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A MODIFIED, ONCOLYTIC, NON-LABORATORY HSV STRAIN AND ITS USE IN TREATING CANCER

Field of the Invention

The present invention relates to non-laboratory virus strains, for example of herpes simplex viruses, with improved oncolytic capabilities as compared to laboratory virus strains.

Background of the Invention

Viruses have been suggested or demonstrated to have utility in a variety of applications in biotechnology and medicine on many occasions. Each is due to the unique ability of viruses to enter cells at high efficiency. This is followed in such applications by either virus gene expression and replication and/or expression of an inserted heterologous gene. Thus viruses can either deliver and express genes in cells (either viral or other genes) which may be useful in for example gene therapy or the development of vaccines, or they may be useful in selectively killing cells by lytic replication or the action of a delivered gene in for example cancer.

Herpes simplex virus (HSV) has been suggested to be of use both as a gene delivery vector in the nervous system and elsewhere and for the oncolytic treatment of cancer. In both applications the virus must however be disabled such that it is no longer pathogenic but such that it can still enter cells and perform the desired function. Thus for non-toxic gene delivery to target cells using HSV it has become apparent that in most cases immediate early gene expression must be prevented/ minimised from the virus. For the oncolytic treatment of cancer, which may also include the delivery of gene(s) enhancing the therapeutic effect, a number of mutations to HSV have been identified which still allow the virus to replicate in culture or in actively dividing cells *in vivo* (e.g. in tumours), but which prevent significant replication in normal tissue. Such mutations include disruption of the genes encoding ICP34.5, ICP6 and thymidine kinase. Of these, viruses with mutations to ICP34.5, or ICP34.5 together with mutations of e.g. ICP6 have so far shown the most favourable safety profile. Viruses deleted for only ICP34.5 have

been shown to replicate in many tumour cell types *in vitro* and to selectively replicate in artificially induced brain tumours in mice while sparing surrounding tissue. Early stage clinical trials have also shown their safety in man.

However, while promise has been shown for various viruses including HSV for gene delivery/therapy or for the oncolytic treatment of cancer, the majority of this work has used virus strains which have been maintained in tissue culture cells for many years. In applications where the virus merely needs enter cells to deliver genes this may not prove problematical as maintenance in cell culture also requires the virus to enter cells, albeit often cells of a different type or species in comparison to the likely target cells for a vector. However, in applications where other properties are required, the use of laboratory virus strains may not allow the full potential of a virus in a particular application to be utilised.

HSV has the unique ability amongst viruses currently under development as vectors in that it has naturally evolved to infect and remain latent in neurons. HSV has also evolved to be highly efficiently transported along nerves from the site of infection, usually at the periphery, to the neuronal cell body, usually in the spinal ganglia. Such capabilities are not required in cell culture and as such capabilities require specific evolved properties of HSV, further adaption to growth in culture may have resulted in optimally efficient axonal transport capabilities to have been lost. HSV vectors for gene delivery to the central or peripheral nervous system are likely to show maximum effectiveness if axonal transport properties have been retained at maximum efficiency. Here, inoculation at a peripheral site would then allow maximally efficient gene delivery to peripheral neuron cell bodies, and inoculation in the brain would allow maximally efficient gene delivery to multiple connected sites. Current vectors based on laboratory strains of HSV may not allow this to occur at the maximum efficiency possible. Indeed, because of HSV's high capacity to be transported along nerves, there is potentially a particularly large discrepancy between the properties which it is desired to conserve and those likely to be retained in culture.

HSV and other viruses such as adeno- or rheovirus also have potential utility in the oncolytic treatment of cancer. However, again viruses under development for

such purposes have previously been extensively maintained in culture. As the oncolytic treatment of cancer requires active replication in often relatively slowly growing human tumour cells, it would be anticipated that adaptation of laboratory virus strains to growth in particular cultured cells may have reduced the efficiency with which such lytic replication in human tumour cells, or infection of human tumour cells, could optimally occur.

Summary of the Invention

The present invention provides the opportunity to develop viruses with improved *in vivo* capabilities of lytic destruction of tumour cells. Here, virus strains are constructed appropriate for these purposes based on recent clinical isolates of the appropriate virus rather than the serially passaged laboratory strains which have previously been used. The present invention therefore has the potential to provide viruses with improved capabilities of infecting human cells *in vivo* and improved replicative/lytic capability in such cells.

We have shown that two clinical isolates of HSV1 (strains JS1 and BL1) have enhanced replication in some human tumour cell lines as compared to HSV1 strain 17+ (a standard laboratory strain).

We have deleted ICP34.5 from the clinical isolate JS1 strain and again compared replicative potential in human tumour cell types in comparison to HSV1 strain 17+ (a standard laboratory strain) in which ICP34.5 was also deleted. This strain (JS1/ICP34.5-) is a modified strain derived from a clinical isolate, and is thus a modified non-laboratory strain of the invention.

JS1 with ICP34.5 deleted showed enhanced growth in some human tumour cells tested as compared to HSV1 ICP34.5 deleted strain 17+, i.e. a laboratory strain virus with the same modification. However, as compared to the laboratory strain

derived from strain 17+, cell killing capabilities were enhanced with the JS1/ICP34.5- virus in all tumour cell lines tested.

Thus, the use of non-laboratory virus strains can be seen to enhance the anti-tumour capabilities of such viruses and was evident in all the tumour cell lines tested so far. This will have applicability for cancer treatment in human patients.

Further enhanced activity may also be anticipated if these viruses are then used to deliver genes with anti-tumour activity. Such genes include those encoding pro-drug activators, tumour suppressor or pro-apoptotic factors, or immune stimulatory proteins.

For this purpose, we have produced an ICP34.5 deleted clinical isolate of HSV1 which expresses human GMCSF. This virus is designed to enhance anti-tumour immune responses following intra-tumoral injection.

The invention also provides viruses of the invention which carry a heterologous gene/genes. The term heterologous gene is intended to embrace any gene not found in the viral genome. The heterologous gene may be any allelic variant of a wild-type gene, or it may be a mutant gene. Heterologous genes are preferably operably linked to a control sequence permitting expression of said heterologous gene in a cell *in vivo*. Viruses of the invention may thus be used to deliver a heterologous gene/genes to a cell *in vivo* where it will be expressed. For oncolytic virus therapy, such genes typically encode proteins capable of enhancing the tumour destroying properties of the virus. These genes may encode proteins which are themselves cytotoxic, are pro-drug activating, or which may be capable of stimulating /enhancing an anti-tumour immune response.

In all cases, single or multiple heterologous genes may be carried by a single virus.

Accordingly the invention provides:

Use of a modified, oncolytic, herpes simplex virus (HSV) strain in the manufacture of a medicament for the oncolytic treatment of cancer, which HSV strain:

(a) has a greater ability than HSV1 17+, HSV1 F and HSV1 KOS strains with equivalent modifications to replicate in or kill tumour cells; and

(b) is produced by isolating an HSV strain from a host and modifying the isolated HSV strain to provide for selective replication in tumour cells.

A method of determining the suitability of an HSV strain for use in the production of a modified, oncolytic HSV strain, said method comprising:

(i) isolating an HSV strain from a host;

(ii) assessing the ability of the HSV to replicate in or kill one or more types of tumour cell;

(iii) determining whether the HSV has a greater ability than one or more of HSV1 strain 17+, HSV1 strain F and HSV1 strain KOS to replicate in or kill tumour cells; and optionally

(iv) selecting the HSV strain for modification, wherein the HSV strain has said greater ability.

A method of producing a modified, oncolytic HSV strain, said method comprising:

(i) providing an HSV strain selected by a method comprising:

(a) isolating an HSV strain from a host;

(b) assessing the ability of the HSV to replicate in or kill one or more types of tumour cell;

(c) determining whether the HSV has a greater ability than one or more of HSV1 strain 17+, HSV1 strain F and HSV1 strain KOS to replicate in or kill tumour cells; and

(d) selecting the HSV strain for modification, wherein the HSV strain has said greater ability;

(ii) modifying said HSV strain to provide for selective replication in tumour cells; and

(iii) assessing the ability of said modified, oncolytic HSV strain to kill tumour cells.

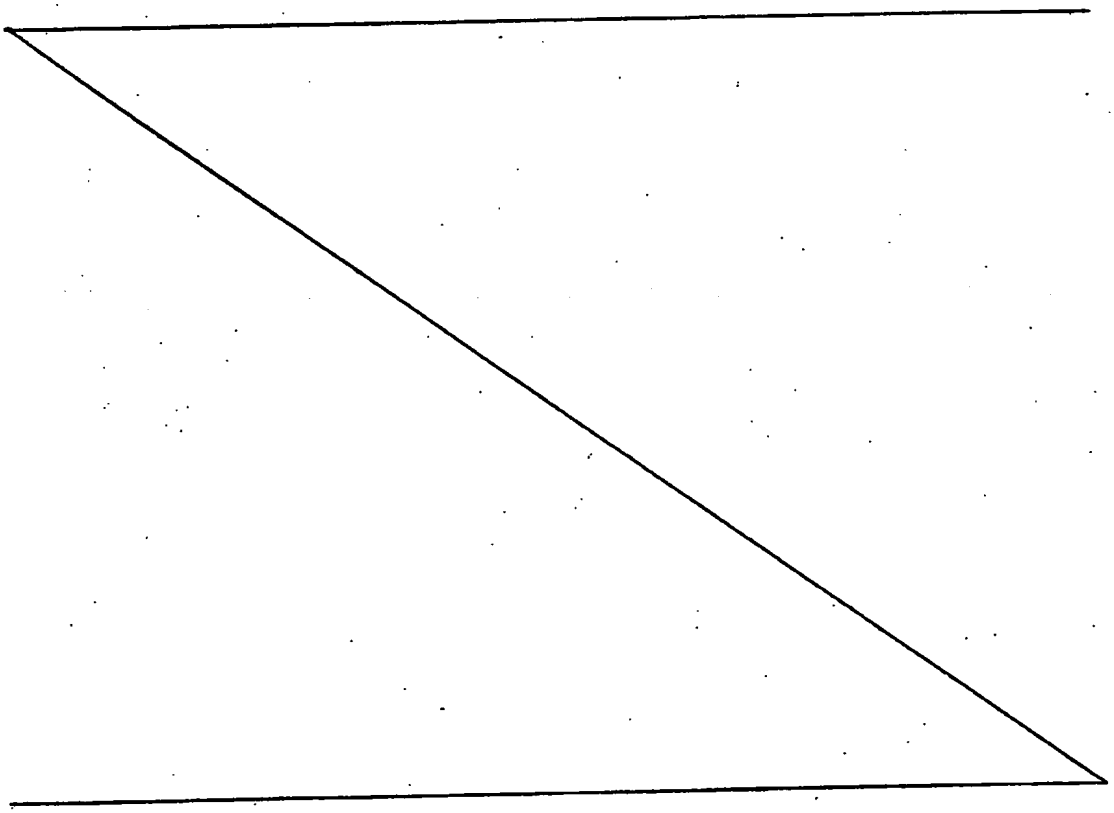
A method of determining whether a gene enhances the anti-tumour effects of a HSV comprising:

(i) providing a modified, oncolytic HSV strain as defined herein;

(ii) inserting said gene into said HSV strain; and

(iii) assessing the ability of said modified, oncolytic HSV strain to replicate in or kill tumour cells compared to the ability of the precursor strain provided in step (i).

An oncolytic HSV strain selected by or produced by a method according to the invention, which HSV strain has a greater ability than HSV1 17+, HSV1 F and HSV1 KOS strains with equivalent modifications to kill tumour cells.



A pharmaceutical composition comprising a modified oncolytic HSV strain of the invention and a modified oncolytic HSV strain of the invention for use in the treatment of the human or animal body.

Brief Description of the Drawings

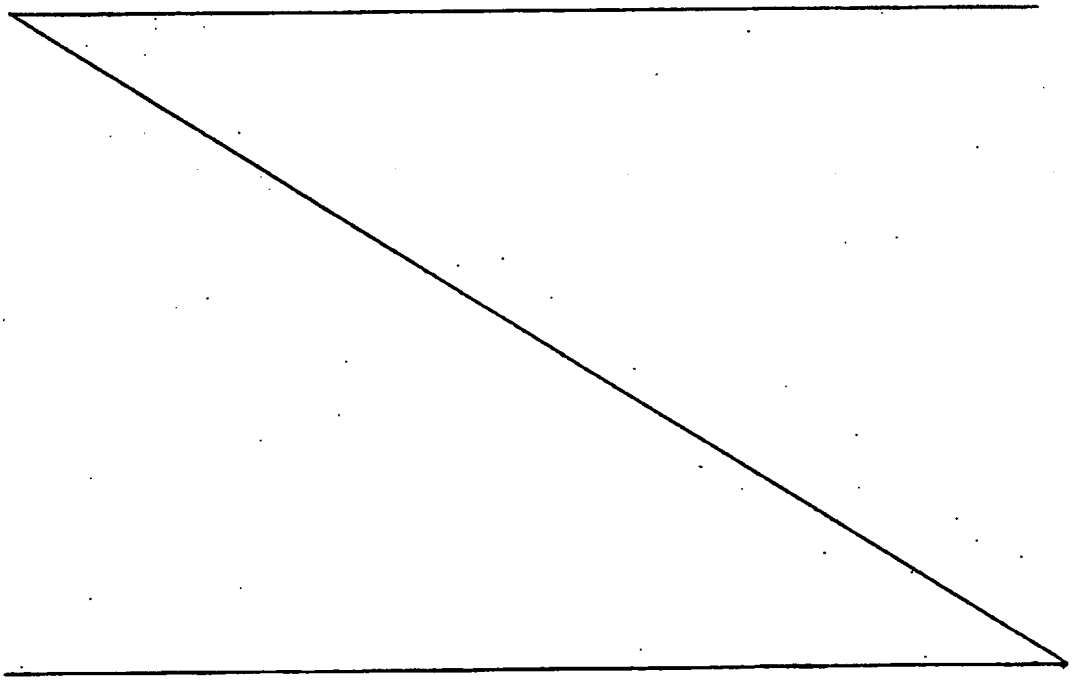
Fig 1. Viruses

From top to bottom, diagrams show: laboratory HSV1 strain 17+, clinical strain BL1, clinical strain JS1, 17+/ICP34.5-, JS1/ICP34.5-, JS1/ICP34.5-/ICP47-/hGMCSF.

Fig 2. Clinical isolates show enhanced growth in tumour cells

(1) Growth of 17+, BL1 and JS1. Left hand diagram: U87 cells. Right-hand diagram: LNCaP cells.

(2) Growth of ICP34.5- 17+ and JS1 on tumour cells. Left-hand diagram: LNCaP cells. Right-hand diagram: MDA-MB-231 cells.



(3) JS1/34.5- does not grow on cells non-permissive for HSV ICP34.5 mutants. Left-hand diagram: 3T6 cells – 17+, JS1. Right-hand diagram: 3T6 cells – 17+, JS1 ICP34.5-.

Fig 3. An ICP34.5 deletcd HSV clinical isolate shows enhanced lysis in all tumour cells tested

Tumour cell lines were either mock infected, infected with HSV1 strain 17+/34.5-, or infected with HSV1 strain JS1/34.5- at the indicated MOI and stained with crystal violet at time points after infection to allow visualisation of cells. Each block of photographs relates to a cell type. From top to bottom, these are HT29 colorectal adenocarcinoma, LNCaP.FGC prostate adenocarcinoma, MDA-MB-231 breast adenocarcinoma, SK-MEL-28 malignant melanoma and U-87 MG glioblastoma astrocytoma. Left-hand blocks relate to results for HSV1 strain 17+/34.5-. Right-hand blocks relate to results for HSV1 strain JS1/34.5-. Central blocks represent mock infected cells. Within each block, the top row represents a 24 hour time-point, the second a 48 hour time-point and the third a 72 hour time-point within each block, the left-hand column represents MOI=0.2, the central column MOI=0.1 and the right-hand column MOI=5.

Detailed Description of the Invention

A. Viruses

Virus Strains of the Invention

A virus strain of the invention will be a strain of a herpes simplex virus (HSV), typically a strain of HSV1 or HSV2.

The virus may be derived from, for example HSV1 or HSV2 strains, or derivatives thereof, preferably HSV1. Derivatives include inter-type recombinants containing DNA from HSV1 and HSV2 strains. Such inter-type recombinants are described in the art, for example in Thompson *et al* (1998) and Meignier *et al* (1988). Derivatives preferably have at least 70% sequence homology to either the HSV1 or HSV2 genomes, more preferably at least 80%, even more preferably at least 90 or 95%. More preferably, a derivative has at least 70% sequence identity to either the HSV1 or HSV2 genome, more preferably at least 80% identity, even more preferably at least 90%, 95% or 98% identity.

For example the UWGCG Package provides the BESTFIT program which can be used to calculate homology (for example used on its default settings) (Devereux *et al.* (1984) *Nucleic Acids Research* 12, p387-395). The PILEUP and BLAST algorithms can be used to calculate homology or line up sequences (typically on their default settings), for example as described in Altschul (1993) J. Mol. Evol. 36:290-300; Altschul *et al.* (1990) J. Mol. Biol. 215:403-10.

Software for performing BLAST analyses is publicly available through the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pair (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score threshold (Altschul *et al.*, 1990). These initial neighbourhood word hits act as seeds for initiating searches to find HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the

BLOSUM62 scoring matrix (see Henikoff and Henikoff (1992) *Proc. Natl. Acad. Sci.* USA 89: 10915-10919) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

The BLAST algorithm performs a statistical analysis of the similarity between two sequences; see e.g., Karlin and Altschul (1993) *Proc. Natl. Acad. Sci.* USA 90: 5873-5787. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a sequence is considered similar to another sequence if the smallest sum probability in comparison of the first sequence to the second sequence is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

A derivative may have the sequence of a HSV1 or HSV2 genome modified by nucleotide substitutions, for example from 1, 2 or 3 to 10, 25, 50 or 100 substitutions. The HSV1 or HSV2 genome may alternatively or additionally be modified by one or more insertions and/or deletions and/or by an extension at either or both ends.

Properties of Virus Strain of the Invention

Virus strains of the invention are "non-laboratory" strains. They can also be referred to as "clinical" strains. A person of skill in the art will readily be able to distinguish between a laboratory strain and a non-laboratory, or clinical, one. Further guidance on the properties likely to be exhibited by virus strains is given below.

The key distinction between a laboratory and non-laboratory strain is that laboratory strains currently in common use have been maintained for long periods, many years in somc cases, in culture. The culture of viruses such as HSV involves a technique known as serial passage. To grow and maintain viruses, suitable cells are infected with the virus, the virus replicates within the cell and the virus is then harvested; fresh cells are then re-infected. this process constitutes one cycle of serial passage. Each such cycle may take, for example, a few days in the case of HSV. As

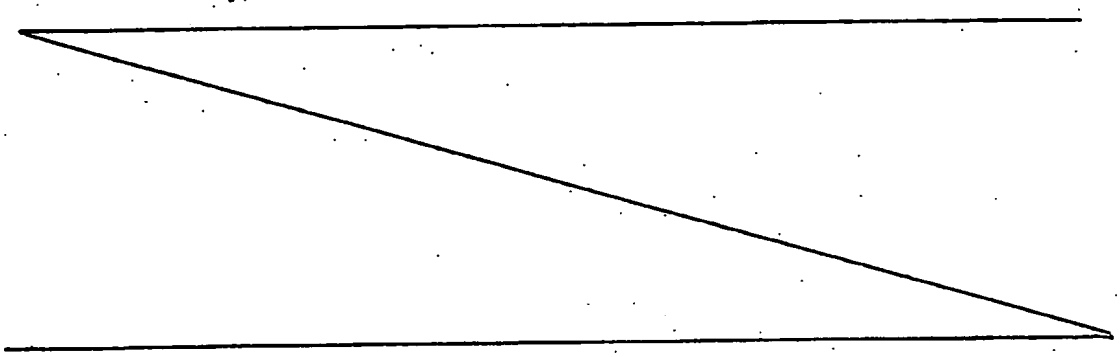
discussed above, such serial passaging may lead to changes in the properties of the virus strain, in that selection takes places for properties that favour growth in culture (e.g. rapid replication), as opposed to properties useful for practical applications.

Virus strains of the invention are non-laboratory strains in that they are derived from strains recently isolated from infected individuals. Strains of the invention are modified compared to the original clinical isolates, and may have spent a time in culture, but any time spent in culture will be comparatively short. Strains of the invention are prepared in such a manner as to retain substantially the desirable properties of the original clinical isolates from which they are derived.

A virus of the invention is capable of efficiently infecting target human cells. Such a virus is recently isolated from an infected individual and then screened for the desired ability of enhanced replication in tumour cells *in vitro* and/or in vivo in comparison to standard laboratory strains. Such viruses with improved properties as compared to laboratory virus strains are viruses of the invention. Identified viruses with such desired improved properties can then be engineered such that they can selectively kill tumour cells by the mutation of appropriate gene(s). These modified viruses are also viruses of the invention. Alternatively, virus strains may be isolated from an infected individual and mutations anticipated to be appropriate for oncolytic therapy may be made. These modified viruses are then screened for the desired improved properties as compared to laboratory strains, viruses with such improved properties providing further viruses of the invention.

Further guidance on the likely properties of the virus strains of the invention is provided as follows.

Preferably, a virus strain of the invention has undergone



two months or less, one month or less, two weeks or less, or one week or less in culture. By these definitions of time in culture, is meant time actually spent in culture. Thus, for example, it is a common practice to freeze virus strains in order to preserve them. Evidently, preserving by freezing or in an equivalent manner does not qualify as maintaining the strain in culture. Thus, time spent frozen or otherwise preserved is not included in the above definitions of time spent in culture. Time spent in culture is typically time actually spent undergoing serial passage, i.e. time during which selection for undesirable characteristics can occur.

Preferably, a virus strain of the invention has undergone 10 or less cycles of serial passage since isolation of its unmodified clinical precursor strain from its host.

Preferably, a virus of the invention has a greater ability, as measured by standard statistical tests, than a reference laboratory strain with the equivalent modifications to perform certain functions useful in the application at hand. For example, in the case of an oncolytic virus for tumour treatment, a virus strain of the invention will preferably have a greater ability than a reference laboratory strain with equivalent modifications to infect or replicate any tumour cell, to kill tumour cells or to spread between cells in tissue. More preferably, such greater ability is a statistically significantly greater ability. For example, according to the invention, a may have up to 1.1 fold, 1.2 fold, 1.5 fold, 2 fold, 5 fold, 10 fold, 20 fold, 50 fold, or 100 fold the capacity of the reference strain in respect of the property being tested.

Preferably, a virus of the invention has, i.e. retains, substantially the ability of its unmodified clinical precursor strain in respect of one or more of the properties characteristic of usefulness in the application at hand. For example, in the case of an oncolytic virus intended for the treatment of tumours, a virus strain of the invention preferably has substantially the ability of its unmodified clinical precursor strain to infect or replicate a tumour cell, kill tumour cells or to spread between cells in tissue.

Preferably, according to the invention, a virus retains substantially the properties of its unmodified clinical precursor strain if, in a quantitative test, it retains 75%, more preferably 80, 90, 95, 98, 99 or 100% of the capacity of the unmodified clinical precursor strain in respect of the property being tested. More preferably, in respect of the property being tested, any differences between the unmodified clinical precursor strain and the modified strain of the invention will not be statistically significant.

Statistical analysis of the properties described herein may be carried out by standard tests, for example, t-tests, ANOVA, or Chi squared tests. Typically, statistical significance will be measured to a level of $p = 0.05$ (5%), more preferably $p = 0.01$, $p = 0.001$, $p = 0.0001$, $p = 0.000001$.

Modifications

Viruses of the invention are typically modified as compared to their precursor clinical strains. In particular, certain genes will typically be rendered non-functional, and the viruses may also comprise a heterologous gene(s). Typically, viruses of the invention are attenuated.

Viral regions altered for the purposes described herein may be either eliminated (completely or partly), or made non-functional, or substituted by other sequences, in particular by a heterologous gene sequence. One or more genes may be rendered non-functional, and one or more heterologous genes inserted.

Oncolytic Viruses of the Invention

In one embodiment, viruses of the invention are modified, oncolytic, non-laboratory viruses. These will be useful in the oncolytic treatment of cancer. Such viruses infect and replicate in tumour cells, subsequently killing the tumour cells. Thus, such viruses are replication competent. Preferably, they are selectively replication competent in tumour cells. This means that either they replicate in tumour cells and not in non-tumour cells, or that they replicate more effectively in

tumour cells than in non-tumour cells. Measurement of selective replication competence can be carried out by the tests described herein for measurement of replication and tumour cell-killing capacity, and also analysed by the statistical techniques mentioned herein if desired.

An oncolytic virus of the invention preferably has a greater ability than a reference laboratory strain with the same modifications to infect or replicate in a tumour cell, to kill tumour cells or to spread between cells in tissues. Preferably, this ability is a statistically significantly greater ability as described herein. The properties of the virus strain in respect of tumour cells can be measured in any manner known in the art.

For example, the capacity of a virus to infect a tumour cell can be quantified by measuring the dose of virus required to measure a given percentage of cells, for example 50% or 80% of cells. The capacity to replicate in a tumour cell can be measured by growth measurements such as those carried out in the Examples (see Figure 2), e.g. by measuring virus growth in cells over a period of 6, 12, 24, 36, 48 or 72 hours or longer.

The ability of a virus to kill tumour cells can be roughly quantitated by eye (see Figure 3) or more exactly quantitated by counting the number of live cells that remain over time for a given time point and MOI for given cell type. For example, comparisons may be made over 24, 48 or 72 hours and using any known tumour cell type. In particular, HT29 colorectal adenocarcinoma, LNCaP.FGC prostate adenocarcinoma, MDA-MB-231 breast adenocarcinoma, SK-MEL-28 malignant melanoma or U-87 MG glioblastoma astrocytoma cells can be used. Any one of these cell types or any combination of these cell types can be used, as may other tumour cell types. It may be desirable to construct a standard panel of tumour cell types for this purpose. To count the number of live cells remaining at a given time point, the number of trypan blue-excluding cells (i.e. live cells) can be counted. Quantitation may also be carried out by fluorescence activated cell sorting (FACS) or MTT assay. Tumour cell-killing ability may also be measured *in vivo*, e.g. by measuring the reduction in tumour volume engendered by a particular virus.

The ability of a virus to spread in tissue, especially solid tissue, can be measured by determining the number of cells at sites connected to the site of the original infection.

In order to determine the properties of viruses of the invention, it will generally be desirable to use a standard laboratory reference strain for comparison. The standard laboratory reference strain may be one or more of HSV1 strain 17+, HSV1 strain F and HSV1 strain KOS. The reference strain will typically have equivalent modifications to the strain of the invention being tested. Thus, the reference strain will typically have equivalent modifications, such as gene deletions and/or heterologous gene insertions. For example, in the case of an HSV strain, if the ICP34.5 and ICP47-encoding genes have been rendered non-functional in the virus of the invention, then they will also be rendered non-functional in the reference strain. The modifications made to the reference strain may be identical to those made to the strain of the invention. By this, it is meant that the gene disruptions in the reference strain will be in exactly equivalent positions to those in the strain of the invention, e.g. deletions will be of the same size and in the same place. Similarly, in these embodiments, heterologous genes will be inserted in the same place, driven by the same promoter, etc. However, it is not essential that identical modifications be made. What is important is that the reference gene has functionally equivalent modifications, e.g. that the same genes are rendered non-functional and/or the same heterologous gene or genes is inserted.

In an oncolytic virus of the invention, suitable modifications will be made to the virus to confer oncolytic activity, if it is not naturally present, and preferably to confer selective oncolytic activity.

Such mutations allowing selective oncolytic activity include mutation to the genes encoding ICP34.5, ICP6 and/or thymidine kinase (TK), preferably ICP34.5. Such mutations to the ICP34.5-encoding gene in laboratory strains of HSV are described in Chou *et al* 1990, Maclean *et al* 1991, although any mutation in which ICP34.5 is non-functional may be used.

Accordingly, the HSV strain is preferably modified such that it lacks one or more of a functional ICP34.5-encoding gene, a functional ICP6-

encoding gene, a functional glycoprotein H-encoding gene, a functional thymidine kinase-encoding gene.

More preferably, the virus lacks a functional ICP34.5-encoding gene.

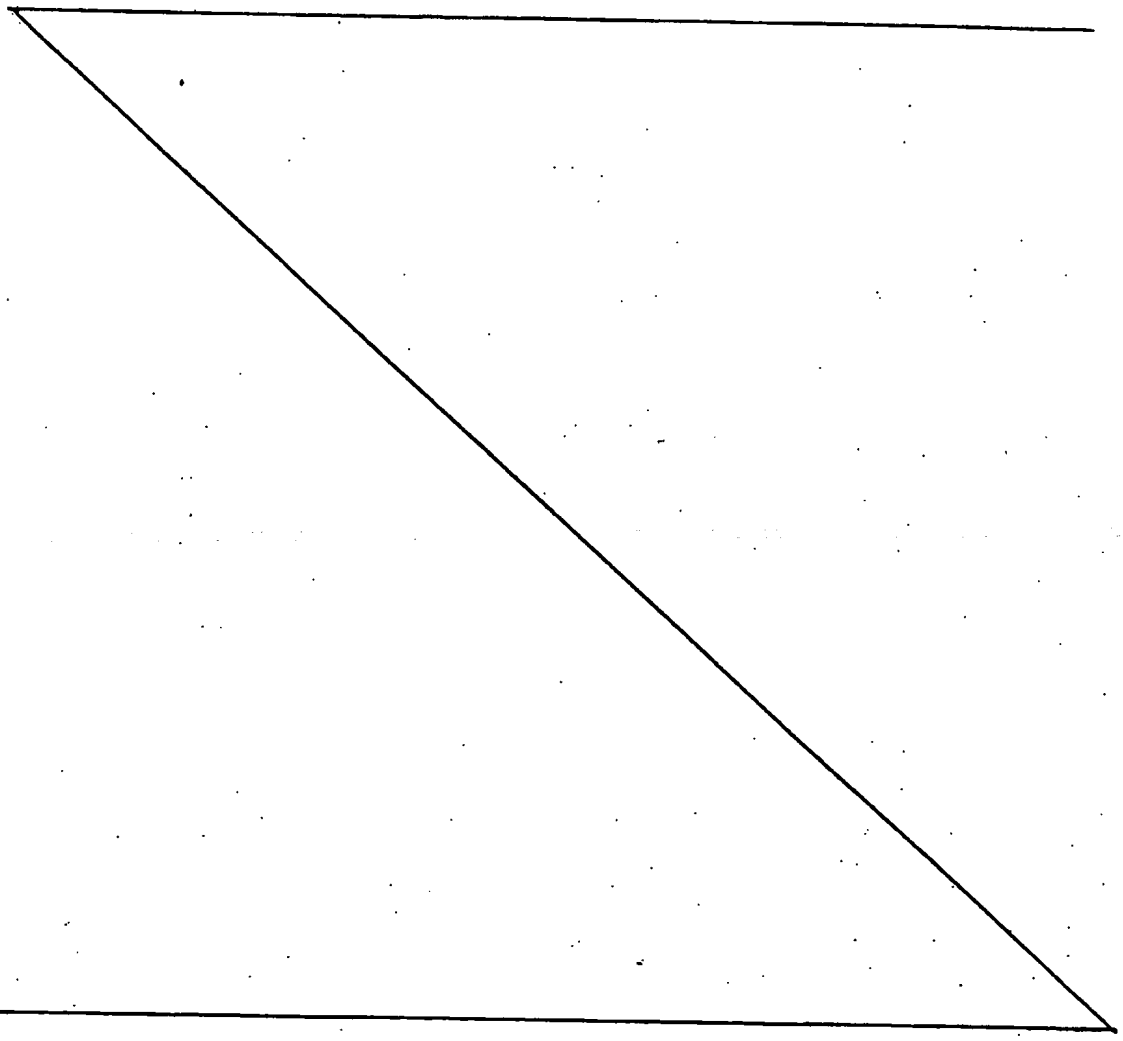
Other modifications may also be made. In particular, the HSV may be modified such that it lacks a functional ICP47 gene. This is because ICP47 usually functions to block antigen presentation in HSV-infected cells so its disruption leads to a virus that does not confer on infected tumour cells particular properties that might protect such HSV infected cells from the host's immune system.

Viruses with any other genes deleted/mutated which provide oncolytic properties (ie selective replication in tumours compared to surrounding tissue) are also viruses of the invention as those skilled in the art will recognise that the above list is not exhaustive and identification of the function of other genes in any of the viruses above may suggest the construction of new viruses which are also viruses of the invention.

Heterologous gene(s) may also be inserted into such viruses of the invention by techniques known in the art and/or described herein. In an oncolytic virus, the heterologous gene will typically be one that enhances the capacity of the virus to counteract tumours. Any genes conferring on the virus anti-tumour properties may thus be inserted. In particular, the heterologous gene may be a gene capable of modifying immune response to the tumour cells in a beneficial manner, especially an immune stimulatory polypeptide such as CD40L, granulocyte macrophage-colony-stimulating factor (GMCSF), another cytokine or chemokine (e.g.RANTES), B7.1 or B7.2 or IL12. Alternatively, the heterologous gene may encode a pro-drug activator, such as nitroreductase or cytochrome P450. In this context, combined treatment of tumours with the pro-drug activated by the pro-drug activator and a virus of the invention is envisaged. Alternatively, the heterologous gene may encode a tumour suppressor, such as p53.

B. Complementing cell lines

When the virus of the invention is a herpes simplex virus which lacks a particular functional essential gene, for example a gene encoding ICP4 or ICP27, the virus of the invention is propagated on a cell line expressing that essential gene. For example, when the virus lacks a functional ICP27 gene, the virus may be propagated on V27 cells (Rice and Knipe, 1990), 2-2 cells (Smith *et al*, 1992) or B130/2 cells (Howard *et al*, 1998). When the virus lacks a functional ICP4 gene the virus may be propagated on a cell line expressing ICP4, for example E5 cells (DeLuca *et al*, 1985). When the virus lacks a functional ICP4 gene and a functional ICP27 gene the virus is propagated on a cell line expressing both ICP4 and ICP27 (such as E26 cells;



Samaniego *et al*, 1995), and when the virus additionally lacks a functional vmw65 gene the virus may be propagated on a cell line also containing a non-HSV homologue of vmw65 (e.g. from equine herpes virus as in Thomas *et al*, 1999). Mutations to vmw65 may also be partially compensated for by inclusion of hexamethylene bisacetamide (HMBA) in the media used for virus growth (MacFarlane *et al*, 1992).

ICP27-expressing cell lines can be produced by co-transfecting mammalian cells, for example the Vero or BHK cells, with a vector, preferably a plasmid vector, comprising a functional HSV ICP27 gene capable of being expressed in said cells, and a vector, preferably a plasmid vector, encoding a selectable marker, for example neomycin resistance. Clones possessing the selectable marker are then screened further to determine which clones also express functional ICP27, for example on the basis of their ability to support the growth of ICP27- HSV strains, using methods known to those skilled in the art (for example, as described in Rice and Knipe, 1990).

Cell lines which do not allow reversion of an ICP27- mutant HSV strain to a strain with functional ICP27 are produced as described above, ensuring that the vector comprising a functional ICP27 gene does not contain sequences that overlap with (i.e. are homologous to) sequences remaining in the ICP27- mutant virus.

Where HSV strains of the invention comprise inactivating modifications in other essential genes, for example ICP4, complementing cell lines will comprise a functional HSV gene which complements the modified essential gene in the same manner as described for ICP27. For example, in the case of HSV strains comprising mutations in both ICP27 and ICP4, a cell line expressing both ICP27 and ICP4 is used (such as described in Samaniego *et al*, 1995 or in Thomas *et al*, 1999). HSV strains expressing other essential genes can be constructed in a similar manner to that described for ICP27. Here again, if it is ensured there is no sequence overlap between the remaining virus DNA and that inserted into the cell line for virus growth, the possibility of reversion of the virus to a less disabled form during growth will be minimised.

C. Methods of mutation

The various viral genes referred to may be rendered functionally inactive by several techniques well known in the art. For example, they may be rendered functionally inactive by deletion(s), substitution(s) or insertion(s), preferably by deletion. A deletion may remove a portion of the genes or the entire gene. For example, deletion of only one nucleotide may be made, resulting in a frame shift. However, preferably a larger deletion is made, for example at least 25%, more preferably at least 50% of the total coding and non-coding sequence (or alternatively, in absolute terms, at least 10 nucleotides, more preferably at least 100 nucleotides, most preferably at least 1000 nucleotides). It is particularly preferred to remove the entire gene and some of the flanking sequences. An inserted sequence may include one or more of the heterologous genes described below. In the case of the vmw65 gene, the entire gene is not deleted since it encodes an essential structural protein, but a small inactivating mutation is made which abolishes the ability of vmw65 to activate transcriptionally IE genes (e.g. as in Ace *et al*, 1989 or Smiley *et al*, 1997).

Mutations are made in the herpes viruses by homologous recombination methods well known to those skilled in the art. For example, HSV genomic DNA is transfected together with a vector, preferably a plasmid vector, comprising the mutated sequence flanked by homologous HSV sequences. The mutated sequence may comprise a deletion(s), insertion(s) or substitution(s), all of which may be constructed by routine techniques. Insertions may include selectable marker genes, for example lacZ or GFP, for screening recombinant viruses by, for example β-galactosidase activity or fluorescence.

D. Heterologous genes and promoters

The viruses of the invention may be modified to carry a heterologous gene/genes. The term "heterologous gene" encompasses any gene. Although a heterologous gene is typically a gene not present in the genome of a herpes virus, herpes gene/genes may be used provided that the coding sequence is not operably

linked to the viral control sequences with which it is naturally associated. The heterologous gene may be any allelic variant of a wild-type gene, or it may be a mutant gene. The term "gene" is intended to cover nucleic acid sequences which are capable of being at least transcribed. Thus, sequences encoding mRNA, tRNA and rRNA are included within this definition. However, the present invention is concerned with the expression of polypeptides rather than tRNA and rRNA. Sequences encoding mRNA will optionally include some or all of 5' andor 3' transcribed but untranslated flanking sequences naturally, or otherwise, associated with the translated coding sequence. It may optionally further include the associated transcriptional control sequences normally associated with the transcribed sequences, for example transcriptional stop signals, polyadenylation sites and downstream enhancer elements.

The heterologous gene/genes may be inserted into the viral genome by homologous recombination of HSV strains with, for example plasmid vectors carrying the heterologous gene/genes flanked by HSV sequences. The heterologous gene/genes may be introduced into a suitable plasmid vector comprising herpes viral sequences using cloning techniques well-known in the art. The heterologous gene/genes may be inserted into the viral genome at any location provided that the virus can still be propagated. It is preferred that the heterologous gene/genes is inserted into an essential gene. Heterologous genes may be inserted at multiple sites within the virus genome.

The transcribed sequence of the heterologous gene/genes is preferably operably linked to a control sequence permitting expression of the heterologous gene/genes in mammalian cells, preferably a tumour cell or a cell of the nervous system. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequence.

The control sequence comprises a promoter allowing expression of the heterologous gene/genes and a signal for termination of transcription. The promoter

is selected from promoters which are functional in mammalian, preferably human, cells of the nervous system or in tumours or in cells of the immune system. The promoter/promoters may be derived from promoter sequences of eukaryotic genes. For example, promoters may be derived from the genome of a cell in which expression of the heterologous gene is to occur, prefcrably a mammalian, preferably human cell. With respect to eukaryotic promoters, they may be promoters that function in a ubiquitous manner (such as promoters of β-actin, tubulin) or, alternatively, a tissue-specific manner, such as the neuron-specific enolase (NSE) promoter. They may also be promoters that respond to specific stimuli, for example promoters that bind steroid hormone receptors. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV) LTR promoter or other retroviral promoters, the human or mouse cytomegalovirus (CMV) IE promoter, or promoters of herpes virus genes including those driving expression of the latency associated transcripts.

Expression cassettes and other suitable constructs comprising the heterologous gene/genes and control sequences can be made using routine cloning techniques known to persons skilled in the art (see, for example Sambrook *et al*, 1989, Molecular Cloning – A laboratory manual: Cold Spring Harbor Press).

It may also be advantageous for the promoters to be inducible so that the levels of expression of the heterologous gene can be regulated during the life-time of the cell. Inducible means that the levels of expression obtained using the promoter can be regulated. For example, in a preferred embodiment where more than one heterologous gene is inserted into the HSV genome, one promoter would comprise a promoter responsive to the tet repressor/VP16 transcriptional activator fusion protein previously reported (Gossen and Bujard, 1992, Grossen *et al*, 1995), and driving the heterologous gene the expression of which is to be regulated. The second promoter would comprise a strong promoter (e.g. the CMV IE promoter) driving the expression of the tet repressor/VP16 fusion protein. Thus, in this example, expression of the first heterologous gene would depend on the presence or absence of tetracycline.

Heterologous genes will typically encode polypeptides of therapeutic use. In oncolytic applications, heterologous genes may encode proteins which are themselves cytotoxic, encode pro-drug activating enzymes or which are capable of stimulating or enhancing an anti-tumour immune response.

Heterologous genes may also include marker genes (for example, encoding β-galactosidase or green fluorescent protein or other fluorescent proteins) or genes whose products regulate the expression of other genes (for example, transcriptional regulatory factors including the tet repressor/vmw65 transcriptional activator fusion protein described above).

Therapeutic applications may well require the administration of multiple genes. Herpes viruses are uniquely appropriate as they do not have the limited packaging capabilities of other viral vector systems. Thus, multiple heterologous genes can be accommodated within its genome. For example, from 2 to 5 genes may be inserted into the genome.

There are, for example, at least two ways in which this could be achieved. For example, more than one heterologous gene and associated control sequences could be introduced into a particular HSV strain either at a single site or at multiple sites in the virus genome. It would also be possible to use pairs of promoters (the same or different promoters) facing in opposite orientations away from each other, these promoters each driving the expression of a heterologous gene (the same or different heterologous gene) as described above.

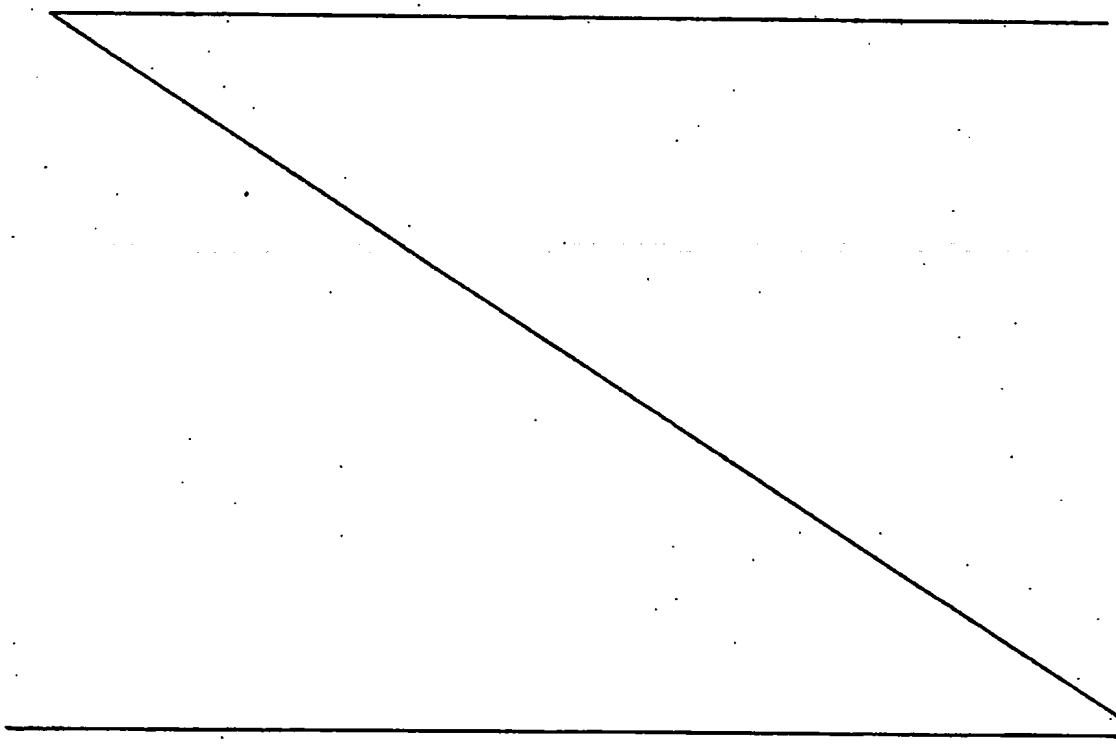
E. Therapeutic uses

Viruses of the invention may be used in methods of therapy. In particular, oncolytic viruses of the invention may be used in applications including the oncolytic treatment of cancer, e.g. by direct intra-tumour injection. Where the virus comprises a heterologous gene encoding a prodrug activator, additional pro-drug therapy may

be carried out. Additionally, treatment may be combined with or stimulation of an immune response by any means known in the art. Viruses of the invention may be used in the therapeutic treatment of any solid tumour in a mammal, preferably in a human. For example viruses of the invention may be administered to a subject with prostate, breast, lung, liver, endometrial, bladder, colon or cervical carcinoma; adenocarcinoma; melanoma; lymphoma; glioma; or sarcomas such as soft tissue and bone sarcomas.

F. Administration

The viruses of the invention may thus be used in a patient, preferably a human patient, in need of treatment. Viruses of the invention may be used for the oncolytic treatment of cancer. The aim of therapeutic treatment is to improve the condition of the patient. Typically therapeutic treatment using a virus of the invention will allieviate the symptoms of the disease or condition of the patient being treated. A method of treatment according to the invention therefore comprises administering a therapeutically effective amount of a



virus of the invention to a patient suffering from cancer.

Administration of an oncolytic virus of the invention to a patient suffering from a tumour will typically kill the cells of the tumour thus decreasing the size of the tumour and/or preventing the spread of malignant cells from the tumour.

One method of administering therapy involves combining the virus with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline.

Oncolytic treatment may then be carried out following direct injection of the vector composition into target tissue. The amount of virus administered is in the case of HSV in the range of from 10^4 to 10^{10} pfu, preferably from 10^5 to 10^8 pfu, more preferably about 10^6 to 10^8 pfu. When injected for oncolytic treatment, typically up to 500 µl, typically from 1-200 µl, preferably from 1-10 µl of a pharmaceutical composition consisting esentially of the virus and a pharmaceutically acceptable suitable carrier or diluent, would be used for injection. However, for some oncolytic therapy applications larger volumes up to 10ml may also be used, depending on the tumour and inoculation site.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage. The dosage may be determined according to various parameters, especially according to the age, weight and condition of the patient to be treated, the severity of the disease or condition and the route of administration.

The preferred route of administration to a patient suffering from cancer is by direct injection into the tumour. The virus may also be administered systemically or by injection into a blood vessel supplying the tumour. The optimum route of

administration will depend on the location and size of the tumour.The dosage may be determined according to various parameters, especially according to the location of the tumour, the size of the tumour, the age, weight and condition of the patient to be treated and the route of administration.

G. Non -Therapeutic aspects

Also provided are methods of identifying suitable clinical strains for modification according to the invention. In addition, methods of target validation are provided. These concern the identification of genes suitable for use in the therapeutic applications of the invention as described above.

Methods of production of viruses of the invention are also provided.

The following Examples illustrate the invention.

Herpes simplex type-1 virus (HSV1) in which the neurovirulence factor ICP34.5 is inactivated has previously been shown to direct tumour specific cell lysis in tumour models both *in vitro* and *in vivo*. Such viruses have also been shown to be safe in Phase I clinical trials by direct intra-cerebral injection in late stage glioma patients.

Previous work has used serially passaged laboratory isolates of HSV1 (viruses derived from HSV1 strain 17+ or HSV1 strain F) which might be anticipated to be attenuated in their lytic capability in human tumour cells as compared to more recent clinical isolates.

In work aimed at producing ICP34.5 deleted HSV with enhanced oncolytic and anti-tumour potential, we have deleted ICP34.5 from an HSV1 clinical isolate and compared replicative and lytic potential in a number of human tumour cell types in comparison to HSV1 strain 17+ (a standard laboratory strain).

Virus Construction (see Fig 1)

The viruses used were either based on HSV1 strain 17+ (a standard laboratory strain) or two clinical isolates derived from cold sores from frequent re-activators of HSV1. These strains were named BL1 and JS1. ICP34.5 was completely deleted from strain 17+ and JS1 together with the insertion of a CMV-GFP cassette. JS1 was also further engineered by the insertion of human or mouse GM-CSF so as to replace the ICP34.5 gene. BL1 and JS1 are thus clinical isolates, or "non-laboratory" strains. The derivatives of JS1 discussed herein are also non-laboratory strains, i.e. modified non-laboratory strains of the invention.

Virus Growth in Tumour Cells (see Fig 2)

JS1 and BL1 showed enhanced growth in some human tumour cells tested as compared to HSV1 ICP34.5 deleted strain 17+ when tested over a 72 hour period (Fig 2). JS1 was selected for further study and the modifications described above (see Fig 1, and above) were made to it.

Lytic Capabilities of Viruses (see Fig. 3)

Lytic (cell killing) capabilities were enhanced with the JS1-derived non-laboratory strains derived virus in all tumour cell lines tested. More particularly, with reference to Figure 3, the JS1/34.5- virus, i.e. JS1 with ICP34.5 removed by deletion, showed enhanced lytic capabilities in HT29 colorectal adenocarcinoma, LNCaP.FGC prostate adenocarcinoma, MDA-MB-231 breast adenocarcinoma, SK-MEL-28 malignant melanoma and U-87 MG glioblastoma astrocytoma cells.

Lytic capabilities were also assessed in SK-MEL-28, MDA-MB-231 and HT29 cells by trypan blue exclusion assay of infected cells at various doses and times after infection with BL1, JS1 as compared to strain 17+. Trypan blue is excluded from live cells and so numbers of live cells remaining in a culture can be assessed by this means. Tumour cell lines cultured in duplicate wells of six well

dishes were infected for 24, 48 or 72 hrs at an MOI of 0.1 or 1 with either 17+, BL1 or JS1 and numbers of live cells were counted. The percentage of the number of live cells in equivalent uninfected control wells are shown in Table 1.

Thus, as in all cases more tumour cells are killed with the clinical isolate viruses BL1 and JS1 than the laboratory isolate 17+, to provide increased oncolytic activity, the use of recent clinical virus strains is likely to enhance the anti-tumour capabilities of such viruses modified to give tumour selective replication (e.g. by the deletion of ICP34.5) when used in human patients for cancer treatment.

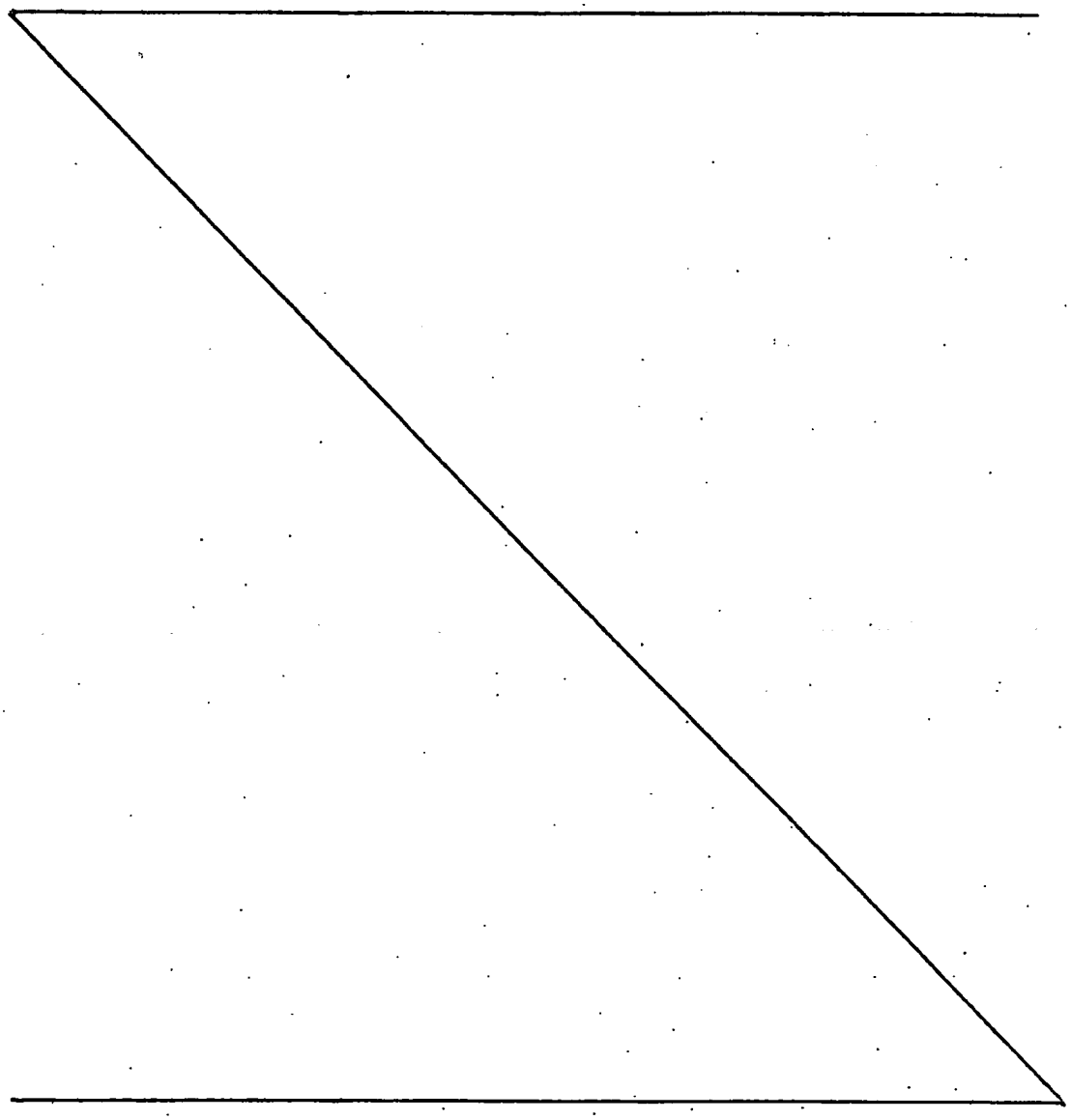


Table 1

Cell line	time after infection	JS1 MOI=0.1	JS1 MOI=1	17 MOI=0.1	17 MOI=1	BL1 MOI=0.1	BL1 MOI=1
		percentage of number of live cells in uninfected control wells					
SK-MEL-28	24h	41	8	57.3	19	43.7	6.67
	duplicate samples	33.7	7	62.6	19.3	39	6.33
	48h	5.51	1.9	7.4	3.7	4.5	0.8
		5.05	0.8	7.1	2.6	4.8	1.1
	72h	0	0	0	0	0	0
		0	0	0	0	0	0
MDA-MB-231	24h	44.91	16.7	69.37	36.34	55.63	26.79
		44.02	16.96	65.8	34.55	60.45	25.27
	48h	14.1	4.7	27.9	8.3	18	6.7
		13.5	3.8	27	8.5	20	8.3
	72h	0	0	2.91	0.73	1.46	0
		0	0	2.91	1.27	1.64	0
HT-29	24h	37.53	15	47.28	23.61	42.22	22.15
		39.24	15	45.76	24.24	43.04	21.33
	48h	13.2	2.3	29.4	4.2	18.4	4.4
		14	3	27.7	4.7	21.2	3.7
	72h	0	0	1.57	0	1.64	0
		0	0	1.89	0	1.57	0

Further Enhanced Anti-Tumour Activity

Further enhanced activity may also be anticipated if these viruses are then used to deliver genes with anti-tumour activity. Such genes include those encoding pro-drug activators or immune stimulatory protcins.

For this purpose, we have produced from JS1 an ICP34.5 deleted clinical isolated of HSV1 which expresses human or mouse GM-CSF GM-CSF is a potent immune stimulator. This virus is designed to enhance anti-tumour immune responses following intra-tumoral injection.

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Deposit Information

HSV1 Strain JS1 has been deposited at the European Collection of Cell Cultures (ECACC), CAMR, Salisbury, Wiltshire SP4 0JG, United Kingdom, on 2 January 2001 under provisional accession number 01010209.

CLAIMS

1. Use of a modified, oncolytic, herpes simplex virus (HSV) strain in the manufacture of a medicament for the oncolytic treatment of cancer, which HSV strain:

(a) has a greater ability than HSV1 17+, HSV1 F and HSV1 KOS strains with equivalent modifications to replicate in or kill tumour cells; and

(b) is produced by isolating an HSV strain from a host and modifying the isolated HSV strain to provide for selective replication in tumour cells.

2. Use according to claim 1 wherein said greater ability is a statistically significant greater ability.

3. Use according to claim 1 or 2 wherein said HSV strain is a strain of HSV1 or HSV2.

4. Use according to any one of the preceding claims wherein said HSV strain is modified such that it lacks one or more of a functional ICP34.5-encoding gene, a functional ICP6-encoding gene, a functional glycoprotein H-encoding gene and a functional thymidine kinase-encoding gene.

5. Use according to claim 4 wherein the HSV strain lacks a functional ICP34.5-encoding gene.

6. Use according to claim 5 wherein the HSV strain further lacks a functional ICP47 gene.

7. Use according to any one of claims 1 to 4 wherein the HSV strain further comprises a heterologous gene.

8. Use according to claim 7, wherein said heterologous gene is a gene capable of modifying immune responses.

9. Use according to claim 8, wherein the gene capable of modifying immune responses encodes an immune stimulatory polypeptide or another gene product capable of modifying immune responses, a prodrug activator, a tumour suppressor or a pro-apoptotic gene product.

10. Use according to claim 9, wherein the immune stimulatory polypeptide is granulocyte macrophage colony-stimulating factor (GMCSF), another cytokine or chemokine, RANTES, B7.1 or B7.2 or IL12, wherein the prodrug activator is nitroreductase or cytochrome p45 or wherein the tumour suppressor is p53.

11. Use according to any one of the preceding claims, wherein the HSV strain is derived from HSV1 strain JS1 as deposited at the European Collection of Cell Cultures (ECACC) under provisional number 01010209.

12. A method of determining whether a gene enhances the anti-tumour effects of an HSV comprising:

(i) providing a modified, oncolytic HSV strain as defined in any one of the preceding claims;

(ii) inserting said gene into said HSV strain; and

(iii) assessing the ability of said modified, oncolytic HSV strain to replicate in or kill tumour cells compared to the ability of the precursor strain provided in step (i).

13. A method of determining the suitability of an HSV strain for use in the production of a modified, oncolytic HSV strain, said method comprising:

(i) isolating an HSV strain from a host;

(ii) assessing the ability of the HSV to replicate in or kill one or more types of tumour cell;

(iii) determining whether the HSV has a greater ability than one or more of HSV1 strain 17+, HSV1 strain F and HSV1 strain KOS to replicate in or kill tumour cells; and optionally

(iv) selecting the HSV strain for modification, wherein the HSV strain has said greater ability.

14. A method according to claim 13 wherein said greater ability is determined relative to HSV1 strain 17+, HSV1 strain F and HSV1 strain KOS.

15. A method of producing a modified, oncolytic HSV strain, said method comprising:

(i) providing an HSV strain selected by a method according to claim 13 or 14;

(ii) modifying said HSV strain to provide for selective replication in tumour cells; and

(iii) assessing the ability of said modified, oncolytic HSV strain to kill tumour cells.

16. A method according to claim 15 wherein the HSV strain is modified by rendering non-functional one or more genes selected from the genes encoding ICP34.5, ICP6, glycoprotein H and thymidine kinase.

17. An oncolytic HSV strain which is selected by or produced by a method according to any one of claims 13 to 16, which HSV strain has a greater ability than HSV1 17+, HSV1 F and HSV1 KOS strains with equivalent modifications to kill tumour cells.

18. A pharmaceutical composition comprising a modified oncolytic HSV strain as defined in claim 17 and a pharmaceutically acceptable carrier or diluent.

19. A modified oncolytic HSV strain as defined in claim 17 for use in the treatment of the human or animal body.

20. Use of modified oncolytic HSV strain as defined in claim 17 in the manufacture of a medicament for the oncolytic treatment of cancer.

21. A modified, oncolytic HSV strain according to claim 17 substantially as herein described and with reference to the Examples.

22. Use according to claim 1 substantially as herein described and with reference to the Examples in the manufacture of a medicament for the treatment of cancer.

23. A method according to claim 15 which method is substantially as herein described.

24. A method according to claim 13 which method is substantially as herein described.

25. A method according to claim 12 which method is substantially as herein described.