Genetically Engineered Human Herpes Simplex Virus in the Treatment of Brain Tumours

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GENETICALLY ENGINEERED HERPES SIMPLEX VIRUSES REPRESENT AN ADVANCE IN THE EXPERIMENTAL APPROACH TO THE MANAGEMENT OF INCURABLE TUMOURS. AN ABILITY TO REPLICATE IN TUMOUR CELLS AND A LARGE CODING CAPACITY, PROVIDE A MODEL FOR BOTH DIRECT ONCOLYTIC ACTIVITY AND FOR THE DELIVERY OF FOREIGN PROTEINS

KEY WORDS

HERPES SIMPLEX VIRUS (HSV) ▮ *FATAL BRAIN TUMOUR* ▮ *GLIOMA* ▮ *GENE THERAPY* ▮ *VIRUS THERAPY*

SUMMARY

Central nervous system malignancies – particularly *glioblastoma multiforme* – pose significant problems for the development of novel therapeutics. In the absence of advances with standard surgical and chemotherapeutic approaches, the utilization of genetically engineered viruses – both as direct oncolytic agents (virus therapy) and for the delivery of foreign proteins (gene therapy) – represents a significant advance in the experimental approach to the management of patients with incurable tumours. Among other viruses, herpes simplex virus (HSV) offers an opportunity to influence the replication of tumour cells directly within the central nervous system. The propensity for HSV to replicate in tumour cells, and its large coding capacity, provide an experimental model for the development of novel therapeutics. The status of these experimental approaches and Phase I studies are summarized.

Background

GLIOMAS ARE THE MOST COMMON primary tumour arising in the human brain. Despite aggressive surgical therapy, radiotherapy and chemotherapy, malignant gliomas are almost always fatal; the median survival rate for glioblastoma, the most malignant glioma, is approximately 52 weeks. The course of the disease is marked by local tumour recurrence with relentless regrowth, causing neurological dysfunction and, ultimately, death. Systemic metastases are extremely rare. Current therapy for malignant glioma usually involves surgery, radiotherapy and, ultimately, chemotherapy. Patients not thought to be candidates for open craniotomy due to age, tumour location or overall medical condition, generally undergo stereotactic biopsy to establish diagnosis. This approach may slow tumour progression but is not curative.[1]

Advances in molecular biology have defined entirely new approaches to cancer therapy, utilizing molecular interventions. Neoplasms not responsive to current oncological treatment have become prime candidates for molecular-based therapies. We have been developing a molecular-based strategy for the treatment of malignant glioma utilizing genetically engineered herpes simplex virus (HSV).

Comparison of Virus and Gene Therapy

Two predominant therapeutic approaches to the treatment of malignant glioma have been developed recently by investigators utilizing the tools of molecular biology. We will refer to these approaches as virus and gene therapy:

- Virus therapy utilizes the inherent destructive effects resulting from viral gene expression by cytolytic viruses, with possible additional contributions from the host immune response to infection, in order to induce tumour cell killing (Figure 1);
- Gene therapy of gliomas involves the insertion of additional genetic material into the tumour cells which is then transcribed and translated into proteins. These proteins either directly or indirectly interfere with tumour cell replication and survival (Figure 2).

Herpes Simplex Virus as a Potential Antiglioma Agent

Experimental work to date in virus therapy of brain tumours has chiefly utilized HSV which is a neurotropic DNA virus with a well-studied genome. It is ubiquitous in humans: 90% of the population over 30 years old have acquired antibodies to HSV, indicating prior infection. Wild-type HSV can invade and replicate in both neurons and glia,[2–4] resulting in necrotizing encephalitis and widespread haemorrhagic necrosis throughout infected brain parenchyma. In recent years several laboratories have constructed recombinant viruses that can neither invade the normal central nervous system (CNS) nor replicate efficiently upon direct inoculation into the CNS of species highly susceptible to HSV infection. These engineered viruses lack the ability to replicate in normal neurons, but retain the ability to replicate in and potentially destroy other types of tissue, including gliomas and other brain tumours.

Engineered HSV Constructs

Genetically engineered HSV constructs studied for glioma therapy have included those with mutations in one or more of the following viral genes: thymidine kinase (TK); DNA polymerase; uracil DNA glycosylase; ribonucleotide reductase; and $\gamma_1$34.5.[5,6] These mutations all act to decrease the toxicity of HSV infection on the

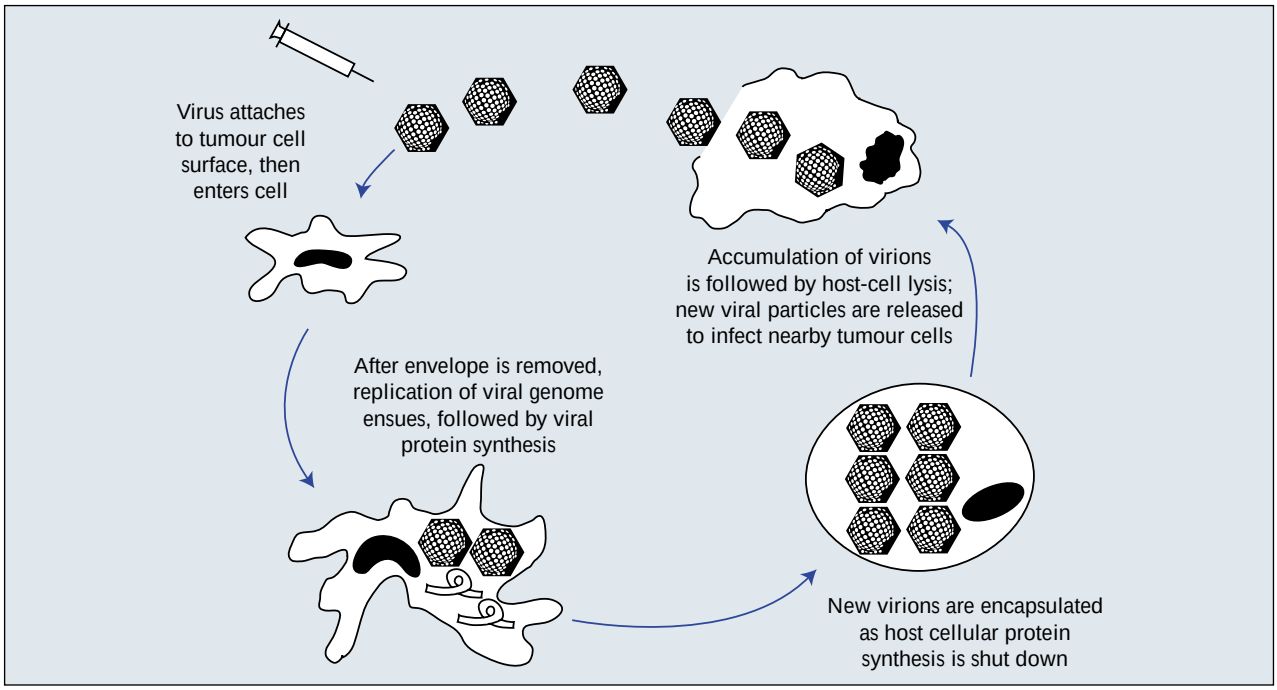

Figure 1:
With oncolytic viral therapy, the virus attaches to the tumour cell surface antigens and is internalized by the cell. Thereafter, the normal viral life cycle proceeds. Hundreds or even thousands of new viral particles are replicated. Replication of these viral particles results in shutdown of normal tumour cells' housekeeping function. Eventually sufficient viral particles are generated to result in tumour cell lysis or destruction. Newly created viral particles then infect nearby tumour cells. Genetically engineered mutations in the virus limit toxicity to normal tissue.

normal central nervous system.[7–10] TK, DNA polymerase, uracil DNA glycosylase and ribonucleotide reductase are all viral enzymes necessary for successful nucleotide synthesis and replication. As a rule, non-dividing cells – such as the post-mitotic neurons of the adult CNS – do not support replication of viruses containing these mutations. Replicating cells, however, can supply cellular homologues to these enzymes *in trans* and allow viral replication to take place. Thus, viruses with these mutations utilize cellular enzymes and factors present in dividing tumour cells. Conversely, the absence of these proteins in non-dividing neurons precludes viral replication in these cells and contributes to the non-neurovirulent phenotype of these viruses.

Herpes simplex viruses constructed to yield deletions in the $\gamma_1$34.5 gene ($\gamma_1$34.5$^-$) are unique. Unlike the viral genes previously described, there are two copies of $\gamma_1$34.5 existing on opposite strands in the two inverted repeat regions. These genes encode a critical bifunctional protein containing 263 amino acids. The first function encoded by this protein enables the virus to replicate in neurons and, therefore, in the absence of the protein the virus fails to induce significant cytopathology even when injected in high amounts into

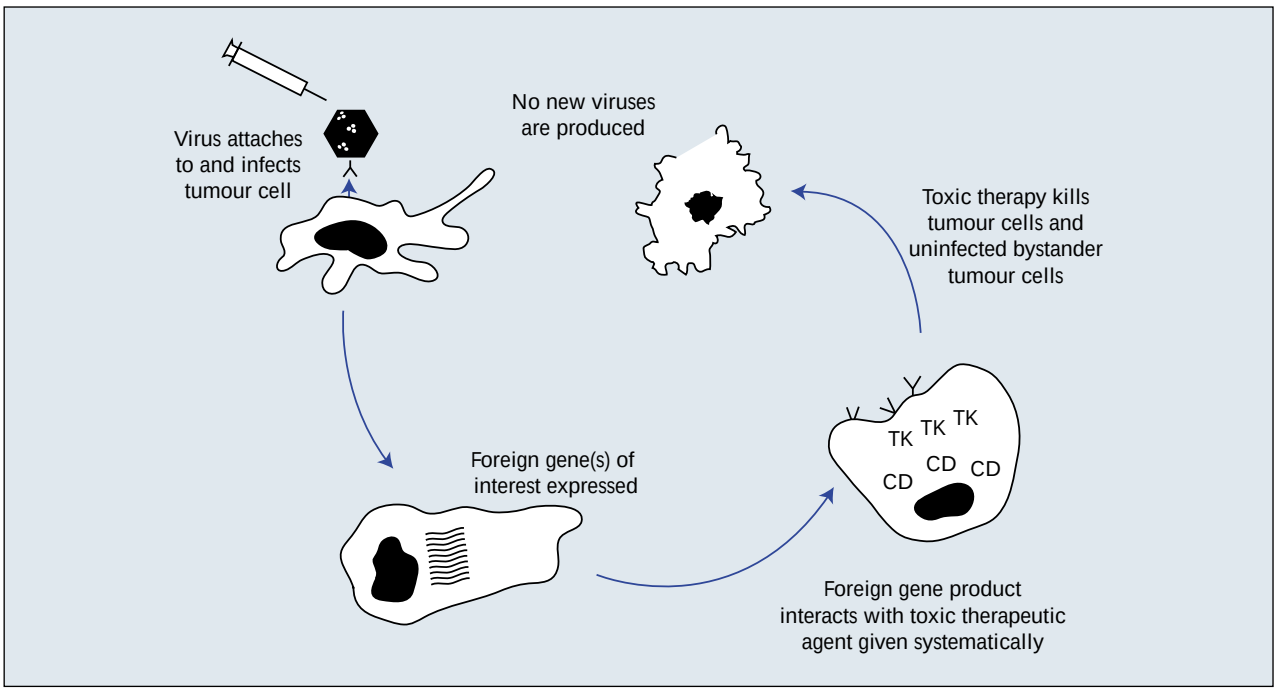

Figure 2:
Gene therapy may or may not use viruses to shuttle the gene of interest into the tumour cell. Regardless, the foreign gene is introduced into the cell, followed by transcription and expression of its protein. The protein that is produced then directly produces injury to the tumour cell or interacts with an externally administered compound such as a prodrug or antibody-directed toxin to produce tumour destruction. TK, thymidine kinase; CD, cytosine deaminase.

the CNS of susceptible animal models. The second function blocks a normal host response to infection. In the absence of the protein the virus induces a host response that totally blocks protein synthesis in the infected cell. Perhaps just as critical to the non-neurovirulence of $\gamma_1$34.5$^-$ mutants is the fact that a portion of the latency-activated transcripts is encoded on the DNA strand opposite the $\gamma_1$34.5 genes. Thus, not only is the function of the $\gamma_1$34.5 gene compromised in these mutants, but their capacity to express genes necessary for the virus to establish latency is lost. Survival of $\gamma_1$34.5$^-$ HSV is therefore restricted to replicating cells and the extent of viral replication appears to be tied to the rate of cellular proliferation (unpublished observations).

A variety of genetically engineered HSV constructs have been evaluated both *in vitro* and *in vivo* for the treatment of brain tumours (Table 1). These studies began when the contribution of the $\gamma_1$34.5 gene to the neurovirulence of HSV was defined.[11] Viruses either deleted in both copies of this gene (R3616) or with a stop codon after codon 27 (R4009) were totally avirulent upon direct intracerebral inoculation into the mouse. These viruses have been found to lyse both human and murine tumour cells *in vitro*. In key studies, these mutants were studied in a *scid* mouse model of human glioma, utilizing both a Winn-type assay (simultaneous inoculation of tumour cells and virus) and intra-tumoural inoculation of these genetically engineered HSV constructs.[8] Intra-tumoural inoculation of virus resulted in a median survival of 120 days; all control animals died by day 35. Histopathological evaluation of the brain of treated mice indicated haemorrhagic necrosis of the tumour. Virological assessment of brain tissue obtained from animals sacrificed at 120 days allowed retrieval of the genetically engineered HSV in 30% of animals at very low levels, indicating that reactive astrocytes, proliferating in response to tissue injury, may provide a modest reservoir. While it can be argued that this indicates a probable need for further attenuation, these are TK+ viruses that can be completely eradicated in the mouse brain by systemic administration of ganciclovir 50 mg/kg/day for 7 days.[9]

The development of a virus containing mutations in two different genes contributing to neurovirulence added an extra margin of safety to potential clinical applications of HSV. Mineta *et al.*[12] combined mutations in the $\gamma_1$34.5 neurovirulence gene with a destructive *lacZ* insertion in the large subunit of the ribonucleotide reductase gene inactivating the ICP6 gene (UL39). Both mutations had been utilized independently in animal models previously. By placing both mutations in the single virus – G207 – the investigators felt the chances of a wild-type reversion or recombination occurring during clinical usage could be minimized.[13]

Primate Studies

In order to evaluate the safety of HSV mutants fully, before proceeding to clinical studies, such therapy needed to be assessed in two relevant species. Studies utilizing the $\gamma_1$34.5 mutants had confirmed their lack of neurovirulence in the susceptible mouse strain BALB/c.[11] Martuza's group chose to evaluate the multiple mutant G207 in the highly susceptible simian primate, *Aotus nancymai* (formerly thought to represent *A. trivirgatus).* Intra-cranial challenge with 10^7 plaque forming units (pfu) of virus did not produce any clinical effects. Histopathological examination of the brains after these animals were sacrificed revealed changes associated with the injection only, with no evidence of post-encephalitic changes. Subsequent studies with intra-cranial inoculation of up to 10^9 pfu of virus into *Aotus* have had similar findings.[14]

Oncolytic HSV as a Vector for Antitumour Gene Therapy

To increase the antiglioma effects of genetically engineered HSV therapy, a new treatment paradigm has emerged by which the HSV construct can be used as a vector for insertion of antineoplastic genes in addition to its native tumouricidal effects. Specifically, foreign genes – such as cytokine genes – can be engineered into HSV in order to enhance the killing of tumour cells. Use of these HSV constructs as vectors for gene therapy has been explored primarily for diseases of the central and peripheral nervous systems, due to:

- Their ability to both infect a variety of neurons and persist in a latent state;
- The size of the genome – 152 kilobase pairs (Kbp) – which may allow transfer of genes 30 Kbp or more in size.[15]

Selection of the correct combination of the engineered HSV construct and gene(s) for transfer should, theoretically, allow maximal exploitation of the advantages of each technique, i.e. direct destruction of the tumour by virus replication and its cell-to-cell spread within the tumour followed by subsequent foreign gene expression, allowing for the added destruction of any surviving tumour cells. This 'one-two punch' system of tumour kill potentially gives genetically engineered HSV a distinct advantage over other available vectors for the treatment of gliomas. Since the constructs selected for their decreased neurovirulence have generally included deletions of a portion of the genome, insertion of the desired foreign gene may be accomplished with minimal additional engineering of HSV.

In an initial effort to examine HSV as a vector for

Table 1: Detailed description of virus construct genotypes

Virus name	Deletions/mutations*	Foreign gene insertions*/promoter
R3616	1000 base pair (bp) deletion in $\gamma_1$34.5 gene	–
R4009	Stop codon after codon 27 in $\gamma_1$34.5 gene	–
G207	1000 bp deletion in $\gamma_1$34.5 gene Disabling *lacZ* insertion in UL39 locus	–
R8304	1000 bp deletion in $\gamma_1$34.5 gene	Murine IL-4, Egr-1 promoter
R8306	1000 bp deletion in $\gamma_1$34.5 gene	Murine IL-10, Egr-1 promoter
R7020	Deletion of 'a' repeat sequence Single intact copy of $\gamma_1$34.5 gene retained	HSV-2 sequence replaces native 'a' sequence (no specific $\gamma_1$34.5 insertion)
M002	1000 bp deletion in $\gamma_1$34.5 gene	p35 and p40 coding sequences of murine IL-12 with intervening IRES sequence, Egr-1 promoter

*Unless otherwise noted: all $\gamma_1$34.5 mutations are present in both copies of gene, and foreign gene insertions are into both copies of $\gamma_1$34.5 locus.

gene therapy, our laboratory examined two HSV constructs using murine interleukin-4 (IL-4), IL-10 and IL-12 genetic inserts.[16,17] These vectors contained the appropriate gene inserted under the Egr-1 promoter, which was placed in a deletion in the $\gamma_1$34.5 gene. Since this gene is located in the 'a' repeat region of the HSV-1 genome, each construct actually contains two copies of the cytokine insert (Figure 3). Each vector produced physiologically significant quantities of cytokine that were 1300–1900-fold higher than background values. The deletion mutant R3616, on the other hand, did not produce any significant change in expression of these cytokines when compared to background, indicating that the production of these cytokines was indeed the result of the genetic inserts and not simply a by-product of HSV-1 infection.

To test the therapeutic effects of these viruses, murine glioma Gl261 was implanted intra-cranially in syngeneic immunocompetent C57BL/6 mice, then treated 5 days later with intra-tumoural injections of virus. The HSV/IL-10 virus produced no difference in survival when compared to the untreated animals, a finding similar to R3616 in this highly aggressive tumour model. However, the HSV/IL-4 virus produced statistically significant increases in survival. To examine the potential role of the immune system in producing this survival advantage, immunohisto-chemistry was performed on brain sections from a separate cohort of animals similarly treated. At 3 and 7 days post-treatment, tumours treated with the IL-4 mutant demonstrated infiltrates of macrophages, CD8-positive and CD4-positive T cells. The IL-10 mutant demonstrated a less abundant infiltrate of CD4-positive T cells and a virtual absence of CD8-positive T cells, similar to what was seen in R3616-treated animals (Figure 4). Thus, the difference in survival seen in those animals treated with the IL-4 mutant may be related to these differences in immune response.

The HSV/IL-12 virus was studied in A/J mice with intra-cranial implants of the syngeneic neuroblastoma Neuro2α. This model may be even more stringent, as:

- A/J mice are more sensitive to HSV-1 infection than C57/BL6 mice;[18]
- Neuro2α is not an immunogenic tumour (unpublished observations).

Mice treated with HSV/IL-12 had marked improvements in survival when compared with mice treated with vehicle or with the parent HSV that did not express any cytokines. Immune infiltrates of the tumours were similar to those seen with the IL-4 virus.[17]

To assist in the development of superior candidate viruses, the minimal packaging capacity of the HSV genome is being determined to allow for the construction of engineered viruses to encode multiple foreign genes. Foreign genes such as those encoding cytokines, suicide enzymes for use with prodrugs, and antiangiogenesis proteins, among others, are also being investigated. Additionally, a variety of promoter systems – including controllable gene expression systems – are under evaluation, which may allow more specific expression of gene products.

Human Investigation

A Phase I clinical trial using an HSV-l mutant for the treatment of recurrence or progression of malignant glioma, despite conventional therapy with surgery or biopsy and external beam radiotherapy, has recently been completed.[19] The construct utilized in the trial, G207, is described above and is both $\gamma_1$34.5$^-$ and UL39$^-$. The virus is also hypersensitive to aciclovir, adding an additional margin of safety in case any patients developed signs of HSV encephalitis. It is replication-deficient at temperatures >39.5 °C so that the development of significant fevers, as seen in encephalitis, halts the spread of viral infection.

Twenty-one patients were enrolled in the trial. Dose escalation started at 1 x 10^6 pfu, and three patients were enrolled at each dose level. No evidence of encephalitis or other toxicity that could be linked to G207 was seen,

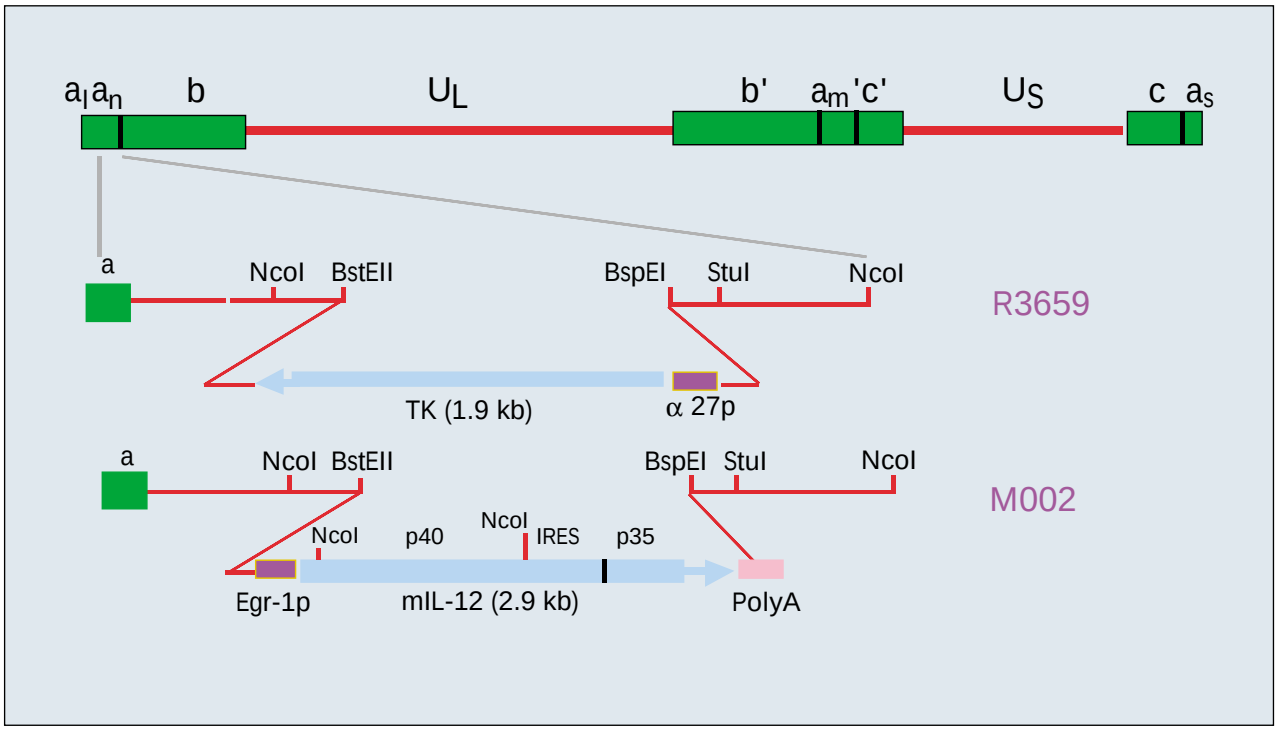

Figure 3:
Schematic linear diagram of the HSV-1 genome showing the three inverted repeats and the unique long and unique short segments. The entire genome is 152 Kbp in length. Also shown is the technique for introducing foreign genes via a thymidine kinase (TK) intermediate that has been utilized in producing mutant viruses studied in the treatment of experimental glioma. The cytokine-expressing viruses, including the one pictured, M002, contain 1 kb deletions in both copies of $\gamma_1$34.5. Note that the $\gamma_1$34.5 gene is diploid, because it is present in a repeat region, and so both copies have been deleted. Each cytokine gene is also present in two copies, with an expression cassette inserted at each $\gamma_1$34.5 locus. IL, interleukin.

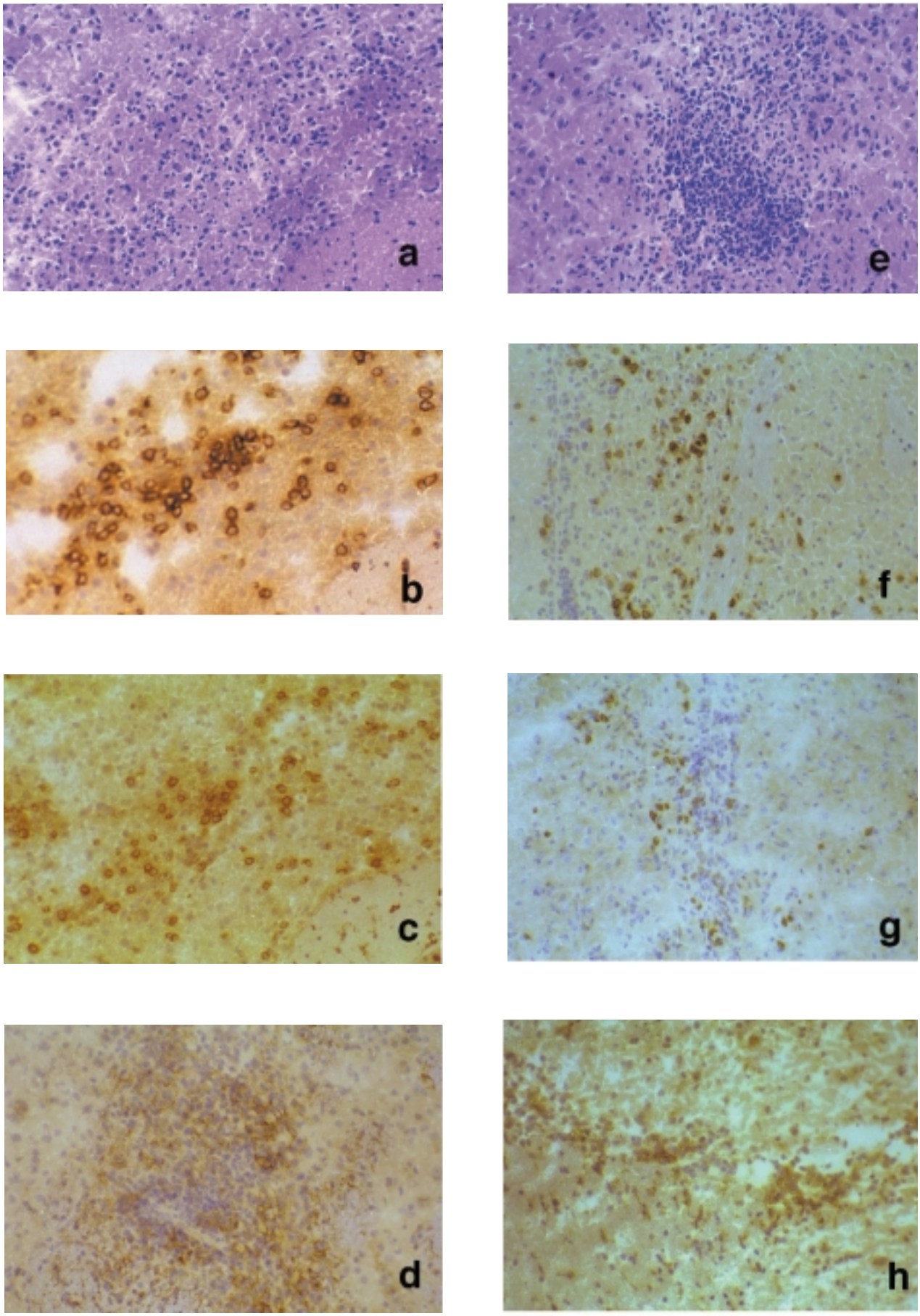

Figure 4:
Immunohistological identification of inflammatory cell infiltrates. A/J female mice were injected intra-cerebrally with Neuro2α cells (1 x 10^5/5 µl) and 5 days later were injected intra-tumourally with 1 x 10^7 pfu of HSV M002 (a, b, c and d) or HSV R3659 (e, f, g and h). After 6 days, the mice were killed and their brains were removed intact and embedded in optimum cutting temperature medium for preparation of frozen sections. Serial 10 mm thick sections were stained with haematoxylin-eosin (a and e) or were reacted with rat monoclonal antibodies to CD4+ (b and f) or CD8+ (c and g) cells or macrophages (d and h); antibody binding was detected by using horseradish peroxidase-labelled antirat antibody, and sections were counterstained with Mayer's haematoxylin.

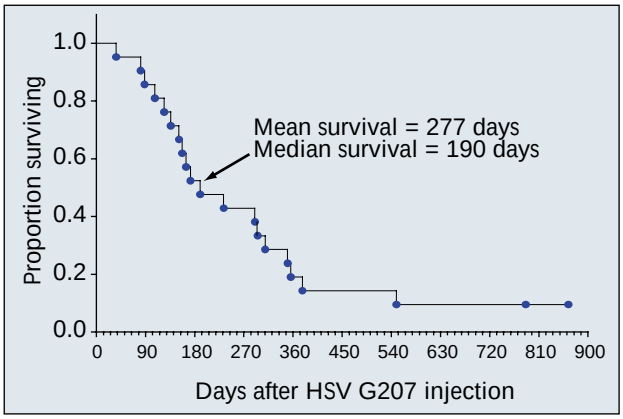

Figure 5:
Kaplan-Meier survival plot of patients with recurrent glioma and treated with escalating doses of G207 after previous surgery, radiation and, in some cases, chemotherapy. Details of the trial are included in the text. Conclusions about efficacy cannot be drawn from this trial; the curve reflects all patients treated, from the initial dose level of 10^6 pfu to 3 x 10^9 pfu.

even at the highest dose level tested, 3 x 10^9 pfu. While no claims of efficacy can be made from the results of a Phase I trial such as this, anecdotal evidence suggesting antiglioma effects of G207 treatment was observed (Figure 5).[19] Another similar trial using the $\gamma_1$34.5 mutant 1716 was recently reported, with safety demonstrated at inoculations up to 10^5 pfu.[20] Future studies with these and other mutants are planned.

Future Directions

Our current strategy envisages a multiple modality treatment approach for malignant gliomas using current adjunctive therapies combined logically with those derived from the tools of molecular medicine. This novel treatment regimen aims to achieve synergistic tumouricidal effects produced by genetically engineered HSV that express foreign antitumour genes. Important issues currently under consideration to improve the efficacy and safety of HSV therapy include evaluation of the therapeutic gene inserts described above, improvement of vector delivery and increasing the specificity of targeting viral therapy to tumour cells.

The combination of viral therapy utilizing engineered HSV-1 with gene therapy is a promising new development in the experimental treatment of malignant brain tumours and, potentially, other human cancers. It is hoped that continued study of this new therapy will lead to an improvement in outcome for patients diagnosed in the future with malignant glioma.

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