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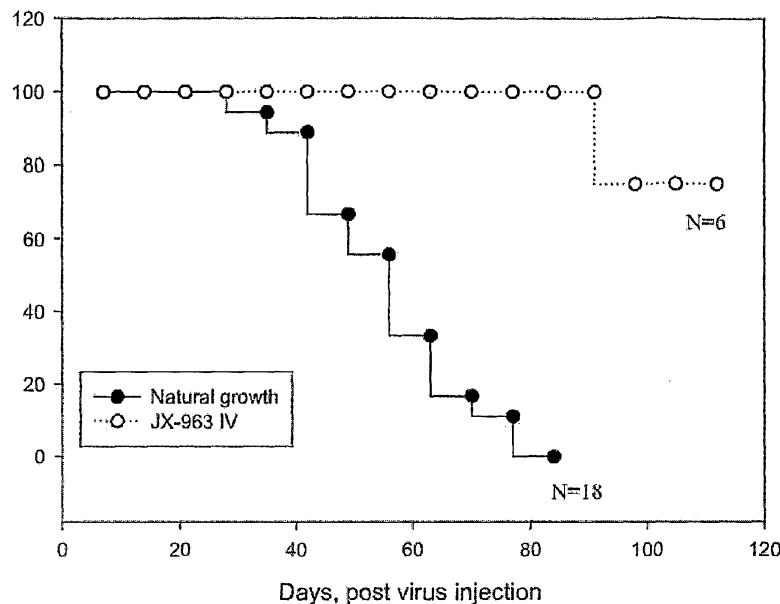
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(54) Title: SYSTEMIC TREATMENT OF METASTATIC AND/OR SYSTEMICALLY-DISSEMINATED CANCERS USING GM-CSF-EXPRESSING POXVIRUSES



A3

(57) Abstract: The present invention concerns methods and compositions for the treatment of cancer and cancer cells using intravascular administration of a vaccinia virus. In some embodiments, methods and compositions involve a replicative vaccinia virus that encodes GM-CSF.

DESCRIPTION**SYSTEMIC TREATMENT OF METASTATIC AND/OR SYSTEMICALLY-DISSEMINATED CANCERS USING GM-CSF-EXPRESSING POXVIRUSES**

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This application claims priority to U.S. Provisional Patent Application Serial Number 60/714,679 filed September 7, 2005, which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION**I. FIELD OF THE INVENTION**

10 The present invention relates generally to the fields of oncology and virology. More particularly, it concerns vaccinia viruses that express GM-CSF and their use in systemic administration to treat cancer.

II. DESCRIPTION OF RELATED ART

15 Normal tissue homeostasis is a highly regulated process of cell proliferation and cell death. An imbalance of either cell proliferation or cell death can develop into a cancerous state (Solyanik *et al.*, 1995; Stokke *et al.*, 1997; Mumby and Walter, 1991; Natoli *et al.*, 1998; Magi-Galluzzi *et al.*, 1998). For example, cervical, kidney, lung, pancreatic, colorectal and brain cancer are just a few examples of the many cancers that can result (Erlandsson, 1998; Kolmel, 1998; Mangray and King, 1998; Gertig and Hunter, 1997; Mougin *et al.*, 1998). In 20 fact, the occurrence of cancer is so high that over 500,000 deaths per year are attributed to cancer in the United States alone.

25 The maintenance of cell proliferation and cell death is at least partially regulated by proto-oncogenes and tumor suppressors. A proto-oncogene or tumor suppressor can encode proteins that induce cellular proliferation (e.g., sis, erbB, src, ras and myc), proteins that inhibit cellular proliferation (e.g., Rb, p16, p19, p21, p53, NF1 and WT1) or proteins that regulate programmed cell death (e.g., bcl-2) (Ochi *et al.*, 1998; Johnson and Hamdy, 1998; Liebermann *et al.*, 1998). However, genetic rearrangements or mutations of these proto-oncogenes and tumor suppressors result in the conversion of a proto-oncogene into a potent 30 cancer-causing oncogene or of a tumor suppressor into an inactive polypeptide. Often, a single point mutation is enough to achieve the transformation. For example, a point mutation

in the p53 tumor suppressor protein results in the complete loss of wild-type p53 function (Vogelstein and Kinzler, 1992).

Currently, there are few effective options for the treatment of many common cancer types. The course of treatment for a given individual depends on the diagnosis, the stage to 5 which the disease has developed and factors such as age, sex and general health of the patient. The most conventional options of cancer treatment are surgery, radiation therapy and chemotherapy. Surgery plays a central role in the diagnosis and treatment of cancer. Typically, a surgical approach is required for biopsy and to remove cancerous growth. 10 However, if the cancer has metastasized and is widespread, surgery is unlikely to result in a cure and an alternate approach must be taken. Radiation therapy, chemotherapy, and immunotherapy are alternatives to surgical treatment of cancer (Mayer, 1998; Ohara, 1998; Ho *et al.*, 1998). Radiation therapy involves a precise aiming of high energy radiation to 15 destroy cancer cells and much like surgery, is mainly effective in the treatment of non-metastasized, localized cancer cells. Side effects of radiation therapy include skin irritation, difficulty swallowing, dry mouth, nausea, diarrhea, hair loss and loss of energy (Curran, 1998; Brizel, 1998).

Chemotherapy, the treatment of cancer with anti-cancer drugs, is another mode of 20 cancer therapy. The effectiveness of a given anti-cancer drug therapy often is limited by the difficulty of achieving drug delivery throughout solid tumors (el-Kareh and Secomb, 1997). Chemotherapeutic strategies are based on tumor tissue growth, wherein the anti-cancer drug is targeted to the rapidly dividing cancer cells. Most chemotherapy approaches include the 25 combination of more than one anti-cancer drug, which has proven to increase the response rate of a wide variety of cancers (U.S. Patents 5,824,348; 5,633,016 and 5,798,339, incorporated herein by reference). A major side effect of chemotherapy drugs is that they also affect normal tissue cells, with the cells most likely to be affected being those that divide rapidly in some cases (*e.g.*, bone marrow, gastrointestinal tract, reproductive system and hair follicles). Other toxic side effects of chemotherapy drugs can include sores in the mouth, 30 difficulty swallowing, dry mouth, nausea, diarrhea, vomiting, fatigue, bleeding, hair loss and infection.

Immunotherapy, a rapidly evolving area in cancer research, is yet another option for the treatment of certain types of cancers. Theoretically, the immune system may be stimulated to identify tumor cells as being foreign and targets them for destruction.

Unfortunately, the response typically is not sufficient to prevent most tumor growth. However, recently there has been a focus in the area of immunotherapy to develop methods that augment or supplement the natural defense mechanism of the immune system. Examples of immunotherapies currently under investigation or in use are immune adjuvants (e.g., 5 *Mycobacterium bovis*, *Plasmodium falciparum*, dinitrochlorobenzene and aromatic compounds) (U.S. Patents 5,801,005 and 5,739,169; Hui and Hashimoto, 1998; Christodoulides *et al.*, 1998), cytokine therapy (e.g., interferons (IL-1, GM-CSF and TNF) (Bukowski *et al.*, 1998; Davidson *et al.*, 1998; Hellstrand *et al.*, 1998), and gene therapy (e.g., TNF, IL-1, IL-2, p53) (Qin *et al.*, 1998; Austin-Ward and Villaseca, 1998; U.S. Patents 10 5,830,880 and 5,846,945) and monoclonal antibodies (e.g., anti-ganglioside GM2, anti-HER-2, anti-p185) (Pietras *et al.*, 1998; Hanibuchi *et al.*, 1998; U.S. Patent 5,824,311). Such methods, while showing some promise, have demonstrated limited success.

15 Replication-selective oncolytic viruses hold promise for the treatment of cancer (Kirm *et al.*, 2001). These viruses can cause tumor cell death through direct replication-dependent and/or viral gene expression-dependent oncolytic effects (Kirm *et al.*, 2001). In addition, viruses are able to enhance the induction of cell-mediated antitumoral immunity within the host (Todo *et al.*, 2001; Sinkovics *et al.*, 2000). These viruses also can be engineered to expressed therapeutic transgenes within the tumor to enhance antitumoral efficacy (Hermiston, 2000).

20 However, major limitations exist to this therapeutic approach. Although a degree of natural tumor-selectivity can be demonstrated for some virus species, new approaches are still needed to engineer and/or enhance tumor-selectivity for oncolytic viruses in order to maximize safety. This selectivity will become particularly important when intravenous administration is used, and when potentially toxic therapeutic genes are added to these viruses 25 to enhance antitumoral potency; gene expression will need to be tightly limited in normal tissues. In addition, increased antitumoral potency through additional mechanisms such as induction of antitumoral immunity or targeting of the tumor-associated vasculature is highly desirable.

30 Therefore, more effective and less toxic therapies for the treatment of cancer are needed. The use of oncolytic viruses and immunotherapy present areas that can be developed, however, the limitations discussed above need to be overcome. Thus, the present invention addresses those limitations.

SUMMARY OF THE INVENTION

Thus, in accordance with the present invention there is provided a method of killing a cancer cell in a subject comprising administering to the subject an effective amount of a replicative vaccinia virus having an expression region with a promoter directing expression of 5 a nucleic acid encoding granulocyte-macrophage colony stimulating factor (GM-CSF), wherein the administration is intravascular. It is specifically contemplated that the nucleic acid encodes human GM-CSF.

The vaccinia virus may be administered intravenously or intraarterially, for example, using intravenous drip or bolus, or using a pump. The vaccinia virus may be dispersed in a 10 pharmaceutically acceptable formulation. The subject may be administered between about 10^5 , 10^6 10^7 , 10^8 and about 10^9 , 10^{10} , 10^{12} , 10^{13} pfu of virus, or between about 10^7 and about 15 10^{10} pfu of virus. The subject may be administered the vaccinia virus multiple times (1, 2, 3, 4, 5, 6, 7 days or weeks of a first treatment, or wherein the second treatment occurs within 1, 2, 3, 4, 5, 6, 7 days or weeks of a first treatment. The same or a different dose may be administered. The cancer cell may 20 be a metastasized cancer cell. The subject may have brain cancer, head & neck cancer, renal cancer, ovarian cancer, testicular cancer, uterine cancer, stomach cancer, lung cancer, colorectal cancer, breast cancer, prostate cancer, pancreatic cancer, hepatocellular cancer, leukemia, lymphoma, myeloma, or melanoma.

20 The vaccinia virus may have a deletion in its genome or a mutation in one or more genes. The thymidine kinase gene of the vaccinia virus may have been deleted. The vaccinia virus may have a mutation in a gene encoding (a) vaccinia virus growth factor; (b) a functional interferon-modulating polypeptide, wherein the interferon-modulating polypeptide directly binds interferon; (c) a complement control polypeptide, wherein the mutation results 25 in the virus lacking at least one functional complement control polypeptide; (d) a TNF-modulating polypeptide, wherein the mutation results in the virus lacking at least one functional TNF-modulating polypeptide; (e) a serine protease inhibitor, wherein the mutation results in the virus lacking at least one functional serine protease inhibitor; (f) an IL-1 β modulator polypeptide, wherein the mutation results in the virus lacking at least one functional IL-1 β modulator polypeptide; (g) a functional A41L, B7R, N1L or vCKBP 30 chemokine binding polypeptide or C11R EGF-like polypeptide, wherein the mutation results in the virus lacking at least one function of A41L, B7R, N1L, vCKBP, or C11R; or (h) a

polypeptide, wherein the mutation results in an increase in infectious EEV form of vaccinia virus. In addition, the vaccinia virus may comprise a mutation in a vaccinia virus growth factor. The vaccinia virus may be the Wyeth or Western Reserve (WR) strain. The promoter may be a vaccinia virus promoter, a synthetic promoter, a promoter that directs transcription 5 during at least the early phase of infection, or a promoter that directs transcription during at least the late phase of infection.

In another embodiment, there is provided a method for treating cancer in a subject comprising administering to the subject an effective amount of a replicative vaccinia virus having an expression region with a promoter directing expression of a nucleic acid encoding 10 granulocyte-macrophage colony stimulating factor (GM-CSF), wherein the administration is intravascular.

In yet another embodiment, there is provided a method for treating one or more metastases in a subject comprising administering to the subject an effective amount of a replicative vaccinia having an expression region with a promoter directing expression of a 15 nucleic acid encoding granulocyte-macrophage colony stimulating factor (GM-CSF), wherein the administration is intravascular.

In other embodiments it is contemplated that methods involving a replication-competent vaccinia virus that is administered intravascularly may contain a nucleic acid 20 encoding a protein or RNA other than GM-CSF. In particular embodiments, the nucleic acid encodes another cytokine. In certain embodiments, the nucleic acid encodes other immunostimulatory cytokines or chemokines, such as IL-12, IL-2 and others. In additional embodiments, the nucleic acid may encode thymidine deaminase or tumor necrosis factor 25 (TNF), such as TNF- α . Moreover, it is contemplated that replicative vaccinia viruses may express more than one heterologous sequence. It may express, for example, GM-CSF protein and another protein or RNA molecule.

It is specifically contemplated that any embodiment discussed with respect to a particular method or composition may be implemented with respect to other methods and compositions of the invention.

The use of the word "a" or "an" when used in conjunction with the term "comprising" 30 in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the 5 spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better 10 understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1 - JX-594 intravenous (IV) treatment of spontaneous rat hepatocellular carcinoma (HCC). Rats received the mutagen N-Nitrosomorpholine (NMM) in their drinking water (175 mg/L) for a period of 8 weeks and were then followed by ultrasound (US) 15 until HCC tumors had formed and were 300-400 mm³ (typically after 16-20 weeks). Animals then received 3 intravenous doses (one every two weeks, arrows) of either PBS (n=17) or 1x10⁸ PFU of JX-594 virus (n=6). Subsequent tumor volumes were then calculated based on tumor measurements from US imaging.

FIGS. 2A-2B - JX-594 Intravenous dose treatment of VX2 liver tumors in rabbits, efficacy against primary tumor and metastases. VX2 cells (from a dissociated 1 mm³ tumor) were implanted into the liver of New Zealand white rabbits and tumor growth followed by ultrasound (US) and CT scan. Once tumors reached 2-4 cm³ animals were treated with a single dose of PBS (n=7) or JX-594 (1x10⁹ PFU), via intravenous or US guided IT injection (n=3/group). (FIG. 2A) Subsequent tumor volume in the liver was measured 7 weeks later by CT scan and (FIG. 2B) number of detectable tumor metastases in the lungs 25 were counted following CT scan at weeks 6 and 7.

FIG. 3 - JX-594 and JX-963 lower dose intravenous repeat treatments of VX2 liver tumors in rabbits. Tumor cells were implanted as described in (FIGS. 2A-2B). Animals were treated intravenously 3 times (every two weeks, arrows) after tumors reached 30 2-4 cm³ with 1x10⁸ PFU of JX-594, JX-963 or vvDD (n=6/group), or PBS (n=18). Subsequent primary tumor volume was followed by CT scan.

FIG. 4 - Effects of JX-594, vvDD and JX-963 on lung metastases in rabbits bearing VX2 liver tumors. Animals (from studies described in FIG. 3) were examined for liver metastases by CT scan at weekly intervals after the beginning of therapy. The mean number of detectable metastases per animal in each group is shown.

5 **FIG. 5 - Survival of rabbits bearing VX2 liver tumors after IV delivery of JX-963.** Animals bearing liver tumors were treated with 3 doses of 1×10^8 PFU of JX-963 as described in FIG. 3. A Kaplan-Meier survival curve of these animals and the control treated group are shown. As the JX-594 and vvDD groups did not show significant differences in survival, JX594 and vvDD groups were not included.

10 **FIGS. 6A-6C - Burst ratio of vaccinia strains, cytopathic effect, and systemic delivery of viral strains to tumors.** (FIG. 6A) Burst ratio of vaccinia strains in tumor to normal cells. Different vaccinia strains were used to infect both primary normal cells (NHBE) and a tumor cell line (A2780) at a Multiplicity of infection (MOI) of 1.0 Plaque Forming Unit (PFU)/cell. Virus collected 48h later was titered by plaque assay and the ratio of virus produced (per cell) in tumor to normal cells is represented. (FIG. 6B) Cytopathic effect produced by viral infection. Western Reserve, Adenovirus serotype 5 and Adenovirus strain *dl1520* (ONYX-015) (in some assays) were added to cell lines at ranges of MOIs (PFU/cell), and cell viability measured after 72 hours using MTS (Promega). The MOI of virus (PFU/cell) needed to reduce the cell viability to 50% of untreated control wells (ED₅₀) is plotted. (FIG. 6C) Systemic delivery of viral strains to tumors. 1×10^9 PFU of vaccinia strain Western Reserve or Adenovirus serotype 5 were delivered intravenously to immunocompetent mice bearing subcutaneous CMT 64 or JC tumors. Mice were sacrificed after 48 or 72 hours and immunohistochemistry performed against viral coat proteins on paraffin embedded sections of tumor tissue. Graphs show scoring of positive cells in each tumour (*=none detectable). For each condition results are based on tumours from 3 mice, and for each tumour, ten randomly chosen fields of view were scored.

25 **FIG. 7 - Cytopathic effect of WR and vvDD on a panel of human tumor cell lines.** EC₅₀ values were determined 72h following infection of tumor cell lines with WR or vvDD. The MOI of virus (PFU/cell) needed to reduce the cell viability to 50% of untreated control wells (ED50) is plotted

30 **FIGS. 8A-8C - Effects of overexpression of H-Ras on viral replication, biodistribution of WR and vvDD following systemic delivery to tumor bearing mice, and**

viral gene expression quantified by light production. (FIG. 7A) Effects of overexpression of H-Ras on viral replication. NIH 3T3 cells, and NIH 3T3 cells expressing activated H-Ras, either proliferating or serum starved, were infected with different strains of vaccinia at an MOI of 1.0 PFU/cell. Viral strains were parental Western Reserve (WR), and WR containing 5 deletions or insertions in either the Thymidine Kinase (TK) gene (vJS6), the viral growth factor (VGF) gene (vSC20), or containing deletions in both these genes (vvDD). Infectious virus was titered by plaque assay after 48h. (FIG. 7B). Biodistribution of WR and vvDD following systemic delivery to tumor bearing mice. Athymic CD1 nu/nu mice bearing subcutaneous human HCT 116 tumors (arrowed) were treated with 1×10^7 PFU of vaccinia 10 strains via tail vein injection. Viral strains (WR and vvDD) expressed luciferase, and the subsequent biodistribution of viral gene expression was detected by bioluminescence imaging in an IVIS100 system (Xenogen Corp, Alameda) following addition of the substrate luciferin 15 at the times indicated after treatment. (FIG. 7C) Viral gene expression, as quantified by light production, was plotted over time for the regions of interest covering the whole body (ventral image)(dashed line, open symbols), or from the tumor only (dorsal view)(solid line, filled symbols) for BALB/c mice bearing subcutaneous JC tumors (n=5 mice/group) and treated with 1×10^7 PFU of either virus by tail vein injection.

FIGS. 9A-9C - Rabbits bearing VX2 tumors implanted into the liver were followed by CT imaging at times after tumor implantation, CTL assay against VX2 tumor cells, and Rabbits re-treated with JX-963. (FIG. 9A) Rabbits bearing VX2 tumors implanted into the liver were followed by CT imaging at times after tumor implantation. 1×10^9 PFU of viruses vvDD and JX-963 were delivered by ear vein injection at 2, 3 and 4 weeks after tumor implantation (arrows), when tumors measured 5 cm^3 . The number of detectable lung metastases was also measured in these animals (representative CT images of 20 primary liver tumors are shown at 8 weeks) (n=18 for control treated animals; n=6 for vvDD treated; n=6 for JX-963 treated). (FIG. 9B) CTL assay targeting VX2 tumor cells. CTL assay was performed by FACS analysis using pre-labeled VX2 cells mixed with 12.5x; 25x and 50x 25 unlabelled peripheral blood lymphocytes from rabbits bearing VX2 tumors and treated with JX-963; from untreated animals with VX2 tumors; and from naïve animals. Cell death was quantified by the ACT1 assay (Cell Technology, Mountain View). (FIG. 9C) Four Rabbits 30 treated as in (A) with JX-963 were re-treated with 1×10^9 PFU of JX-963 at Day 42 after implantation (arrow), subsequent tumor volume was followed by CT scan.

FIGS. 10A-10B – Viral production of cell lines infected with either WR or Ad5, and cytopathic effect produced by viral infection. (FIG. 10A) Different cell lines were infected with either Western Reserve or Adenovirus serotype 5 at an MOI of 1.0 PFU/cell. Amounts of virus produced (Infectious Units/cell) 48h later were titered by plaque assay.

5 (FIG. 10B) Mice treated as in Fig. 1C were sacrificed and tumor sections stained for viral coat proteins. Representative photographs show sections at 72h and 10 days post-treatment.

FIG. 11 - Viral production of cell lines infected with either WR or Ad5. Human tumor cell lines (Panc-1 and MCF-7) or human immortalized but non-transformed cell lines (Beas-2B and MRC-5), either proliferating or grown to contact inhibition (N.B. tumor cells 10 did not become contact inhibited), were treated with different strains of vaccinia at an MOI of 1.0 PFU/cell. Strains used were Western Reserve (WR) and WR containing deletions in either the Thymidine Kinase (TK) gene (vJS6), the viral growth factor (VGF) gene (vSC20), or containing deletions in both these genes (vvDD). Virus produced after 48h was titered by plaque assay.

15 **FIG. 12 – Recovery of systemically delivered vvDD.** Recovery of vvDD delivered systemically (intraperitoneal injection of 1×10^9 PFU) to C57B/6 mice bearing subcutaneous MC38 tumors. Mice were sacrificed on days 5 or 8 after treatment (n=8/group) and different tissues recovered and viral infectious units (PFU/mg tissue) titered by plaque assay (*=below the limits of detection).

20 **FIGS. 13A-13B - Efficacy of vvDD following delivery by different routes into tumor bearing mouse models.** (FIG. 13A) Single intravenous injections of 1×10^9 PFU of viral strain vvDD or vaccinia Wyeth strain bearing a Thymidine Kinase deletion were delivered to immunocompetent mice bearing subcutaneous TIB 75 tumors ($50-100 \text{ mm}^3$). Tumor volume was measured by calipers, (n=8/group). (FIG. 13B) 1×10^9 PFU of vvDD was 25 delivered intratumorally (IT) or intraperitoneally (IP) to either SCID mice bearing subcutaneous HT29 tumors or C57B/6 mice bearing subcutaneous MC38 tumors and subsequent tumor volume compared to an uninfected control group (n=8/group).

30 **FIG. 14 - Formation of neutralizing antibodies following treatment of VX2 tumor bearing rabbits with JX-963 (1×10^8 PFU).** Dilutions of plasma obtained from rabbits at indicated times were incubated with a known number of viral PFU, and dilutions required to retain 50% of the plaques are shown (n=3).

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

GM-CSF-expressing poxviruses demonstrated efficacy and safety following intratumoral injection in animals and in patients with melanoma (Mastrangelo *et al.*, 1999; 2000). Localized injection site and distant effects were seen, including tumor regressions and 5 stabilizations (Mastrangelo *et al.*, 1999). Efficacy was proposed to be due to induction of an immune response to the cancer cells in the mammal. U.S. Patents 6,093,700 and 6,475,999 propose the use of poxviruses to deliver GM-CSF to tumors by intratumoral injection in humans in order to immunize *in situ* against melanoma, head and neck cancer, prostate cancer and bladder (Mastrangelo *et al.*, 2002). These cancers were selected because of their 10 superficial nature and access to direct intratumoral injection. These cancers are also reportedly sensitive to immunotherapy approaches that induce systemic tumor-specific, cytotoxic T-lymphocytes. GM-CSF was demonstrated to be a potent inducer of tumor-specific cytotoxic T-lymphocytes (CTLs) (Dranoff *et al.*, 1993). Since viruses induce immune cell infiltration and proinflammatory cytokines, viruses may constitute an ideal method of 15 delivering and expressing GM-CSF within a tumor mass.

The intratumoral route of injection has major limitations, however. This limits treatment to tumors that are accessible to safe intratumoral injection, usually superficial tumors, such as those listed above. In addition, efficacy against non-injected tumors requires induction of sufficiently potent tumor-specific, cytotoxic T-lymphocytes. These weaknesses 20 limit the utility of this approach to cancers that are superficial and are able to induce sufficiently potent tumor-specific, cytotoxic T-lymphocytes systemically. Such tumors are rare. The only clear example of applicability to this approach is metastatic melanoma. Even in this tumor type however, distant responses were slow and were limited to superficial skin metastases. Organ-based or visceral tumors did not respond. Since these tumors are the cause 25 of almost all cancer-related morbidity and deaths, systemic cytotoxic T-lymphocytes do not appear to be sufficient to induce lasting systemic, visceral responses or cures. Therefore, the vast majority of human tumors are not amenable to successful efficacy with this approach.

Intravascular administration (*i.e.*, intravenous, intra-arterial) with delivery to metastatic tumors and immune tissues (*e.g.*, reticuloendothelial cells) has numerous 30 theoretical advantages. First, delivery of GM-CSF-expressing poxviruses to the majority of tumor sites, or all tumor sites, in the body of the mammal allows for systemic tumor destruction by both local intratumoral effects (due to poxvirus replication and GM-CSF

effects locally within the infected tumors) and tumor-specific, cytotoxic T-lymphocytes induction and subsequent efficacy both at the infected tumor site and at a distance (in tumor sites that were not directly infected with the initial dosage). Delivery of virus to tumors through the bloodstream also allows for more uniform, widespread infection of cancer cells in the tumor. Therefore, the inventors now propose that multi-focal, disseminated, metastatic tumors can be effectively treated by intravascular poxviruses expressing GM-CSF. Many cancer types and/or stages that would not be amenable to the intratumoral approach would also be potentially treatable with the intravascular injection approach. Examples would include, but would not be limited to, lung, colorectal, breast, prostate, pancreatic, hepatocellular, leukemias, lymphomas, myelomas, and melanomas.

However, the safe and effective use of intravascular poxviruses expressing GM-CSF was viewed by the skilled artisan as being negatively impacted by potential problems. First, safety concerns were significant. After intravascular administration, numerous normal tissues would be exposed to poxvirus infection. Subsequent expression of a potent proinflammatory cytokine like GM-CSF would be predicted to potentially lead to significant inflammation in numerous organs such as liver, lung, kidney, heart, brain and others. In addition, systemic exposure to viremia can potentially induce sepsis and its associated complications (e.g., hypotension). Poxvirus infection in the brain of mice or humans, for example, can lead to clinically-significant, even fatal, encephalitis. GM-CSF expression could potentially significantly worsen this complication due to enhancement of inflammation.

Second, systemic induction of tumor-specific CTLs through GM-CSF expression was previously performed only through localized GM-CSF expression either through direct intratumoral injection (e.g., Vaccinia-GM-CSF, HSV-GM-CSF) or through *ex vivo* infection/transfection of autologous or allogeneic tumor cells to express GM-CSF (e.g., GVAX approach) followed by injection of GM-CSF cells into the skin.

Despite these potential drawbacks, the inventors now have demonstrated that two different GM-CSF-expressing poxviruses were well-tolerated intravenously and highly effective against disseminated tumors and metastases. In addition, a GM-CSF-expressing poxvirus had significantly better efficacy against both primary tumors and lung metastases than its non-GM-CSF-expressing control after intravenous administration. Also, this virus had significantly better efficacy against both primary tumors and lung metastases than a comparable virus (Wyeth vaccine strain) despite an additional deletion in the vgf gene not

present in the other virus. Therefore, intravenous administration with a vaccinia expressing human GM-CSF resulted in significantly better efficacy over the same vaccinia without GM-CSF, and intravascular administration of a WR strain deletion mutant expressing human GM-CSF was significantly better than a standard vaccine strain expressing GM-CSF. These 5 viruses were well-tolerated after intravenous administration to both tumor-bearing (rats and rabbits) and normal animals (rabbits). Treated animals did not lose weight while tumor-bearing animals that did not receive treatment lost weight. Survival was increased following intravenous treatment. No significant organ toxicity was noted by blood testing or histopathology. The only reproducible histopathological findings were multiple sites of 10 lymphoid hyperplasia that were noted following treatment (consistent with systemic immunostimulation). No significant toxicities were noted on histopathology. Therefore, these GM-CSF expressing poxviruses are well-tolerated at doses that were highly effective against systemic cancer.

I. POXVIRUSES

15 A. Vaccinia Virus

Vaccinia virus is a mystery to virology. It is not known whether vaccinia virus is the product of genetic recombination, if it is a species derived from cowpox virus or variola virus by prolonged serial passage, or if it is the living representative of a now extinct virus. 20 Vaccinia virus was used for smallpox vaccination via inoculation into the superficial layers of the skin of the upper arm. However, with the eradication of smallpox, routine vaccination with vaccinia virus has ceased. Recent interest in vaccinia has focused on its possible usage as a vector for immunization against other viruses and gene therapy.

Vaccinia virus is a member of the family *Poxviridae*, the subfamily *Chordopoxvirinae* and the genus *Orthopoxvirus*. The virions contain RNA polymerase, early transcription 25 factor, poly(A) polymerase, capping enzyme complex, RNA (nucleoside-2') methyltransferase, nucleoside triphosphate phosphohydrolase II, nick-joining enzyme, DNA topoisomerase and protein kinase. The genome is a double-stranded DNA of just over 180,000 base pairs characterized by a 10 kB inverted terminal repeat. The virus enters cells through pH-independent fusion with the plasma membrane or a low pH-dependent endosomal 30 route.

B. Other Poxviruses

The genus *Orthopoxvirus* is relatively more homogeneous than other members of the *Chordopoxvirinae* subfamily and includes 11 distinct but closely related species, which includes vaccinia virus, variola virus (causative agent of smallpox), cowpox virus, buffalopox virus, monkeypox virus, mousepox virus and horsepox virus species as well as others (see 5 Moss, 1996). Certain embodiments of the invention, as described herein, may be extended to other members of *Orthopoxvirus* genus as well as the *Parapoxvirus*, *Avipoxvirus*, *Capripoxvirus*, *Leporipoxvirus*, *Suipoxvirus*, *Molluscipoxvirus*, and *Yatapoxvirus* genus. A 10 genus of poxvirus family is generally defined by serological means including neutralization and cross-reactivity in laboratory animals. Various members of the *Orthopoxvirus* genus, as well as other members of the *Chordovirinae* subfamily utilize immunomodulatory molecules, examples of which are provided herein, to counteract the immune responses of a host 15 organism. Thus, the invention described herein is not limited to vaccinia virus, but may be applicable to a number of viruses.

15 II. ENGINEERING OF POXVIRUSES

Viruses are frequently inactivated, inhibited or cleared by immunomodulatory molecules such as interferons (- α , - β , - γ) and tumor necrosis factor- α (TNF) (Moss, 1996). Host tissues and inflammatory/immune cells frequently secrete these molecules in response to 20 viral infection. These molecules can have direct antiviral effects and/or indirect effects through recruitment and/ or activation of inflammatory cells and lymphocytes. Given the importance of these immunologic clearance mechanisms, viruses have evolved to express 25 gene products that inhibit the induction and/or function of these cytokines/chemokines and interferons. For example, vaccinia virus (VV; and some other poxviruses) encodes the secreted protein vCKBP (B29R) that binds and inhibits the CC chemokines (e.g., RANTES, eotaxin, MIP-1-alpha) (Alcami *et al.*, 1998). Some VV strains also express a secreted viral 30 protein that binds and inactivates TNF (e.g., Lister A53R) (Alcami *et al.*, 1999). Most poxvirus strains have genes encoding secreted proteins that bind and inhibit the function of interferons- α / β (e.g., B18R) or interferon- γ (B8R). vC12L is an IL-18-binding protein that prevents IL-18 from inducing IFN- γ and NK cell/cytotoxic T-cell activation.

Most poxvirus virulence research has been performed in mice. Many, but not all, of 35 these proteins are active in mice (B18R, for example, is not). In situations in which these proteins are active against the mouse versions of the target cytokine, deletion of these genes

leads to reduced virulence and increased safety with VV mutants with deletions of or functional mutations in these genes. In addition, the inflammatory/immune response to and viral clearance of these mutants is often increased compared to the parental virus strain that expresses the inhibitory protein. For example, deletion of the T1/35kDa family of poxvirus-secreted proteins (chemokine-binding/-inhibitory proteins) can lead to a marked increase in leukocyte infiltration into virus-infected tissues (Graham *et al.*, 1997). Deletion of the vC12L gene in VV leads to reduced viral titers/toxicity following intranasal administration in mice; in addition, NK cell and cytotoxic T-lymphocyte activity is increased together with IFN- γ induction (Smith *et al.*, 2000). Deletion of the Myxoma virus T7 gene (able to bind IFN- γ and a broad range of chemokines) results in reduced virulence and significantly increased tissue inflammation/infiltration in a toxicity model (Upton *et al.*, 1992; Mossman *et al.*, 1996). Deletion of the M-T2 gene from myxoma virus also resulted in reduced virulence in a rabbit model (Upton *et al.* 1991). Deletion of the B18R anti-interferon- α - β gene product also leads to enhanced viral sensitivity to IFN-mediated clearance, reduced titers in normal tissues and reduced virulence (Symons *et al.*, 1995; Colamonici *et al.*, 1995; Alcami *et al.*, 2000). In summary, these viral gene products function to decrease the antiviral immune response and inflammatory cell infiltration into virus-infected tissues. Loss of protein function through deletion/mutation leads to decreased virulence and/or increased proinflammatory properties of the virus within host tissues. See PCT/US2003/025141, which is hereby incorporated by reference.

Cytokines and chemokines can have potent antitumoral effects (Vicari *et al.*, 2002; Homey *et al.*, 2002). These effects can be on tumor cells themselves directly (*e.g.*, TNF) or they can be indirect through effects on non-cancerous cells. An example of the latter is TNF, which can have antitumoral effects by causing toxicity to tumor-associated blood vessels; this leads to a loss of blood flow to the tumor followed by tumor necrosis. In addition, chemokines can act to recruit (and in some cases activate) immune effector cells such as neutrophils, eosinophils, macrophages and/or lymphocytes. These immune effector cells can cause tumor destruction by a number of mechanisms. These mechanisms include the expression of antitumoral cytokines (*e.g.*, TNF), expression of fas-ligand, expression of perforin and granzyme, recruitment of natural killer cells, *etc.* The inflammatory response can eventually lead to the induction of systemic tumor-specific immunity. Finally, many of these cytokines (*e.g.*, TNF) or chemokines can act synergistically with chemotherapy or radiation therapy to destroy tumors.

Clinically effective systemic administration of recombinant versions of these immunostimulatory proteins is not feasible due to (1) induction of severe toxicity with systemic administration and (2) local expression within tumor tissue is needed to stimulate local infiltration and antitumoral effects. Approaches are needed to achieve high local 5 concentrations of these molecules within tumor masses while minimizing levels in the systemic circulation. Viruses can be engineered to express cytokine or chemokine genes in an attempt to enhance their efficacy. Expression of these genes from replication-selective vectors has potential advantages over expression from non-replicating vectors. Expression from replicating viruses can result in higher local concentrations within tumor masses; in 10 addition, replicating viruses can help to induce antitumoral immunity through tumor cell destruction/oncolysis and release of tumor antigens in a proinflammatory environment. However, there are several limitations to this approach. Serious safety concerns arise from 15 the potential for release into the environment of a replication-competent virus (albeit tumor-selective) with a gene that can be toxic if expressed in high local concentrations. Viruses that express potent pro-inflammatory genes from their genome may therefore pose safety risks to the treated patient and to the general public. Even with tumor-targeting, replication-selective viruses expressing these genes, gene expression can occur in normal tissues resulting in toxicity. In addition, size limitations prevent expression of multiple and/or large genes from 20 viruses such as adenovirus; these molecules will definitely act more efficaciously in combination. Finally, many of the oncolytic viruses in use express anti-inflammatory proteins and therefore these viruses will counteract the induction of a proinflammatory milieu within the infected tumor mass. The result will be to inhibit induction of antitumoral immunity, antivascular effects and chemotherapy-/radiotherapy-sensitization.

A. Vaccinia Virus Products

25 1. Interferon-Modulating Polypeptides

Interferon- α - β blocks viral replication through several mechanisms. Interferon- γ has weaker direct viral inhibitory effects but is a potent inducer of cell-mediated immunity through several mechanisms. Viruses have evolved to express secreted gene products that are able to counteract the antiviral effects of interferons. For example, vaccinia virus (and other 30 poxviruses) encodes the secreted proteins B8R and B18R which bind interferon- γ and - α - β , respectively (Smith *et al.*, 1997; Symons *et al.*, 1995; Alcami *et al.*, 2000). An additional example of a vaccinia gene product that reduces interferon induction is the caspase-1 inhibitor

B13R which inhibits activation of the interferon- γ -inducing factor IL-18. Interferon modulating polypeptides include, but are not limited to, B18R, which may be termed B19R in other viral strains, such as the Copenhagen strain of Vaccinia virus; B8R; B13R; vC12L; A53R; E3L and other viral polypeptides with similar activities or properties. IFN modulating 5 polypeptides may be divided into the non-exclusive categories of those that preferentially modulate IFN α and/or β pathways (such as B18R, B8R, B13R, or vC12L) and those that modulate IFN γ pathways(for example B8R, B13R, or vC12L).

Cancer cells are frequently resistant to the effects of interferons. A number of mechanisms are involved. These include the fact that ras signal transduction pathway 10 activation (e.g., by ras mutation, upstream growth factor receptor overexpression/mutation, etc.), a common feature of cancer cells, leads to PKR inhibition. In addition, lymphocytes are often inhibited in tumor masses by a variety of mechanisms including IL-10 production and fas-L expression by tumor cells. Since lymphocytes are a major source of interferon- γ production, lymphocyte inhibition leads to a decrease in interferon- γ production in tumors. 15 Therefore, tumor masses tend to be sanctuaries from the effects of interferons. In addition, interferons themselves can have antitumoral effects. For example, IFN- γ can increase MHC class-I-associated antigen presentation; this will allow more efficient CTL-mediated killing of tumor cells. IFN- α/β , for example, can block angiogenesis within tumor masses and thereby block tumor growth.

20 2. Complement Control Polypeptides

A major mechanism for the clearance of viral pathogens is the killing of infected cells within the host or of virions within an organism by complement-dependent mechanisms. As 25 the infected cell dies it is unable to continue to produce infectious virus. In addition, during apoptosis intracellular enzymes are released which degrade DNA. These enzymes can lead to viral DNA degradation and virus inactivation. Apoptosis can be induced by numerous mechanisms including the binding of activated complement and the complement membrane attack complex. Poxviruses such as vaccinia have evolved to express gene products that are able to counteract the complement-mediated clearance of virus and/or virus-infected cells. These genes thereby prevent apoptosis and inhibit viral clearance by complement-dependent 30 mechanisms, thus allowing the viral infection to proceed and viral virulence to be increased. For example, vaccinia virus complement control proteins (VCP; e.g., C21L) have roles in the prevention of complement-mediated cell killing and/or virus inactivation (Isaacs *et al.*, 1992).

VCP also has anti-inflammatory effects since its expression decreases leukocyte infiltration into virally-infected tissues. Complement control polypeptides include, but are not limited to, VCP, also known as C3L or C21L.

5 Cancer cells frequently overexpress cellular anti-complement proteins; this allows cancer cells to survive complement attack +/- tumor-specific antibodies (Caragine *et al.*, 2002; Durrant *et al.*, 2001; Andoh *et al.* 2002). Therefore, agents that preferentially target tumor cells due to their inherent resistance to complement-mediated killing would have selectivity and potential efficacy in a wide range of human cancers (Durrant *et al.*, 2001). In addition, one of the hallmarks of cancer cells is a loss of normal apoptotic mechanisms (Gross 10 *et al.*, 1999). Resistance to apoptosis promotes carcinogenesis as well as resistance to antitumoral agents including immunologic, chemotherapeutic and radiotherapeutic agents (Eliopoulos *et al.*, 1995). Apoptosis inhibition can be mediated by a loss of pro-apoptotic molecule function (*e.g.*, bax), an increase in the levels/function of anti-apoptotic molecules (*e.g.*, bcl-2) and finally a loss of complement sensitivity.

15 **3. TNF-Modulating Polypeptides**

One of the various mechanisms for the clearance of viral pathogens is the killing of infected cells within the host by the induction of apoptosis, as described above. Apoptosis can be induced by numerous mechanisms including the binding of TNF and lymphotoxin-alpha (LT α) to cellular TNF receptors, which triggers intracellular signaling cascades. 20 Activation of the TNF receptors function in the regulation of immune and inflammatory responses, as well as inducing apoptotic cell death (Wallach *et al.*, 1999)

Various strains of poxviruses, including some vaccinia virus strains, have evolved to express gene products that are able to counteract the TNF-mediated clearance of virus and/or virus-infected cells. The proteins encoded by these genes circumvent the proinflammatory 25 and apoptosis inducing activities of TNF by binding and sequestering extracellular TNF, resulting in the inhibition of viral clearance. Because viruses are not cleared, the viral infection is allowed to proceed, and thus, viral virulence is increased. Various members of the poxvirus family express secreted viral TNF receptors (vTNFR). For example, several poxviruses encode vTNFRs, such as myxoma (T2 protein), cowpox and vaccinia virus strains, 30 such as Lister, may encode one or more of the CrmB, CrmC (A53R), CrmD, CrmE, B28R proteins and/or equivalents thereof. These vTNFRs have roles in the prevention of TNF-mediated cell killing and/or virus inactivation (Saraiva and Alcami, 2001). TNF modulatory

polypeptides include, but are not limited to, A53R, B28R (this protein is present, but may be inactive in the Copenhagen strain of vaccinia virus) and other polypeptides with similar activities or properties.

One of the hallmarks of cancer cells is aberrant gene expression, which may lead to a 5 loss of sensitivity to a number of molecular mechanisms for growth modulation, such as sensitivity to the anti-cancer activities of TNF. Thus, viral immunomodulatory mechanisms may not be required for the propagation of a virus within the tumor microenvironment.

4. Serine Protease Inhibitors

A major mechanism for the clearance of viral pathogens is the induction of apoptosis 10 in infected cells within the host. As the infected cell dies it is unable to continue to produce infectious virus. In addition, during apoptosis intracellular enzymes are released which degrade DNA. These enzymes can lead to viral DNA degradation and virus inactivation. Apoptosis can be induced by numerous mechanisms including the binding of cytokines (e.g., tumor necrosis factor), granzyme production by cytotoxic T-lymphocytes or fas-ligand 15 binding; caspase activation is a critical part of the final common apoptosis pathway. Viruses have evolved to express gene products that are able to counteract the intracellular signaling cascade induced by such molecules including fas-ligand or tumor necrosis factor (TNF)/TNF-related molecules (e.g., E3 10.4/ 14.5, 14.7 genes of adenovirus (Wold *et al.*, 1994); E1B-19kD of adenovirus (Boyd *et al.*, 1994); crmA from cowpox virus; B13R from vaccinia virus) 20 (Dobbelstein *et al.*, 1996; Kettle *et al.*, 1997). These gene products prevent apoptosis by apoptosis-inducing molecules and thus allow viral replication to proceed despite the presence of antiviral apoptosis-inducing cytokines, fas, granzyme or other stimulators of apoptosis.

VV SPI-2/B13R is highly homologous to cowpox CrmA; SPI-1 (VV) is weakly 25 homologous to CrmA (Dobbelstein *et al.*, 1996). These proteins are serpins (serine protease inhibitors) and both CrmA and SPI-2 have roles in the prevention of various forms of apoptosis. Inhibition of interleukin-1 β -converting enzyme (ICE) and granzyme, for example, can prevent apoptosis of the infected cell. These gene products also have anti-inflammatory effects. They are able to inhibit the activation of IL-18 which in turn would decrease IL-18-mediated induction of IFN- γ . The immunostimulatory effects of IFN- γ on cell-mediated 30 immunity are thereby inhibited (Kettle *et al.*, 1997). SPIs include, but are not limited to, B13R, B22R, and other polypeptides with similar activities or properties.

One of the hallmarks of cancer cells is a loss of normal apoptotic mechanisms (Gross *et al.*, 1999). Resistance to apoptosis promotes carcinogenesis as well as resistance to antitumoral agents including immunologic, chemotherapeutic and radiotherapeutic agents (Eliopoulos *et al.*, 1995). Apoptosis inhibition can be mediated by a loss of pro-apoptotic molecule function (*e.g.*, bax) or an increase in the levels/function of anti-apoptotic molecules (*e.g.*, bcl-2).

5. IL-1 β -Modulating Polypeptides

IL-1 β is a biologically active factors that acts locally and also systemically. Only a few functional differences between IL-1 β and IL-1 α have been described. The numerous 10 biological activities of IL-1 β is exemplified by the many different acronyms under which IL-1 has been described. IL-1 does not show species specificity with the exception of human IL-1 β that is inactive in porcine cells. Some of the biological activities of IL-1 are mediated indirectly by the induction of the synthesis of other mediators including ACTH (Corticotropin), PGE2 (prostaglandin E2), PF4 (platelet factor-4), CSF (colony stimulating 15 factors), IL-6, and IL-8. The synthesis of IL-1 may be induced by other cytokines including TNF- α , IFN- α , IFN- β and IFN- γ and also by bacterial endotoxins, viruses, mitogens, and antigens. The main biological activity of IL-1 is the stimulation of T-helper cells, which are induced to secrete IL-2 and to express IL-2 receptors. Virus-infected macrophages produce 20 large amounts of an IL-1 inhibitor that may support opportunistic infections and transformation of cells in patients with T-cell maturation defects. IL-1 acts directly on B-cells, promoting their proliferation and the synthesis of immunoglobulins. IL-1 also functions as one of the priming factors that makes B-cells responsive to IL-5. IL-1 stimulates the proliferation and activation of NK-cells and fibroblasts, thymocytes, glioblastoma cells.

Blockade of the synthesis of IL-1 β by the viral protein is regarded as a viral strategy 25 allowing systemic antiviral reactions elicited by IL-1 to be suppressed or diminished. Binding proteins effectively blocking the functions of IL-1 with similar activity as B15R have been found also to be encoded by genes of the cowpox virus. Vaccinia virus also encodes another protein, designated B8R, which behaves like a receptor for cytokines (Alcami and Smith, 1992; Spriggs *et al.*, 1992). IL-1 modulating polypeptides include, but are not limited to, 0 B13R, B15R, and other polypeptides with similar activities or properties.

One of the hallmarks of cancer cells is aberrant gene expression, which may lead to a loss of sensitivity to a number of molecular mechanisms for growth modulation, such as

sensitivity to the anti-cancer activities of IL-1. Thus, viral immunomodulatory mechanisms may not be required for the propagation of a virus within the tumor microenvironment.

6. EEV Form

5 Viral spread to metastatic tumor sites, and even spread within an infected solid tumor mass, is generally inefficient (Heise *et al.*, 1999). Intravenous administration typically results in viral clearance or inactivation by antibodies (e.g., adenovirus) (Kay *et al.*, 1997) and/or the complement system (e.g., HSV) (Ikeda *et al.*, 1999). In addition to these immune-mediated mechanisms, the biodistribution of these viruses results in the vast majority of intravenous virus depositing within normal tissues rather than in tumor masses. Intravenous adenovirus, 10 for example, primarily ends up within the liver and spleen; less than 0.1% of the input virus depositing within tumors, even in immunodeficient mice (Heise *et al.*, 1999). Therefore, although some modest antitumoral efficacy can be demonstrated with extremely high relative doses in immunodeficient mouse tumor models, intravenous delivery is extremely inefficient and significantly limits efficacy.

15 Vaccinia virus has the ability to replicate within solid tumors and cause necrosis. In addition, thymidine kinase-deletion mutants can infect tumor masses and ovarian tissue and express marker genes preferentially in mouse tumor model systems (Gnant *et al.*, 1999). However, since these studies generally determined tumor targeting based on marker gene expression after ≥ 5 days, it is unclear whether the virus preferentially deposits in, expresses 20 genes in or replicates in tumor/ovary tissue (Puhlmann *et al.*, 2000). Regardless of the mechanism, the antitumoral efficacy of this virus without additional transgenes was not statistically significant (Gnant *et al.*, 1999). In contrast, intratumoral virus injection had significant antitumoral efficacy (McCart *et al.* 2000). Therefore, i.v. efficacy could be improved if i.v. delivery to the tumor were to be improved.

25 Vaccinia virus replicates in cells and produces both intracellular virus (IMV, intracellular mature virus; IEV, intracellular enveloped virus) and extracellular virus (EEV, extracellular enveloped virus; CEV, cell-associated extracellular virus) (Smith *et al.*, 1998). IMV represents approximately 99% of virus yield following replication by wild-type vaccinia virus strains. This virus form is relatively stable in the environment, and therefore it is 30 primarily responsible for spread between individuals; in contrast, this virus does not spread efficiently within the infected host due to inefficient release from cells and sensitivity to complement and/or antibody neutralization. In contrast, EEV is released into the extracellular

milieu and typically represents only approximately 1% of the viral yield (Smith *et al.*, 1998). EEV is responsible for viral spread within the infected host and is relatively easily degraded outside of the host. Importantly, EEV has developed several mechanisms to inhibit its neutralization within the bloodstream. First, EEV is relatively resistant to complement 5 (Vanderplasschen *et al.*, 1998); this feature is due to the incorporation of host cell inhibitors of complement into its outer membrane coat plus secretion of Vaccinia virus complement control protein (VCP) into local extracellular environment. Second, EEV is relatively resistant to neutralizing antibody effects compared to IMV (Smith *et al.*, 1997). EEV is also released at earlier time points following infection (*e.g.*, 4-6 hours) than is IMV (which is only 10 released during/ after cell death), and therefore spread of the EEV form is faster (Blasco *et al.*, 1993).

Unfortunately, however, wild-type vaccinia strains make only very small amounts of EEV, relatively. In addition, treatment with vaccinia virus (*i.e.*, the input dose of virus) has been limited to intracellular virus forms to date. Standard vaccinia virus (VV) manufacturing 15 and purification procedures lead to EEV inactivation (Smith *et al.*, 1998), and non-human cell lines are frequently used to manufacture the virus; EEV from non-human cells will not be protected from complement-mediated clearance (complement inhibitory proteins acquired from the cell by EEV have species restricted effects). Vaccinia virus efficacy has therefore been limited by the relative sensitivity of the IMV form to neutralization and by its inefficient 20 spread within solid tumor masses; this spread is typically from cell to adjacent cell. IMV spread to distant tumor masses, either through the bloodstream or lymphatics, is also inefficient.

Therefore, the rare EEV form of vaccinia virus has naturally acquired features that make it superior to the vaccinia virus form used in patients to date (IMV); EEV is optimized 25 for rapid and efficient spread through solid tumors locally and to regional or distant tumor sites. Since EEV is relatively resistant to complement effects, when it is grown in a cell type from the same species, this virus form will have enhanced stability and retain activity longer in the blood following intravascular administration than standard preparations of vaccinia virus (which contain exclusively IMV) (Smith *et al.*, 1998). Since EEV is resistant to 30 antibody-mediated neutralization, this virus form will retain activity longer in the blood following intravascular administration than standard preparations of vaccinia virus (which contain almost exclusively IMV) (Vanderplasschen *et al.*, 1998). This feature will be particularly important for repeat administration once neutralizing antibody levels have

increased; all approved anti-cancer therapies require repeat administration. Therefore, the EEV form of vaccinia, and other poxviruses, will result in superior delivery of therapeutic viruses and their genetic payload to tumors through the bloodstream. This will lead to enhanced systemic efficacy compared with standard poxvirus preparations. Finally, the risk
5 of transmission to individuals in the general public should be reduced significantly since EEV is extremely unstable outside of the body. Polypeptides involved in the modulation of the EEV form of a virus include, but are not limited to, A34R, B5R, and various other proteins that influence the production of the EEV form of the poxviruses. A mutation at codon 151 of A34R from a lysine to a aspartic acid (K151D mutation) renders the A34R protein less able to tether the EEV form to the cell membrane. B5R is an EEV-membrane bound polypeptide that may bind complement. The total deletion of A43R may lead to increased EEV release, but markedly reduced infectivity of the viruses, while the K151D mutation increases EEV release while maintaining infectivity of the released viruses. B5R has sequence homology to VCP (anti-complement), but complement inhibition has not yet been proven.

15 Briefly, one method for identifying a fortified EEV form is as follows. EEV are diluted in ice-cold MEM and mixed (1:1 volume) with active or heat-inactivated (56°C, 30 min, control) serum diluted in ice-cold MEM (final dilution of serum 1/10, 1/20, or 1/30). After incubation or 75 minutes at 7°C, samples are cooled on ice and mAb 5B4/2F2 is added to fresh EEV samples to neutralize any contaminates (IMV and ruptured EEV). Virions are
20 then bound to RK13 cells for one hour on ice, complement and unbound virions are washed away, and the number of plaques are counted two days later. The higher the plaque number the greater the resistance to complement. Vanderplasschen *et al.* (1998), herein incorporated by reference. Exemplary methods describing the isolation of EEV forms of Vaccinia virus can be found in Blasco *et al.* (1992) (incorporated herein by reference).

25 7. Other Polypeptides

Other viral immunomodulatory polypeptides may include polypeptides that bind other mediators of the immune response and/or modulate molecular pathways associated with the immune response. For example, chemokine binding polypeptides such as B29R (this protein is present, but may be inactive in the Copenhagen strain of Vaccinia virus), C23L, vCKBP,
30 A41L and polypeptides with similar activities or properties. Other vaccinia virus proteins such as the vaccinia virus growth factor (*e.g.*, C11L), which is a viral EGF-like growth factor, may also be the target for alteration in some embodiments of the invention. Other

polypeptides that may be classified as viral immunomodulatory factors include, but are not limited to B7R, N1L, or other polypeptides that whose activities or properties increase the virulence of a poxvirus.

8. **Vaccinia Virus-Induced Cell Fusion**

5 In certain embodiments of the invention an alteration, deletion, or mutation of A56R or K2L encoding nucleic genes may lead to cell-cell fusion or syncitia formation induced by VV infection. Vaccinia virus-induced cell fusion will typically increase antitumoral efficacy of VV due to intratumoral viral spread. Intratumoral viral spreading by cell fusion will typically allow the virus to avoid neutralizing antibodies and immune responses. Killing and
10 infection of adjacent uninfected cells (*i.e.*, a “bystander” effect) may be more efficient in VV with mutations in one or both of these genes, which may result in improved local antitumoral effects.

B. **Virus Propagation**

Vaccinia virus may be propagated using the methods described by Earl and Moss in
15 Ausbel *et al.*, Current Protocols in Molecular Biology, pages 16.15.1 to 16.18.10, which is incorporated by reference herein.

III. **PROTEINACEOUS AND NUCLEIC ACID COMPOSITIONS**

The present invention concerns poxviruses that are advantageous in the study and treatment of cancer cells and cancer in a patient. It concerns vaccinia viruses, optionally
20 constructed with one or more mutations compared to wild-type such that the virus has desirable properties for use against cancer cells, while being less toxic or non-toxic to non-cancer cells. Such poxviruses are described in PCT/US2003/025141, which is incorporated herein by reference. The teachings described below provide various protocols, by way of example, of implementing methods and compositions of the invention. They provide
25 background for generating mutated viruses through the use of recombinant DNA technology.

A. **Proteinaceous Compositions**

In certain embodiments, the present invention concerns generating vaccinia virus, optionally those that lack one or more functional polypeptides or proteins and/or generating
30 poxviruses that have the ability to release more of a particular form of the virus, such as an infectious EEV form. In other embodiments, the present invention concerns poxviruses and

their use in combination with proteinaceous composition as part of a pharmaceutically acceptable formulation.

As used herein, a “protein” or “polypeptide” refers to a molecule comprising at least one amino acid residue. In some embodiments, a wild-type version of a protein or polypeptide are employed, however, in many embodiments of the invention, a viral protein or polypeptide is absent or altered so as to render the virus more useful for the treatment of a cancer cells or cancer in a patient. The terms described above may be used interchangeably herein. A “modified protein” or “modified polypeptide” refers to a protein or polypeptide whose chemical structure is altered with respect to the wild-type protein or polypeptide. In some embodiments, a modified protein or polypeptide has at least one modified activity or function (recognizing that proteins or polypeptides may have multiple activities or functions). The modified activity or function may be reduced, diminished, eliminated, enhanced, improved, or altered in some other way (such as specificity) with respect to that activity or function in a wild-type protein or polypeptide. It is specifically contemplated that a modified protein or polypeptide may be altered with respect to one activity or function yet retain wild-type activity or function in other respects. Alternatively, a modified protein may be completely nonfunctional or its cognate nucleic acid sequence may have been altered so that the polypeptide is no longer expressed at all, is truncated, or expresses a different amino acid sequence as a result of a frameshift.

In certain embodiments the size of a mutated protein or polypeptide may comprise, but is not limited to, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975, 1000, 1100, 1200, 1300, 1400, 1500, 1750, 2000, 2250, 2500 or greater amino molecule residues, and any range derivable therefrom. It is contemplated that polypeptides may be mutated by truncation, rendering them shorter than their corresponding wild-type form.

As used herein, an “amino molecule” refers to any amino acid, amino acid derivative or amino acid mimic as would be known to one of ordinary skill in the art. In certain

embodiments, the residues of the proteinaceous molecule are sequential, without any non-amino molecule interrupting the sequence of amino molecule residues. In other embodiments, the sequence may comprise one or more non-amino molecule moieties. In particular embodiments, the sequence of residues of the proteinaceous molecule may be 5 interrupted by one or more non-amino molecule moieties.

Accordingly, the term “proteinaceous composition” encompasses amino molecule sequences comprising at least one of the 20 common amino acids in naturally synthesized proteins, or at least one modified or unusual amino acid.

Proteinaceous compositions may be made by any technique known to those of skill in 10 the art, including the expression of proteins, polypeptides or peptides through standard molecular biological techniques, the isolation of proteinaceous compounds from natural sources, or the chemical synthesis of proteinaceous materials. The nucleotide and protein, polypeptide and peptide sequences for various genes have been previously disclosed, and may be found at computerized databases known to those of ordinary skill in the art. One such 15 database is the National Center for Biotechnology Information's GenBank and GenPept databases (www.ncbi.nlm.nih.gov). The coding regions for these known genes may be amplified and/or expressed using the techniques disclosed herein or as would be known to those of ordinary skill in the art.

1. Functional Aspects

20 When the present application refers to the function or activity of viral proteins or polypeptides, it is meant to refer to the activity or function of that viral protein or polypeptide under physiological conditions, unless otherwise specified. For example, an interferon-modulating polypeptide refers to a polypeptide that affects at least one interferon and its activity, either directly or indirectly. The polypeptide may induce, enhance, raise, increase, 25 diminish, weaken, reduce, inhibit, or mask the activity of an interferon, directly or indirectly. An example of directly affecting interferon involves, in some embodiments, an interferon-modulating polypeptide that specifically binds to the interferon. Determination of which molecules possess this activity may be achieved using assays familiar to those of skill in the art. For example, transfer of genes encoding products that modulate interferon, or variants 30 thereof, into cells that are induced for interferon activity compared to cells with such transfer of genes may identify, by virtue of different levels of an interferon response, those molecules having a interferon-modulating function.

It is specifically contemplated that a modulator may be a molecule that affects the expression proteinaceous compositions involved in the targeted molecule's pathway, such as by binding an interferon-encoding transcript. Determination of which molecules are suitable modulators of interferon, IL-1 β , TNF, or other molecules of therapeutic benefit may be 5 achieved using assays familiar to those of skill in the art—some of which are disclosed herein—and may include, for example, the use of native and/or recombinant viral proteins.

2. Variants of Viral Polypeptides

Amino acid sequence variants of the polypeptides of the present invention can be substitutional, insertional or deletion variants. A mutation in a gene encoding a viral polypeptide 10 may affect 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 275, 300, 325, 350, 15 375, 400, 425, 450, 475, 500 or more non-contiguous or contiguous amino acids of the polypeptide, as compared to wild-type. Various polypeptides encoded by Vaccinia Virus may be identified by reference to Rosel *et al.* (1986), Goebel *et al.* (1990) and GenBank Accession Number NC_001559, each of which is incorporated herein by reference.

Deletion variants lack one or more residues of the native or wild-type protein. Individual 20 residues can be deleted or all or part of a domain (such as a catalytic or binding domain) can be deleted. A stop codon may be introduced (by substitution or insertion) into an encoding nucleic acid sequence to generate a truncated protein. Insertional mutants typically involve the addition of material at a non-terminal point in the polypeptide. This may include the insertion of an immunoreactive epitope or simply one or more residues. Terminal additions, called fusion 25 proteins, may also be generated.

Substitutional variants typically contain the exchange of one amino acid for another at one or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide, with or without the loss of other functions or properties. Substitutions may be conservative, that is, one amino acid is replaced with one of similar shape and charge. 30 Conservative substitutions are well known in the art and include, for example, the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to

asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine. Alternatively, substitutions may be non-conservative such that a function or activity of the polypeptide is affected. Non-conservative changes typically involve substituting a residue with one that is chemically dissimilar, such as a polar or charged amino acid for a nonpolar or uncharged amino acid, and vice versa.

The term “functionally equivalent codon” is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine, and also refers to codons that encode biologically equivalent amino acids (see Table 1, below).

It also will be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5' or 3' sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein activity where protein expression is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, *i.e.*, introns, which are known to occur within genes.

Table 1: Codon Table

Amino Acids			Codons				
Alanine	Ala	A	GCA	GCC	GCG	GCU	
Cysteine	Cys	C	UGC	UGU			
Aspartic acid	Asp	D	GAC	GAU			
Glutamic acid	Glu	E	GAA	GAG			
Phenylalanine	Phe	F	UUC	UUU			
Glycine	Gly	G	GGA	GGC	GGG	GGU	
Histidine	His	H	CAC	CAU			
Isoleucine	Ile	I	AUA	AUC	AUU		
Lysine	Lys	K	AAA	AAG			
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG
Methionine	Met	M	AUG				CUU
Asparagine	Asn	N	AAC	AAU			
Proline	Pro	P	CCA	CCC	CCG	CCU	
Glutamine	Gln	Q	CAA	CAG			
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG
Threonine	Thr	T	ACA	ACC	ACG	ACU	
Valine	Val	V	GUA	GUC	GUG	GUU	
Tryptophan	Trp	W	UGG				
Tyrosine	Tyr	Y	UAC	UAU			

The following is a discussion based upon changing of the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions 5 of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid substitutions can be made in a protein sequence, and in its underlying DNA coding sequence, and nevertheless produce a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the DNA sequences of genes without appreciable loss of 10 their biological utility or activity, as discussed below. Table 1 shows the codons that encode particular amino acids.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982). It is accepted that the 15 relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

It also is understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by 20 reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned 25 to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine *-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still produce a biologically equivalent and immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity 30 values are within ±2 is preferred, those that are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions generally are based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take into consideration the various foregoing characteristics are well known to those of skill in the art and include: 5 arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

IV. NUCLEIC ACID MOLECULES

A. Polynucleotides Encoding Native Proteins or Modified Proteins

The present invention concerns polynucleotides, isolatable from cells, that are capable 10 of expressing all or part of a protein or polypeptide. In some embodiments of the invention, it concerns a viral genome that has been specifically mutated to generate a virus that lacks certain functional viral polypeptides. The polynucleotides may encode a peptide or polypeptide containing all or part of a viral amino acid sequence or they be engineered so they do not encode such a viral polypeptide or encode a viral polypeptide having at least one 15 function or activity reduced, diminished, or absent. Recombinant proteins can be purified from expressing cells to yield active proteins. The genome, as well as the definition of the coding regions of vaccinia virus may be found in Rosel *et al.* (1986), Goebel *et al.* (1990) and/or GenBank Accession Number NC_00159, each of which is incorporated herein by reference.

20 As used herein, the term “DNA segment” refers to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a DNA segment encoding a polypeptide refers to a DNA segment that contains wild-type, polymorphic, or mutant polypeptide-coding sequences yet is isolated away from, or purified free from, total mammalian or human genomic DNA. Included within the term “DNA segment” are a 25 polypeptide or polypeptides, DNA segments smaller than a polypeptide, and recombinant vectors, including, for example, plasmids, cosmids, phage, viruses, and the like.

As used in this application, the term “poxvirus polynucleotide” refers to a nucleic acid 30 molecule encoding a poxvirus polypeptide that has been isolated free of total genomic nucleic acid. Similarly, a “vaccinia virus polynucleotide” refers to a nucleic acid molecule encoding a vaccinia virus polypeptide that has been isolated free of total genomic nucleic acid. A “poxvirus genome” or a “vaccinia virus genome” refers to a nucleic acid molecule that can be provided to a host cell to yield a viral particle, in the presence or absence of a helper virus.

The genome may or may have not been recombinantly mutated as compared to wild-type virus.

The term "cDNA" is intended to refer to DNA prepared using messenger RNA (mRNA) as template. The advantage of using a cDNA, as opposed to genomic DNA or DNA polymerized from a genomic, non- or partially-processed RNA template, is that the cDNA primarily contains coding sequences of the corresponding protein. There may be times when the full or partial genomic sequence is preferred, such as where the non-coding regions are required for optimal expression or where non-coding regions such as introns are to be targeted in an antisense strategy.

It also is contemplated that a particular polypeptide from a given species may be represented by natural variants that have slightly different nucleic acid sequences but, nonetheless, encode the same protein (see Table 1 above).

Similarly, a polynucleotide comprising an isolated or purified wild-type or mutant polypeptide gene refers to a DNA segment including wild-type or mutant polypeptide coding sequences and, in certain aspects, regulatory sequences, isolated substantially away from other naturally occurring genes or protein encoding sequences. In this respect, the term "gene" is used for simplicity to refer to a functional protein, polypeptide, or peptide-encoding unit (including any sequences required for proper transcription, post-translational modification, or localization). As will be understood by those in the art, this functional term includes genomic sequences, cDNA sequences, and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides, domains, peptides, fusion proteins, and mutants. A nucleic acid encoding all or part of a native or modified polypeptide may contain a contiguous nucleic acid sequence encoding all or a portion of such a polypeptide of the following lengths: 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 441, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800, 810, 820, 830, 840, 850, 860, 870, 880, 890, 900, 910, 920, 930, 940, 950, 960, 970, 980, 990, 1000, 1010, 1020, 1030, 1040, 1050, 1060, 1070, 1080, 1090, 1095, 1100, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 9000, 10000, or more nucleotides, nucleosides, or base pairs.

In particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences that encode a wild-type or mutant poxvirus polypeptide or peptide that includes within its amino acid sequence a contiguous amino acid sequence in accordance with, or essentially corresponding to a native polypeptide.

5 Thus, an isolated DNA segment or vector containing a DNA segment may encode, for example, a TNF modulator or TNF-modulating polypeptide that can inhibit or reduce TNF activity. The term "recombinant" may be used in conjunction with a polypeptide or the name of a specific polypeptide, and this generally refers to a polypeptide produced from a nucleic acid molecule that has been manipulated *in vitro* or that is the replicated product of such a 10 molecule.

In other embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences that encode a polypeptide or peptide that includes within its amino acid sequence a contiguous amino acid sequence in accordance with, or essentially corresponding to the polypeptide.

15 The nucleic acid segments used in the present invention, regardless of the length of the coding sequence itself, may be combined with other nucleic acid sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, 20 with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol.

It is contemplated that the nucleic acid constructs of the present invention may encode 25 full-length polypeptide from any source or encode a truncated version of the polypeptide, for example a truncated vaccinia virus polypeptide, such that the transcript of the coding region represents the truncated version. The truncated transcript may then be translated into a truncated protein. Alternatively, a nucleic acid sequence may encode a full-length 30 polypeptide sequence with additional heterologous coding sequences, for example to allow for purification of the polypeptide, transport, secretion, post-translational modification, or for therapeutic benefits such as targeting or efficacy. As discussed above, a tag or other heterologous polypeptide may be added to the modified polypeptide-encoding sequence, wherein "heterologous" refers to a polypeptide that is not the same as the modified polypeptide.

In a non-limiting example, one or more nucleic acid constructs may be prepared that include a contiguous stretch of nucleotides identical to or complementary to the a particular gene, such as the B18R gene. A nucleic acid construct may be at least 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 400, 500, 600, 700, 5 800, 900, 1,000, 2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, 15,000, 20,000, 30,000, 50,000, 100,000, 250,000, 500,000, 750,000, to at least 1,000,000 nucleotides 10 in length, as well as constructs of greater size, up to and including chromosomal sizes (including all intermediate lengths and intermediate ranges), given the advent of nucleic acids constructs such as a yeast artificial chromosome are known to those of ordinary skill in the art. It will be readily understood that "intermediate lengths" and "intermediate ranges," as used herein, means any length or range including or between the quoted values (*i.e.*, all integers including and between such values).

The DNA segments used in the present invention encompass biologically functional equivalent modified polypeptides and peptides, for example, a modified gelonin toxin. Such 15 sequences may arise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. 20 Changes designed by human may be introduced through the application of site-directed mutagenesis techniques, *e.g.*, to introduce improvements to the antigenicity of the protein, to reduce toxicity effects of the protein *in vivo* to a subject given the protein, or to increase the efficacy of any treatment involving the protein.

In certain other embodiments, the invention concerns isolated DNA segments and 25 recombinant vectors that include within their sequence a contiguous nucleic acid sequence from that shown in sequences identified herein (and/or incorporated by reference). Such sequences, however, may be mutated to yield a protein product whose activity is altered with respect to wild-type.

It also will be understood that this invention is not limited to the particular nucleic 30 acid and amino acid sequences of these identified sequences. Recombinant vectors and isolated DNA segments may therefore variously include the poxvirus-coding regions themselves, coding regions bearing selected alterations or modifications in the basic coding

region, or they may encode larger polypeptides that nevertheless include poxvirus-coding regions or may encode biologically functional equivalent proteins or peptides that have variant amino acids sequences.

The DNA segments of the present invention encompass biologically functional equivalent poxvirus proteins and peptides. Such sequences may arise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by man may be introduced through the application of site-directed mutagenesis techniques, *e.g.*, to introduce improvements to the antigenicity of the protein.

B. Mutagenesis of Poxvirus Polynucleotides

In various embodiments, the poxvirus polynucleotide may be altered or mutagenized. Alterations or mutations may include insertions, deletions, point mutations, inversions, and the like and may result in the modulation, activation and/or inactivation of certain pathways or molecular mechanisms, as well as altering the function, location, or expression of a gene product, in particular rendering a gene product non-functional. Where employed, mutagenesis of a polynucleotide encoding all or part of a Poxvirus may be accomplished by a variety of standard, mutagenic procedures (Sambrook *et al.*, 1989). Mutation is the process whereby changes occur in the quantity or structure of an organism. Mutation can involve modification of the nucleotide sequence of a single gene, blocks of genes or whole chromosome. Changes in single genes may be the consequence of point mutations which involve the removal, addition or substitution of a single nucleotide base within a DNA sequence, or they may be the consequence of changes involving the insertion or deletion of large numbers of nucleotides.

Mutations may be induced following exposure to chemical or physical mutagens. Such mutation-inducing agents include ionizing radiation, ultraviolet light and a diverse array of chemical such as alkylating agents and polycyclic aromatic hydrocarbons all of which are capable of interacting either directly or indirectly (generally following some metabolic biotransformations) with nucleic acids. The DNA damage induced by such agents may lead to modifications of base sequence when the affected DNA is replicated or repaired and thus to

a mutation. Mutation also can be site-directed through the use of particular targeting methods.

1. Random Mutagenesis

a. Insertional Mutagenesis

5 Insertional mutagenesis is based on the inactivation of a gene via insertion of a known DNA fragment. Because it involves the insertion of some type of DNA fragment, the mutations generated are generally loss-of-function, rather than gain-of-function mutations. However, there are several examples of insertions generating gain-of-function mutations. Insertion mutagenesis has been very successful in bacteria and Drosophila (Cooley *et al.*, 10 1988) and recently has become a powerful tool in corn (Arabidopsis; (Marks *et al.*, 1991; Koncz *et al.* 1990); and Antirrhinum (Sommer *et al.*, 1990). Insertional mutagenesis may be accomplished using standard molecular biology techniques.

b. Chemical Mutagenesis

15 Chemical mutagenesis offers certain advantages, such as the ability to find a full range of mutations with degrees of phenotypic severity, and is facile and inexpensive to perform. The majority of chemical carcinogens produce mutations in DNA. Benzo[a]pyrene, N-acetoxy-2-acetyl aminofluorene and aflotoxin B1 cause GC to TA transversions in bacteria and mammalian cells. Benzo[a]pyrene also can produce base substitutions such as AT to TA. N-nitroso compounds produce GC to AT transitions. Alkylation of the O4 position of 20 thymine induced by exposure to n-nitrosoureas results in TA to CG transitions.

c. Radiation Mutagenesis

25 Biological molecules are degraded by ionizing radiation. Adsorption of the incident energy leads to the formation of ions and free radicals, and breakage of some covalent bonds. Susceptibility to radiation damage appears quite variable between molecules, and between different crystalline forms of the same molecule. It depends on the total accumulated dose, and also on the dose rate (as once free radicals are present, the molecular damage they cause depends on their natural diffusion rate and thus upon real time). Damage is reduced and controlled by making the sample as cold as possible. Ionizing radiation causes DNA damage, generally proportional to the dose rate.

30 In the present invention, the term "ionizing radiation" means radiation comprising particles or photons that have sufficient energy or can produce sufficient energy to produce ionization (gain or loss of electrons). An exemplary and preferred ionizing radiation is an x-

radiation. The amount of ionizing radiation needed in a given cell or for a particular molecule generally depends upon the nature of that cell or molecule and the nature of the mutation target. Means for determining an effective amount of radiation are well known in the art.

d. *In Vitro* Scanning Mutagenesis

5 Random mutagenesis also may be introduced using error prone PCR. The rate of mutagenesis may be increased by performing PCR in multiple tubes with dilutions of templates.

10 One particularly useful mutagenesis technique is alanine scanning mutagenesis in which a number of residues are substituted individually with the amino acid alanine so that the effects of losing side-chain interactions can be determined, while minimizing the risk of 15 large-scale perturbations in protein conformation (Cunningham *et al.*, 1989).

15 *In vitro* scanning saturation mutagenesis provides a rapid method for obtaining a large amount of structure-function information including (i) identification of residues that modulate ligand binding specificity, (ii) a better understanding of ligand binding based on the 20 identification of those amino acids that retain activity and those that abolish activity at a given location, (iii) an evaluation of the overall plasticity of an active site or protein subdomain, (iv) identification of amino acid substitutions that result in increased binding.

2. Site-Directed Mutagenesis

20 Structure-guided site-specific mutagenesis represents a powerful tool for the dissection and engineering of protein-ligand interactions (Wells, 1996; Braisted *et al.*, 1996). The technique provides for the preparation and testing of sequence variants by introducing one or 25 more nucleotide sequence changes into a selected DNA.

25 Site-specific mutagenesis uses specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent, unmodified nucleotides. In this way, a primer sequence is provided with sufficient size and complexity to form a stable duplex on both sides of the deletion junction being traversed. A primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

30 The technique typically employs a bacteriophage vector that exists in both a single-stranded and double-stranded form. Vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use

is generally well known to those skilled in the art. Double-stranded plasmids are also routinely employed in site-directed mutagenesis, which eliminates the step of transferring the gene of interest from a phage to a plasmid.

5 In general, one first obtains a single-stranded vector, or melts two strands of a double-stranded vector, which includes within its sequence a DNA sequence encoding the desired protein or genetic element. An oligonucleotide primer bearing the desired mutated sequence, synthetically prepared, is then annealed with the single-stranded DNA preparation, taking into account the degree of mismatch when selecting hybridization conditions. The hybridized product is subjected to DNA polymerizing enzymes such as *E. coli* polymerase I (Klenow 10 fragment) in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed, wherein one strand encodes the original non-mutated sequence, and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate host cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

15 Comprehensive information on the functional significance and information content of a given residue of protein can best be obtained by saturation mutagenesis in which all 19 amino acid substitutions are examined. The shortcoming of this approach is that the logistics of multiresidue saturation mutagenesis are daunting (Warren *et al.*, 1996, Zeng *et al.*, 1996; Burton and Barbas, 1994; Yelton *et al.*, 1995; Hilton *et al.*, 1996). Hundreds, and possibly 20 even thousands, of site specific mutants must be studied. However, improved techniques make production and rapid screening of mutants much more straightforward. See also, U.S. Patents 5,798,208 and 5,830,650, for a description of "walk-through" mutagenesis. Other methods of site-directed mutagenesis are disclosed in U.S. Patents 5,220,007; 5,284,760; 5,354,670; 5,366,878; 5,389,514; 5,635,377; and 5,789,166.

25 **C. Vectors**

The term "vector" is used to refer to a carrier nucleic acid molecule into which an exogenous nucleic acid sequence can be inserted for introduction into a cell where it can be replicated. A nucleic acid sequence can be "exogenous," which means that it is foreign to the cell into which the vector is being introduced or that the sequence is homologous to a 30 sequence in the cell but in a position within the host cell nucleic acid in which the sequence is ordinarily not found. Vectors include plasmids, cosmids, viruses (bacteriophage, animal viruses, and plant viruses), and artificial chromosomes (e.g., YACs). One of skill in the art

would be well equipped to construct a vector through standard recombinant techniques, which are described in Sambrook *et al.* (1989) and Ausubel *et al.* (1994), both incorporated herein by reference. In addition to encoding a modified polypeptide such as modified gelonin, a vector may encode non-modified polypeptide sequences such as a tag or targeting molecule.

5 Useful vectors encoding such fusion proteins include pIN vectors (Inouye *et al.*, 1985), vectors encoding a stretch of histidines, and pGEX vectors, for use in generating glutathione S-transferase (GST) soluble fusion proteins for later purification and separation or cleavage. A targeting molecule is one that directs the modified polypeptide to a particular organ, tissue, cell, or other location in a subject's body.

10 The term "expression vector" refers to a vector containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. In other cases, these sequences are not translated, for example, in the production of antisense molecules or ribozymes. Expression vectors can contain a variety of "control sequences," which refer to 15 nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host organism. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described *infra*.

20 In accordance with the present invention, vaccinia virus is itself an expression vector. There are other viral and non-viral vectors that may also be used to engineer the vaccinia viruses of the present invention. In one embodiment, such vectors may be engineered to express GM-CSF.

1. Promoters and Enhancers

25 A "promoter" is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind such as RNA polymerase and other transcription factors. The phrases "operatively positioned," "operatively linked," "under control," and "under transcriptional control" mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or 30 expression of that sequence. A promoter may or may not be used in conjunction with an "enhancer," which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

A promoter may be one naturally associated with a gene or sequence, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other prokaryotic, viral, or eukaryotic cell, and promoters or enhancers not "naturally occurring," *i.e.*, containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™, in connection with the compositions disclosed herein (see U.S. Patent 4,683,202, U.S. Patent 5,928,906, each incorporated herein by reference). Furthermore, it is contemplated the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

Naturally, it may be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the cell type, organelle, and organism chosen for expression. In certain embodiments of the invention, the promoter is a vaccinia virus promoter that is active during the replication cycle of vaccinia virus. In particular, the promoter may be the vaccinia virus late promoter—expression under control of late promoter ties expression to later stage in replication cycle, resulting in enhanced cancer-selectivity (because late gene expression should be minimal or non-existent in normal tissues). Gene expression could also be controlled under the synthetic early-late promoter to maximize the duration and the level of gene expression. Mastrangelo *et al.*, 1999, which is hereby incorporated by reference.

Those of skill in the art of molecular biology generally know the use of promoters, enhancers, and cell type combinations for protein expression, for example, see Sambrook *et al.* (1989), incorporated herein by reference. The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level

expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous. Particular promoters are those that can be active in the cytoplasm because the virus replicates in the cytoplasm.

5 Table 2 lists several elements/promoters that may be employed, in the context of certain embodiments of the present invention, to regulate the expression of a gene. This list is not intended to be exhaustive of all the possible elements involved in the promotion of expression but, merely, to be exemplary thereof. Table 3 provides examples of inducible elements, which are regions of a nucleic acid sequence that can be activated in response to a
10 specific stimulus.

TABLE 2
Promoter and/or Enhancer

Promoter/Enhancer	References
Immunoglobulin Heavy Chain	Banerji <i>et al.</i> , 1983; Gilles <i>et al.</i> , 1983; Grosschedl <i>et al.</i> , 1985; Atchinson <i>et al.</i> , 1986, 1987; Imler <i>et al.</i> , 1987; Weinberger <i>et al.</i> , 1984; Kiledjian <i>et al.</i> , 1988; Porton <i>et al.</i> ; 1990
Immunoglobulin Light Chain	Queen <i>et al.</i> , 1983; Picard <i>et al.</i> , 1984
T-Cell Receptor	Luria <i>et al.</i> , 1987; Winoto <i>et al.</i> , 1989; Redondo <i>et al.</i> ; 1990
HLA DQ α and/or DQ β	Sullivan <i>et al.</i> , 1987
β -Interferon	Goodbourn <i>et al.</i> , 1986; Fujita <i>et al.</i> , 1987; Goodbourn <i>et al.</i> , 1988
Interleukin-2	Greene <i>et al.</i> , 1989
Interleukin-2 Receptor	Greene <i>et al.</i> , 1989; Lin <i>et al.</i> , 1990
MHC Class II 5	Koch <i>et al.</i> , 1989
MHC Class II HLA-DR α	Sherman <i>et al.</i> , 1989
β -Actin	Kawamoto <i>et al.</i> , 1988; Ng <i>et al.</i> ; 1989
Muscle Creatine Kinase (MCK)	Jaynes <i>et al.</i> , 1988; Horlick <i>et al.</i> , 1989; Johnson <i>et al.</i> , 1989
Prealbumin (Transthyretin)	Costa <i>et al.</i> , 1988
Elastase I	Omitz <i>et al.</i> , 1987
Metallothionein (MTII)	Karin <i>et al.</i> , 1987; Culotta <i>et al.</i> , 1989
Collagenase	Pinkert <i>et al.</i> , 1987; Angel <i>et al.</i> , 1987
Albumin	Pinkert <i>et al.</i> , 1987; Tronche <i>et al.</i> , 1989, 1990
α -Fetoprotein	Godbout <i>et al.</i> , 1988; Campere <i>et al.</i> , 1989
γ -Globin	Bodine <i>et al.</i> , 1987; Perez-Stable <i>et al.</i> , 1990
β -Globin	Trudel <i>et al.</i> , 1987
c-fos	Cohen <i>et al.</i> , 1987
c-HA-ras	Triesman, 1986; Deschamps <i>et al.</i> , 1985
Insulin	Edlund <i>et al.</i> , 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsh <i>et al.</i> , 1990

TABLE 2
Promoter and/or Enhancer

Promoter/Enhancer	References
α_1 -Antitrypsin	Latimer <i>et al.</i> , 1990
H2B (TH2B) Histone	Hwang <i>et al.</i> , 1990
Mouse and/or Type I Collagen	Ripe <i>et al.</i> , 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang <i>et al.</i> , 1989
Rat Growth Hormone	Larsen <i>et al.</i> , 1986
Human Serum Amyloid A (SAA)	Edbrooke <i>et al.</i> , 1989
Troponin I (TN I)	Yutzey <i>et al.</i> , 1989
Platelet-Derived Growth Factor (PDGF)	Pech <i>et al.</i> , 1989
Duchenne Muscular Dystrophy	Klamut <i>et al.</i> , 1990
SV40	Banerji <i>et al.</i> , 1981; Moreau <i>et al.</i> , 1981; Sleigh <i>et al.</i> , 1985; Firak <i>et al.</i> , 1986; Herr <i>et al.</i> , 1986; Imbra <i>et al.</i> , 1986; Kadesch <i>et al.</i> , 1986; Wang <i>et al.</i> , 1986; Ondek <i>et al.</i> , 1987; Kuhl <i>et al.</i> , 1987; Schaffner <i>et al.</i> , 1988
Polyoma	Swartzendruber <i>et al.</i> , 1975; Vasseur <i>et al.</i> , 1980; Katinka <i>et al.</i> , 1980, 1981; Tyndell <i>et al.</i> , 1981; Dandolo <i>et al.</i> , 1983; de Villiers <i>et al.</i> , 1984; Hen <i>et al.</i> , 1986; Satake <i>et al.</i> , 1988; Campbell <i>et al.</i> , 1988
Retroviruses	Kriegler <i>et al.</i> , 1982, 1983; Levinson <i>et al.</i> , 1982; Kriegler <i>et al.</i> , 1983, 1984a, b, 1988; Bosze <i>et al.</i> , 1986; Miksicek <i>et al.</i> , 1986; Celander <i>et al.</i> , 1987; Thiesen <i>et al.</i> , 1988; Celander <i>et al.</i> , 1988; Chol <i>et al.</i> , 1988; Reisman <i>et al.</i> , 1989
Papilloma Virus	Campo <i>et al.</i> , 1983; Lusky <i>et al.</i> , 1983; Spandidos and Wilkie, 1983; Spalholz <i>et al.</i> , 1985; Lusky <i>et al.</i> , 1986; Cripe <i>et al.</i> , 1987; Gloss <i>et al.</i> , 1987; Hirochika <i>et al.</i> , 1987; Stephens <i>et al.</i> , 1987
Hepatitis B Virus	Bulla <i>et al.</i> , 1986; Jameel <i>et al.</i> , 1986; Shaul <i>et al.</i> , 1987; Spandau <i>et al.</i> , 1988; Vannice <i>et al.</i> , 1988
Human Immunodeficiency Virus	Muesing <i>et al.</i> , 1987; Hauber <i>et al.</i> , 1988; Jakobovits <i>et al.</i> , 1988; Feng <i>et al.</i> , 1988; Takebe <i>et al.</i> , 1988; Rosen <i>et al.</i> , 1988; Berkhout <i>et al.</i> , 1989; Laspia <i>et al.</i> , 1989; Sharp <i>et al.</i> , 1989; Braddock <i>et al.</i> , 1989
Cytomegalovirus (CMV)	Weber <i>et al.</i> , 1984; Boshart <i>et al.</i> , 1985; Foecking <i>et al.</i> , 1986
Gibbon Ape Leukemia Virus	Holbrook <i>et al.</i> , 1987; Quinn <i>et al.</i> , 1989

TABLE 3
Inducible Elements

Element	Inducer	References
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter <i>et al.</i> , 1982; Haslinger <i>et al.</i> , 1985; Searle <i>et al.</i> , 1985; Stuart <i>et al.</i> , 1985; Imagawa <i>et al.</i> , 1987, Karin <i>et al.</i> , 1987; Angel <i>et al.</i> , 1987b; McNeall <i>et al.</i> , 1989
MMTV (mouse mammary	Glucocorticoids	Huang <i>et al.</i> , 1981; Lee <i>et</i>

TABLE 3 Inducible Elements		
Element	Inducer	References
tumor virus)		<i>al.</i> , 1981; Majors <i>et al.</i> , 1983; Chandler <i>et al.</i> , 1983; Lee <i>et al.</i> , 1984; Ponta <i>et al.</i> , 1985; Sakai <i>et al.</i> , 1988
β-Interferon	poly(rI)x poly(rC)	Tavernier <i>et al.</i> , 1983
Adenovirus 5 E2	ElA	Imperiale <i>et al.</i> , 1984
Collagenase	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987a
Stromelysin	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987b
SV40	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	Hug <i>et al.</i> , 1988
GRP78 Gene	A23187	Resendez <i>et al.</i> , 1988
α-2-Macroglobulin	IL-6	Kunz <i>et al.</i> , 1989
Vimentin	Serum	Rittling <i>et al.</i> , 1989
MHC Class I Gene H-2 κ b	Interferon	Blanar <i>et al.</i> , 1989
HSP70	ElA, SV40 Large T Antigen	Taylor <i>et al.</i> , 1989, 1990a, 1990b
Proliferin	Phorbol Ester-TPA	Mordacq <i>et al.</i> , 1989
Tumor Necrosis Factor	PMA	Hensel <i>et al.</i> , 1989
Thyroid Stimulating Hormone α Gene	Thyroid Hormone	Chatterjee <i>et al.</i> , 1989

The identity of tissue-specific promoters or elements, as well as assays to characterize their activity, is well known to those of skill in the art. Examples of such regions include the human LIMK2 gene (Nomoto *et al.* 1999), the somatostatin receptor 2 gene (Kraus *et al.*, 1998), murine epididymal retinoic acid-binding gene (Lareyre *et al.*, 1999), human CD4 (Zhao-Emonet *et al.*, 1998), mouse alpha2 (XI) collagen (Tsumaki, *et al.*, 1998), D1A dopamine receptor gene (Lee, *et al.*, 1997), insulin-like growth factor II (Wu *et al.*, 1997), human platelet endothelial cell adhesion molecule-1 (Almendro *et al.*, 1996), and the SM22 α promoter.

Also contemplated as useful in the present invention are the dectin-1 and dectin-2 promoters. Additional viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the present invention are listed in Tables 2 and 3. Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of structural genes encoding oligosaccharide processing enzymes, protein folding accessory proteins, selectable marker proteins or a heterologous protein of interest. Alternatively, a tissue-specific promoter

for cancer gene therapy (Table 4) or the targeting of tumors (Table 5) may be employed with the nucleic acid molecules of the present invention.

TABLE 4:
Candidate Tissue-Specific Promoters for Cancer Gene Therapy

Tissue-specific promoter	Cancers in which promoter is active	Normal cells in which promoter is active
Carcinoembryonic antigen (CEA)*	Most colorectal carcinomas; 50% of lung carcinomas; 40-50% of gastric carcinomas; most pancreatic carcinomas; many breast carcinomas	Colonic mucosa; gastric mucosa; lung epithelia; eccrine sweat glands; cells in testes
Prostate-specific antigen (PSA)	Most prostate carcinomas	Prostate epithelium
Vasoactive intestinal peptide (VIP)	Majority of non-small cell lung cancers	Neurons; lymphocytes; mast cells; eosinophils
Surfactant protein A (SP-A)	Many lung adenocarcinomas cells	Type II pneumocytes; Clara
Human achaete-scute homolog (hASH)	Most small cell lung cancers	Neuroendocrine cells in lung
Mucin-1 (MUC1)**	Most adenocarcinomas (originating from any tissue)	Glandular epithelial cells in breast and in respiratory, gastrointestinal, and genitourinary tracts
Alpha-fetoprotein	Most hepatocellular carcinomas; possibly many testicular cancers	Hepatocytes (under certain conditions); testis
Albumin	Most hepatocellular carcinomas	Hepatocytes
Tyrosinase	Most melanomas	Melanocytes; astrocytes; Schwann cells; some neurons
Tyrosine-binding protein (TRP)	Most melanomas	Melanocytes; astrocytes, Schwann cells; some neurons
Keratin 14	Presumably many squamous cell carcinomas (e.g., Head and neck cancers)	Keratinocytes
EBV LD-2	Many squamous cell carcinomas of head and neck	Keratinocytes of upper digestive tract
Glial fibrillary acidic protein (GFAP)	Many astrocytomas	Astrocytes
Myelin basic protein (MBP)	Many gliomas	Oligodendrocytes
Testis-specific angiotensin-converting enzyme (Testis-specific ACE)	Possibly many testicular cancers	Spermatazoa
Osteocalcin	Possibly many osteosarcomas	Osteoblasts

TABLE 5:
Candidate Promoters for Use with a Tissue-Specific Targeting of Tumors

Promoter	Cancers in which Promoter is active	Normal cells in which Promoter is active
E2F-regulated promoter	Almost all cancers	Proliferating cells
HLA-G	Many colorectal carcinomas; many melanomas; possibly many other cancers	Lymphocytes; monocytes; spermatocytes; trophoblast
FasL	Most melanomas; many pancreatic carcinomas; most astrocytomas possibly many other cancers	Activated leukocytes: neurons; endothelial cells; keratinocytes; cells in immunoprivileged tissues; some cells in lungs, ovaries, liver, and prostate
Myc-regulated promoter	Most lung carcinomas (both small cell and non-small cell); most colorectal carcinomas	Proliferating cells (only some cell-types): mammary epithelial cells (including non-proliferating)
MAGE-1	Many melanomas; some non-small cell lung carcinomas; some breast carcinomas	Testis
VEGF	70% of all cancers (constitutive overexpression in many cancers)	Cells at sites of neovascularization (but unlike in tumors, expression is transient, less strong, and never constitutive)
bFGF	Presumably many different cancers, since bFGF expression is induced by ischemic conditions	Cells at sites of ischemia (but unlike tumors, expression is transient, less strong, and never constitutive)
COX-2	Most colorectal carcinomas; many lung carcinomas; possibly many other cancers	Cells at sites of inflammation
IL-10	Most colorectal carcinomas; many lung carcinomas; many squamous cell carcinomas of head and neck; possibly many other cancers	Leukocytes
GRP78/BiP	Presumably many different cancers, since GRP78 expression is induced by tumor-specific conditions	Cells at sites of ischemia
CarG elements from Egr-1	Induced by ionization radiation, so conceivably most tumors upon irradiation	Cells exposed to ionizing radiation; leukocytes

2. Initiation Signals and Internal Ribosome Binding Sites

A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences.

Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be “in-frame” with the reading frame of the desired coding sequence to ensure translation of the entire 5 insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

In certain embodiments of the invention, the use of internal ribosome entry sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are 10 able to bypass the ribosome scanning model of 5'-methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple 15 open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message (see U.S. Patent 5,925,565 and 5,935,819, herein incorporated by reference).

20 3. Multiple Cloning Sites

Vectors can include a multiple cloning site (MCS), which is a nucleic acid region that 25 contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector (see Carbonelli *et al.*, 1999, Levenson *et al.*, 1998, and Cocea, 1997, incorporated herein by reference). “Restriction enzyme digestion” refers to catalytic cleavage of a nucleic acid molecule with an enzyme that functions only at specific locations in a nucleic acid molecule. Many of these restriction enzymes are commercially available. Use of such enzymes is widely understood by those of skill in the art. Frequently, a vector is linearized or fragmented using a restriction enzyme that cuts within the MCS to enable exogenous sequences to be ligated to the vector. 30 “Ligation” refers to the process of forming phosphodiester bonds between two nucleic acid fragments, which may or may not be contiguous with each other. Techniques involving

restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

4. Splicing Sites

5 Most transcribed eukaryotic RNA molecules will undergo RNA splicing to remove introns from the primary transcripts. Vectors containing genomic eukaryotic sequences may require donor and/or acceptor splicing sites to ensure proper processing of the transcript for protein expression. (See Chandler *et al.*, 1997, incorporated herein by reference.)

5. Termination Signals

10 The vectors or constructs of the present invention will generally comprise at least one termination signal. A "termination signal" or "terminator" is comprised of the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain embodiments a termination signal that ends the production of an RNA transcript is contemplated. A terminator may be necessary *in vivo* to achieve desirable message levels.

15 In eukaryotic systems, the terminator region may also comprise specific DNA sequences that permit site-specific cleavage of the new transcript so as to expose a polyadenylation site. This signals a specialized endogenous polymerase to add a stretch of about 200 A residues (polyA) to the 3' end of the transcript. RNA molecules modified with this polyA tail appear to more stable and are translated more efficiently. Thus, in other 20 embodiments involving eukaryotes, it is preferred that that terminator comprises a signal for the cleavage of the RNA, and it is more preferred that the terminator signal promotes polyadenylation of the message. The terminator and/or polyadenylation site elements can serve to enhance message levels and/or to minimize read through from the cassette into other sequences.

25 Terminators contemplated for use in the invention include any known terminator of transcription described herein or known to one of ordinary skill in the art, including but not limited to, for example, the termination sequences of genes, such as for example the bovine growth hormone terminator or viral termination sequences, such as for example the SV40 terminator. In certain embodiments, the termination signal may be a lack of transcribable or 30 translatable sequence, such as due to a sequence truncation.

6. Polyadenylation Signals

In expression, particularly eukaryotic expression, one will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, 5 and/or any such sequence may be employed. Preferred embodiments include the SV40 polyadenylation signal and/or the bovine growth hormone polyadenylation signal, convenient and/or known to function well in various target cells. Polyadenylation may increase the stability of the transcript or may facilitate cytoplasmic transport.

7. Origins of Replication

10 In order to propagate a vector in a host cell, it may contain one or more origins of replication sites (often termed "ori"), which is a specific nucleic acid sequence at which replication is initiated. Alternatively an autonomously replicating sequence (ARS) can be employed if the host cell is yeast.

8. Selectable and Screenable Markers

15 In certain embodiments of the invention, cells containing a nucleic acid construct of the present invention may be identified *in vitro* or *in vivo* by including a marker in the expression vector. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression vector. Generally, a selectable marker is one that confers a property that allows for selection. A positive selectable marker is one in 20 which the presence of the marker allows for its selection, while a negative selectable marker is one in which its presence prevents its selection. An example of a positive selectable marker is a drug resistance marker.

25 Usually the inclusion of a drug selection marker aids in the cloning and identification of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the implementation of conditions, other types of markers including screenable markers such as GFP, whose basis is colorimetric analysis, are also contemplated. Alternatively, screenable enzymes such as herpes simplex virus thymidine kinase (*tk*) or chloramphenicol 30 acetyltransferase (CAT) may be utilized. One of skill in the art would also know how to employ immunologic markers, possibly in conjunction with FACS analysis. The marker used

is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable and screenable markers are well known to one of skill in the art.

D. Nucleic Acid Detection

5 In addition to their use in directing the expression of poxvirus proteins, polypeptides and/or peptides, the nucleic acid sequences disclosed herein have a variety of other uses. For example, they have utility as probes or primers for embodiments involving nucleic acid hybridization. They may be used in diagnostic or screening methods of the present invention. Detection of nucleic acids encoding poxvirus or poxvirus polypeptide modulators are
10 encompassed by the invention.

1. Hybridization

The use of a probe or primer of between 13 and 100 nucleotides, preferably between 17 and 100 nucleotides in length, or in some aspects of the invention up to 1-2 kilobases or more in length, allows the formation of a duplex molecule that is both stable and selective. Molecules
15 having complementary sequences over contiguous stretches greater than 20 bases in length are generally preferred, to increase stability and/or selectivity of the hybrid molecules obtained. One will generally prefer to design nucleic acid molecules for hybridization having one or more complementary sequences of 20 to 30 nucleotides, or even longer where desired. Such fragments may be readily prepared, for example, by directly synthesizing the fragment by chemical means
20 or by introducing selected sequences into recombinant vectors for recombinant production.

Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of DNAs and/or RNAs or to provide primers for amplification of DNA or RNA from samples. Depending on the application envisioned, one would desire to employ varying conditions of hybridization to achieve varying
25 degrees of selectivity of the probe or primers for the target sequence.

For applications requiring high selectivity, one will typically desire to employ relatively high stringency conditions to form the hybrids. For example, relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.10 M NaCl at temperatures of about 50°C to about 70°C. Such high stringency conditions tolerate little, if any, mismatch
30 between the probe or primers and the template or target strand and would be particularly suitable for isolating specific genes or for detecting specific mRNA transcripts. It is generally

appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide.

For certain applications, for example, site-directed mutagenesis, it is appreciated that lower stringency conditions are preferred. Under these conditions, hybridization may occur even 5 though the sequences of the hybridizing strands are not perfectly complementary, but are mismatched at one or more positions. Conditions may be rendered less stringent by increasing salt concentration and/or decreasing temperature. For example, a medium stringency condition could be provided by about 0.1 to 0.25 M NaCl at temperatures of about 37°C to about 55°C, while a low stringency condition could be provided by about 0.15 M to about 0.9 M salt, at 10 temperatures ranging from about 20°C to about 55°C. Hybridization conditions can be readily manipulated depending on the desired results.

In other embodiments, hybridization may be achieved under conditions of, for example, 15 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 1.0 mM dithiothreitol, at temperatures between approximately 20°C to about 37°C. Other hybridization conditions utilized could include approximately 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, at temperatures ranging from approximately 40°C to about 72°C.

In certain embodiments, it will be advantageous to employ nucleic acids of defined sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, 20 including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of being detected. In preferred embodiments, one may desire to employ a fluorescent label or an enzyme tag such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmentally undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known that can be employed to provide a detection means that is visibly or 25 spectrophotometrically detectable, to identify specific hybridization with complementary nucleic acid containing samples.

In general, it is envisioned that the probes or primers described herein will be useful as reagents in solution hybridization, as in PCR™, for detection of expression of corresponding genes, as well as in embodiments employing a solid phase. In embodiments involving a solid 30 phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to hybridization with selected probes under desired conditions. The conditions selected will depend on the particular circumstances

(depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, *etc.*). Optimization of hybridization conditions for the particular application of interest is well known to those of skill in the art. After washing of the hybridized molecules to remove non-specifically bound probe molecules, hybridization is 5 detected, and/or quantified, by determining the amount of bound label. Representative solid phase hybridization methods are disclosed in U.S. Patents 5,843,663, 5,900,481 and 5,919,626. Other methods of hybridization that may be used in the practice of the present invention are disclosed in U.S. Patents 5,849,481, 5,849,486 and 5,851,772. The relevant portions of these and other references identified in this section of the Specification are 10 incorporated herein by reference.

2. Amplification of Nucleic Acids

Nucleic acids used as a template for amplification may be isolated from cells, tissues or other samples according to standard methodologies (Sambrook *et al.*, 1989). In certain embodiments, analysis is performed on whole cell or tissue homogenates or biological fluid 15 samples without substantial purification of the template nucleic acid. The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to first convert the RNA to a complementary DNA.

The term "primer," as used herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. 20 Typically, primers are oligonucleotides from ten to twenty and/or thirty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded and/or single-stranded form, although the single-stranded form is preferred.

Pairs of primers designed to selectively hybridize to nucleic acids corresponding to sequences of genes identified herein are contacted with the template nucleic acid under 25 conditions that permit selective hybridization. Depending upon the desired application, high stringency hybridization conditions may be selected that will only allow hybridization to sequences that are completely complementary to the primers. In other embodiments, hybridization may occur under reduced stringency to allow for amplification of nucleic acids contain one or more mismatches with the primer sequences. Once hybridized, the template-primer complex is contacted with one or more enzymes that facilitate template-dependent 30 nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

The amplification product may be detected or quantified. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of incorporated radiolabel or fluorescent label or even via a system using electrical and/or 5 thermal impulse signals (Bellus, 1994).

A number of template dependent processes are available to amplify the oligonucleotide sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (referred to as PCRTM) which is described in detail in U.S. Patents 4,683,195, 4,683,202 and 4,800,159, and in Innis *et al.*, 10 1988, each of which is incorporated herein by reference in their entirety.

A reverse transcriptase PCRTM amplification procedure may be performed to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known (see Sambrook *et al.*, 1989). Alternative methods for reverse transcription utilize thermostable DNA polymerases. These methods are described in WO 90/07641. Polymerase chain reaction methodologies are well known in the art. Representative methods of RT-PCR 15 are described in U.S. Patent 5,882,864.

Another method for amplification is ligase chain reaction ("LCR"), disclosed in European Application No. 320 308, incorporated herein by reference in its entirety. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target 20 sequence. A method based on PCRTM and oligonucleotide ligase assay (OLA), disclosed in U.S. Patent 5,912,148, may also be used.

Alternative methods for amplification of target nucleic acid sequences that may be used in the practice of the present invention are disclosed in U.S. Patents 5,843,650, 5,846,709, 5,846,783, 5,849,546, 5,849,497, 5,849,547, 5,858,652, 5,866,366, 5,916,776, 25 5,922,574, 5,928,905, 5,928,906, 5,932,451, 5,935,825, 5,939,291 and 5,942,391, GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety.

Qbeta Replicase, described in PCT Application No. PCT/US87/00880, may also be used as an amplification method in the present invention. In this method, a replicative 30 sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence which may then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[alpha-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention (Walker *et al.*, 1992). Strand Displacement 5 Amplification (SDA), disclosed in U.S. Patent 5,916,779, is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation.

Other nucleic acid amplification procedures include transcription-based amplification 10 systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR (Kwoh *et al.*, 1989; PCT Application WO 88/10315, incorporated herein by reference in their entirety). European Application No. 329 822 disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention.

PCT Application WO 89/06700 (incorporated herein by reference in its entirety) 15 disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter region/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR" (Frohman, 1990; Ohara *et al.*, 1989).

20 3. Detection of Nucleic Acids

Following any amplification, it may be desirable to separate the amplification product 25 from the template and/or the excess primer. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods (Sambrook *et al.*, 1989). Separated amplification products may be cut out and eluted from the gel for further manipulation. Using low melting point agarose gels, the separated band may be removed by heating the gel, followed by extraction of the nucleic acid.

Separation of nucleic acids may also be effected by chromatographic techniques known in art. There are many kinds of chromatography which may be used in the practice of the present invention, including adsorption, partition, ion-exchange, hydroxylapatite, 30 molecular sieve, reverse-phase, column, paper, thin-layer, and gas chromatography as well as HPLC.

In certain embodiments, the amplification products are visualized. A typical visualization method involves staining of a gel with ethidium bromide and visualization of bands under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the separated amplification products can be 5 exposed to x-ray film or visualized under the appropriate excitatory spectra.

In one embodiment, following separation of amplification products, a labeled nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, or another binding partner 10 carrying a detectable moiety.

In particular embodiments, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those of skill in the art (see Sambrook *et al.*, 1989). One example of the foregoing is described in U.S. Patent 5,279,721, incorporated by reference herein, which discloses an apparatus and method 15 for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

Other methods of nucleic acid detection that may be used in the practice of the instant invention are disclosed in U.S. Patents 5,840,873, 5,843,640, 5,843,651, 5,846,708, 20 5,846,717, 5,846,726, 5,846,729, 5,849,487, 5,853,990, 5,853,992, 5,853,993, 5,856,092, 5,861,244, 5,863,732, 5,863,753, 5,866,331, 5,905,024, 5,910,407, 5,912,124, 5,912,145, 5,919,630, 5,925,517, 5,928,862, 5,928,869, 5,929,227, 5,932,413 and 5,935,791, each of which is incorporated herein by reference.

4. Other Assays

Other methods for genetic screening may be used within the scope of the present invention, for example, to detect mutations in genomic DNA, cDNA and/or RNA samples. Methods used to detect point mutations include denaturing gradient gel electrophoresis (“DGGE”), restriction fragment length polymorphism analysis (“RFLP”), chemical or enzymatic cleavage methods, direct sequencing of target regions amplified by PCRTM (see 25 above), single-strand conformation polymorphism analysis (“SSCP”) and other methods well known in the art.

One method of screening for point mutations is based on RNase cleavage of base pair mismatches in RNA/DNA or RNA/RNA heteroduplexes. As used herein, the term "mismatch" is defined as a region of one or more unpaired or mispaired nucleotides in a double-stranded RNA/RNA, RNA/DNA or DNA/DNA molecule. This definition thus 5 includes mismatches due to insertion/deletion mutations, as well as single or multiple base point mutations.

U.S. Patent 4,946,773 describes an RNase A mismatch cleavage assay that involves annealing single-stranded DNA or RNA test samples to an RNA probe, and subsequent treatment of the nucleic acid duplexes with RNase A. For the detection of mismatches, the 10 single-stranded products of the RNase A treatment, electrophoretically separated according to size, are compared to similarly treated control duplexes. Samples containing smaller fragments (cleavage products) not seen in the control duplex are scored as positive.

Other investigators have described the use of RNase I in mismatch assays. The use of 15 RNase I for mismatch detection is described in literature from Promega Biotech. Promega markets a kit containing RNase I that is reported to cleave three out of four known mismatches. Others have described using the MutS protein or other DNA-repair enzymes for detection of single-base mismatches.

Alternative methods for detection of deletion, insertion or substitution mutations that 20 may be used in the practice of the present invention are disclosed in U.S. Patents 5,849,483, 5,851,770, 5,866,337, 5,925,525 and 5,928,870, each of which is incorporated herein by reference in its entirety.

E. Methods of Gene Transfer

Suitable methods for nucleic acid delivery to effect expression of compositions of the 25 present invention are believed to include virtually any method by which a nucleic acid (*e.g.*, DNA, including viral and nonviral vectors) can be introduced into an organelle, a cell, a tissue or an organism, as described herein or as would be known to one of ordinary skill in the art. Such methods include, but are not limited to, direct delivery of DNA such as by injection (U.S. Patents 5,994,624, 5,981,274, 5,945,100, 5,780,448, 5,736,524, 5,702,932, 5,656,610, 5,589,466 and 5,580,859, each incorporated herein by reference), including microinjection 30 (Harlan and Weintraub, 1985; U.S. Patent 5,789,215, incorporated herein by reference); by electroporation (U.S. Patent 5,384,253, incorporated herein by reference); by calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et*

al., 1990); by using DEAE-dextran followed by polyethylene glycol (Gopal, 1985); by direct sonic loading (Fechheimer *et al.*, 1987); by liposome mediated transfection (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987; Wong *et al.*, 1980; Kaneda *et al.*, 1989; Kato *et al.*, 1991); by microparticle bombardment (PCT Application Nos. WO 94/09699 5 and 95/06128; U.S. Patents 5,610,042; 5,322,783 5,563,055, 5,550,318, 5,538,877 and 5,538,880, and each incorporated herein by reference); by agitation with silicon carbide fibers (Kaepller *et al.*, 1990; U.S. Patents 5,302,523 and 5,464,765, each incorporated herein by reference); by *Agrobacterium*-mediated transformation (U.S. Patents 5,591,616 and 10 5,563,055, each incorporated herein by reference); or by PEG-mediated transformation of protoplasts (Omirulleh *et al.*, 1993; U.S. Patents 4,684,611 and 4,952,500, each incorporated herein by reference); by desiccation/inhibition-mediated DNA uptake (Potrykus *et al.*, 1985). Through the application of techniques such as these, organelle(s), cell(s), tissue(s) or organism(s) may be stably or transiently transformed.

5. Lipid Components and Moieties

15 In certain embodiments, the present invention concerns compositions comprising one or more lipids associated with a nucleic acid, an amino acid molecule, such as a peptide, or another small molecule compound. In any of the embodiments discussed herein, the molecule may be either a poxvirus polypeptide or a poxvirus polypeptide modulator, for example a nucleic acid encoding all or part of either a poxvirus polypeptide, or alternatively, an amino 20 acid molecule encoding all or part of poxvirus polypeptide modulator. A lipid is a substance that is characteristically insoluble in water and extractable with an organic solvent. Compounds than those specifically described herein are understood by one of skill in the art as lipids, and are encompassed by the compositions and methods of the present invention. A lipid component and a non-lipid may be attached to one another, either covalently or non- 25 covalently.

30 A lipid may be naturally occurring or synthetic (*i.e.*, designed or produced by man). However, a lipid is usually a biological substance. Biological lipids are well known in the art, and include for example, neutral fats, phospholipids, phosphoglycerides, steroids, terpenes, lysolipids, glycosphingolipids, glucolipids, sulphatides, lipids with ether and ester-linked fatty acids and polymerizable lipids, and combinations thereof.

A nucleic acid molecule or amino acid molecule, such as a peptide, associated with a lipid may be dispersed in a solution containing a lipid, dissolved with a lipid, emulsified with a lipid, mixed with a lipid, combined with a lipid, covalently bonded to a lipid, contained as a suspension in a lipid or otherwise associated with a lipid. A lipid or lipid/poxvirus-associated 5 composition of the present invention is not limited to any particular structure. For example, they may also simply be interspersed in a solution, possibly forming aggregates which are not uniform in either size or shape. In another example, they may be present in a bilayer structure, as micelles, or with a "collapsed" structure. In another non-limiting example, a lipofectamine(Gibco BRL)-poxvirus or Superfect (Qiagen)-poxvirus complex is also 10 contemplated.

In certain embodiments, a lipid composition may comprise about 1%, about 2%, about 3%, about 4% about 5%, about 6%, about 7%, about 8%, about 9%, about 10%, about 11%, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 26%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about 33%, about 34%, about 35%, about 36%, about 37%, about 38%, about 39%, about 40%, about 41%, about 42%, about 43%, about 44%, about 45%, about 46%, about 47%, about 48%, about 49%, about 50%, about 51%, about 52%, about 53%, about 54%, about 55%, about 56%, about 57%, about 58%, about 59%, about 60%, about 61%, about 62%, about 63%, about 64%, about 65%, about 66%, about 67%, about 68%, about 69%, about 70%, about 71%, about 72%, about 73%, about 74%, about 75%, about 76%, about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, about 100%, or any range 15 derivable therein, of a particular lipid, lipid type or non-lipid component such as a drug, protein, sugar, nucleic acids or other material disclosed herein or as would be known to one of skill in the art. In a non-limiting example, a lipid composition may comprise about 10% to about 20% neutral lipids, and about 33% to about 34% of a cerebroside, and about 1% cholesterol. In another non-limiting example, a liposome may comprise about 4% to about 20% 25 terpenes, wherein about 1% of the micelle is specifically lycopene, leaving about 3% to about 11% of the liposome as comprising other terpenes; and about 10% to about 35% phosphatidyl choline, and about 1% of a drug. Thus, it is contemplated that lipid 30

compositions of the present invention may comprise any of the lipids, lipid types or other components in any combination or percentage range.

V. GM-CSF

In a particular aspect of the invention, the vaccinia viruses will carry a gene encoding for GM-CSF. GM-CSF is granulocyte-macrophage colony-stimulating factor, a substance that helps make more white blood cells, especially granulocytes, macrophages, and cells that become platelets. It is a cytokine that belongs to the family of drugs called hematopoietic (blood-forming) agents, and is also known as sargramostim. GM-CSF was first cloned and sequence in 1985 by Cantrell *et al.* (1985). Human GM-CSF is a 144-amino acid glycoprotein encoded by a single open-reading frame with a predicted molecular mass of 16,293 daltons. It exhibits a 69% nucleotide homology and 54% amino acid homology to mouse GM-CSF and exists as a single-copy gene.

GM-CSF is produced by a number of different cell types (including activated T cells, B cells, macrophages, mast cells, endothelial cells and fibroblasts) in response to cytokine or immune and inflammatory stimuli. Besides granulocyte-macrophage progenitors, GM-CSF is also a growth factor for erythroid, megakaryocyte and eosinophil progenitors. On mature hematopoietic cells, GM-CSF is a survival factor for and activates the effector functions of granulocytes, monocytes/macrophages and eosinophils. GM-CSF has also been reported to have a functional role on non-hematopoietic cells. It can induce human endothelial cells to migrate and proliferate.

GM-CSF is species specific and human GM-CSF has no biological effects on mouse cells. GM-CSF exerts its biological effects through binding to specific cell surface receptors. The high affinity receptors required for human GM-CSF signal transduction have been shown to be heterodimers consisting of a GM-CSF-specific α chain and a common β chain that is shared by the high-affinity receptors for IL-3 and IL-5.

Although GM-CSF can stimulate the proliferation of a number of tumor cell lines, including osteogenic sarcoma, carcinoma and adenocarcinoma cell lines, clinical trials of GM-CSF (alone or with other immunotherapies) are in progress for people with melanoma, leukemia, lymphoma, neuroblastoma, Kaposi sarcoma, mesothelioma, lung cancer, breast cancer, prostate cancer, colorectal cancer, brain tumors, kidney cancer and cervical cancer. Common side effects of GM-CSF include flu-like symptoms (fever, headaches, muscle aches), rashes, facial flushing, and bone pain.

VI. OTHER HETEROLOGOUS GENES

In some embodiments, the vaccinia virus used in methods of the invention contains a nucleic acid sequence that expresses a heterologous sequence that does not encode GM-CSF but encodes another heterologous sequence. In certain embodiments, the heterologous sequence encodes another cytokine. Alternatively or additionally, the vaccinia virus may contain a nucleic acid that encodes for IL-12, thymidine deaminase, TNF, and the like. In addition, any gene product discussed herein may be encoded by a nucleic acid contained within a vaccinia virus and used in methods of the invention.

VII. PHARMACEUTICAL FORMULATIONS, DELIVERY, AND TREATMENT

REGIMENS

In an embodiment of the present invention, a method of treatment for a hyperproliferative disease, such as cancer, by the delivery of a vaccinia virus, is contemplated. Examples of cancer contemplated for treatment include lung cancer, head and neck cancer, breast cancer, pancreatic cancer, prostate cancer, renal cancer, uterine cancer, bone cancer, testicular cancer, cervical cancer, gastrointestinal cancer, lymphomas, pre-neoplastic lesions in the lung, colon cancer, melanoma, bladder cancer and any other cancers or tumors that may be treated.

An effective amount of the pharmaceutical composition, generally, is defined as that amount sufficient to detectably and repeatedly to ameliorate, reduce, minimize or limit the extent of the disease or its symptoms. More rigorous definitions may apply, including elimination, eradication or cure of disease.

Preferably, patients will have adequate bone marrow function (defined as a peripheral absolute granulocyte count of $> 2,000 / \text{mm}^3$ and a platelet count of $100,000 / \text{mm}^3$), adequate liver function (bilirubin $< 1.5 \text{ mg / dl}$) and adequate renal function (creatinine $< 1.5 \text{ mg / dl}$).

Cancer cells that may be treated by methods and compositions of the invention include cells from the bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, gastrointestinal, gum, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, testis, tongue, or uterus. In addition, the cancer may specifically be of the following histological type, though it is not limited to these: neoplasm, malignant; carcinoma; carcinoma, undifferentiated; giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma;

pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma, malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial 5 polyposis coli; solid carcinoma; carcinoid tumor, malignant; bronchiolo-alveolar adenocarcinoma; papillary adenocarcinoma; chromophobe carcinoma; acidophil carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma; follicular adenocarcinoma; papillary and follicular adenocarcinoma; nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometroid carcinoma; 10 skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous adenocarcinoma; mucoepidermoid carcinoma; cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous adenocarcinoma; signet ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma; inflammatory carcinoma; paget's disease, mammary; acinar 15 cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; thymoma, malignant; ovarian stromal tumor, malignant; thecoma, malignant; granulosa cell tumor, malignant; androblastoma, malignant; sertoli cell carcinoma; leydig cell tumor, malignant; lipid cell tumor, malignant; paraganglioma, malignant; extra-mammary paraganglioma, malignant; pheochromocytoma; glomangiosarcoma; malignant melanoma; 20 amelanotic melanoma; superficial spreading melanoma; malig melanoma in giant pigmented nevus; epithelioid cell melanoma; blue nevus, malignant; sarcoma; fibrosarcoma; fibrous histiocytoma, malignant; myxosarcoma; liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar rhabdomyosarcoma; stromal sarcoma; mixed tumor, malignant; mullerian mixed tumor; nephroblastoma; hepatoblastoma; carcinosarcoma; 25 mesenchymoma, malignant; brenner tumor, malignant; phyllodes tumor, malignant; synovial sarcoma; mesothelioma, malignant; dysgerminoma; embryonal carcinoma; teratoma, malignant; struma ovarii, malignant; choriocarcinoma; mesonephroma, malignant; hemangiosarcoma; hemangioendothelioma, malignant; Kaposi's sarcoma; hemangiopericytoma, malignant; lymphangiosarcoma; osteosarcoma; juxtacortical 30 osteosarcoma; chondrosarcoma; chondroblastoma, malignant; mesenchymal chondrosarcoma; giant cell tumor of bone; Ewing's sarcoma; odontogenic tumor, malignant; ameloblastic odontosarcoma; ameloblastoma, malignant; ameloblastic fibrosarcoma; pinealoma, malignant; chordoma; glioma, malignant; ependymoma; astrocytoma; protoplasmic astrocytoma;

fibrillary astrocytoma; astroblastoma; glioblastoma; oligodendrogloma; oligodendroblastoma; primitive neuroectodermal; cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma; retinoblastoma; olfactory neurogenic tumor; meningioma, malignant; neurofibrosarcoma; neurilemmoma, malignant; granular cell tumor, malignant; malignant lymphoma; hodgkin's disease; hodgkin's; paragranuloma; malignant lymphoma, small lymphocytic; malignant lymphoma, large cell, diffuse; malignant lymphoma, follicular; mycosis fungoides; other specified non-hodgkin's lymphomas; malignant histiocytosis; multiple myeloma; mast cell sarcoma; immunoproliferative small intestinal disease; leukemia; lymphoid leukemia; plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; 10 myeloid leukemia; basophilic leukemia; eosinophilic leukemia; monocytic leukemia; mast cell leukemia; megakaryoblastic leukemia; myeloid sarcoma; and hairy cell leukemia.

The present invention contemplates methods for inhibiting or preventing local invasiveness and/or metastasis of any type of primary cancer. For example, the primary cancer may be melanoma, non-small cell lung, small-cell lung, lung, hepatocarcinoma, 15 retinoblastoma, astrocytoma, glioblastoma, gum, tongue, leukemia, neuroblastoma, head, neck, breast, pancreatic, prostate, renal, bone, testicular, ovarian, mesothelioma, cervical, gastrointestinal, lymphoma, brain, colon, or bladder. In certain embodiments of the present invention, the primary cancer is lung cancer. For example, the lung cancer may be non-small cell lung carcinoma.

20 Moreover, the present invention can be used to prevent cancer or to treat pre-cancers or premalignant cells, including metaplasias, dysplasias, and hyperplasias. It may also be used to inhibit undesirable but benign cells, such as squamous metaplasia, dysplasia, benign prostate hyperplasia cells, hyperplastic lesions, and the like. The progression to cancer or to a more severe form of cancer may be halted, disrupted, or delayed by methods of the invention 25 involving GM-CSF polypeptides or other polypeptide(s) encoded by a vaccinia virus, as discussed herein.

A. Administration

To kill cells, inhibit cell growth, inhibit metastasis, decrease tumor or tissue size and otherwise reverse or reduce the malignant phenotype of tumor cells, using the methods and 30 compositions of the present invention, one would generally contact a hyperproliferative cell with the therapeutic compound such as a polypeptide or an expression construct encoding a polypeptide. The routes of administration will vary, naturally, with the location and nature of

the lesion, and include, *e.g.*, intradermal, transdermal, parenteral, intravenous, intramuscular, intranasal, subcutaneous, regional, percutaneous, intratracheal, intraperitoneal, intraarterial, intravesical, intratumoral, inhalation, perfusion, lavage, direct injection, and oral administration and formulation.

5 The present invention specifically concerns intravascular administration of a vaccinia virus of the invention. The term "intravascular" is understood to refer to delivery into the vasculature of a patient, meaning into, within, or in a vessel or vessels of the patient. In certain embodiments, the administration is into a vessel considered to be a vein (intravenous), while in others administration is into a vessel considered to be an artery. Veins include, but are not limited to, the internal jugular vein, a peripheral vein, a coronary vein, a hepatic vein, the portal vein, great saphenous vein, the pulmonary vein, superior vena cava, inferior vena cava, a gastric vein, a splenic vein, inferior mesenteric vein, superior mesenteric vein, cephalic vein, and/or femoral vein. Arteries include, but are not limited to, coronary artery, pulmonary artery, brachial artery, internal carotid artery, aortic arch, femoral artery, 10 peripheral artery, and/or ciliary artery. It is contemplated that delivery may be through or to 15 an arteriole or capillary.

Injection into the tumor vasculature is specifically contemplated for discrete, solid, accessible tumors. Local, regional or systemic administration also may be appropriate. For tumors of >4 cm, the volume to be administered will be about 4-10 ml (preferably 10 ml), while for tumors of <4 cm, a volume of about 1-3 ml will be used (preferably 3 ml). Multiple 20 injections delivered as single dose comprise about 0.1 to about 0.5 ml volumes. The viral particles may advantageously be contacted by administering multiple injections to the tumor, spaced at approximately 1 cm intervals.

In the case of surgical intervention, the present invention may be used preoperatively, 25 to render an inoperable tumor subject to resection. Alternatively, the present invention may be used at the time of surgery, and/or thereafter, to treat residual or metastatic disease. For example, a resected tumor bed may be injected or perfused with a formulation comprising a poxvirus polypeptide or a poxvirus comprising a mutation that renders the poxvirus advantageous for treatment of cancer or cancer cells. The perfusion may be continued post-resection, for example, by leaving a catheter implanted at the site of the surgery. Periodic 30 post-surgical treatment also is envisioned.

Continuous administration also may be applied where appropriate, for example, where a tumor is excised and the tumor bed is treated to eliminate residual, microscopic disease. Delivery *via* syringe or catheterization is preferred. Such continuous perfusion may take place for a period from about 1-2 hours, to about 2-6 hours, to about 6-12 hours, to about 12-24 5 hours, to about 1-2 days, to about 1-2 wk or longer following the initiation of treatment. Generally, the dose of the therapeutic composition *via* continuous perfusion will be equivalent to that given by a single or multiple injections, adjusted over a period of time during which the perfusion occurs. It is further contemplated that limb perfusion may be used to administer therapeutic compositions of the present invention, particularly in the treatment of melanomas 10 and sarcomas.

Treatment regimens may vary as well, and often depend on tumor type, tumor location, disease progression, and health and age of the patient. Obviously, certain types of tumor will require more aggressive treatment, while at the same time, certain patients cannot tolerate more taxing protocols. The clinician will be best suited to make such decisions based 15 on the known efficacy and toxicity (if any) of the therapeutic formulations.

In certain embodiments, the tumor being treated may not, at least initially, be resectable. Treatments with therapeutic viral constructs may increase the resectability of the tumor due to shrinkage at the margins or by elimination of certain particularly invasive portions. Following treatments, resection may be possible. Additional treatments subsequent 20 to resection will serve to eliminate microscopic residual disease at the tumor site.

A typical course of treatment, for a primary tumor or a post-excision tumor bed, will involve multiple doses. Typical primary tumor treatment involves a 6 dose application over a two-week period. The two-week regimen may be repeated one, two, three, four, five, six or more times. During a course of treatment, the need to complete the planned dosings may be 25 re-evaluated.

The treatments may include various "unit doses." Unit dose is defined as containing a predetermined-quantity of the therapeutic composition. The quantity to be administered, and the particular route and formulation, are within the skill of those in the clinical arts. A unit dose need not be administered as a single injection but may comprise continuous infusion over a set period of time. Unit dose of the present invention may conveniently be described in terms of plaque forming units (pfu) for a viral construct. Unit doses range from 10^3 , 10^4 , 10^5 , 30 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} , 10^{11} , 10^{12} , 10^{13} pfu and higher. Alternatively, depending on the kind

of virus and the titer attainable, one will deliver 1 to 100, 10 to 50, 100-1000, or up to about 1 x 10⁴, 1 x 10⁵, 1 x 10⁶, 1 x 10⁷, 1 x 10⁸, 1 x 10⁹, 1 x 10¹⁰, 1 x 10¹¹, 1 x 10¹², 1 x 10¹³, 1 x 10¹⁴, or 1 x 10¹⁵ or higher infectious viral particles (vp) to the patient or to the patient's cells.

B. Injectable Compositions and Formulations

5 The preferred method for the delivery of an expression construct or virus encoding all or part of a poxvirus genome to cancer or tumor cells in the present invention is via intratumoral injection. However, the pharmaceutical compositions disclosed herein may alternatively be administered parenterally, intravenously, intradermally, intramuscularly, transdermally or even intraperitoneally as described in U.S. Patent 5,543,158; U.S. Patent 10 5,641,515 and U.S. Patent 5,399,363 (each specifically incorporated herein by reference in its entirety).

15 Injection of nucleic acid constructs may be delivered by syringe or any other method used for injection of a solution, as long as the expression construct can pass through the particular gauge of needle required for injection. A novel needleless injection system has recently been described (U.S. Patent 5,846,233) having a nozzle defining an ampule chamber for holding the solution and an energy device for pushing the solution out of the nozzle to the site of delivery. A syringe system has also been described for use in gene therapy that permits 20 multiple injections of predetermined quantities of a solution precisely at any depth (U.S. Patent 5,846,225).

25 Solutions of the active compounds as free base or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U.S. Patent 30 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof,

and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, 5 chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

For parenteral administration in an aqueous solution, for example, the solution should 10 be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous, intratumoral and intraperitoneal administration. In this connection, sterile aqueous media that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of 15 isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. 20 Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by 25 incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously 30 sterile-filtered solution thereof.

The compositions disclosed herein may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino

groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and 5 such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like.

10 As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the 15 therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The phrase "pharmaceutically-acceptable" or "pharmacologically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The preparation of an aqueous composition that 20 contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared.

C. Combination Treatments

25 The compounds and methods of the present invention may be used in the context of hyperproliferative diseases/conditions including cancer and atherosclerosis. In order to increase the effectiveness of a treatment with the compositions of the present invention, such as attenuated vaccinia viruses, it may be desirable to combine these compositions with other agents effective in the treatment of those diseases and conditions. For example, the treatment of a cancer may be implemented with therapeutic compounds of the present invention and 30 other anti-cancer therapies, such as anti-cancer agents or surgery.

Various combinations may be employed; for example, an attenuated poxvirus, such as vaccinia virus, is "A" and the secondary anti-cancer therapy is "B":

A/B/A B/A/B B/B/A A/A/B A/B/B B/A/A A/B/B/B B/A/B/B
B/B/B/A B/B/A/B A/A/B/B A/B/A/B A/B/B/A B/B/A/A
B/A/B/A B/A/A/B A/A/A/B B/A/A/A A/B/A/A A/A/B/A

Administration of the therapeutic expression constructs of the present invention to a patient will follow general protocols for the administration of that particular secondary therapy, taking into account the toxicity, if any, of the poxvirus treatment. It is expected that the treatment cycles would be repeated as necessary. It also is contemplated that various standard therapies, as well as surgical intervention, may be applied in combination with the described cancer or tumor cell therapy.

10 1. Anti-Cancer Therapy

An “anti-cancer” agent is capable of negatively affecting cancer in a subject, for example, by killing cancer cells, inducing apoptosis in cancer cells, reducing the growth rate of cancer cells, reducing the incidence or number of metastases, reducing tumor size, inhibiting tumor growth, reducing the blood supply to a tumor or cancer cells, promoting an immune response against cancer cells or a tumor, preventing or inhibiting the progression of cancer, or increasing the lifespan of a subject with cancer. Anti-cancer agents include biological agents (biotherapy), chemotherapy agents, and radiotherapy agents. More generally, these other compositions would be provided in a combined amount effective to kill or inhibit proliferation of the cell. This process may involve contacting the cells with the expression construct and the agent(s) or multiple factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the expression construct and the other includes the second agent(s).

25 Tumor cell resistance to chemotherapy and radiotherapy agents represents a major problem in clinical oncology. One goal of current cancer research is to find ways to improve the efficacy of chemo- and radiotherapy by combining it with gene therapy. For example, the herpes simplex-thymidine kinase (HS-tK) gene, when delivered to brain tumors by a retroviral vector system, successfully induced susceptibility to the antiviral agent ganciclovir (Culver *et al.*, 1992). In the context of the present invention, it is contemplated that poxvirus therapy could be used similarly in conjunction with chemotherapeutic, radiotherapeutic,

immunotherapeutic or other biological intervention, in addition to other pro-apoptotic or cell cycle regulating agents.

Alternatively, the gene therapy may precede or follow the other agent treatment by intervals ranging from minutes to weeks. In embodiments where the other agent and expression construct are applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent and expression construct would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one may contact the cell with both modalities within about 12-24 h of each other and, more preferably, within about 6-12 h of each other. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

a. Chemotherapy

Cancer therapies also include a variety of combination therapies with both chemical and radiation based treatments. Combination chemotherapies include, for example, cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, busulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, raloxifene, estrogen receptor binding agents, taxol, gemcitabien, navelbine, farnesyl-protein transferase inhibitors, transplatinum, 5-fluorouracil, vincristine, vinblastine and methotrexate, Temazolomide (an aqueous form of DTIC), or any analog or derivative variant of the foregoing. The combination of chemotherapy with biological therapy is known as biochemotherapy.

b. Radiotherapy

Other factors that cause DNA damage and have been used extensively include what are commonly known as γ -rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that all of these factors effect a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 wk), to single doses of 2000 to 6000

roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

5 The terms "contacted" and "exposed," when applied to a cell, are used herein to describe the process by which a therapeutic construct and a chemotherapeutic or radiotherapeutic agent are delivered to a target cell or are placed in direct juxtaposition with the target cell. To achieve cell killing or stasis, both agents are delivered to a cell in a combined amount effective to kill the cell or prevent it from dividing.

c. Immunotherapy

10 Immunotherapeutics, generally, rely on the use of immune effector cells and molecules to target and destroy cancer cells. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually effect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, *etc.*) and serve merely as a targeting agent. 15 Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells. The combination of therapeutic modalities, *i.e.*, direct cytotoxic activity and inhibition or reduction of certain poxvirus polypeptides would provide therapeutic benefit in the treatment of cancer.

20 Immunotherapy could also be used as part of a combined therapy. The general approach for combined therapy is discussed below. In one aspect of immunotherapy, the tumor cell must bear some marker that is amenable to targeting, *i.e.*, is not present on the majority of other cells. Many tumor markers exist and any of these may be suitable for targeting in the context of the present invention. Common tumor markers include 25 carcinoembryonic antigen, prostate specific antigen, urinary tumor associated antigen, fetal antigen, tyrosinase (p97), gp68, TAG-72, HMFG, Sialyl Lewis Antigen, MucA, MucB, PLAP, estrogen receptor, laminin receptor, *erb* B and p155. An alternative aspect of immunotherapy is to anticancer effects with immune stimulatory effects. Immune stimulating molecules also exist including: cytokines such as IL-2, IL-4, IL-12, GM-CSF, IFN γ , 30 chemokines such as MIP-1, MCP-1, IL-8 and growth factors such as FLT3 ligand. Combining immune stimulating molecules, either as proteins or using gene delivery in

combination with a tumor suppressor such as mda-7 has been shown to enhance anti-tumor effects (Ju *et al.*, 2000).

As discussed earlier, examples of immunotherapies currently under investigation or in use are immune adjuvants (e.g., *Mycobacterium bovis*, *Plasmodium falciparum*, 5 dinitrochlorobenzene and aromatic compounds) (U.S. Patent 5,801,005; U.S. Patent 5,739,169; Hui and Hashimoto, 1998; Christodoulides *et al.*, 1998), cytokine therapy (e.g., interferons α , β and γ ; IL-1, GM-CSF and TNF) (Bukowski *et al.*, 1998; Davidson *et al.*, 1998; Hellstrand *et al.*, 1998) gene therapy (e.g., TNF, IL-1, IL-2, p53) (Qin *et al.*, 1998; Austin-Ward and Villaseca, 1998; U.S. Patent 5,830,880 and U.S. Patent 5,846,945) and 10 monoclonal antibodies (e.g., anti-ganglioside GM2, anti-HER-2, anti-p185) (Pietras *et al.*, 1998; Hanibuchi *et al.*, 1998; U.S. Patent 5,824,311). Herceptin (trastuzumab) is a chimeric (mouse-human) monoclonal antibody that blocks the HER2-neu receptor. It possesses anti-tumor activity and has been approved for use in the treatment of malignant tumors (Dillman, 1999). Combination therapy of cancer with herceptin and chemotherapy has been shown to 15 be more effective than the individual therapies. Thus, it is contemplated that one or more anti-cancer therapies may be employed with the poxvirus-related therapies described herein.

Passive Immunotherapy. A number of different approaches for passive immunotherapy of cancer exist. They may be broadly categorized into the following: injection of antibodies alone; injection of antibodies coupled to toxins or chemotherapeutic 20 agents; injection of antibodies coupled to radioactive isotopes; injection of anti-idiotype antibodies; and finally, purging of tumor cells in bone marrow.

Preferably, human monoclonal antibodies are employed in passive immunotherapy, as they produce few or no side effects in the patient. However, their application is somewhat limited by their scarcity and have so far only been administered intralesionally. Human 25 monoclonal antibodies to ganglioside antigens have been administered intralesionally to patients suffering from cutaneous recurrent melanoma (Irie and Morton, 1986). Regression was observed in six out of ten patients, following, daily or weekly, intralesional injections. In another study, moderate success was achieved from intralesional injections of two human monoclonal antibodies (Irie *et al.*, 1989).

It may be favorable to administer more than one monoclonal antibody directed against 30 two different antigens or even antibodies with multiple antigen specificity. Treatment protocols also may include administration of lymphokines or other immune enhancers as

described by Bajorin *et al.* (1988). The development of human monoclonal antibodies is described in further detail elsewhere in the specification.

Active Immunotherapy. In active immunotherapy, an antigenic peptide, polypeptide or protein, or an autologous or allogenic tumor cell composition or "vaccine" is administered, generally with a distinct bacterial adjuvant (Ravindranath and Morton, 1991; Morton *et al.*, 1992; Mitchell *et al.*, 1990; Mitchell *et al.*, 1993). In melanoma immunotherapy, those patients who elicit high IgM response often survive better than those who elicit no or low IgM antibodies (Morton *et al.*, 1992). IgM antibodies are often transient antibodies and the exception to the rule appears to be anti-ganglioside or anti-carbohydrate antibodies.

Adoptive Immunotherapy. In adoptive immunotherapy, the patient's circulating lymphocytes, or tumor infiltrated lymphocytes, are isolated *in vitro*, activated by lymphokines such as IL-2 or transduced with genes for tumor necrosis, and readministered (Rosenberg *et al.*, 1988; 1989). To achieve this, one would administer to an animal, or human patient, an immunologically effective amount of activated lymphocytes in combination with an adjuvant-incorporated antigenic peptide composition as described herein. The activated lymphocytes will most preferably be the patient's own cells that were earlier isolated from a blood or tumor sample and activated (or "expanded") *in vitro*. This form of immunotherapy has produced several cases of regression of melanoma and renal carcinoma, but the percentage of responders were few compared to those who did not respond.

20 d. Genes

In yet another embodiment, the secondary treatment is a gene therapy in which a therapeutic polynucleotide is administered before, after, or at the same time as an attenuated poxvirus is administered. Delivery of a poxvirus in conjunction with a vector encoding one of the following gene products will have a combined anti-cancer effect on target tissues. Alternatively, the poxvirus may be engineered as a viral vector to include the therapeutic polynucleotide. A variety of proteins are encompassed within the invention, some of which are described below. Table 7 lists various genes that may be targeted for gene therapy of some form in combination with the present invention.

30 Inducers of Cellular Proliferation. The proteins that induce cellular proliferation further fall into various categories dependent on function. The commonality of all of these proteins is their ability to regulate cellular proliferation. For example, a form of PDGF, the sis oncogene, is a secreted growth factor. Oncogenes rarely arise from genes encoding

growth factors, and at the present, sis is the only known naturally-occurring oncogenic growth factor. In one embodiment of the present invention, it is contemplated that anti-sense mRNA directed to a particular inducer of cellular proliferation is used to prevent expression of the inducer of cellular proliferation.

5 The proteins FMS, ErbA, ErbB and neu are growth factor receptors. Mutations to these receptors result in loss of regulatable function. For example, a point mutation affecting the transmembrane domain of the Neu receptor protein results in the neu oncogene. The erbA oncogene is derived from the intracellular receptor for thyroid hormone. The modified oncogenic ErbA receptor is believed to compete with the endogenous thyroid hormone 10 receptor, causing uncontrolled growth.

15 The largest class of oncogenes includes the signal transducing proteins (e.g., Src, Abl and Ras). The protein Src is a cytoplasmic protein-tyrosine kinase, and its transformation from proto-oncogene to oncogene in some cases, results via mutations at tyrosine residue 527. In contrast, transformation of GTPase protein ras from proto-oncogene to oncogene, in one example, results from a valine to glycine mutation at amino acid 12 in the sequence, reducing ras GTPase activity.

The proteins Jun, Fos and Myc are proteins that directly exert their effects on nuclear functions as transcription factors.

20 **Inhibitors of Cellular Proliferation.** The tumor suppressor oncogenes function to inhibit excessive cellular proliferation. The inactivation of these genes destroys their inhibitory activity, resulting in unregulated proliferation. The tumor suppressors p53, p16 and C-CAM are described below.

25 In addition to p53, which has been described above, another inhibitor of cellular proliferation is p16. The major transitions of the eukaryotic cell cycle are triggered by cyclin-dependent kinases, or CDK's. One CDK, cyclin-dependent kinase 4 (CDK4), regulates progression through the G₁. The activity of this enzyme may be to phosphorylate Rb at late G₁. The activity of CDK4 is controlled by an activating subunit, D-type cyclin, and by an inhibitory subunit, the p16^{INK4} has been biochemically characterized as a protein that specifically binds to and inhibits CDK4, and thus may regulate Rb phosphorylation (Serrano 30 *et al.*, 1993; Serrano *et al.*, 1995). Since the p16^{INK4} protein is a CDK4 inhibitor (Serrano, 1993), deletion of this gene may increase the activity of CDK4, resulting in hyperphosphorylation of the Rb protein. p16 also is known to regulate the function of CDK6.

5 p16^{INK4} belongs to a newly described class of CDK-inhibitory proteins that also includes p16^B, p19, p21^{WAF1}, and p27^{KIP1}. The p16^{INK4} gene maps to 9p21, a chromosome region frequently deleted in many tumor types. Homozygous deletions and mutations of the p16^{INK4} gene are frequent in human tumor cell lines. This evidence suggests that the p16^{INK4}
10 gene is a tumor suppressor gene. This interpretation has been challenged, however, by the observation that the frequency of the p16^{INK4} gene alterations is much lower in primary uncultured tumors than in cultured cell lines (Caldas *et al.*, 1994; Cheng *et al.*, 1994; Hussussian *et al.*, 1994; Kamb *et al.*, 1994; Kamb *et al.*, 1994; Mori *et al.*, 1994; Okamoto *et al.*, 1994; Nobori *et al.*, 1994; Orlow *et al.*, 1994; Arap *et al.*, 1995). Restoration of wild-type p16^{INK4} function by transfection with a plasmid expression vector reduced colony formation by some human cancer cell lines (Okamoto, 1994; Arap, 1995).

15 Other genes that may be employed according to the present invention include Rb, APC, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, zac1, p73, VHL, MMAC1 / PTEN, DBCCR-1, FCC, rsk-3, p27, p27/p16 fusions, p21/p27 fusions, anti-thrombotic genes (e.g., COX-1, TFPI), PGS, Dp, E2F, *ras*, *myc*, *neu*, *raf*, *erb*, *fms*, *trk*, *ret*, *gsp*, *hst*, *abl*, E1A, p300, genes involved in angiogenesis (e.g., VEGF, FGF, thrombospondin, BAI-1, GDAIF, or their receptors) and MCC.

20 **Regulators of Programmed Cell Death.** Apoptosis, or programmed cell death, is an essential process for normal embryonic development, maintaining homeostasis in adult tissues, and suppressing carcinogenesis (Kerr *et al.*, 1972). The Bcl-2 family of proteins and ICE-like proteases have been demonstrated to be important regulators and effectors of apoptosis in other systems. The Bcl-2 protein, discovered in association with follicular lymphoma, plays a prominent role in controlling apoptosis and enhancing cell survival in response to diverse apoptotic stimuli (Bakhshi *et al.*, 1985; Cleary and Sklar, 1985; Cleary *et al.*, 1986; Tsujimoto *et al.*, 1985; Tsujimoto and Croce, 1986). The evolutionarily conserved Bcl-2 protein now is recognized to be a member of a family of related proteins, which can be categorized as death agonists or death antagonists.

25 Subsequent to its discovery, it was shown that Bcl-2 acts to suppress cell death triggered by a variety of stimuli. Also, it now is apparent that there is a family of Bcl-2 cell death regulatory proteins which share in common structural and sequence homologies. These different family members have been shown to either possess similar functions to Bcl-2 (e.g.,

Bcl_{XL}, Bcl_W, Bcl_S, Mcl-1, A1, Bfl-1) or counteract Bcl-2 function and promote cell death (e.g., Bax, Bak, Bik, Bim, Bid, Bad, Harakiri).

D. Surgery

Approximately 60% of persons with cancer will undergo surgery of some type, which 5 includes preventative, diagnostic or staging, curative and palliative surgery. Curative surgery is a cancer treatment that may be used in conjunction with other therapies, such as the treatment of the present invention, chemotherapy, radiotherapy, hormonal therapy, gene therapy, immunotherapy and/or alternative therapies.

Curative surgery includes resection in which all or part of cancerous tissue is 10 physically removed, excised, and/or destroyed. Tumor resection refers to physical removal of at least part of a tumor. In addition to tumor resection, treatment by surgery includes laser surgery, cryosurgery, electrosurgery, and microscopically controlled surgery (Mohs' surgery). It is further contemplated that the present invention may be used in conjunction with removal of superficial cancers, precancers, or incidental amounts of normal tissue.

Upon excision of part of all of cancerous cells, tissue, or tumor, a cavity may be 15 formed in the body. Treatment may be accomplished by perfusion, direct injection or local application of the area with an additional anti-cancer therapy. Such treatment may be repeated, for example, every 1, 2, 3, 4, 5, 6, or 7 days, or every 1, 2, 3, 4, and 5 weeks or every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months. These treatments may be of varying 20 dosages as well.

E. Other agents

It is contemplated that other agents may be used in combination with the present 25 invention to improve the therapeutic efficacy of treatment. These additional agents include immunomodulatory agents, agents that affect the upregulation of cell surface receptors and GAP junctions, cytostatic and differentiation agents, inhibitors of cell adhesion, agents that increase the sensitivity of the hyperproliferative cells to apoptotic inducers, or other biological agents. Immunomodulatory agents include tumor necrosis factor; interferon α , β , and γ ; IL-2 and other cytokines; F42K and other cytokine analogs; or MIP-1, MIP-1 β , MCP-1, RANTES, and other chemokines. It is further contemplated that the upregulation of cell surface 30 receptors or their ligands such as Fas / Fas ligand, DR4 or DR5 / TRAIL (Apo-2 ligand) would potentiate the apoptotic inducing abilities of the present invention by establishment of

an autocrine or paracrine effect on hyperproliferative cells. Increases intercellular signaling by elevating the number of GAP junctions would increase the anti-hyperproliferative effects on the neighboring hyperproliferative cell population. In other embodiments, cytostatic or differentiation agents can be used in combination with the present invention to improve the 5 anti-hyperproliferative efficacy of the treatments. Inhibitors of cell adhesion are contemplated to improve the efficacy of the present invention. Examples of cell adhesion inhibitors are focal adhesion kinase (FAKs) inhibitors and Lovastatin. It is further contemplated that other agents that increase the sensitivity of a hyperproliferative cell to apoptosis, such as the antibody c225, could be used in combination with the present invention 10 to improve the treatment efficacy.

Apo2 ligand (Apo2L, also called TRAIL) is a member of the tumor necrosis factor (TNF) cytokine family. TRAIL activates rapid apoptosis in many types of cancer cells, yet is not toxic to normal cells. TRAIL mRNA occurs in a wide variety of tissues. Most normal cells appear to be resistant to TRAIL's cytotoxic action, suggesting the existence of 15 mechanisms that can protect against apoptosis induction by TRAIL. The first receptor described for TRAIL, called death receptor 4 (DR4), contains a cytoplasmic "death domain"; DR4 transmits the apoptosis signal carried by TRAIL. Additional receptors have been identified that bind to TRAIL. One receptor, called DR5, contains a cytoplasmic death domain and signals apoptosis much like DR4. The DR4 and DR5 mRNAs are expressed in 20 many normal tissues and tumor cell lines. Recently, decoy receptors such as DcR1 and DcR2 have been identified that prevent TRAIL from inducing apoptosis through DR4 and DR5. These decoy receptors thus represent a novel mechanism for regulating sensitivity to a pro-apoptotic cytokine directly at the cell's surface. The preferential expression of these inhibitory receptors in normal tissues suggests that TRAIL may be useful as an anticancer agent that 25 induces apoptosis in cancer cells while sparing normal cells. (Marsters *et al.*, 1999).

There have been many advances in the therapy of cancer following the introduction of cytotoxic chemotherapeutic drugs. However, one of the consequences of chemotherapy is the development/acquisition of drug-resistant phenotypes and the development of multiple drug resistance. The development of drug resistance remains a major obstacle in the treatment of 30 such tumors and therefore, there is an obvious need for alternative approaches such as gene therapy.

Another form of therapy for use in conjunction with chemotherapy, radiation therapy or biological therapy includes hyperthermia, which is a procedure in which a patient's tissue is exposed to high temperatures (up to 106°F). External or internal heating devices may be involved in the application of local, regional, or whole-body hyperthermia. Local 5 hyperthermia involves the application of heat to a small area, such as a tumor. Heat may be generated externally with high-frequency waves targeting a tumor from a device outside the body. Internal heat may involve a sterile probe, including thin, heated wires or hollow tubes filled with warm water, implanted microwave antennae, or radiofrequency electrodes.

A patient's organ or a limb is heated for regional therapy, which is accomplished 10 using devices that produce high energy, such as magnets. Alternatively, some of the patient's blood may be removed and heated before being perfused into an area that will be internally heated. Whole-body heating may also be implemented in cases where cancer has spread throughout the body. Warm-water blankets, hot wax, inductive coils, and thermal chambers may be used for this purpose.

15 Hormonal therapy may also be used in conjunction with the present invention or in combination with any other cancer therapy previously described. The use of hormones may be employed in the treatment of certain cancers such as breast, prostate, ovarian, or cervical cancer to lower the level or block the effects of certain hormones such as testosterone or estrogen. This treatment is often used in combination with at least one other cancer therapy as 20 a treatment option or to reduce the risk of metastases.

Table 6. Oncogenes

Gene	Source	Human Disease	Function
Growth Factors			
<i>HST/KS</i>	Transfection		FGF family member
<i>INT-2</i>	MMTV promoter Insertion		FGF family member
<i>INT1/WNT1</i>	MMTV promoter Insertion		Factor-like
<i>SIS</i>	Simian sarcoma virus		PDGF B
Receptor Tyrosine Kinases			
<i>ERBB/HER</i>	Avian erythroblastosis virus; ALV promoter insertion; amplified human tumors	Amplified, deleted Squamous cell Cancer; glioblastoma	EGF/TGF- α / Amphiregulin/ Hetzellulin receptor
<i>ERBB-2/NEU/HER-2</i>	Transfected from rat Glioblastomas	Amplified breast, Ovarian, gastric cancers	Regulated by NDF/ Hetzellulin and EGF- Related factors
<i>FMS</i>	SM feline sarcoma virus		CSF-1 receptor
<i>KIT</i>	HZ feline sarcoma virus		MGF/Steel receptor

Table 6. Oncogenes

<i>Gene</i>	<i>Source</i>	<i>Human Disease</i>	<i>Function</i>
<i>TRK</i>	Transfection from human colon cancer		Hematopoiesis NGF (nerve growth Factor) receptor
<i>MET</i>	Transfection from human osteosarcoma		Scatter factor/HGF Receptor
<i>RET</i>	Translocations and point mutations	Sporadic thyroid cancer; Familial medullary thyroid cancer; multiple endocrine neoplasias 2A and 2B	Orphan receptor Tyr Kinase
<i>ROS</i>	URII avian sarcoma Virus		Orphan receptor Tyr Kinase
<i>PDGF</i> receptor	Translocation	Chronic Myelomonocytic Leukemia	TEL(ETS-like Transcription factor)/ PDGF receptor gene Fusion
<i>TGF-β</i> receptor		Colon carcinoma Mismatch mutation target	
NONRECEPTOR TYROSINE KINASES			
<i>ABI</i>	Abelson Mul.V	Chronic myelogenous Leukemia translocation with BCR	Interact with RB, RNA Polymerase, CRK, CBL
<i>FPS/FES</i>	Avian Fujinami SV;GA FeSV		
<i>LCK</i>	Mul.V (murine leukemia virus) promoter insertion		Src family; T cell Signaling; interacts CD4/CD8 T cells
<i>SRC</i>	Avian Rous sarcoma Virus		Membrane-associated Tyr kinase with signaling function; activated by receptor kinases
<i>YES</i>	Avian Y73 virus		Src family; signaling
SER/THR PROTEIN KINASES			
<i>AKT</i>	AKT8 murine retrovirus		Regulated by PI(3)K?; regulate 70-kd S6 k?
<i>MOS</i>	Maloney murine SV		GVBD; cystostatic factor; MAP kinase kinase
<i>PIM-1</i>	Promoter insertion Mouse		
<i>RAF/MIL</i>	3611 murine SV; MH2 avian SV		Signaling in RAS Pathway
MISCELLANEOUS CELL SURFACE			
<i>APC</i>	Tumor suppressor	Colon cancer	Interacts with catenins
<i>DCC</i>	Tumor suppressor	Colon cancer	CAM domains
E-cadherin	Candidate tumor Suppressor	Breast cancer	Extracellular homotypic binding; intracellular interacts with catenins
<i>PTC/NBCCS</i>	Tumor suppressor and <i>Drosophila</i> homology	Nevvoid basal cell cancer Syndrome (Gorline syndrome)	12 transmembrane domain; signals through Gli homologue CI to antagonize

Table 6. Oncogenes

Gene	Source	Human Disease	Function
<i>TAN-1</i> homologue	Notch Translocation	T-ALI.	Hedgehog pathway Signaling
MISCELLANEOUS SIGNALING			
<i>BCL-2</i>	Translocation	B-cell lymphoma	Apoptosis
<i>CBL</i>	Mu Cas NS-1 V		Tyrosine- Phosphorylated RING finger interact Abl
<i>CRK</i>	CT1010 ASV		Adapted SH2/SH3 interact Abl
<i>DPC4</i>	Tumor suppressor	Pancreatic cancer	TGF- β -related signaling Pathway
<i>MAS</i>	Transfection and Tumorigenicity		Possible angiotensin Receptor
<i>NCK</i>			Adaptor SH2/SH3
GUANINE NUCLEOTIDE EXCHANGERS AND BINDING PROTEINS			
<i>BCR</i>		Translocated with ABL in CML	Exchanger; protein Kinase
<i>DBL</i>	Transfection		Exchanger
<i>GSP</i>			
<i>NF-1</i>	Hereditary tumor Suppressor	Tumor suppressor Neurofibromatosis	RAS GAP
<i>OST</i>	Transfection		Exchanger
Harvey-Kirsten, N-	HaRat SV; Ki RaSV; Balb-MoMuSV;		Signal cascade
<i>RAS</i>	Transfection	Point mutations in many human tumors	
<i>VAV</i>	Transfection		S112/S113; exchanger
NUCLEAR PROTEINS AND TRANSCRIPTION FACTORS			
<i>BRCA1</i>	Heritable suppressor	Mammary Cancer/ovarian cancer	Localization unsettled
<i>BRCA2</i>	Heritable suppressor	Mammary cancer	Function unknown
<i>ERBA</i>	Avian erythroblastosis Virus		Thyroid hormone receptor (transcription)
<i>ETS</i>	Avian E26 virus		DNA binding
<i>EVII</i>	MuLV promotor Insertion	AML	Transcription factor
<i>FOS</i>	FBI/FBR murine osteosarcoma viruses		Transcription factor with c-JUN
<i>GLI</i>	Amplified glioma	Glioma	Zinc finger; cubitus Interrup tus homologue is in hedgehog signaling pathway; inhibitory link PTC and hedgehog
<i>HMGI/LIM</i>	Translocation <i>t</i> (3;12) <i>t</i> (12;15)	Lipoma	Gene fusions high mobility group HMGI-C (XT-hook) and transcription factor LIM or acidic domain
<i>JUN</i>	ASV-17		Transcription factor AP-1 with FOS
<i>MLL/VHRX</i> <i>ELI/MEN</i>	+ Translocation/fusion ELL with MLL	Acute myeloid leukemia	Gene fusion of DNA- binding and methyl

Table 6. Oncogenes

<i>Gene</i>	<i>Source</i>	<i>Human Disease</i>	<i>Function</i>
	Trithorax-like gene		transferase MLL with ELI RNA pol II
			Elongation factor
<i>MYB</i>	Avian myeloblastosis Virus		DNA binding
<i>MYC</i>	Avian MC29; Translocation B-cell Lymphomas; promoter Insertion avian leukosis Virus	Burkitt's lymphoma	DNA binding with MAX partner; cyclin Regulation; interact RB?; regulate Apoptosis?
<i>N-MYC</i>	Amplified	Neuroblastoma	
<i>L-MYC</i>		Lung cancer	
<i>REL</i>	Avian Reticuloendotheliosis Virus		NF- κ B family Transcription factor
<i>SKI</i>	Avian SKV770 Retrovirus		Transcription factor
<i>VHL</i>	Heritable suppressor	Von Hippel-Landau Syndrome	Negative regulator or elongin; transcriptional elongation complex
<i>WT-1</i>		Wilm's tumor	Transcription factor
CELL CYCLE/DNA DAMAGE RESPONSE			
<i>ATM</i>	Hereditary disorder	Ataxia-telangiectasia	Protein/lipid kinase Homology; DNA damage response upstream in P53 pathway
<i>BCL-2</i>	Translocation	Follicular lymphoma	Apoptosis
<i>FACC</i>	Point mutation	Fanconi's anemia group C (predisposition Leukemia	
<i>FHIT</i>	Fragile site 3p14.2	Lung carcinoma	Histidine triad-related Diadenosine 5',3'''-P ¹ .P ⁴ tetraphosphate Asymmetric hydrolase
<i>hMLI/MutL</i>		HNPCC	Mismatch repair; MutL Homologue
<i>HMSH2/MutS</i>		HNPCC	Mismatch repair; MutS Homologue
<i>HPMS1</i>		HNPCC	Mismatch repair; MutL Homologue
<i>HPMS2</i>		HNPCC	Mismatch repair; MutL Homologue
<i>INK4/MTS1</i>	Adjacent INK-4B at 9p21; CDK complexes	Candidate MTS1 Suppressor and MLM melanoma gene	p16 CDK inhibitor
<i>INK4B/MTS2</i>		Candidate suppressor	
<i>MDM-2</i>	Amplified	Sarcoma	p15 CDK inhibitor
<i>p53</i>	Association with SV40 T antigen	Mutated >50% human Tumors, including hereditary Li-Fraumeni syndrome	Negative regulator p53 Transcription factor; Checkpoint control; apoptosis
<i>PRAD1/BCL1</i>	Translocation with Parathyroid hormone or IgG	Parathyroid adenoma; B-CLL	Cyclin D

Table 6. Oncogenes

<i>Gene</i>	<i>Source</i>	<i>Human Disease</i>	<i>Function</i>
<i>RB</i>	Hereditary Retinoblastoma; Association with many DNA virus tumor Antigens	Retinoblastoma; Osteosarcoma; breast Cancer; other sporadic Cancers	Interact cyclin/cdk; regulate E2F transcription factor
<i>XPA</i>		Xeroderma Pigmentosum; skin Cancer predisposition	Excision repair; photo- product recognition; zinc finger

VIII. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

10

EXAMPLE 1: Material and Methods

Viruses and cell lines. The panel of wild type poxvirus strains (Wyeth, Western Reserve (WR), USSR, Tian Tan, Tash Kent, Patwadangar, Lister, King, IHD-W, IHD-J and Evans) was kindly provided by Dr Geoff Smith, Imperial College, London. Human Adenovirus serotype 5 (Ad5) was obtained from ATCC. The Viral growth factor (VGF) deleted strain of WR (vSC20) was kindly provided by Dr Bernie Moss, NIH. The thymidine kinase deleted strain of WR (vJS6) and the TK-, VGF- double deleted strain of WR (vvDD) are described in Puhlmann *et al.* (2000) and McCart *et al.* (2001). WR strain expressing firefly luciferase was kindly provided by Dr Gary Luker, (Uni Michigan).

Vaccinia strain JX-963 was constructed by recombination of a version of the pSC65 plasmid containing the *E.coli* gpt and human GM-CSF genes (under the control of the p7.5 and pSE/L promoters respectively) into the thymidine kinase gene of the vSC20 (VGF deleted) strain of WR. Further selection of white plaques after propagation of the virus in X-Gal produced a virus with non-functioning lacZ (lacZ is expressed from within VGF in vSC20). Correct insertion into the TK gene and loss of lacZ function was verified by sequencing and GM-CSF production verified by ELISA.

The vvDD expressing luciferase was constructed by insertion of a version of the pSC65 plasmid with luciferase under control of the p7.5 promoter into vSC20. Bioluminescence was verified using an IVIS 50 system (Xenogen, Alameda).

The human tumor cell lines include A2780 (Ovarian, obtained from ECACC), A549 (lung, obtained from ECACC), HCT 116, HT-29 and SW620 (colon, obtained from ATCC), HT-1080 (fibrosarcoma, obtained from ATCC), LNCaP (prostate, obtained from ATCC),

PANC-1 (pancreatic, obtained from ATCC), MCF-7 (breast, obtained from ATCC). Non-transformed cells include MRC-5 (lung fibroblast, obtained from ATCC), Beas-2B (bronchial epithelial, kindly provided by Tony Reid, UCSD) and the primary, normal cells NHBE (Normal human bronchial epithelial) and SAEC (Small airway bronchial epithelial), both 5 obtained from Clonetics (Walkersville, MD).

The mouse tumor cell lines include CMT 64 (C57/B6 lung, obtained from Cancer Research UK), JC (BALB/c mammary, obtained from ATCC), MC38 (C57/B6 colon, obtained from NIH) and TIB-75 (BNL 1ME A.7R.1)(BALB/c hepatic, obtained from ATCC). The cell lines NIH 3T3 and NIH 3T3 overexpressing H-Ras were kindly provided by Richard 10 Marais (ICR, London). The rabbit tumor cell line VX2 has been described previously (Kidd, 1940; Tjernberg, 1962; Chen *et al.*, 2004).

In vitro replication and cytopathic effect assays. Cell lines are seeded into 6-well plates at 5×10^6 cells/well and left overnight. Virus was then added at a multiplicity of infection (MOI) of 1.0 Plaque forming units (PFU)/cell and allowed to infect for 2h. At the 15 end of the infection the media was changed and plates incubated for 48h, the cells were then scraped into the media and collected. Cells were lysed by three rounds of freezing and thawing followed by sonication before serial dilutions of the crude viral lysate was added to BSC-1 cells to titer the virus. Plaque assay was performed as described previously (Earl *et al.*, 1998). Adenovirus was titered on A549 cells (Earl *et al.*, 1998). Studies are typically run 20 in triplicate.

In order to assess the cytopathic effect (CPE) of the virus, cells were seeded at 1000 cells/well in 96-well plates and allowed to attach overnight. Serial dilutions of the viruses to be tested were then added to the plates in triplicate (MOI range from 100 to 0.001) and the plates incubated for a further 72h. After this time media was replaced with media without 25 serum and MTS (Promega) added to the plates. After 2-4h incubation the absorbance at 450nm was read on an ELISA plate reader. Cytopathic effect was determined as reduction in viability of a test well relative to both untreated wells containing cells only (100% viable) and cell-free wells (0% viable). Results were represented as the MOI at which 50% of the cell layer was viable (effective concentration 50%, EC50).

30 **Mouse syngeneic and xenograft tumor model studies.** Immunocompetent mice are implanted subcutaneously with syngeneic tumor cells (1×10^6 cells/mouse), such that JC and TIB-75 cells are implanted into BALB/c mice and MC38 and CMT 64 cells are implanted

into C57/B6 mice. Certain human xenograft models involve 1×10^7 HT29 cells implanted subcutaneously into SCID mice (all mice are aged 8-10 weeks and sex matched). Once tumors reached 50-100 mm³ animals are regrouped and treated as indicated. Tumor sizes were followed by caliper measurement.

5 Mice treated with luciferase expressing virus can be imaged using an IVIS 100 system (Xenogen, Alameda). Mice are injected intraperitoneally with luciferin (30 mg/kg) and anesthetized (2% isoflurane) prior to imaging.

10 Some mice are sacrificed at times indicated post-treatment and organs are recovered for viral biodistribution or immunohistochemical studies. For viral biodistribution, organs are snap frozen and ground before plaque assays are performed as described. For immunohistochemistry studies, organs are fixed in formalin before embedding in paraffin blocks for sectioning. Sections are stained with hematoxylin and eosin (H & E) and with viral coat proteins (polyclonal anti-vaccinia antibody or polyclonal antihexon antibody for adenovirus treated animals).

15 **Rabbit model.** The implantation of VX2 tumors into the livers of New Zealand White rabbits and the measurement of tumor progression and metastasis to the lungs by CT and ultrasound scans has been described previously (Paeng *et al.*, 2003).

20 **Cytotoxic T-lymphocyte (CTL) assay.** This is performed by mixing labeled peripheral blood lymphocytes (PBLs) obtained from rabbits treated as indicated with VX2 tumor cells. After a 4h period cell apoptosis was measured by propidium iodide staining and flow cytometry.

25 **Neutralizing Antibody assay.** Production of anti-vaccinia neutralizing antibody is measured in the plasma obtained from rabbits post-treatment. Dilutions of plasma are mixed with 1000 PFU of vaccinia overnight before addition to a 96-well plate containing A2780 cells. After 72h cell viability is measured by MTS assay. Viral neutralization is measured as the dilution of plasma required to prevent viral inactivation.

Statistical analyses. Kaplan-Meier curves are compared using the Generalized Wilcoxon test. Tumor response rates and metastasis-free rates are typically compared with Fisher's exact test.

EXAMPLE 2: Rat Tumor Model

Rats (Sprague-Dawley, Males) were exposed to carcinogen (N-Nitrosomorpholine, NNM) in their drinking water (175 mg/L) for a period of 8 weeks, during which time liver cirrhosis developed, followed by *in situ* development of tumors (hepatocellular carcinoma or 5 cholangiocarcinoma) within the liver between weeks 16–20 on average (model previously described in Oh *et al.*, 2002). Tumor detection and evaluation was performed by an experienced ultrasonographer using ultrasound imaging. Tumor sizes were approximately 0.75 - 1.5 cm. in diameter at baseline immediately prior to treatment initiation; tumor volumes were not significantly different at baseline between the control and treatment groups 10 (estimated mean volumes were 400-500 mm³). Control animals (n=17) received no treatment, whereas treated animals (n=6) received intravenous injections (via tail vein) with a poxvirus (Wyeth strain; thymidine kinase gene deletion present) expressing human GM-CSF from a synthetic early-late promoter (virus construct described in Mastrangelo *et al.*, 1999). Virus was administered at a dose of 10⁸ plaque-forming units (titered as in Earl *et al.*, 1998) in a 15 total volume of 0.75 ml; (virus suspension mixed with 10 mM Tris up to the desired volume) intravenously by tail vein over 60 seconds. Treatment was repeated every two weeks for three total doses (day 1, 15 and 29).

Over ten weeks following the initiation of treatment, the control tumors increased in size significantly until reaching a mean of approximately 3000 mm³ (S.E. 500) (FIG. 1). 20 Control animals needed to be sacrificed for ethical reasons due to tumor progression at this time. All tumors had increased in size significantly. In contrast, five of the six treated tumors regressed completely (below the limit of detection by ultrasound). The mean tumor volume in the treated group was approximately 50 mm³ (S.E., <10; p<0.01 vs. controls).

EXAMPLE 3: Rabbit VX2 Tumor Model

25 A study was performed in a VX2 rabbit carcinoma model (as described in Paeng *et al.*, 2003). Rabbit was selected as a species because human GM-CSF was previously demonstrated to have significant biological activity in rabbits (in contrast to mice). VX2 tumors were grown in muscle of New Zealand white rabbits and cells from a 1 - 2 mm³ fragment of tumor were dissociated, resuspended in 0.1 ml normal saline and were injected 30 beneath the liver capsule (21 gauge needle; injection site covered with surgical patch with a purse-string tie) and allowed to grow for 14 days until primary tumors were established (mean diameter, 1.5 - 2.0 cm; est. volume 2-4 cm³). VX2 cells were demonstrated to be infectable

by vaccinia poxvirus *ex vivo* in a standard burst assay. Tumor sizes were monitored over time by CT scanning and by ultrasound. Over the following seven weeks, control (untreated) animals (n=18) developed tumor progression within the liver, with estimated mean tumor volumes reaching approximately 100 cm³ (S.E. approximately 20). In addition, numerous 5 tumor metastases progressed and became detectable within the lungs and livers over time (FIGS. 2A-B). By week 7, control animals all had detectable metastases, with a mean number of lung metastases of 17 (S.E. 2.3). The median survival of these control animals was 55 days (post-treatment initiation in treated animals), and all were dead within 80 days.

Treated animals (n=3) in the first experiment received a single intravenous injection 10 (via tail vein) with a poxvirus (Wyeth strain; thymidine kinase gene deletion present) expressing human GM-CSF from a synthetic early-late promoter (virus construct described in Mastrangelo *et al.*, 1999). Virus was administered at a dose of 10⁹ plaque-forming units (titered as in Earl *et al.*, 1998) in a total volume of 7 ml; (virus suspension mixed with 10 mM Tris up to the desired volume) intravenously by ear vein over 60 seconds. By week seven, in 15 contrast to controls, treated animals had no lung metastases detectable by CT scanning (FIGs. 2A - 2B). Survival was significantly increased, also. By 110 days post-treatment initiation, the median survival had not been reached, and approximately 70% were still alive.

Treated animals (n=6 per group) in a second experiment received three weekly 20 intravenous injections (via tail vein) with either JX-594, a poxvirus (Wyeth strain; thymidine kinase gene deletion present) expressing human GM-CSF from a synthetic early-late promoter (virus construct described in Mastrangelo *et al.*, 1999, which is hereby incorporated by reference), vvDD, a vaccinia WR strain with deletions in thymidine kinase and vaccinia growth factor genes (vvDD as described by McCart *et al.*, 1999), or JX-963, vaccinia WR strain with deletions in thymidine kinase and vaccinia growth factor genes and expressing 25 human GM-CSF from a synthetic early-late promoter. Virus was administered at a dose of 10⁸ plaque-forming units (titered as in Earl *et al.*, 1998) in a total volume of 7 ml; (virus suspension mixed with 10 mM Tris up to the desired volume) intravenously by ear vein over 60 seconds. By week seven, in contrast to controls, JX-963 treated animals had no lung metastases detectable by CT scanning (p<0.01 vs. controls) (FIG. 4). JX-594-treated animals 30 had a mean of 8 lung tumors (S.E. 2; p<0.05 vs controls). vvDD-treated animals had a mean of 5 lung tumors (S.E. 2; p<0.05 vs controls). Of note, JX-963 and vvDD also had significant efficacy against the primary tumor growth in the liver, in contrast to JX-594 at this dose (FIG. 3) and JX-963 dramatically increased the survival of these animals (FIG. 5).

The GM-CSF-expressing virus JX-963 had significantly better efficacy against both primary tumors and lung metastases than its non-GM-CSF-expressing control vvDD; 2) the GM-CSF-expressing virus JX-963 had significantly better efficacy against both primary tumors and lung metastases than its GM-CSF-expressing Wyeth strain control (despite an additional deletion in the vgf gene not present in JX-594). Therefore, intravenous administration with a vaccinia expressing human GM-CSF resulted in significantly better efficacy over the same vaccinia without GM-CSF, and intravascular administration of a WR strain deletion mutant expressing human GM-CSF was significantly better than a Wyeth strain (standard vaccine strain) deletion mutant expressing GM-CSF.

10 **EXAMPLE 4: Systemic cancer efficacy with JX-963**

Targeted therapies hold great promise for the treatment of cancer, but novel agents are still needed as resistance frequently develops through mutation of the target molecules and/ or tumor escape through pathway redundancies. Oncolytic viruses are viruses that have their replication restricted to malignant cell types, either inherently or through genetic engineering (Thorne *et al.*, 2005)¹. Selective intratumoral replication leads to virus multiplication, killing of the infected cancer cell by unique and apoptosis-independent mechanisms (oncolysis) and spread of the virus to other tumor cells. Virotherapeutics therefore have the potential to effectively treat refractory cancers and clinical proof-of-concept has been achieved with local or regional administration for several oncolytic viruses (Parato *et al.*, 2005)². However, for oncolytic viruses to have a major impact on patient survival, systemic efficacy and intravenous delivery will be needed.

The inventor has therefore undertaken a stepwise design and development strategy to create a more effective systemic agent. First, the inventor identified poxviruses such as vaccinia as a virus species that has evolved for systemic dissemination and resistance to clearance by complement and antibodies (Smith *et al.*, 1997; Buller and Palumbo, 1991). Vaccinia has well-defined mechanisms to allow for transport in the blood without inactivation and can spread rapidly within tissues, it also has a long history of human use during the smallpox eradication campaign. A panel of vaccinia viruses used during the vaccination program, and some related strains were screened for their ability to replicate in normal (NHBE) and tumor (A2780) cells. All vaccinia strains replicated to higher levels in the tumor cell line than in the normal cells (FIG. 6A), but the therapeutic index (tumor to normal cell replication ratio) varied between strains. Strains used extensively in the laboratory (such as

Western Reserve (WR)) tended to display greater inherent tumor selectivity *in vitro* than their parental vaccine strains (Wyeth). This is the first time that wild type vaccinia strains have been shown to display inherent superior replication in tumor cell lines relative to normal cells. This is not true for all viruses however, as Adenovirus serotype 5 (Ad5) (the backbone for the 5 majority of oncolytic viruses in the clinic) did not display such selectivity (FIG. 10A).

Another desirable attribute for an oncolytic agent is rapid intratumoral spread (Wein *et al.*, 2003). This can be achieved through a short replication cycle and early release of virus from infected cells. The ability of the WR strain of vaccinia to destroy tumor cells was therefore examined at early time points (72h) after infection and compared to Ad5 and the 10 oncolytic adenovirus strain *dl1520* (ONYX-015) (Heise *et al.*, 1997) (FIG. 6B). WR displayed up to 5-logs of increased killing potential in tumor cells at this time relative to both Ad5 and *dl1520*, as well as greater tumor selectivity than either adenoviral strain.

The major limitation of most oncolytic viruses tested to date is an inability to 15 efficiently infect tumors following systemic delivery, as seen when 1×10^9 plaque forming units (PFU) of Ad5 were delivered intravenously to subcutaneous tumor models in mice (FIG. 6C and FIG. 10B); this equates to a dose of 3.5×10^{12} PFU in a 70 kg human, higher than ever given to a patient. Little or no replicating virus was evident in tumors (as detected by immunohistochemical staining for viral coat proteins 48 and 72h after viral delivery). Vaccinia strain WR however could effectively traffic to and infect the tumors in these same 20 models, with up to 50% of the tumor cells staining positive within 48h of treatment. Furthermore, vaccinia was able to persist in the tumor for at least 10 days (FIG. 10B), despite the fact an immune response would have been initiated by this time.

In order to maximize safety, particularly for intravenous administration in 25 immunodeficient cancer patients, attenuating and tumor-targeting genetic deletions were introduced into the virus. The inventor has previously described preferential tumor-expression of viral genes with insertions into the vaccinia thymidine kinase (TK) gene and of TK and viral growth factor (VGF) double deletions (Puhlmann *et al.*, 1999; McCart *et al.*, 2001). Although the targeting mechanisms of these deletions were not previously demonstrated, the rationale was to restrict virus replication and oncolysis to cancer cells with 30 elevated E2F levels (as E2F drives production of the cellular thymidine kinase gene product (Hengstschlager *et al.* 1994)) and activation of the epidermal growth factor (EGF) receptor pathway (as activation of this pathway by VGF is necessary for efficient viral replication

(Andrade *et al.*, 2004)). Here it is shown that the TK and VGF double deleted virus (vvDD) displayed an impressive ability to destroy a wide range of tumor cells of different origins (FIG. 7). It was also found that single deletions in either the vaccinia TK or the VGF genes attenuated the ability of vaccinia to replicate in non-proliferating, non-transformed human cell 5 lines, while the double deleted virus (vvDD) was further attenuated (FIG. 11). None of these strains were attenuated in their ability to replicate in human tumor cells.

It was further found that the block in the ability of the VGF-deleted virus to replicate in non-proliferating, non-transformed cells could be overcome in cells expressing activated H-ras (FIG. 8A). It was found that H-ras activation led to increased replication of even WR 10 (p=0.0094), and that VGF deletion did not inhibit viral replication in H-ras activated cells, whereas the TK deletion did (p=0.016). This indicates that the tumor selectivity introduced by the gene deletions in vvDD is more than a simple preference for proliferating cells, since slowly proliferating or even non-proliferating cells could be targeted if they contained mutations in the EGF-R/ Ras/ MAP Kinase signaling pathway.

15 In order to determine whether the double deleted vaccinia (vvDD) might produce toxicity by targeting normal proliferating cells (such as gut epithelial, bone marrow or ovarian cells), *in vivo* viral gene expression was studied by non-invasive bioluminescence imaging (FIG. 8B) and viral biodistribution was examined post mortem (FIG. 12). Bioluminescence imaging following IV delivery of 1×10^7 PFU of WR or vvDD expressing luciferase showed 20 that both viruses displayed similar initial infection and viral gene expression patterns (including spleen, lung, liver and tumor) (FIG. 8B). However, the bioluminescent signal from vvDD was rapidly cleared from most organs other than the tumor, even in immunodeficient mice, while WR continued to replicate in the target organs and spread to other tissues, including bone marrow, skin and brain (FIGS. 8B and 8C). Although vvDD did produce 25 some points of infection outside of the tumor, these appeared transiently and late, indicating secondary spread without replication (data not shown). Recovery of infectious viral units from tissues of mice treated IV with 1×10^9 PFU of vvDD (a lethal dose for WR) revealed that by day 8 after treatment the tumor displayed increasing viral titer, with over 1,000-fold more 30 viral copies per mg tissue than any other organ, while all normal tissues were below the limits of detection or showed falling viral titers (FIG. 12).

The anti-tumor effects of vvDD were then analyzed in immunocompetent mouse models. vvDD had significantly greater anti-tumor effects than a Wyeth TK deleted vaccinia

strain (the most common vaccinia strain in clinical trials, usually used as a vaccine) when both were delivered intravenously (FIG. 13). Further studies showed that 1×10^9 PFU of vvDD was capable of significant anti-tumor effects when delivered by either systemic or intratumoral injection to both immunodeficient mice carrying human tumor xenografts and 5 immunocompetent mice bearing syngeneic tumors (FIG. 13).

In order to increase the anti-tumor potential of vvDD, and to suppress the outgrowth of microscopic tumor deposits that are not vascularized at the time of IV dosing, the cytokine GM-CSF was inserted into the site of the TK gene (under the control of the synthetic E/L promoter); this virus was designated JX-963. Because human GM-CSF is not active in 10 rodents but is active in rabbits (Cody *et al.*, 2005), and in order to assess the activity against much larger primary tumors that reproducibly metastasize, JX-963 was used in a rabbit model with primary (VX2) liver tumors and lung metastases (Kim *et al.*, 2006). As in the mouse models, 1×10^9 PFU of intravenous vvDD had significant anti-tumor effects (FIG. 9A). The vvDD virus was also capable of inhibiting the outgrowth of microscopic lung metastases. In 15 order to assess additional efficacy due to concomitant GM-CSF expression, JX-963 was compared directly to vvDD. JX-963 produced greater efficacy against the primary tumor, and completely blocked outgrowth of lung metastases. GM-CSF was detected in the plasma of JX-963 treated mice by ELISA (data not shown). In addition to direct oncolytic effects, JX-963 was also found to cross-protect the animal against the tumor by raising a CTL response 20 against the VX2 tumor cells (FIG. 9B).

One concern in using vaccinia virus as an anti-tumor agent is that, even though systemic delivery to the tumor is initially possible in naïve individuals, the immune response raised by prior exposure to the virus may inhibit the efficacy of subsequent treatment. A 25 strong anti-viral antibody response was raised within 3 weeks of initial infection in the rabbits tested (FIG. 14). To study the feasibility of repeat dosing after neutralizing antibody formation, four rabbits that had initially responded to treatment but had tumor progression after four weeks off of therapy were re-treated. 1×10^9 PFU of JX-963 delivered intravenously at 6 weeks after the initial treatment resulted in a decrease in primary tumor size in 3 of 4 animals treated (FIG. 9C).

30 Therefore, by selecting vaccinia virus, that has evolved to spread through the hematopoietic system, and screening strains for tumor selective replication the inventor was able to find a virus capable of systemic tumor delivery with rapid oncolytic effects. In order

to improve the safety of this virus several deletions capable of increasing its therapeutic index were introduced, their mechanism of action described and their biodistribution examined *in vivo*. Dramatic therapeutic effects against large primary tumors following systemic delivery were demonstrated. Finally, because it is unlikely all tumor cells will be infected, even following systemic viral delivery, GM-CSF was expressed from this viral backbone. The addition of GM-CSF was found to increase the effectiveness of this virus against primary tumors, prevent the outgrowth of micrometastases, and produced an anti-tumor CTL response. This indicates that this virus, JX-963, is capable of systemic delivery to tumors, where it rapidly and efficiently destroys tumor tissue, while sparing normal organs, and at the same time induces an immune response within the tumor that is capable of recognizing tumor antigens produced *in situ*. Repeat dosing was further shown to produce additional anti-tumor effects, either by direct oncolysis or by boosting the anti-tumor immune response. JX-963 therefore has the potential to effectively treat a variety of tumors.

* * * * *

5 All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method 10 described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention 25 as defined by the appended claims.

Throughout the specification and the claims, unless the context requires otherwise, the word "comprise" and its variations, such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

30 The reference to any prior art in this specification is not, and should not be taken as an acknowledgement or any form of suggestion that such art forms part of the common general knowledge in Australia.

IX. REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference:

- 5 U. S. Patent 4,554,101
- U. S. Patent 4,683,195
- U. S. Patent 4,683,202
- U. S. Patent 4,684,611
- U. S. Patent 4,800,159
- 10 U. S. Patent 4,879,236
- U. S. Patent 4,883,750
- U. S. Patent 4,946,773
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- U. S. Patent 5,220,007
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- U. S. Patent 5,284,760
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- 20 U. S. Patent 5,366,878
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CLAIMS

1. A method of killing a cancer cell in a human subject comprising administering to the human subject an effective amount of a replicative vaccinia virus lacking functional thymidine kinase and vaccinia growth factor genes and having an expression region with a promoter directing expression of a nucleic acid encoding granulocyte-macrophage colony stimulating factor (GM-CSF), wherein the administration is intravascular.
2. The method of claim 1, wherein the vaccinia virus is administered intravenously.
3. The method of claim 1, wherein the vaccinia virus is administered intraarterially.
4. The method of claim 1, wherein the vaccinia virus is in a pharmaceutically acceptable formulation.
5. The method of claim 1, wherein at least the thymidine kinase gene has been deleted.
6. The method of claim 1, wherein the vaccinia virus has a mutation in a gene encoding:
 - 20 a) vaccinia virus growth factor;
 - b) a functional interferon-modulating polypeptide, wherein the interferon-modulating polypeptide directly binds interferon;
 - c) a complement control polypeptide, wherein the mutation results in the virus lacking at least one functional complement control polypeptide;
 - 25 d) a TNF-modulating polypeptide, wherein the mutation results in the virus lacking at least one functional TNF-modulating polypeptide;
 - e) a serine protease inhibitor, wherein the mutation results in the virus lacking at least one functional serine protease inhibitor;
 - 30 f) an IL-1 β modulator polypeptide, wherein the mutation results in the virus lacking at least one functional IL-1 β modulator polypeptide;

- g) a functional A41L, B7R, N1L or vCKBP chemokine binding polypeptide or C11R EGF-like polypeptide, wherein the mutation results in the virus lacking at least one function of A41L, B7R, N1L, vCKBP, or C11R; or
- h) a polypeptide, wherein the mutation results in an increase in infectious EEV form of vaccinia virus.

7. The method of claim 5, wherein the vaccinia virus growth factor gene has been deleted.

8. The method of claim 1, wherein the vaccinia virus is the Wyeth or Western Reserve (WR) strain.

9. The method of claim 8, wherein the vaccinia virus is the WR strain.

10. The method of claim 1, wherein the promoter is a vaccinia virus promoter or a synthetic promoter.

11. The method of claim 10, wherein the promoter directs transcription during at least the early phase of infection.

12. The method of claim 10, wherein the promoter directs transcription during at least the late phase of infection.

13. The method of claim 1, wherein the subject is administered the vaccinia virus multiple times.

14. The method of claim 13, wherein a second treatment occurs within 3 weeks of a first treatment.

15. The method of claim 14, wherein the second treatment occurs within 2 weeks of the first treatment.

16. The method of claim 13, wherein the same dose is administered.
17. The method of claim 1, wherein the subject is administered between about 10^5 and about 10^{13} pfu of virus.
18. The method of claim 1, wherein the subject is administered between about 10^7 and about 10^{10} pfu of virus.
19. The method of claim 1, wherein the administration occurs intravascularly by injection, intravenous drip or bolus or using a pump.
20. The method of claim 1, wherein the cancer cell is a metastasized cancer cell.
21. The method of claim 1, wherein the subject has lung cancer, colorectal cancer, breast cancer, prostate cancer, pancreatic cancer, hepatocellular cancer, leukemia, lymphoma, myeloma, or melanoma.
22. A method for treating cancer in a human subject comprising administering to the human subject an effective amount of a replicative vaccinia virus lacking functional thymidine kinase and vaccinia growth factor genes and having an expression region with a promoter directing expression of a nucleic acid encoding granulocyte-macrophage colony stimulating factor (GM-CSF), wherein the administration is intravascular.
23. A method for treating one or more metastases in a human subject comprising administering to the human subject an effective amount of a replicative vaccinia virus lacking functional thymidine kinase and vaccinia growth factor genes and having an expression region with a promoter directing expression of a nucleic acid encoding granulocyte-macrophage colony stimulating factor (GM-CSF), wherein the administration is intravascular.
24. The method of claim 22 or claim 23, wherein the vaccinia virus is the WR strain.
25. The method of claim 1, substantially as hereinbefore described with reference to any one of the Examples.

26. The method of claim 22 or 23, substantially as hereinbefore described with reference to any one of the Examples.

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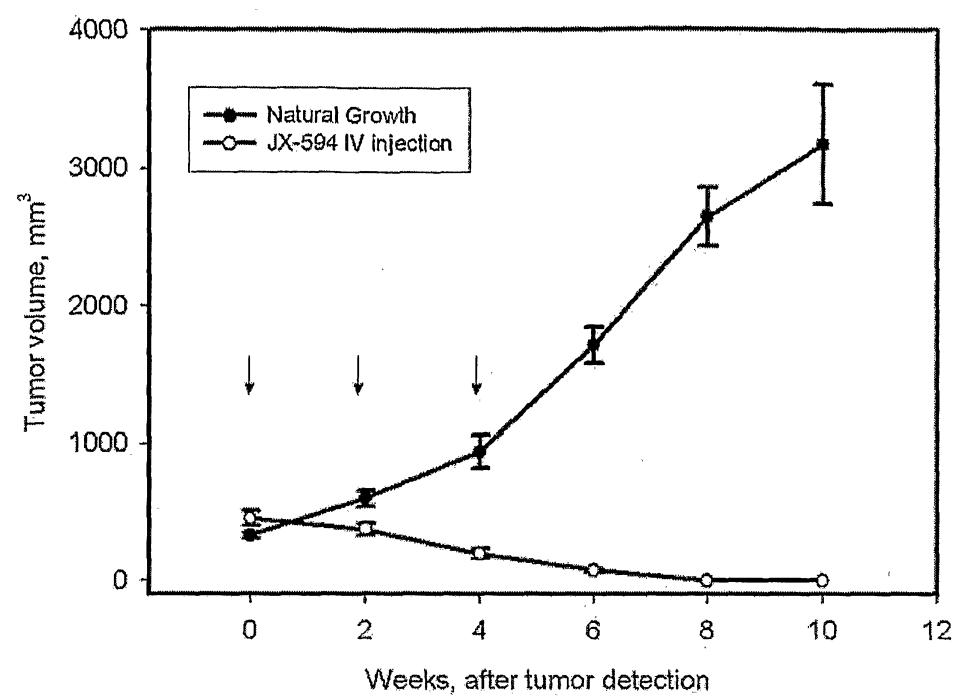


FIG. 1

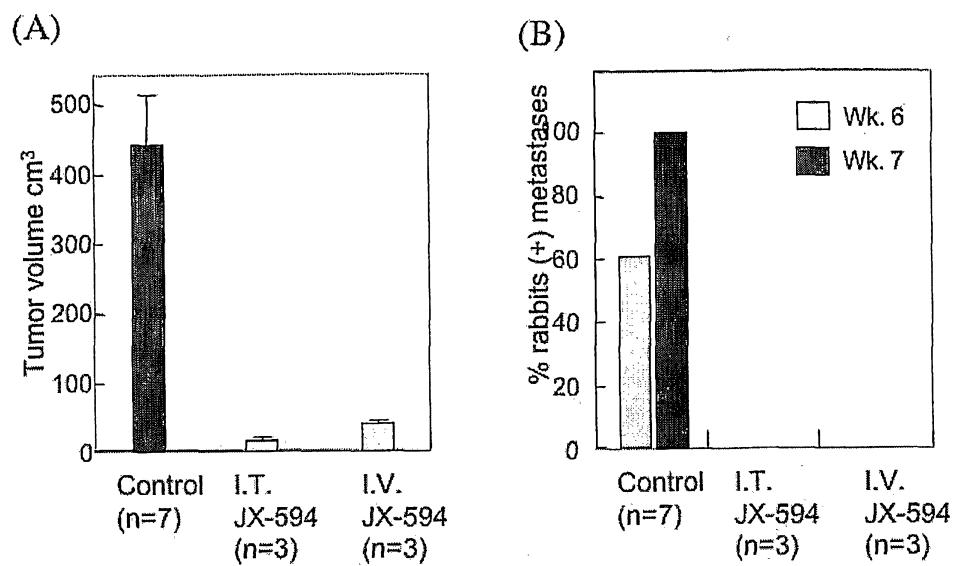


FIG. 2

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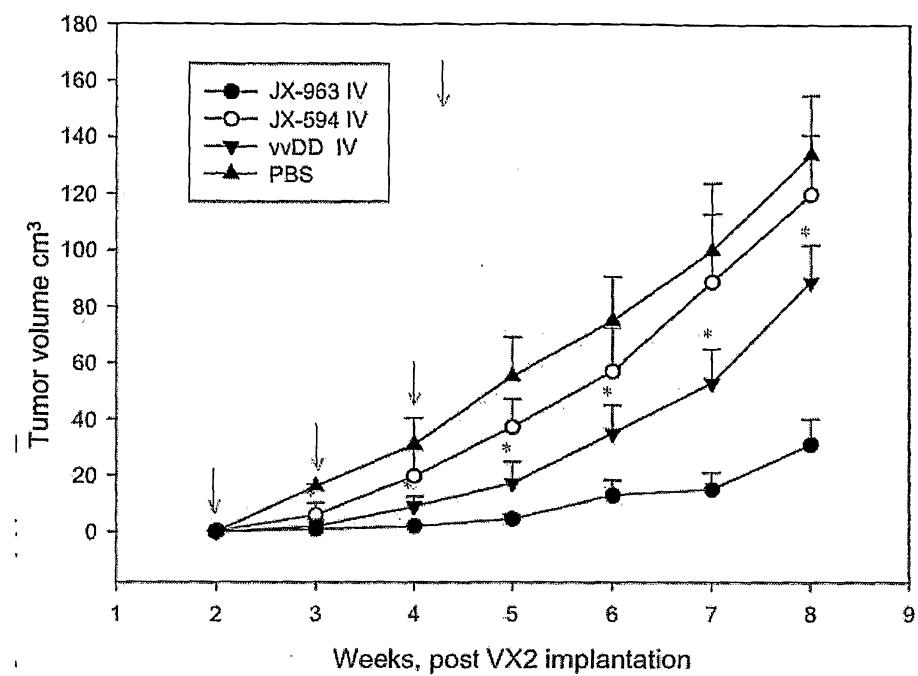
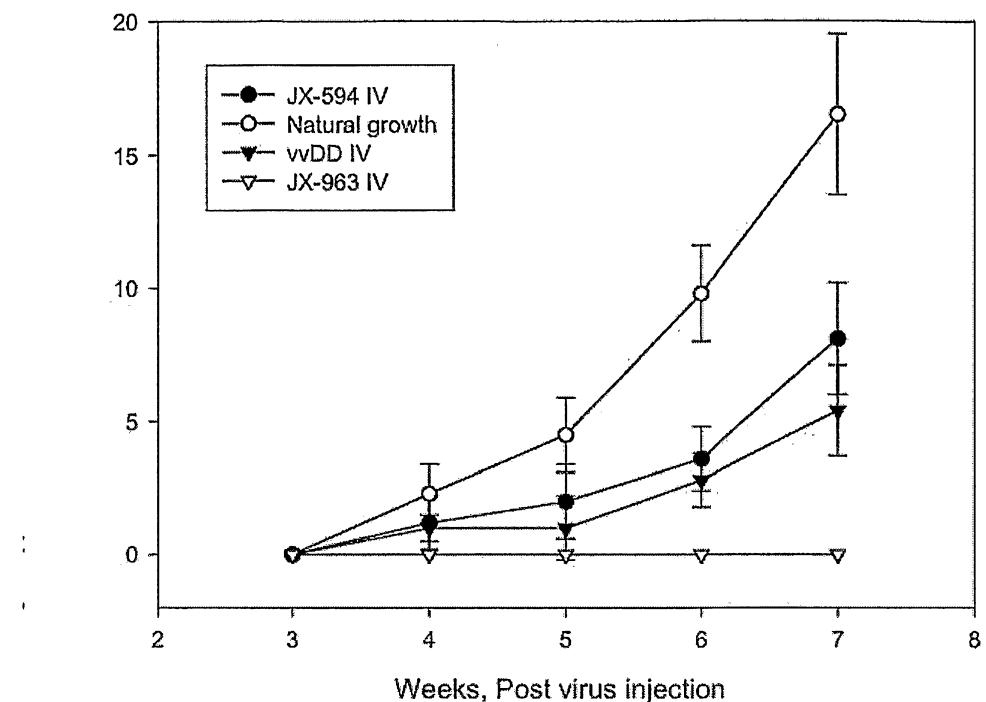


FIG. 3

**FIG. 4**

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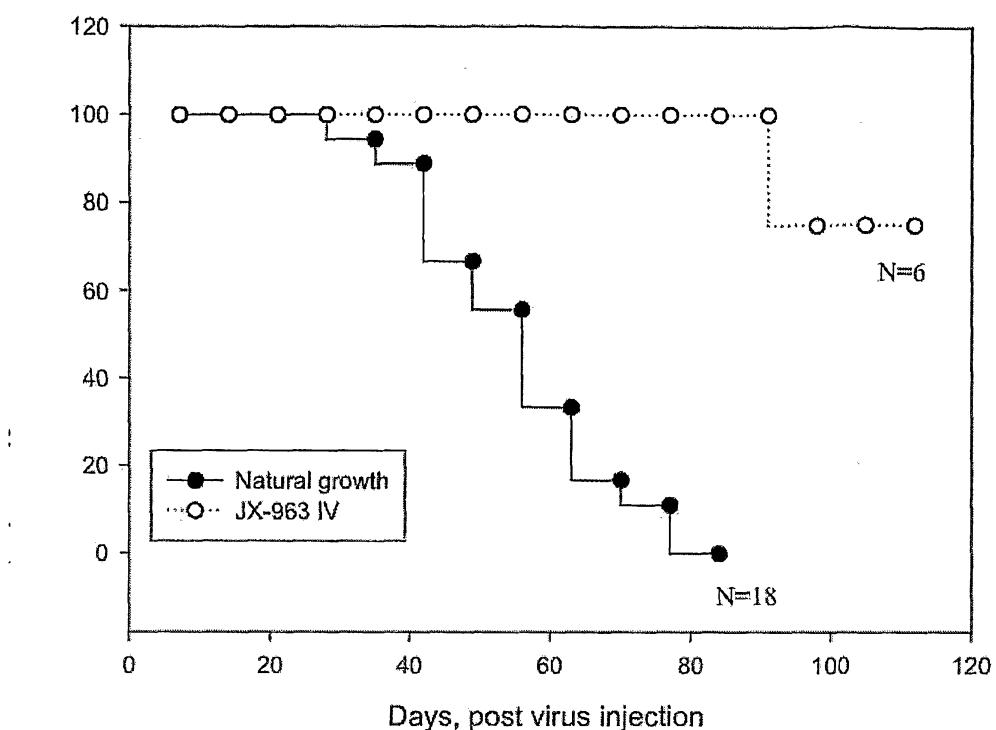


FIG. 5

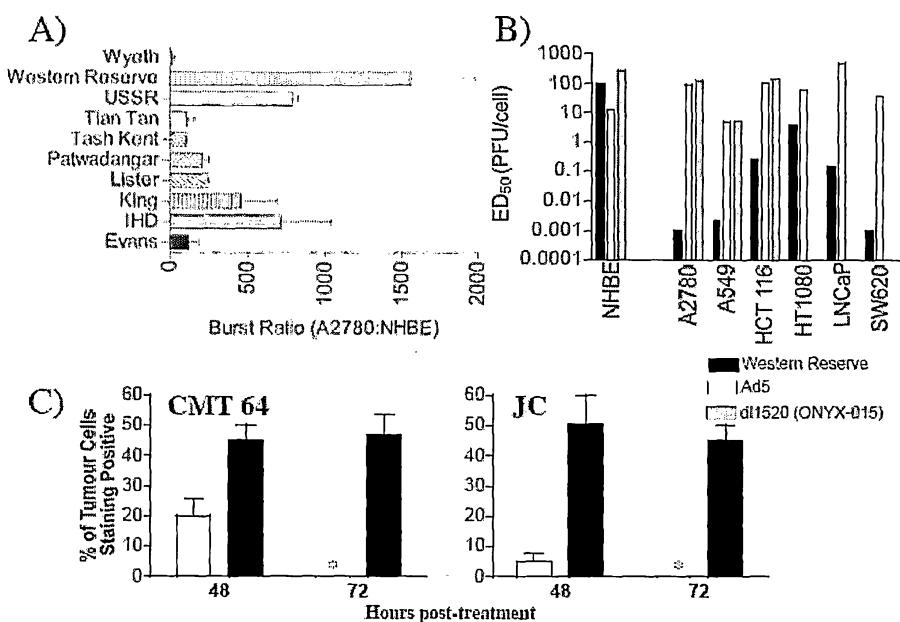


FIG. 6

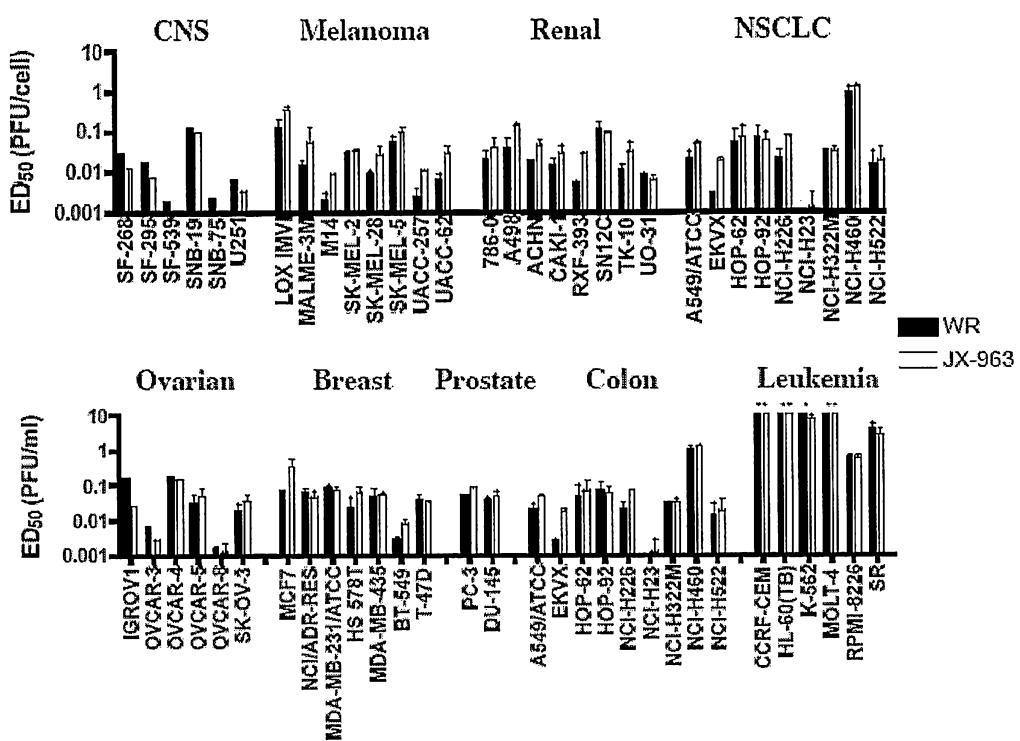


FIG. 7

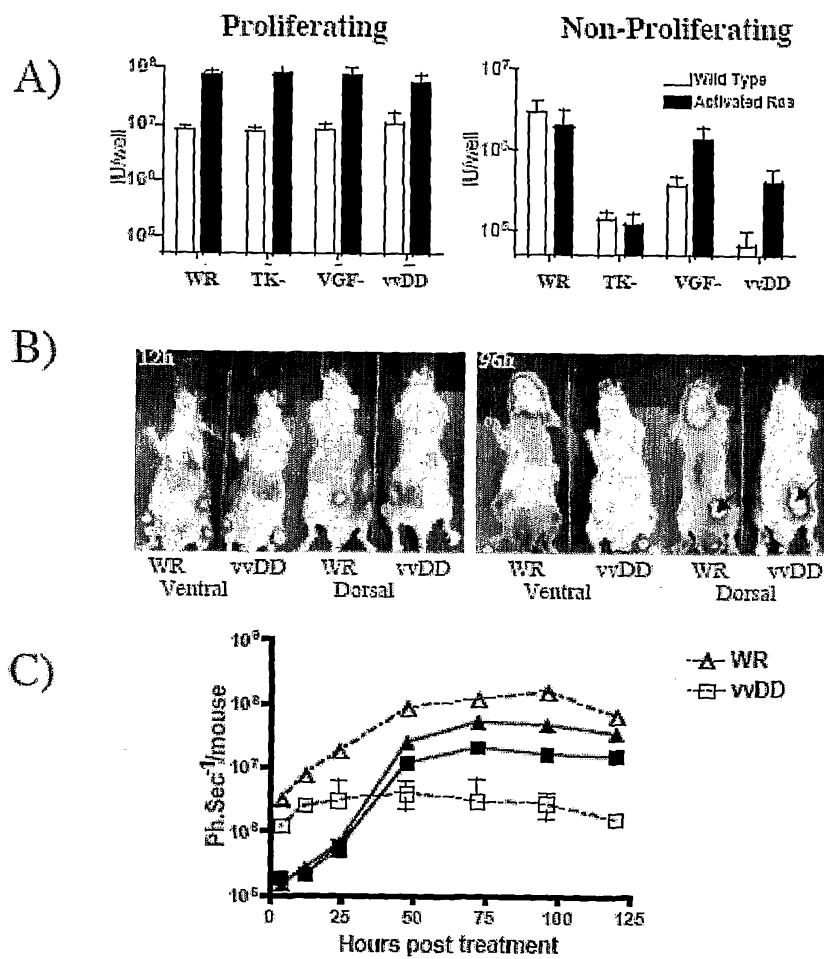


FIG. 8

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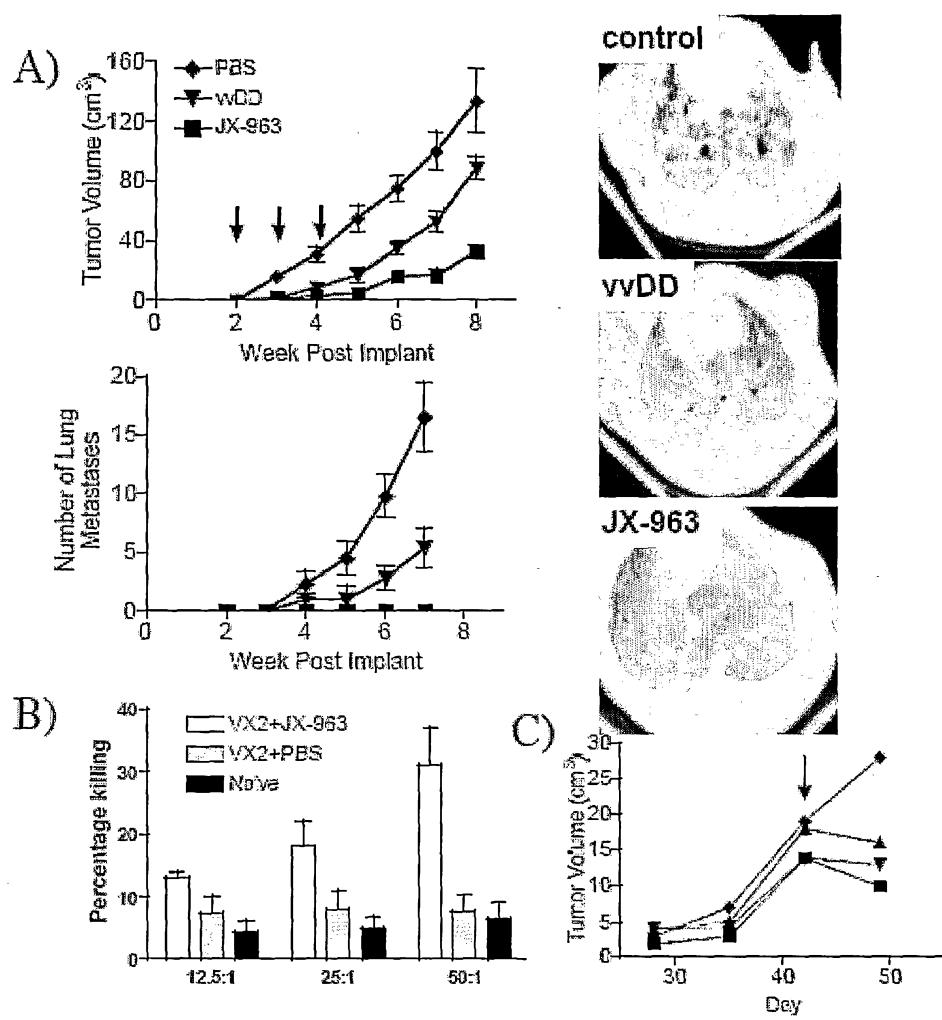


FIG. 9

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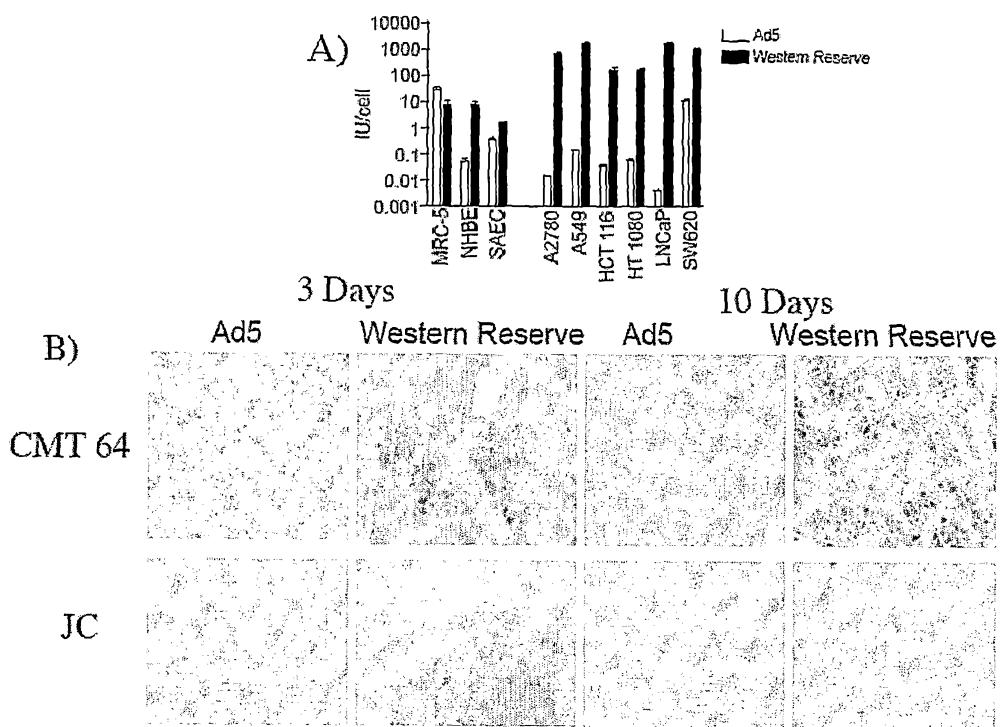


FIG. 10

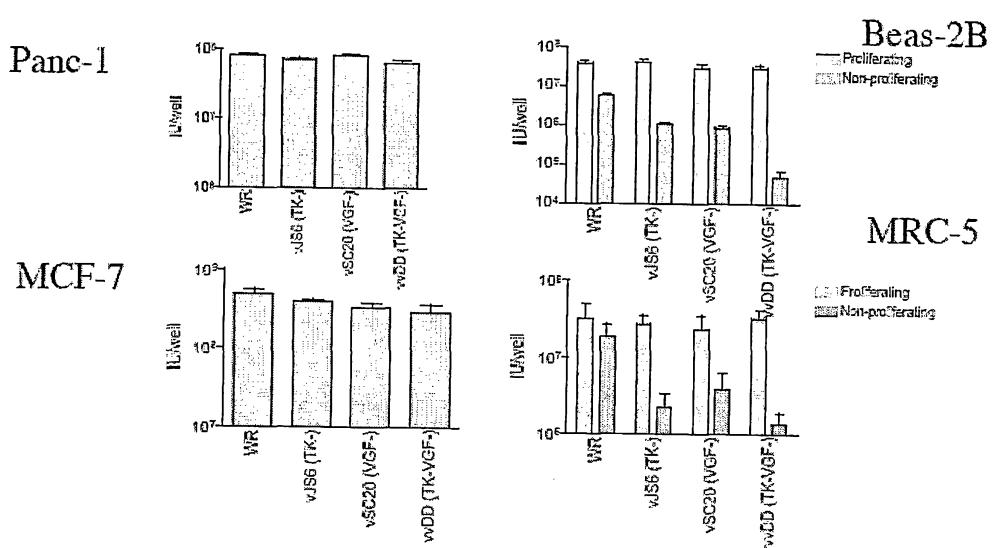


FIG. 11

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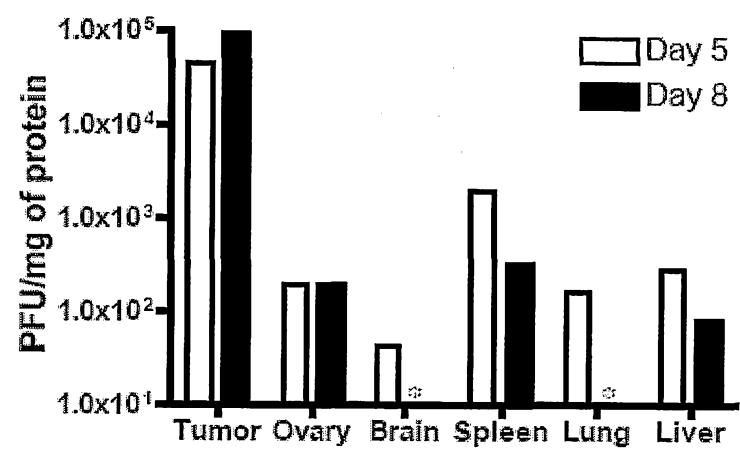


FIG. 12

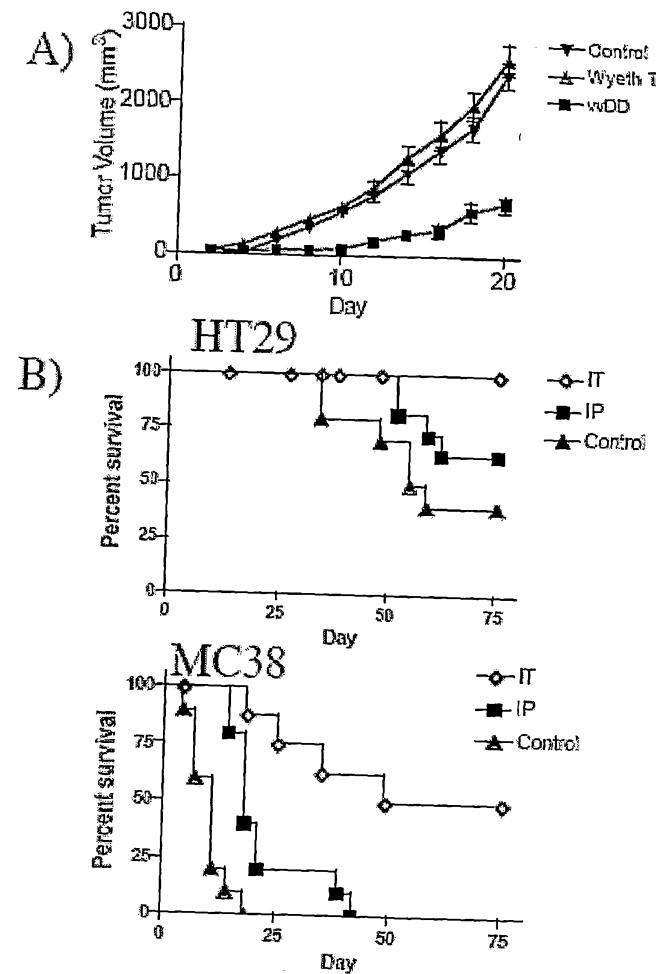
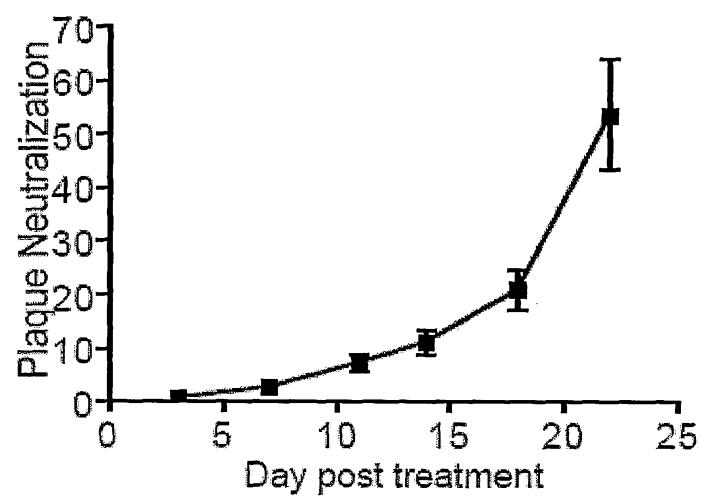


FIG. 13

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**FIG. 14**