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(54) Title: METHODS FOR THE TREATMENT OF CELLULAR PROLIFERATIVE DISORDERS

(57) **Abrégé/Abstract:**

The present invention relates to methods of identifying the susceptibility of cells to reovirus infection by measuring constitutive MAP kinase pathway signaling. The invention also pertains to methods using reovirus for the treatment of cellular proliferative disorders, and particularly cellular proliferative disorders wherein the proliferating cells exhibit constitutive MEK phosphorylation, in mammals. In particular, the methods provide for reovirus treatment of mammals to treat proliferative disorders, including breast tumors, a subset of tumors in which mutation of the Ras gene is not believed to play a significant role.



ABSTRACT OF THE DISCLOSURE

The present invention relates to methods of identifying the susceptibility of cells to reovirus infection by measuring constitutive MAP kinase pathway signaling. The invention also pertains to methods using reovirus for the treatment of cellular proliferative disorders, and particularly cellular proliferative disorders wherein the proliferating cells exhibit constitutive MEK phosphorylation, in mammals. In particular, the methods provide for reovirus treatment of mammals to treat proliferative disorders, including breast tumors, a subset of tumors in which mutation of the Ras gene is not believed to play a significant role.

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METHODS FOR THE TREATMENT OF CELLULAR PROLIFERATIVE DISORDERS

Field of the Invention

[0001] The present invention relates to methods of identifying the susceptibility of cells to reovirus infection by measuring constitutive mitogen activated protein (MAP) kinase pathway signaling. The invention also pertains to methods for using reovirus for the treatment of cellular proliferative disorders, and particularly cellular proliferative disorders wherein the proliferating cells exhibit constitutive MAP kinase phosphorylation, in mammals. The methods of the invention provide for reovirus treatment of mammals to treat proliferative disorders, which include tumors in which Ras mutations are not believed to play a significant role.

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[0002] The following publications, patent applications, and patents are cited in this application:

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[0003] All of the publications, patent applications, and patents, cited above or elsewhere in this application, are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent application or patent was specifically and individually indicated to be incorporated by reference in its entirety.

State of the Art

[0004] Normal cell proliferation is regulated by a balance between growth-promoting proto-oncogenes and growth-constraining tumor-suppressor genes. Tumorigenesis can be caused by genetic alterations to the genome that result in the mutation of those cellular elements that govern the interpretation of cellular signals, such as potentiation of proto-oncogene activity or inactivation of tumor suppression. It is believed that the interpretation of these signals ultimately influences the growth and differentiation of a cell, and that misinterpretation of these signals can result in neoplastic growth (neoplasia).

[0005] Genetic alteration of the proto-oncogene Ras is believed to contribute to approximately 30% of all human tumors (Wiesmuller and Wittinghofer (1994); Barbacid (1987)). The role that Ras plays in the pathogenesis of human tumors is specific to the type of tumor. Activating mutations in Ras itself are found in most types of human malignancies, for example pancreatic cancer (80%), sporadic colorectal carcinomas (40-50%), human lung adenocarcinomas (15-24%), thyroid tumors (50%) and myeloid leukemia (30%) (Mills *et al.* (1995); Chaubert *et al.* (1994); Bos (1989)), and ovarian carcinomas (20-47%) (Sherman (2000); Dokianakis *et al.* (1999)).

[0006] Ras is known to interact with a number of downstream effectors, including members of the Raf family (*e.g.*, Raf1, B-raf, and A-raf), members of the RalGEF family (*e.g.*, RalGDS, RGL1, RGL2, and Rlf), and phosphatidylinositol-3-kinases (PI-3 kinases) (*e.g.*, isoforms of protein kinase C, AKT kinase/protein kinase B, p70-S6 kinase, and RacGEFs). The mitogen activated protein (MAP) kinase pathway is perhaps the best studied of the downstream Ras-

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effector pathways. The MAP kinase pathway (or simply the MAP pathway) comprises Ras, Raf, MEK (MAP/ERK kinase, formerly known as MAP kinase kinase), and ERK (extracellular signal related kinase or MAP kinase), wherein Ras activates Raf, which activates MEK, which activates MAP kinase. Phosphorylated MAP kinase (ERK) translocates to the cell nucleus and modulates the activity of transcription factors, for example c-Myc and Elk-1, through further phosphorylation (Ward *et al.* (2001); Sebolt-Leopold (2000); Lewin (1997)), thereby affecting gene expression.

[0007] Ras activation also results as a consequence of upstream mitogenic signaling, notably by activation of tyrosine kinase receptors, including but not limited to the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors. Tyrosine kinase receptors activate Ras through adapter proteins such as Grb2 and Sos (*e.g.*, Lewin (1997)). These upstream elements, if amplified or overexpressed, result in elevated Ras activity and subsequent activation downstream Ras effectors. An example of activation of elements upstream of Ras includes overexpression of c-erbB-2/neu PDGFR (Levitzki (1994); Janes *et al.* (1994); Bos (1989)). HER-2/neu (erbB-2) is overexpressed in approximately 30% of all breast cancers (Spandidos (1987); Zhou *et al.* (1989); Archer *et al.* (1995)) and is associated with poor patient prognosis (Slamon *et al.* (1989)). When overexpressed in NIH-3T3 cells, HER-2 mediates transformation; however, there does appear to be a threshold level of overexpression that is required for this transformation to occur (Jardines *et al.* (1993); Clark *et al.* (1995)). It appears that this transforming ability of HER-2 is dependent upon Ras activity as cell lines overexpressing HER-2 exhibit a dramatic increase in MAP kinase activity, the latter being reflective of Ras activity (Janes *et al.* (1994)). The growth factor receptor EGFR that is closely related to, but distinct from HER-2, has also been observed to be overexpressed in breast tumors and has also been correlated strongly with a poor patient prognosis (Shackney *et al.* (1998); Koenders *et al.* (1991)).

[0008] Additional elements upstream of Ras have also been implicated in the etiology of breast cancer. The non-receptor tyrosine kinase c-Src has been implicated as being an important candidate in promoting the progression of breast cancer. A number of studies indicate a 4- to 30-fold increase of c-Src activity in primary breast cancer tumors when compared to normal breast tissue (Verbeeks *et al.* (1996); Jacobs *et al.* (1983)). c-Src has been suggested to also play an important role in the transmission of signals from both the estrogen and progesterin receptors via

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the Ras/MAP kinase pathway. A number of groups have observed that the treatment of breast tumor derived MCF-7 cells with estradiol results in the activation of c-Src kinase activity with resultant activation of MAP kinase (Di Domenico *et al.* (1996); Migliaccio *et al.* (1996)). More recently, Migliaccio *et al.* (1998), have demonstrated in T47D breast cancer cells that progestin stimulated cell proliferation is dependent upon Src/Ras/MAP kinase signaling. The fact that these steroid receptors can utilize Ras for their signaling suggests that Ras may play a more pivotal role in the promotion of growth in ER and PR positive breast tumors.

[0009] MEK is also activated in a Ras-independent manner through MEK kinase, which is activated through largely unknown or uncharacterized G-proteins (Lewin (1997)). Accordingly, MEK is a common factor in Ras-dependent and Ras-independent MAP kinase pathway activation.

[0010] It has previously been demonstrated that reovirus is replication competent in cells that possess an activated Ras signaling pathway, either through direct mutation of Ras itself or via upstream elements that result in its activation (Strong *et al.* (1993); Strong *et al.* (1998)). However, it would be useful to be able to treat cells with other types of proliferative disorders, for example disorders not associated with activated Ras, using oncolytic reovirus.

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SUMMARY OF THE INVENTION

[0011] The present invention is directed to methods of determining the susceptibility of cells to reovirus infection by measuring constitutive MAP kinase pathway signaling in the cell, wherein the presence of constitutive signaling indicates susceptibility to infection by reovirus. According to one aspect of the invention, MAP kinase pathway signaling is measured by determining the state of phosphorylation of MEK, for example using an antibody specific for phosphorylated MEK (phospho-MEK). The transformed, reovirus-susceptible cells may harbor normal Ras and cell activation may not be a result of Ras activation. In one aspect of the invention, cell transformation results from activation or mutation of one or more elements in the MAP kinase pathway, including but not limited to Raf, MEK, and MAP kinase. In another aspect of the invention, cell transformation results from activation or mutation of MEK kinase.

[0012] Cells used in the practice of the invention may be comprised in a biological specimen, particularly one obtained from a mammal suspected of having a proliferative disorder. Examples of biological specimens useful for practicing the invention include but are not limited to cell pellets from cultured immortalized cells lines, cultured primary tumor cells, tumor biopsy specimens, and post-mortem tumor specimens. Examples of proliferative disorders include but are not limited to neurofibromatosis, lung cancer, prostate cancer, colorectal cancer, thyroid cancer, renal cancer, adrenal cancer, liver cancer, pancreatic cancer, breast cancer and central, peripheral nervous system cancer, or a hematopoietic neoplasm. The proliferative disorder may be a neoplasm, including a solid neoplasm. The neoplasm may be metastatic.

[0013] The invention may be practiced using a mammal. In one aspect of the invention, the mammal is selected from the group consisting of dogs, cats, sheep, goats, cattle, horses, pigs, mice, non-human primates, and humans. The mammal may be immunocompetent or immunocompromised.

[0014] Another aspect of the invention is the treatment of a proliferative disorder in a mammal, which disorder is characterized by constitutive MEK phosphorylation. The method comprises administering to the proliferating cells in the mammal an effective amount of one or more reoviruses under conditions that result in substantial lysis of the proliferating cells. The method may further comprising a step selected from the group consisting of: administering to the proliferating cells in said mammal an effective amount of an immune suppressive agent;

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removing B-cells or T-cells from said mammal; removing anti-reovirus antibodies from said mammal; removing antibodies from said mammal; administering anti-antireovirus antibodies to said mammal; and/or suppressing the immune system of the mammal.

[0015] The reovirus used to practice the invention may be a mammalian reovirus or an avian reovirus. The reovirus may be a human reovirus, including but not limited to serotype 1 reovirus, serotype 2 reovirus and serotype 3 reovirus. The reovirus may be one or more recombinant reovirus. The reovirus may be a field isolate. One or more different strain and/or types of reovirus may be administered. The reovirus may be immunoprotected or encapsulated in a micelle. The reovirus may be treated with a protease prior to administration. In one aspect of the invention, approximately 1 to 10^{15} plaque forming units of reovirus/kg body weight are administered. The reovirus may be administered in a single dose or in more than one dose. An effective amount of a chemotherapeutic agent may be administered along with the reovirus.

[0016] The reovirus may be administered by one or more of a number of different routes, including but not limited to intravascular, intrathecal, intravenous, intramuscular, subcutaneous, intraperitoneal, topical, oral, rectal, vaginal, nasal, and intratumoral. The reovirus may also be administered by injection into or near the solid neoplasm. Administration of reovirus may be preceded by surgical removal of the substantially all of the neoplasm, followed by administration of the reovirus to the surgical site in an amount sufficient to result in substantial oncolysis of any remaining neoplastic cells.

[0017] Also provided is a pharmaceutical composition comprising an effective amount of a reovirus and a pharmaceutically acceptable excipient. The pharmaceutical composition may comprise an immunosuppressant or immunoinhibitant, along with a reovirus and a pharmaceutically acceptable excipient. Kits comprising a reovirus and, optionally, an immunosuppressant or immunoinhibitant are also provided.

[0018] In another aspect of the invention, suspected tumorigenic cells are identified in a biological specimen or population of cells by lysing the cells, incubating the cell material with phospho-MEK-specific antibody, and detecting the presence of the phospho-MEK-specific antibody. To facilitate antibody detection, the phospho-MEK-specific antibody may be conjugated to a tag, including but not limited to a fluorescent tag, a radioactive tag, and an enzyme (*e.g.*, horseradish peroxidase or alkaline phosphatase).

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[0019] In another aspect of the invention, constitutive MEK phosphorylation is determined using a MEK-specific antibody that does not recognize the phosphorylation status of MEK. Total MEK is determined by immunoblot analysis, immunoprecipitation, radioimmunoassay (RIA), or 2D-electrophoreses. Total MEK is determined, for example, in cell lysates from normal cells and in cell lysates from cells with a suspected proliferative disorder. The relative amount of phospho-MEK is then determined by pulse labeling cells, for example, with a [³²P]- or [³³P]-containing molecule, prior to determining total MEK using a MEK-specific antibody. The relative amount of label incorporated into MEK is then compared in normal cells and suspected tumorigenic cells, normalized for the total amount of MEK in the respective specimen. These assays are performed in the absence of mitogens, thus an increased proportion of phospho-MEK in biological specimens comprising cells with suspected proliferative disorders, compared to the relative amount of phospho-MEK in specimens comprising normal cells, indicates constitutive MEK phosphorylation, the presence of a cell proliferative disorder, and susceptibility to reovirus infection.

[0020] The methods and pharmaceutical compositions of the invention provide an effective means to treat neoplasia, without the side effects associated with other forms of cancer therapy. When used, inhibition or suppression of the immune system increases the availability of reovirus to infect and lyse proliferating cells which exhibit constitutive MEK phosphorylation because anti-reovirus antibodies are not formed. Because reovirus is not known to be associated with disease, any safety concerns associated with deliberate administration of a virus are minimized.

[0021] The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred embodiments of the invention, as illustrated in the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] Figure 1 shows the results of immunoblot analyses used to determine relative amounts of GTP-bound Ras, total Ras, phosphorylated MEK, and total MEK, in various normal and tumorigenic colon (A) and ovarian (B) cells.

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[0023] Figure 2 shows the change in tumor size (mm^2) over time (days) in animals receiving colon (A-C) or ovarian (D) tumor xenografts followed by live (open circles) or UV-inactivated (closed circles) reovirus.

DETAILED DESCRIPTION OF THE INVENTION

A. Definitions

[0024] *Administration to a proliferating cell or neoplasm:* Administration of reovirus in a manner so that it contacts the proliferating cells or cells of the neoplasm (also referred to herein as neoplastic cells). The route by which the reovirus is administered, as well as the formulation, carrier or vehicle, will depend on the location as well as the type of the neoplasm. Routes of inoculation include but are not limited to direct tumor injection; intravenous, intravascular, intrathecal, intramuscular, subcutaneous, intraperitoneal, topical, orally, rectal, vaginal, or nasal administration; or by inhalation spray.

[0025] *Anti-antireovirus antibodies:* Antibodies directed against anti-reovirus antibodies.

[0026] *Antibody specific for MEK or MEK-specific antibody:* An antibody that specifically recognizes phosphorylated and non-phosphorylated forms of MEK.

[0027] *Antibody specific for phosphorylated MEK or phospho-MEK-specific antibody:* An antibody that specifically recognizes a phosphorylated form of MEK, for example, the antibody MEK1/2.

[0028] *Anti-reovirus antibody:* An antibody which binds to reovirus.

[0029] *Biological specimen:* Any cell- or cell material-comprising specimen obtained directly or indirectly from animals, including but not limited to cultured immortalized cells, cultured primary cells, tumor biopsy specimens, and post-mortem tumor specimens.

[0030] *Constitutive MEK phosphorylation:* MEK phosphorylation in the absence of mitogens. Herein expressed as “the level of constitutive MEK phosphorylation or” the “relative amount of MEK phosphorylation” with respect to total MEK (*i.e.*, phosphorylated and non-phosphorylated MEK) or using equivalent terminology.

[0031] *Elements in the MAP kinase pathway:* Factors in the MAP kinase pathway, including but not limited to Ras, Raf, MEK, and MAP kinase.

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[0032] *Elements in the Ras pathway*: Factors in the Ras pathway, including but not limited to the EGF and PDGF receptors, Grb2, Sos, and Ras.

[0033] *Elements upstream of MEK*: Factors in the Ras, MAP kinase, or any other pathways that directly or indirectly activate or suppress MEK, for example, through phosphorylation. Examples include but are not limited to ligands that activate MEK-containing signal transduction pathways, cell surface receptors such as tyrosine kinase receptors (e.g., the EGF and PDGF receptors), Grb2, Sos, Ras, Raf, and MEK kinase.

[0034] *Humanized antibodies*: Antibody molecules in which the amino acid sequence in the non-antigen binding regions has been altered so that the antibody more closely resembles a human antibody, and still retains its original binding ability.

[0035] *Immunosuppressants or immune suppressive agents*: Include conventional immunosuppressants, immunoinhibitors, antibodies, and conditions such as radiation therapy or HIV infection which result in compromise of the immune system.

[0036] *Mammal suspected of having a proliferative disorder*: A mammal having a proliferative disorder or tumor, that has been diagnosed with a proliferative disorder or tumor, that has been previously diagnosed with a proliferative disorder or tumor, or that has had substantially all of a tumor surgically removed and is suspected of harboring some residual tumor cells.

[0037] *MAP kinase pathway (or MAP pathway)*: The mitogen-activated protein (MAP) kinase pathway is a downstream Ras-effector pathway comprising (in order of activation) Ras, Raf, MEK (MAP/ERK kinase, formerly known as MAP kinase kinase), ERK (extracellular signal related kinase or MAP kinase), and transcription factors, such as c-Myc and Elk-1.

[0038] *MAPK, MAP kinase*: MAP (mitogen-activated protein) kinase or ERK (extracellular signal related kinase).

[0039] *MEK kinase*. Mitogen-activated extracellular signal-regulated kinase. A kinase activated by G-proteins, likely in response to activation of serpentine receptors. MEK kinase is part of a MAP kinase pathway-independent method of MEK activation.

[0040] *MEK*: MAP/ERK kinase, formerly called MAP kinase kinase.

[0041] *Naturally-occurring reovirus*: Reovirus that can be isolated from a source in nature and has not been intentionally modified by humans in the laboratory. For example, reovirus from a field source or a patient.

[0042] *Neoplasm*: An abnormal benign or malignant tissue growth, generally forming a distinct mass that grows by cellular proliferation more rapidly than normal tissue growth. Neoplasms show partial or total lack of structural organization and functional coordination with normal tissue. Neoplasms arising from epithelial structures are called carcinomas, malignant neoplasms that originate from connective tissues such as muscle, cartilage, fat or bone are called sarcomas and malignant tumors affecting hematopoietic structures (structures pertaining to the formation of blood cells) including components of the immune system, are called leukemias and lymphomas. A tumor is the neoplastic growth of the disease cancer. As used herein, a "neoplasm," also referred to as a "tumor," is intended to encompass hematopoietic neoplasms as well as solid neoplasms.

[0043] *Normal cells*: Cells not manifesting proliferative disorders or a transformed phenotype.

[0044] *Normal mammal*: An animal not manifesting a cell proliferative disorder.

[0045] *Normal Ras*: Ras having no mutations associated with cell transformation.

[0046] *Phospho-MAPK*: Phosphorylated-MAP kinase.

[0047] *Phospho-MEK*: Phosphorylated MEK..

[0048] *Proliferating cell*: A cell that is proliferating more rapidly than normal cells.

[0049] *Proliferative disorder*: Any cellular disorder in which the cells proliferate more rapidly than normal tissue growth. Proliferative disorders include but are not limited to neoplasms. Other proliferative disorders include, but are not limited to neurofibromatosis.

[0050] *Pulse labeling cells*: The addition of a labeling agent, typically a radioactive isotope-containing precursor molecule for cell macromolecule (*e.g.*, protein or nucleic acid), to growing cells, causing the cells to incorporate label into macromolecules that are synthesized during the labeling period. Common labeling agents include but are not limited to [³⁵S]-methionine, [³⁵S]-cysteine and ∇ or ([³²P]-nucleotides and ribonucleotides.

[0051] Biological specimens that have been pulse-labeled may be referred to as "labeled" while corresponding or equivalent biological specimens that have not been pulse-labeled may be referred to as "unlabeled."

[0052] *Ras activation*: Activation of Ras, including but not limited to activation by upstream elements in the Ras pathway.

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[0053] *Ras pathway:* In mammals, the Ras signal transduction pathway involves (in order of activation) tyrosine receptor kinases such as the EGF and PDGF receptors, an adaptor protein such as Grb2, Sos, and Ras (a monomeric guanine nucleotide-binding protein). GTP-bound Ras may then activate downstream Ras effector pathways, including but not limited to the MAP kinase pathway.

[0054] *Reovirus or reoviruses:* Reoviruses are viruses with a double-stranded, segmented RNA genome. The virions measure 60-80 nm in diameter and possess two concentric capsid shells, each of which is icosahedral. The genome consists of double-stranded RNA in 10-12 discrete segments with a total genome size of 16-27 kbp. The individual RNA segments vary in size. Three distinct but related types of reovirus have been recovered from many species. All three types share a common complement-fixing antigen.

[0055] The life cycle and biology of reoviruses make them ideally suited for practicing the embodiments of the invention. The name reovirus (Respiratory and enteric orphan virus) is a descriptive acronym suggesting that these viruses, although not associated with any known disease state in humans, can be isolated from both the respiratory and enteric tracts (Sabin (1959)). The term "reovirus" refers to all viruses classified in the reovirus genus.

[0056] The human reovirus consists of three serotypes: type 1 (strain Lang or T1L), type 2 (strain Jones, T2J) and type 3 (strain Dearing or strain Abney, T3D). The three serotypes are easily identifiable on the basis of neutralization and hemagglutinin-inhibition assays (Sabin (1959); Fields *et al.* (1996); Rosen (1960); Stanley (1967)).

[0057] Although reovirus is not known to be associated with any particular disease, many people have been exposed to reovirus by the time they reach adulthood (*i.e.*, fewer than 25% in children <5 years old, to greater than 50% in those 20-30 years old (Jackson and Muldoon (1973); Stanley (1974))).

[0058] *Resistance to reovirus infection:* Those cells that do not demonstrate induction of cytopathic effects, viral protein synthesis, and/or virus production following exposure to reovirus.

[0059] *Substantial lysis:* Lysis of at least 10%, more preferably at least 30%, and most preferably at least 60%, of proliferating cells. The percentage of lysis can be determined for tumor cells by measuring the reduction in the size of the tumor in the mammal or the lysis of the tumor cells *in vitro*.

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[0060] *Susceptible to reovirus infection:* Those cells that demonstrate induction of cytopathic effects, viral protein synthesis, and/or virus production following exposure to reovirus.

[0061] *Total MEK or Total level of MEK:* The amount or level of phosphorylated MEK in addition to non-phosphorylated MEK in biological specimen.

[0062] *Unit dosage forms:* Physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a predetermined quantity of reovirus calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical excipient.

B. Detailed Description of The Invention

[0063] As described herein, Applicants have discovered that cells which exhibit constitutive MAP kinase pathway phosphorylation, independent of the presence of Ras mutation or the presence of elevated levels of GTP-bound Ras, are susceptible to reovirus infection. Resistance of cells to reovirus infection indicates that infection of the cells with the virus does not result in significant viral production or yield. Resistance to reovirus infection was found to be at the level of gene translation, rather than at early transcription: while viral transcripts were produced, virus proteins were not expressed.

[0064] In the treatment methods of the invention, reovirus is administered to proliferating cells in the individual mammal exhibiting constitutive MEK phosphorylation. In one embodiment of this invention a course of reovirus therapy is administered one or more times.

[0065] In the methods of the invention, reovirus is administered to proliferating cells in the individual mammal exhibiting constitutive MAP kinase phosphorylation. Representative types of human reovirus that can be used include type 1 (*e.g.*, strain Lang or T1L); type 2 (*e.g.*, strain Jones or T2J); and type 3 (*e.g.*, strain Dearing or strain Abney, T3D or T3A); other strains of reovirus can also be used. In a preferred embodiment, the reovirus is human reovirus serotype 3, more preferably the reovirus is human reovirus serotype 3, strain Dearing. Alternatively, the reovirus can be a non-human mammalian reovirus (*e.g.*, non-human primate reovirus, such as baboon reovirus; equine; or canine reovirus), or a non-mammalian reovirus (*e.g.*, avian reovirus).

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A combination of different serotypes and/or different strains of reovirus, such as reovirus from different species of animal, can be used. The reovirus may be naturally occurring or modified.

[0066] The reovirus may be modified but still capable of lytically infecting a mammalian cell exhibiting constitutive MAP kinase pathway phosphorylation. The reovirus may be chemically or biochemically pretreated (*e.g.*, by treatment with a protease, such as chymotrypsin or trypsin) prior to administration to the proliferating cells. Pretreatment with a protease removes the outer coat or capsid of the virus and may increase the infectivity of the virus. The reovirus may be coated in a liposome or micelle (Chandran and Nibert (1998)) to reduce or prevent an immune response from a mammal which has developed immunity to the reovirus. For example, the virion may be treated with chymotrypsin in the presence of micelle forming concentrations of alkyl sulfate detergents to generate a new infectious subviral particle (ISVP). An ISVP may be used either alone or in combination with whole virus to provide an agent that is either poorly recognized or has not been previously prevented by the patient's immune system.

[0067] The reovirus may be a recombinant reovirus resulting from the recombination/reassortment of genomic segments from two or more genetically distinct reoviruses. Recombination/reassortment of reovirus genomic segments may occur in nature following infection of a host organism with at least two genetically distinct reoviruses. Recombinant virions can also be generated in cell culture, for example, by co-infection of permissive host cells with genetically distinct reoviruses (Nibert *et al.* (1995)).

[0068] Accordingly, the invention contemplates the use of recombinant reovirus resulting from reassortment of genome segments from two or more genetically distinct reoviruses, including but not limited to, human reovirus, such as type 1 (*e.g.*, strain Lang), type 2 (*e.g.*, strain Jones), and type 3 (*e.g.*, strain Dearing or strain Abney), non-human mammalian reoviruses, or avian reovirus. The invention further contemplates the use of recombinant reoviruses resulting from reassortment of genome segments from two or more genetically distinct reoviruses wherein at least one parental virus is genetically engineered, comprises one or more chemically synthesized genomic segment, has been treated with chemical or physical mutagens, or is itself the result of a recombination event. The invention further contemplates the use of recombinant reovirus that have undergone recombination in the presence of chemical mutagens, including but not limited to dimethyl sulfate and ethidium bromide, or physical mutagens, including but not limited to ultraviolet light and other forms of radiation.

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[0069] The invention further contemplates the use of recombinant viruses that comprise deletions or duplications in one or more genome segments, that comprise additional genetic information as a result of recombination with a host cell genome, or that comprise synthetic genes.

[0070] The reovirus may be modified by incorporation of mutated coat proteins, such as for example, into the virion outer capsid. The proteins may be mutated by replacement, insertion or deletion. Replacement includes the insertion of different amino acids in place of the native amino acids. Insertions include the insertion of additional amino acid residues into the protein at one or more locations. Deletions include deletions of one or more amino acid residues in the protein. Such mutations may be generated by methods known in the art. For example, oligonucleotide site directed mutagenesis of the gene encoding for one of the coat proteins could result in the generation of the desired mutant coat protein. Expression of the mutated protein in reovirus infected mammalian cells *in vitro* such as COS1 cells will result in the incorporation of the mutated protein into the reovirus virion particle (Turner *et al.* (1992); Duncan *et al.* (1991); Mah *et al.* (1990)).

[0071] The reovirus is preferably a reovirus modified to reduce or eliminate an immune reaction to the reovirus. Such a modified reovirus is termed an "immunoprotected reovirus". The modifications could include packaging of the reovirus in a liposome, a micelle or other vehicle to mask the reovirus from the host immune system. Alternatively, the outer capsid of the reovirus virion particle may be removed since the proteins present in the outer capsid are the major determinant of the host humoral and cellular responses. In addition to reducing or eliminating immune responses, the modifications may also reduce non-specific uptake of the virus in normal tissues. As discussed above, reovirus is capable of binding to a multitude of cell types, presumably due to the ubiquitous nature of its receptor. Therefore, by masking the reovirus, non-specific binding and uptake can be reduced.

[0072] It has previously been shown that reovirus could be used as an effective oncolytic agent in a mouse model (WO 99/08692). It has additionally been shown that cellular susceptibility to reovirus infection can be determined by measuring constitutive MAP signaling of the cell, wherein the presence of elevated levels of phosphorylated MAP kinase indicates activation of Ras, for example by one or more mutations in the Ras gene or by activation of upstream elements in the Ras pathway (U.S. Patent Application Serial No. 09/985,756).

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[0073] Evidence is presented herein to show that reovirus is useful as an oncolytic agent against various colon and ovarian tumors and that the susceptibility of these cells to reovirus infection is not a function of activation or overexpression of regulatory elements in the Ras pathway, or the existence of activating mutations in Ras itself.

[0074] The invention is based, *inter alia*, on the observation that reovirus infected different human colon and ovarian cancer cells independent of whether the cells harbored mutated Ras or whether Ras was activated in the cells. For example, Caco-2, DLD-1, HCT-116, HT-29, and SW-48 are all human colon cancer cell lines. While DLD-1 and HCT-116 are known to harbor activating mutations of K-ras (Shirasawa *et al.* (1993)), Caco-2 and HT-29 harbor normal Ras (Lorentz *et al.* (1999)). Reovirus was able to infect all cell lines, independent of the presence of activating Ras mutations. Moreover, the ability of reovirus to infect the different cells did not correspond with the levels of GTP-bound Ras in the cells, indicating that reovirus susceptibility can result from activation or overexpression of downstream elements in the Ras pathway, in addition to Ras or upstream elements of the Ras pathway.

[0075] In all cases, the cells that were susceptible to reovirus infection demonstrated elevated constitutive levels of phosphorylated MEK (*i.e.*, MAP/ERK kinase), based on immunoblot analysis using a phospho-MEK-specific antibody designated MEK1/2. A constitutive level of phospho-MEK is that which is present in the absence of a mitogen. These results suggest that an assay for detecting the relative levels of phospho-MEK, compared with non-phosphorylated MEK, can be useful in identifying cells that are susceptible to reovirus infection.

[0076] One embodiment of the invention is the identification of reovirus susceptible cells using a phospho-MEK-specific antibody, for example, in the phospho-MEK immunoblot assay described above and in the following Examples. This assay is useful for screening cells comprised in a biological specimen, for example, cultured immortalized cells lines, cultured primary tumor cells, tumor biopsy specimens, and post-mortem tumor specimens for susceptibility to reovirus. In one particular embodiment of the invention, tumor biopsy specimens or cultured primary tumor cells can be screened for reovirus susceptibility to determine whether reovirus administration could be used to treat the particular tumors of an animal.

[0077] In another embodiment of the invention, the phospho-MEK immunoblot assay could be used to determine whether non-tumor cells of the same animal were resistant to reovirus

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infection, thereby determining whether reovirus could be administered to the animal without concern about infecting non-tumor cells. In a preferred embodiment of the invention, tumor cells and cells adjacent to the tumor could be screened for reovirus susceptibility, to confirm that the tumor cells would be specifically infected upon the administration of reovirus.

[0078] In one embodiment of the invention, phospho-MEK-specific antibodies are used to determine the level of phospho-MEK in a specimen and MEK-specific antibodies are used to determine the total amount of MEK in a specimen. This embodiment is useful, for example, for determining the relative amount of phospho-MEK, compared to the total amount of MEK, in a specimen. Multiple specimens may be analyzed in this manner wherein relative levels of phospho-MEK in different specimens are normalized for total MEK, allowing for meaningful comparison of phospho-MEK levels among different specimens.

[0079] For example, using a MEK-specific antibody that recognizes phosphorylated and unphosphorylated MEK, the total amount of MEK in a biological specimen suspected of comprising cells with a proliferative disorder and in a biological specimen comprising normal cells is determined by standard antibody-based assays. The amounts of phospho-MEK in the same or equivalent biological specimens are determined using a phospho-MEK-specific antibody. The relative amounts of phosph-MEK, normalized for the total amount of MEK, are then compared in the biological specimen suspected of comprising cells with a proliferative disorder and in the biological specimen comprising normal cells; wherein an increased relative amount of phospho-MEK in the biological specimen suspected of comprising cells with a proliferative disorder is indicative of a cell proliferative disorder and susceptibility to reovirus infection.

[0080] In another embodiment of the invention, the relative amount of phospho-MEK in a specimen, in the absence of mitogen, is determined using only a MEK-specific antibody. This embodiment of the invention is particularly useful when the biological specimens comprise cultured cells or biological specimen from animals into which radionuclides may be administered. As above, the total amount of MEK in each biological specimen is determined using a MEK-specific antibody. However, in this embodiment, the amounts of phospho-MEK in each biological are determined by pulse labeling the specimens, for example, with ^{32}P or ^{33}P , then quantitating the amount of labeled MEK. As above, the relative amounts of phospho-MEK in each biological specimen, normalized for the total amount of MEK in the respective biological

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specimens, indicate the levels of constitutive MEK phosphorylation in each specimen. An increased proportion of phospho-MEK in the biological specimen suspected of comprising cells with a proliferative disorder indicates susceptibility to reovirus infection.

[0081] For example, using a MEK-specific antibody that recognizes phosphorylated and unphosphorylated forms of MEK, the total amount of MEK in a biological specimen suspected of comprising cells with a proliferative disorder and in a biological specimen comprising normal cells is determined by standard antibody-based assays. The amounts of phospho-MEK in the same biological specimens are determined using a MEK-specific antibody in equivalent biological specimens that have been pulse labeled with a suitable radionuclide or other labeling agent. The relative amounts of phosph-MEK, normalized for the total amount of MEK, are then compared in the biological specimen suspected of comprising cells with a proliferative disorder and in the biological specimen comprising normal cells; wherein an increased relative amount of phospho-MEK in the biological specimen suspected of comprising cells with a proliferative disorder is indicative of a cell proliferative disorder and susceptibility to reovirus infection.

[0082] The MEK-specific and phospho-MEK-specific antibodies described herein can be used in combination with virtually any antibody-based assay known in the art. These assays include but are not limited to, immunoblot analysis, immunoprecipitation, radioimmunoassay, and 2D-electrophoresis.

[0083] The results described above and in the Examples further suggest that elevated levels of phospho-MEK are more consistently associated with tumor cells than elevated levels of GTP-bound. Accordingly, a phospho-MEK-specific biological assay is likely to be more reliable in identifying tumor cells (*i.e.*, cells with proliferative disorders) than assays based on the level of GTP-bound Ras in a cell or biological specimen. Accordingly, in another embodiment of the invention, the phospho-MEK-specific and MEK-specific antibodies and assays described above and in the Examples, below, are useful for identifying tumor cells, present, for example, in immortalized cells lines, cultured primary tumor cells, tumor biopsy specimens, and post-mortem tumor specimens. In a preferred embodiment of the invention, the assays described herein can be used to determine both (i) whether biological specimens comprise cells having elevated levels of phospho-MEK, indicating the presence of tumorigenic cells in the biological specimens, and (ii) whether the cells are susceptible to reovirus infection.

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[0084] The phospho-MEK-specific or MEK-specific antibodies of the invention, may be conjugated with a tag to facilitate the detection of the antibody. Antibody tags are well known in the art and include but are not limited to fluorescent tags (*e.g.*, green fluorescent protein or fluorescein isothiocyanate), radioactive tags (*e.g.*, [³⁵S] or [¹²⁵I]), and enzyme tags (*e.g.*, horseradish peroxidase or alkaline phosphatase). The presence of a tag facilitates visualization and/or quantitation of the antibody and/or decreases the number of steps required to visualize the antibody, thereby simplifying the practice of the instant invention.

[0085] The instant invention is useful, for example, for detecting a variety of proliferative disorders, in which a subpopulation of cells proliferate more rapidly than normal cells or normal tissues. Cell proliferative disorders include neoplasms. Other proliferative disorders include, but are not limited to neurofibromatosis.

[0086] Upon determining that a population of cells has a proliferative disorder associated with constitutive phosphorylation of MEK, reovirus is then administered in a manner so that it contacts the proliferating cells or cells of the neoplasm (also referred to herein as "neoplastic cells"). The route by which the reovirus is administered, as well as the formulation, carrier or vehicle, will depend on the location as well as the type of the neoplasm. A wide variety of administration routes can be employed. For example, for a solid neoplasm that is accessible, the reovirus can be administered by injection directly to the neoplasm. For a hematopoietic neoplasm, for example, the reovirus can be administered intravenously or intravascularly. For neoplasms that are not easily accessible within the body, such as metastases or brain tumors, the reovirus is administered in a manner such that it can be transported systemically through the body of the mammal and thereby reach the neoplasm (*e.g.*, intrathecally, intravenously or intramuscularly). Alternatively, the reovirus can be administered directly to a solid neoplasm, where it then is carried systemically through the body to metastases. The reovirus can also be administered subcutaneously, intraperitoneally, topically (*e.g.*, for melanoma), orally (*e.g.*, for oral or esophageal neoplasm), rectally (*e.g.*, for colorectal neoplasm), vaginally (*e.g.*, for cervical or vaginal neoplasm), nasally or by inhalation spray (*e.g.*, for lung neoplasm).

[0087] Reovirus can be administered systemically to mammals which are immune compromised or which have not developed immunity to the reovirus epitopes. In such cases, reovirus administered systemically, *i.e.*, by intravenous injection, will contact the proliferating

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cells resulting in lysis of the cells. Where the mammals to be treated have higher titers of anti-reovirus antibodies, more reovirus must be administered to be effective.

[0088] Immunocompetent mammals previously exposed to a reovirus subtype may have developed humoral and/or cellular immunity to that reovirus subtype. Nevertheless, it has been found that direct injection of the reovirus into a solid tumor in immunocompetent mammals will result in the lysis of the neoplastic cells. On the other hand, when the reovirus is administered systemically to immunocompetent mammals, the mammals may produce an immune response to the reovirus. Such an immune response may be avoided if the reovirus is of a subtype to which the mammal has not developed immunity, or the reovirus has been modified as previously described herein such that it is immunoprotected, for example, by protease digestion of the outer capsid or packaging in a micelle.

[0089] Alternatively, it is contemplated that the immunocompetency of the mammal against the reovirus may be suppressed either by the prior or co-administration of pharmaceuticals known in the art to suppress the immune system in general (Cuff *et al.* (1998)) or alternatively the administration of such immunoinhibitors as anti-antireovirus antibodies. The humoral immunity of the mammal against reovirus may also be temporarily reduced or suppressed by plasmaphoresis of the mammal's blood to remove the anti-reovirus antibodies. The humoral immunity of the mammal against reovirus may additionally be temporarily reduced or suppressed by the intravenous administration of non-specific immunoglobulin to the mammal.

[0090] It is contemplated that the reovirus may be administered to immunocompetent mammals immunized against the reovirus in conjunction with the administration of immunosuppressants and/or immunoinhibitors. Such immunosuppressants and immunoinhibitors are known to those of skill in the art and include such agents as cyclosporin, rapamycin, tacrolimus, mycophenolic acid, azathioprine and their analogs, and the like. Other agents are known to have immunosuppressant properties as well (see, *e.g.*, Gilman *et al.* at 1242, the disclosure of which is incorporated herein by reference). Such immunoinhibitors also include anti-antireovirus antibodies. Such antibodies can be made by methods known in the art (see, *e.g.*, Harlow and Lane (1988)). Such anti-antireovirus antibodies may be administered prior to, at the same time or shortly after the administration of the reovirus. Preferably an effective amount of the anti-antireovirus antibodies are administered in sufficient time to reduce or eliminate an immune response by the mammal to the administered reovirus.

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[0091] This invention also includes pharmaceutical compositions containing, as the active ingredient, one or more immunosuppressants or immunoinhibitors and one or more of the reoviruses associated with pharmaceutically acceptable carriers or excipients. In making the compositions of this invention, the active ingredients/ immunosuppressant or immunoinhibitor and reovirus are usually mixed with an excipient, diluted by an excipient or enclosed within such a carrier which can be in the form of a capsule, sachet, paper or other container. When the pharmaceutically acceptable excipient serves as a diluent, it can be a solid, semi-solid, or liquid material, which acts as a vehicle, carrier or medium for the active ingredient. Thus, the compositions can be in the form of tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups, aerosols (as a solid or in a liquid medium), ointments containing, for example, up to 10% by weight of the active compound, soft and hard gelatin capsules, suppositories, sterile injectable solutions, and sterile packaged powders.

[0092] Some examples of suitable excipients include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, tragacanth, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, sterile water, syrup, and methyl cellulose. The formulations can additionally include: lubricating agents such as talc, magnesium stearate, and mineral oil; wetting agents; emulsifying and suspending agents; preserving agents such as methyl- and propylhydroxy-benzoates; sweetening agents; and flavoring agents. The compositions of the invention can be formulated so as to provide quick, sustained or delayed release of the active ingredient after administration to the patient by employing procedures known in the art.

[0093] For preparing solid compositions such as tablets, the principal active ingredients/ immunosuppressant or immunoinhibitor and reovirus are mixed with a pharmaceutical excipient to form a solid preformulation composition containing a homogeneous mixture of a compound of the present invention. When referring to these preformulation compositions as homogeneous, it is meant that the active ingredient is dispersed evenly throughout the composition so that the composition may be readily subdivided into equally effective unit dosage forms such as tablets, pills and capsules.

[0094] The tablets or pills of the present invention may be coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action. For example, the tablet or pill can comprise an inner dosage and an outer dosage component, the latter being in the form of

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an envelope over the former. The two components can be separated by an enteric layer which serves to resist disintegration in the stomach and permit the inner component to pass intact into the duodenum or to be delayed in release. A variety of materials can be used for such enteric layers or coatings, such materials including a number of polymeric acids and mixtures of polymeric acids with such materials as shellac, cetyl alcohol, and cellulose acetate.

[0095] The liquid forms in which the novel compositions of the present invention may be incorporated for administration orally or by injection include aqueous solutions, suitably flavored syrups, aqueous or oil suspensions, and flavored emulsions with edible oils such as corn oil, cottonseed oil, sesame oil, coconut oil, or peanut oil, as well as elixirs and similar pharmaceutical vehicles.

[0096] Compositions for inhalation or insufflation include solutions and suspensions in pharmaceutically acceptable, aqueous or organic solvents, or mixtures thereof, and powders. The liquid or solid compositions may contain suitable pharmaceutically acceptable excipients as described herein. Preferably the compositions are administered by the oral or nasal respiratory route for local or systemic effect. Compositions in preferably pharmaceutically acceptable solvents may be nebulized by use of inert gases. Nebulized solutions may be inhaled directly from the nebulizing device or the nebulizing device may be attached to a face mask tent, or intermittent positive pressure breathing machine. Solution, suspension, or powder compositions may be administered, preferably orally or nasally, from devices which deliver the formulation in an appropriate manner.

[0097] Another preferred formulation employed in the methods of the present invention employs transdermal delivery devices ("patches"). Such transdermal patches may be used to provide continuous or discontinuous infusion of the reovirus of the present invention in controlled amounts. The construction and use of transdermal patches for the delivery of pharmaceutical agents is well known in the art. See, for example, U.S. Patent 5,023,252, herein incorporated by reference. Such patches may be constructed for continuous, pulsatile, or on demand delivery of pharmaceutical agents.

[0098] Other suitable formulations for use in the present invention can be found in *Remington's Pharmaceutical Sciences*, the disclosure of which is incorporated herein by reference.

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[0099] The immunosuppressant or immunoinhibitor and reovirus or the pharmaceutical composition comprising the immunosuppressant or immunoinhibitor and reovirus may be packaged into convenient kits providing the necessary materials packaged into suitable containers. Such kits may comprise, for example, one or more types or strains of reovirus suspended or lyophilized in a suitable pharmaceutical excipient, any necessary sterile or sterilizable solutions and/or reagents for resuspending or otherwise preparing the reovirus for administration to an animal, syringes or alternative delivery devices for administering the reovirus, and instructions for the use of the kit. It is contemplated that the kits may also include, for example, chemotherapeutic agents, antibodies, immunosuppressant agents, and any additional pharmacological agents disclosed herein.

[00100] The immunosuppressant or immunoinhibitor is administered in an appropriate amount and using an appropriate schedule of administration sufficient to result in immunosuppression or immunoinhibition of the mammal's immune system. Such amounts and schedules are well known to those of skill in the art.

[00101] The reovirus is administered in an amount that is sufficient to treat the proliferative disorder (*e.g.*, an "effective amount"). A proliferative disorder is "treated" when administration of reovirus to the proliferating cells effects lysis of the proliferating cells. This may result in a reduction in size of the neoplasm, or in a complete elimination of the neoplasm. The reduction in size of the neoplasm, or elimination of the neoplasm, is generally caused by lysis of neoplastic cells ("oncolysis") by the reovirus. Preferably the effective amount is that amount able to inhibit tumor cell growth. Preferably the effective amount is from about 1.0 pfu/kg body weight to about 10^{15} pfu/kg body weight, more preferably from about 10^2 pfu/kg body weight to about 10^{13} pfu/kg body weight. For example, for treatment of a human, approximately 10^2 to 10^{17} plaque forming units (PFU) of reovirus can be used, depending on the type, size and number of tumors present. The effective amount will be determined on an individual basis and may be based, at least in part, on consideration of the type of reovirus; the chosen route of administration; the individual's size, age, gender; the severity of the patient's symptoms; the size and other characteristics of the neoplasm; and the like. The course of therapy may last from several days to several months or until diminution of the disease is achieved.

[00102] The immunosuppressant or immunoinhibitor and reovirus can be administered in a single dose, or multiple doses (*i.e.*, more than one dose). The multiple doses can be administered

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concurrently, or consecutively (*e.g.*, over a period of days or weeks). The reovirus can also be administered to more than one neoplasm in the same individual.

[00103] The compositions are preferably formulated in a unit dosage form, each dosage containing an appropriate amount of immunosuppressant or immunoinhibitor and from about 10^2 pfus to about 10^{13} pfus of the reovirus.

[00104] As mentioned above, it has been found that the reovirus is effective for the treatment of solid neoplasms in immunocompetent mammals. Administration of unmodified reovirus directly to the neoplasm results in oncolysis of the neoplastic cells and reduction in the size of the tumor in immunocompetent animals. When animals are rendered immunosuppressed or immunodeficient in some way, systemic administration of reovirus will be more effective in producing oncolysis.

[00105] It is contemplated that the reovirus may be administered in conjunction with surgery or removal of the neoplasm. Therefore, provided herewith are methods for the treatment of a solid neoplasm comprising surgical removal of the neoplasm and administration of a reovirus at or near to the site of the neoplasm.

[00106] It is contemplated that the reovirus may be administered in conjunction with or in addition to radiation therapy which renders the mammal immunosuppressed.

[00107] It is further contemplated that the reovirus of the present invention may be administered in conjunction with or in addition to known anticancer compounds or chemotherapeutic agents. Chemotherapeutic agents are compounds which may inhibit the growth of tumors. Such agents, include, but are not limited to, 5-fluorouracil, mitomycin C, methotrexate, hydroxyurea, cyclophosphamide, dacarbazine, mitoxantrone, anthracyclins (Epirubicin and Doxorubicin), antibodies to receptors, such as herceptin, etoposide, pregnasome, platinum compounds such as carboplatin and cisplatin, taxanes such as taxol and taxotere, hormone therapies such as tamoxifen and anti-estrogens, interferons, aromatase inhibitors, progestational agents and LHRH analogs.

[00108] The reovirus and immunosuppressants of the present invention have been found to reduce the growth of tumors that are metastatic. In an embodiment of the invention, a method is provided for reducing the growth of metastatic tumors in a mammal comprising administering an effective amount of a reovirus to the immunosuppressed mammal.

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[00109] The practitioner will recognize that other viruses that selectively lyse Ras-activated cells may also be used to practice the invention. For example, the viruses described in U.S. Patent Application Publication No. 20010048919 may be used to practice the invention.

[00110] The practitioner will understand that for the purposes of the instant disclosure, "ras" or "Ras" refer to the *Ras* gene or the Ras polypeptide as appropriate in context (as is the case with other gene/protein names, including but not limited to, Ral, Raf, MEK, and ERK). These terms are used for convenience and no limitations of the scope of the invention should be construed based on specific instances of capitalization or italics. Moreover, the practitioner will understand that "MAP kinase," MAPK," and "MEK" refer to MAP kinase and "Ras-MAP pathway," Ras-MAP kinase pathway," "Ras-MAPK pathway," "MAP pathway," "MAP kinase pathway," and "MAPK pathway" all refer to the same biochemical pathway unless otherwise dictated by context.

Utility

[00111] The diagnostic methods of the present invention may be used to identify the susceptibility of cells to reovirus infection by measuring constitutive MEK phosphorylation. This will be useful to determine in what cases reovirus treatment of cellular proliferative disorders is likely to be effective.

[00112] The reoviruses and immunosuppressants of the present invention may be used for a variety of purposes. They may be used in methods for treating proliferative disorders which exhibit constitutive MEK phosphorylation in a mammal. They may be used to reduce or eliminate neoplasms. They may be used in methods for treating metastases. They may be used in conjunction with known treatments for cancer including surgery, chemotherapy and radiation.

[00113] In order to further illustrate the present invention and advantages thereof, the following specific examples are given but are not meant to limit the scope of the claims in any way.

EXAMPLES

[00114] In the examples below, all temperatures are in degrees Celsius (unless otherwise indicated) and all percentages are weight percentages (also unless otherwise indicated).

[00115] In the examples below, the following abbreviations have the following meanings. If an abbreviation is not defined, it has its generally accepted meaning:

μ M	=	micromolar
mM	=	millimolar
M	=	molar
ml	=	milliliter
μ l	=	microliter
mg	=	milligram
μ g	=	microgram
PAGE	=	polyacrylamide gel electrophoresis
FBS	=	fetal bovine serum
DTT	=	dithiothriitol
SDS	=	sodium dodecyl sulfate
PBS	=	phosphate buffered saline
DMEM	=	Dulbecco's modified Eagle's medium
MEM	=	modified Eagle's medium
2-ME	=	2-mercaptoethanol
MOI or moi	=	multiplicity of infection
PFU or pfu	=	plaque forming unit(s)
MAPK	=	MAP (mitogen-activated protein) kinase or ERK
phospho-MAPK	=	phosphorylated-MAP kinase
MEK	=	MAP/ERK kinase, formerly called MAP kinase kinase
phospho-MEK	=	phosphorylated MEK
ERK	=	extracellular signal related kinase or MAP kinase
HRP	=	horseradish-peroxidase
EGFR	=	epidermal growth factor receptor

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MEK kinase	=	mitogen-activated extracellular signal-regulated kinase
SCID	=	severe combined immune deficiency
i.v.	=	intravenous
i.p.	=	intraperitoneal
i.m.	=	intramuscular
s.c.	=	subcutaneous

EXAMPLE 1. Use of reovirus as an oncolytic agent in cells harboring K-ras or a normal Ras proto oncogene

[00116] Normal human colon fibroblasts (CCD-18Co), human colon cancer cells (Caco-2, DLD-1, HCT-116, HT-29, and SW-48), normal human ovarian fibroblasts (NOV-31), and human ovarian cancer cells (MDAH 2774, PA-1, SKOV3, and SW626) were obtained from the ATCC (Rockville, MD) and maintained according to ATCC protocols. Cells were infected at an MOI of 20 pfu/cell in six-well plates. Reovirus infections and assays for reovirus replication were performed essentially as described in U.S. Patent No. 6,136,307, incorporated by reference in its entirety.

[00117] Morphological changes following infection were nominal in normal colon and ovarian cells (*i.e.*, CCD-18Co and NOV-31) while >95% of the cells of each tumor cell line were destroyed by reovirus infection. Pulse labeling of infected cells with [³⁵S]-methionine, followed by analysis by SDS-PAGE of the labeled proteins, demonstrated minimal reovirus protein synthesis in normal cells and significant levels of reovirus protein synthesis in cancers cells, providing additional evidence that cell death was the result of reovirus replication.

[00118] HCT-116 and DLD-1 are known to possess activating mutations of K-ras while Caco-2 and HT-29 possess a normal Ras proto oncogene. The status of Ras in the ovarian cancer cell lines was unknown although Ras mutations occur in only about 20-47% of ovarian cancers Sherman (2000); Dokianakis *et al.* (1999)). Thus the ability of reovirus to infect all five colon and all four ovarian cancer cell lines tested in this study suggests that an activating Ras mutation is not essential for reovirus infectivity.

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EXAMPLE 2. Use of a phospho-MEK-specific antibody to determine the phosphorylation status of MEK in cells harboring K-ras or a normal Ras proto-oncogene

[00119] The cells of Example 1 were assayed for relative levels of GTP-bound Ras compared to normal Ras, and phosphorylated MEK compared to nonphosphorylated MEK. The amount of GTP-bound Ras was determined by incubating cell lysates from each of the cell lines with the Raf-1 Ras-binding domain conjugated to agarose beads. The beads and associated polypeptides were collected by centrifugation, washed, resuspended in Laemmli sample buffer, and heated. The resulting samples were resolved by SDS-PAGE and subjected to immunoblot analysis using Ras-specific antibodies. The total amount of Ras was determined by subjecting cell lysates to immunoblot analysis without first using Raf-1 Ras-binding domain-conjugated beads to select for GTP-bound Ras.

[00120] Relative amounts of phosphorylated MEK were determined by performing immunoblot analysis on cell lysates using either a MEK-specific antibody or a phospho-MEK-specific antibody and determining the relative amounts of phosphorylated MEK in each specimen.

[00121] The results of immunoblot analysis are shown in Figure 1. The relative levels of GTP-bound-Ras and phospho-MEK are significantly higher in all tumor cell lines compared to normal cell lines. However, the relative levels of phospho-MEK do not in all cases correspond to the relative levels of GTP-bound Ras in the same cell lysates. For example, HCT-116 and SW626 cells show high levels of GTP-bound-Ras but intermediate levels of phospho-MEK. Conversely, DLD-1 and MDAH 2774 cells show intermediate levels of GTP-bound-Ras but high level of phospho-MEK. These results suggest that phosphorylation of MEK is not solely a function of Ras activation and that overexpression and/or activation of other elements may also affect MEK phosphorylation and susceptibility to reovirus infection.

EXAMPLE 3. Use of a MEK-specific antibody to determine the relative amount of phospho-MEK in cells

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[00122] MEK phosphorylation is also determined using a MEK-specific antibody that does not recognize the phosphorylation status of MEK. Total MEK is determined by immunoblot analysis, immunoprecipitation, radioimmunoassay (RIA), 2D-electrophoreses, or any other antibody-based detection method known in the art. Total MEK is determined, for example, in cell lysates from normal cells and in cell lysates from cells with a suspected proliferative disorder.

[00123] The relative amount of phospho-MEK is then determined by pulse labeling cells, for example, with a [^{32}P]- or [^{33}P]-containing molecule, prior to determining total MEK using a MEK-specific antibody. The relative amount of label incorporated into MEK is then compared in normal cells and suspected tumorigenic cells and normalized for the total amount of MEK in the respective specimens. These assays are performed in the absence of mitogens, thus an increased proportion of phospho-MEK in specimens comprising cells with suspected proliferative disorders, compared to the relative amount of phospho-MEK in specimens comprising normal cells, indicates constitutive MEK phosphorylation.

[00124] Constitutive MEK phosphorylation is indicative of a cell proliferative disorder as well as susceptibility to reovirus infection. In this manner, a practitioner can determine whether a certain population of cells has a proliferative disorder and whether such cells can be destroyed by reovirus infection.

EXAMPLE 4. Use of reovirus as an oncolytic agent in tumors resulting from colon or ovarian cancer xenografts.

[00125] Colon tumor xenografts were established by implanting DLD-1, HCT-116, or HT-29 cells subcutaneously in 6-8 week-old female SCID/NOD mice ($n = 6-7$) obtained from the Cross Cancer Institute (Edmonton, Alberta, Canada). Established tumors of $20-25 \text{ mm}^2$ were injected with about 10^7 pfu live reoviruses or UV-inactivated reovirus and tumor size was monitored for about 4 weeks.

[00126] Ovarian tumor xenografts were established in a manner similar to the above by implanting SKOV3 cells in the hind flanks of 6-8 week-old female CD-1 nude mice ($n = 6-7$) obtained from the Charles River Laboratory (Montreal, Quebec, Canada). Established tumors of $20-25 \text{ mm}^2$ were injected with about 5×10^8 pfu live reoviruses or UV-inactivated reovirus. The

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injection was repeated two weeks later. Tumor size was monitored for up to about 7 weeks following the first intratumoral injection.

[00127] Figure 2 shows the change in tumor size over the course of the experiment. In all cases, tumor size in animals receiving intratumoral live reovirus injections (open circles) was significantly reduced compared to tumor size in animals receiving intratumoral UV-inactivated reovirus injections (closed circles). The maximum reduction in tumor sizes at the termination of the experiment were about 10-fold, 4-fold, 6-fold, and 15-fold in the cases of HCT-116 (Fig. 2A), HT-29 (Fig. 2B), DLD-1 (Fig. 2C), and SKOV3 (Fig. 2D) cells, respectively.

[00128] These data established that intratumoral injection of reovirus reduces the size of tumors derived from cells harboring K-ras (DLD-1 and HCT-116 cells) or normal Ras (HT-29 cells), further suggesting that reovirus susceptibility, and the efficacy of reovirus administration in reducing tumor size, is not dependent upon the presence of Ras mutations.

EXAMPLE 5. Use of reovirus as an oncolytic agent in ascites ovarian cancer model.

Description of the model, animals, and inoculations:

[00129] Data obtained from the experiment described in Example 2 indicate that MDAH 2774 cells possess high levels of phospho-MEK but only intermediate levels of GTP-bound Ras (Figure 1), suggesting that the high levels of phospho-MEK in these cells results from upregulation or overexpression of a Ras pathway regulatory element other than, or in addition to, Ras itself. The introduction of these cells into the peritoneal cavity of mice, and the subsequent spread of the cells to distant loci, approximate the clinical manifestations of stage III and IV (based on the International Federation of Gynecology and Obstetrics classification system) ovarian cancer.

[00130] 6-8 week-old female CD-1 mice (n = 20) obtained from the Charles River Laboratory were injected i.p. with about 2×10^6 MDAH 2774 ovarian carcinoma cells. Animals then received either live or UV-inactivated reovirus (5×10^8 pfu) at days 5 and 19 days following MDAH 2774 injection (n = 10 for each group). Body weight was monitored weekly and mice were sacrificed if distressed or if tumor mass exceeded 20% of body weight.

Histology and immunohistochemistry:

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[00131] For histological analysis, tumors or remaining masses were fixed in 10% neutral buffered formalin and imbedded in paraffin. Sections were immersed in xylene followed by decreasing concentrations of ethanol. Sections were exposed to 3% hydrogen peroxide in methanol for about 15 minutes to inactivate endogenous peroxidase activity. Following washes, sections were incubated with a primary rabbit anti-reovirus polyclonal antibody, washed, then developed using HRP-conjugated secondary antibody by methods well known to those skilled in the art. Sections were counterstained with hematoxylin prior to analysis.

Results:

[00132] Mice receiving UV-inactivated reovirus rapidly gained weight (*e.g.*, were 15-20% heavier than mice receiving live reovirus at 4 weeks following MDAH 2774 injection) and died by day 42 following MDAH 2774 injection. In contrast, 90% of mice receiving live reovirus appeared healthy even after 100 days when they were eventually sacrificed. These results suggested that reovirus was effective in treating a stage III/IV ovarian cancer-like disease caused by tumor cells having elevated levels of phospho-MEK that was not a result of activating mutation in the Ras polypeptide or in activation or overexpression of upstream elements in the Ras/MAP kinase pathway.

[00133] While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

CLAIMS

What is claimed is:

1. A method of determining susceptibility of a cell to reovirus infection, the method comprising measuring the level of constitutive MEK phosphorylation in the cell, wherein constitutive MEK phosphorylation indicates susceptibility to infection by reovirus.
2. The method of claim 1 wherein the cell is comprised in a biological specimen from a mammal suspected of having a proliferative disorder.
3. The method of claim 2 wherein constitutive MEK phosphorylation in the biological specimen from the mammal suspected of having a proliferative disorder is compared to constitutive MEK phosphorylation in a biological specimen from a normal mammal.
4. The method of claim 1 wherein constitutive MEK phosphorylation is determined using an antibody specific for phosphorylated MEK.
5. The method of claim 1 wherein constitutive MEK phosphorylation is determined by pulse labeling cells with a radioactive isotope of phosphorus then measuring incorporation of the radioactive isotope into MEK.
6. The method of claim 5 wherein the radioactive isotope is ^{32}P or ^{33}P .
7. The method of claim 5 wherein incorporation of the radioactive isotope into MEK is determined by a method selected from the group consisting of immunoblot analysis, immunoprecipitation, radioimmunoprecipitation, isoelectric focusing, two-dimensional gel electrophoresis, or affinity chromatography, wherein the amount of radioactive isotope incorporated into MEK indicates constitutive MEK phosphorylation in the cell.

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8. The method of claim 1 wherein the cell comprises normal Ras.
9. The method of claim 1 wherein cell transformation is not a result of Ras activation.
10. The method of claim 1 wherein the cell comprises at least one mutation in one of more elements in the MAP kinase pathway selected from the group consisting of Raf, MEK, and MAP kinase.
11. The method of claim 1 wherein the cell comprises at least one mutation in MEK kinase.
12. The method of claim 1 wherein the cell comprises at least one mutations in one or more elements upstream of MEK.
13. The method of claim 2 wherein the proliferative disorder is neurofibromatosis.
14. The method of claim 2 wherein the proliferative disorder is a solid neoplasm.
15. The method of claim 2 wherein the proliferative disorder is selected from the group consisting of lung cancer, prostate cancer, colorectal cancer, thyroid cancer, renal cancer, adrenal cancer, liver cancer, pancreatic cancer, breast cancer and central and peripheral nervous system cancer.
16. The method of claim 2 wherein the proliferative disorder is breast cancer.
17. The method of claim 2 wherein the mammal is selected from the group consisting of dogs, cats, sheep, goats, cattle, horses, pigs, mice, non-human primates, and humans.
18. The method of claim 2 wherein the mammal is human.

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19. A method of treating a proliferative disorder in a mammal, which disorder is characterized by proliferating cells which exhibit constitutive MEK phosphorylation, comprising the steps of:
 - a) identifying a mammal having a proliferative disorder characterized by proliferating cells exhibiting constitutive MEK phosphorylation, and
 - b) administering to the proliferating cells in said mammal an effective amount of one or more reoviruses under conditions which result in substantial lysis of the proliferating cells.
20. The method of claim 19 further comprising a step selected from the group consisting of:
 - a) administering to the proliferating cells in said mammal an effective amount of an immune suppressive agent;
 - b) removing B-cells or T-cells from said mammal;
 - c) removing anti-reovirus antibodies from said mammal;
 - d) removing antibodies from said mammal;
 - e) administering anti-antireovirus antibodies to said mammal; and
 - f) suppressing the immune system of the mammal.
21. The method of claim 19, wherein the reovirus is selected from the group consisting of a mammalian reovirus and an avian reovirus.
22. The method of claim 21, wherein the reovirus is a mammalian reovirus.
23. The method of claim 22, wherein the reovirus is a human reovirus.
24. The method of claim 23, wherein the reovirus is selected from the group consisting of serotype 1 reovirus, serotype 2 reovirus and serotype 3 reovirus.
25. The method of claim 22, wherein the reovirus is serotype 3 reovirus.
26. The method of claim 21, wherein the reovirus is an avian reovirus.

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27. The method of claim 19, wherein the reovirus is a recombinant reovirus.
28. The method of claim 19, wherein more than one type of reovirus is administered.
29. The method of claim 19, wherein more than one strain of reovirus is administered.
30. The method of claim 19, wherein the reovirus is a field isolate.
31. The method of claim 19, wherein the proliferative disorder is a neoplasm.
32. The method of claim 19, wherein the proliferative disorder is neurofibromatosis.
33. The method of claim 26, wherein the neoplasm is a solid neoplasm.
34. The method of claim 31, wherein the neoplasm is selected from the group consisting of lung cancer, prostate cancer, colorectal cancer, thyroid cancer, renal cancer, adrenal cancer, liver cancer, pancreatic cancer, breast cancer and central and peripheral nervous system cancer.
35. The method of claim 31, wherein the neoplasm is a central nervous system cancer.
36. The method of claim 31, wherein the neoplasm is breast cancer.
37. The method of claim 31, wherein the neoplasm is a hematopoietic neoplasm.
38. The method of claim 33, wherein the reovirus is administered by injection into or near the solid neoplasm.

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39. The method of claim 19 wherein the reovirus is administered by a route selected from the group consisting of intravascular, intrathecal, intravenous, intramuscular, subcutaneous, intraperitoneal, topical, oral, rectal, vaginal, nasal, and intratumoral.
40. The method of claim 19 wherein the mammal is immunocompetent.
41. The method of claim 19 wherein the reovirus is immunoprotected.
42. The method of claim 19 wherein the reovirus is encapsulated in a micelle.
43. The method of claim 19, wherein approximately 1 to 10^{15} plaque forming units of reovirus/kg body weight are administered.
44. The method of claim 19, wherein the reovirus is administered in a single dose.
45. The method of claim 19, wherein the reovirus is administered in more than one dose.
46. The method of claim 31, wherein the neoplasm is metastatic.
48. The method of claim 19 further comprising the administration of an effective amount of a chemotherapeutic agent.
49. The method of claim 19, wherein the reovirus is treated with a protease prior to administration.
50. The method of claim 34, wherein the neoplasm is pancreatic cancer.
51. The method of claim 38 further comprising surgical removal of the substantially all of the neoplasm and administration of the reovirus to the surgical site in an amount sufficient to result in substantial oncolysis of any remaining neoplastic cells.

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52. A method of inhibiting metastasis of a neoplasm in a mammal, which neoplasm is characterized by proliferating cells which exhibit constitutive MEK phosphorylation, comprising the steps of:

- a) identifying a mammal having a neoplasm characterized by proliferating cells exhibiting constitutive MEK phosphorylation, and
- b) administering to the mammal a reovirus in an amount sufficient to result in substantial lysis of the neoplastic cells.

53. The method of claim 52 further comprising a step selected from the group consisting of:

- a) administering to the proliferating cells in said mammal an effective amount of an immune suppressive agent;
- b) removing B-cells or T-cells from said mammal;
- c) removing anti-reovirus antibodies from said mammal;
- d) removing antibodies from said mammal;
- e) administering anti-antireovirus antibodies to said mammal; and
- f) suppressing the immune system of the mammal.

54. A method of treating a suspected neoplasm in a mammal, which neoplasm is characterized by proliferating cells which exhibit constitutive MEK phosphorylation, comprising the steps of:

- a) identifying a mammal having a neoplasm characterized by proliferating cells exhibiting constitutive MEK phosphorylation,
- b) surgically removing substantially all of the neoplasm from one or more surgical sites in said animal, and
- c) administering an effective amount of reovirus at or near to the one or more surgical sites to result in oncolysis of any remaining neoplastic cells.

55. The method of claim 54 further comprising a step selected from the group consisting of:

- a) administering to the proliferating cells in said mammal an effective amount of an immune suppressive agent;
- b) removing B-cells or T-cells from said mammal;

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- c) removing anti-reovirus antibodies from said mammal;
 - d) removing antibodies from said mammal;
 - e) administering anti-antireovirus antibodies to said mammal; and
 - f) suppressing the immune system of the mammal.
56. A method of identifying tumorigenic cells, the method comprising measuring the level of constitutive MEK phosphorylation in the cell, wherein constitutive MEK phosphorylation indicates tumorigenicity.
57. The method of claim 56 wherein the cell is comprised in a biological specimen from a mammal suspected of having a proliferative disorder.
58. The method of claim 57 wherein constitutive MEK phosphorylation in the biological specimen from the mammal suspected of having a proliferative disorder is compared to constitutive MEK phosphorylation in a biological specimen from a normal mammal.
59. The method of claim 58 wherein constitutive MEK phosphorylation is determined using an antibody specific for phospho-MEK.
60. The method of claim 58 wherein constitutive MEK phosphorylation is determined by pulse labeling cells with a radioactive isotope of phosphorus then measuring incorporation of the radioactive isotope into MEK using an MEK-specific antibody.
61. The method of claim 60 wherein the radioactive isotope is ^{32}P or ^{33}P .
62. The method of claim 58 wherein incorporation of the radioactive isotope into MEK is determined by a method selected from the group consisting of immunoblot analysis, immunoprecipitation, isoelectric focusing, radioimmunoassay, two-dimensional gel electrophoresis, or affinity chromatography, wherein the amount of radioactive isotope incorporated into MEK indicates constitutive MEK phosphorylation in the cell.

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63. The method of claim 54 wherein the cell comprises normal Ras.
64. The method of claim 54 wherein cell transformation is not a result of Ras activation.
65. The method of claim 54 wherein the cell comprises at least one mutation in one of more elements in the MAP kinase pathway selected from the group consisting of Raf, MEK, and MAP kinase.
66. The method of claim 54 wherein the cell comprises at least one mutation in MEK kinase.
67. The method of claim 54 wherein the cell comprises at least one mutations in one of more elements upstream of MEK.
68. A method of determining susceptibility of a biological specimen to reovirus infection, wherein the biological specimen is suspected of comprising neoplastic cells, comprising:
- a) determining the amount of total MEK in the biological specimen;
 - b) pulse-labeling the biological specimen and determining the amount of phospho-MEK in the pulse-labeled biological specimen;
 - c) obtaining the relative amount of phospho-MEK by dividing the amount of phospho-MEK of step (b) with the amount of total MEK of step (a);
 - d) providing a normal biological specimen, which comprises normal cells;
 - e) determining the relative amount of phospho-MEK in the normal biological specimen by repeating steps (a)-(c) using the normal biological specimen; and
 - f) comparing the relative amounts of phospho-MEK of steps (c) and (e), wherein an increased relative amount in step (c) compared to that of step (e) is indicative of the presence of neoplastic cells in, and reovirus susceptibility of, the biological specimen.
69. The method of claim 68 wherein the biological specimen is pulse labeled with an isotope selected from the group consisting of ^{32}P and ^{33}P .

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70. The method of claim 68 further comprising the use of one or more methods selected from the group consisting of immunoblot analysis, immunoprecipitation, radioimmunoassay, and 2D-electrophoreses, for quantitation of MEK in the biological specimens.
71. A method of determining susceptibility of a biological specimen to reovirus infection, wherein the biological specimen is suspected of comprising neoplastic cells, comprising:
- a) determining the amount of total MEK in the biological specimen;
 - b) determining the amount of phospho-MEK in the biological specimen;
 - c) obtaining the relative amount of phospho-MEK by dividing the amount of phospho-MEK of step (b) with the amount of total MEK of step (a);
 - d) providing a normal biological specimen, which comprises normal cells;
 - e) determining the relative amount of phospho-MEK in the normal biological specimen by repeating steps (a)-(c) using the normal biological specimen; and
 - f) comparing the relative amounts of phospho-MEK of steps (c) and (e), wherein an increased relative amount in step (c) compared to that of step (e) is indicative of the presence of neoplastic cells in, and reovirus susceptibility of, the biological specimen.
72. The method of claim 71 further comprising the use of one or more methods selected from the group consisting of immunoblot analysis, immunoprecipitation, radioimmunoassay, and 2D-electrophoreses, for quantitation of MEK and phospho-MEK in the biological specimens.

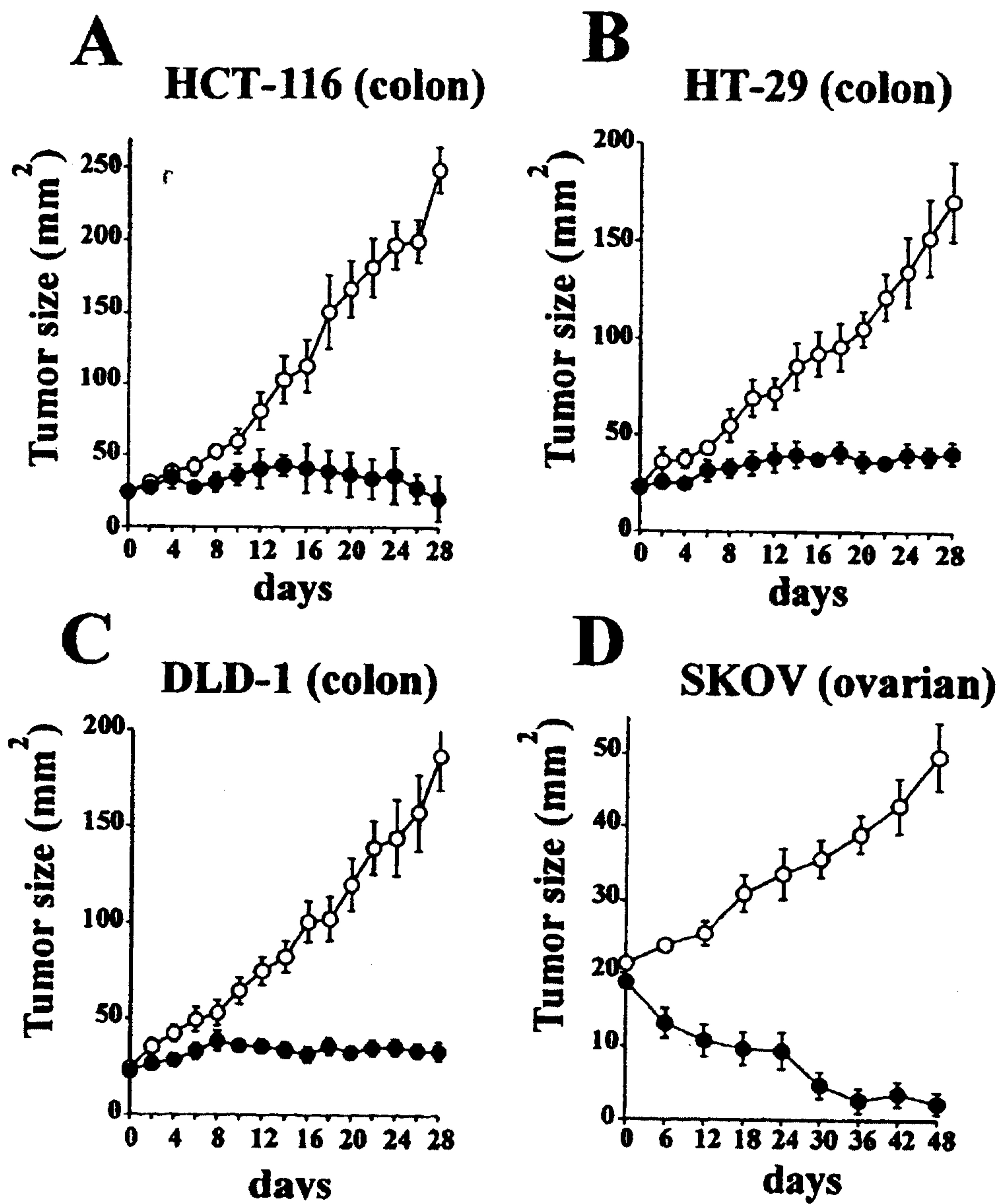
Application number - numéro de demande: 2422245

Figures: one

Pages: _____

Unscannable items
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(Request original documents in File Prep. Section on the 10th floor)

Documents reçu avec cette demande ne pouvant être balayés
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Figure 2*Marks & Clerk*