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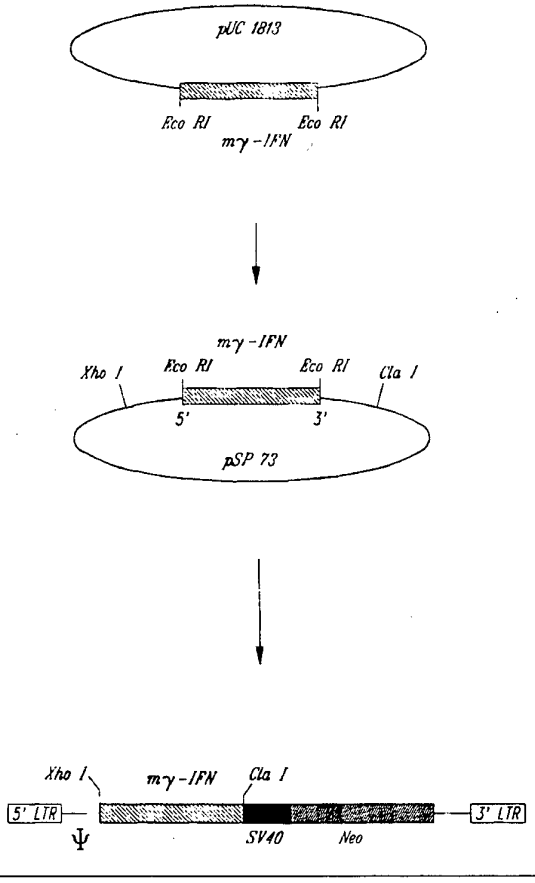
(54) **Title:** INHIBITION OF SELECTED TUMOR GROWTH WITH RECOMBINANT VIRAL SECTOR

(57) **Abstract**

The present invention provides methods for inhibiting the growth of selected tumors utilizing recombinant viral vectors. Briefly, within one aspect of the present invention, a method for inhibiting the growth of a selected tumor is provided comprising the step of directly administering to a warm-blooded animal a vector construct which directs the expression of at least one anti-tumor agent, such that the growth of said tumor is inhibited. Representative examples of anti-tumor agents include immune activators and tumor proliferation inhibitors.



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Description

INHIBITION OF SELECTED TUMOR GROWTH WITH RECOMBINANT VIRAL SECTOR

Technical Field

The present invention relates generally to the field of cancer immunotherapeutics, and more specifically, to methods of inhibiting the growth of a selected tumor utilizing vector constructs.

Background of the Invention

Cancer accounts for one-fifth of the total mortality in the United States, and is the second leading cause of death. Cancer is typically characterized by the uncontrolled division of a population of cells. This uncontrolled division typically leads to the formation of a tumor, which may subsequently metastasize to other sites.

Primary solid tumors can generally be treated by surgical resection. However, the majority of patients which have solid tumors also possess micrometastases beyond the primary tumor site. If treated with surgery alone, approximately 70% of these patients will experience recurrence of the cancer. In addition to surgery, many cancers are now also treated with a combination of therapies involving cytotoxic chemotherapeutic drugs (*e.g.*, vincristine, vinblastine, cisplatin, methotrexate, 5-FU, etc.) and/or radiation therapy. One difficulty with this approach however, is that radiotherapeutic and chemotherapeutic agents are toxic to normal tissues, and often create life-threatening side effects. In addition, these approaches often have extremely high failure/remission rates (up to 90% depending upon the type of cancer).

In addition to chemo- and radiation therapies, many have attempted to bolster or augment an individual's own immune system in order to eliminate the cancer cells. Several immunotherapies have utilized bacterial or viral components as adjuvants, in order to stimulate the immune system to destroy the tumor cells. Examples of such components include BCG, endotoxin, mixed bacterial vaccines, interferons (α, β, and γ), interferon inducers (*e.g.*, Brucella abortus, and various viruses), and thymic factors (*e.g.*, thymosin fraction 5, and thymosin alpha-1) (*see generally* "Principles of Cancer Biotherapy," Oldham (ed.), Raven Press, New York, 1987). Such agents have generally been useful as adjuvants and as nonspecific stimulants in animal tumor models, but have not yet proved to be generally effective in humans.

Lymphokines have also been utilized in the treatment of cancer. Briefly, lymphokines are secreted by a variety of cells, and generally have an effect on specific cells in the generation of an immune response. Examples of lymphokines include Interleukins (IL)-1, -2, -3, and -4, as well as colony stimulating factors such as G-CSF, GM-CSF, and M-CSF. Recently, one group has utilized IL-2 to stimulate peripheral blood cells in order to expand and produce large quantities of cells which are cytotoxic to tumor cells (Rosenberg et al., *N. Engl. J. Med. 313*:1485-1492, 1985).

Others have suggested the use of antibody-mediated anti-cancer therapies. Briefly, antibodies may be developed which recognize certain cell surface antigens that are either unique, or more prevalent on cancer cells compared to normal cells. These antibodies, or "magic bullets," may be utilized either alone or conjugated with a toxin in order to specifically target and kill tumor cells (Dillman, "Antibody Therapy," *Principles of Cancer Biotherapy*, Oldham (ed.), Raven Press, Ltd., New York, 1987). For example, Ball et al. (*Blood 62*:1203-1210, 1983) treated several patients with acute myelogenous leukemia with one or more of several monoclonal antibodies specific for the leukemia, resulting in a marked decrease in circulating leukemia cells during treatment. Similarly, others have utilized toxin-conjugated antibodies therapeutically to treat a variety of tumors, including, for example, melanomas, colorectal carcinomas, prostate carcinomas, breast carcinomas, and lung carcinomas (*see* Dillman, *supra*). One difficulty however, is that most monoclonal antibodies are of murine origin, and thus hypersensitivity against the murine antibody may limit its efficacy, particularly after repeated therapies. Common side effects include fever, sweats and chills, skin rashes, arthritis, and nerve palsies.

Therefore, compositions and methods which augment natural host defenses against tumor induction or progression without the cytotoxic side effects of prior methods, may increase remission rates and enhance survival of patients with cancer. The present invention provides such compositions and methods, and further provides other related advantages.

Summary of the Invention

Briefly stated, the present invention is directed towards methods for inhibiting the growth of a selected tumor. Within one aspect of the invention, a method is provided for inhibiting the growth of a selected tumor in a warm-blooded animal, comprising the step of directly administering to the tumor a vector construct which directs the expression of at least one anti-tumor agent, such that the growth of the tumor is inhibited. Within one embodiment of the invention, the vector construct is carried by

a recombinant viral vector. Within a preferred embodiment, the recombinant viral vector is a recombinant retroviral vector.

Within one embodiment of the invention, the anti-tumor agent is selected from the group consisting of immune activators and tumor proliferation inhibitors. Immune activators include, for example, immune modulators and lymphokines. Representative examples of immune modulators include CD3, ICAM-1, ICAM-2, LFA-1, LFA-3, β-2-microglobulin, chaperones, alpha interferon and gamma interferon, B7/BB1, B7-2 and major histocompatibility complex (MHC). Representative examples of lymphokines include gamma interferon tumor necrosis factor, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, GM-CSF, CSF-1, and G-CSF. As noted above, within other embodiments of the invention, anti-tumor agents include tumor proliferation inhibitors such as, for example, toxins and antisense sequences. Representative examples of toxins include ricin, abrin, diphtheria toxin, cholera toxin, gelonin, pokeweed, antiviral protein, tritin, Shigella toxin, and Pseudomonas exotoxin A, herpes simplex virus thymidine kinase (HSVTK), and *E. coli* guanine phosphoribosyl transferase. Representative examples of antisense sequences include antisense thymidine kinase, antisense dihydrofolate reductase, antisense HER2, antisense ABL, antisense Myc, and antisense *ras*.

Within a particularly preferred embodiment of the invention, the anti-tumor agent is a membrane anchor - gamma interferon fusion protein. Within another embodiment, the anti-tumor agent is a gamma interferon - Interleukin-2 fusion protein.

Within additional aspects of the present invention, isolated DNA sequences are provided which encode membrane anchor - gamma interferon fusion proteins and membrane anchor - anti-tumor agent fusion proteins, as well as vector constructs which direct the expression of these sequences, and recombinant viral and retroviral vectors which carry the vector construct. Also provided are recombinant viral vectors and recombinant retroviral vectors carrying a vector construct which directs the expression of an Interleukin-2 - gamma interferon fusion protein.

Also provided by the present invention are target cells infected with the recombinant retroviral vectors discussed above, as well as pharmaceutical compositions comprising the above described recombinant viral or retroviral vectors, in combination with a pharmaceutically acceptable carrier or diluent.

Within another aspect of the present invention, methods for inhibiting the growth of a selected tumor in a warm-blooded animal are provided, comprising the step of delivering to a warm-blooded animal a recombinant retroviral or viral vector as described above, such that the growth of the tumor is inhibited.

Within another aspect of the present invention, additional methods for inhibiting the growth of a selected tumor in a warm-blooded animal are provided, comprising the steps of (a) removing tumor cells associated with the selected tumor from a warm-blooded animal, (b) infecting the removed cells with a recombinant retroviral or viral vector as described above, and (c) delivering the infected cells to a warm-blooded animal, such that the growth of the selected tumor is inhibited. Within various embodiments of the invention, prior to the step of delivering, fibroblasts may be depleted from the removed cells. In addition, prior to delivering the infected cells to a warm-blooded animal, the infected cells may be inactivated.

Within yet another aspect of the present invention, a method for inhibiting the growth of a selected tumor in a warm-blooded animal is provided, comprising the steps of (a) removing tumor cells associated with the selected tumor from a warm-blooded animal, (b) contacting the cells with a vector construct which directs the expression of an anti-tumor agent such that the cells are capable of expressing said anti-tumor agent, and (c) delivering the cells from step (b) to an allogeneic warm-blooded animal, such that the growth of the selected tumor is inhibited.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1 schematically illustrates the cloning of murine gamma-interferon into a replication defective retroviral vector.

Figure 2 is a Western blot which depicts MHC Class I protein expression in L33 and B16F10 cell lines.

Figure 3 is a Western blot which depicts MHC Class I expression in L33 and B16F10 cells treated with recombinant murine gamma-interferon *in vitro*.

Figure 4A is a graph which illustrates the induction of anti-B16F10 CTL response in Black 6 mice.

Figure 4B is a graph which illustrates the induction of anti-B16F10 murine gamma interferon #4 CTL response in Black 6 mice.

Figure 5 is a graph which illustrates the induction of anti-B16F10 murine gamma interferon #4 CTL response in mice, following either i.p. or i.m. injection.

Figure 6 is a bar graph which illustrates tumor growth in Black 6 mice which were injected with either B16F10, or B16F10 mγ-IFN #4 cells.

Figure 7 is a bar graph which illustrates tumor growth in Black 6 mice challenged with B16F10 cells, after vaccination with either irradiated B16F10 cells or irradiated B16F10 murine gamma-interferon #4 cells.

Figure 8 is a bar graph which illustrates tumor growth following general different vaccination regimens with either irradiated B16F10 cells or irradiated B16F10 murine gamma interferon #4 cells.

Figure 9 is a graph which illustrates tumor growth in Balb/C mice which were injected with either 6×10^6 L33 cells, L33 cells treated with recombinant murine gamma-interferon, or L33 murine gamma-interferon #15 cells.

Figure 10 is a graph which illustrates tumor growth in Balb/C mice which were injected with either 6×10^6 L33 cells, L33 cells treated with recombinant murine gamma-interferon, or L33 murine gamma-interferon #15 cells.

Figure 11 is a graph which illustrates tumor growth in Balb/C nude mice which were injected with either L33 cells or L33 murine gamma-interferon #15 cells.

Figure 12 is a graph which illustrates tumor growth in Balb/C mice injected which were with either CT 26 cells, or pooled CT 26 murine gamma-interferon expressing cells.

Figure 13 is a graph which illustrates CTL induction by pooled CT 26 murine gamma-interferon expressing cells (a non-clonal pool) in non-tumor bearing animals.

Figure 14 is a graph which illustrates CTL induction by irradiated CT 26 murine gamma-interferon #10 expressing cells.

Figure 15 is a graph which illustrates CTL specificity of CT 26 murine gamma-interferon #10 for CT 26 target cells.

Figure 16 is a graph which illustrates the effect of murine gamma-interferon expression by CT 26 murine gamma-interferon #10 cells, on CT 26 CTL lysis.

Figure 17 is a graph which illustrates tumor growth in C57Bl/6 mice which were injected with either LLT cells or LLT murine gamma-interferon expressing cells.

Figure 18 is a graph which illustrates CTL induction by irradiated LLT murine gamma-interferon expressing cells.

Figure 19 is a graph which illustrates CTL specificity of LLT murine gamma-interferon effector T-cells for LLT target cells.

Figure 20 is a graph which illustrates the effect of murine gamma-interferon expression by LLT murine gamma-interferon cells on LLT CTL lysis.

Figure 21 is a series of graphs which depicts the FACS analysis of MHC levels in L33 cells, L33 murine gamma-interferon #13 cells, L33 murine gamma-interferon #15 cells, and L33 murine gamma-interferon expressing cells (a non-clonal pool).

Figure 22 is a series of Western Blots which shows the increase in HLA Class I in γ-IFN-expressing human melanomas.

Figure 23 is a graph which depicts the percent transduction vs. Multiplicity of Infection ("MOI") for melanomas DM252, DM6, and DM92.

Figure 24 is a photograph of a 6-well plate containing melanoma cells, which shows the transduction of human melanomas with concentrated unpurified vector.

Figure 25 is a high magnification photograph of melanoma cells transduced with vector.

Figure 26 is a photograph of 4 tissue culture plates which shows that human melanoma is easily transfected within 24 hours after induction into tissue culture.

Detailed Description of the Invention

As noted above, the present invention is directed generally towards methods of inhibiting the growth of a selected tumor utilizing vector constructs which direct the expression of an anti-tumor agent. Briefly, the ability to recognize and defend against foreign pathogens such as tumor cells is central to the function of the immune system. This system, through immune recognition, is capable of distinguishing "self" from "nonself" (foreign), and is essential to ensure that defensive mechanisms are directed towards invading entities rather than against host tissues. The methods which are described in greater detail below provide an effective means of inducing MHC unrestricted response, potent Class I-restricted or Class II-restricted protective and therapeutic CTL responses, as well as humoral responses.

In particular, the present invention provides methods for inhibiting the growth of a selected tumor in a warm-blooded animal, comprising the step of directly administering to the tumor a vector construct which directs the expression of at least one anti-tumor agent, such that the growth of the tumor is inhibited. Within the context of the present invention, "inhibiting the growth of a selected tumor" refers to either (1) the direct inhibition of tumor cell division, or (2) immune cell mediated tumor cell lysis, or both, which leads to a suppression in the net expansion of tumor cells. Inhibition of tumor growth by either of these two mechanisms may be readily determined by one of ordinary skill in the art based upon a number of well known methods. For example, tumor inhibition may be determined by measuring the actual tumor size over a period of

time. Alternatively, tumor inhibition may be determined by estimating the size of a tumor (over a period of time) utilizing methods well known to those of skill in the art. More specifically, a variety of radiologic imaging methods (*e.g.*, single photon and positron emission computerized tomography; *see generally*, "Nuclear Medicine in Clinical Oncology," Winkler, C. (ed.) Springer-Verlag, New York, 1986), may be utilized to estimate tumor size. Such methods may also utilize a variety of imaging agents, including for example, conventional imaging agents (*e.g.*, Gallium-67 citrate), as well as specialized reagents for metabolite imaging, receptor imaging, or immunologic imaging (*e.g.*, radiolabeled monoclonal antibody specific tumor markers). In addition, non-radioactive methods such as ultrasound (*see*, "Ultrasonic Differential Diagnosis of Tumors", Kossoff and Fukuda, (eds.), Igaku-Shoin, New York, 1984), may also be utilized to estimate the size of a tumor.

In addition to the *in vivo* methods for determining tumor inhibition discussed above, a variety of *in vitro* methods may be utilized in order to predict *in vivo* tumor inhibition. Representative examples include lymphocyte mediated anti-tumor cytolytic activity determined for example, by a ^{51}Cr release assay, tumor dependent lymphocyte proliferation (Ioannides et al., *J. Immunol. 146*(5):1700-1707, 1991), *in vitro* generation of tumor specific antibodies (Herlyn et al., *J. Immunol. Meth. 73*:157-167, 1984), cell (*e.g.*, CTL, helper T cell) or humoral (*e.g.*, antibody) mediated inhibition of cell growth *in vitro* (Gazit et al., *Cancer Immunol. Immunother 35*:135-144, 1992), and, for any of these assays, determination of cell precursor frequency (Vose, *Int. J. Cancer 30*:135-142 (1982).

Alternatively, for other forms of cancer, inhibition of tumor growth may be determined based upon a change in the presence of a tumor marker. Examples include prostate specific antigen ("PSA") for the detection of prostate cancer (*see* U.S. Patent No. Re. 33,405), and Carcino-Embryonic Antigen ("CEA") for the detection of colorectal and certain breast cancers. For yet other types of cancers such as leukemia, inhibition of tumor growth may be determined based upon the decreased numbers of leukemic cells in a representative blood cell count.

A variety of tumors may be selected for treatment in accordance with the methods described herein. In general, solid tumors are preferred, although leukemias and lymphomas may also be treated if they have developed a solid mass, or if suitable tumor associated markers exist such that the tumor cells can be physically separated from nonpathogenic normal cells. For example, acute lymphocytic leukemia cells may be sorted from other lymphocytes with the leukemia specific marker "CALLA". Representative examples of suitable tumors include melanomas, colorectal carcinomas,

lung carcinomas (including large cell, small cell, squamous and adeno-carcinomas), renal cell carcinomas and breast adeno-carcinomas.

As noted above, a variety of anti-tumor agents may be utilized in accordance with the present invention. Within the context of the present invention, "anti-tumor agents" are understood to refer to compounds or molecules which inhibit the growth of a selected tumor as discussed above. Representative examples of anti-tumor agents include immune activators and tumor proliferation inhibitors. Briefly, immune activators function by improving immune recognition of tumor-specific antigens such that the immune system becomes "primed." Priming may consist of lymphocyte proliferation, differentiation, or evolution to higher affinity interactions. The immune system thus primed will more effectively inhibit or kill tumor cells. Immune activation may be subcategorized into immune modulators (molecules which affect the interaction between lymphocyte and tumor cell) and lymphokines, that act to proliferate, activate, or differentiate immune effector cells. Representative examples of immune modulators include CD3, ICAM-1, ICAM-2, LFA-1, LFA-3, β-2-microglobulin, chaperones, alpha interferon and gamma interferon, B7/BB1, B7-2 and major histocompatibility complex (MHC). Representative examples of lymphokines include gamma interferon, tumor necrosis factor, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, GM-CSF, CSF-1, and G-CSF.

Tumor proliferation inhibitors act by directly inhibiting cell growth, or by directly killing the tumor cell. Representative examples of tumor proliferation inhibitors include toxins such as ricin (Lamb et al., *Eur. J. Biochem. 148*:265-270, 1985), abrin (Wood et al., *Eur. J. Biochem. 198*:723-732, 1991; Evensen, et al., *J. of Biol. Chem. 266*:6848-6852, 1991: Collins et al., *J. of Biol. Chem. 265*:8665-8669, 1990; Chen et al., *Fed. of Eur. Biochem Soc. 309*:115-118, 1992), diphtheria toxin (Tweten et al., *J. Biol. Chem. 260*:10392-10394, 1985), cholera toxin (Mekalanos et al., *Nature 306*:551-557, 1983; Sanchez & Holmgren, *PNAS 86*:481-485, 1989), gelonin (Stirpe et al., *J. Biol. Chem. 255*:6947-6953, 1980), pokeweed (Irvin, *Pharmac. Ther. 21*:371-387, 1983), antiviral protein (Barbieri et al., *Biochem. J. 203*:55-59, 1982; Irvin et al., *Arch. Biochem. & Biophys. 200*:418-425, 1980; Irvin, *Arch. Biochem. & Biophys. 169*:522-528, 1975), tritin, Shigella toxin (Calderwood et al., *PNAS 84*:4364-4368, 1987; Jackson et al., *Microb. Path. 2*:147-153, 1987), and Pseudomonas exotoxin A (Carroll and Collier, *J. Biol. Chem. 262*:8707-8711, 1987), herpes simplex virus thymidine kinase (HSVTK) (Field et al., *J. gen. Virol. 49*:115-124, 1980), and *E. coli.* guanine phosphoribosyl transferase. Additional examples of tumor proliferation inhibitors include antisense sequences which inhibit tumor cell growth by preventing the cellular

synthesis of critical proteins needed for cell growth. Examples of such antisense sequences include antisense thymidine kinase, antisense dihydrofolate reductase (Maher and Dolnick, *Arch. Biochem. & Biophys. 253*:214-220, 1987; Bzik et al., *PNAS 84*:8360-8364, 1987), antisense HER2 (Coussens et al., *Science 230*:1132-1139, 1985), antisense ABL (Fainstein, et al., *Oncogene 4*:1477-1481, 1989), antisense Myc (Stanton et al., *Nature 310*:423-425, 1984) and antisense *ras*, as well as antisense sequences which block any of the enzymes in the nucleotide biosynthetic pathway. Finally, tumor proliferation inhibitors also include tumor suppressors such as p53, retinoblastoma (Rb), and MCC and APC for colorectal carcinoma.

In addition, within a further embodiment of the invention antisense RNA may be utilized as an anti-tumor agent in order to induce a potent Class I restricted response. Briefly, in addition to binding RNA and thereby preventing translation of a specific mRNA, high levels of specific antisense sequences are believed to induce the increased expression of interferons (including gamma-interferon), due to the formation of large quantities of double-stranded RNA. The increased expression of gamma interferon, in turn, boosts the expression of MHC Class I antigens. Preferred antisense sequences for use in this regard include actin RNA, myosin RNA, and histone RNA. Antisense RNA which forms a mismatch with actin RNA is particularly preferred.

Sequences which encode the above-described anti-tumor agents may be obtained from a variety of sources. For example, plasmids that contain sequences which encode anti-tumor agents may be obtained from a depository such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford England). Representative sources sequences which encode the above-noted anti-tumor agents include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids), BBG 6 (which contains sequences encoding gamma interferon), ATCC No. 39656 (which contains sequences encoding TNF), ATCC No. 20663 (which contains sequences encoding alpha interferon), ATCC Nos. 31902, 31902 and 39517 (which contains sequences encoding beta interferon), ATCC No 67024 (which contains a sequence which encodes Interleukin-1β), ATCC Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2), ATCC Nos. 59399, 59398, and 67326 (which contain sequences encoding Interleukin-3), ATCC No. 57592 (which contains sequences encoding Interleukin-4), ATCC Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and ATCC No. 67153 (which contains sequences encoding Interleukin-6).

Alternatively, known cDNA sequences which encode anti-tumor agents may be obtained from cells which express or contain the sequences. Briefly, within one embodiment mRNA from a cell which expresses the gene of interest is reverse transcribed with reverse transcriptase using oligo dT or random primers. The single stranded cDNA may then be amplified by PCR (*see* U.S. Patent Nos. 4,683,202, 4,683,195 and 4,800,159. See also PCR Technology: Principles and Applications for DNA Amplification, Erlich (ed.), Stockton Press, 1989) utilizing oligonucleotide primers complementary to sequences on either side of desired sequences. In particular, a double stranded DNA is denatured by heating in the presence of heat stable Taq polymerase, sequence specific DNA primers, ATP, CTP, GTP and TTP. Double-stranded DNA is produced when synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of the desired DNA.

Sequences which encode the above-described anti-tumor agents may also be synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (*e.g.*, ABI DNA synthesizer model 392 (Foster City, California)).

In addition to the anti-tumor agents described above, the present invention also provides anti-tumor agents which comprise a fusion protein of, for example, two or more cytokines, immune modulators, toxins or differentiation factors. Preferred anti-tumor agents in this regard include alpha interferon - Interleukin-2, Interleukin-12, GM-CSF - IL-4, GM-CSF - IL-2, GM-CSF - IL-3 (*see* U.S. Patent Nos. 5,082,927 and 5,108,910), GM-CSF - gamma interferon, and gamma interferon - IL-4. Within a particularly preferred embodiment, the anti-tumor agent is a gamma interferon-Interleukin-2 fusion protein. Within other preferred embodiments, multiple anti-tumor agents (*e.g.*, two or more lymphokines) may be expressed from a single vector construct utilizing internal ribosome binding sites, as discussed in more detail below. The construction of these anti-tumor agent(s) may be readily accomplished given the disclosure provided herein. The construction of a particularly preferred anti-tumor agent, gamma interferon - Interleukin-2, is described in more detail below in Example 1F.

Within other embodiments of the invention, the anti-tumor agent may further comprise a membrane anchor, and may be constructed, for example, as an anti-tumor agent - membrane anchor fusion protein. Briefly, the membrane anchor aspect of the fusion protein may be selected from a variety of sequences, including, for example, the transmembrane domain of well known molecules. Generally, membrane anchor sequences are regions of a protein that bind the protein to a membrane. Customarily, there are two types of anchor sequences that attach a protein to the outer surface of a

cell membrane: (1) transmembrane regions that span the lipid bilayer of the cell membrane, and interact with the hydrophobic center region (proteins containing such regions are referred to integral membrane proteins), and (2) domains which interact with an integral membrane protein or with the polar surface of the membrane (such proteins are referred to as peripheral, or extrinsic, proteins).

Membrane anchors for use within the present invention may contain transmembrane domains which span the membrane one or more times. For example, in glycophorin and guanylyl cyclase, the membrane binding region spans the membrane once, whereas the transmembrane domain of rhodopsin spans the membrane seven times, and that of the photosynthetic reaction center of Rhodopseudomonas viridis spans the membrane eleven times (*see* Ross et al., *J. Biol. Chem. 257*:4152, 1982; Garbers, *Pharmac. Ther. 50*:337-345, 1991; Engelman et al., *Proc. Natl. Acad. Sci. USA 77*:2023, 1980; Heijne and Manoil, *Prot. Eng. 4*:109-112, 1990). Regardless of the number of times the protein crosses the membrane, the membrane spanning regions typically have a similar structure. More specifically, the 20 to 25 amino-acid residue portion of the domain that is located inside the membrane generally consists almost entirely of hydrophobic residues (*see* Eisenberg et al., *Ann. Rev. Biochem. 53*:595-623, 1984). For example, 28 of the 34 residues in the membrane spanning region of glycophorin are hydrophobic (*see* Ross et al.; Tomita et al., *Biochemistry 17*:4756-4770, 1978). In addition, although structures such as beta sheets and barrels do occur, the membrane spanning regions typically have an alpha helical structure, as determined by X-ray diffraction, crystallography and cross-linking studies (*see* Eisenberg et al. at 20; Heijne and Manoil at 109). The location of these transmembrane helices within a given sequence can often be predicted based on hydrophobicity plots. Stryer et al., *Biochemistry*, 3rd. ed. 304, 1988. Particularly preferred membrane anchors for use within the present invention include naturally occurring cellular proteins (that are non-immunogenic) which have been demonstrated to function as membrane signal anchors (such as glycophorin).

Within a preferred aspect of the present invention, a DNA sequence is provided which encodes a membrane anchor - gamma interferon fusion protein. Within one embodiment, this fusion protein may be constructed by genetically fusing the sequence which encodes the membrane anchor of the gamma-chain of the Fc receptor, to a sequence which encodes gamma-interferon. The construction of such an anti-tumor agent is set forth in more detail below in Example 1.

Once a sequence encoding at least one anti-tumor agent has been obtained, it is necessary to ensure that the sequence encodes a non-tumorigenic protein.

Various assays are known and may easily be accomplished which assess the tumorigenicity of a particular cellular component. Representative assays include tumor formation in nude mice or rats, colony formation in soft agar, and preparation of transgenic animals, such as transgenic mice.

Tumor formation in nude mice or rats is a particularly important and sensitive method for determining the tumorigenicity of an anti-tumor agent. Nude mice lack a functional cellular immune system (*i.e.*, do not possess CTLs), and therefore provide a useful *in vivo* model in which to test the tumorigenic potential of cells. Normal non-tumorigenic cells do not display uncontrolled growth properties if injected into nude mice. However, transformed cells will rapidly proliferate and generate tumors in nude mice. Briefly, in one embodiment the vector construct is delivered to syngeneic murine cells, followed by injection into nude mice. The mice are visually examined for a period of 2 to 8 weeks after injection in order to determine tumor growth. The mice may also be sacrificed and autopsied in order to determine whether tumors are present. (Giovanella et al., *J. Natl. Cancer Inst. 48*:1531-1533, 1972; Furesz et al., "Tumorigenicity testing of cell lines considered for production of biological drugs," *Abnormal Cells*, New Products and Risk, Hopps and Petricciani (eds.), Tissue Culture Association, 1985; and Levenbook et al., *J. Biol. Std. 13*:135-141, 1985).

Tumorigenicity may also be assessed by visualizing colony formation in soft agar (MacPherson and Montagnier, *Vir. 23*:291-294, 1964). Briefly, one property of normal non-tumorigenic cells is "contact inhibition" (*i.e.*, cells will stop proliferating when they touch neighboring cells). If cells are plated in a semi-solid agar support medium, normal cells rapidly become contact inhibited and stop proliferating, whereas tumorigenic cells will continue to proliferate and form colonies in soft agar.

Transgenic animals, such as transgenic mice, may also be utilized to assess the tumorigenicity of an anti-tumor agent (*e.g.*, Stewart et al., *Cell 38*:627-637, 1984; Quaife et al., *Cell 48*:1023-1034, 1987; and Koike et al., *Proc. Natl. Acad. Sci. USA 86*:5615-5619, 1989). In transgenic animals, the gene of interest may be expressed in all tissues of the animal. This unregulated expression of the transgene may serve as a model for the tumorigenic potential of the newly introduced gene.

In addition to tumorgenicity studies, it is generally preferable to determine the toxicity of the anti-tumor agent(s), prior to administration. A variety of methods well known to those of skill in the art may be utilized to measure such toxicity, including for example, clinical chemistry assays which measure the systemic levels of various proteins and enzymes, as well as blood cell volume and number.

Once an anti-tumor agent has been selected, it is placed into a vector construct which directs its expression. Within the context of the present invention, a "vector construct" is understood to refer to an assembly which is capable of directing the expression of the sequence(s) or gene(s) of interest. The vector construct must include transcriptional promoter element(s), and preferably includes a signal which directs polyadenylation. In addition, the vector construct must include a sequence which, when transcribed, is operably linked to the sequence(s) or gene(s) of interest and acts as a translation initiation sequence. Optionally, the vector construct may also include a selectable marker such as Neo, TK, hygromycin, phleomycin, histidinol, or DHFR, as well as one or more restriction sites and a translation termination sequence. In addition, if the vector construct is placed into a retrovirus, the vector construct must include a packaging signal, long terminal repeats (LTRs), and positive and negative strand primer binding sites appropriate to the retrovirus used (if these are not already present).

As noted above, within one aspect of the present invention recombinant retroviruses are provided which carry a vector construct capable of directing the expression of an anti-tumor agent. The construction of such recombinant retroviral vectors is described in greater detail in an application entitled "Recombinant Retroviruses" (U.S.S.N. 07/586,603, filed September 21, 1990, which is hereby incorporated by reference in its entirety). These recombinant retroviral vectors may be used to generate transduction competent retroviral vector particles by introducing them into appropriate packaging cell lines (see U.S.S.N. 07/800,921). In addition, Examples 1, 3 and 4 describe the preparation of recombinant retroviral vectors, as well as vector constructs which direct the expression of several anti-tumor agents.

Vector constructs of the present invention may also be developed and utilized with other viral carriers including, for example, poliovirus (Evans et al., *Nature 339*:385-388, 1989, and Sabin, *J. of Biol. Standardization 1*:115-118, 1973); rhinovirus; pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch et al., *PNAS 86*:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci. 569*:86-103, 1989; Flexner et al., *Vaccine 8*:17-21, 1990; U.S. Patent Nos. 4,603,112 and 4,769,330; WO 89/01973); SV40 (Mulligan et al., *Nature 277*:108-114, 1979); influenza virus (Luytjes et al., *Cell 59*:1107-1113, 1989; McMicheal et al., *The New England Journal of Medicine 309*:13-17, 1983; and Yap et al., *Nature 273*:238-239, 1978); adenovirus (Berkner, *Biotechniques 6*:616-627, 1988, and Rosenfeld et al., *Science 252*:431-434, 1991); parvovirus such as adeno-associated virus (Samulski et al., *Journal of Virology 63*:3822-3828, 1989, and Mendelson et al., *Virology 166*:154-165, 1988); herpes (Kit, *Adv. Exp. Med. Biol. 215*:219-236, 1989); SV40; HIV; measles (EP 0 440,219); and

Sindbis virus (Xiong et al., *Science 234*:1188-1191, 1989), and corona virus. In addition, viral carriers may be homologous, non-pathogenic (defective), replication competent virus (*e.g.*, Overbaugh et al., *Science 239*:906-910, 1988), and nevertheless induce cellular immune responses, including CTL.

As noted above, a vector construct which directs the expression of at least one anti-tumor agent is directly administered to the tumor. Various methods may be utilized within the context of the present invention in order to directly administer the vector construct to the tumor. For example, within one embodiment a small metastatic lesion may be located, and the vector injected several times in several different locations within the body of tumor. Alternatively, arteries which serve a tumor may be identified, and the vector injected into such an artery, in order to deliver the vector directly into the tumor. Within another embodiment, a tumor which has a necrotic center may be aspirated, and the vector injected directly into the now empty center of the tumor. Within yet another embodiment, the vector construct may be directly administered to the surface of the tumor, for example, by application of a topical pharmaceutical composition containing the vector construct, or preferably, a recombinant retroviral vector carrying the vector construct.

Within another aspect of the present invention, methods are provided for inhibiting the growth of a selected tumor, comprising the step of delivering to a warm-blooded animal a vector construct which directs the expression of at least one anti-tumor agent, such that the growth of the tumor is inhibited. Within preferred embodiments, the vector construct is carried by a recombinant viral or retroviral vector.

In addition to vector constructs, nucleic acids which encode anti-tumor agent(s) may also be administered to a patient by a variety of methods. Representative examples include transfection by various physical methods, such as lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA 84*:7413-7417, 1989), direct DNA injection (Acsadi et al., *Nature 352*:815-818, 1991); microprojectile bombardment (Williams et al., *PNAS 88*:2726-2730, 1991); liposomes (Wang et al., *PNAS 84*:7851-7855, 1987); $CaPO_4$ (Dubensky et al., *PNAS 81*:7529-7533, 1984); DNA ligand (Wu et al, *J. of Biol. Chem. 264*:16985-16987, 1989); or administration of DNA linked to killed adenovirus (Curiel et al., *Hum. Gene Ther. 3*: 147-154, 1992). A variety of methods for administering recombinant retroviral vectors may also be utilized within the context of the present invention, such methods are described in greater detail in an application entitled "Recombinant Retroviruses" (U.S.S.N. 07/586,603), which is herein expressly incorporated by reference.

In addition, a cellular response (including CTL) may also be generated by administration of a bacteria which expresses an anti-tumor agent such as those discussed above, on its cell surface. Representative examples include BCG (Stover, *Nature 351:*456-458, 1991) and Salmonella (Newton et al., *Science 244:*70-72, 1989).

Within another aspect of the present invention, a method is provided for inhibiting the growth of a selected tumor in a warm-blooded animal, comprising the steps of (a) removing tumor cells associated with the selected tumor from a warm-blooded animal, (b) infecting the removed cells with a vector construct which directs the expression of at least one anti-tumor agent, and (c) delivering the infected cells to a warm-blooded animal, such that the growth of the selected tumor is inhibited by immune responses generated against the gene-modified tumor cell. Within one embodiment of the present invention, subsequent to removing tumor cells from a warm-blooded animal, a single cell suspension may be generated by, for example, physical disruption or proteolytic digestion. In addition, division of the cells may be increased by addition of various factors such as melanocyte stimulating factor for melanomas or epidermal growth factor for breast carcinomas, in order to enhance uptake, genomic integration and expression of the recombinant viral vector.

Within the context of the present invention, it should be understood that the removed cells may not only be returned to the same animal, but may also be utilized to inhibit the growth of selected tumor cells in another, allogeneic, animal. In such a case it is generally preferable to have histocompatibility matched animals (although not always, *see, e.g.*, Yamamoto et al., "Efficacy of Experimental FIV Vaccines," 1st International Conference of FIV Researchers, University of California at Davis, September 1991). Therefore, within yet another aspect of the present invention, a method for inhibiting the growth of a selected tumor in a warm-blooded animal is provided, comprising the steps of (a) removing tumor cells associated with the selected tumor from a warm-blooded animal, (b) contacting the cells with a vector construct which directs the expression of an anti-tumor agent such that the cells are capable of expressing said anti-tumor agent, and (c) delivering the cells from step (b) to an allogeneic warm-blooded animal, such that the growth of the selected tumor is inhibited.

In addition, it should be understood that a variety of cells (target cells) may be utilized within the context of the present invention, including for example, human, macaque, dog, rat, and mouse cells.

Cells may be removed from a variety of locations including, for example, from a selected tumor. In addition, within other embodiments of the invention, a vector construct may be inserted into non-tumorigenic cells, including for example, cells from

the skin (dermal fibroblasts), or from the blood (*e.g.*, peripheral blood leukocytes). If desired, particular fractions of cells such as a T cell subset or stem cells may also be specifically removed from the blood (*see,* for example, PCT WO 91/16116, an application entitled "Immunoselection Device and Method"). Vector constructs may then be contacted with the removed cells utilizing any of the above-described techniques, followed by the return of the cells to the warm-blooded animal, preferably to or within the vicinity of a tumor.

The above-described methods may additionally comprise the steps of depleting fibroblasts or other non-contaminating tumor cells subsequent to removing tumor cells from a warm-blooded animal, and/or the step of inactivating the cells, for example, by irradiation.

As noted above, within certain aspects of the present invention, several anti-tumor agents may be administered either concurrently or sequentially, in order to inhibit the growth of a selected tumor in accordance with the methods of the present invention. For example, within one embodiment of the invention, an anti-tumor agent such as γ-IFN may be co-administered or sequentially administered to a warm-blooded animal along with other anti-tumor agents such as IL-2, or IL-12, in order to inhibit or destroy a pathogenic agent. Such therapeutic compositions may be administered directly utilizing a single vector construct which directs the expression of at least two anti-tumor agents, or, within other embodiments, expressed from independent vector constructs. Alternatively, one anti-tumor agent (*e.g.*, γ-IFN) may be administered utilizing a vector construct, while other tumor agents (*e.g.*, IL-2) are administered directly (*e.g.*, as a pharmaceutical composition intravenously).

Within a particularly preferred embodiment, vector or VCLs which deliver and express both a γ-IFN gene and another gene encoding IL-2, may be administered to the patient. In such a construct, one gene may be expressed from the vector LTR and the other may utilize an additional transcriptional promoter found between the LTRs, or may be expressed as a polycistronic mRNA, possibly utilizing an internal ribosome binding site. After *in vivo* gene transfer, the patient's immune system is activated due to the expression of γ-IFN. Infiltration of the dying tumor with inflammatory cells, in turn, increases immune presentation and further improves the patient's immune response against the tumor.

As noted above, within preferred embodiments of the present invention, pharmaceutical compositions are provided comprising a recombinant retrovirus or virus carrying one of the above-described vector constructs, in combination with a pharmaceutically acceptable carrier or diluent. The composition may be prepared either

as a liquid solution, or as a solid form (*e.g.*, lyophilized) which is suspended in a solution prior to administration. In addition, the composition may be prepared with suitable carriers or diluents for either surface administration, injection, oral, nasal, vaginal, sub-linguinal, inhalant or rectal administration.

Pharmaceutically acceptable carriers or diluents are nontoxic to recipients at the dosages and concentrations employed. Representative examples of carriers or diluents for injectable solutions include water, isotonic saline solutions which are preferably buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline), mannitol, dextrose, glycerol, and ethanol, as well as polypeptides or proteins such as human serum albumin. A particularly preferred composition comprises a vector or recombinant virus in 10 mg/ml mannitol, 1 mg/ml HSA, 20 mM Tris, pH 7.2, and 150 mM NaCl. In this case, since the recombinant vector represents approximately 1 μg of material, it may be less than 1% of high molecular weight material, and less than 1/100,000 of the total material (including water). This composition is stable at -70°C for at least six months.

Pharmaceutical compositions of the present invention may also additionally include factors which stimulate cell division, and hence, uptake and incorporation of a recombinant retroviral vector. Representative examples include Melanocyte Stimulating Hormone (MSH), for melanomas or epidermal growth factor for breast or other epithelial carcinomas. Pharmaceutical compositions of the present invention may be injected via a variety of routes (*e.g.*, intradermally ("i.d."), intracranially ("i.c."), intraperitoneally ("i.p."), intrathecally ("i.t."), intravenously ("i.v."), subcutaneously ("s.c."), intramuscularly ("i.m.") or preferably, directly into the tumor. The individual doses normally used are 10^7 to 10^9 c.f.u. (colony forming units of neomycin resistance titered on HT1080 cells). These are administered at one to four week intervals for three or four doses initially. Subsequent booster shots may be given as one or two doses after 6-12 months, and thereafter annually.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1

PREPARATION OF MURINE RETROVIRAL PROVECTOR DNA

A. Preparation of the Retroviral Backbone KT-3

An EcoR I - EcoR I fragment containing a 5' long terminal repeat ("LTR") and gag sequences from a Moloney murine leukemia virus ("MoMLV") in an N2 vector (Armentano et. al., *J. Vir. 61*:1647-1650, 1987; Eglitis et. al., *Science 230*:1395-1398, 1985) contained in pUC31, is ligated into the plasmid SK^+ (Stratagene, Calif.). (The plasmid pUC31 is derived from pUC19 (Stratagene, Calif.) in which additional restriction sites Xho I, Bgl II, BssH II and Nco I are inserted between the EcoR I and Sac I sites of the polylinker.) The resulting construct is designated N2R5. The N2R5 construct is mutated by *in vitro* site-directed mutagenesis to change the ATG start codon to ATT in order to prevent gag expression. This mutagenized fragment is 200 base pairs (bp) in length, and flanked by Pst I restriction sites. The Pst I-Pst I mutated fragment is purified from the SK^+ plasmid and inserted into the Pst I site of N2 MoMLV 5' LTR in pUC31 in order to replace the non-mutated 200 bp fragment. This construct is designated pUC31/N2R5g^M.

The 1.0 kilobase (Kb) MoMLV 3' LTR EcoR I - EcoR I fragment from N2 is cloned into plasmid SK^+ resulting in a construct designated N2R3$^-$. A 1.0 Kb Cla I-Hind III fragment is purified from this construct.

The Cla I-Cla I dominant selectable marker gene fragment from pAFVXM retroviral vector (Kriegler et. al., *Cell 38*:483, 1984; St. Louis et. al., *PNAS 85*:3150-3154, 1988), comprising a SV40 early promoter driving expression of the neomycin phosphotransferase gene, is cloned into plasmid SK^+. A 1.3 Kb Cla I-BstB I neo gene fragment is purified from this plasmid.

The expression vector is constructed by a three-part ligation in which the Xho I-Cla I fragment containing a gene of interest as described below, and the 1.0 Kb MoMLV 3' LTR Cla I-Hind III fragment are inserted into the Xho I-Hind III site of pUC31/N2R5g^M plasmid. The 1.3 Kb Cla I-BstB I neo gene fragment is then inserted into the Cla I site of this plasmid in the sense orientation.

B. Cloning of mγ-IFN Into KT-3

A mγ-IFN cDNA is cloned into the EcoR I site of pUC1813 essentially as set forth below. Briefly, pUC1813 (containing a sequence encoding γ-IFN) is prepared as essentially described by Kay et. al., *Nucleic Acids Research 15*:2778, 1987; and Gray et. al., *PNAS 80*:5842, 1983) (Figure 1A). The mγ-IFN cDNA is retrieved by EcoR I digestion of pUC1813, and the isolated fragment is cloned into the EcoR I site of phosphatase-treated pSP73 (Promega; Madison, Wisc.) (Figure 1B). This construct is designated SP mγ-IFN. The orientation of the cDNA is verified by appropriate restriction enzyme digestion and DNA sequencing. In the sense orientation, the 5' end of the cDNA is adjacent to the Xho I site of the pSP73 polylinker and the 3' end adjacent to the Cla I site. The Xho I-Cla I fragment containing the mγ-IFN cDNA in either sense or antisense orientation is retrieved from SP mγ-IFN construct and cloned into the Xho I-Cla I site of the KT-3 retroviral backbone. This construct is designated KT mγ-IFN (Figure 1).

C. Cloning of hγ-IFN Into KT-3

1. Preparation Of Sequences Encoding hγ-IFN Utilizing PCR

(a) PHA Stimulation Of Jurkat Cells

Jurkat cells (ATCC No. CRL 8163) are resuspended at a concentration of 1 x 10^6 cells/ml in RPMI growth media (Irvine Scientific; Santa Ana, Calif.) with 5% fetal bovine serum (FBS) to a final volume of 158.0 ml. Phytohemoagglutinin ("PHA") (Curtis Mathes Scientific, Houston, Texas) is added to the suspension to a final concentration of 1%. The suspension is incubated at 37°C in 5% CO_2 overnight. The cells are harvested on the following day and aliquoted into three 50.0 ml centrifuge tubes. The three pellets are combined in 50 ml 1x phosphate buffered saline (PBS, 145 mM, pH 7.0) and centrifuged at 1000 rpm for 5 minutes. The supernatant is decanted and the cells are washed with 50.0 ml PBS. The cells are collected for RNA isolation.

(b) RNA Isolation

The PHA stimulated Jurkat cells are resuspended in 22.0 ml guanidinium solution (4 M guanidinium thiocyanate; 20 mM sodium acetate, pH 5.2; 0.1 M dithiothreitol, 0.5% sarcosyl). This cell-guanidinium suspension is then passed through a 20 gauge needle six times in order to disrupt cell membranes. A CsCl solution (5.7 M CsCl, 0.1 M EDTA) is then overlaid with 11.0 ml of the disrupted cell-guanidinium solution. The solution is centrifuged for 24 hours at 28,000 rpm in a SW28.1 rotor (Beckman, Fullerton, Calif.) at 20°C. After centrifugation the supernatant is carefully

aspirated and the tubes blotted dry. The pellet is resuspended in a guanidinium-HCl solution (7.4 M guanidinium-HCl; 25 mM Tris-HCl, pH 7.5; 5 mM dithiothreitol) to a final volume of 500.0 µl. This solution is transferred to a microcentrifuge tube. Twelve and one-half microliters of concentrated Glacial acetic acid (HAc) and 250 µl of 100% EtOH are added to the microfuge tube. The solution is mixed and stored for several days at -20°C to precipitate RNA.

After storage, the solution is centrifuged for 20 minutes at 14,000 rpm, 4 °C. The pellet is then resuspended in 75% EtOH and centrifuged for 10 minutes in a microfuge at 14,000 rpm, 4°C. The pellet is dried by centrifugation under vacuum, and resuspended in 300 µl deionized (DI) H_2O. The concentration and purity of the RNA is determined by measuring optical densities at 260 and 280 nm.

(c) Reverse Transcription Reaction

Immediately before use, 5.0 µl (3.4 mg/ml) of purified Jurkat RNA is heat treated for 5 minutes at 90°C, and then placed on ice. A solution of 10.0 µl of 10x PCR buffer (500 mM KCl; 200 mM Tris-HCl, pH 8.4; 25 mM $MgCl_2$; 1 mg/ml bovine serum albumin (BSA)); 10.0 µl of 10 mM dATP, 10.0 µl of 10 mM dGTP, 10.0 µl of 10 mM dCTP, 10.0 µl of 10 mM dTTP, 2.5 µl RNasin (40,000 U/ml, Promega; Madison, Wis.) and 33.0 µl DI H_20, is added to the heat treated Jurkat cell RNA. To this solution 5.0 µl (108 nmol/ml) of V-OLI #56 (Sequence ID No. 1), and 5.0 µl (200,000 U/ml) MoMLV reverse transcriptase (Bethesda Research Laboratories, EC 3.1.27.5, Maryland) is mixed in a microfuge tube and incubated at room temperature for 10 minutes. Following the room temperature incubation, the reaction mixture is incubated for 1 hour at 37°C, and then incubated for 5 minutes at 95°C. The reverse transcription reaction mixture is then placed on ice in preparation for PCR.

(d) PCR Amplification

The PCR reaction mixture contains 100.0 µl reverse transcription reaction; 356.0 µl DI H_20; 40.0 µl 10x PCR buffer; 1.0 µl (137 nmol/ml) V-OLI #5 (Sequence ID No. 2); 0.5 µl (320 nmol/ml) V-OLI #6 (Sequence ID No. 3), and 2.5 µl, 5,000 U/ml, Taq polymerase (EC 2.7.7.7, Perkin-Elmer Cetus, Calif.). One hundred microliters of this mixture is aliquoted into each of 5 tubes.

V-OLI #56 (Sequence ID No. 1)

5' - 3': TAA TAA ATA GAT TTA GAT TTA

This primer is complementary to a sequence of the mγ-IFN cDNA 30 base pairs downstream of the stop codon.

V-OLI #5 (Sequence ID No. 2)

5' - 3': GC CTC GAG ACG ATG AAA TAT ACA AGT TAT ATC TTG

This primer is complementary to the 5' coding region of the mγ-IFN gene, beginning at the ATG start codon. The 5' end of the primer contains a Xho I restriction site.

V-OLI #6 (Sequence ID No. 3)

5' - 3': GA ATC GAT CCA TTA CTG GGA TGC TCT TCG ACC TGG

This primer is complementary to the 3' coding region of the mγ-IFN gene, ending at the TAA stop codon. The 5' end of the primer contains a Cla I restriction site.

Each tube was overlaid with 100.0 µl mineral oil, and placed into a PCR machine (Ericomp Twin Block System, Ericomp, Calif.). The PCR program regulates the temperature of the reaction vessel first at 95°C for 1 minute, next at 67°C for 2 minutes and finally at 72°C for 2 minutes. This cycle is repeated 40 times. The last cycle regulates the temperature of the reaction vessel first at 95°C for 1 minute, next at 67°C for 2 minutes and finally at 72°C for 7 minutes. The completed PCR amplification reactions are stored at 4°C for 1 month in preparation for PCR DNA isolation.

(e) Isolation Of PCR DNA

The aqueous phase from the PCR amplification reactions are transferred into a single microfuge tube. Fifty microliters of 3 M sodium acetate and 500.0 µl of chloroform:isoamyl alcohol (24:1) is added to the solution. The solution is vortexed and then centrifuged at 14,000 rpm at room temperature for 5 minutes. The upper aqueous phase is transferred to a fresh microfuge tube and 1.0 ml of 100% EtOH is added. This solution is incubated for 4.5 hours at -20°C and then centrifuged at 14,000 rpm for 20 minutes. The supernatant is decanted, and the pellet is rinsed with 500.0 µl of 70% EtOH. The pellet is dried by centrifugation under a vacuum. The isolated hγ-IFN PCR DNA is resuspended in 10.0 µl DI H_2O.

2. Construction Of hγ-IFN Retroviral Vectors

(a) Creation And Isolation Of Blunt-Ended hγ-IFN PCR DNA Fragments

The hγ-INF PCR DNA is blunt ended using T4 DNA polymerase. Specifically, 10.0 µl of PCR amplified DNA; 2.0 µl, 10x, T4 DNA polymerase buffer (0.33 M Tris-acetate, pH 7.9, 0.66 M potassium acetate, 0.10 M magnesium acetate, 5 mM dithiothreitol, 1 mg/ml bovine serum albumin (BSA)); 1.0 µl, 2.5 mM dNTP (a mixture containing equal molar concentrations of dATP, dGTP, dTTP and dCTP); 7.0 µl DI H_2O; 1.0 µl, 5000 U/ml, Klenow fragment (EC 2.7.7.7, New England Biolabs, Mass.); and 1.0 µl, 3000 U/ml, T4 DNA polymerase (EC 2.7.7.7, New England Biolabs, Mass.) are mixed together and incubated at 37°C for 15 minutes. The reaction mixture is then incubated at room temperature for 40 minutes and followed by an incubation at 68°C for 15 minutes.

The blunt ended hγ-INF is isolated by agarose gel electrophoresis. Specifically, 2.0 µl of loading dye (0.25% bromophenol blue; 0.25% xylene cyanol; and 50% glycerol) is added to reaction mixture and 4.0 µl is loaded into each of 5 lanes of a 1% agarose/Tris-borate-EDTA (TBE) gel containing ethidium bromide. Electrophoresis of the gel is performed for 1 hour at 100 volts. The desired DNA band containing hγ-INF, approximately 500 base pairs in length, is visualized under ultraviolet light.

This band is removed from the gel by electrophoretic transfer onto NA 45 paper (Schleicher and Schuell, Keene, New Hampshire). The paper is incubated at 68°C for 40 minutes in 400.0 µl of high salt NET buffer (1 M NaCl; 0.1 mM EDTA; and 20 mM Tris, pH 8.0) to elute the DNA. The NA 45 paper is removed from solution and 400.0 µl of phenol:chloroform:isoamyl alcohol (25:24:1) is added. The solution is vortexed and centrifuged at 14,000 for 5 minutes. The upper aqueous phase is transferred to a fresh tube and 400.0 µl of chloroform:isoamyl alcohol (24:1) is added. The mixture is vortexed and centrifuged for 5 minutes. The upper aqueous phase is transferred, a second time, to a fresh tube and 700.0 µl of 100% EtOH is added. The tube is incubated at -20°C for 3 days. Following incubation, the DNA is precipitated from the tube by centrifugation for 20 minutes at 14,000 rpm. The supernatant is decanted and the pellet is rinsed with 500.0 µl of 70% EtOH. The pellet, containing blunt ended hγ-INF DNA, is dried by centrifugation under vacuum and resuspended in 50.0 µl of DI H_2O.

The isolated blunt ended hγ-IFN DNA is phosphorylated using polynucleotide kinase. Specifically, 25.0 µl of blunt-ended hγ-INF DNA, 3.0 µl of 10x kinase buffer (0.5 M Tris-HCl, pH 7.6; 0.1 M $MgCl_2$; 50 mM dithiothreitol; 1 mM

spermidine; 1 mM EDTA), 3.0 µl of 10 mM ATP, and 1.0 µl of T4 polynucleotide kinase (10,000 U/ml, EC 2.7.1.78, New England Biolabs, Maryland) is mixed and incubated at 37°C for 1 hour 45 minutes. The enzyme is then heat inactivated by incubating at 68°C for 30 minutes.

(b) Ligation Of hγ-IFN PCR DNA Into The SK^+ Vector

An SK^+ plasmid is digested with Hinc II restriction endonuclease and purified by agarose gel electrophoresis as described below. Specifically, 5.9 µl (1.7 mg/ml) SK^+ plasmid DNA (Stratagene; San Diego, Calif.); 4.0 µl 10x Universal buffer (Stratagene, Calif.); 30.1 µl DI H_2O, and 4.0 µl Hinc II, 10,000 U/ml, are mixed in a tube and incubated for 7 hours at 37°C. Following incubation, 4.0 µl of loading dye is added to the reaction mixture and 4.0 µl of this solution is added to each of 5 lanes of a 1% agarose/TBE gel containing ethidium bromide. Electrophoresis of the gel is performed for 2 hours at 105 volts. The Hinc II cut SK^+ plasmid, 2958 base pairs in length, is visualized with ultraviolet light. The digested SK^+ plasmid is isolated from the gel using the method described in Example 1C, Section 2(a).

Dephosphorylation of the Hinc II cleavage site of the plasmid is performed using calf intestine alkaline phosphatase. Specifically, 50.0 µl digested SK^+ plasmid; 5.0 µl 1 M Tris, pH 8.0; 2.0 µl 5 mM EDTA, pH 8.0; 43.0 µl H_2O and 2.0 µl, 1,000 U/ml, calf intestinal phosphatase ("CIP") (Boehringer Mannheim, Indianapolis, Indiana) are mixed in a tube and incubated at 37°C for 15 minutes. Following incubation, 2.0 µl CIP is added. and the solution is incubated at 55°C for 90 minutes. Following this incubation, 2.5 µl 20% sodium dodecyl sulfate ("SDS"), 1.0 µl 0.5 M EDTA, pH 8.0, and 0.5 µl, 20 mg/ml, proteinase K (EC 3.4.21.14, Boehringer Mannheim, Indianapolis, Indiana) are added, and the solution is incubated at 55°C for 2 hours. This solution is cooled to room temperature, and 110.0 µl phenol:chloroform:isoamyl alcohol (25:24:1) is added. The mixture is vortexed and centrifuged at 14,000 rpm for 5 minutes. The upper aqueous phase is transferred to a fresh tube and 200.0 µl of 100% EtOH is added. This mixture is incubated at 70°C for 15 minutes. The tube is centrifuged and the pellet is rinsed with 500.0 µl of 70% EtOH. The pellet was then dried by centrifugation under a vacuum. The dephosphorylated SK^+ plasmid is resuspended in 40 µl DI H_2O.

The hγ-INF PCR DNA is ligated into the SK^+ plasmid using T4 DNA ligase. Specifically, 30.0 µl blunt ended, phosphorylated, hγ-IFN PCR DNA reaction mixture, 2.0 µl dephosphorylated SK^+ plasmid and 1.0 µl T4 DNA ligase are combined in a tube and incubated overnight at 16°C. DNA was isolated using a minprep

procedure. More specifically, the bacterial strain DH5a (Gibco BRL, Gaithersburg, MD) is transformed with 15.0 µl of ligation reaction mixture, plated on Luria-Bertani agar plates (LB plates) containing ampicillin and 5-bromo-4-chloro-3-indolyl-b-D-galactoside (X-gal, Gold Biotechnology; St. Louis, Missouri), and incubated overnight at 37°C. DNA is isloated from white bacterial colonies using the procedure described by Sambrook et al. (*Molecular Cloning*, Cold Springs Harbor Press, 1989). The presence of the hγ-IFN gene is determined by restriction endonuclease cleavage with Xho I, Cla I, Ava II, Dra I, and Ssp I. The expected endonuclease restriction cleavage fragment sizes for plasmids containing the hγ-IFN gene are presented in Table 1. The isolated DNA plasmid is designated SK hγ-IFN and used in constructing the retroviral vectors.

Table 1

Enzyme	Fragment Size (bp)
Xho I and Cla I	500, 2958
Ava II	222, 1307, 1937
Dra I	700, 1149, 1500
Ssp I	750, 1296, 2600

(c) Ligation Of hγ-IFN Gene Into Retroviral Vector

The interferon gene is removed from SK hγ-IFN vector by digestion with Xho I and Cla I restriction endonucleases. The resulting fragment containing the hγ-IFN gene is approximately 500 bp in length, and is isolated in a 1% agarose/TBE gel electrophoresis as described in Example 1C, 2(b). The Xho I-Cla I hγ-IFN fragment is then ligated into the KT-3 retroviral backbone. This construct is designated KT hγ-IFN. The structure and presence expression of hγ-IFN is determined by transforming DH5a bacterial strain with the KT hγ-IFN construct. Specifically, the bacteria is transformed with 15.0 µl of ligation reaction mixture. The transformed bacterial cells are plated on LB plates containing ampicillin. The plates are incubated overnight at 37°C and bacterial colonies are selected. The DNA is isolated as described in (b) above, and

digested with Xho I, Cla I, Dra I, Nde I, and Ssp I. The expected endonuclease restriction cleavage fragment sizes for plasmids containing the hγ-IFN gene are presented in Table 2.

Table 2

Enzyme	Fragment Size (bp)
Xho I and Cla I	500, 6500
Nde I	1900, 5100
Dra I	692, 2700, 3600
Ssp I	541, 1700, 4700

D. Cloning of hIL-2 Into KT-3

The method for cloning hIL-2 into KT-3 retroviral vector is essentially identical to the procedure for cloning hγ-IFN into KT-3, with the exception that different primers are required for amplification of the hIL-2 DNA sequence. The following hIL-2 PCR primer sequences are used:

V-OLI #55 (Sequence ID No. 4)

5'-3': ATA AAT AGA AGG CCT GAT ATG

This primer is complimentary to a sequence of the hIL-2 cDNA downstream of the stop codon.

V-OLI #1 (Sequence ID No. 5)

5'-3': GC CTC GAG ACA ATG TAC AGG ATG CAA CTC CTG TCT

This primer is the sense sequence of the hIL-2 gene complimentary to the 5' coding region beginning at the ATG start codon. The 5' end of the primer contains a Xho I restriction site.

V-OLI #2 (Sequence ID No. 6)

5'-3': GA ATC GAT TTA TCA AGT CAG TGT TGA GAT GAT GCT

The primer is the anti-sense sequence of the hIL-2 gene complimentary to the 3' coding region ending at the TAA stop codon. The 5' end of the primer contains the Cla I restriction site.

E. Cloning of Membrane-Bound human γ-IFN Into KT-3

1. Methods For Constructing Membrane-Bound Human γ-IFN

(a) Sequence Selection For Membrane-Bound hγ-IFN

The cell surface membrane-bound hγ-IFN protein is a chimeric protein comprising the complete cDNA of hγ-IFN, the transmembrane region of human Fc receptor γ-chain and a modified cytoplasmic region of Fc receptor γ-chain. The modification of the cytoplasmic region consists of an internal deletion and is intended to block any signal transduction associated with an Fc receptor.

Modification and splicing of DNA is achieved by the overlap extension PCR method of Horton et al. (*Gene 77*:61-68, 1978). The carboxyl terminus of hγ-IFN is joined to the Fc receptor γ-chain at the amino end of the transmembrane region. The underlined amino acids identify the transmembrane region sequence.

Human γ-IFN . . .G R R A S Q COOH
(Sequence ID No. 7)

Human Fc
receptor γ-chain NH_2 . . . . L G E P Q <u>L C Y I L D A</u> COOH
(Sequence ID No. 8)

Hybrid NH_2 . . . G R R A S Q <u>L C Y I L D A</u> COOH
(Sequence ID No. 9)

The addition, of several hydrophilic amino acids between hγ-IFN and the transmembrane region may be employed to extend the active region away from the cell surface (Kuster, et. al., *J. Bio. Chem. 265*:6448, 1990). The sequence modification of the transmembrane region of the human Fc receptor γ-chain involves the deletion of amino acids S^{57}-Y^{76}. The underlined amino acids identify the deleted sequence.

Human Fc receptor γ-chain intracellular region

(Sequence ID Nos. 10 and 36)

...KAAITS^{57}YEK...QETY^{76}ETLK... COOH

Modified intracellular region

(Sequence ID No. 11)

...KAAITETLK... COOH

(b) PCR Amplification

A polymerase chain reaction (PCR) mixture is prepared according to procedures specified by Perkin-Elmer-Cetus, Calif. More specifically, the reaction mixture contains 0.5 µg purified plasmid, 5.0 µl of 10x PCR reaction buffer, 5.0 µl 2.5 mM of each dATP, dCTP, dGTP, and dTTP, 1.0 µl; 0.5 µg of each primer, 0.5 µl of 2.5 units/100.0 µl Taq polymerase and 8.0 µl of 10 mM $MgCl_2$. The reaction mixture is then brought to 50.0 µl with DI H_2O.

Each reaction mixture is overlaid with 100.0 µl mineral oil, and placed into a PCR machine (Ericomp Twin Block System). The PCR program regulates the temperature of the reaction vessel first at 95°C for 1 minute, next at 67°C for 2 minutes and finally at 72°C for 2 minutes. This cycle is repeated 40 times. The last cycle regulates the temperature of the reaction vessel first at 95°C for 1 minute, next at 67°C for 2 minutes and finally at 72°C for 7 minutes. The completed PCR reactions are stored at 4°C for about 1 month.

(c) Construction Of Hybrid γ-IFN/Fc Receptor Retroviral Vector

The following hγ-IFN PCR primer sequences are used:

(Sequence ID No. 12)

5'-3': CAG GAC CCA TAT GTA AAA GAA GCA GAA AAC C

This primer is the sense sequence of hγ-IFN corresponding to a region at the carboxy terminus of the protein, and is designated hγ-IFN/P1.

(Sequence ID No. 13)

5'-3': GCA GAG CTG GGA TGC TCT TCG ACC TCG

This primer is the anti-sense sequence of hγ-IFN corresponding to a region at the carboxy terminus of the protein, and is designated hγ-IFN/P2.

The following γ-chain Fc receptor primer sequences are used:

(Sequence ID No. 14)

5'-3': GCA TCC CAG CTC TGC TAT ATC CTG GAT GCC

This primer is the sense sequence of Fc receptor γ-chain, and is designated γ-chain Fc/P3.

(Sequence ID No. 15)

5'-3': GGC ATG CAG GCA TAT GTG ATG CCA ACC

This primer is the anti-sense sequence of Fc receptor γ-chain, and is designated γ-chain Fc/P4.

The hγ-IFN template DNA from Example 1C 1(e), hγ-IFN/P1 primer DNA, hγ-IFN/P2 primer DNA, γ-chain Fc receptor template DNA, γ-chain Fc/P3 primer DNA and γ-chain Fc/P4 primer DNA are combined, denatured at 95°C for 1 minute, and extended in the presence of PCR reaction mix without additional primers. The 3' end of the template-primer hγ-IFN DNA will anneal to the 5' end of the template-primer γ-chain Fc DNA and extension will produce the hybrid product designated hγ-IFN/Fc-Rec. The primers hγ-IFN/P1 and γ-chain Fc/P4 are then added and 40 cycles of PCR are performed to amplify the hγ-IFN/Fc-Rec product.

The hγ-IFN/Fc-Rec product contains only a short fragment of the hγ-IFN gene. This fragment extends from the Nde I restriction endonuclease site within the

gene to the end of the gene sequence. hγ-IFN/Fc-Rec is digested with Nde I and cloned into KT hγ-IFN containing the leader portion of hγ-IFN. This vector is designated KT hγ -IFN/Fc-Rec.

(d) Construction Of Hybrid hγ-IFN/Modified Fc Receptor Intracellular Region Retroviral Vector

The γ-chain of the Fc receptor is modified to eliminate the biological activity of the protein. The following modified Fc receptor intracellular region PCR primer sequences are utilized:

(Sequence ID No. 16)

5'-3': CAG AGT CTC GGT TAT AGC TGC CTT TCG CAC

This primer is the sense sequence of the modified Fc receptor intracellular region, and is designated mFc/P5.

(Sequence ID No. 17)

5'-3': GCT ATA ACC GAG ACT CTG AAG CAT GAG

This primer is the anti-sense sequence of the modified Fc receptor intracellular region, and is designated mFc/P6.

The hγ-IFN/Fc-Rec template DNA from Example 1C 3(a), hγ-IFN/P1 primer DNA, mFc/P5 primer DNA, mFc/P6 primer DNA and γ-chain Fc/P4 primer DNA are combined, denatured at 95°C for 1 minute and extended in the presence of PCR reaction mix without additional primers. The 3' end of the template-primer (hγ-IFN/Fc-Rec template DNA bound hγ-IFN primer DNA and mFc/P5 primer DNA) will anneal to the 5' end of the template-primer (hγ-IFN/Fc-Rec template DNA bound mFc/P6 primer DNA and γ-chain Fc/P4 primer DNA) and extension will produce a modified Fc receptor γ-chain omitting 20 amino acid codons. Primers mFc/P6 and γ-chain Fc/P4 are then added and 40 cycles of PCR are performed to amplify the modified product. The PCR product is designated mFcIR.

The KT hγ-IFN/Fc-Rec vector is digested with Nde I. The Nde I-Nde I fragment removed from this vector is replaced with the Nde I-Nde I mFcIR PCR DNA fragment. This vector is designated KT hγ-IFN/mFc-Rec.

F. Cloning of Human Gamma IFN/Human IL-2 Into KT-3

1. Method For Constructing hγ-IFN/hIL-2 Hybrid

(a) Sequence Selection for hγ-IFN/hIL-2 Hybrid

Two methods are described below for the construction of hγ-IFN/hIL-2 hybrid proteins. The first method describes the construction of a hybrid protein having γ -IFN at the carboxyl terminus and the second method describes a hybrid protein having human interleukin-2 (hIL-2) at the carboxyl terminus.

Modification and splicing of DNA is achieved by the overlap extension PCR method of Horton et al. (*Gene* 77:61-68, 1989). In the first method of construction the complete hγ-IFN coding sequence, including the hγ-IFN signal sequence, is linked to the complete hIL-2 coding sequence except for the hIL-2 signal sequence. DNA sequences are obtained from Genebank, Washington D.C., hγ-IFN (HUMIFNINI). The underlined sequence indicates the signal peptide sequence that was excluded.

Human γ-IFN NH_2. . . G R R A S Q COOH
(Sequence ID No. 18)

Human IL-2 NH_2. . . . <u>L V T N S</u> A P T S S S . .COOH
(Sequence ID No. 19)

Hybrid amino
acid sequence
NH_2. . . G R R A S Q A P T S S S . .COOH
(Sequence ID No. 20)

In the second method the complete hIL-2 coding sequence, including the hIL-2 signal sequence is linked to the complete hγ-IFN coding sequence excluding the h γ-IFN signal sequence. DNA sequences are obtained from Genebank; hIL-2 (HUMIL2S1, HUMIL2S2 and HUMIL2S3). The underlined sequence indicates the signal peptide sequence.

Human IL-2 NH_2. . . I I S T L T COOH
(Sequence ID No. 21)

Human γ-IFN NH_2. . <u>V L G S L G</u> C Y C Q D . . . .COOH
(Sequence ID No. 22)

Hybrid amino

acid sequence

NH_2. . . I I S T L T C Y C Q D . . . .COOH

(Sequence ID No. 23)

(b) Construction of Hybrid hγ-IFN/hIL-2 Retroviral Vector

The following hγ-IFN primer sequences are used:

(Sequence ID No. 24)

5'-3': CAG GAC CCA TAT GTA AAA GAA GCA GAA G

This primer is the sense sequence of the hγ-IFN corresponding to a region at the carboxy terminus of the hγ-IFN protein, and is designated hγ-IFN/P7.

(Sequence ID No. 25)

5'-3': GG TGC ACT CTG GGA TGC TCT TCG ACC TCG

This primer is the anti-sense sequence of the hγ-IFN corresponding to a region at the carboxy terminus of the hγ-IFN protein, and is designated hγ-IFN/P8.

The following hIL-2 primer sequences are used:

(Sequence ID No. 26)

5'-3': CC CAG GCA CCT ACT TCA AGT TCT ACA AAG

This primer is the sense sequence of the hIL-2 corresponding to a region at the amino terminus of the hIL-2 protein, and is designated hIL-2/P9.

(Sequence ID No. 27)

5'-3': GGG TCT TAA GTG AAA GTT TTT GCT TTG AGC

This primer is the anti-sense sequence of the hIL-2 corresponding to a region at the amino terminus of the hIL-2 protein, and is designated hIL-2/P10.

The hγ-IFN template DNA from Example 1C 1(e), hγ-IFN/P7 primer DNA, hγ-IFN/P8 primer DNA, hIL-2 template DNA from hIL-2/P9 primer DNA and hIL-2/P10 primer DNA are combined, denatured at 95°C for 1 minute, and extended in the presence of PCR reaction mix without additional primers. The 3' end of the template-primer hγ-IFN DNA will anneal to the 5' end of the template-primer hIL-2 DNA and extension will produce a 673 bp fragment designated hγ-IFN/hIL-2. The

primers hγ-IFN/P7 and hIL-2/P10 are then added and 40 cycles of PCR are performed to amplify the hγ-IFN/hIL-2 product.

The hybrid hγ-IFN/hIL-2 vector is constructed by a three part ligation. The 5' end of KT hγ-IFN retroviral vector is isolated from Nde I restriction endonuclease digestion. This hγ-IFN/hIL-2 product is digested with Nde 1 and Afl II to yield a 656 base pair fragment. This fragment is ligated to the Nde I site of the isolated 5' end of KT hγ-IFN. The 3' end of KT hIL-2 retroviral vector is isolated from Afl II restriction endonuclease digestion. The Afl II restriction site of the 3' KT hIL-2 is ligated to the Afl II restriction site of the construct. This retroviral construct is designated KT hγ-IFN/hIL-2.

(c) Construction of Hybrid hIL-2/hγ-IFN Retroviral Vector

The following hIL-2 primer sequences are used:

(Sequence ID No. 28)

5'-3': CAT CTT CAG TGT CTA GAA GAA GAA CTC

This primer is the sense sequence of hIL-2 corresponding to a region at the carboxy terminus of the hIL-2 protein, and is designated hIL-2/P11.

(Sequence ID No. 29)

5'-3': G GCA GTA ACA AGT CAG TGT TGA GATVGAT GC

This primer is the anti-sense sequence hIL-2 corresponding to a region of the carboxy terminus of the hIL-2 protein, and is designated hIL-2/P12.

The following hγ-IFN primer sequences are used:

(Sequence ID No. 30)

5'-3': GT GAC TGA TGT TAC TGC CAG GAC CCA TAT G

This primer is the sense sequence corresponding to a region of the amino terminus of the hγ-IFN protein, and is designated hγ-IFN/P13.

(Sequence ID No. 31)

5'-3': CGA ATA ATT AGT CAG CTT TTC GAA GTC

This primer is the anti-sense sequence corresponding to a region of the amino terminus of the hγ-IFN protein, and is designated hγ-IFN/P14.

The hIL-2 template DNA from hIL-2/P11 primer DNA, hIL-2/P12 primer DNA, hγ-IFN/P13 template DNA from Example 1C 1(e), hγ-IFN/P13 primer DNA and hγ-IFN/P14 primer DNA are combined, denatured at 95°C for 1 minute and extended in the presence of PCR reaction mix without additional primers. The 3' end of the template-primer hIL-2 DNA will anneal to the 5' end of the template-primer hγ-IFN DNA and extension will produce a 541 bp fragment designated hIL-2/hγ-IFN. The primers hIL-2/P11 and hγ-IFN/P14 are then added, and 40 cycles of PCR are performed to amplify the hIL-2/hγ-IFN product.

The hybrid hIL-2/hγ-IFN vector is constructed by a three part ligation. The 5' end of KT hIL-2 retroviral vector is isolated from Xba I restriction endonuclease digestion. This hγ-IFN/hIL-2 product is digested with Xba I and BstB I to yield a 507 base pair fragment. This fragment is ligated to the Xba I site of the isolated 5' end of KT hIL-2. The 3' end of KT hγ-IFN retroviral vector is isolated from BstB I restriction endonuclease digestion. The BstB I restriction site of the 3' KT hγ-IFN is ligated to the BstB I restriction site of the construct. This retroviral construct is designated KT hIL-2/hγ-IFN.

Example 2

THE REPAIR OF THE hγ-IFN GENE

Subsequent sequencing of KT hγ-IFN, the retroviral vector, revealed the presence of a one base pair deletion within the hγ-IFN gene. This deletion is reversed using multi-step PCR procedure.

A. Sequence Selection

Sequences are obtained from IBI Pustell sequence analysis program (Int. Biotech, Inc., New Haven, Conn.).

The following hγ-IFN primer sequences are used:

(Sequence ID No. 32)

5'-3': G CCT CGA GCT CGA GCG ATG AAA TAT ACA AGT TAT ATC TTG

This primer is the sense sequence complimentary to the start codon ATG region extending seven codons upstream of hγ-IFN gene, and is designated hγ-IFN 1b.

(Sequence ID No. 33)

5'-3': GTC ATC TCG TTT CTT TTT GTT GCT ATT

This primer is the anti-sense sequence complimentary to codons 106 to 120 of the hγ-IFN gene, and is designated hγ-IFN Rep B.

(Sequence ID No. 34)

5'-3': AAT AGC AAC AAA AAG AAA CGA GAT GAC

This primer is the sense sequence complimentary to codons 106 to 120 of the hγ-IFN gene, and is designated hγ-IFN Rep A.

(Sequence ID No. 35)

5'-3': G CAT CGA TAT CGA TCA TTA CTG GGA TGC TCT TCG ACC TCG

This primer is the anti-sense sequence complimentary to the stop codon ATT region and extending seven codons upstream of the hγ-IFN gene, and is designated hγ-IFN 3b.

B. Initial PCR

A solution of 1 x 10^6 KT hγ-IFN plasmid molecules in 398.0 µl, DI H_2O; 50 µl, 10x PCR buffer (500 mM KCl and 200 mM Tris-HCl, pH 8.4; 25 mM $MgCl_2$; 1.0 mg/ml BSA); 5.0 µl, 2.5 mM dATP; 5.0 µl, 2.5 mM dGTP; 5.0 µl, 2.5 mM dCTP; 5.0 µl, 2.5 mM dTTP; 12.0 µl, 18.6 nmol/ml, oligonucleotide hγ-IFN 1b; 15.0 µl, 24.6 nmol/ml, oligonucleotide hγ-IFN RepB; and 2.5 µl, Taq polymerase is mixed in a microfuge tube and 50 µl is aliquoted into 10 tubes. Similarly, a solution of 1 x 10^6 KT hγ-IFN plasmid molecules in 395.0 µl, DI H_2O; 50.0 µl, 10x PCR buffer (500 mM KCl; 200 mM Tris-HCl, pH 8.4; 25 mM $MgCl_2$; 1 mg/ml BSA); 5.0 µl, 2.5 mM dATP; 5.0 µl, 2.5 mM dGTP; 5.0 µl, 2.5 mM dCTP; 5.0 µl, 2.5 mM dTTP; 13 µl, 23.4 nmol/ml, oligonucleotide hγ-IFN RepA; 17.0 µl, 18.0 nmol/ml, oligonucleotide hγ-IFN 3b; and 2.5 µl Taq polymerase is mixed in a microfuge tube and 50.0 µl is aliquoted into 10 tubes. The 20 tubes are placed in a PCR machine (Model 9600, Perkin Elmer Cetus; Los Angeles, Calif.). The PCR program regulates the temperature of the reaction vessel in the first cycle at 94°C for 2 minutes. The next 35 cycles are regulated at 94°C for 0.5 minutes, then at 55°C for 0.5 minutes and finally at 72°C for 1 minute. The final cycle is regulated at 72°C for 10 minutes. This cycling program is designated Program 10.

Following PCR amplification, 225.0 µl of each reaction tube is mixed with 25.0 µl loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 50%

glycerol, agarose gel loading dye) and loaded into the wells of a 2% agarose gel containing ethidium bromide. The gel is electrophoresed at approximately 90 volts for 1 hour. Ultraviolet light is used to visualize the DNA band separation. Two bands are isolated, one fragment of 250 bp in size and the other of 150 bp in size by electrophoretic transfer onto NA 45 paper as previously described in Example 1C 2(a). Following precipitation, each of the two DNA pellets is resuspended in 20.0 µl DI H_2O and prepared for further PCR amplification.

C. Annealing and Second Round PCR

A solution of 20.0 µl of the 150 bp PCR DNA; 20.0 µl of the 350 bp PCR DNA: 161.5 µl, DI H_2O; 25.0 µl, 10x PCR buffer (500 mM KCl; 200 mM Tris-HCl, pH 8.4; 25 mM $MgCl_2$; and 1 mg/ml BSA); 2.5 µl, 2.5 mM dATP; 2.5 µl, 2.5 mM dGTP; 2.5 µl, 2.5 mM dCTP; 2.5 µl, 2.5 mM dTTP; and 1.25 µl Taq polymerase is mixed in a microfuge tube and 47.3 µl aliquoted into each of 5 tubes. Each tube is placed in a PCR machine (Model 9600, Perkin-Elmer-Cetus, Calif.). The PCR program regulates the temperature of the reaction vessel for 5 cycles at 94°C for 0.5 minutes. The next cycle is regulated at 55°C for 1 minute. Following this cycle, 1.2 µl hγ-IFN 1b and 1.5 µl hγ-IFN 3b are added to the reaction mixture. The tubes are then PCR amplified using program 10. The product is designated rhγ-IFN.

D. Creation and Isolation of Blunt-Ended rhγ-IFN PCR DNA Fragment

The PCR amplified rhγ-IFN DNA is blunt ended using T4 polymerase. Specifically, 120.0 µl rhγ-IFN PCR solution is mixed with 1.25 µl, 2.5 mM dATP; 1.25 µl, 2.5 mM dGTP; 1.25 µl, 2.5 mM dCTP; 1.25 µl, 2.5 mM dTTP; 1 µl, T4 DNA polymerase; and 1.0 µl Klenow fragment. This mixture is incubated at room temperature for 10 minutes. Following incubation, 13.0 µl of agarose gel loading dye is added to the mixture and this solution is loaded into a 1% agarose gel. The gel is electrophoresed at approximately 90 volts for 1 hour. Ultraviolet light is used to visualize the DNA banding. A 500 bp band is isolated by electrophoretic transfer onto NA 45 paper as described in Example 1C 2(a). Following precipitation, the DNA pellet is resuspended in 12.0 µl DI H_2O.

The isolated 500 bp fragment is blunt ended using T4 polynucleotide kinase. Specifically, 1.0 mg of this fragment is mixed with 1.5 µl 10x kinase buffer (0.5 mM Tris-HCl, pH 7.6; 0.1 mM $MgCl_2$; 50 mM dithiothreitrol; 1 mM spermidine; 1 mM EDTA); 1.5 µl, 10 mM ATP; and 1.0 µl, T4 polynucleotide kinase, and incubated at 37°C for 30 minutes.

E. Ligation of rhγ -IFN PCR DNA Into the SK^+ Vector

The rhγ-IFN PCR DNA is ligated into the SK^+ vector as described in Example 1C 2(b). A solution of 2.0 µl rhγ-IFN PCR DNA-kinase reaction mixture; 2.0 µl CIP treated SK^+ vector; and 1.0 µl, T4 DNA ligase is incubated at 16°C for 4 hours. DH5a bacteria is transformed as described in Example 1C 2(c).

F. Ligation of rhγ-IFN Gene Into Retroviral Vector

Ligation of rhγ-IFN gene into retroviral vector is performed as described in Example 1C 2(c). The new vector is designated KT rhγ-IFN.

Example 3

TRANSDUCTION OF PACKAGING CELL LINES (CA AND DA) AND MURINE TUMOR CELL LINES (B16F10 AND L33) WITH mγ-IFN RETROVIRAL VECTOR

A. Plasmid DNA Transfection

293 2-3 cells (a cell line derived from 293 cells ATCC No. CRL 1573, WO 92/05266) 5 x 10^5 cells are seeded at approximately 50% confluence on a 6 cm tissue culture dish. The following day, the media is replaced with 4 ml fresh media 4 hours prior to transfection. A standard calcium phosphate-DNA coprecipitation is performed by mixing 10.0 µg of KT mγ-IFN plasmid and 10.0 µg MLP G plasmid with a 2M CaCl solution, adding a 1x Hepes buffered saline solution, pH 6.9, and incubating for 15 minutes at room temperature. The calcium phosphate-DNA coprecipitate is transferred to the 293 2-3 cells, which are then incubated overnight at 37°C, 5% CO_2. The following morning, the cells are rinsed 3 times in 1x PBS, pH 7.0. Fresh media is added to the cells, followed by overnight incubation at 37°C, 10% CO_2. The following day, the media is collected off the cells and passed through a 0.45 µm filter. This supernatant is used to transduce packaging and tumor cell lines.

B. Packaging Cell Line Transduction

CA cells (an amphotropic cell line derived from CF-2 cells, ATCC No. 6574, WO 92/05266) are seeded at 1 x 10^5 cells/6 cm dish. One-half milliliter of the freshly collected 293 2-3 supernatant is added to the CA cells. The following day, G418 is added to these cells and a drug resistant pool is generated over a period of a week. This pool of cells is dilution cloned by adding 0.8 -1.0 cells to each well of 96 well plates. Twenty-four clones were expanded to 24 well plates, then to 6 well plates, at

which time cell supernatants are collected for titering. CA clones are selected for vector production. A CA clone having a titer of approximately 5 x 10^6 cfu/ml is selected, and designated mγ-IFN #23.

DA cells (referred to as DA2 in WO 92/05266), an amphotropic cell line derived from D 17 cells ATCC No. CCL-183, are seeded at 5 x 10^5 cells/10 cm dish. 0.5 ml of the 293 2-3 supernatant stored at -70°C is added to the DA cells. The following day, G418 is added to these cells and a drug resistant pool is generated over the period of a week. DA clones are selected for vector production.

C. Murine Tumor Cell Line Transductions

1. L33

L33 cells (Dennert, USC Comprehensive Cancer Center, Los Angeles, Calif., Patek, et. al., *Int. J. of Cancer 24*:624-628, 1979) are seeded at 1 x 10^5 cells/6 cm dish. 1.0 ml of the 293 2-3 supernatant stored at -70°C is added to the L33 cells. The following day, G418 is added to these cells and a drug resistant pool is generated over a period of a week. This pool of cells is dilution cloned by adding 1.0 cell to each well of 96 well plates, followed by the expansion of clones to 24 well plates, and then 6 well plates, after which cell lysates are prepared for analysis of major histocompatability complex (MHC) expression. A clone, L33/mγ-IFN #15, which had significantly increased levels of MHC expression is used in subsequent mouse studies.

2. B16F10

B16F10 cells (Dennert, USC Comprehensive Cancer Center, Los Angeles, Calif.; Warner, et. al., *Nature 300:*113-121, 1982) are seeded at 2 x 10^5 cell/10 cm dish with a 10 μg/ml polybrene (1,5-dimethyl-1,5-diazaundeca-methylene polymethobromide, Sigma, St. Louis, Missouri). 0.1 ml of supernatant from the CA mγ-IFN pool is added to the cells and incubated for 6 hours at 37°C, 10% CO_2. G418 is added after incubation and a drug resistant pool is generated. This pool is dilution cloned by adding 1.0 cells to each well of 96 well plates. Twenty-four clones are expanded to 24 well plates, then to 6 well plates, at which time cell lysates are made for analysis of MHC expression. A clone, B16F10/mγ-IFN #4, having significantly increased levels of MHC expression is used in subsequent mouse studies.

3. CT 26 And Lewis Lung Tumor Cells

Colon tumor 26 (CT 26) (Brattain, Baylor College of Medicine, Houston Texas) and Lewis lung tumor (LLT) (Waude, Southern Research Institute, Birmingham,

Alabama, ATCC No. CRL 1642) cells are seeded 1 x 10^5 cells/6 cm plate for each cell line in DMEM with 10% FBS and 4 μg/ml polybrene and incubated for 24 hours at 37° C, 10% CO_2. After incubation, 1.0 ml of KT mγ-IFN retroviral vector (9 x 10^6 cfu/ml) is added to each respective cell line and incubated for 24 hours at 37°C, 10% CO_2. Following incubation, the medium is changed and replaced with DMEM with 10% FBS and 400 μg/ml G418. These cell lines are kept under G418 selection for approximately two weeks. Selected CT 26 and LLT resistant pools are dilution cloned by adding 1.0 cell to each well of 96 well plates. Two 96 well plates are seeded for each G418-selected pool. CT 26 and LLT mγ-IFN expressing clones are expanded into 24 well plates and then to 6 well plates. Lysates are prepared of each clone and analyzed for up-regulated MHC protein expression by Western blot analysis. A clone, CT 26/mγ-IFN #10, having up-regulated MHC protein expression is selected. All LLT studies are conducted using the non-clonal pool of the mγ-IFN expressing LLT cells.

Example 4

TRANSDUCTION OF PACKAGING CELL LINE AND HUMAN MELANOMA CELL LINES WITH hγ-IFN RETROVIRAL VECTOR

A. Plasmid DNA Transfection

5 x 10^5 293 2-3 cells (described in patent application WO 92/05266) are seeded at approximately 50% confluence on a 6 cm tissue culture dish. The following day, the media is replaced with 3 ml fresh media 4 hours prior to transfection. At the time of transfection, 5.0 μl of KT hγ-IFN plasmid is mixed with 2.0 μg MLP G plasmid in 0.1x Tris-EDTA, 150 mM, pH 7.4. A standard calcium phosphate-DNA coprecipitation is performed mixing the DNA with a 2M CaCl solution, adding a 1x Hepes buffered saline solution, 2M, pH 6.9, and incubating for 15 minutes at room temperature. The calcium phosphate-DNA coprecipitate is transferred to the 293 2-3 cells, which are then incubated overnight at 37°C, 5% CO_2. The following morning, the cells are rinsed 3 times in 1x PBS, pH 7.0. Fresh media is added to the cells, followed by overnight incubation at 37°C in 10% CO_2. The following day, media is collected off the cells and passed through a 0.45 μm filter. The filtered supernatant is stored at -70°C for use in packaging cell transductions.

B. Packaging Cell Transduction

CA 6BM cells (CA cells described in patent application WO 92/05266 cured of mycoplasma by 6 cycles of BM cycline) are seeded at 1 x 10^5 cells/6 cm dish

with 4 μg/ml polybrene. The following day, 0.2 ml of the supernatant collected off the 293 2-3 transiently transfected cells in Example 4A is added to the media of the CA cells. These cells are then incubated overnight at 37°C, 5% CO_2. The following day, the media is replaced with fresh media containing 800 μg/ml G418. Cells are grown to confluence and expanded under G418 selection. Upon subsequent confluence, a majority of the cells are frozen while a culture is maintained free of G418. When cells once again reached confluence, the supernatant is collected for analysis of the presence of hγ-IFN by viral inhibition assay. The pool of cells is dilution cloned by adding 1.0 cell to each well of 96 plates. G418 is included in the culture media. Twenty-four clones are expanded and analyzed for titer, the presence of helper virus, expression, and functional transfer of expression.

C. Second Generation Transfection/Transduction

1. CA 6BM Transduction

5.0×10^5 DX cells (a cell line derived from D 17 cells ATCC No. 183, WO 92/05266) are seeded at approximately 50% confluence on a 6 cm tissue culture dish. The following day, the media is replaced with 4 ml fresh media 3.5 hours prior to transfection. A standard calcium phosphate-DNA coprecipitation is performed by mixing 10.0 μg of KT hγ-IFN plasmid with a 120 ml 2M CaCl solution, adding a 240 ml of a 2M 1x Hepes buffered saline solution, pH 6.9, and incubating for 15 minutes at room temperature. The calcium phosphate-DNA coprecipitate is transferred to the DX cells, which are then incubated overnight at 37°C, 5% CO_2. The following morning, the cells are rinsed 3 times with 3 ml of 145 mM 1x PBS, pH 7.0. Fresh media is added to the cells, followed by overnight incubation at 37°C, 10% CO_2. The following day, the media is collected off the cells, passed through a 0.45 μm filter, and stored at -70°C.

Three days later, CA 6BM cells are seeded with 4 μg/ml polybrene at 1×10^5 cells/6 cm dish. The following day, 5.0 and 1.0 ml of the supernatant collected from the DX transfected cells is added to the CA 6BM cells. These mixtures are incubated for 4 hours at 37°C, 10% CO_2. Following the incubation, the cells are dilution cloned in the presence of 800 μg/ml G418 at 10 and 30 cells/well of 96 well plates. Forty clones are expanded to 24 well plates and then to 6 well plates. The cell supernatants are collected and titered. Clones with titers of at least 1×10^6 cfu/ml are placed in roller bottles and monitored for the generation of helper virus. This packaging cell line is designated CA/hγ-IFN.

2. DA Transduction

5.0 x 10^5 DX cells are seeded at approximately 50% confluence on a 6 cm tissue culture dish. The following day, the media is replaced with 4 ml fresh media 4 hours prior to transfection. A standard calcium phosphate-DNA coprecipitation is performed by mixing 2.0 µl, 6.0 µg, of KT hγ-IFN plasmid with a 120 ml 2M CaCl solution, adding 240 ml of a 2M 1x Hepes buffered saline solution, pH 6.9, and incubating for 15 minutes at room temperature. The calcium phosphate-DNA coprecipitate is transferred to the DX cells, which are then incubated overnight at 37°C, 5% CO_2. The following morning, the cells are rinsed 3 times with 3ml of 145 mM 1x PBS, pH 7.0. Fresh media is added to the cells and followed by overnight incubation at 37°C, 10% CO_2. The following day, the media is collected off the cells and passed through a 0.45 µm filter.

The previous day, DA cells (previously described in patent application WO 92/05266 as DA2) are seeded at 1 x 10^5 cells/6 cm dish. 1.0 ml of the freshly collected DX supernatant is added to the DA cells. The following day, G418 is added to these cells and a drug resistant pool is generated over a 2-week period. The pool of cells is dilution cloned by adding 1.0 cell to each well of 96 well plates. Twenty-four clones are expanded to 24 well plates, then to 6 well plates. The cell supernatants are collected for titering and clones with titers of at least 5 x 10^5 cfu/ml are selected. A DA clone hIFNr #15 is selected and designated DA/hγ-IFN.

D. Human Melanoma Transductions

Melanoma cell lines DM6, DM92, DM252, DM265, DM262 and DM259 were established from human tumor biopsies (Dr. Hilliard Seigler, Duke University and Viagene, Inc.) by mincing the tumor into 1 mm chunks or grinding the tumor through a Cellector mesh and plating them on a tissue culture flask. Cells were repeatedly passaged by differential trypsinization, where the cells are trypsinized and the tumor cells are removed before the fibroblasts lift off the flask. The cells were carried until constant growth was observed and sufficient cell numbers were generated and frozen.

After establishment, each cell line was seeded at 10^6 cells/10 cm dish with 4 µg/ml polybrene. The following day, 5-10 mls of filtered supernatant from the DA/hγ-IFN pool was added to each of the cell cultures. This corresponds to a multiplicity of infection (MOI) of 5-10. The next day, the cells were selected with 800 µg/ml of G418. Samples of the supernatants of all transduced cell lines were saved twice weekly. The supernatants were filtered through a 0.45 µm filter and stored at -70°C until assayed for γ-IFN expression as described in Example 6. The cultures were

maintained until selection was complete and sufficient cell numbers were generated and frozen.

Example 5

MHC CLASS I EXPRESSION

A. Determination of Mouse MHC Class I Expression by Western Blot Analysis

RIPA lysates are prepared from confluent plates of cells. Specifically, the media is first aspirated off the cells. Depending upon the size of the culture plate containing the cells, a volume of 100.0 to 500.0 µl ice cold RIPA lysis buffer (10 mM Tris, pH 7.4; 1% Nonidet P40 (Calbiochem, Calif); 0.1% SDS; 150 mM NaCl) is added to the cells. Cells are scrapped from plates using a micropipet and the mixture is transferred to a microfuge tube. The tube is centrifuged for 5 minutes to precipitate cellular debris and the lysate supernatant is transferred to another tube. The lysates are electrophoresed on a 10% SDS-PAGE gel and the protein bands are transferred to an Immobilon membrane in CAPS buffer (10 mM CAPS, pH 11.0; 10% methanol) at 10 to 60 volts for 2 to 18 hours. The membrane is transferred from the CAPS buffer to 5% Blotto (5% nonfat dry milk; 50 mM Tris, pH 7.4; 150 mM NaCl; 0.02% sodium azide, and 0.05% Tween 20) and probed with a rat IgM antibody, 72.14S (Richard Dutton, UCSD, San Diego, Calif.). This antibody probe is directed against a conserved intracellular region of the mouse MHC Class I molecule. Antibody binding to the membrane is detected by the use of ^{125}I-Protein A.

B. Analysis of MHC Expression in Murine Tumor Cell Lines With and Without mγ-IFN Retroviral Vector

MHC expression is confirmed by Western blot and FACS analysis. Specifically, L33, CT 26, and LLT parent cell lines express relatively normal levels of MHC Class I protein and the B16F10 parent cell line has down-regulated levels of MHC Class I protein. The mγ-IFN-transduced pools and clones of these cell lines express greater levels of MHC Class I than their corresponding parent cell lines which is demonstrated by Western blot and FACS analysis. Western blot of lysates of various CT 26 mγ-IFN, LLT mγ-IFN subclones and the parent CT 26 and LLT cell lines show up-regulated MHC Class I expression. A Western blot analysis of two L33 mγ-IFN subclones, two B16F10 mγ-IFN subclones, parent L33 cell line, and parent B16F10 cell line illustrates the up-regulated MHC Class I expression of the mγ-IFN clones as compared to the parent cells, Figure 2. FACS analysis of L33 and two L33 mγ-IFN

subclones illustrates that the subclones have considerable more MHC Class I expressed on the surface as compared to the parent cells. FACS analysis is performed on harvested cells. Specifically, cells are incubated with an MHC Class I specific antibody 34.4 anti-D^d antibody (Dutton, UCSD; San Diego, Calif.). This bound antibody is detected by incubating the 34.4 anti-D^d bound cells with fluoroscene conjugated rabbit anti-mouse IgG antibody (Capell, North Carolina). Fluorescent emission from the cell bound antibody-fluoroscene conjugate is detected and quantitated by FACS, Figure 21.

C. MHC Expression in Tumor Cells Treated With Recombinant mγ-IFN In Vitro

1.0×10^6 cells treated with recombinant mγ-IFN *in vitro* are plated the day before treatment so that 50% confluency is reached by the next day. Recombinant mγ-IFN (Genzyme, Cambridge, MA) is added at concentrations ranging from 0 to 500 U/ml to duplicate plates of the cells under study. The plates are incubated for 48 hours at 37°C and cells are lysed and analyzed by Western blot to determine an increase in MHC Class I expression. The data is presented in Figure 3.

In order to study the effects of recombinant mγ-IFN on MHC expression levels of exogenously treated cells following removal of the IFN, multiple plates of L33 cells are seeded the day before treatment so that 50% confluency is reached the next day. Recombinant mγ-IFN is added at a final concentration of 200 U/ml, and the cells are incubated for 48 hours. The cells are then washed with PBS, fresh media is added, and the cells are lysed 0, 24, 48, and 72 hours following the PBS wash. The cell extracts are analyzed by Western blot for MHC Class I expression. Results indicate that by 48 hours after removal of the IFN there is a significant decrease in cellular MHC Class I expression and this decrease continues with time (Figure 3A).

Example 6

DETERMINATION OF γ IFN ACTIVITY

A. mγ-IFN Assay

The activity of γ-IFN is quantified by Lee Biomolecular, San Diego, Calif., as the measurement of the protective effect against cytocidal infection with encephalomyocarditis (EMC) virus. A mouse cell line L929, ATCC CCL 1, is used to assay for mγ-IFN. The filtered supernatants are added to the cells at different concentrations and then the cells are challenged with the EMC virus. Mouse γ-IFN

samples are co-assayed with the appropriate NIH, reference reagents and the results are normalized to NIH reference units (U/ml) (Brennan et al., *Biotechniques 1:*78, 1983).

Samples of the supernatants of all transduced cell lines are saved when the cells are fed twice weekly. The supernatant is filtered through a 0.45 μm filter and stored at -70°C until testing. Activities are recorded for the cell types CT 26, BC10ME, LLT, and B16F10. This data is presented in Tables 3 and 4.

Table 3

γ-IFN PRODUCTION IN VARIOUS BALB/C CELL LINES

Cell Type	U/ml
rCT26	3.5
CT26 IFN pool	3400
CT26 IFN clone #10	4500
BC10ME	22
BC10ME IFN pool	110
L33	< 0.3
L33 α-IFN	7.7

ND = Not Done

Table 4

γ-IFN PRODUCTION IN VARIOUS C57BL/6 CELL LINES

Cell Type	U/ml
LLT	< 0.69
LLT IFN pool	82
LLT IFN #21	40
LLT IFN #28	2.1
LLT IFN pool tumor	21
LLT IFN pool lg met	11

B16F10	< 2.6
B16F10 IFN #4	90

Example 7

TUMORIGENICITY OF B16F10 AND B16F10/mγ-IFN #4 CELLS

Parental B16F10 and B16F10/mγ-IFN#4 cells are harvested, counted, and resuspended to a concentration of 8 x 10^5 cells/ml in Hanks buffered salt solution (HBSS, Irvine Scientific, Calif.). Two Black 6 mice (Harlan Sprague-Dawley, Indianapolis, Indiana) are injected intravenously (i.v.) with 0.5 ml of the B16F10 cell suspension (3 x 10^5 cells). Five C57 Bl/6 mice are injected i.v. with 0.5 ml of the B16F10 IFN#4 cell suspension. Fourteen days after injection the lungs are removed from the mice, stained and preserved in Bouin's Solution (Sigma, St. Louis, Missouri). The 4 lobes of the lungs are separated, examined under 10x magnification, and the number of black tumors present on each is determined.

The average number of tumors per lung for each group and the standard deviation is shown in Figure 6.

Example 8

B16F10 CTL ASSAYS

A. Experiment 1

B16F10/mγ-IFN #4 cells are irradiated with 10,000 rad of ^{60}Co at the Salk Institute. Two Black 6 mice are injected intraperitoneally (i.p.) with 1 x 10^7 irradiated cells in 1.0 ml HBSS. Three weeks later, the mice are injected i.v. with 3 x 10^5 live B16F10 cells in 0.5 ml HBSS. Two Black 6 control mice are also injected with the same dose. Fourteen days later, the lungs and spleens are removed from the mice. The lungs are stained and preserved in Bouin's Solution. The 4 lobes of the lungs are separated, examined under 10x magnification and the number of black tumors present in each is determined. No tumors were visible on any of the lungs. Splenocytes are removed from the spleens, washed three times in HBSS, and resuspended in CTL media containing RPMI and 5% heat inactivated FBS at 3 x 10^7 cells/10ml in T-25 flasks. Sixty thousand B16F10 cells irradiated with 10,000 rad of ^{60}Co at the Salk Institute are added to the flasks. The cells are incubated for 6 days at 37°C, 5% CO_2. After incubation, a standard 5-hour CTL assay is performed using both B16F10 and B16F10/hγ-IFN #4 cells as targets. The data is presented in Figure 4.

Six- to eight-week-old female Balb/C mice (Harlan Sprague-Dawley, Indianapolis, Indiana) are injected twice i.p. with 1 x 10^7 vector transduced cells irradiated with 10,000 rads at room temperature. Animals are sacrificed 7 days later and 3 x 10^6 splenocytes/ml are cultured *in vitro* with 6 x 10^4 irradiated syngeneic transduced cells/ml in T-25 flasks. Culture medium consists of RPMI 1640; 5% FBS, heat-inactivated; 1 mM pyruvate; 50 mg/ml gentamycin and 10^{-5} M 2-mercaptoethanol. Effector cells are harvested 4-7 days later and tested using various effector:target cell ratios in 96 well microtiter plates in a standard 4-6 hour assay. The assay employs $Na_2{}^{51}CrO_4$-labeled, 100 uCi, 1 hr at 37°C (Amersham, Arlington Heights, Illinois), target cells at 1 x 10^4 cells/well in a final volume of 200.0 µl. Following incubation, 100.0 µl of culture medium is removed and analyzed in a Beckman gamma spectrometer (Beckman; Dallas Texas). Spontaneous release (SR) is determined as CPM from targets plus medium and maximum release (MR) is determined as CPM from targets plus 1M HCl. Percent target cell lysis is calculated as: [(Effector cell + target CPM) - (SR)/(MR) - (SR)] x 100. Spontaneous release values of targets are typically 10%-20% of the MR.

B. Experiment 2

B16F10/mγ-IFN#4 cells are harvested, resuspended in HBSS, and irradiated with 20,000 rad of ^{60}Co at the Salk Institute. Cells are aliquoted at 2 different concentrations: 1 x 10^7 cells/ml and 1 x 10^7 cells/0.1 ml. Three groups consisting of 4 Black 6 mice are injected. The first group of three mice receives no cells, the second group receives 1.0 ml i.p., 1.0 x 10^7 total cells and the third group receives 0.1 ml i.m., 1 x 10^7 total cells.

Seven days after injection, all mice are i.v. injected with 0.5 ml live B16F10 cells at 8 x 10^5 cells/ml HBSS. Fourteen days after i.v. injection, the spleens are removed from the mice. The splenocytes are isolated from the spleens, washed three times in HBSS, and resuspended in CTL media at 3 x 10^7 cells/10 ml in T-25 flasks. Sixty thousand B16F10 cells irradiated with 20,000 rad of ^{60}Co at the Salk Institute are added to the flasks. The cells are incubated for 5 days at 37°C 5% CO_2. After incubation, at which time a standard 6.5 hour CTL assay is performed using B16F10 cells as targets. The data is presented in Figure 5.

Example 9

B16F10 VACCINE STUDIES

A. Experiment 1

B16F10 and B16F10/mγ-IFN#4 cells are harvested and resuspended in HBSS. The cells are irradiated with 10,000 rad of ^{60}Co. The cell concentration for both cell suspensions is adjusted to 8×10^5 cells/ml. Three groups of three Black 6 mice are injected. The first group is injected i.v. with 0.5 ml, 4.0×10^5 irradiated B16F10 cells. The second group is injected i.v. with 0.5 ml of the irradiated B16F10/mγ-IFN#4 cells and the final group did not receive cells. Ten days after injection, all 9 mice are injected i.v. with 0.5 ml live B16F10 cells at 6.0×10^5 cells/ml HBSS. Fourteen days after i.v. injection, the lungs are removed from the mice and stained and preserved in Bouin's Solution. The 4 lobes of the lungs are separated, examined under 10x magnification, and the number of black tumors present in each is determined.

The average number of tumors per lung for each group and the standard deviation is shown in Figure 7.

B. Experiment 2

B16F10/hγ-IFN#4 cells are harvested, resuspended in HBSS, and irradiated with 10,000 rad of ^{60}Co. Cells are aliquoted at 5 different concentrations: 4×10^5 cells/0.5 ml, 5×10^5 cells/ml, 1×10^7 cells/ml, 5×10^6 cells/0.1 ml, and 1×10^7 cells/0.1 ml. Six groups of 4 Black 6 mice are injected. Group one is not injected with cells. Group two is injected with 0.5 ml i.v., 4×10^5 B16F10/mγ-IFN#4 cells. The third group is injected with 1.0 ml i.p., 5×10^6 B16F10/mγ-IFN#4 cells. The fourth group is injected with 1.0 ml i.p., 1×10^7 B16F10/mγ-IFN#4 cells. The fifth group is injected with 0.1 ml intramuscularly (i.m.), 5×10^6 B16F10/mγ-IFN#4 cells and the final group is injected with 0.1 ml i.m., 1.0×10^7 B16F10/mγ-IFN#4 cells. Seven days after injection all mice are injected i.v. with 0.5 ml live B16F10 cells at 6.0×10^5 cells/ml HBSS. Fourteen days after i.v. injection the lungs are removed from the mice, stained and preserved in Bouin's Solution. The 4 lobes of the lungs are separated, examined under 10x magnification, and the number of black tumors in each lung is determined.

The average number of tumors per lung for each group and the standard deviation, is shown in Figure 8.

Example 10

COMPARISON OF TUMORIGENICITY PROPERTIES OF CELLS TREATED WITH mγ-IFN

AND RECOMBINANT mγ-IFN PROTEIN

A. Experiment 1

The following experiment is performed to determine whether tumor cells treated with mγ-IFN would grow differently *in vivo* as compared with tumor cells that continually express mγ-IFN. Three separate groups of ten Balb/C mice are subcutaneously injected with either 3 x 10^6 L33 cells, 3 x 10^6 L33 cells treated with 400 units of recombinant mγ-IFN for three days *in vitro* prior to injection, or 3 x 10^6 cells of a clone of mγ-IFN vector-modified L33 tumor cells, L33/mγ-IFN #15. Prior to injection, these cells are grown in 10 cm Falcon tissue culture dishes using DMEM with 10% FBS. The cells are harvested with Versene (Irvine Scientific, Calif.) and resuspended in HBSS at a concentration of 1.5 x 10^7 per ml. Three million cells of each of the L33 subtypes stated above are injected subcutaneously near the sternum of each animal in a total volume of 0.2 ml. Tumor growth is recorded weekly. The volume of each tumor is determined by measuring the length, width and height of the tumor using a Castroviejo Caliper from Roboz Instruments, Germany. The average tumor size is compared with the average of tumor growth in the other two groups.

The data indicate that the L33 cells treated with recombinant mγ-IFN grow similarly to the L33 parent tumor line. In contrast, the L33/mγ-IFN #15 clone that consistently expresses mγ-IFN is rejected, Figures 9 and 21. In summary, the tumor cells expressing mγ-IFN can induce a more potent and complete immune response than the tumor cells treated *in vitro* with mγ-IFN.

B. Experiment 2

This experiment is identical to Experiment 1, with the following exception. Instead of injecting 3 x 10^6 cells of either L33, L33 treated *in vitro* with recombinant mγ-IFN or L33/mγ-IFN #15, 6 x 10^6 cells of each type are used as the inoculum in the animals. The data indicates that when twice the number of cells are injected, tumor cells expressing mγ-IFN, induce a more potent and complete immune response than the cells treated *in vitro* with mγ-IFN, Figure 10.

C. Tumorgenicity of L33 Cells Expressing mγ-IFN in Nude Mice

Tumorgenicity is determined by monitoring L33/mγ-IFN #15 cell growth in mice with impaired T-cell mediated immunity. Two groups of 7 Balb/C nude mice are injected with either 6 x 10^6 L33 or 6 x 10^6 L33/mγ-IFN #15. Tumor growth is monitored and average measurements are compared between the two groups.

The data indicate that the L33 mγ-IFN #15 cells are not rejected in nude mice, Figure 11. In addition, the mγ-IFN-expressing cells grow approximately 40% slower than do the parent L33 cells. Apparently a T-cell mediated component in the murine immune system is needed for the rejection of the L33 tumor cells. This response is induced in mice with a normal immune system but not in mice with impaired T-cell mediated immunity.

Example 11

DETERMINATION OF ENHANCED IMMUNITY IN BALB/C MICE BY mγ-IFN EXPRESSING CT 26 TUMOR CELLS

A. Tumorgenicity of mγ-IFN Expressing CT 26 Tumor Cells

Tumorgenicity is determined by observing tumor growth in normal Balb/C mice injected with CT 26/mγ-IFN. Two groups of 10 mice are subcutaneously injected with either parent CT 26 or mγ-IFN expressing CT 26 pool. This pool is a non-clonal population of G418 selected transduced cells. These cell types are grown in 10 cm Falcon tissue culture dishes using DMEM and 10% FBS. The cells are harvested using Versene and resuspended in HBSS at a concentration of 2.5×10^6 cells/ml. A total of 0.1 ml of cell suspension is subcutaneously injected near the sternum in each mouse. Tumor measurements of each animal are recorded weekly. The average tumor measurements are compared between the three groups. Figure 12 presents tumor growths over a 30-day period.

The data indicate that mγ-IFN expressing CT 26 cells are rejected whereas no rejection is observed in unmodified CT 26 cells. In summary, an immune response is induced by lymphokine-expressing cells that is not induced by parent tumor cells.

B. Splenocyte Cytolytic Activity in CT 26 AND CT 26/mγ-IFN Expressing Tumor-Bearing Animals

Mice that have rejected their respective mγ-IFN expressing CT 26 tumors are used to determine whether the observed rejection is due to an augmented immune response. Specifically, splenocytes are harvested from animals who had rejected their respective mγ-IFN expressing CT 26 tumor. These tumors are induced by a single subcutaneous injection of each tumor cell type of 2.0×10^5 cells near the sternum of each animal. The process of splenocyte recovery is briefly described. The spleen of a mouse is removed by making a longitudinal incision through the outer fur coat and inner

abdominal wall using a pair of scissors. The spleen is then aseptically dissected away from the adjoining connective tissue and placed in HBSS. The spleen is then placed in a 10 cm plate with 2 ml of fresh HBSS. The splenocytes are removed from the spleen by creating a small tear at one end followed by a gentle stroking of the spleen using the flattened surface of a 23 gauge needle. The splenocytes are collected by adding 7 mls of HBSS to the plate. The cell suspension is collected with a pipet and passed through a Nytex screen (Tetco, Elmsford, New York) to break up lumps. Seven milliliters of additional medium is used to rinse the plate of remaining splenocytes and the mixture is passed through the Nytex screen. The resulting splenocytes are centrifuged in a 15 ml polypropylene tube at 1600 RPM for 5 minutes at room temperature. The pelleted splenocytes are resuspended in 14 ml of HBSS and centrifuged at 1600 rpm for 5 minutes at room temperature. The pelleted splenocytes are resuspended in 10 ml of HBSS and centrifuged at 1600 rpm for 5 minutes at room temperature. Prior to centrifugation, a dilution of an aliquot of the resuspended splenocytes is removed and counted in Trypan Blue (Irvine Scientific; Santa Ana, Calif.). The concentration of non-blue cells is determined. The splenocytes are then resuspended in RPMI and 5% heat-inactivated FBS at a concentration of 3×10^7 splenocytes/ml. The splenocytes are then restimulated *in vitro*. Specifically, 3×10^7 splenocytes are mixed with 6×10^5 irradiated CT 26, or other appropriate restimulator, in a T-25 cm flask with 10 ml RPMI and 5% FBS at an effector:restimulator cell ratio of 50:1 and incubated at 37°C, 5% CO_2, for 5-7 days. After incubation, these effectors are removed from the flask, counted and incubated with various ratios with ^{51}chromium (^{51}Cr)-labeled target cells in a 96 well plate for 4 hours at 37°C. After incubation, 100 ul of supernatant from each well of each effector:target cell ratio is placed in a tube and the release of ^{51}Cr is determined using a gamma counter. The percentage of lysis is determined using the following formula.

$$\% \text{ Lysis} = \frac{(\text{Experimental Release, CPM}) - (\text{Spontaneous Release, CPM})}{(\text{Maximum Release, CPM}) - (\text{Spontaneous Release, CPM})} x100$$

The data indicates that a potent immune response is induced in animals that rejected their mγ-IFN expressing CT 26 cells, Figure 13. In summary, the mγ-IFN expressing CT 26 cells can induce an immune response that the parent tumor cannot induce.

Example 12

INDUCTION OF SPLENOCYTE CYTOLYTIC ACTIVITY IN BALB/C MICE USING IRRADIATED CT 26 OR CT 26/mγ-IFN EXPRESSING TUMOR CELLS

A. Generation of Splenocyte Cytolytic Activity Using Two Injections of CT 26 or CT 26/mγ-IFN Expressing Cells

A series of injections are performed to determine whether irradiated mγ-IFN CT 26 cells could enhance immune activation against CT 26 more than irradiated unmodified CT 26. Two mice are injected with two weekly doses of 1.0×10^7 irradiated CT 26 or CT 26/mγ-IFN #10 cells per dose. After two weeks, the spleens are removed, stimulated *in vitro* with their respective inducers and used against chromium labeled CT 26 targets in a ^{51}Cr release assay. The data indicates that the cytotoxicity of the CT 26/mγ-IFN effectors are more potent against CT 26 than CT 26 effectors, Figure 14.

B. Specificity of Splenocytes Induced by the Two Injections of CT 26 or CT 26/mγ -IFN Expressing Tumor Cells

The splenocyte effectors generated by a two dose regimen of 1×10^7 CT 26/mγ-IFN #10 cells are used in a ^{51}Cr release assay against several non-CT 26 targets to demonstrate specificity for CT 26. BC10ME, a syngeneic line in Balb/C mice, and B16F10, a tumor cell line obtained from a different strain of mice C57B1/6 (Harlan Sprague-Dawley, Indianapolis, Indiana) and not Balb/C, are selected. The data indicate that, when CT 26 cells expressing mγ-IFN are used as stimulators, the response induced is specific to CT 26 and not against other cell types of either the same strain of mouse or of an unrelated strain, Figure 15.

C. Enhanced Cytolysis of CT 26 γ-IFN Expressing Tumor Cells

mγ-IFN expressing ^{51}Cr labeled CT 26 target cells show enhanced lysis in chromium release assays when using effector splenocytes generated by the two dose regimen of 1×10^7 irradiated CT 26 cells. The data in Figure 16 indicates that CT 26 expressing mγ-IFN cells serve as better targets than unmodified CT 26 cells. It is possible that the mγ-IFN expressed by these modified CT 26 targets may induce greater affinity for the CT 26 effectors which results in more efficient cytolysis by these effectors. The enhanced level of MHC molecules on the surface of these cells may also contribute to the enhanced lysis.

Example 13

DETERMINATION OF ENHANCED IMMUNITY IN C57BL/6 MICE BY mγ-IFN EXPRESSING LLT CELLS

A. Tumorigenicity of mγ-IFN Expressing LLT Cells

Tumorgenicity of mγ-IFN expressing LLT cells is determined by injection into normal C57B1/6 mice. Two groups of ten mice are subcutaneously injected with either parent LLT or mγ-IFN-expressing LLT pool. These cells are grown as described in Example 3 C 3. The cells are harvested, as described in Example 13 A, and resuspended in HBSS at a concentration of 2.5×10^6 cells/ml. One tenth of a milliliter of cell suspension is subcutaneously injected near the sternum of each mouse. Tumor measurements are recorded weekly and the average tumor sizes are compared between the two groups. Figure 17 represents tumor growth over a period of 21 days.

The data indicate that both the mγ-IFN expressing LLT cells grow significantly slower than the unmodified LLT cells. In summary, the data implies that a partial immune response is induced by mγ-IFN-expressing cells that is not induced by parent LLT cells.

B. Generation of Splenocyte Cytolytic Activity Using Two Injections of mγ-IFN Expressing LLT Cells

A series of injections in C57B1/6 mice are performed in order to determine whether irradiated mγ-IFN modified LLT cells enhance a greater immune activation against LLT cells than irradiated LLT cells. Mice are injected with two weekly doses of 1×10^7 irradiated LLT or LLT/mγ-IFN pool. Two weeks following the last injection, spleens are removed, stimulated *in vitro* with their respective inducer, and used against ^{51}Cr-labeled LLT targets in a chromium release assay. The data indicates that the cytotoxicity of the LLT/mγ-IFN effectors are more potent against LLT than LLT effectors or unmodified stimulators demonstrating the greater effectiveness of mγ-IFN expressing stimulator cells, Figure 18.

C. Specificity of Splenocytes Induced by Two Injections of γ-IFN Expressing LLT Cells

The splenocyte effectors generated by a two dose regimen of 1×10^7 LLT mγ-IFN pool cells are used in a chromium release assay against several non-LLT targets to demonstrate specificity for LLT. B16F10, a syngeneic tumor line in C57B1/6 mice, and CT 26, a tumor cell line in Balb/C mice but not C57B1/6, are selected. The

data indicates that, by using LLT cells expressing mγ-IFN as stimulators, the response induced is specific to LLT and not against other cell types of either the same strain of mouse or of an unrelated strain, Figure 19.

D. Enhanced Cytolysis of LLT mγ-IFN Expressing Tumor Cells

mγ-IFN expressing ^{51}Cr-labeled LLT target cells show enhanced lysis in chromium release assays when using effector splenocytes generated by the two dose regimen of 1×10^7 irradiated LLT cells, Figure 20. The data indicates that LLT expressing mγ-IFN cells serve as better targets than unmodified LLT cells. It is possible that the mγ-IFN that are expressed by these modified LLT targets induce greater affinity for the LLT effectors which result in more efficient cytolysis by these effectors.

Example 14

HLA Class I and hγ-IFN Expression in Transduced Human Melanomas

A. Determination of Human MHC (HLA) Class 1 Expression By Western Blot Analysis.

HLA expression is determined essentially as described in Example 5A for murine MHC except that the HLA Class I specific antibody W6/32 is used.

B. Analysis and HLA Expression In Human Melanomas With and Without hγ-IFN Retroviral Vector

DM92, DM252, or DM265 are treated with hγ-IFN vector or hIL-2 as a control for vector transduction. The data in Figure 22 indicates that hγ-IFN vector increases the level of HLA compared with the non-transduced cells whereas, those transduced with IL-2 did not. Transduction of DM265 results in increased HLA even though there is little or no hγ-IFN secreted into the medium. (Table 4).

hγ-IFN is quantified by viral inhibition of encephalomyocarditis virus on a chimpanzee cell line A549, ATCC CCL 185. Activity is determined by comparison with authentic NIH reference reagents and normalized to NIH reference units (U/mL) (Brennan et al., *Biotechniques 1*:78, 1983). The data for several human melanoma cell lines are reported in Table 4. These data indicate that all human melanomas transduced with the hγ-IFN retroviral vector express readily detectable levels of biologically active h γ-IFN. This expression is often stable with time (Table 5, DM252 and DM92) but sometimes decreases with time in culture (Table 4, DM6 and DM265). This time

dependent decrease in hγ-IFN may indicate that expression of the gene is somewhat toxic, thus resulting in a selective advantage for cells expressing low levels of hγ-IFN.

Table 5

mγ-IFN ACTIVITY IN HUMAN MELANOMA CELL LINES

hγ-IFN Production	Day	U/ml
DM262		<1.1
DM262/Dhγ-IFN	20	20
DM252		<6.6
DM252/Dhγ-IFN	2	710
	15	890
	20	1700
	27	400
	34	470
	73	370
	84	240
DM6		<5.0
DM6/Dhγ-IFN	6	54
	13	63
	20	37
	34	<7.7
	73	<1.1
DM92		<5.0
DM92/Dhγ-IFN	6	83
	13	54
	20	83
	30	45
	34	56
DM265		<4.4
DM265/Dhγ-IFN	13	45
	16	45
	20	<7.7
	37	<1.1
	58	4.6

	70	<1.1
DM259		<1.1
DM259/Dhγ-IFN	30	8.1

Example 15

TRANSDUCIBILITY OF HUMAN MELANOMA CELL LINES BY RETROVIRAL VECTORS

A. Transduction of Human Melanoma Cell Lines with Unconcentrated VectorSupernatants

Human melanoma cell lines, DM6, DM92 and DM252 were transduced at different MOI with retroviral vector which expresses *E. coli* β-galactosidase gene (CB βgal). CBβgal is a vector made by replacing the HIV IIIB *gag/prot* gene of KT-3 with the *E. coli* β-galactosidase gene. Producer cell lines were generated in a manner analogous to that described in Examples 1, 3 and 4. Three days after transduction with CBβgal the cells were stained with X-gal (Gold Biotechnology, St. Louis, MO), the number of blue cells were enumerated and the percent transduction was calculated (Norton, et al., *Molec. and Cell. Biol. 5*:218-290, 1985). The results indicate that the three human melanoma cell lines were all easily transducible, approaching 100% transduction.

B. Transduction Efficiencies of Human Melanomas Using Concentrated Vector

Amphotropic CBβgal vector was harvested from CA producer cell lines and concentrated 40-fold by tangential flow concentration. Six well plates of DM252, DM6 and DM92 are set up at 4×10^4 cells/well. The next day, day 0, each melanoma cell line is transduced at an MOI of 50, 25, 10, 5, 1 and 0 with CBβgal vector that is concentrated but not purified. The next day, (day 1) the vector is removed from the cells and the cells are rinsed with media. On day 5, cells are stained with X-gal. These cells are not selected with G418. The transduction efficiency of human melanomas decreased with increasing MOIs when concentrated vector is used (Figure 24) suggesting the presence of an inhibitor to transduction. Therefore, purification of vector may be crucial for direct injection of vector into tumors which will require concentrated vector. Purification methods may include methods typically used by those skilled in the art for protein purification such as gel filtration or ion exchange chromatography. Microscopic inspection of samples, MOI = 1.0 for the above experiment indicated that

even under these conditions 25-90% of the cell population can be transduced without the aid of G418 selection (Figure 25).

C. Transduction Time Experiment After Culture Initiation

Chunks from human tumor biopsy DM262 which had been frozen, are thawed, minced with scalpels, ground through a mesh and plated in ten 6.0 cm tissue culture plates. On days 1, 2, 3, 6, 7, 8, 9, 10 and 13 after culture initiation, one plate is transduced with 1.0×10^6 cfu of unpurified CBβgal vector. On day 20, all of the plates are stained with X-gal. These cells are not selected with G418. The data indicate, Figure 26, that the tumor is rapidly transducible with a high efficiency as soon as one day after culture initiation. Transduction immediately after culture initiation will allow the melanomas to be returned to patients rapidly and with minimum effects due to hγ-IFN toxicity or antigenic drift while in culture. The high efficiency of transduction so soon after plating suggests that *in vivo* transduction by direct injection of vector into tumors may be effective.

Example 16

DIRECT ADMINISTRATION OF VECTOR INTO TUMOR BEARING ANIMALS

A. Direct Administration of Vector into Mice

Mouse tumor systems may be utilized to show that cell mediated immune responses can be enhanced by direct administration of a vector construct which expresses at least one anti-tumor agent. For example, six to eight week old female Balb/C or C57B1/6 mice are injected subcutaneously with 1×10^5 to 2×10^5 tumor cells which are allowed to grow within the mice for one to two weeks. The resulting tumors can be of variable size (usually 1-4 mm^3 in volume) as long as the graft is not compromised by either infection or ulceration. One-tenth to two-tenths of a milliliter of a vector construct which expresses an anti-tumor agent such as γ-IFN, (minimum titer 10^6 cfu/ml) is then injected intratumorally (with or without polybrene or promatine sulfate to increase efficiency of transduction). Multiple injections of the vector are given to the tumor every two to three days.

Depending on the parameters of the particular experiment, the nature of the vector preparations can be variable as well. The vector can be from filtered or unfiltered supernatant from vector producing cell lines (VCL), or may be processed further by filtration, concentration or dialysis and formulation. Other standard

purification techniques, such as gel filtration and ion exchange chromatography, may also be utilized to purify the vector. For example, dialysis can be used to eliminate γ -interferon that has been produced by the VCL itself (and which, if administered, may effect tumor growth). Dialysis may also be used to remove possible inhibitors of transduction. Another option is to perform intratumor injections of the γ-interferon VCL itself, in order to more extensively introduce the vector. Briefly, cells are injected after being spun down from culture fluid and resuspended in a pharmaceutically acceptable medium (e.g., PBS plus 1 mg/ml HSA). As few as 10^5 cells may be used within this aspect of the invention.

Efficacy of the vector construct may be determined by measuring the reduction in primary tumor growth, the reduction in tumor burden (as determined by decreased tumor volume), or by the induction of increased T-cell activity against tumor target cells (as measured in an *in vitro* assay system using lymphocytes isolated from the spleens of these tumor bearing cells). In a metastatic murine tumor model, efficacy may also be determined by first injecting tumor cells that are metastatic, and, when the tumor is 1-4 mm^3 in volume, injecting vector several times into that tumor. The primary tumor graft may then be surgically removed after 2-3 weeks, and the reduction in metastases to the established target organ (lung, kidney, liver, etc.) counted. To measure the change in metastases in a target organ, the organ can be removed, weighed, and compared to a non-tumor bearing organ. In addition, the amount of metastases in the target organ can be measured by counting the number of visible metastatic nodules by using a low powered dissecting microscope.

B. Direct Administration of Vector into Humans

For humans, the preferred location for direct administration of a vector construct depends on the location of the tumor or tumors. The human γ-interferon gene or other sequences which encode anti-tumor agents can be introduced directly into solid tumors by vector administration (the vectors may be purified as previously described). They may also be delivered to leukemias, lymphomas or ascites tumors. For skin lesions such as melanomas, the vector may be directly injected into or around the lesion. At least 10^5 cfu of vector particles should be administered, preferably more than 10^6 cfu in a pharmaceutically acceptable formulation (*e.g.*, 10 mg/ml mannitol, 1 mg/ml HSA, 25 mM Tris pH 7.2 and 105 mM NaCl). For internal tumor lesions, the effected tumor can be localized by X-ray, CT scan, antibody imaging or other methods known to those skilled in the art of tumor localization. Vector injection can be through the skin into internal lesions, or by adaptations of bronchoscopy (for

lungs), sigmoidoscopy (for colorectal or esophageal tumors) or intra-arterial or intra-blood vessel catheter (for many types of vascularized solid tumors). The injection can be into or around the tumor lesion. The efficiency of induction of a biological response may be measured by CTL assay or by delayed type hypersensitivity (DTH) reactions to the tumor. Efficacy and clinical responses may be determined by measuring the tumor burden using X-ray, CT scan or antibody imaging or other methods known to those skilled in the art of tumor localization.

EXAMPLE 17

INJECTION OF β-GAL DIRECT VECTOR INTO MICE INJECTED WITH CT26 TUMOR CELLS

To assess whether tumor cells could become transduced *in vivo* by the direct injection of vector, a reporter vector that expresses the *E. coli* β-galatosidase gene (CB β-gal) was used. Briefly, five groups of two mice each are injected S.C. with 2 x 10^5 CT 26 tumor cells. Another group of two mice are S.C. injected with the 2 x 10^5 CT 26 β-gal-expressing cells as a control for β-gal staining. The injection area on each mouse was circled with water-resistant marker. Two days after tumor cell inoculation, mice are injected with 0.2 ml of either PBS plus polybrene(4 μg/ml), CB β-gal (5 x 10^6 colony forming units (CFU) ml) with and without polybrene, or DAhγ-IFN #15 with and without polybrene. Mice in each group are injected with their respective inoculant every two days within the area marked by water-resistant marker. Each group receives a total of four injections. Two days after the last injections, tumors from each of the groups of mice are removed, minced, and seeded into two 10 cm^2 plates containing DMEM plus 10% FBS and antibiotics. These tumor explants are allowed to grow *in vitro* for one week. After one week one the cells are harvested, fixed with 2% formaldehyde and stained with X-gal overnight. The results of this experiment are presented below:

Tumor Type and Treatment	Total Number of Cells	Number of Blue Cells	% Blue
1. CT26 (PBS+ polybrene)	7.4 x 10^6	2.4 x 10^5	3.2%
2. CT26 β-gal	6.2 x 10^6	2.0 x 10^6	31.6%
3. CT26 + β-gal vector w/o poly	6.6 x 10^6	8.8 x 10^5	13.3%
4. CT26 + β-gal vector w/poly	6.4 x 10^6	1.3 x 10^6	20.0%
5. CT26 + DA hγIFN #15 w/o poly	6.9 x 10^6	2.0 x 10^5	2.9%
6. CT26 + DA hγIFN #15 w/poly	1.3 x 10^6	3.0 x 10^4	2.7%

Due to the overnight staining, there was a substantial background staining of the unmodified CT26 or CT26 injected with hγ-IFN vector as a staining control (3%). Under these conditions, a maximum net of 17% of the cells stained (20 minus the 3% background). Given that the positive control (100% transduced) was only 28.6% stained itself, the 17% stain indicates that (17/28.6) x 100 or 60% of the cells were transduced *in vivo*. It is important to note that the tumor cells being transduced were in log phase and that the multiplicity of infection (M.O.I.) therefore decreased with tumor growth.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: COMPOSITION AND METHODS FOR CANCER IMMUNOTHERAPY

(iii) NUMBER OF SEQUENCES: 36

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
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(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TAATAAATAG ATTTAGATTT A 21

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCCTCGAGAC GATGAAATAT ACAAGTTATA TCTTG 35

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAATCGATCC ATTACTGGGA TGCTCTTCGA CCTGG 35

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATAAATAGAA GGCCTGATAT G 21

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCCTCGAGAC AATGTACAGG ATGCAACTCC TGTCT 35

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GAATCGATTT ATCAAGTCAG TGTTGAGATG ATGCT 35

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gly Arg Arg Ala Ser Gln

1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Leu Gly Glu Pro Gln Leu Cys Tyr Ile Leu Asp Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gly Arg Arg Ala Ser Gln Leu Cys Tyr Ile Leu Asp Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Lys Ala Ala Ile Thr Ser Tyr Glu Lys
1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Lys Ala Ala Ile Thr Glu Thr Leu Lys
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CAGGACCCAT ATGTAAAAGA AGCAGAAAAC C 31

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCAGAGCTGG GATGCTCTTC GACCTCG 27

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCATCCCAGC TCTGCTATAT CCTGGATGCC 30

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGCATGCAGG CATATGTGAT GCCAACC 27

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CAGAGTCTCG GTTATAGCTG CCTTTCGCAC 30

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCTATAACCG AGACTCTGAA GCATGAG 27

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Gly Arg Arg Ala Ser Gln
1 5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Leu Val Thr Asn Ser Ala Pro Thr Ser Ser Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Gly Arg Arg Ala Ser Gln Ala Pro Thr Ser Ser Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Ile Ile Ser Thr Leu Thr
1 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Val Leu Gly Ser Leu Gly Cys Tyr Cys Gln Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Ile Ile Ser Thr Leu Thr Cys Tyr Cys Gln Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CAGGACCCAT ATGTAAAAGA AGCAGAAG 28

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GGTGCACTCT GGGATGCTCT TCGACCTCG 29

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCCAGGCACC TACTTCAAGT TCTACAAAG 29

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GGGTCTTAAG TGAAAGTTTT TGCTTTGAGC 30

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CATCTTCAGT GTCTAGAAGA AGAACTC 27

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GGCAGTAACA AGTCAGTGTT GAGATGATGC 30

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GTGACTGATG TTACTGCCAG GACCCATATG 30

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CGAATAATTA GTCAGCTTTT CGAAGTC 27

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GCCTCGAGCT CGAGCGATGA AATATACAAG TTATATCTTG 40

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GTCATCTCGT TTCTTTTTGT TGCTATT 27

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

ATTAGCAACA AAAAGAAACG AGATGAC 27

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GCATCGATAT CGATCATTAC TGGGATGCTC TTCGACCTCG 40

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Gln Glu Thr Tyr Glu Thr Leu Lys
1 5

Claims

1. A method for inhibiting the growth of a selected tumor in a warm-blooded animal, comprising directly administering to said tumor a vector construct which directs the expression of at least one anti-tumor agent, such that the growth of said tumor is inhibited.

2. The method of claim 1 wherein said vector construct is carried by a recombinant viral vector.

3. The method of claim 2 wherein said recombinant viral vector is a recombinant retroviral vector.

4. The method of claim 1 wherein said anti-tumor agent is selected from the group consisting of immune activators and tumor proliferation inhibitors.

5. The method of claim 4 wherein said immune activators are selected from the group consisting of immune modulators and lymphokines.

6. The method of claim 5 wherein said immune modulator is selected from the group consisting of CD3, ICAM-1, ICAM-2, LFA-1, LFA-3, β -2-microglobulin, chaperones, alpha interferon, gamma interferon, B7/BB1, B7-2 and major histocompatibility complex (MHC).

7. The method of claim 5 wherein said lymphokine is selected from the group consisting of gamma interferon, tumor necrosis factor, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, GM-CSF, CSF-1, and G-CSF.

8. The method of claim 4 wherein said tumor proliferation inhibitor is selected from the group consisting of toxins and antisense sequences.

9. The method of claim 8 wherein said toxin is selected from the group consisting of ricin, abrin, diphtheria toxin, cholera toxin, gelonin, pokeweed, antiviral protein, tritin, Shigella toxin, and Pseudomonas exotoxin A, herpes simplex virus thymidine kinase (HSVTK), and *E. coli.* guanine phosphoribosyl transferase.

10. The method of claim 8 wherein said antisense sequence is selected from the group consisting of antisense thymidine kinase, antisense dihydrofolate reductase, antisense HER2, antisense ABL, antisense Myc, and antisense *ras*.

11. The method of claim 1 wherein said anti-tumor agent is a gamma interferon - interleukin-2 fusion protein.

12. An isolated DNA sequence encoding a membrane anchor - anti-tumor agent fusion protein.

13. An isolated DNA sequence encoding a membrane anchor - gamma interferon fusion protein.

14. A vector construct which directs the expression of a DNA sequence according to claims 12-13.

15. A recombinant viral vector carrying a vector construct according to claim 14.

16. A recombinant retroviral vector carrying a vector construct according to claim 14.

17. A recombinant viral vector carrying a vector construct which directs the expression of an Interleukin-2 - gamma interferon fusion protein.

18. A recombinant retroviral vector carrying a vector construct which directs the expression of an Interleukin-2 - gamma interferon fusion protein.

19. Target cells infected with the recombinant retroviral vector of claims 16 or 18.

20. A pharmaceutical composition comprising a recombinant viral vector according to claims 15 or 17, in combination with a pharmaceutically acceptable carrier or diluent.

21. A pharmaceutical composition comprising a recombinant viral vector according to claims 16 or 18, in combination with a pharmaceutically acceptable carrier or diluent.

22. A method for inhibiting the growth of a selected tumor in a warm-blooded animal, comprising delivering to a warm-blooded animal a recombinant viral or retroviral vector according to any of claim 15 to 18, such that the growth of said tumor is inhibited.

23. A method for inhibiting the growth of a selected tumor in a warm-blooded animal comprising:

(a) removing tumor cells associated with the selected tumor from a warm-blooded animal;

(b) infecting the removed cells with a recombinant retroviral or viral vector according to any of claims 15 to 18; and

(c) delivering said infected cells to a warm-blooded animal, such that the growth of said selected tumor is inhibited.

24. The method of claim 22 wherein the removed tumor cells include fibroblasts and wherein, prior to the step of delivering, fibroblasts are depleted from said removed cells.

25. The method of claim 22 wherein, prior to delivering the infected cells to a warm-blooded animal, said infected cells are inactivated.

26. A method for inhibiting the growth of a selected tumor in a warm-blooded animal comprising:

(a) removing tumor cells associated with the selected tumor from a warm-blooded animal;

(b) contacting said cells with a vector construct which directs the expression of an anti-tumor agent such that said cells are capable of expressing said anti-tumor agent; and

(c) delivering the cells from step (b) to an allogeneic warm blooded animal, such that the growth of said selected tumor is inhibited.

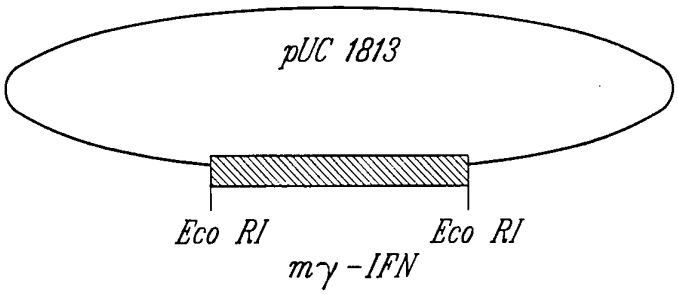


FIG. 1A

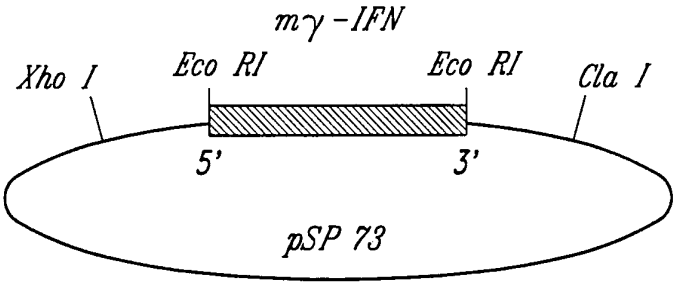


FIG. 1B

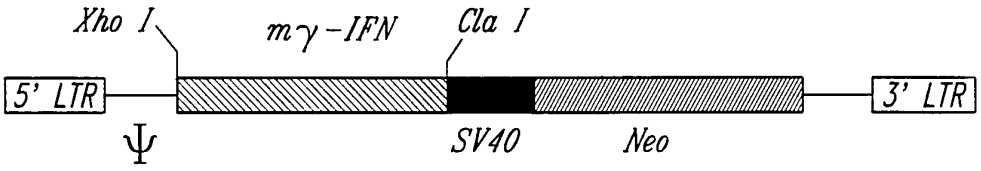


FIG. 1C

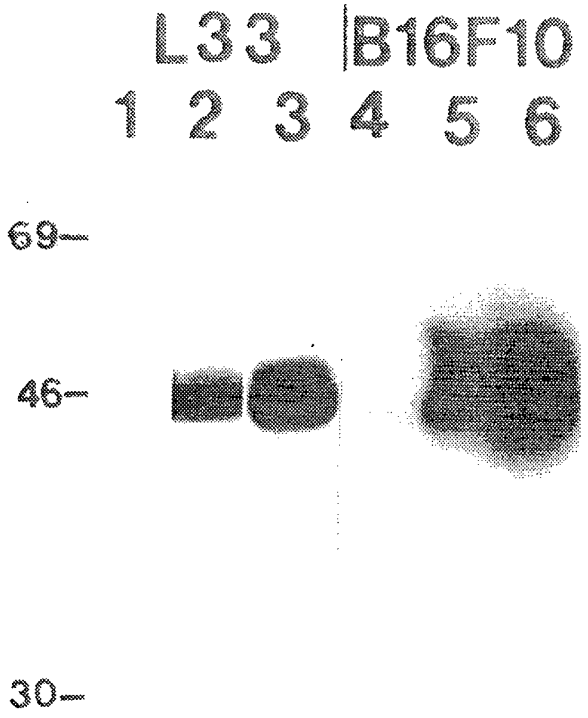


FIG. 2

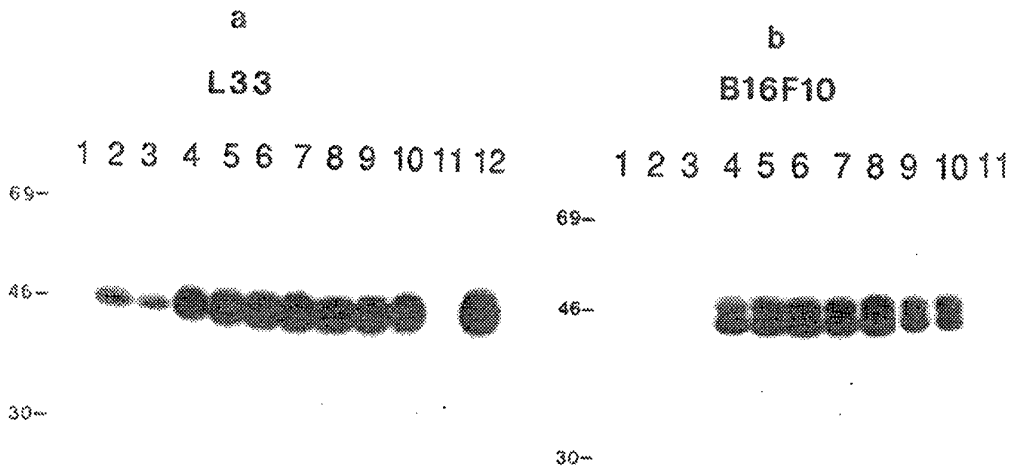

a
L33
1 2 3 4 5 6 7 8 9 10 11 12
69-
46-
30-
b
B16F10
1 2 3 4 5 6 7 8 9 10 11
69-
46-
30-

FIG. 3A FIG. 3B

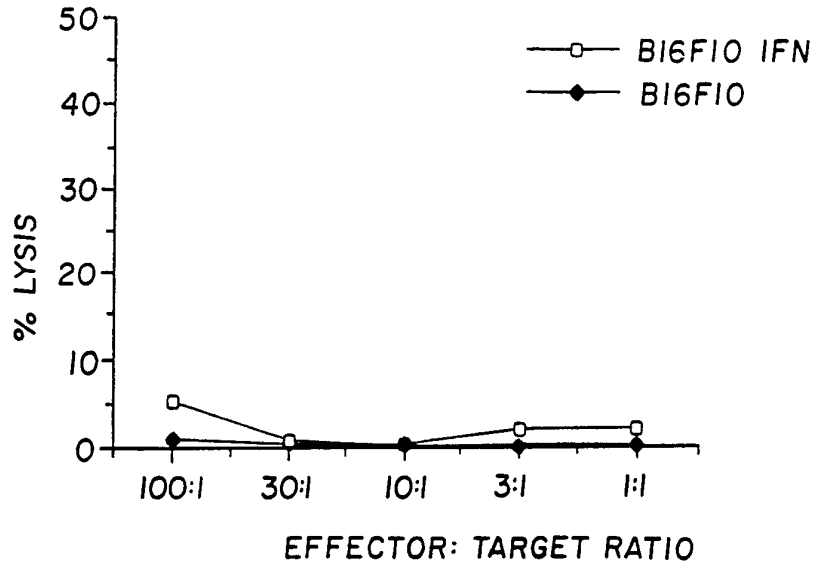

50
40
30
20
10
0
% LYSIS
B16F10 IFN
B16F10
100:1
30:1
10:1
3:1
1:1
EFFECTOR: TARGET RATIO

FIG. 4A

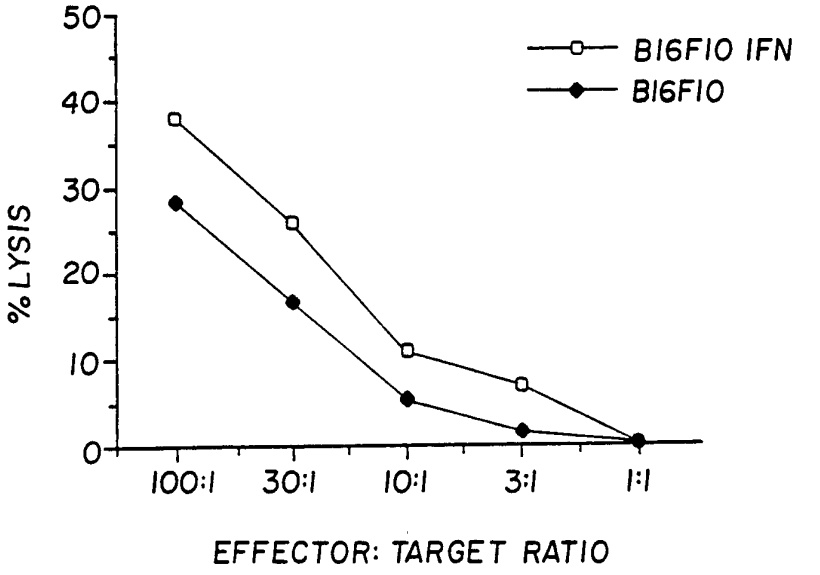

50
40
30
20
10
0
% LYSIS
B16F10 IFN
B16F10
100:1
30:1
10:1
3:1
1:1
EFFECTOR: TARGET RATIO

FIG. 4B

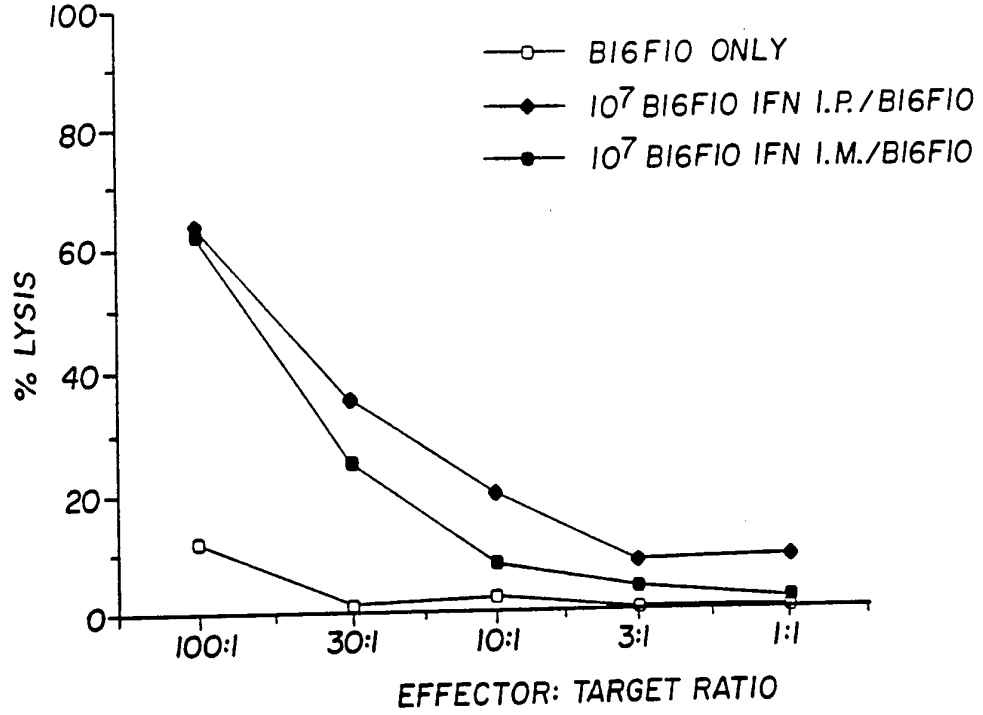

100
80
60
40
20
0
% LYSIS
B16F10 ONLY
10^7 B16F10 IFN I.P./B16F10
10^7 B16F10 IFN I.M./B16F10
100:1
30:1
10:1
3:1
1:1
EFFECTOR: TARGET RATIO

FIG. 5

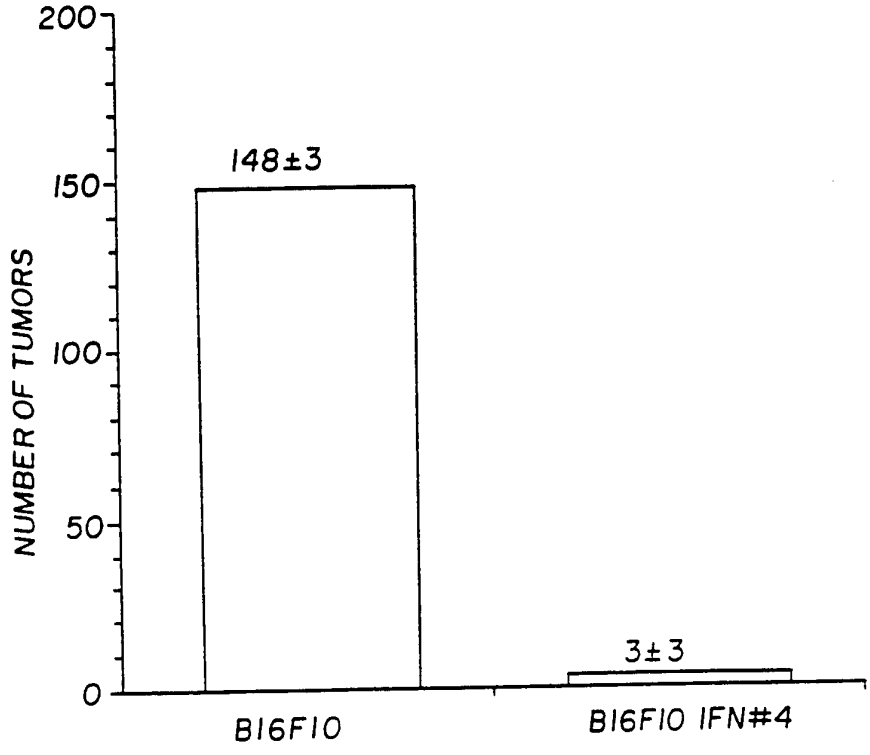

200
150
100
50
0
NUMBER OF TUMORS
148±3
3±3
B16F10
B16F10 IFN#4

FIG. 6

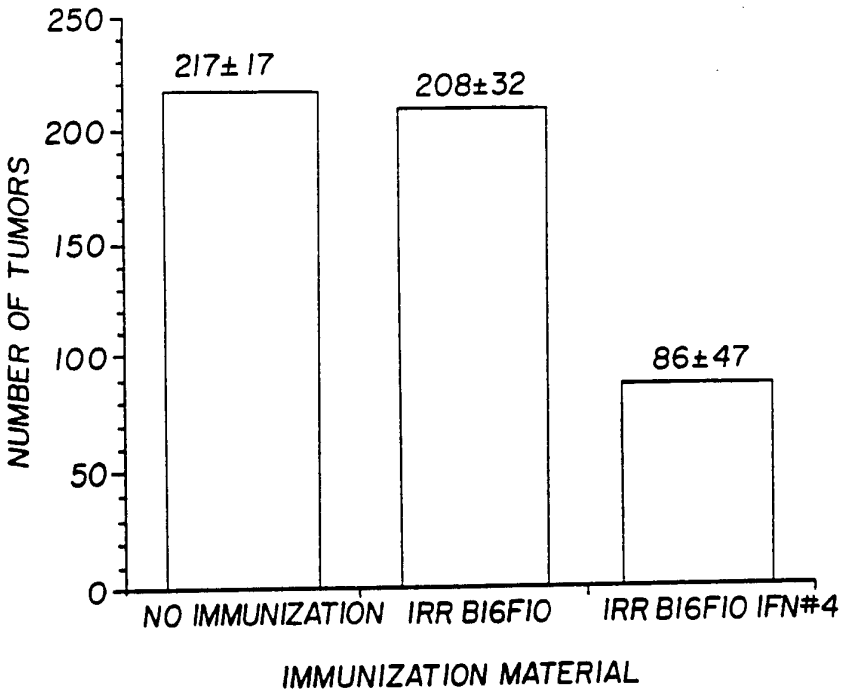

250
200
150
100
50
0
217±17
208±32
86±47
NUMBER OF TUMORS
NO IMMUNIZATION
IRR B16F10
IRR B16F10 IFN#4
IMMUNIZATION MATERIAL

FIG. 7

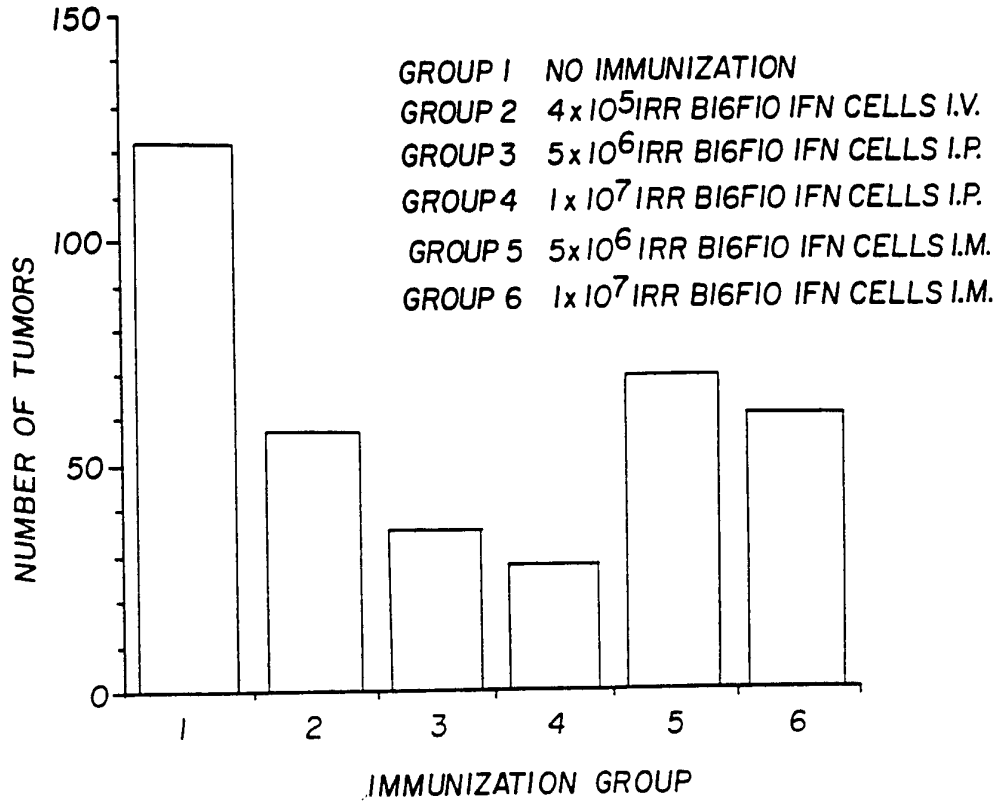

150
100
50
0
NUMBER OF TUMORS
GROUP 1 NO IMMUNIZATION
GROUP 2 4x10^5 IRR B16F10 IFN CELLS I.V.
GROUP 3 5x10^6 IRR B16F10 IFN CELLS I.P.
GROUP 4 1x10^7 IRR B16F10 IFN CELLS I.P.
GROUP 5 5x10^6 IRR B16F10 IFN CELLS I.M.
GROUP 6 1x10^7 IRR B16F10 IFN CELLS I.M.
1
2
3
4
5
6
IMMUNIZATION GROUP

FIG. 8

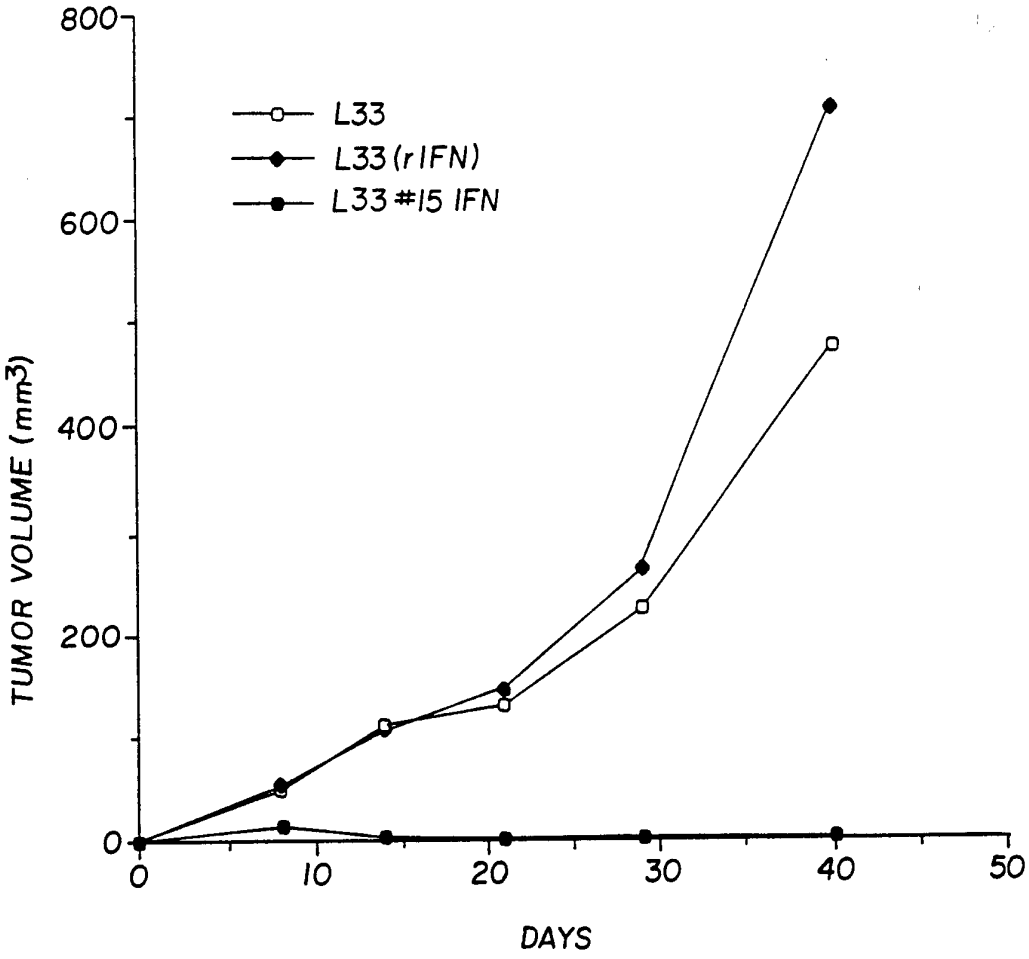

800
600
400
200
0
TUMOR VOLUME (mm3)
L33
L33 (rIFN)
L33 #15 IFN
0
10
20
30
40
50
DAYS

FIG. 9

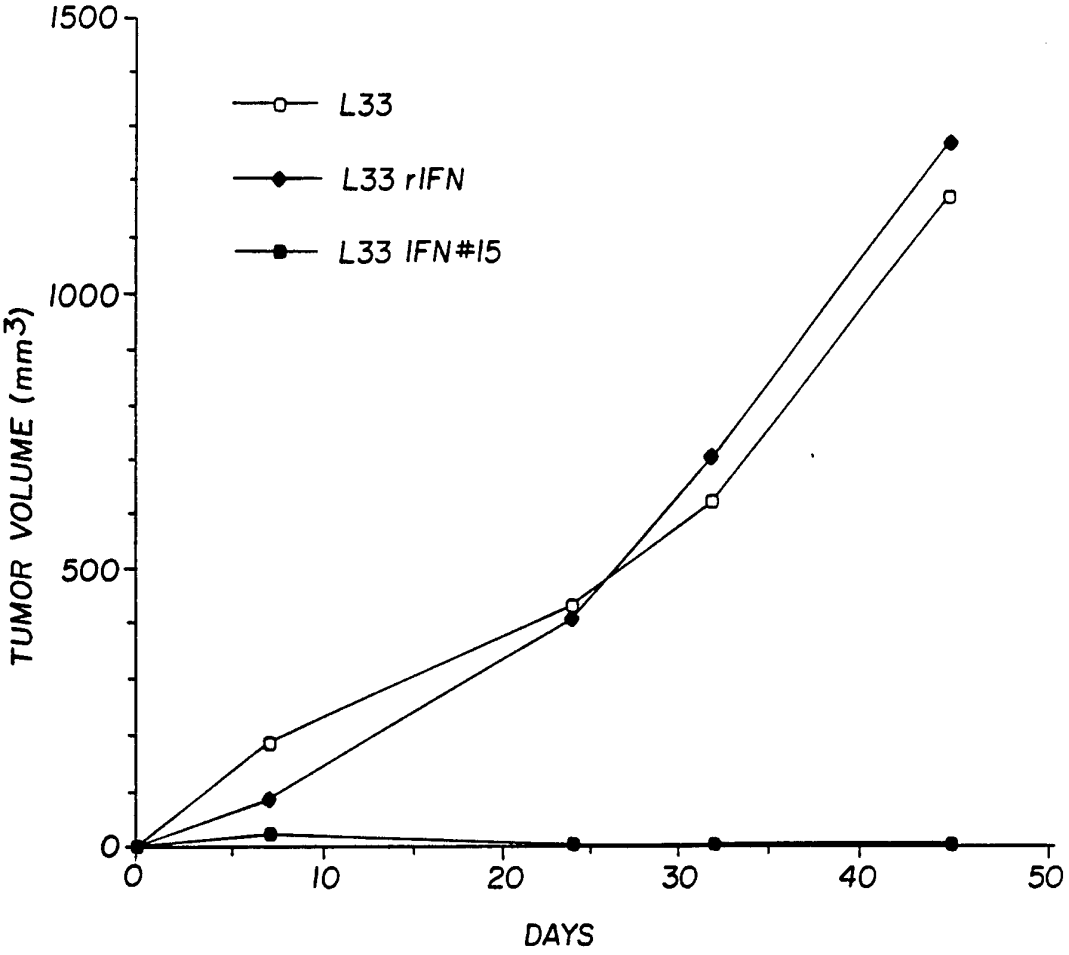

1500
1000
500
0
TUMOR VOLUME (mm3)
L33
L33 rIFN
L33 IFN#15
0
10
20
30
40
50
DAYS

FIG. 10

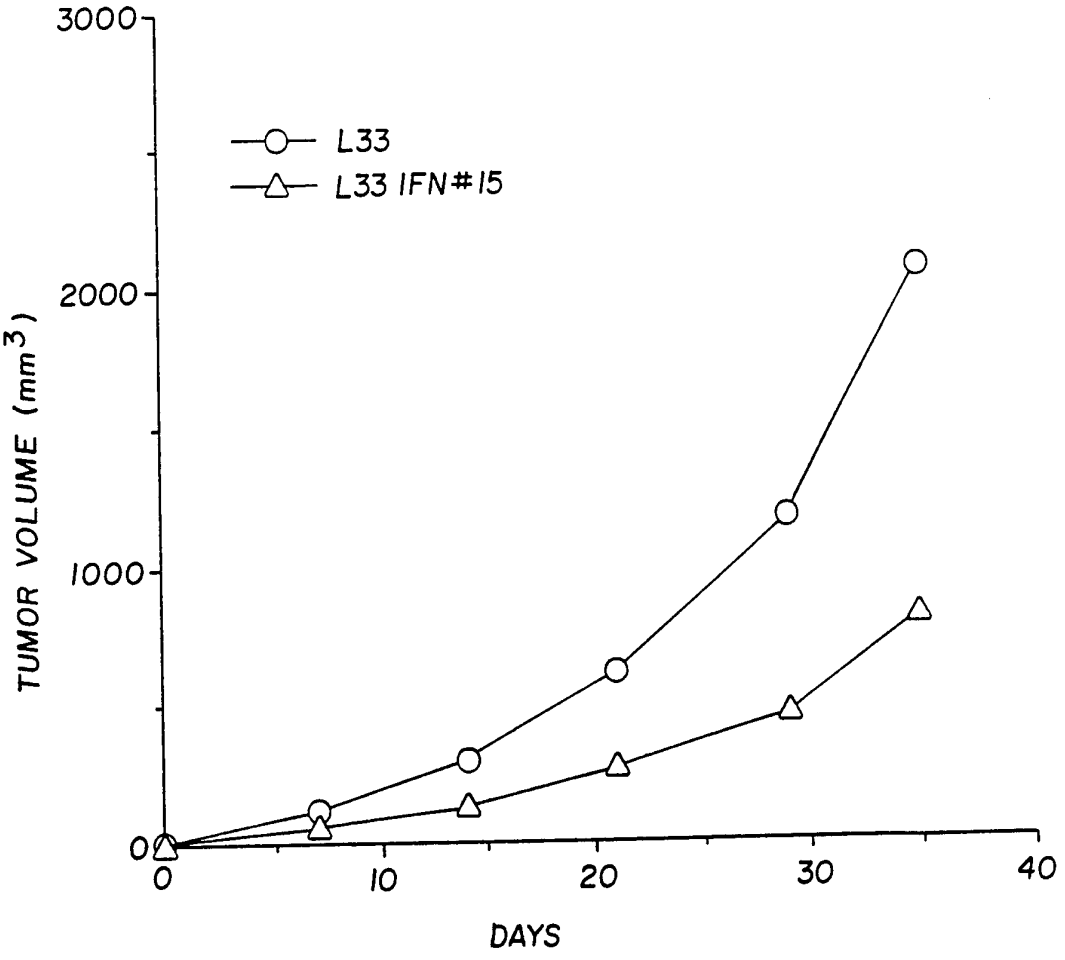

3000
2000
1000
0
TUMOR VOLUME (mm^3)
L33
L33 IFN#15
0
10
20
30
40
DAYS

FIG. 11

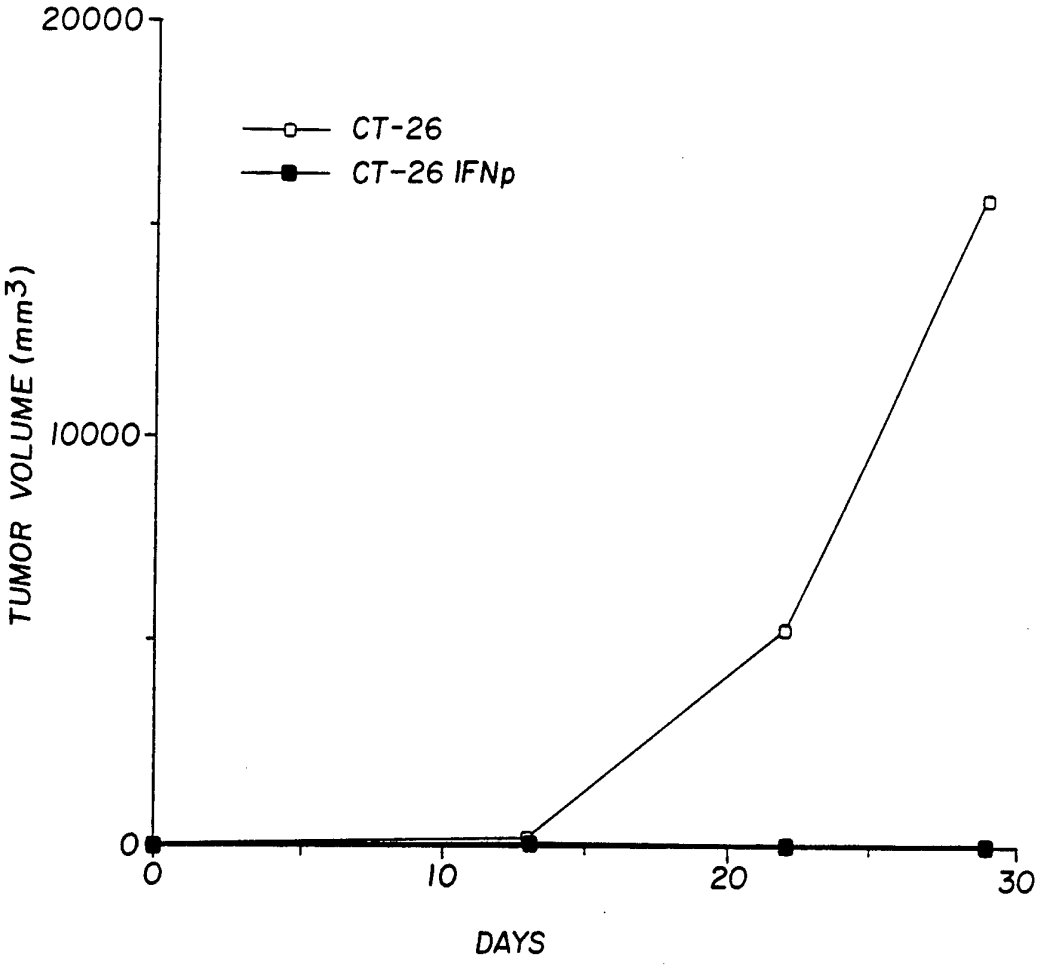


FIG. 12

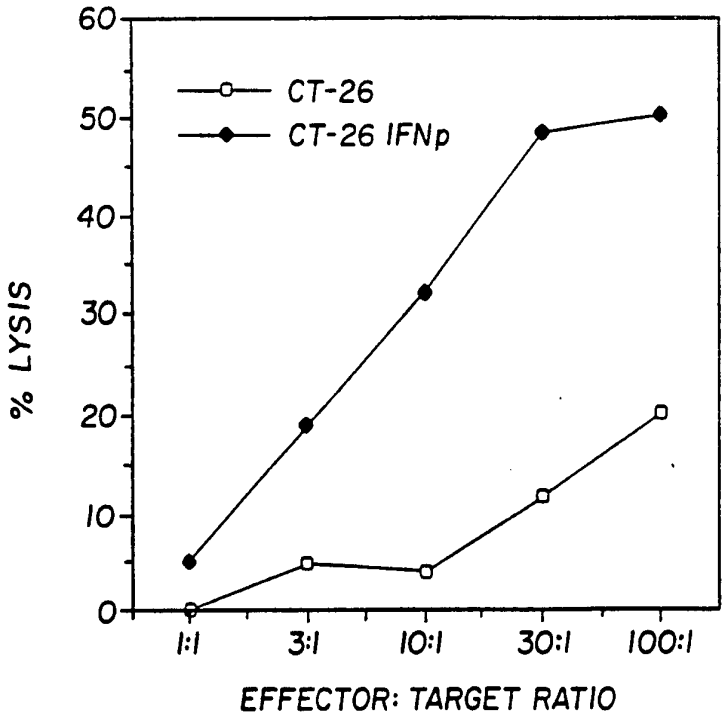

60
50
40
30
20
10
0
% LYSIS
CT-26
CT-26 IFNp
1:1
3:1
10:1
30:1
100:1
EFFECTOR: TARGET RATIO

FIG. 13

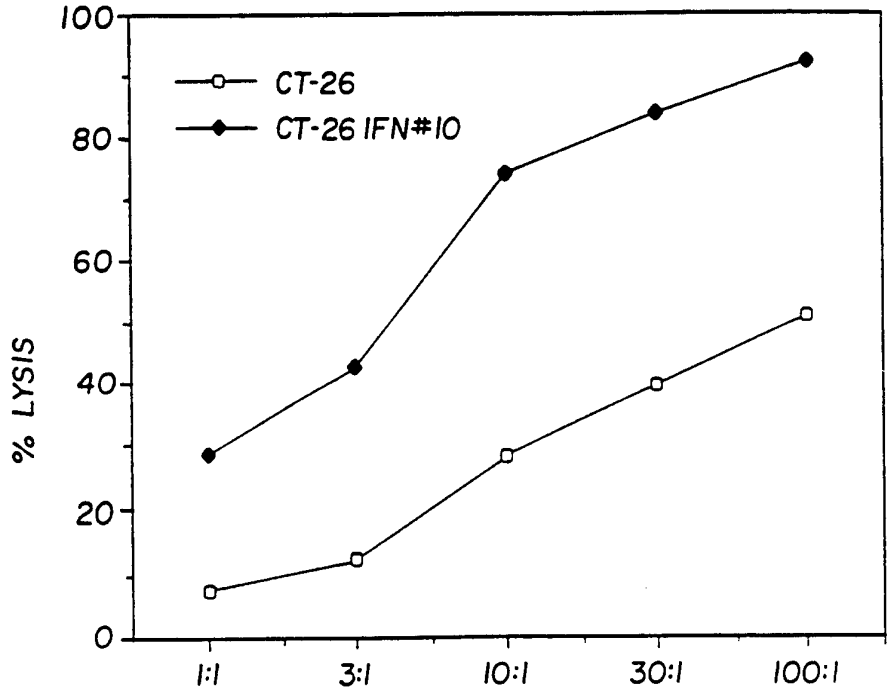

100
80
60
40
20
0
% LYSIS
CT-26
CT-26 IFN#10
1:1
3:1
10:1
30:1
100:1
EFFECTOR: TARGET RATIO

FIG. 14

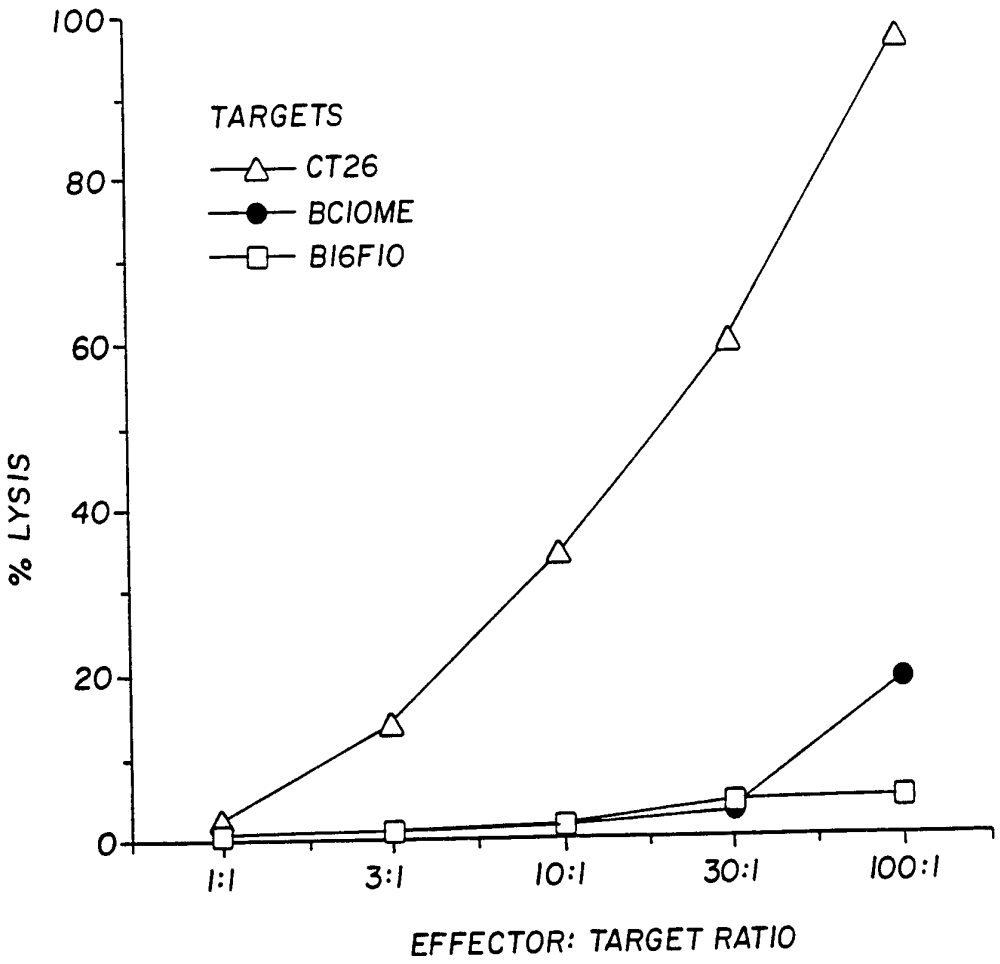

100
80
60
40
20
0
% LYSIS
TARGETS
CT26
BCIOME
BI6FIO
1:1
3:1
10:1
30:1
100:1
EFFECTOR: TARGET RATIO

FIG. 15

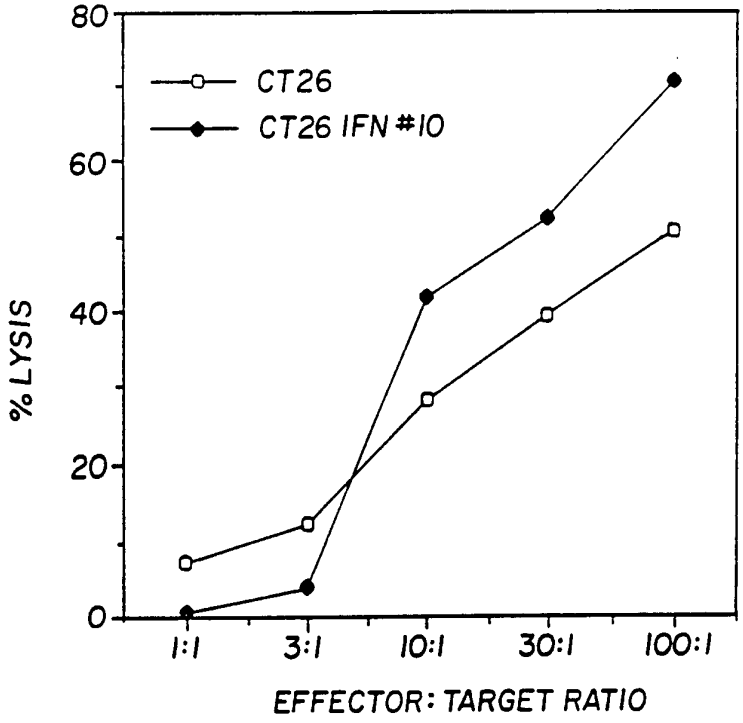

80
60
40
20
0
% LYSIS
CT26
CT26 IFN #10
1:1
3:1
10:1
30:1
100:1
EFFECTOR: TARGET RATIO

FIG. 16

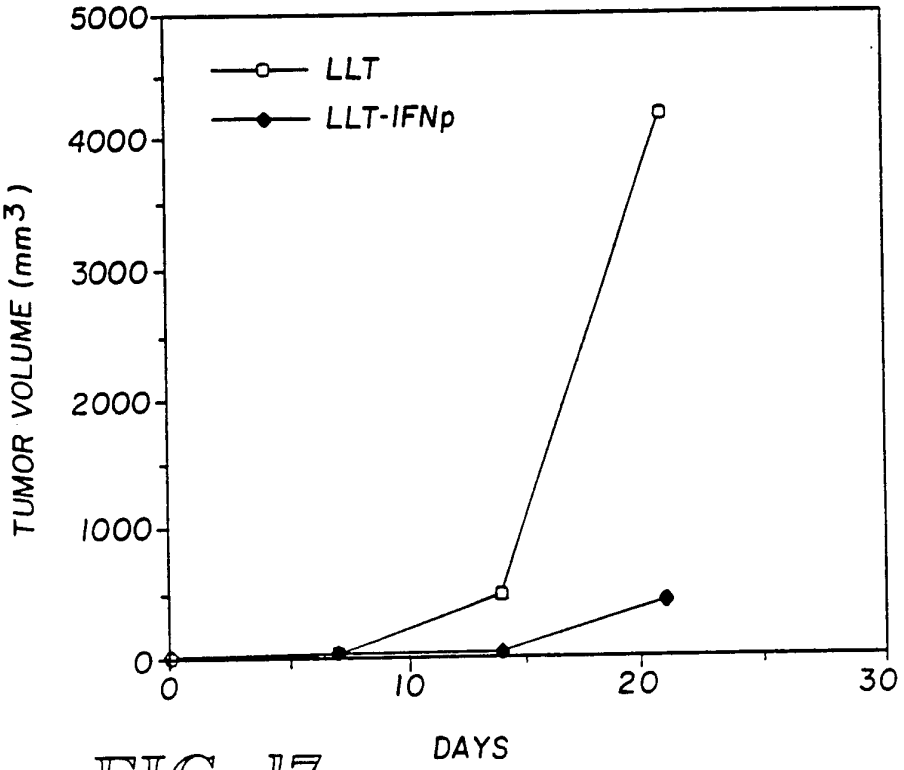

5000
4000
3000
2000
1000
0
TUMOR VOLUME (mm3)
LLT
LLT-IFNp
0
10
20
30
DAYS

FIG. 17

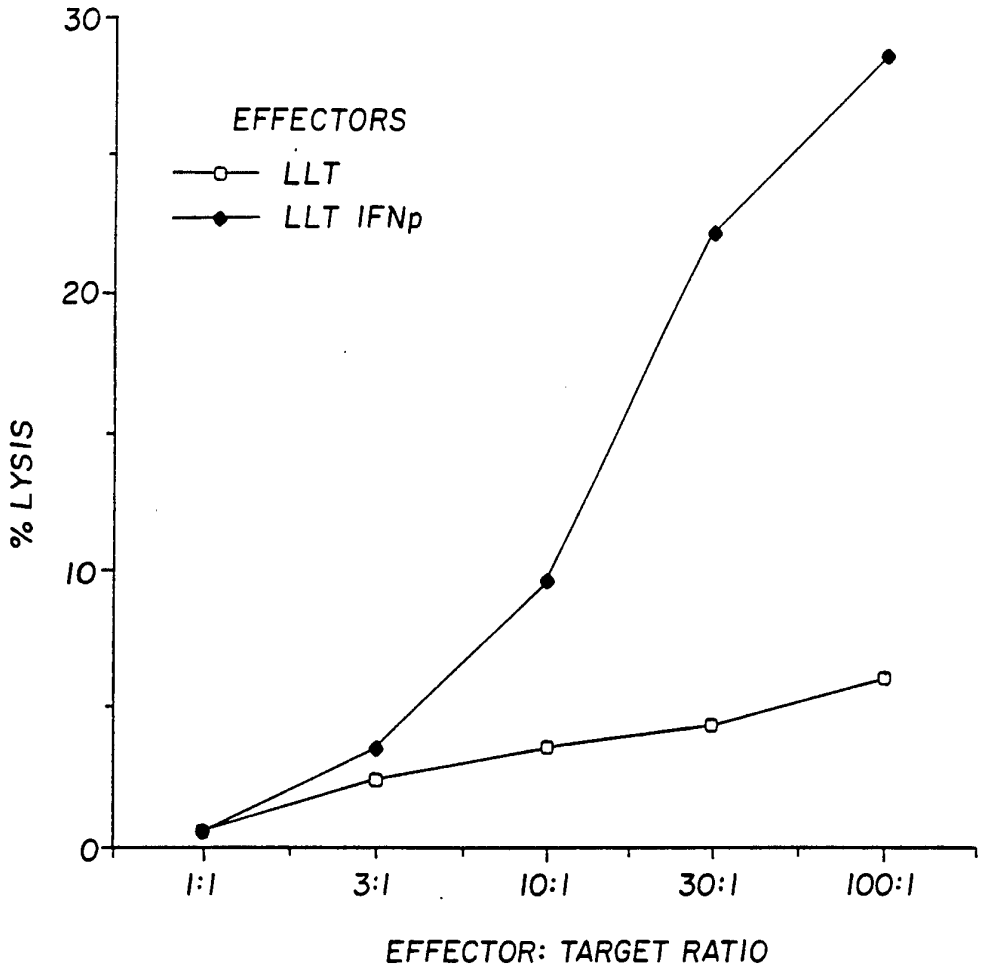

30
20
10
0
% LYSIS
EFFECTORS
LLT
LLT IFNp
1:1
3:1
10:1
30:1
100:1
EFFECTOR: TARGET RATIO

FIG. 18

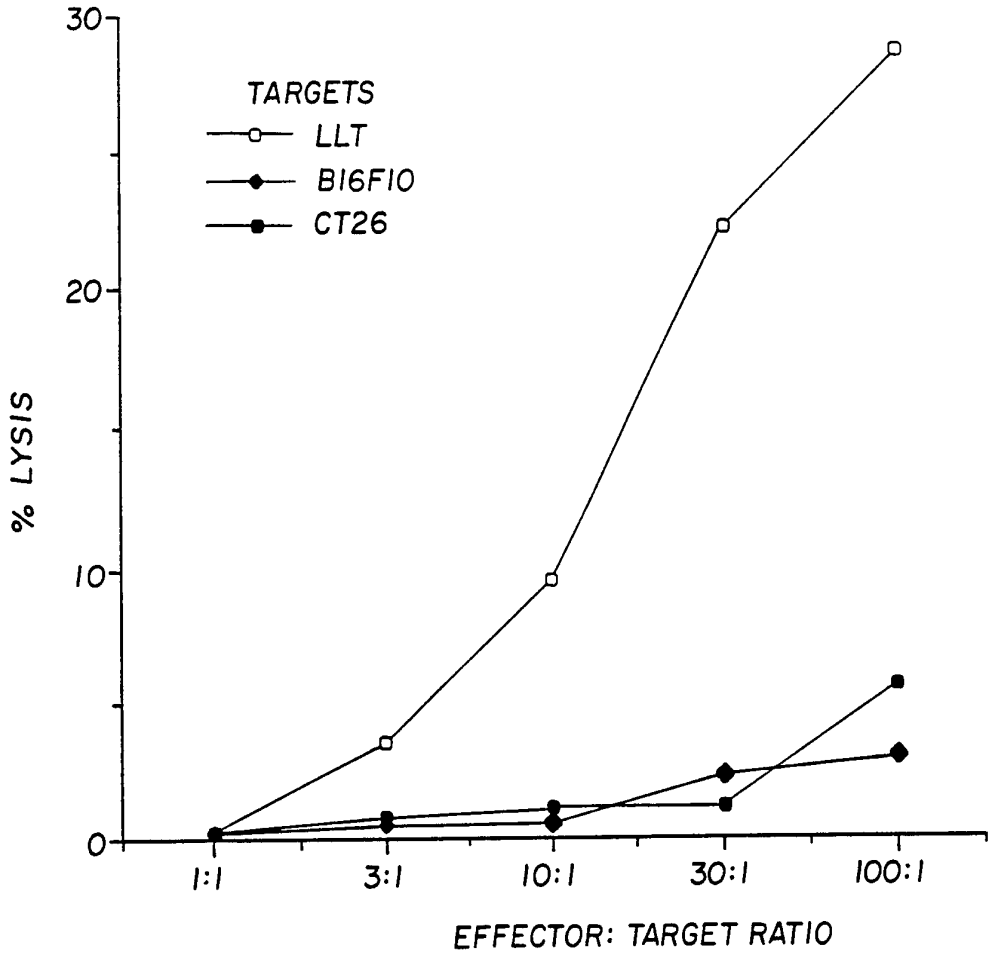

30
20
10
0
% LYSIS
TARGETS
LLT
B16F10
CT26
1:1
3:1
10:1
30:1
100:1
EFFECTOR: TARGET RATIO

FIG. 19

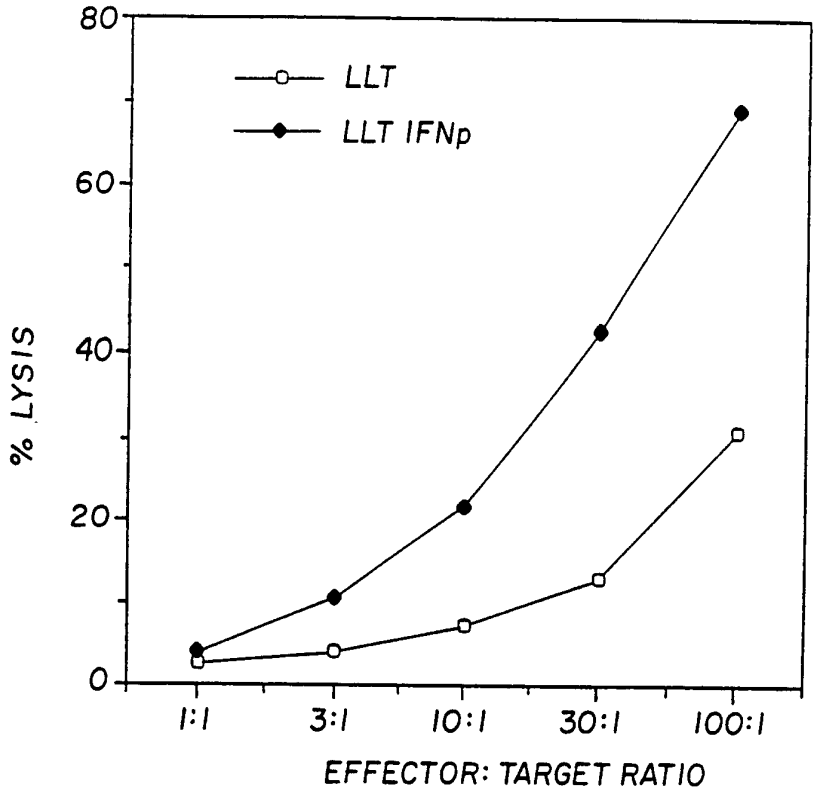
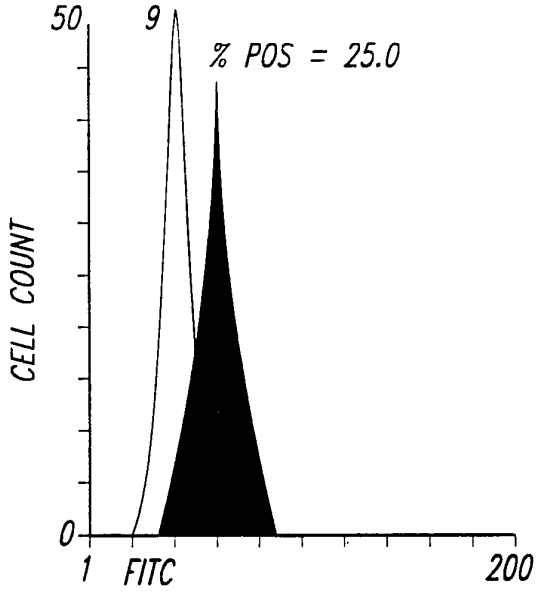
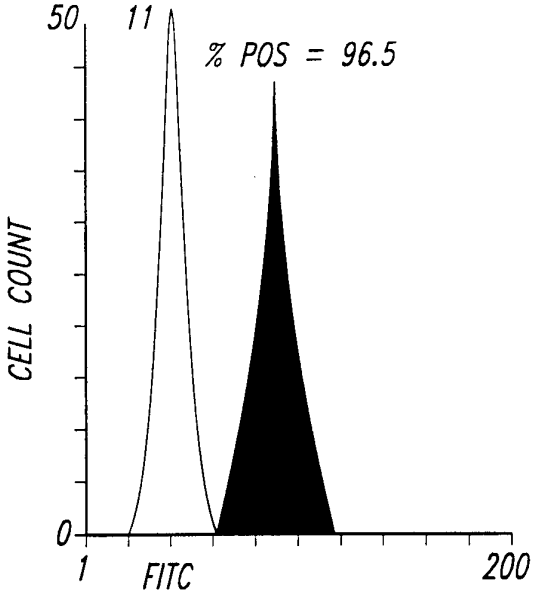

80
60
40
20
0
% LYSIS
LLT
LLT IFNp
1:1
3:1
10:1
30:1
100:1
EFFECTOR: TARGET RATIO

FIG. 20



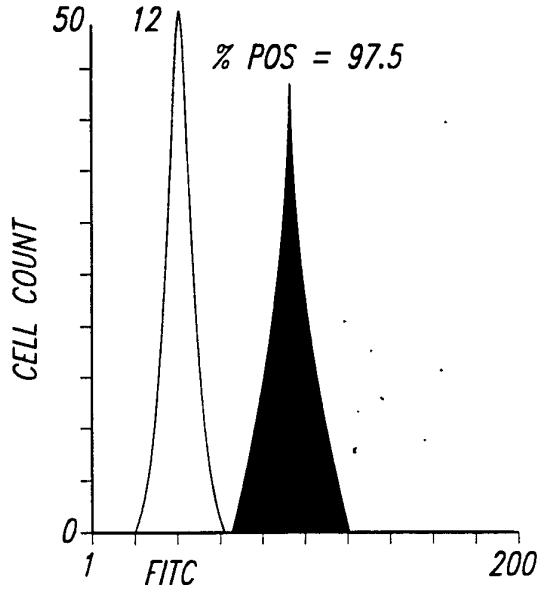
L33

FIG. 21A



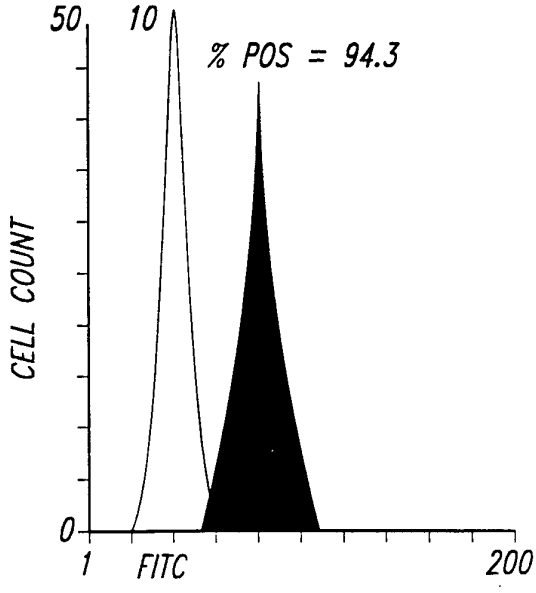
L33 mγ-IFN #13

FIG. 21B



L33 mγ-IFN #15

FIG. 21C



POOLED L33
EXPRESSING CELLS

FIG. 21D

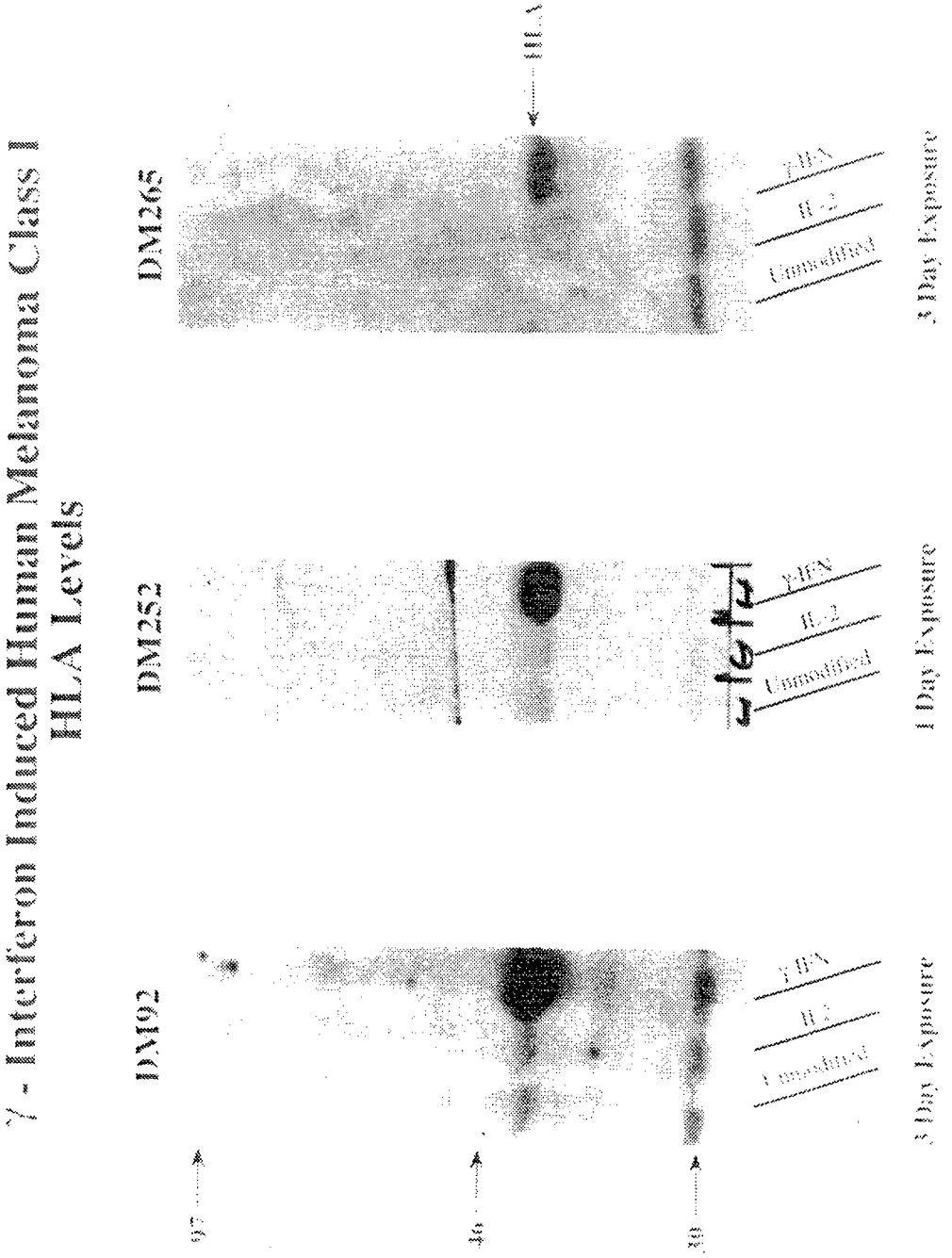

γ - Interferon Induced Human Melanoma Class I
HLA Levels
DM92
DM252
DM265
γIFN
IL-2
Unmodified
3 Day Exposure
1 Day Exposure
3 Day Exposure
97
46
30

FIG. 22

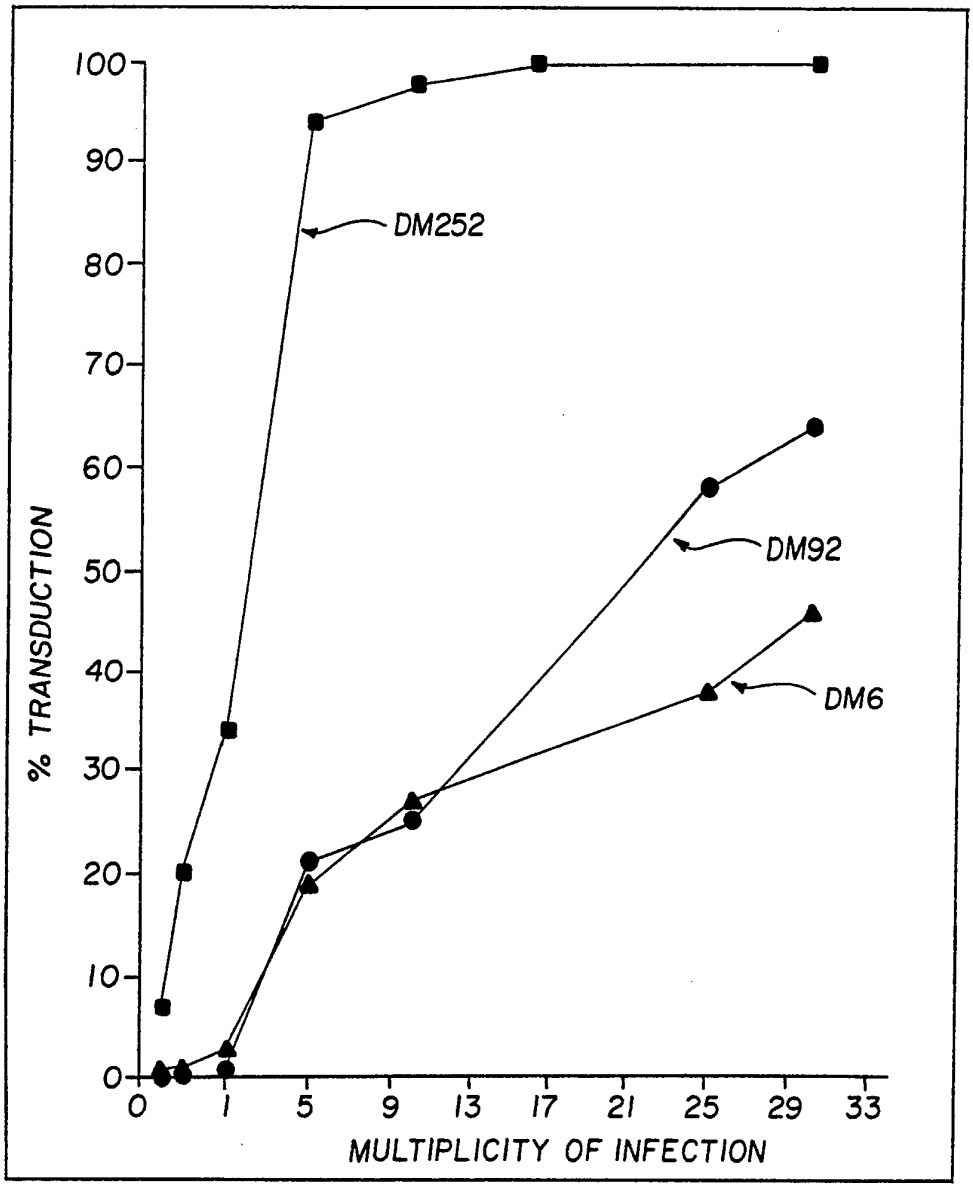

100
90
80
70
60
50
40
30
20
10
0
% TRANSDUCTION
DM252
DM92
DM6
0
1
5
9
13
17
21
25
29
33
MULTIPLICITY OF INFECTION

FIG. 23

Multiplicity of Infection of Melanoma Cells with Concentrated, Unpurified Vector

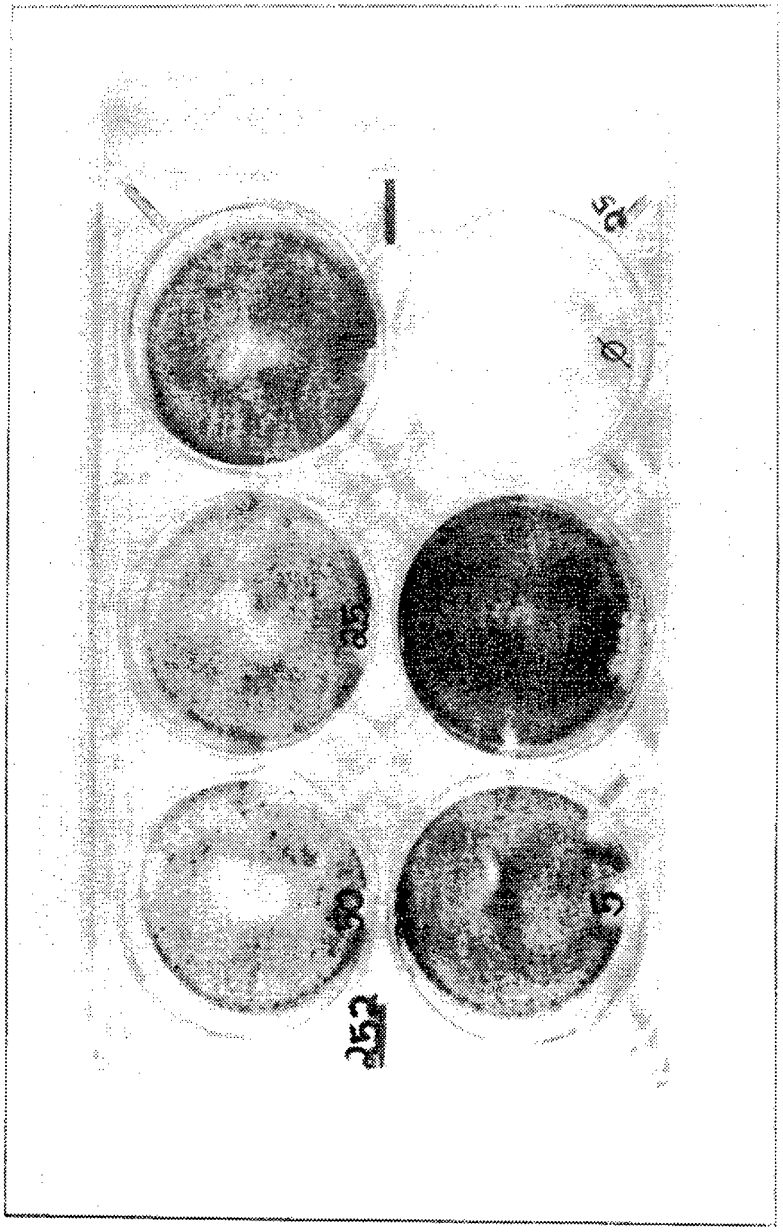
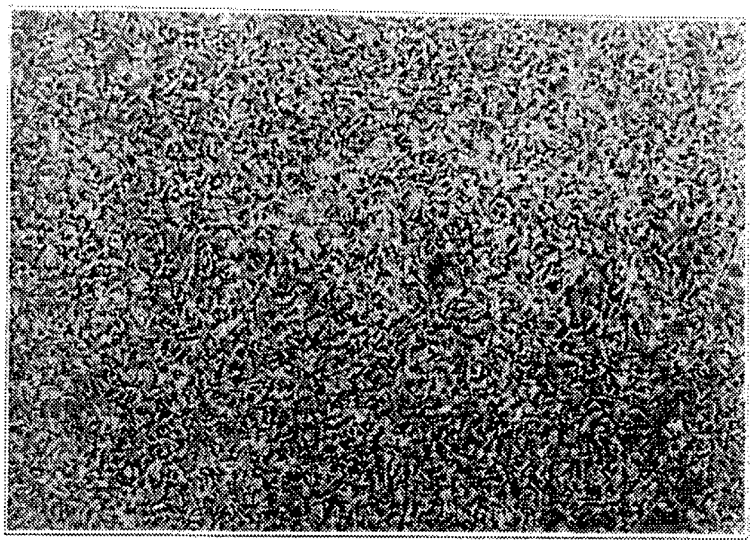
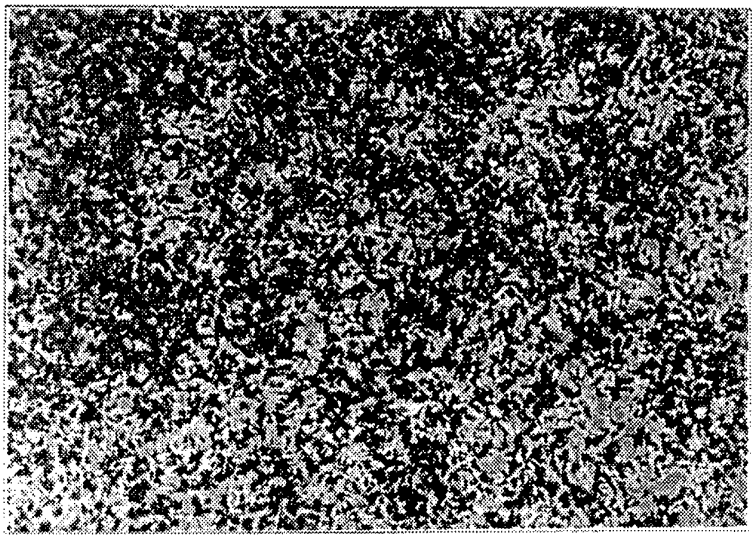


FIG. 24

Human Melanoma Transducibility - DM252



Non-transduced



Transduced at MOI of 1

FIG. 25

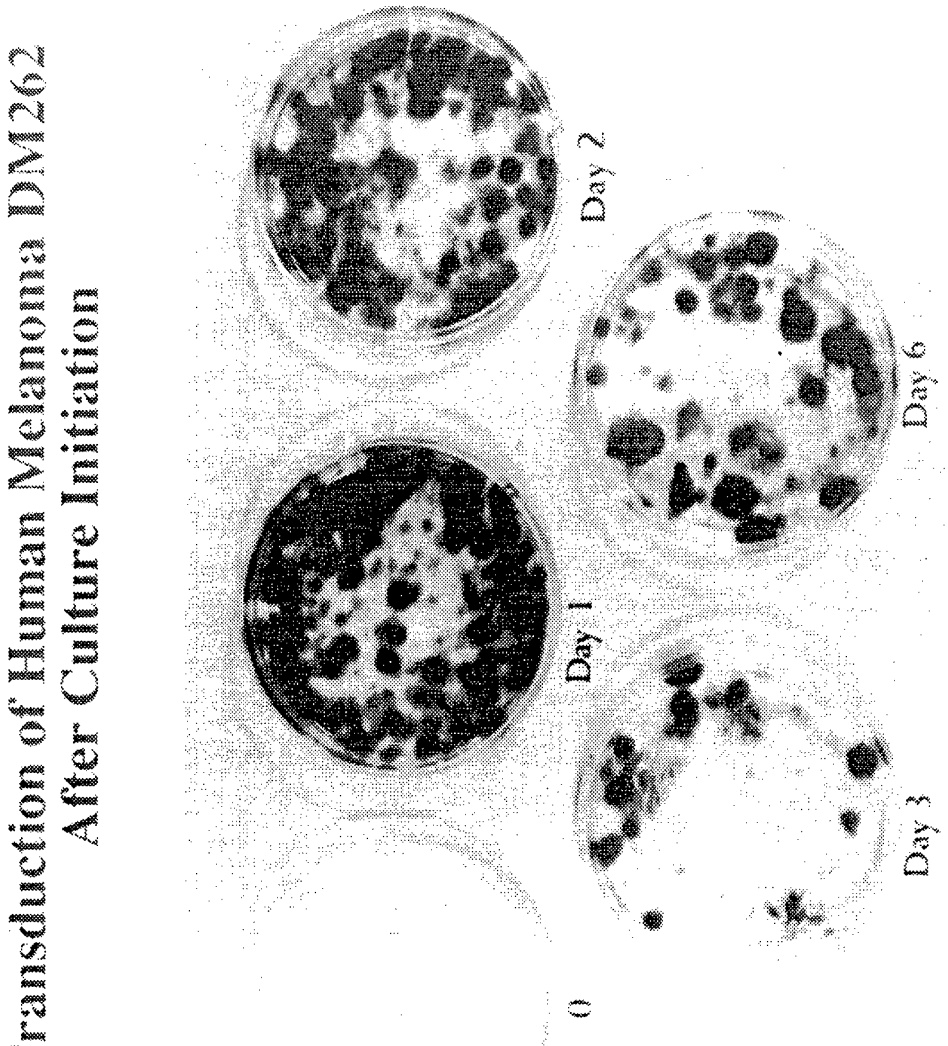

Transduction of Human Melanoma DM262
After Culture Initiation
Day 0
Day 1
Day 2
Day 3
Day 6

FIG. 26