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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/63, A61K 38/17, 31/70, 48/00, C12N 5/10, A01K 67/027, C12N 15/00

A1

(11) International Publication Number: WO 95/12681

(43) International Publication Date: 11 May 1995 (11.05.95)

(21) International Application Number: PCT/US94/12701

(22) International Filing Date: 4 November 1994 (04.11.94)

(30) Priority Data:
08/147,285 5 November 1993 (05.11.93) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/147,285 (CIP)
Filed on 5 November 1993 (05.11.93)

(71) Applicants *(for all designated States except US)*: NEW YORK UNIVERSITY [US/US]; 550 First Avenue, New York, NY 10016 (US). UNIVERSITA DEGLI STUDI "G., D'ANNUNZIO"-CHIETI [IT/IT]; Via del Vestini, I-66100 Cheti (IT).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: IACOBELLI, Stefano [IT/IT]; Via Chiana, 48, I-00198 Roma (IT). JALLAL, Bahija [MA/MA]; 515 Glaveston Drive, Redwood City, CA 94063-4720 (US). NATOLI, Clara [IT/IT]; Via Conca d'Oro, 285, I-00141 Roma (IT). POWELL, Jeff [US/US]; 1063 Morse Avenue 6-204, Sunnyvale, CA 94089 (US). ULLRICH, Axel [DE/DE]; Adalbertstrasse 108, D-8000 Munich 40 (DE).

(74) Agents: WARBURG, Richard, J. et al.; Lyon & Lyon, First Interstate World Center, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90017-2066 (US).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).

**Published**
*With international search report.*
*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: METHODS RELATING TO IR-95

(57) Abstract

The invention provides a method for treating a disorder by administering a therapeutically effective amount of IR-95 to a patient in need of such treatment. The disorders treated include cancers, bacterial infections, and viral infections. The invention also provides a method for suppressing an immune response by admininstering a therapeutically effective amount of an IR-95 antagonist to an organism in need of such treatment, for example a patient suffering from an autoimmune disorder.

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# DESCRIPTION

## Methods Relating to IR-95

### Related Application

This application is a continuation-in-part of U.S. Application No. 08/147,285, filed November 5, 1993 (pending), which is a continuation-in-part of U.S. Application No. 08/019,190, filed February 17, 1993, (abandoned), which is a continuation-in-part of Italian Patent Application No. RM92A000099, filed February 17, 1992.

### Field of the Invention

The present invention relates generally to the fields of medicine and molecular and cellular biology and more specifically to the field of therapeutic antigens and antigen related products. In particular, the present invention relates to the field of treating disorders, such as cancer, bacterial infection, parasitic infection, viral infection, autoimmune disorders, rheumatoid arthritis, allergy and organ transplant rejection.

### Background of the Invention

The following is a description of relevant art, none of which is admitted to be prior art to the present invention.

Many tumor cells are poorly immunogenic, and therefore do not elicit an immune response sufficient for elimination of the tumor.

The insufficient immune response of tumor cells may be due to low expression of foreign ("tumor-specific") antigens. Thus, identification of tumor-specific antigens has been a major focus of tumor immunologists for decades. Many putative tumor-specific antigens are currently being examined as potential vaccines, with limited success (Urban and Schreiber, *Ann. Rev. Immunol.* 10:617-44, 1992; Livingston, *Current Opinion in Immunology* 4:624-9, 1992).

Alternatively, the failure of the immune system to recognize and react to tumor antigens may be due to improper antigen presentation or insufficient costimulation (Baskar et al., *Proc. Natl. Acad. Sci. USA* 90:5687-5690, 1993; Fenton et al., AACR Symposium on Molecular Approaches to Cancer Immunotherapy, Page B-1, 1993).

Tumor cells also produce and secrete proteins which directly affect the host immune response to the tumor, either positively or negatively. A tumor-associated protein with possible immunoregulatory properties was identified by Iacobelli et al. (Iacobelli et al., *Cancer Res.* 46:3005-10, 1986), who generated monoclonal antibodies to supernatants of CG-5 human breast cancer cells. One of the MAbs produced in this manner recognized a 90kDa protein produced by 80% of breast cancers but not by normal breast tissue. Subsequent studies detected this protein, designated IR-95, in the sera of normal as well as tumor-bearing patients (Iacobelli et al., *Breast Cancer Research and Treatment* 11:19-30, 1993).

IR-95 is a secreted glycoprotein that was originally identified in the culture supernatant of human breast cancer cells[1] and by cDNA cloning was identified as a member of the scavenger receptor cysteine-rich domain family of proteins[2,3]. Although IR-95 is found in the serum of healthy individuals, its concentration in the serum of patients with various types of cancer, autoimmune disorder, or HIV infections is frequently increased[1,4-6]. In mammary carcinomas, 60% of the tumors analysed displayed an inverse correlation between the expression of the IR-95 gene and the mRNA levels of the HER2/neu oncogene, a well-established indicator of aggressive disease progression and poor prognosis for patients with breast and ovarian cancer[7,8].

Background information relating to IR-95 is reviewed in PCT Application Number WO 93/16181, which was published on August 19, 1993, incorporated herein by reference in its entirety, including any drawings. Koths et al. (Koths

et al., *J. Biol. Chem.* 268:14245-14249, 1993), incorporated herein by reference in its entirety, including any drawings, independently cloned a gene encoding a protein with an identical sequence, which they designated Mac-2-binding protein. Mac-2-BP was shown to inhibit the proliferative response of peripheral blood mononuclear cells stimulated with the T-cell mitogen PHA, and this inhibition was attributed to competitive binding and neutralization of the mitogen, rather than to a direct effect of Mac-2-BP on the responding cell population.

IR-95 has been shown to enhance the *in vitro* generation of cytotoxic effector cells (NK and LAK) from PBMC of apparently healthy donors, and to enhance IL-2 production by peripheral blood mononuclear cells (PBMC) stimulated with suboptimal doses of Concanavalin A (Ullrich et al., *J. Biol. Chem.* 269:18401-18407, 1994), incorporated herein by reference in its entirety, including any drawings.

T-cell activation, an important step in many immune responses, requires crosslinking of the T-cell receptor:CD3 complex by HLA-peptide complexes on the surface of antigen presenting cells. However, presentation of HLA-peptide complexes in the absence of certain costimulatory signals results in T-cell anergy rather than activation (Johnson and Jenkins, *Immunologic Res.* 12:48-64, 1993).

One such costimulatory molecule is B7, the ligand for the T-cell molecule CD28 (Harding et al., *Nature* 356:607-9, 1992; Norton et al., *J. Immunol.* 149:1556-61, 1992; Linsley and Ledbetter, *Ann. Rev. Immuno* 11:191-212, 1993). Antigen presentation by B7-negative APC results in T-cell anergy, while the introduction of B7 into these deficient APC restores their ability to activate T-cells following antigen presentation (Gimmi et al., *Proc. Natl. Acad. Sci.* 90:6586-90, 1993).

Soluble mediators are also important costimulatory signals in T-cell activation. For example, IL-1 supports the antigen-driven activation of T-cells (Rotteveel

et al., *Cellular Immunol.* 138:245-50, 1991), and the production of lymphokines such as IL-9 (Schmitt et al., *J. Immunol.* 147:3848-54, 1991). IL-6 exhibits a number of effects on T-cell activation, including stimulation of thymocyte proliferation, enhancement of cytotoxic T-cell differentiation in mixed lymphocyte reactions, and T-cell activation in conjunction with TCR crosslinking or mitogen stimulation (See Van Snick, *Ann. Rev. Immunol.* 8:253-78, 1990).

The inability of some tumor cells to provide adequate costimulation for T-cell activation may play a role in the escape of the tumor from immune surveillance. For example, certain B7-negative tumor cells are non-immunogenic and lethal when implanted into mice. When engineered to express B7, the tumor cells become immunogenic, eliciting an immune response that recognizes not only the B7-positive immunogenic tumor, but also the B7-negative nonimmunogenic parental tumor (Baskar et al., *Proc. Natl. Acad. Sci. USA* 90:5687-5690, 1993; Fenton et al., AACR Symposium on Molecular Approaches to Cancer Immunotherapy, Page B-1, 1993).

Likewise, the engineered expression of certain cytokines by tumor cells results in a more vigorous immune response to the tumor. Genes encoding IL-2 (Bubenik, *Intl J. Oncology* 2:1049-1052, 1993; Tsai et al., *JNCI* 85:546-53, 1993; Porgador et al., *J. Immunotherapy* 14:191-201, 1993), IL-4 (Yu et al., *Cancer Res.* 53:3125-8, 1993; Hunt et al., *J. Immunotherapy* 14:314-21, 1993), GM-CSF (Dranoff et al., *Proc. Natl. Acad. Sci. USA* 90:3539-43, 1993), and IFN (Porgador et al., *J. Immunotherapy* 14:191-201, 1993; Ogasawara et al., *Cancer Res.* 53:3561-8, 1993) have been expressed in various tumor cell lines. The engineered expression of cytokines by tumor cells increases their immunogenicity and/or impairs their ability to grow *in vivo*.

Certain cytokines, when administered systemically or locally, can lead to successful immune recognition of the

tumor, and mobilization of an effective immune response (Sivanandham et al., *Annals of Plastic Surgery* 28:114-118, 1992; Dipierro et al., *Medical Oncology and Pharmacotherapy* 10:53-59, 1993). Recent advances in gene transfer technology have allowed the insertion of genes encoding the cytokines of interest into non-immunogenic tumor cells. Such cytokine-engineered tumor cells elicit a strong tumor-specific immune response that is capable of recognizing and eradicating the parental, non-immunogenic tumor cells (Dranoff et al., *Proc. Natl. Acad. Sci. USA* 90:3539-43, 1993; Patel et al., *J. Immunotherapy* 14:310-3, 1993; Bubenik, *Intl J. Oncology* 2:1049-1052, 1993).

Summary of the Invention

The present invention relates to therapeutic uses of IR-95, for example its use in reducing the volume of a tumor or in decreasing the growth rate of cells in the tumor, preferably *in vivo*. IR-95 increases the production of cytokines IL-1 and IL-6 by human peripheral blood lymphocytes, and acts synergistically with Concanavalin A (ConA, a potent T-cell mitogen) to increase the production of IL-2. It has also been discovered that the development of tumors in nude mice is decreased (slower tumor growth and decreased tumor volume) when the mice are implanted with cells that express IR-95.

In a particularly preferred embodiment, the invention relates to IR-95 gene transfer and gene therapy products and methods. The in vivo data presented herein, surprisingly, demonstrates that IR-95 gene therapy methods are effective at inhibiting tumor growth and reducing tumor volume. In view of the present disclosure, those skilled in art will recognize that various modifications and alterations of the exemplary gene therapy techniques described herein will also be effective and useful.

Thus, in a first aspect the invention features a pharmaceutical composition having a therapeutically

effective amount of IR-95 and a physiologically acceptable carrier or diluent.

By "therapeutically effective amount" is meant agents of this invention have a "therapeutic effect" which generally refers to either: (a) the inhibition to some extent of growth of cells causing or contributing to a particular disorder; or (b) relief of symptoms or the inhibition to some extent of the causes or contributors of such a disorder. The doses of IR-95 and antagonist(s) thereof which are useful as a treatment are "therapeutically effective amounts", which as used herein, means an amount of the protein, fragment or antagonist thereof, which produces the desired therapeutic effect. This amount can be routinely determined by one of skill in the art and will vary depending upon several factors such as the particular illness from which the patient suffers and the severity thereof, as well as the patient's height, weight, sex, age, and medical history.

A "therapeutic effect" relieves to some extent one or more of the symptoms of the disorder. In reference to the treatment of a cancer, a therapeutic effect refers to one or more or the following: 1) reduction in tumor size; 2) inhibition (i.e., slowing to some extent, preferably stopping) of tumor metastasis; 3) inhibition, to some extent, of tumor growth; and/or 4) relieving to some extent one more or the symptoms associated with the disorder. In reference to other disorders, a therapeutic effect refers to the ability to remove or reduce one or more symptoms associated with the disorder and includes the ability to inhibit the growth and/or survival of a foreign organism. Other such symptoms are well known to members of the medical community.

By "IR-95" is meant the 90K-tumor associated antigen described in PCT patent application No. WO 93/16181, published August 19, 1993, and the functional derivatives of IR-95 described herein. The term IR-95 is meant to refer to the full-length protein and any active fragments

thereof. Fragments which are active may be identified *in vitro* or by testing for the ability to affect tumor growth in nude mice.

Physiologically acceptable carriers and diluents are known in the art and are described in detail herein. In preferred embodiments the carrier or diluent is not a material that is commonly used to buffer the pH of a solution, such as Tris buffer.

The therapeutically effective amount is preferably between about 5 to about 5,000 mg/dose/week/patient and more preferably between 50 and 500 mg/dose/week/patient.

In a related aspect the invention features the use of IR-95 in the manufacture of a medicant for the treatment of a disorder, preferably cancer, a bacterial infection or a viral infection.

By "disorder" is meant a state in an organism (e.g., a human) which is recognized as abnormal by members of the medical community. The disorder may be an infection by a foreign organism or uncontrolled cell proliferation (e.g., tumor growth). Examples of disorders to be treated by the present invention include cancers (e.g., breast or ovarian cancer), immune disorders (e.g., autoimmune disorders) and infections (e.g., bacterial or viral infections, including retroviral infections such as HIV). These and other disorders are often characterized by conditions such as unwanted cell differentiation or proliferation, an unwanted immune response or lack of immune response, abnormally low levels of cytokines (for example IR-95) and/or the formation of tumors.

In another aspect the invention features a method of treating a disorder. The method involves administering a therapeutically effective amount of IR-95 to an organism in need of such treatment. In preferred embodiments, the disorder is either cancer, a bacterial infection, a parasitic infection, or a viral infection, and the therapeutically effective amount is between about 5 to about 5,000

mg/dose/wk/patient, more preferably between 50 and 500 mg/dose/wk/patient.

By "organism" is meant any living creature. The term includes mammals, and specifically humans. Preferred organisms include mice, as the ability to treat or diagnose mice is often predictive of the ability to function in other organisms such as humans.

By "in need of such treatment" is meant an organism having a disorder that is effectively treated by administration of IR-95. Organisms in need of treatment may be identified using routine diagnostic methodologies, for example by detecting the symptoms characteristic of the particular disorder to be treated (e.g., cancer, bacterial infection, or viral infection). Patients in need of treatment may also be identified by detecting the level of IR-95 in the organism as an indication of need for treatment.

In preferred embodiments administering a therapeutically effective amount of IR-95 to an organism involves implanting the organism with cells capable of expressing IR-95; the volume of a tumor in the organism is reduced or the growth rate of cells in the tumor is decreased; cytokine (e.g., IL-1 or IL-6) production is increased; and the IR-95 is present a concentration of 10 mg/ml to 20 mg/ml.

In another aspect the invention features a pharmaceutical composition having a therapeutically effective amount of an IR-95 antagonist and a physiologically acceptable carrier or diluent.

By "antagonist" is meant any agent that decreases the effect of IR-95 *in vivo* or *in vitro*. For example, a compound that decreases the effect of IR-95 on cytokine production or immune cell activation. IR-95 antagonists include antisense and ribozyme agents as described herein.

In yet another aspect the invention features the use of an IR-95 antagonist in the manufacture of a medicant

for the treatment of an autoimmune disorder, rhuematoid arthritis, allergy, or organ transplant rejection.

By "autoimmune disorder" is meant any disease, disease state or pathology mediated in whole or in part by cells of the immune system causing damage to or compromising function of tissues or cells present endogenously in an organism. This may include pathologies mediated by T lymphocytes causing damage to body tissues or pathologies mediated by antibodies secreted extracellularly from cells of the immune system. Examples of autoimmune disorders to be treated by the present invention include Addison's disease, autoimmune hemolytic anemia, Crohne's disease, Goodpasture's syndrome, Graves' disease, Hashimoto's thyroiditis, idiopathic thrombocytopenic purpura, insulin-dependent diabetes mellitus, multiple sclerosis, myasthenia gravis, pemphigus vulgaris, pernicious anemia, poststreptococcal glomrulonephritis, psoriasis, rheumatoid arthritis, scleroderma, Sjögren's syndrome, spontaneous infertility, and systemic lupus erythematosus.

In another aspect, the invention features a method of suppressing an immune response. The method involves administering a therapeutically effective amount of a IR-95 antagonist to an organism in need of such treatment. In preferred embodiments the patient is suffering from an autoimmune disorder, rheumatoid arthritis, allergy, or organ transplant rejection, and the therapeutically effective amount is between about 5 to about 5,000 mg/dose/wk/patient, more preferably between 50 and 500 mg/dose/wk/patient.

By "suppressing" is meant that the immune response is decreased or eliminated. Suppressing an immune response may preferably remove an abnormal level of immune response that is associated with a disorder and/or cause the level of immune response to approach a level associated with normal or healthy individuals.

By "immune response" is meant the response of the whole or part of an immune system of an organism, i.e., cellular and humoral systems *in vivo or in vitro*. In preferred embodiments, the immune response is either the proliferation or differentiation of a cell selected from the group that consists of T-lymphocytes, B-lymphocytes, natural killer cells, monocytes and granulocytes. An immune response may thus be measured using techniques well known in the art.

For the treatment of autoimmune disease, rheumatoid arthritis, allergy, rejection of organ transplants, and other pathological situations where the immune system is activated and needs to be suppressed, a IR-95 antagonist can be administered. The appropriate doses of the antagonist can be routinely determined by one of skill in the art as described herein. Generally the antagonist(s) of IR-95 is preferably provided at a dose of between about 5 to about 5000 mg/dose/week/patient. More specifically, one preferable dose range is from 50 to 500 mg/dose/week/patient.

In another aspect the invention features a phamaceutical composition comprising a therapeutically effective amount of nucleic acid encoding IR-95 and a physiologically acceptable carrier or diluent. Nucleic acid encoding IR-95 is described in PCT Application No WO 93/16181, published August 19, 1993.

The invention also provides the use of nucleic acid encoding IR-95 in the manufacture of a medicant for the treatment of cancer, a bacterial infection, a parasitic infection or a viral infection.

In yet another aspect the invention features a pharmaceutical composition comprising cells capable of expressing IR-95 and a physiologically acceptable carrier or diluent.

The invention also features the use of cells capable of expressing IR-95 in the manufacture of a medicant for

the treatment of cancer, a bacterial infection, a parasitic infection or a viral infection.

Also provided is a method of treating a disorder comprising implanting an organism in need of treatment with cells capable of expressing IR-95.

In preferred embodiments the volume of a tumor in said organism is reduced or the growth rate of cells in said tumor is decreased. In other preferred embodiments cytokine (for example IL-1 or IL-6) production in a blood cell is increased and IR-95 is present in a concentration of 10 mg/ml to 20 mg/ml.

The invention provides a vector in a liposome, said vector comprising nucleic acid encoding IR-95 and being adapted to cause expression of said IR-95.

By "vector" is meant a nucleic acid, *e.g.*, DNA derived from a plasmid, cosmid, phagemid or bacteriophage, into which fragments of nucleic acid may be inserted or cloned. The vector can contain one or more unique restriction sites for this purpose, and may be capable of autonomous replication in a defined host or organism such that the cloned sequence is reproduced. The vector molecule can confer some well-defined phenotype on the host organism which is either selectable or readily detected. Some components of a vector may be a DNA molecule further incorporating a DNA sequence encoding a therapeutic or desired product, and regulatory elements for transcription, translation, RNA stability and replication. A viral vector in this sense is one that contains a portion of a viral genome, *e.g.*, a packaging signal, and is not merely DNA or a located gene within a viral article.

In preferred embodiments expression of said IR-95 results in the production of functional IR-95 and the vector comprises a retroviral vector. In a preferred embodiment, a vector comprising nucleic acid encodes IR-95, wherein the IR-95 vector is adapted to cause expression of a IR-95. Expression includes the efficient transcription of an inserted gene or nucleic acid sequence

within the vector. Expression products may be proteins, polypeptides or RNA. By "expression of IR-95" is meant that a complete or functional partial IR-95 protein is produced from the vector containing the nucleic acid encoding IR-95.

Also provided is a transfected cell line containing a vector comprising nucleic acid encoding IR-95. In preferred embodiments the IR-95 is expressed as a secreted protein. Yet an additional preferred embodiment, comprises a cell stably transfected with a IR-95 vector. A cell may be co-transfected with a vector containing a selectable marker. This selectable marker is used to select those cells which have become transfected. Types of selectable markers which may be used are well known to those of ordinary skill in the art. Alternatively, a transfected cell containing a IR-95 vector may only be transiently transfected, resulting in transient expression of a IR-95.

The term "transfected" as used herein refers to a cell having undergone the process of introduction of nucleic acid or a nucleic acid vector into a cell. Various methods of transfecting a cell are possible including microinjection, $CaPO_4$ precipitation, lipofection (liposome fusion), electroporation and use of a gene gun.

The term "stable" as used herein refers to the introduction of a gene into the chromosome of the targeted cell where it integrates and becomes a permanent component of the genetic material in that cell. An episomal transfection is a variant of stable transfection in which the introduced gene is not incorporated in the host cell chromosomes but rather is replicated as an extrachromosomal element. This can lead to apparently stable transfection of the characteristics of a cell.

The term "transient" as used herein relates to the introduction of a gene into a cell to express a IR-95, where the introduced gene is not integrated into the host cell genome and is accordingly eliminated from the cell

over a period of time. Transient expression relates to the expression of a gene product during a period of transient transfection.

The invention also provides a transformed cell line containing a vector comprising nucleic acid encoding IR-95. In yet a further preferred embodiment, there is provided a cell stably transformed with a IR-95 vector. Cells may be co-transformed with a vector containing a selectable marker. This selectable marker is used to select those cells which have become transformed. Types of selectable markers which may be used are well known to those of ordinary skill in the art.

The term "transformed" as used herein refers to a process or mechanism of inducing transient or permanent changes in the characteristics (expressed phenotype) of a cell by the mechanism of gene transfer whereby DNA or RNA is introduced into a cell in a form where it expresses a specific gene product or alters the expression or effect of endogenous gene products.

The invention features a transgenic non-human animal containing IR-95. An additional preferred embodiment, provides for a transgenic animal containing a IR-95 vector. In preferred embodiments the transgenic animal is a mammal, for example a mouse.

By "transgenic animal" is meant an animal whose genome contains an additional copy or copies of the gene from the same species or it contains the gene or genes of another species, such as a gene encoding a IR-95 introduced by genetic manipulation or cloning techniques, as described herein and as known in the art. The transgenic animal can include the resulting animal in which the vector has been inserted into the embryo from which the animal developed or any progeny of that animal.

The term "progeny" as used herein includes direct progeny of the transgenic animal as well as any progeny of succeeding progeny. Thus, one skilled in the art will readily recognize that if two different transgenic animals

have been made each utilizing a different gene or genes and they are mated, the possibility exists that some of the resulting progeny will contain two or more introduced genes. One skilled in the art will readily recognize that by controlling the matings, transgenic animals containing multiple introduced genes can be made.

Similarly, the invention features a method for introducing a continuous supply of IR-95 into an animal, comprising the step of administering an effective amount of a vector of the present invention into said animal. The invention also provides a method for introducing a continuous supply of IR-95 into a tissue culture, comprising the step of administering an effective amount of a vector of the present invention into said tissue culture. In another preferred embodiment, a method of introducing a continuous supply of IR-95 into an animal or a tissue culture by administering an effective amount of a vector is provided.

By "continuous" is meant that the IR-95 is constitutively expressed without the need for the addition of an exogenously administered activating compound to initiate expression.

The term "effective amount" means an amount sufficient to give expression of some amount of IR-95 in the muscle or tissue culture.

In yet another aspect, the invention features a method of screening compounds for their pharmacological effects on an immune response comprising the steps of administering a compound to a transgenic animal expressing IR-95 and measuring said immune response in said transgenic animal.

Also provided is a method of administering a nucleic acid sequence encoding a IR-95 to an animal comprising the steps of removing cells from said animal, transfecting said cells with said IR-95 nucleic acid sequence, and reimplanting said transfected cells into said animal.

In another preferred embodiment, there is provided a method of introducing into an animal a IR-95 vector containing a IR-95 of another species and capable of expressing in whole or in part or in modified form this other species IR-95. This transgenic animal is useful in screening compounds for their pharmacological effects on an immune response comprising the steps of administering compounds to the transgenic animal and measuring the immune response in the transgenic animal.

In another preferred embodiment, an *in vivo* method of administering a nucleic acid sequence is provided, as described below. In a further preferred embodiment, naked DNA may be administered.

The term "naked DNA" means substantially pure DNA which is not associated with protein, lipid, carbohydrate or contained within a cell or an artificial delivery system such as a liposome. A tissue or cell may also be transduced with a IR-95 vector.

The term "transfected or transfection" as used herein refers to the process of introducing a recombinant virus into a cell by infecting the cell with the virus particle. The virus may be administered substantially simultaneously, *i.e.*, the IR-95 nucleic acid sequence and the virus may administered in the same composition or that the administration of one may follow the other by about up to one hour.

In an additional preferred embodiment a method of administering IR-95 nucleic acid sequence through cell surface receptor mediated endocytosis is provided. That is, the biological process whereby cell surface receptors which have bound a ligand cluster together on the cell surface followed by invagination of the cell membrane containing the clustered receptors and formation of an intercellular vesicle containing the receptor ligand complexes.

A "cell surface receptor" is a specific chemical grouping on the surface of a cell to which a ligand can

attach. Cell surface receptors which may be used in the present invention include the folate receptor, the biotin receptor, the lipoic acid receptor, the low density lipoprotein receptor, the asialoglycoprotein receptor, IgG antigenic sites, insulin-like growth factor type II/cation-independent mannose-6-phosphate receptor, calcitonin gene-related peptide receptor, insulin-like growth factor I receptor, nicotinic acetylcholine receptor, hepatocyte growth factor receptor, endothelin receptor, bile acid receptor. Further, incorporating DNA into macromolecular complexes that undergo endocytosis increases the range of cell types that will take up foreign genes from the extracellular space. Such complexes may include lipids, polylysine, viral particles, ligands for specific cell-surface receptors or nuclear proteins.

The term "DNA transporter" refers to a molecular complex which is capable of non-covalently binding to DNA and efficiently transporting the DNA through the cell membrane. Although not necessary, it is preferable that the transporter also transport the DNA through the nuclear membrane. The methods and material set forth in International Publication No. WO 93/18759, filed March 19, 1993 and published September 30, 1993 are hereby incorporated by reference.

In another preferred embodiment, a two-component system of administering a IR-95 nucleic acid sequence is provided.

The term "two-component system" means a system utilizing a packaging cell which produces a viral vector. In a preferred embodiment, a partial hepatectomy may be performed prior to administration of the packaging cell.

The term "hepatectomy" or "partial hepatectomy" is used as is commonly understood in the art.

In an additional preferred embodiment, a retroviral vector containing a modified retroviral envelope glyco-

protein is provided. The term retroviral envelope glycoprotein is used as is commonly understood in the art.

The invention features a method of treating a disorder comprising the steps of:

(a) inserting an expression vector comprising nucleic acid encoding IR-95 into a cell;

(b) growing said cell in vitro; and

(c) infusing said cell to an organism in need of treatment.

The invention also features a method of administering a IR-95 nucleic acid sequence utilizing an *in vivo* approach comprising the steps of administering directly to an animal said IR-95 nucleic acid sequence selected from the group of methods of administration consisting of intravenous injection, intramuscular injection, or by catheterization and direct delivery of said IR-95 nucleic acid sequence via the blood vessels supplying a target organ.

In preferred embodiments the target organ is selected from the group consisting of heart, skeletal muscle, adipose tissues, spleen, lung, brain, kidney, testis, adrenal or small intestine; the IR-95 nucleic acid sequence is administered as naked DNA; the IR-95 nucleic acid sequence is contained in a viral vector such as papovaviruses, adenovirus, vaccinia virus, adeno-associated virus, herpesviruses and retroviruses of avian, murine or human origin.

The invention features a method of administering a IR-95 nucleic acid sequence in a two-component system comprising the steps of administering a packaging cell, wherein said packaging cell produces a viral vector.

In preferred embodiments the packaging cell is administered to cells *in vitro*.

Also featured is a method of administering a IR-95 nucleic acid sequence comprising the step of administering to an animal an adenovirus vector, wherein an E1 region of said adenovirus vector is replaced with said IR-95 nucleic

acid sequence and administering said adenovirus vector by a method of administration selected from the group consisting of intravenous injection, intramuscular injection, intraportal injection or intra-arterial injection.

In another embodiment, the invention provides a gene therapy produce comprising a therapeutically effective amount of nucleic acid encoding IR-95 and a means for administering said nucleic acid. The means for administering may be a needle for microinjection, a gene gun, a vector, a device for electroporation, a liposome, a small projectile used on particle bondediment, a protein used in receptor mediated endocytosis, a DNA transporter, or any other means for providing such nucleic acid. The present invention is thus based on the surprising discovery that administration of nucleic acid encoding IR-95 is capable of producing a therapeutic effect when administered to an organism.

In another aspect, the invention provides a gene transfer method for administering nucleic acid encoding IR-95 to an organism.

In another aspect, the invention provides a gene therapy method for administering a therapeutically effective amount of nucleic acid encoding IR-95 to an organism in need of such treatment. As explained above, the present invention demonstrates that IR-95 gene therapy is effective in vivo. In view of the description and examples provided herein, those skilled in the art will now recognize that effective IR-95 gene therapy may take various forms and thus, many modifications of the exemplary techniques are encompassed by the present invention.

The present invention also features kits useful for carrying out the methods described herein. Such kits would all share the common features of containing nucleic acid encoding, IR-95, and one or more components required to administer the gene therapy produce. In various preferred embodiments, the kits may also include other components such as the vectors useful in expressing IR-95,

a needle or syringe useful for microinjection, transfected or transformed cells, an I.V., a catheter, a packaging cell, materials useful for transfection, electorporation, lipofection, particle bombardment, or coupling of chemically modified proteins. Those skilled in the art will recognize that such kits may contain various combinations of the above components.

Any terms which are used herein and are not specifically defined herein are used as they would be by one of ordinary skill in the art(s) to which the invention pertains. Abbreviations used herein include the following: CM, conditioned medium; GM-CSF, granulocyte-macrophage colony-stimulating factor; LAK, lymphokine-activated killer cell; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide. Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Drawings

Figure 1 shows inhibition of tumor growth in nude mice. In panels a and *b*, For each line tested, 2 x $10^6$ cells were subcutaneously implanted above the right hind leg in nude mice. Each experimental group consisted of 8 animals, which were independently injected. The control groups for each experiment represent parental cells before or after pLXSN virus infection. Data points represent average tumor volumes of all experimental animals in one group. P. Values were calculated by Student's t-test comparison of mean tumor volumes to that of the appropriate control group. Figure 1a shows formation of solid tumors by virus-infected MM5 cells. □; control MM5/LXSN; ■ MM5/90-1 (p <0.00001).O; MM5/90-2 (p <0.00001). Figure 1b shows tumor formation by virus-infected B-29 cells. □; control B-29/LXSN; ■; polyclonal line B-29/90P (p <0.002); O; clonal line B29/90-1 )p <0.002).

In panels c and d, Coimplantation of IR-95-expressing and parental MM5 cells' domain. Animals were either injected with $10^5$ parental MM5 cells alone or together with $10^6$ LXSN-IR-95-infected cells (MM5/90-1). Each experimental group consisted of four animals. Figure 1c shows tumor growth in animals implanted with either parental MM5 cells (□) or IR-95-expressing cells (O) ($p = 0.0065$) alone, or coimplanted with both cell lines above the right hind leg (■) ($p = 0.01$). Figure 1d shows tumor formation in mice implanted with parental and IR-95-expressing MM5 cells at different sites. Animals were implanted with MM5 cells above the right hind leg (■) ($p = 0.13$) and with MM5/90-1 cells above the left hind leg (O) ($p = 0.082$). □; tumor growth in control animals implanted with MM5 cells only.

Figure 1e shows the effect of IR-95 expression on tumor formation by SF7763t cells.

METHODS. IR-95-expressing cell lines were established as described in Example 9. Parental cells and retrovirus-infected cells were trypsinized and washed several times in phosphate buffered saline (PBS). For implantation, cells were resuspended in 100 μl of PBS and implanted subcutaneously above the left or right hind leg of four-to-eight-week-old nude mice. Starting at the time when developing tumors were first visible, measurements of tumor volumes were done at regular intervals twice a week. The animals were sacrificed when the tumor reached a volume greater than 2500 $mm^3$. The inhibitory effect of IR-95 production on tumor growth was calculated by comparison of the average tumor volume produced by IR-95-secreting cells in 8 animals with the volume of tumors from animals of the respective control group.

The human glioblastoma SF763t cell line was infected with amphotropic viruses which were obtained after infection of PA317 cells with ecotropic virus. Cells ($10^7$/mouse) were subcutaneously implanted in nude mice as described above.

Description of the Preferred Embodiments

The present invention provides therapeutic methods relating to IR-95. Methods for isolating and purifying the antigen, for producing monoclonal or polyclonal antibodies, for sequencing the protein, for producing probes, and methods for modifying and expressing the disclosed nucleotide and amino acid sequences are described in PCT Application Publication WO 93/16181, published August 19, 1993.

The purified protein and antibodies thereto as well as its genetic sequences are useful in therapeutic methods. In particular, IR-95 and antagonists thereof are useful as therapeutic agents for cancer, including breast, ovarian and other malignancies, viral infection, including HIV, bacterial infection, parasitic infection, inflammation, autoimmune disease, aging, and different physiopathological conditions which are characterized by a variable degree of immune deficit associated with immune activation or elevated serum IR-95 levels.

We have examined the effects of IR-95 on cytokine production by human PBMC. PBMC were stimulated with suboptimal doses of the accessory cell-dependent T-cell mitogen ConA in the presence or absence of purified recombinant IR-95, and the culture supernatants were analyzed for the accessory cell-derived cytokines IL-1 and IL-6, and the T-cell-derived cytokine IL-2. As shown previously (Ullrich et al., *J. Biol. Chem.* 269:18401-18407, 1994, incorporated herein by reference in its entirety including any drawings), levels of IL-2 were higher in the supernatants of PBMC costimulated with ConA and IR-95, compared to stimulation with either agent alone. We now extend these findings to include positive effects of IR-95 on production of the accessory cell-derived cytokines IL-1 and IL-6, expression of the adhesion molecule ICAM-1, and expression of accessory function of monocytes. The increased response of IL-2 was dose-dependent with respect to both ConA and IR-95, and

was dependent on the presence of the T-cell mitogen. Similarly, secretion of IL-1 and IL-6 were enhanced by the addition of IR-95 to the ConA-stimulated PBMC.

In contrast to the ConA dependency of the IL-2 response, IL-1 and IL-6 production were induced by IR-95 alone, indicating that the actual target of activation was the accessory cell, not the T-cell. Indeed, T-cells enriched by passage over nylon wool failed to respond to ConA alone or in combination with IR-95, while CD14-enriched cells produced IL-1 and IL-6 when stimulated with IR-95 alone, regardless of the presence or absence of the T-cell mitogen ConA. These data indicate that it is the CD14+ accessory cells which produce IL-1 and IL-6 in response to IR-95, and that IR-95 acts upon accessory cells independently of ConA. IR-95 was also found to increase the expression on monocytes of ICAM-1, an important adhesion molecule involved in costimulation of T-cells.

T-cells depleted of accessory cells failed to respond to ConA alone, IR-95 alone, or the combination of ConA and IR-95, indicating that this protein does not have a direct effect on T-cells. In fact, IR-95 alone (i.e., without ConA) was sufficient to induce production of IL-1 and IL-6 by unfractionated PBMC or by CD14-enriched PBMC. In addition, expression of ICAM-1 was increased on a human monocytic cell line cultured with purified IR-95 in the absence of any other stimulus. This IR-95-induced upregulation of ICAM-1 expression was accompanied by an increased accessory function of the monocytes, as determined by their ability to support ConA-induced activation of peripheral blood T-cells.

IR-95 thus activates accessory cells, resulting in the secretion of cytokines, activation of helper T-cells and effector cells such as NK and LAK, and expression of adhesion molecules, which in turn act as costimulatory signals for T-cell activation. The activated T-cells then produce cytokines such as IL-2, which lead to a more

vigorous cell-mediated immune response to tumor cells and virus-infected cells. Thus, IR-95 may be used as an immunotherapeutic reagent for diseases such as cancer and viral infection, due to its ability to enhance cell-mediated immune responses.

The positive effect of IR-95 on ConA-induced T-cell activation may be due to its ability to directly activate accessory cell secretion of IL-1 and IL-6, both of which are involved in T-cell activation. Therefore, we examined the effect of IR-95 on adhesion molecule expression and accessory function of a human monocytic cell line, THP-1. In agreement with the experiments with CD14+ PBMC, we found that IR-95 had a direct positive effect on THP-1 cells, evidenced as an increase in ICAM-1 expression following an overnight culture with IR-95. Not only did IR-95 upregulate ICAM-1 expression, but it also caused an increase in the ability of the THP-1 cells to support T-cell activation: IR-95-pulsed THP-1 cells caused a 2-fold increase in T-cell proliferation compared to non-pulsed cells. The activated T-cells may then produce lymphokines (in addition to IL-2) which act in a feedback loop to increase accessory cell activation and cytokine secretion; this mechanism could explain why in some experiments, the addition of both IR-95 and ConA appeared to synergistically increase the levels of cytokines produced.

IR-95 appears to be a soluble costimulatory molecule, which acts at the level of the accessory cell, indirectly supporting T-cell activation. Secretion of IR-95 by tumor cells or normal cells (Ullrich et al., *J. Biol. Chem.* 269:18401-18407, 1994) presumably triggers the first step in a cascade which ultimately results in enhanced cell-mediated immunity. While the current results indicate that IR-95 induces cytokine production by accessory cells, the effect of IR-95 on expression of adhesion molecules may be just as important in ultimate T-cell activation. Indeed, we present evidence that incubation with IR-95 increases expression of ICAM-1 (also known as CD54) on a

human monocytic cell line, concomitant with an increase in the ability of the THP-1 cells to support T-cell activation.

Additionally, experiments have demonstrated that IR-95 is capable of altering tumor cell growth *in vivo*. Clones which express high amounts of human IR-95 were developed from murine 6378 cells (derived from mouse mammary gland carcinoma) and used for tumor growth experiments. In these experiments female nude mice, 4 to 5 weeks old, were divided into 4 groups of 4 individuals. Each individual in groups 1, 2 and 3 was subcutaneously implanted with a clone of murine 6378 cells which produced human IR-95 ($2 \times 10^6$ cells / mouse). The animals in group 4 were implanted with 6378 cells which do not produce human IR-95. The tumor volume in each animal was measured twice a week and the tumor growth curve determined. Mice implanted with the 6378 cells expressing human IR-95 showed significantly slower tumor growth than mice implanted with control cells. Twenty-three days post implantation, the tumor volume in mice implanted with the cells expressing IR-95 was reduced by 40% (group 1), and 70% (group 2, 3) as compared to the control group.

Using different experimental systems and approaches, IR-95-encoding cDNA has now been cloned four times. While we[2] and Koths et al.[3] identified human IR-95 as a tumor-associated secreted antigen or the Mac-2-BP, respectively, the putative mouse homologue of IR-95 was independently isolated as either cyclophilin C-associated protein (CyCAP)[16] or murine adherent macrophage (MAMA) protein[17]. The common denominator of the four independent investigations that converged on the discovery of the IR-95 (IR-95/Mac-2-BP/CyCAP/MAMA) protein appears to be a function in the body's immune surveillance and defense against cancer cells and likely other pathogenic agents. The diverse scenarios in which IR-95 and its homologues were found to play a role, together with our previous observations of IR-95-stimulated NK and LAK activities *in vitro* as well as

induction of IL-2 secretion in mitogen-stimulated PBMC[2], suggest multiple modes of IR-95 interaction with cellular components of the immune system. Moreover, observations in cancer patients and *in vitro* demonstrate that IR-95 itself is induced by $\alpha$ and $\gamma$ interferon[4,6,18,19] and TNF$\alpha$[17], indicating that the stimulatory actions of these factors on cellular host defense systems may involve this secreted glycoprotein. The results presented here strongly support an important role of IR-95 in the body's cellular immune defense against cancer cells. Moreover, as suggested by constitutive high expression levels in epithelia such as those of the intestinal mucosa[3]. IR-95 is likely to be involved in shielding the body from invasion by pathogens such as bacteria and parasites. We have thus shown that the ectopically-enhanced expression of IR-95 by human and murine tumor cells inhibits *in vivo* tumorigenesis in athymic mice.

Given the above findings, IR-95 or its genetic sequences will be useful in therapy as an immunoregulatory agent. For example, patients who suffer from a particular cancer which does not induce over expression of IR-95 may be treated by infusion with IR-95. Furthermore, those patients with cancers that generate elevated levels of IR-95 in their serum, may be supplied additional IR-95 by infusion.

Gene Therapy

IR-95 or its genetic sequences will also be useful in gene therapy (reviewed in Miller, *Nature* 357:455-460, (1992). Miller states that advances have resulted in practical approaches to human gene therapy that have demonstrated positive initial results. An *in vivo* model of gene therapy for human severe combined immunodeficiency is described in Ferrari, et al., *Science* 251:1363-1366, (1991). The basic science of gene therapy is described in Mulligan, *Science* 260:926-931, (1993).

In one preferred embodiment, an expression vector containing the IR-95 coding sequence is inserted into cells, the cells are grown *in vitro* and then infused in large numbers into patients. In another preferred embodiment, a DNA segment containing a promoter of choice (for example a strong promoter) is transferred into cells containing an endogenous IR-95 in such a manner that the promoter segment enhances expression of the endogenous IR-95 gene (for example, the promoter segment is transferred to the cell such that it becomes directly linked to the endogenous IR-95 gene).

The ability of IR-95 to induce cytokine production and adhesion molecule expression by lymphocytes makes it useful for gene therapy of tumors. Tumor cells which do not express IR-95 may be engineered to do so, thereby at least partially restoring the immunogenicity of the tumor cells. Local production of IR-95 at the site of the tumor could lead to activation of infiltrating monocytes and macrophages. Increased secretion of pro-inflammatory cytokines such as IL-1 and IL-6 could result in a greater degree of infiltrating, activated Th cells and effector cells (CTL, NK/LAK). Increased expression of adhesion molecules by accessory cells could lead to more vigorous T-cell activation *in situ*. The net effect would be a more vigorous anti-tumor immune response which should provide therapeutic benefit to the host. The engineered expression of IR-95 in non-expressing tumor cell lines inhibits the ability of the cells to grow as xenografts in athymic mice.

The gene therapy may involve the use of an adenovirus containing IR-95 cDNA targeted to a tumor, systemic IR-95 increase by implantation of engineered cells, injection with IR-95 virus, or injection of naked IR-95 DNA into appropriate tissues.

Target cell populations (e.g., haematopoietic, muscle, or immune cells) may be modified by introducing altered forms of IR-95 in order to modulate the activity

of such cells. For example, by reducing or inhibiting an immune activity within target cells, an abnormal immune response leading to a condition may be decreased, inhibited, or reversed. Deletion or missense mutants of IR-95, that retain the ability to interact with other components of the immune system but cannot participate in normal immune response function may be used to inhibit an abnormal, deleterious immune response.

Expression vectors derived from viruses such as retroviruses, vaccinia virus, adenovirus, adeno-associated virus, herpes viruses, several RNA viruses, or bovine papilloma virus, may be used for delivery of nucleotide sequences (e.g., cDNA) encoding recombinant IR-95 protein into the targeted cell population (e.g., tumor cells). Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors containing coding sequences. See, for example, the techniques described in Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. (1989), and in Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, recombinant nucleic acid molecules encoding protein sequences can be used as naked DNA or in reconstituted system e.g., liposomes or other lipid systems for delivery to target cells (See e.g., Felgner et al., *Nature* 337:387-8, 1989). Several other methods for the direct transfer of plasmid DNA into cells exist for use in human gene therapy and involve targeting the DNA to receptors on cells by complexing the plasmid DNA to proteins. See, Miller, supra.

In its simplest form, gene transfer can be performed by simply injecting minute amounts of DNA into the nucleus of a cell, through a process of microinjection. Capecchi MR, Cell 22:479-88 (1980). Once recombinant genes are introduced into a cell, they can be recognized by the cells normal mechanisms for transcription and translation,

and a gene product will be expressed. Other methods have also been attempted for introducing DNA into larger numbers of cells. These methods include: transfection, wherein DNA is precipitated with $CaPO_4$ and taken into cells by pinocytosis (Chen C. and Okayama H, Mol. Cell Biol. 7:2745-52 (1987)); electroporation, wherein cells are exposed to large voltage pulses to introduce holes into the membrane (Chu G. et al., Nucleic Acids Res., 15:1311-26 (1987)); lipofection/liposome fusion, wherein DNA is packaged into lipophilic vesicles which fuse with a target cell (Felgner PL., et al., Proc. Natl. Acad. Sci. USA. 84:7413-7 (1987)); and particle bombardment using DNA bound to small projectiles (Yang NS. et al., Proc. Natl. Acad. Sci. 87:9568-72 (1990)). Another method for introducing DNA into cells is to couple the DNA to chemically modified proteins.

It has also been shown that adenovirus proteins are capable of destabilizing endosomes and enhancing the uptake of DNA into cells. The admixture of adenovirus to solutions containing DNA complexes, or the binding of DNA to polylysine covalently attached to adenovirus using protein crosslinking agents substantially improves the uptake and expression of the recombinant gene. Curiel DT et al., Am. J. Respir. Cell. Mol. Biol., 6:247-52 (1992).

As used herein "gene transfer" means the process of introducing a foreign nucleic acid molecule into a cell. Gene transfer is commonly performed to enable the expression of a particular product encoded by the gene. The product may include a protein, polypeptide, anti-sense DNA or RNA, or enzymatically active RNA. Gene transfer can be performed in cultured cells or by direct administration into animals. Generally gene transfer involves the process of nucleic acid contact with a target cell by non-specific or receptor mediated interactions, uptake of nucleic acid into the cell through the membrane or by endocytosis, and release of nucleic acid into the cytoplasm from the plasma membrane or endosome. Expression

may require, in addition, movement of the nucleic acid into the nucleus of the cell and binding to appropriate nuclear factors for transcription.

As used herein "gene therapy" is a form of gene transfer and is included within the definition of gene transfer as used herein and specifically refers to gene transfer to express a therapeutic product from a cell *in vivo* or *in vitro*. Gene transfer can be performed *ex vivo* on cells which are then transplanted into a patient, or can be performed by direct administration of the nucleic acid or nucleic acid-protein complex into the patient.

In another preferred embodiment, a vector having nucleic acid sequences encoding IR-95 is provided in which the nucleic acid sequence is expressed only in specific tissue. Methods of achieving tissue-specific gene expression as set forth in International Publication No. WO 93/09236, filed November 3, 1992 and published May 13, 1993.

In all of the preceding vectors set forth above, a further aspect of the invention is that the nucleic acid sequence contained in the vector may include additions, deletions or modifications to some or all of the sequence of the nucleic acid, as defined above.

In another preferred embodiment, a method of gene replacement is set forth. "Gene replacement" as used herein means supplying a nucleic acid sequence which is capable of being expressed *in vivo* in an animal and thereby providing or augmenting the function of an endogenous gene which is missing or defective in the animal.

Antisense and Ribozyme Approaches

Various antisense or ribozyme approaches may be used to regulate an immune response. Included in the scope of the invention are oligoribonucleotides, including anti-sense RNA and DNA molecules and ribozymes that function to inhibit translation of IR-95. Anti-sense RNA and DNA

molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. With respect to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between -10 and +10 regions of the relevant nucleotide sequence, are preferred.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific interaction of the ribozyme molecule to complementary target RNA, followed by a endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead or other motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of RNA sequences encoding protein complex components.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features, such as secondary structure, that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays. See, Draper PCT WO 93/23569.

Both anti-sense RNA and DNA molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of RNA molecules. See, Draper, id. hereby incorporated by reference herein. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the

antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Various modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribo- or deoxy- nucleotides to the 5′ and/or 3′ ends of the molecule or the use of phosphorothioate or 2′ O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

Pharmaceutical Formulations and Modes of Administration

The particular antagonist compound, antisense or ribozyme molecule or IR-95 protein of the present invention that affects the disorder of interest can be administered to a patient either by themselves, or in pharmaceutical compositions where it is mixed with suitable carriers or excipient(s).

IR-95 or antagonists thereof can routinely be prepared as therapeutic agent(s) by one of skill in the art using standard techniques and references which are well known in the art (see, for example, *Remington's Pharmaceutical Sciences*, 18th ed., (A.R. Gennaro, Ed.), Mack Publishing Comp., Easton, PA, USA 18042 (1990), especially chapters 8 (Pharmaceutical Preparations and Their Manufacture) and 4 (Testing and Analysis), thereof). Appropriate and optimum routes of administration can also be routinely determined by one of skill in the art. The former include the oral, intravenous, intramuscular, subcutaneous, transdermal, *in situ* and bucal routes of administration among others.

In treating a patient exhibiting disorder of interest, a therapeutically effective amount of a agent or agents such as these is administered. A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms or a prolongation of survival in a patient.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the $LD_{50}$ (the dose lethal to 50% of the population) and the $ED_{50}$ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio $LD_{50}/ED_{50}$. Compounds which exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the $ED_{50}$ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the $IC_{50}$ as determined in cell culture (i.e., the concentration of the test compound which achieves a half-maximal inhibition of the cellular level and/or activity of IR-95). Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by HPLC.

The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g. Fingl et al., in The Pharmacological Basis of Therapeutics, 1975, Ch. 1

p. 1). It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administrated dose in the management of the oncogenic disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

Depending on the specific conditions being treated, such agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, PA (1990). Suitable routes may include oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, just to name a few.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice

of the invention into dosages suitable for systemic administration is within the scope of the invention. With proper choice of carrier and suitable manufacturing practice, the compositions of the present invention, in particular, those formulated as solutions, may be administered parenterally, such as by intravenous injection. The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes, then administered as described above. Liposomes are spherical lipid bilayers with aqueous interiors. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external microenvironment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules may be directly administered intracellularly.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used

pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

The nucleic acid sequence encoding IR-95 can be administered prophylactically, or to patients having a disorder listed above, *e.g.*, by exogenous delivery of the nucleic acid sequence encoding IR-95 as naked DNA, DNA associated with specific carriers, or in a nucleic acid expression vector to a desired tissue by means of an appropriate delivery vehicle, *e.g.*, a liposome, by use of iontophoresis, electroporation and other pharmacologically approved methods of delivery. Routes of administration may include intramuscular, intravenous, aerosol, oral (tablet or pill form), topical, systemic, ocular, as a suppository, intraperitoneal and/or intrathecal.

Some methods of delivery that may be used include:

a. encapsulation in liposomes,

b. transduction by retroviral vectors,

c. localization to nuclear compartment utilizing nuclear targeting site found on most nuclear proteins,

d. transfection of cells *ex vivo* with subsequent reimplantation or administration of the transfected cells,

e. a DNA transporter system.

A IR-95 nucleic acid sequence may be administered utilizing an *ex vivo* approach whereby cells are removed from an animal, transduced with the IR-95 nucleic acid sequence and reimplanted into the animal. The liver can be accessed by an *ex vivo* approach by removing hepatocytes from an animal, transducing the hepatocytes *in vitro* with the IR-95 nucleic acid sequence and reimplanting them into the animal (*e.g.*, as described for rabbits by Chowdhury et al, Science 254: 1802-1805, 1991, or in humans by Wilson, Hum. Gene Ther. 3: 179-222, 1992) incorporated herein by reference.

Many nonviral techniques for the delivery of a IR-95 nucleic acid sequence into a cell can be used, including direct naked DNA uptake (*e.g.*, Wolff et al., Science 247: 1465-1468, 1990), receptor-mediated DNA uptake, *e.g.*, using DNA coupled to asialoorosomucoid which is taken up by the asialoglycoprotein receptor in the liver (Wu and Wu, J. Biol. Chem. 262: 4429-4432, 1987; Wu et al., J. Biol. Chem. 266: 14338-14342, 1991), and liposome-mediated delivery (*e.g.*, Kaneda et al., Expt. Cell Res. 173: 56-69, 1987; Kaneda et al., Science 243: 375-378, 1989; Zhu et al., Science 261: 209-211, 1993). Many of these physical methods can be combined with one another and with viral techniques; enhancement of receptor-mediated DNA uptake can be effected, for example, by combining its use with adenovirus (Curiel et al., Proc. Natl. Acad. Sci. USA 88: 8850-8854, 1991; Cristiano et al., Proc. Natl. Acad. Sci. USA 90: 2122-2126, 1993).

The IR-95 or nucleic acid encoding IR-95 may also be administered via an implanted device that provides a

support for growing cells. Thus, the cells may remain in the implanted device and still provide the useful and therapeutic agents of the present invention.

Vectors

A number of viral vectors can be used to deliver a IR-95 nucleic acid sequence, including papovaviruses, adenovirus, vaccinia virus, adeno-associated virus, herpesviruses, retroviruses of avian, murine, and human origin and other viruses as are known in the art (reviewed by Morgan and Anderson, Ann. Rev. Biochem. 62: 191-217, 1993 incorporated herein by reference.) Retroviral vectors can be used for transducing the IR-95 vector into liver cells or muscle. The advantage of retrovirus as a delivery system is the ability of the virus to integrate into the host cell chromosomes (reviewed by A.D. Miller, Hum. Gene Ther. 1: 5-14, 1990). The IR-95 vector can be delivered by retroviral-mediated gene transfer, a two-component system consisting of the packaging cell and the viral vector. The IR-95 nucleic acid sequence can be inserted into the retroviral vector by molecular cloning (*e.g.*, as described by Wilson, Hum. Gene Ther. 3: 179-222, 1992). The virus particle assembled by the producer cell line (*i.e.*, a packaging cell line containing the IR-95-containing retroviral vector) will be used to transfer the IR-95 nucleic acid sequence to a target organ or tissue such as liver cells *in vivo* (following partial hepatectomy because only dividing cells take up retroviral vectors), isolated hepatocytes *in vitro* or skeletal muscle *in vivo*. The virus particle will bind to the cell and deliver the IR-95 nucleic acid sequence which is integrated into the host genome and result in stable long-term expression of the IR-95.

Two major limitations to the use of retroviral vectors are the restricted host-cell range and the inability to obtain high-titer virus. These limitations have been overcome by Burns et al., Proc. Natl. Acad. Sci.

USA 90: 8033-8037, 1993. They replaced the retroviral envelope glycoprotein with the G glycoprotein of vesicular stomatitis virus. Such vectors can be produced in high titer (>$10^9$ colony-forming units/ml) and can infect diverse cell types. Partial hepatectomy may not be necessary for liver expression using such vectors. The nucleic acid sequence encoding IR-95 can be delivered by using this or a similarly designed vector *in vivo* by intravenous administration.

Another viral vector delivery system that will be used is the adenovirus system. The IR-95 nucleic acid sequence can be used to replace the E1 region of the adenovirus using the method described by Graham and Prevec (Methods Molec. Biol., Vol. 7, E.J. Murray, ed., Humana Press, NJ, pp. 109-128, 1991) using recombination in 293 cells incorporated herein by reference. The replication-defective IR-95 nucleic acid sequence/adenovirus can be injected intravenously, intramuscularly, intraportally or intra-arterially (hepatic artery). To date, adenovirus-mediated expression vectors generally direct the transient expression of the therapeutic gene. Improvements and refinements in vector structure and design may lead to diminished immunogenicity and allow the vector to be administered repeatedly. Other modifications may result in the ability of the IR-95 nucleic acid sequence to be integrated in the host chromosomes allowing for stable expression.

Other viral vector delivery systems as are known in the art will also be used for the targeted transfer of the IR-95 nucleic acid sequence.

Derivatives of IR-95

Also provided herein are functional derivatives of IR-95. By "functional derivative" is meant a "chemical derivative," "fragment," "variant," "chimera," or "hybrid" of IR-95, which terms are defined below. A functional derivative retains at least a portion of the function of

the protein, for example reactivity with an antibody specific for IR-95, which permits its utility in accordance with the present invention.

A "chemical derivative" of IR-95 contains additional chemical moieties not normally a part of the protein. Covalent modifications of the protein or peptides are included within the scope of this invention. Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues, as described below.

Cysteinyl residues most commonly are reacted with alpha-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylprocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Parabromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect or reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing primary amine containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal,

2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high $pK_a$ of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine alpha-amino group.

Tyrosyl residues are well-known targets of modification for introduction of spectral labels by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizol and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction carbodiimide (R'-N-C-N-R') such as 1-cyclohexyl-3-(2-morpholinyl(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residue are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Derivatization with bifunctional agents is useful, for example, for cross-linking IR-95 to a water-insoluble support matrix or to other macromolecular carriers. Commonly used cross-linking agents include, for example, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis-(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[p-azidophenyl) dithiolpropioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively,

reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains (Creighton, T.E., Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and, in some instances, amidation of the C-terminal carboxyl groups.

Such derivatized moieties may improve the stability, solubility, absorption, biological half life, and the like. The moieties may alternatively eliminate or attenuate any undesirable side effect of IR-95 and the like. Moieties capable of mediating such effects are disclosed, for example, in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, PA (1990).

The term "fragment" is used to indicate a polypeptide derived from the amino acid sequence of the protein IR-95 having a length less than the full-length polypeptide from which it has been derived. Such a fragment may, for example, be produced by proteolytic cleavage of the full-length protein. Preferably, the fragment is obtained recombinantly by appropriately modifying the DNA sequence encoding the proteins to delete one or more amino acids at one or more sites of the C-terminus, N-terminus, and/or within the native sequence. Fragments of a protein, when present in an immune complex resembling a naturally occurring immune complex, are useful for screening for compounds that act to modulate immune responses, as described below. It is understood that such fragments, when present in a complex may retain one or more characterizing portions of the native complex. Examples of such retained

characteristics include: substrate specificity; interaction with other molecules in the intact cell; regulatory functions; or binding with an antibody specific for the native complex, or an epitope thereof.

Another functional derivative intended to be within the scope of the present invention is a IR-95 comprising at least one "variant" polypeptide which either lack one or more amino acids or contain additional or substituted amino acids relative to the native polypeptide. The variant may be derived from a naturally occurring IR-95 by appropriately modifying the protein DNA coding sequence to add, remove, and/or to modify codons for one or more amino acids at one or more sites of the C-terminus, N-terminus, and/or within the native sequence. It is understood that such variants having added, substituted and/or additional amino acids retain one or more characterizing portions of the native IR-95, as described above.

A functional derivative of IR-95 comprising proteins with deleted, inserted and/or substituted amino acid residues may be prepared using standard techniques well-known to those of ordinary skill in the art. For example, the modified components of the functional derivatives may be produced using site-directed mutagenesis techniques (as exemplified by Adelman et al., 1983, DNA 2:183) wherein nucleotides in the DNA coding the sequence are modified such that a modified coding sequence is modified, and thereafter expressing this recombinant DNA in a prokaryotic or eukaryotic host cell, using techniques such as those described above. Alternatively, functional derivatives of IR-95 with amino acid deletions, insertions and/or substitutions may be conveniently prepared by direct chemical synthesis, using methods well-known in the art. The functional derivatives of IR-95 typically exhibit the same qualitative biological activity as the native IR-95.

Examples

The examples which follow are for illustrative purposes only and are not intended to limit the scope of the invention. Examples characterizing IR-95, cloning the IR-95 gene, stably and transiently expressing IR-95, purifying IR-95, and enhancing natural killer and lymphokine activated killer cell activity are described in PCT Application Number WO 93/16181, published August 19, 1993. The present examples illustrate the effect of IR-95 on IL-1, IL-2, IL-6, ICAM expression and tumor growth.

Materials and Methods:

Media: RPMI medium was supplemented with 10% heat-inactivated fetal calf serum and glutamine. This formulation will be abbreviated as RPMI+FCS. Minimal essential medium was supplemented with 10% heat-inactivated fetal calf serum and glutamine. This formulation will be abbreviated as MEM+FCS.

Cell lines: HT-2 cells, an IL-2-dependent murine cell line, were passaged every 2 days in RPMI+FCS supplemented with 40 U/ml recombinant murine IL-2 (Genzyme). 7TD1 cells, an IL-6-dependent murine B-cell hybridoma, were passaged twice weekly in RPMI+FCS supplemented with 50 U/ml recombinant murine IL-6 (Genzyme). A375.S2 cells, an IL-1-sensitive human melanoma line, were passaged twice weekly in MEM+FCS. A375.S2 cells grow as adherent cells, and their growth is inhibited by human IL-1. U373 is a human glioblastoma cell line which produces natural IR-95; these cells were passaged twice weekly in MEM+FCS supplemented with non-essential amino acids and sodium pyruvate. BT2A4 cells were derived from NIH.3T3 cells by transfection with an expression vector containing the full length cDNA encoding human IR-95, followed by methotrexate amplification to select a clone which expressed high levels of recombinant IR-95 (Ullrich et al., *J. Biol. Chem.* 269:18401-18407, 1994). THP-1 is a human monocytic leukemia cell line, which was passaged twice weekly in

RPMI+FCS. All cell lines were obtained from the American Type Culture Collection.

Purification of IR-95 from conditioned medium: BT2A4 cells were seeded into cell factories in DMEM + 5% FCS + glutamine. When the cells reached confluence, the FCS concentration was gradually reduced to 0.5%. Conditioned medium (CM) was collected every two days, filter-sterilized, and stored at 4°C. CM was concentrated approximately 20-fold with a 20K cut-off cross filtration unit (Sartorius), followed by precipitation with 45% saturated ammonium sulfate. The ammonium sulfate cut was passed over an affinity column consisting of a purified monoclonal antibody specific for human IR-95 (Iacobelli et al., *Breast Cancer Research and Treatment* 11:19-30, 1993; Ullrich et al., *J. Biol. Chem.* 269:18401-18407, 1994) covalently coupled to Tresyl-activated agarose (Schleicher & Schuell). After extensive washing with PBS to remove non-bound material, the bound material was eluted with 1M ethanolamine (prep 1) or 3M $MgCl_2$ (prep 2). Eluates were immediately neutralized with a citric acid solution, concentrated by ultrafiltration, and dialyzed against PBS containing 1% glycerol. The purified material was aliquoted and stored at -80°C. Affinity-purified, BT2A4-derived IR-95 was further purified by electroelution of the IR-95 band from preparative SDS-PAGE. Electro-eluted IR-95 appeared as a single band when analyzed by silver staining of SDS-PAGE.

SDS-PAGE analysis of affinity-purified and electro-eluted IR-95 was performed. Affinity-purified IR-95 and electroeluted IR-95 were analyzed by SDS-PAGE on a 10-15% gradient gel. Bands were visualized by silver staining using molecular weight standards.

Peripheral blood mononuclear cells: Blood was obtained from apparently healthy donors following informed consent. Peripheral blood mononuclear cells were isolated from the buffy coats by density gradient centrifugation through Histopaque (Sigma). PBMC were washed extensively

with RPMI+FCS. Cells were adjusted to the appropriate concentration and either frozen in 90% FCS + 10% DMSO, or used fresh. Frozen cells were thawed rapidly and washed twice before use. Viability was determined by trypan blue exclusion. Recovery of viable cells from liquid nitrogen storage generally ranged from 50% to 95% of input cells.

T-cell enrichment from PBMC: Nylon wool-enriched T-cells were prepared essentially as described (Hathcock, 3.2.1 through 3.2.4 in Current Protocols in Immunology, Coligan et al., eds., Wiley Interscience (New York 1994)). Briefly, PBMC were resuspended in RPMI + 20% FCS and applied to nylon wool columns which had been washed and equilibrated in the same medium. The columns were incubated at 37°C for one hour. Non-adherent (T-cell-enriched) cells were eluted by slowly washing the columns with warm RPMI + 20% FCS. The enriched T-cells were washed twice in RPMI+FCS, counted, and adjusted to the appropriate volume. Enriched T-cells and unfractionated PBMC were assayed in parallel as described below.

Immunomagnetic enrichment of PBMC: PBMC were enriched for CD3+ and CD14+ cells using the MiniMACS Separation System (Miltenyi Biotec), according to the manufacturer's instructions. Briefly, aliquots of $10^7$ PBMC were stained with phycoerythrin-conjugated monoclonal antibodies to CD3 or CD14 (Leinco Technologies) for 15 minutes on ice. After one wash, magnetic beads conjugated to goat anti-mouse Ig were added to the samples, and incubated on ice for 5 minutes. The cells were then passed over MiniMACS magnetic columns to allow capture of labeled cells. Unstained cells were recovered by washing the columns in the presence of the magnetic source. Stained cells were then recovered by washing the columns after removing the magnetic source. Aliquots of the enriched cells were then analyzed by flow cytometry (FACScan, Becton Dickinson) to gauge the purity of the cells. Each enriched population was resuspended to the same volume as $10^7$ unfractionated PBMC, and all four populations were then cultured with

purified IR-95 for 48 hours as described below. In some assays, CD3-enriched and CD14-enriched cells were mixed together and stimulated with IR-95.

ConA costimulation assay: PBMC (or enriched fractions thereof) were resuspended in RPMI+FCS, and plated in 24-well plates ($4x10^6$/well unfractionated PBMC, an equivalent cell dose of fractionated PBMC), or in 96-well flat-bottom plates ($2x10^5$/well PBMC). RPMI+FCS was added to control wells, and various combinations of ConA (Sigma catalog # C-5275) and IR-95 diluted in RPMI+FCS were added to experimental wells, as indicated in the figure legends. Cultures were incubated at 37°C for 48 hours unless otherwise noted. Supernatants were collected from individual wells, clarified by centrifugation, and frozen at -80°C until assayed for cytokine content.

Cytokine bioassays: Samples and recombinant cytokine controls were serially diluted in 96-well flat bottom plates in a volume of 50 µl/well. The appropriate indicator cells were then added in 50 µl/well: HT-2, $10^4$/well for IL-2; 7TD1, $2x10^3$/well for IL-6; A375.S2, $10^4$/well for IL-1. Following the appropriate incubation period at 37°C (18 hours for HT-2, 96 hours for 7TD1, 72 hours for A375.S2), the plates were pulsed with MTT for 4 hours, and the resulting formazan crystals were solubilized with acidified isopropanol. [Note: for the IL-1 assay, plates were washed three times with PBS to remove non-adherent (growth-inhibited) cells prior to pulsing with MTT]. Optical density was measured on a dual wavelength microplate spectrophotometer (sample wavelength = 570 nm, reference wavelength = 630 nm). Concentrations of each cytokine in the samples were determined by regression analysis and comparison to a standard curve, which consisted of the recombinant cytokine control. Alternatively, the relative titer of cytokine was determined as the reciprocal of the dilution which resulted in a half-maximal response.

Analysis of adhesion molecule expression/function: THP-1 cells, a human monocytic cell line that possesses accessory function, were used to examine the effect of IR-95 on expression of adhesion molecules. Cells were plated in 24-well plates at $4x10^5$/well, in the presence or absence of purified IR-95 at various doses. Following an overnight incubation at 37°C, the cells were harvested and washed three times prior to staining with fluorochrome-labelled monoclonal antibodies to human cell surface antigens. Staining was done in 96-well round-bottom plates on ice for 20 minutes. After washing away unbound antibody, fluorescence was analyzed in a Becton Dickinson FACScan, using LYSIS II software to gate live cells by FSC/SSC parameters.

The accessory function of THP-1 cells was analyzed by their ability support the ConA-induced activation of purified T-cells. THP-1 cells were cultured overnight with or without IR-95, then harvested and washed. The cells were fixed in formalin or treated with mitomycin C prior to being used in a functional assay. CD3+ cells were purified from PBMC by magnetic separation, as described above. THP-1 cells (~$3x10^4$/well) and CD3+ cells ($1.5-3x10^4$/well) were cocultured in 96-well round-bottom plates. ConA was added to final concentrations ranging up to 10 μg/ml. The plates were incubated for 48 hours, then DNA replication was measured in the cultures as described above.

Example 1: Increased IL-2 secretion by PBMC in response to ConA and IR-95-containing conditioned medium:

Effect of IR-95 containing conditioned medium on the PBMC response to ConA was studied. Conditioned media (CM) were collected from U373 (a human glioblastoma which naturally produces IR-95), BT2A4 (IR-95-negative parental cells), and NIH.3T3 cultures three days after passage. The CM were centrifuged and passed through 0.2 μM filters to remove cell debris. Freshly-isolated PBMC ($2x10^6$/well

in 24-well plates in a final volume of 1.0 ml) were cultured with doses of ConA up to 2.5 μg/ml, with or without CM (25% v/v final concentration). After 48 hours, supernatants were harvested and assayed for IL-2 activity.

IL-2 secretion was minimal at the doses of ConA used. However, the addition of CM from BT2A4 or U373 cells enhanced IL-2 secretion 2- to 5-fold in response to these suboptimal doses of the T-cell mitogen, while CM from the non-producing NIH.3T3 cells had no effect. CM alone did not induce IL-2 production by PBMC, suggesting that the increased IL-2 levels seen in the ConA + CM wells were due to a costimulation effect.

Example 2: Effect of purified recombinant IR-95 on lymphokine production by PBMC:

The results described herein demonstrated that IR-95-containing CM could augment the PBMC response to ConA, but it was not clear whether this effect was due to IR-95 itself or some other component of CM. Therefore, recombinant IR-95 affinity-purified from BT2A4 CM was assayed in a similar fashion and the effect of purified IR-95 on lymphokine secretion by human PBMC was studied. PBMC were cultured for 48 hours in 24-well plates ($2x10^6$/well) in a final volume of 1.0 ml with ConA plus or minus purified IR-95 affinity-purified from BT2A4 CM at 20 μg/ml. Supernatants were harvested and assayed for cytokine bioactivity. All experiments were performed at least three times. IL-2 production was detectable at the highest dose of ConA tested, and was significantly augmented (approximately 7-fold) by the addition of IR-95. As in the previous experiment, IR-95 alone failed to induce detectable IL-2 production. Thus, PBMC response appears to be due to IR-95 itself, and not some other component of CM.

These supernatants were also assayed for the accessory cell-derived cytokines IL-1 and IL-6, both of which are important in T-cell activation. PBMC ($2x10^6$/well) were cultured for 48 hours with affinity-purified (AP) or gel-

purified (GP) IR-95. Supernatants were harvested and assayed for IL-1 and IL-6. IL-1 and IL-6 were produced by the ConA-stimulated PBMC, and the levels of each cytokine were higher in cultures which had been costimulated with ConA and IR-95. At the highest dose of ConA, addition of IR-95 to the PBMC cultures increased IL-1 production by approximately 3-fold, and IL-6 production by greater than 6-fold. Similar results were obtained in several experiments with independently-purified batches of recombinant IR-95, using freshly-isolated PBMC from different donors. Thus, the costimulation of ConA-induced lymphokine production does not appear to be due to an unidentified component of CM, or an artifact of a particular batch of purified material.

To attribute the activity observed in previous experiments strictly to IR-95, and not to some unidentified contaminant in the affinity-purified material, we investigated the ability of gel-purified IR-95 to induce secretion of IL-1 and IL-6 by PBMC. PBMC were cultured with gel-purified IR-95 for 48 hours, and the supernatants were harvested and analyzed for IL-1 and IL-6. No IL-1 or IL-6 was detectable in supernatants of resting PBMC, while the addition of IR-95 to the PBMC cultures resulted in secretion of significant levels of both cytokines. Thus, the ability to stimulate cytokine secretion can be attributed solely to the IR-95 protein itself. It should also be noted that culture of PBMC with IR-95 alone (i.e., without ConA) was sufficient to stimulate secretion of IL-1 and IL-6.

Example 3: Response of nylon wool-enriched T-cells to costimulation with ConA and IR-95:

Results of initial experiments showed that IR-95 increased production of the accessory cell-derived cytokines IL-1 and IL-6, and of the T-cell-derived cytokine IL-2. Since these experiments were conducted with unfractionated PBMC populations, it was not possible to

determine which cell type was the direct target of IR-95. Therefore, PBMC were passed over nylon wool columns to deplete B-cells, macrophages, and other accessory cells and the response of nylon wool-enriched T-cells to costimulation with ConA and IR-95 was studied. The nonadherent cells, enriched for T-cells and NK cells, were then examined in the ConA costimulation assay described above; unfractionated PBMC were assayed in parallel. Cultures consisted of $2x10^6$ unfractionated PBMC/well, or the nylon wool-nonadherent cells collected from an equivalent number of PBMC. One-half of the cells were then passed over nylon wool columns to deplete B-cells and accessory cells. The nonadherent cells were harvested, washed, and resuspend to the original volume. Nylon wool-nonadherent cells or unfractionated cells were plated along with ConA IR-95 as indicated. ConA was added at 2.5 μg/ml, and IR-95 was added at 2 and 10 μg/ml. Supernatants were collected after 48 hours and assayed for cytokine bioactivity.

The T-cell-enriched population did not produce IL-2 in response to ConA, as expected, since ConA is an accessory cell-dependent mitogen. The addition of IR-95 to the T-cell population did not restore the ability of these cells to make IL-2 in response to mitogenic stimulation. The unfractionated PBMC did produce IL-2 when stimulated with ConA, and the level of IL-2 produced was higher when IR-95 was added to the cultures.

IL-6 was not produced by the enriched T-cell population, except in wells which contained both ConA and the highest dose of IR-95; this may represent contaminating accessory cells in the nylon wool nonadherent population, or IL-6 production by T-cells. By comparison, the unfractionated PBMC produced IL-6 in the absence of ConA, and this response was increased by the addition of low doses of IR-95. The ConA-independent production of IL-6 in response to IR-95 had been observed before, as described above. These data suggest that IR-95 does not exert an effect directly on T-cells, but rather it activates acces-

sory cells with downstream effects on T-cell activation. This hypothesis was explored in further experiments.

Example 4: Production of IL-6 by CD14-enriched cells stimulated with IR-95 alone:

IL-1 and IL-6 production by fractionated PBMC stimulated with IR-95 was studied. PBMC were enriched for CD3+ and CD14+ cells by immunomagnetic sorting. CD14-enriched fractions and unseparated PBMC were cultured with ConA ± IR-95 for 48 hours. Supernatants were assayed for IL-1 and IL-6 bioactivity. CD3-enriched fractions did not produce detectable levels of either IL-1 or IL-6.

The failure of nylon wool-enriched T-cells to respond to IR-95, and the ability of PBMC to produce IL-1 and IL-6 in response to IR-95 alone, suggested that the immediate target of IR-95 activity was accessory cells rather than T-cells. To test this hypothesis, PBMC were enriched for cells expressing CD3 (T-cells) and CD14 (monocytes/macrophages). These enriched fractions, as well as unseparated PBMC, were cultured with ConA ± IR-95 for 48 hours, and supernatants were assayed for IL-1 and IL-6.

The results demonstrate that IL-1 and IL-6 were produced by unfractionated PBMC in response to IR-95 with or without ConA, in agreement with earlier results. The CD3-enriched cells failed to produce detectable levels of either cytokine. By contrast, CD14-enriched cells produced both IL-1 and IL-6 when stimulated with IR-95 alone. The addition of CD3-enriched cells to CD14-enriched cells did not significantly affect the levels of IL-1 and IL-6 produced in response to IR-95. Thus, the ability of CD14-enriched cells to secrete significant levels of IL-1 and IL-6 when stimulated with IR-95 alone supports the hypothesis that IR-95 exerts a direct effect on this accessory cell population.

Example 5: Effect of IR-95 on expression of ICAM-1:

In addition to secreting soluble cytokines such as IL-1 and IL-6, accessory cells also provide costimulatory signals to T-cells via adhesion molecules such as ICAM-1. To examine the issue of adhesion molecule expression, ICAM-1 expression and accessory function of THP-1 cells pulsed with IR-95 was studied. THP-1 cells were cultured with and without involved IR-95 overnight, then stained with fluorochrome-labelled antibodies to various cell surface molecules. Cells were then harvested, washed, and analyzed for ICAM-1 expression by flow cytometry, and for accessory function by their ability to restore ConA responsiveness to purified CD3+ cells. A fluorescence histogram of ICAM-1 expression was obtained, detected by staining the cells with PE-labelled anti-CD54 (Becton Dickinson), followed by analysis on a BD FACScan using LYSIS II software. $^{3}$H-thymidine incorporation by ConA-stimulated CD3+ cells cocultured for 48 hours with THP-1 cells that had been previously pulsed overnight with and without IR-95 was measured. Of the adhesion molecules examined, only ICAM-1 appeared to be influenced by IR-95. At doses of IR-95 ranging from 2 to 20 mg/ml, ICAM-1 expression was increased up to 4-fold in 24 hours, while other important adhesion molecules, including B7 and LFA-1, were not affected.

Due to the importance of ICAM-1 and other adhesion molecules in T-cell costimulation, we investigated whether the IR-95-induced upregulation of ICAM-1 expression resulted in a functional change in the THP-1 cells. Following an overnight culture in the presence or absence of IR-95, THP-1 cells were harvested and washed, then cocultured with purified CD3+ PBMC and various doses of ConA for 48 hours. Proliferation of the CD3+ cells was measured as described.

THP-1 cells possess endogeneous accessory function, demonstrated by their ability to support ConA-induced T-cell activation, and this function is apparently increased

following culture with IR-95. $^{3}$H-thymidine incorporation by the responding T-cells increased approximately 2-fold when the THP-1 cells had been pulsed overnight with IR-95, compared to THP-1 cells not pulsed with IR-95. This effect is probably not due to secretion of soluble costimulatory molecules such as IL-6, since it was seen when the THP-1 cells were fixed with formalin as well as when they were treated with mitomycin C prior to addition to the CD3+ cultures. Thus, the upregulation of ICAM-1 expression by IR-95 correlates with an increased accessory function of THP-1 cells.

Example 6: The effect of IR-95 on cytokine production in peripheral blood lymphocytes:

The effect of IR-95 on immune cell activation has been examined *in vitro* and the effect of IR-95 (preparation PS-1 and preparation Mg-1) on lymphokine secretion by human peripheral blood lymphocytes (PBL) was studied. Peripheral blood lymphocytes (PBL) were isolated by Ficoll gradient centrifugation of blood obtained from healthy volunteers. The PBL ($2x10^{6}$ cells/well in 24 well plates in a final volume of 1 ml) were cultured with various doses of Concanavalin A (ConA, potent T-cell mitogen), and purified IR-95, either separately or in combination. Unless otherwise indicated supernatants from the PBL cultures were harvested after 48 hours and stored at -80°C until analyzed for cytokine content. The cytokines measured were IL-1, IL-2, and IL-6. IL-1 and IL-6 are primarily derived from macrophages and other accessory cells and are vital in achieving full activation of T-cells. Il-2 is produced by activated T-helper cells.

The sensitivity of PBL response was also examined by utilizing lower doses of ConA, in conjunction with IR-95 (prep. PS-1). The response of PBL to doses of ConA as low as 0.62 ug/ml was enhanced by the addition of IR-95. This was true for all three cytokines measured (IL-1, IL-2, and IL-6) in the PBL supernatants. At a ConA dose of 1.25

ug/ml, which was insufficient to achieve T-cell activation, the addition of IR-95 resulted in T-cell activation, as determined by the production of IL-2.

Cytokine Bioassay. IL-2: Samples and controls were incubated with the IL-2-dependent cell line HT-2 overnight. The plates were pulsed with MTT for 4 hours, and the resulting formazan crystals were solubilized with acidified isopropanol. Optical density was measured on a dual wavelength microplate reader (sample wavelength = 570 nm, reference wavelength = 630nm). Concentrations of IL-2 in the samples were determined by comparison to a standard curve by regression analyses.

IL-6: Samples and controls were incubated with the IL-6-dependent cell line 7TD1 for 4 days, then processed as described in the IL-2 assay.

IL-1: Samples and controls were incubated with IL-1-sensitive cell line A375.52 for 3 days. Non-adherent (growth-inhibited) cells were washed off prior to pulsing with MTT and processed as described for the IL-2 assay above.

Results

The results demonstrate the effect of IR-95 on cytokine production by PBL. At suboptimal doses of the T-cell mitogen ConA (up to 10 ug/ml), IR-95 preparation PS-1 increased the production of the T-cell cytokine IL-2. This response was dose dependent with respect to both ConA and IR-95, suggesting that the mitogen and IR-95 were acting in a synergistic manner to achieve an enhanced T-cell response. IL-6 secretion was enhanced in response to IR-95 alone, without the addition of the T-cell mitogen ConA, suggesting that the effect of IR-95 is on accessory cell, and not directly on the T-cell. Production of IL-1 was also enhanced by the addition of IR-95 to the cultures.

To ensure that the effect noted in the previous experiment was not restricted to one purified batch of

material, the experiment was repeated with fresh PBL and two separate purified batches of IR-95. The IL-1 and IL-6 responses of PBL were enhanced by the addition of either batch (PS-1 or Mg-1) of purified IR-95. The magnitude of the responses were very similar regardless of which batch of material was employed, suggesting that the results obtained are consistent from batch to batch and therefore are not an artifact of the purification process.

The sensitivity of the PBL response was examined by utilizing lower doses of ConA, in conjunction with IR-95 (prep. PS-1). The response of PBL to doses of ConA as low as 0.62 ug/ml was enhanced by the addition of IR-95. This was true for all three cytokines measured (IL-1, IL-2, and IL-6) in the PBL supernatants. In the absence of IR-95, ConA at a dose of 1.25 ug/ml does not achieve T-cell activation as determined by the production of IL-2. However, a significant T-cell activation can be achieved in the presence of 1.25 ug/ml ConA when IR-95 is also present at a dose of 20 ug/ml.

Although the magnitude of the responses vary from one experiment to the next, the pattern is very clear; IR-95 increases the production of IL-1 and IL-6 by human PBL, and acts synergistically with ConA to increase the production of IL-2. Taken together, these data indicate that IR-95 provides an important co-stimulatory signal in T-cell activation.

Example 7: The effect of IR-95 on tumor growth in nude mice.

Materials and Methods

The human IR-95 cDNA was subcloned into the retroviral pLXSN vector (Miller, A.D. and Rosman, G.J., *Biotechniques* 7, 980-992 (1989)). The latter plasmid was transfected into the helper virus-free packaging cell line PA 317 by calcium phosphate precipitation (Chen and Okayama, *Mol. Cell. Biol.*, 7:2745-2752, 1987). After 48

hours, supernatants containing amphotropic virus were collected and used to infect GP86 secondary packaging cells in the presence of 8 ug/ml polybrene. Cells were then selected with G418 (1 mg/ml) and clones were isolated.

The effect of IR-95 on tumor growth in nude mice was studied. Female nude mice were divided into 4 groups of 4 individuals. Each individual in groups 1, 2, and 3 was implanted with clones of murine cell line 6378 which express human IR-95 ($2 \times 10^7$ cells subcutaneously / mouse). The animals in group 4 were implanted with 6378 cells transfected with the control expression vector. The tumor volume in each mouse was measured twice a week and the tumor growth curve determined. Twenty-three days post implantation, the tumor volume in mice implanted with cells expressing IR-95 was reduced by 40% (group 1), and 70% (groups 2 and 3) as compared to the control.

Murine 6378 cells (derived from mouse mammary gland carcinoma) that do not express IR-95 were infected 4 times, about 4 h each time, with 1 ml viral supernatant (titer = $1 \times 10^6$). The cells were then selected with G418 for 15 days and clones were isolated, expanded, and tested for expression. Expression was tested by labeling cells with [$^{35}$S]-methionine *in vivo* for 16 hours, and immunoprecipitation was performed using a specific monoclonal antibody directed against IR-95.

Three clones which expressed high amounts of human IR-95 were chosen for tumor growth experiments. Female nude mice, 4 to 5 weeks old, were divided into 4 groups of 4 individuals. Each individual in groups 1, 2 and 3 was subcutaneously implanted with one of the high expression clones of murine 6378 cells ($2 \times 10^7$ cells / mouse). The animals in group 4 were implanted with 6378 cells infected with the control expression vector. Tumor volume was measured twice a week and the tumor growth curve determined.

Results

Mice in all four groups developed tumors. However, mice implanted with 6378 cells expressing human IR-95 showed significantly slower tumor growth than mice implanted with control transfected 6378 cells which do not produce human IR-95. Twenty-three days post implantation, the tumor volume in mice implanted with cells expressing IR-95 was reduced by 40% (group 1), and 70% (group 2, 3) as compared to the control group.

Example 8: Correlation Between IR-95 Expression and Tumor Formation.

In view of various observations and the demonstration of IR-95 as a stimulator of natural killer (NK) cell activity suggested that this protein represents a molecular alarm signal for the cellular immune defense system against cancer cells and possibly virus infections[2]. We further demonstrate IR-95-mediated suppression of tumorigenicity of mammary carcinoma and glioblastoma cell lines in nude mice. Tumor growth inhibition is achieved by both local and systemic increases in IR-95 levels.

A panel of cell lines derived from different tumor types was characterized with respect to IR-95 expression and their ability to form solid tumors in nude mice. As shown in Table 1, while about 90% of the cell lines that displayed either no detectable or low levels of IR-95 expression formed tumors 10 to 15 days after subcutaneous implantation of $2 \times 10^6$ cells into nude mice, over 60% of the lines that expressed comparatively high levels of IR-95 were not tumorigenic under identical experimental conditions. Mice inoculated with these cells remained tumor-free for at least six weeks. This effect was independent of the tissue origin and the species from which the original tumor was derived. These observations suggested that high level IR-95 expression by tumor cells suppressed tumor formation in nude mice, which, despite their lack of T cells, still possess functional macro-

phages and NK cells, primary mediators of cell-mediated immunity.

Example 9: Retroviral Induced IR-95 Secretion.

For further investigation of the role of IR-95 in tumor suppression, we employed a replication-defective retrovirus containing cDNA coding for human IR-95 under the transcriptional control of mouse sarcoma virus 3' long terminal repeat[9] for the modulation of IR-95 expression on several cancer cell lines, MM5 and B-29, were infected with recombinant LXSN-IR-95 virus or the parental LXSN virus as a control. While neither cell line had detectable endogenous mRNA or IR-95 protein, high levels of secreted IR-95 were detected in the conditioned media after infection with the LXSN-IR-95 virus at a multiplicity of infection (MOI) of 5. For MM5 and B-29 cells, clonal (MM5/90-1, MM5/90-2, B-29/90-1, B-29/90-2) as well as polyclonal (B-29/90P) IR-95-expressing lines were established after LXSN-IR-95 virus infection by selection for neomycin resistance. No induction of IR-95 expression was detected for parental LXSN virus-infected and subsequently G418-selected cells.

Infection of the mouse mammary tumor cell lines with LXSN-IR-95 virus and the thereby enhanced level of human IR-95 production did not affect any of the characteristics of neoplastic transformation, including growth in low serum, loss of contact inhibition, or anchorage-independent growth, as measured by colony formation in semisolid media.

IR-95 protein was produced by retrovirus-infected tumor cells. MM5 and B-29 cells (ATCC, CRL 6444 and CRL 6325, respectively) were infected with the ecotropic retrovirus LXSN-IR-95 or the control virus LXSN. 80% confluent cells seeded in 6-cm dishes were labeled with [$^{35}$S]methionine in 0.1% dialysed FBS. Since IR-95 is secreted into the medium, the conditioned media of the labeled cells was analyzed for the presence of IR-95

protein by immunoprecipitation with the monoclonal antibody 19B5, SDS-polyacrylamide gel electrophoresis, and autoradiography.

METHODS: Generation of the recombinant retrovirus. The IR-95 coding sequence was isolated from a Bluescript ksII vector, containing the full-length IR-95 cDNA[2], as a EcoRV/XhoI restriction fragment and ligated into the retroviral vector pLXSN[9], which was cut with HpaI and XhoI. For the production of recombinant virus stocks, the helper virus-free packaging cell line PA317[20] was transfected with pLXSN-IR-95. The low titer amphotropic viruses produced were used to infect GP+E-86 secondary packaging cells[21]. The GP+E-86 producer clones were obtained after G418 selection.

Retrovirus-mediated gene transfer was performed as follows: Mouse mammary carcinoma cell lines were seeded at a density of $10^5$ cells per 6 cm dish. Cells were incubated with retrovirus-containing culture supernatant from GP+E-86 producer line clones for four hours in the presence of polybrene (4$\mu$/ml; Aldrich) and at a MOI of 5. The infection cycle was repeated up to four time in order to achieve high IR-95 protein levels. After G418 selection, clones and/or polyclonal populations of cells were isolated.

MM5/90-1, MM5/90-2, B-29/90-1, and B-29/90-2 are clonal lines, and B-29/90p is a polyclonal line of B-29 cells infected with LXSN-IR-95. MM5/LXSN and B-29/LXSN are polyclonal kines of MM5 and B-29 cells infected with the control virus LXSN.

Example 10: In Vivo Tumor Inhibition By IR-95

To investigate whether IR-95 expression influenced the ability of MM5 and B-29 cells to form solid tumors *in vivo*, we subcutaneously injected 2 x $10^6$ cells of the clonal and polyclonal lines above the right hind legs of nude mice. In parallel, the same number of cells infected with LXSN virus only were implanted into the animals of

the control group. The growth of developing tumors was subsequently measured at regular intervals. After a lag phase of 10 to 15 days, mice implanted with LXSN control virus-infected MM% or B-29 cells developed tumors, which increased in volume at an exponential rate (Fig. 1a,b). The same mammary carcinoma cells infected with LXSN-IR-95 retrovirus, however, were significantly inhibited in their ability to form solid tumors. This was true for G418 selected clonal lines B-29/90-1 (Fig. 1b) and B-29/90-2, the polyclonal line B-29/90P (Fig. 1b), and the tow independently established cell clones MM5/90-1 and MM5/90-2 (Fig. 1a).

For each of the experimental groups eight mice were implanted with LXSN or LXSN-IR-95 virus-infected B-29 or MM5 cells, respectively. All animals in the control groups, together with six animals with LXSN-IR-95 virus-infected B-29 or MM5 cells, were sacrificed when the tumors of the control animals reached a volume of 2,500 $mm^3$. For both experimental groups of mice implanted with LXSN-IR-95 virus-infected cells, two animals were kept or further surveillance of tumor development. The two mice implanted with B-29/90-1 cells ectopically expressing high levels of human IR-95 remained tumor-free for over three months. While one of these subsequently developed a slow growing tumor, the two experimental animals implanted with MM5/90-1 and MM5/90-2 cells remained tumor-free for over four months after cell implantation.

In comparison with the control, average tumor growth in animals inoculated with IR-95-expressing B-29 cells was inhibited over 80-90% and MM5 cell tumorigenicity was suppressed by 70-80% upon infection with LXSN-IR-95 virus. The somewhat weaker suppression of tumor formation for MM5 cells correlated with relatively lower IR-95 expression levels in MM5/90-1 cells in comparison with B-29/90-1 cells, as determined by immunoblot analysis.

Example 11: In Vivo Tumor Inhibition By Local IR-95 Administration Other Than Tumor Cell Expression.

To investigate whether mediation of the IR-95 effect required expression of the protein by the tumor cell itself or whether an increased local concentration of the factor was sufficient for activation of a cellular immune defense, we coimplanted $10^5$ parental MM5 cells and $10^6$ IR-95-expressing MM5/90-1 cells into nude mice at the same site. As shown in Fig. 1c, tumor growth in animals inoculated with both cell lines above the right hind leg was reduced by about 60% when compared with animals int he control group implanted with the same amount of parental MM5 cells alone. Control animals implanted with IR-95-expressing MM5/90-1 cells alone showed an 80% suppression of tumor growth.

Example 12: In Vivo Tumor Inhibition By Systemic Administration.

Additional experiments addressed the question of whether a systemic increase in IR-95 levels caused by a source distant from a IR-95-deficient tumor would have an effect on its progression. For this purpose, we implanted IR-95-expressing MM5/90-1 cells and the parental MM5 cells on opposite sites of the same experimental animal (Fig. 1d). While MM5/90-1 cells exhibited the same significantly suppressed growth characteristics as in the previous experiments, the growth of the tumors derived from the parental cells was inhibited by 45% in these animals, indicating a clear systemic effect of elevated IR-95 levels in the serum of experimental animals. Analogous inhibition of tumor growth by elevated IR-95 levels as those described for MM5 and B-29 mouse mammary carcinoma cells was observed in experiments with the human blioblastoma cell lines SF763t and U87t after infection with LXSN-IR-95 virus (Fig. 1e).

Example 13: Immunohistochemical Analysis.

Tumors resulting from experiments with B-29 or MM5 controls, or IR-95-expressing B-29/90 or MM5/90 cells, were resected for immunohistochemical analysis. Positive staining with anti-human IR-95 antibodies were found in growth-inhibited tumor tissues of the experimental groups bearing IR-95-expressing cells but not in tumors resected from the control animals. A representative example is for tissues derived from B-29 or B-29/90-1 cell-implanted mice. In sections of the growth-suppressed tumors derived from LXSN-IR-95-infected cells, the presence of IR-95 protein was readily detectable with a monoclonal anti-IR-95 antibody. The staining was relatively uniform and diffuse throughout the tumors, as expected for a secreted protein. In contrast, parental B-29 tumor sections did not display any detectable IR-95 protein. Both control and growth-inhibited tumors showed a similar extent of macrophage infiltration. The possibility remains, however, of differences in macrophage activation state and differential infiltration of other cell types such as NK cells. Neovascularization was detected in both the control and the growth-inhibited tumors. In contrast to tumors derived from LXSN-infected cells, the endothelial cells of blood vessels in IR-95-expressing tissue implants were strongly positive for VCAM-1 and ICAM-1, as examined by immunohistochemical analysis of experimental tissue sections. This finding was subsequently confirmed by the demonstration of ICAM-1 induction *in vitro* by purified IR-95 protein.

While the expression of ICAM-1 and VCAM-1 by vascular endothelium, ICAM-1 expression is low but can be markedly induced by cytokines such as interleukin-1 (IL-1) tumor necrosis factor (TNF), and interferon-$\gamma$ (IFN-$\gamma$), and VCAM-1 is not expressed in resting endothelium, but may be induced by IL-1, IL-4, or TNF[14,15]. We observed induction of ICAM-1 and VCAM-1 in tumor endothelium by elevated local IR-95 concentrations. This may be mediated directly

by IR-95 or through cytokines secreted by accessory cells upon exposure to IR-95. Nevertheless, the increase of ICAM-1 and VCAM-1 expression could be a first step in IR-95 action, which would allow the margination of leukocytes on the vessel wall and migration into the tissue, via interaction of these cell adhesion proteins with their respective ligands, LFA-1 and VLA-4.

Immunohistochemical staining of tumor sections. Tumors were resected from animals which had been implanted with B-29/IR-95 cells or with control-infected cells. Sections were immunohistochemically stained with a monoclonal anti-IR-95 antibody or with monoclonal antibodies directed against VACAM an dICAM-1. IR-95 expression in IR-95-secreting tumors (B-29/90-1 cells), was measured, but at different magnifications. Tumor was resected from the control group, stained with anti-IR-95 antibodies; no positive staining could be detected. IR-95-secretin tumors stained with anti-ICAM-1 antibodies were observed at magnifications of 4 x, 63 x and 40 x.

METHODS: After sacrificing the animals, tumors were resected and embedded in tissue-tek (Miles) and frozen in liquid nitrogen. 5 um thick sections were cut with a cryostat (Leica) and used for immunohistochemical analysis. For the detection of IR-95 the monoclonal mouse antibody 19B5 was used, and ICAM-1 and VCAM were detected using specific biotin labelled monoclonal antibodies (Pharminge, San Diego). Staining was visualized by incubation with horseradish peroxidase-coupled strepavidin followed by 0.05% Diaminobenzidine and 0.03% hydrogen peroxide in PBS.

Table 1 shows the correlation between IR-95 expression and tumor formation in nude mice. Cell lines derived from different tumor types were tested for their ability to grow as xenografts in nude mice. The expression of IR-95 was determined with either Northern analysis and/or Western blotting. Unless designated otherwise, the cell lines are of human origin.

METHODS: For each cell line tested, 2 x $10^6$ to 1 x $10^7$ cells were subcutaneously implanted in nude mice above the right hind leg. For the determination of tumor formation, mice were observed for a period of at least six weeks. IR-95 expression levels of the lines grown in tissue culture were determined by Norther analysis and/or immunoblotting, using specific monoclonal and-IR-95 antibodies. -: no detectable IR-95 expression; +++: high IR-95 expression levels.

Table I. Correlation between 90K expression and tumour formation in nude mice

| Cell Line | Tumour Type | 90K | Tumour Formation |
|---|---|---|---|
| SF763t | glioblastoma | - | + |
| U87t | glioblastoma | - | + |
| U87MG | glioblastoma | -/+ | + |
| U118MG | glioblastoma | -/+ | + |
| U138MG | glioblastoma | - | - |
| U373MG | glioblastoma | +++ | - |
| U1240 | glioblastoma | +++ | - |
| U1242 | glioblastoma | +++ | - |
| T98G | glioblastoma | +++ | - |
| A172 | glioblastoma | +++ | - |
| 9L | glioblastoma (rat) | -/+ | + |
| C6 | glioblastoma (rat) | -/+ | + |
| SKOV-3 | ovarian carcinoma | -/+ | + |
| Ovcar-3 | ovarian carcinoma | -/+ | + |
| HBL100 | mammary carcinoma | +++ | - |
| T-47D | mammary carcinoma | -/+ | + |
| MCF7 | mammary carcinoma | +++ | - |
| ZR-75-1 | mammary carcinoma | ++ | + |
| BT549 | mammary carcinoma | - | - |
| SKBR3 | mammary carcinoma | ++ | - |
| MDA231 | mammary carcinoma | ++ | + |
| B-29 | mammary carcinoma (mouse) | - | + |
| MM5 | mammary carcinoma (mouse) | - | + |
| MM2 MTC | mammary carcinoma (mouse) | ++ | - |
| Colo320 | colon carcinoma | -/+ | + |
| A431 | epidermoid carcinoma | -/+ | + |
| Calu3 | lung carcinoma | -/+ | + |
| Calu6 | lung carcinoma | ++ | + |
| A549 | lung carcinoma | - | + |
| PC3 | prostate carcinoma | +++ | + |

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All publications and patent applications mentioned in this specification are indicative of the level of skill of one in the art to which this invention pertains and are hereby incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, certain changes and modifications may be practiced within the scope of the appended claims. Modifications of the above-described modes for carrying out the invention that are apparent to persons of skill in the art, such as those in the fields of medicine, immunology, hybridoma technology, pharmacology, and/or related fields, are intended to be within the scope of the following claims. Other embodiments are within the appended claims.

Claims

1. A pharmaceutical composition comprising a therapeutically effective amount of IR-95 and a physiologically acceptable carrier or diluent.

2. The pharmaceutical composition of claim 1 wherein said therapeutically effective amount is between about 5 to about 5,000 mg/dose/week/patient.

3. The pharmaceutical composition of claim 2 wherein said therapeutically effective amount is between 50 and 500 mg/dose/week/patient.

4. The use of IR-95 in the manufacture of a medicant for the treatment of cancer, a bacterial infection, a parasitic infection or a viral infection.

5. A method of treating a disorder comprising administering a therapeutically effective amount of IR-95 to an organism in need of such treatment.

6. A pharmaceutical composition comprising a therapeutically effective amount of an IR-95 antagonist and a physiologically acceptable carrier or diluent.

7. Use of an IR-95 antagonist in the manufacture of a medicant for the treatment of an autoimmune disorder, rheumatoid arthritis, allergy, or organ transplant rejection.

8. A method of suppressing an immune response comprising administering a therapeutically effective amount of an IR-95 antagonist to an organism in need of such treatment.

9. A phamaceutical composition comprising nucleic acid encoding IR-95 and a physiologically acceptable carrier or diluent.

10. The use of nucleic acid encoding IR-95 in the manufacture of a medicant for the treatment of cancer, a bacterial infection, a parasitic infection or a viral infection.

11. A phamaceutical composition comprising cells capable of expressing IR-95 and a physiologically acceptable carrier or diluent.

12. The use of cells capable of expressing IR-95 in the manufacture of a medicant for the treatment of cancer, a bacterial infection, a parasitic infection or a viral infection.

13. A method of treating a disorder comprising implanting an organism in need of treatment with cells capable of expressing IR-95.

14. The method of claim 13 wherein the volume of a tumor in said organism is reduced or the growth rate of cells in said tumor is decreased.

15. The method of claim 14 wherein cytokine production in a blood cell is increased.

16. The method of claim 15 wherein said cytokine is IL-1 or IL-6.

17. The method of claim 16 wherein said IR-95 is present in a concentration of 10 mg/ml to 20 mg/ml.

18. A vector comprising nucleic acid encoding IR-95 within a liposome, said vector being adapted to cause expression of said IR-95.

19. The vector of claim 18, wherein expression of said IR-95 results in the production of functional IR-95.

20. The vector of claim 19, wherein said vector comprises a retroviral vector.

21. A transfected cell line containing a vector comprising nucleic acid encoding IR-95.

22. The transfected cell line of claim 21, wherein said IR-95 is expressed as a secreted protein.

23. A transformed cell line containing a vector comprising nucleic acid encoding IR-95.

24. A transgenic non-human animal containing IR-95.

25. The transgenic animal of claim 24, wherein said transgenic animal is a mammal.

26. The transgenic animal of claim 25, wherein said mammal is a mouse.

27. A method for introducing a continuous supply of IR-95 into a tissue culture, comprising the step of administering an effective amount of a vector into said tissue culture, said vector comprising nucleic acid encoding IR-95 and being adapted to cause expression of said IR-95.

28. A method for introducing a continuous supply of IR-95 into an animal, comprising the step of administering an effective amount of a vector into said animal, said

vector comprising nucleic acid encoding IR-95 and being adapted to cause expression of said IR-95.

29. A method of screening compounds for their pharmacological effects on an immune response comprising the steps of administering a compound to a transgenic animal expressing IR-95 and measuring said immune response in said transgenic animal.

30. A method of administering a nucleic acid sequence encoding a IR-95 to an animal comprising the steps of removing cells from said animal, transducing said cells with said IR-95 nucleic acid sequence, and reimplanting said transduced cells into said animal.

31. A method of treating a disorder comprising the steps of:

(a) inserting an expression vector comprising nucleic acid encoding IR-95 into a cell;

(b) growing said cell in vitro; and

(c) infusing said cell to an organism in need of treatment.

32. A method of administering a IR-95 nucleic acid sequence utilizing an *in vivo* approach comprising the steps of administering directly to an animal said IR-95 nucleic acid sequence selected from the group of methods of administration consisting of intravenous injection, intramuscular injection, or by catheterization and direct delivery of said IR-95 nucleic acid sequence via the blood vessels supplying a target organ.

33. The method of claim 32, wherein said target organ is selected from the group consisting of heart, skeletal muscle, adipose tissues, spleen, lung, brain, kidney, testis, adrenal or small intestine.

34. The method of claim 33, wherein said IR-95 nucleic acid sequence is administered as naked DNA.

35. The method of claim 32, wherein said IR-95 nucleic acid sequence is contained in a viral vector.

36. The method of claim 35, wherein said viral vector is selected from the group consisting of papovaviruses, adenovirus, vaccinia virus, adeno-associated virus, herpesviruses and retroviruses of avian, murine or human origin.

37. A method of administering a IR-95 nucleic acid sequence in a two-component system comprising the steps of administering a packaging cell, wherein said packaging cell produces a viral vector.

38. The method of claim 37, wherein said packaging cell is administered to cells *in vitro*.

39. A method of administering a IR-95 nucleic acid sequence comprising the step of administering to an animal an adenovirus vector, wherein an E1 region of said adenovirus vector is replaced with said IR-95 nucleic acid sequence and administering said adenovirus vector by a method of administration selected from the group consisting of intravenous injection, intramuscular injection, intraportal injection or intra-arterial injection.

40. A gene therapy product comprising a therapeutically effective amount of nucleic acid encoding IR-95 and a means for administering said nucleic acid.

41. A gene transfer method for administering nucleic acid encoding IR-95 to an organism.

42. A gene therapy method for administering a therapeutically effective amount of nucleic acid encoding IR-95 to an organism in need of such treatment.

43. A method of manufacturing a pharmaceutical composition comprising the steps of:

(a) inserting an expression vector comprising nucleic acid encoding IR-95, or a functional fragment thereof, into cells;

(b) culturing the cells in vitro; and

(c) harvesting the cells and combining the cells with a pharmaceutically acceptable carrier.

44. A pharmaceutical composition according to claim 9 wherein the nucleic acid encoding Ir-95 comprises a vector according to any of claims 18 to 20.

45. The use of a vector according to any of claims 18 to 20 in the manufacture of a medicament for the treatment of cancer, a bacterial infection or a viral infection.

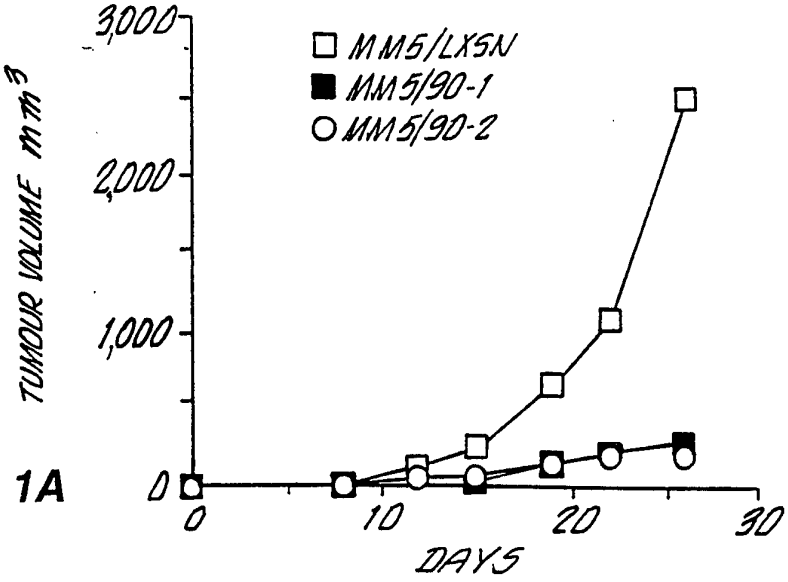


FIG. 1A

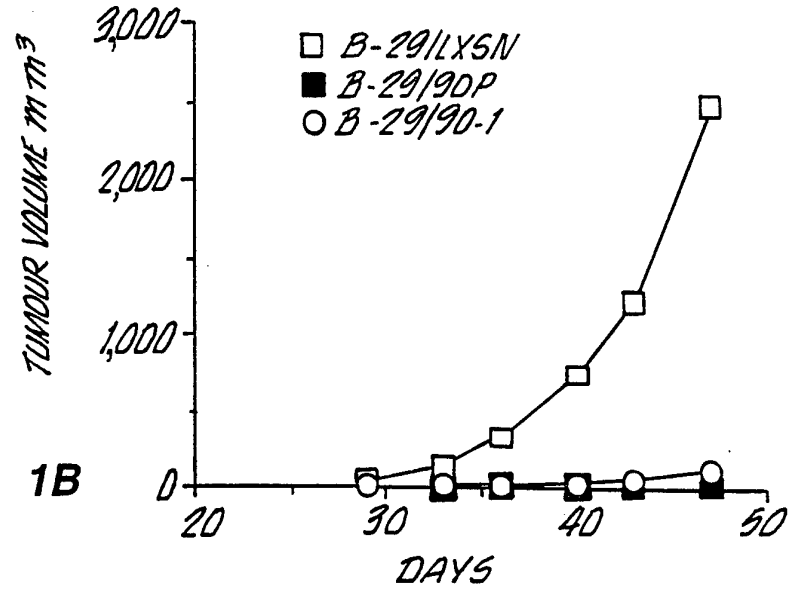


FIG. 1B

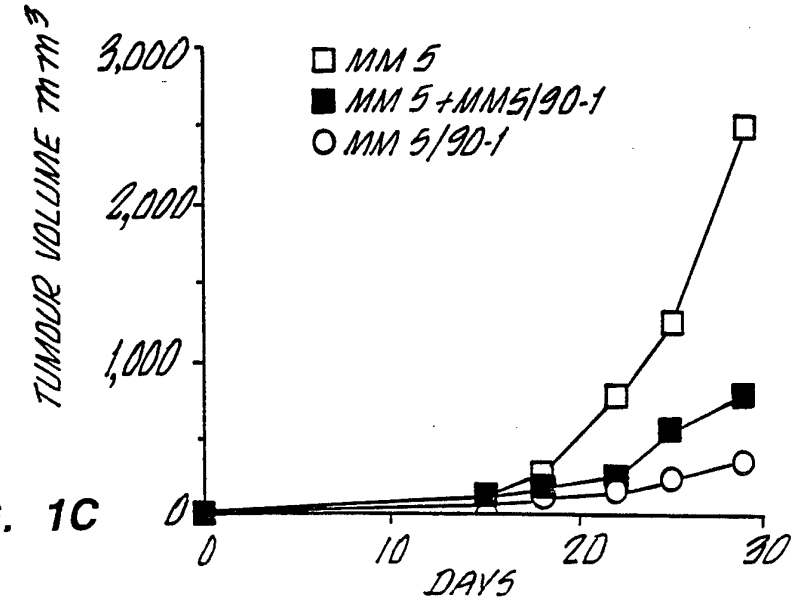


FIG. 1C

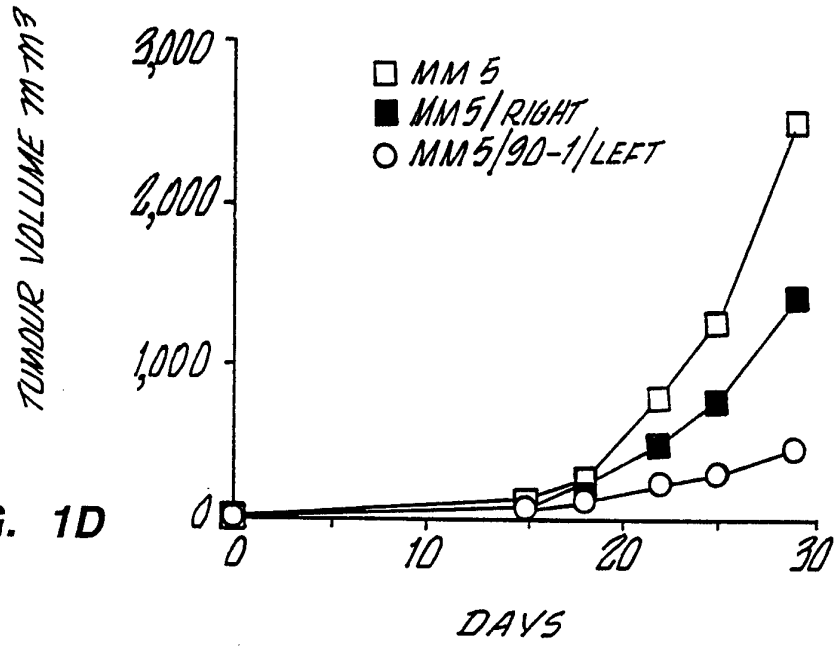
TUMOUR VOLUME MM3
3,000
2,000
1,000
0
MM 5
MM5/RIGHT
MM5/90-1/LEFT
0
10
20
30
DAYS

FIG. 1D

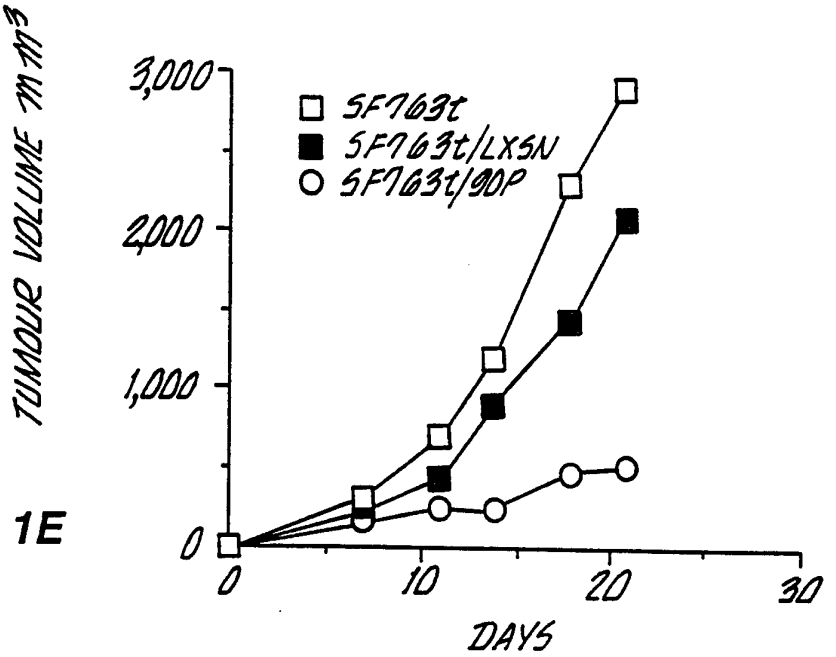
TUMOUR VOLUME MM3
3,000
2,000
1,000
0
SF763t
SF763t/LXSN
SF763t/90P
0
10
20
30
DAYS

FIG. 1E

# INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 94/12701

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/63 A61K38/17 A61K31/70 A61K48/00 C12N5/10
A01K67/027 C12N15/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K C12N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO,A,93 16181 (NEW YORK UNIVERSITY) 19 August 1993<br>cited in the application<br>see the whole document<br>--- | 1-8 |
| X | WO,A,93 16180 (MAX-PLANCK-GESELLSCHAFT ZUR FORDERUNG DER WISSENSCHAFTEN) 19 August 1993<br>cited in the application<br>see the whole document<br>---<br>-/-- | 9-45 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 15 February 1995 | 27. 02. 95 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Moreau, J |

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| P,X | JOURNAL OF BIOLOGICAL CHEMISTRY., vol.269, no.28, 15 July 1994, BALTIMORE, MD US pages 18401 - 18407 ULRICH A., ET AL. 'The Secreted Tumor-associated Antigen 90K is a Potent Immune Stimulator' cited in the application see the whole document | 1-8 |

# INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/US94/12701

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 5, 8, 13-17, 27, 28, 30-39, 41 and 42 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern al Application No
PCT/US 94/12701

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| WO-A-9316181 | 19-08-93 | AU-B- | 3497993 | 03-09-93 |
| | | AU-B- | 3629093 | 13-09-93 |
| | | CN-A- | 1076629 | 29-09-93 |
| | | WO-A- | 9317119 | 02-09-93 |
| WO-A-9316180 | 19-08-93 | AU-B- | 3497893 | 03-09-93 |
| | | CN-A- | 1076489 | 22-09-93 |