gal (Ik and l; IIk and l) which were not evident at the lower dose (Ii and j; IIIi and j). Infiltration of CD4⁺ and CD8⁺ immunoreactive lymphocytes in animals injected with RAd128 (Ie and h; IIe and h) or RAd127 (Ia and d; IIa and d) was increased in animals treated with GCV (Ib, d, f, h; IIb, d, f, h).

absence of GCV (55% of apoptosis) ($P < 0.001$; RAd128 *versus* uninfected, uninfected + GCV, RAd127, RAd127 + GCV). In the presence of GCV, the toxicity of RAd128 was significantly increased (65% apoptosis) ($P < 0.001$; RAd128 + GCV *versus* RAd128).

These results show that *in vitro*, high levels of HSV1-TK and HSV1-ΔTK proteins expressed in neurones and glial cells, are not cytotoxic even after the addition of ganciclovir. Nevertheless, a population of non-neuronal/

non-glial cells was very sensitive to the transduction with adenoviruses encoding HSV1-TK, but much less sensitive to the toxicity of HSV1-ΔTK. This suggests that much of the neuronal and glial toxicity detected *in vivo* could depend on the inflammatory and immune reactions to either the viral vector, and/or transgenes.

We injected two doses of adenoviral vectors, 8×10^6 IU and 8×10^7 IU, which have previously been used by us and others in experimental trials of glioma gene therapy

and gene transfer into the brain. Animals were injected with either dose, of RAD127, RAD128, or RAD β -gal, used as a control. Animals were then treated either with ganciclovir or saline twice daily for 7 days, and killed immediately after the 7th day of treatment to determine whether the short-term inflammatory reaction was dependent on the vectors themselves, the transgene, or the combination of vector expressing the transgene and ganciclovir. Brains were cut serially, and adjacent vibratome sections were stained for Nissl or immunoreacted to detect the presence of transgene (ie using antibodies against HSV1-TK) (Figure 2), activated macrophages/microglial cells (ie using antibodies recognizing ED1), astrocytes (ie using antibodies against with GFAP) (Figure 3), CD4⁺ and CD8⁺ lymphocytes (Figure 4).

No brain lesion (Figure 2 Ii-l) or HSV1-TK immunoreactivity (Figure 2 II i-l) was detected in the brains of animals injected with RAD β -gal, at either dose, either in the absence or presence of ganciclovir. In these brains we detected only an increase in the infiltration of ED1 immunoreactive macrophages/microglial cells that did not increase following the ganciclovir treatment (Figure 3 Ii-l), and an increase in GFAP immunoreactivity (Figure 3 Iii-l). Infiltration of macrophages/microglia (ED1) and astrocytes (GFAP) was higher with the higher viral vector dose (Figure 3 Ik-l and 3 Iik-l). There was a small but significant infiltration of CD4⁺ and CD8⁺ cells at the 8×10^7 IU dose (Figure 4 Ik-l and 4 Iik-l), but not in brains injected with 8×10^6 IU (Figure 4 Ii-j and 4 Iii-j). Infiltration of lymphocytes following injection of RAD β -gal did not increase following ganciclovir treatment.

Following the injection of 8×10^7 IU of RAD127 or RAD128, a morphologically detectable lesion was detected by Nissl staining in brains of animals treated with ganciclovir (Figure 2 Id and h), but not in those treated with saline (Figure 2 Ic and g). At the lower dose of 8×10^6 IU, a lesion was detected only in animals injected with RAD128 and ganciclovir (Figure 2 If), but not in animals injected with RAD127 and ganciclovir (Figure 2 Ib). There were no lesions in animals injected with either virus and saline. Expression of the transgenes was stronger at the higher dose of viral vector and in animals injected with RAD128 (Figure 2 Iia-h). HSV1- Δ TK expression was very low in animals injected with RAD127 at the dose of 8×10^6 IU (Figure 2 Iia and b). The severity of macrophage/microglial infiltration (ED1: Figure 3 Ia-h), astroglial activation (GFAP: Figure 3 Iia-h), and the infiltration of CD4⁺ (Figure 4 Ia-h) and CD8⁺ (Figure 4 Iia-h) lymphocytes followed the same trend of the morphologically evident lesion seen in the Nissl stain (Figure 2 Ia-h), and the transgene expression (Figure 2 Iia-h).

The corresponding influx of macrophages/microglial cells, as well as the infiltration of CD4⁺ and CD8⁺ lymphocytes, was correspondingly higher in animals injected with RAD128 and treated with ganciclovir. The lesion induced *in vivo* was more important in animals injected with RAD128 and treated with ganciclovir (Figures 2-4). Infiltration of ED1 (Figure 3 I) cells, CD4⁺ (Figure 4 I) and CD8⁺ (Figure 4 II) lymphocytes, was stronger in animals injected with RAD Δ TK, compared with animals injected with either RAD Δ TK or RAD β -gal, even in the absence of ganciclovir treatment, indicating that rapid inflammatory infiltration in the brain is not only viral vector and vector dose-dependent, but also depends on the transgene

expressed by the viral vector. Our *in vitro* results suggest that *in vivo* toxicity is not a consequence of direct toxicity to neuronal or glial cells. Therefore, the *in vivo* inflammation could be due to the toxicity towards non-neuronal and non-glial cells, a fact overlooked by previous studies. In addition to the lower direct toxicity elicited by HSV1- Δ TK for brain cell cultures, this gene could also be useful in avoiding or reducing, for example, the liver-associated toxicity showed by HSV1-TK/GCV treatment.¹⁶

In summary, our *in vivo* experiments demonstrated that RAD128 expressing HSV1-TK was more inflammatory than RAD127, expressing a truncated HSV1- Δ TK, in the absence and in the presence of ganciclovir. Our data suggest that the *in vivo* toxicity could be due to inflammatory or immune responses to the vectors, combined with the toxicity for non-neuronal, non-glial cells within normal peri-tumoral tissue, eg endothelial cells. The fact that HSV1- Δ TK can efficiently inhibit tumour growth *in vivo*,¹ and displays significantly reduced toxicity for normal brain cells *in vitro* and *in vivo* represents an advantage for glioma gene therapy.¹⁷ Thus, HSV1- Δ TK encoded within a replication-deficient adenovirus vector could be exploited for the treatment of brain tumours to achieve clinically relevant levels of tumour cell killing, coupled with a reduced cytotoxicity for the surrounding non-tumoral brain tissue.

Acknowledgements

This work was supported by a project grant (SP2332/0101) from the Cancer Research Campaign (UK) (CRC), the BBSRC (34/T08236) and EU-Biomed programmes (Contract No BMH4-CT96-1436; B104-CT98-0297; BMH4-CT98-3277) to MGC and PRL. We would also like to acknowledge the support which our laboratory receives from Action Research, The Wellcome Trust, The MRC, The British Heart Foundation, The Royal Society, The Parkinson's Disease Society, REMEDI, The Sir Halley Stewart Trust, The Lister Institute for Preventive Medicine, and the Faculty of Medicine Bequest Fund. We thank Prof AM Heagerty for his support and encouragement. The skilful secretarial of Mrs Ros Poulton is very gratefully acknowledged. We also wish to thank Roche Products Ltd for their generous gift of Cymeve (GCV) for this study and Michel Janicot from Rhone Poulenc Rorer, France for kindly providing the anti-TK antibodies. TDS is a Research Training Fellow supported by Action Research, AEM was a fellow of CONICET (Argentina), and PRL is a Research Fellow of the Lister Institute of Preventive Medicine.

References

- Dewey RA *et al*. Chronic brain inflammation and persistent HSV1-TK expression in survivors of syngeneic glioma treated by adenovirus gene therapy: implications for clinical trials. *Nature Med* 1999; **11**: 1256-1264.
- Badie B *et al*. Stereotactic delivery of recombinant adenovirus into C6 glioma cell line in a rat brain tumor model. *Neurosurgery* 1994; **35**: 910-916.
- Barba D, Hardin J, Sadelain M, Gage FH. Development of anti-tumor immunity following thymidine kinase-mediated killing of experimental brain tumours. *Proc Natl Acad Sci USA* 1994; **91**: 4348-4352.
- Izquierdo M *et al*. Long-term rat survival after malignant brain tumor regression by retroviral gene therapy. *Gene Therapy* 1995; **2**: 66-69.

- 5 Chen SH *et al*. Gene therapy for brain tumours: regression of experimental gliomas by adenovirus-mediated gene transfer *in vivo*. *Proc Natl Acad Sci USA* 1994; **91**: 3054–3057.
- 6 Culver KW *et al*. *In vivo* gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science* 1992; **256**: 1550–1552.
- 7 Boviatsis EJ *et al*. Long-term survival of rats harbouring brain neoplasms treated with ganciclovir and herpes simplex virus vector that retains an intact thymidine kinase gene. *Cancer Res* 1994; **54**: 5745–5751.
- 8 Kaplitt MG *et al*. Mutant herpes simplex virus induced regression of tumours growing in immunocompetent rats. *J Neurooncol* 1994; **19**: 137–147.
- 9 Marcel T, Grausz JD. The TMC worldwide gene therapy enrolment report, end 1996. *Hum Gene Ther* 1997; **8**: 775–800.
- 10 Ram Z *et al*. Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. *Nature Med* 1997; **3**: 1354–1361.
- 11 Goodman JC *et al*. Adenoviral-mediated thymidine kinase gene transfer into the primate brain followed by systemic ganciclovir: pathologic, radiologic and molecular studies. *Hum Gene Ther* 1996; **7**: 1241–1250.
- 12 Smith JG *et al*. Intracranial administration of adenovirus expressing HSV-TK in combination with ganciclovir produces a dose-dependent, self-limiting inflammatory response. *Hum Gene Ther* 1997; **8**: 943–954.
- 13 Maron A *et al*. Differential toxicity of ganciclovir for rat neurones and astrocytes in primary culture following adenovirus-mediated transfer of the HSVtk gene. *Gene Therapy* 1997; **4**: 25–31.
- 14 Shering AF *et al*. Cell type-specific expression in brain cell cultures from a short human cytomegalovirus major immediate early promoter depends on whether it is inserted into herpesvirus or adenovirus vectors. *J Gen Virol* 1997; **78**: 445–459.
- 15 Morelli AE *et al*. Neuronal and glial cell type-specific promoters within adenovirus recombinants restrict the expression of the apoptosis-inducing molecule Fas ligand to predetermined brain cell types, and abolish peripheral liver toxicity. *J Gen Virol* 1999; **80**: 571–583.
- 16 Brand K *et al*. Liver-associated toxicity of the HSV-tk/GCV approach and adenoviral vectors. *Cancer Gene Ther* 1997; **4**: 9–16.
- 17 Löwenstein PR *et al*. The basic science of brain-tumour gene therapy. *Biochem Soc Trans* 1999; **27**: 873–881.