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(54) Title: ADENO-ASSOCIATED VIRAL LIPOSOMES AND THEIR USE IN TRANSFECTING DENDRITIC CELLS TO STIMULATE SPECIFIC IMMUNITY

(57) Abstract

A composition for genetic manipulation of cells comprises a liposome comprised of lipid material, and adeno-associated viral (AAV) material. Typically, the AAV material is plasmid, and comprises one or more terminal repeats of the AAV genome. Methods are disclosed for introducing DNA into cells using AAV/liposome complexes. The DNA is introduced and expressed in stem cells, T cells, primary tumor cells, tumor cell lines and dendritic cells or other antigen-presenting cells. Such transfected cells are used in therapeutic methods to treat subjects with cancer or microbial infections. Dendritic cells with DNA encoding tumor or viral antigens and are used to treat subjects with tumors or viral infections by administration *in vivo* or by activation of antigen-specific lymphocytes *ex vivo* followed by administration of those lymphocytes to the subject.

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ADENO-ASSOCIATED VIRAL LIPOSOMES AND THEIR USE IN TRANSFECTING DENDRITIC CELLS TO STIMULATE SPECIFIC IMMUNITY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from Provisional Application Nos. 60/001,312, filed July 21, 1995, and 60/007,184, filed November 1, 1995, and is a continuation-in-part of Application No. 08/566,286, filed December 1, 1995, which incorporates subject matter of said provisionals and also claims priority thereof; and this application is also a continuation-in-part of U.S. Application No. 08/305,221, filed September 12, 1994, which is a continuation-in-part of U.S. Application No. 08/120,605, filed September 13, 1993 (abandoned in favor of file wrapper continuation U.S. Application No. 08/482,323, filed June 6, 1995).

BACKGROUND OF THE INVENTION

Field of the Invention

The invention in the fields of molecular biology and medicine relates to improved methods and compositions for transfecting cells, particularly dendritic cells and other antigen presenting cells, through the use of cationic liposomes to facilitate transfection with adeno-associated viral (AAV) plasmids. Transfected cells expressing genes of interest, such as tumor-associated or viral antigens, are used for immunization and therapy.

Description of the Background Art

Transfection of eukaryotic cells has become an increasingly important technique for the study and development of gene therapy. Advances in gene therapy depend in large part upon the development of delivery systems capable of efficiently introducing DNA into a target cell. A number of methods have been developed for the stable or transient expression of heterologous genes in cultured cell types. These include transduction techniques which use a carrier molecule or virus.

Most gene therapy strategies have relied on transduction by transgene insertion into retroviral or DNA virus vectors. However, adenovirus and other DNA viral vectors can produce infectious sequelae, can be immunogenic after repeated administrations, and can only package a limited amount of insert DNA.

Of the viral vector systems, the recombinant adeno-associated viral (AAV) transduction system has proven to be one of the most efficient vector systems for stably and efficiently carrying genes into a variety of mammalian cell types (Lebkowski, J.S. *et al.*, *Mol. Cell. Biol.* (1988) *8*:3988-3996). It has been well-documented that AAV DNA integrates into cellular DNA as one to several tandem copies joined to cellular DNA through inverted terminal repeats (ITRs) of the viral DNA, and that the physical structure of integrated AAV genomes suggest that viral insertions usually appear as multiple copies with a tandem head to tail orientation via the AAV terminal repeats (Kotin, R.M. *et al.*, *Proc. Natl. Acad. Sci. USA* (1990) *87*:2211-2215). Thus, the AAV terminal repeats (ITRs) are an essential part of the AAV transduction system.

Although recombinant adeno-associated viral (AAV) vectors differ from adenoviral vectors, the transgene DNA size limitation and packaging properties are the same as with any other DNA viral vectors.

AAV is a linear single stranded DNA parvovirus, and requires co-infection by a second unrelated virus in order to achieve productive infection. AAV carries two sets of functional genes: *rep* genes, which are necessary for viral

replication, and structural capsid protein genes (Hermonat, P.L. *et al.*, *J. Virol.* (1984) *51*:329-339). The *rep* and capsid genes of AAV can be replaced by a desired DNA fragment to generate AAV plasmid DNA. Transcomplementation of *rep* and capsid genes are required to create a recombinant virus stock. Upon transduction using such virus stock, one recombinant virus uncoats in the nucleus and integrates into the host genome by its molecular ends.

Although extensive progress has been made, transduction techniques suffer from variable efficiency, significant concern about possible recombination with endogenous virus, cellular toxicity and host immune reactions. Thus, there is a need for non-viral DNA transfection procedures.

Liposomes have been used to encapsulate and deliver a variety of materials to cells, including nucleic acids and viral particles (Faller, D.V. *et al.*, *J. Virol.* (1984) *49*:269-272).

Preformed liposomes that contain synthetic cationic lipids have been shown to form stable complexes with polyanionic DNA (Felgner, P.L. *et al.*, *Proc. Natl. Acad. Sci. USA* (1987) *84*:7413-7417). Cationic liposomes, liposomes comprising some cationic lipid, that contain a membrane fusion-promoting lipid dioctadecyldimethyl-ammonium-bromide (DDAB) have efficiently transferred heterologous genes into eukaryotic cells (Rose, J.K. et al., *Biotechniques* (1991) *10*:520-525). Cationic liposomes can mediate high level cellular expression of transgenes, or mRNA, by delivering them into a variety of cultured cell lines (Malone, R. *et al.*,*Proc. Natl. Acad. Sci. USA* (1989) *86*:6077-6081).

Ecotropic and amphotropic packaged retroviral vectors have been shown to infect cultured cells in the presence of cationic liposomes, such as Lipofectin (BRL, Gaithersburg, MD), and in the absence of specific receptors (Innes, C.L. *et al.*, *J. Virol.* (1990) *64*:957-961).

Even though non-viral techniques have overcome some of the problems of the viral systems, the need still remains for improved transfection efficiency in non-viral systems, increased range of cell types that are transfectable, increased duration of expression in transfected cells, and

increased levels of expression following transfection. Some improved efficiency is attained by the use of promoter enhancer elements in the plasmid DNA constructs (Philip, R., *et al.*, *J. Biol. Chem.* (1993) *268*:16087-16090).

Immune Destruction of Tumor Cells

Interleukin-2 (IL-2) has been used to treat neoplasms such as metastatic renal cell carcinoma, as one approach to the immune-mediated destruction of human cancer. Although durable complete remissions have been achieved, the overall response rate has been low.

Testing of recombinant IL-2 (rIL-2) (Chiron Corp., Emeryville, CA) on cancer patients has revealed dose-limiting toxicity which was dependent upon the route and schedule of IL-2 administration. High dose bolus IL-2 administration was associated with significant toxicity involving nearly every organ system. Moreover, a 4% mortality rate in ECOG 0 performance status patients has been found with high dose IL-2. For an overview of ECOG performance status, see, *e.g.*, Oken, *Am. J. Clin. Oncol. (CCT)* 5:649-655 (1982), Table 2, at p. 654).

As distinguished from bolus administration, use of lower dose (1-7 x 10^6 Cetus units/M^2/day) continuous intravenous infusion of IL-2 has demonstrated clinical efficacy and lowered toxicity, suggesting an improved safety profile in adoptive immunotherapy of advanced cancer (West, W. H. *et al.*, (1987) *N. Engl. J. Med. 316*:898).

Cell populations which potentially mediate or promote the immune destruction of tumors when combined with IL-2 include lymphokine activated killer (LAK) cells and cytotoxic T lymphocytes (CTL) in particular tumor-infiltrating lymphocytes (TIL). TIL are primarily T lymphocytes found in close apposition to a tumor mass which can be isolated, expanded, and activated *in vitro*. TIL are of interest in the treatment of neoplasia because of their affinity and presumably their specificity for tumor cells as well as their cytotoxic action. TILs have been reinfused into patients along with exogenous IL-2 (see, *e.g.*, U.S. Patent No. 5,126,132, Rosenberg, 30 June 1992) which, in some instances,

resulted in durable complete remissions of advanced malignancies.

Dendritic Cells

Many attempts have been made to elicit immune responses in subjects that would lead to destruction and removal of unwanted cells, tissues or microorganisms, in particular, tumors or oncogenic viruses such as Epstein-Barr virus (EBV). However, success has been limited. One difficulty is in adequately presenting tumor-associated antigens to the immune system to evoke a cellular immune response. Dendritic cells ("DC"; plural is "DCs") are known to be highly potent antigen-presenting cells ("APC; plural is (APCs"). For a review of DCs and their role in immunogenicity, see Steinman, R., *Annu. Rev. Immunol.* 9:271-296 (1991), which reference is incorporated by reference in its entirety. The present invention employs such cells as a means to present tumor antigens to elicit a specific immune response *in vitro* or *in vivo*.

Antigen-specific CTL have been a subject of active investigation for their potential immunotherapeutic utility in the treatment of cancer or virus infection. Tumor-associated antigens (TAA), identified in a number of different types of tumors, including carcinomas can be used for *in vitro* generation of CTL with lytic activity against cells of the tumor. CEA (carcinoembryonic antigen) is a well-known TAA that is expressed on a majority of colorectal, gastric, pancreatic, non-small cell lung, and breast carcinomas. MART-1 is an example of an antigen associated with melanoma. The present invention targets CEA and melanoma antigens as well as other tumor-associated antigens.

SUMMARY OF THE INVENTION

Cationic liposomes are used to facilitate adeno-associated viral (AAV) plasmid transfections of primary and cultured cell types. AAV plasmid DNA complexed with liposomes results in several-fold higher levels of expression of the DNA than do complexes using standard conventional plasmids. In addition, expression lasts for a period of 30 days without any selection. AAV plasmid:liposome complexes induced levels of transgene expression comparable to those obtained by recombinant AAV transduction. High levels of gene expression were observed in freshly isolated $CD4^+$ and $CD8^+$ T cells, TILs and $CD34^+$ stem cells from normal human peripheral blood.

Primary breast, ovarian and lung tumor cells have been transfected using the AAV plasmid DNA:liposome complexes. Transfected tumor cells also expressed the transgene product after lethal irradiation. Transfection efficiency ranged from 10-50% as assessed by J-galactosidase (*J-gal*) gene expression. The ability to express transgenes in primary tumor cells is utilized to produce tumor vaccines and to generate lymphoid cells that permit highly specific modulations of the cellular immune response in cancer and AIDS, and in gene therapies.

Disclosed herein is a composition for genetic manipulation of host cells which comprises a liposome comprising lipid material and AAV material. The AAV material is preferably a plasmid, such as pMP6-IL2 or pACMV-IL2. The AAV material can comprise an inverted terminal repeat (ITR), or two or more ITRs. Where two ITRs are present in the AAV material, a DNA sequence (or "genetic material") of interest can be integrated between the two ITRs. Moreover, a promoter can be integrated between two ITRs. The promoter can be any of a number of promoters which are active in eukaryotic, preferably mammalian, more preferably human cells, such as a CMV immediate-early promoter, a CMV immediate-late promoter, a CMV early promoter, an ADA promoter, or a TK promoter. The composition preferably comprises a DNA sequence of interest, such as a an IL-2 gene or a *J-gal* gene. The lipid material can comprise a cationic lipid. Also provided are cells

transfected by the composition, including antigen-presenting cells, more preferably dendritic cells.

Disclosed herein is a method for introducing a genetic sequence of interest into a host cell. The method comprises steps of providing a composition comprising liposome AAV material and a genetic sequence of interest and contacting the composition with a host cell (which comprises genetic material) whereby the sequence of interest is introduced into the host cell. The host cell can be a $CD34^+$ stem cell, a T cell, such as a $CD3^+$, $CD4^+$, or $CD8^+$ cell, a cell of a tumor cell line such as a bladder cancer, prostate cancer or B lymphoma cell, or an embryonic kidney cell line. Alternatively, the tumor cell may be a primary tumor cell. The step of providing a composition can comprise providing a liposome that comprises cationic lipid. Also included is providing a composition of AAV material that comprises a plasmid, for example, pMP6-IL2 or pACMV-IL2. The method for introducing the genetic sequence of interest into a cell can further comprise a step of integrating the sequence of interest into the genetic material of the host cell.

Disclosed is a method for treating a subject, preferably a human. The treatment method comprises (a) providing a subject with a condition in need of treatment, and (b) providing a composition comprising liposome AAV material and a genetic sequence of interest. The method further comprises a step of contacting the composition with a host cell, whereby the genetic sequence of interest is introduced into the host cell. The contacting step can be *in vivo*, in which case the host cell is a cell of the subject. Alternatively, the contacting can be *ex vivo*, in which case the method further comprises a step of delivering the host cell which has been transfected with genetic sequence of interest to the subject. The subject of the present method is preferably one having a condition such as a neoplasm (including a malignant neoplasm), an infection, including HIV infection, an autoimmune condition or a genetic abnormality, such as a missing or defective gene.

The genetic sequence of interest may encode a peptide, an anti-sense oligonucleotide, or RNA. Preferred plasmids to

be provided include pMP6-IL2 or pACMV-IL2. The genetic sequence of interest comprises may encode a cytokine, including IL-2 (and may comprise IL-2 genomic DNA, a costimulatory factor, an MHC class I molecule, a tumor-specific or a tumor-associated antigen or the MDR I gene. When the method involves contacting the composition of the invention with a host cell, the host cell may be a neoplastic cell (including a primary tumor cell or a cell of a tumor cell line), a bone marrow hematopoietic cell, a peripheral blood cell or a TIL.

Other preferred plasmids have the pMP6 backbone, as described herein, with any other gene of interest inserted in place of the IL-2 gene of the pMP6-IL2 plasmid

A preferred expression vector comprises a genetic sequence essentially that depicted in Figure 3 (SEQ ID NO:1). Also preferred is an expression vector which comprises a genetic sequence substantially that of SEQ ID NO:1. In another embodiment, the expression vector comprises a genetic sequence which is SEQ ID NO:1. In a preferred embodiment, the expression vector comprises a genetic sequence essentially that of the genetic sequence of plasmid pMP6, or substantially that of the genetic sequence of plasmid pMP6 or the genetic sequence of plasmid pMP6. An expression vector comprising a genetic sequence essentially that of genetic sequence of plasmid pMP6 preferably further comprises a DNA sequence of interest to be introduced into a cell being transfected, for example a tumor cell or a DC.

Also provided is a cell that is genetically modified with an expression vector comprising a genetic sequence essentially that of SEQ ID NO:1 or with any of the expression vectors listed above. The genetically modified cell can be a cell of any of the categories described above.

The present invention provides a method for producing a protein comprising the steps of providing a composition comprising liposome, AAV material and a genetic sequence of interest. The foregoing composition is contacted with a host cell which comprises genetic material, whereby the genetic sequence of interest is introduced into the host cell. The protein production method further comprises a step of

expressing a protein encoded by the genetic sequence of interest. The host cell can be a $CD34^+$ stem cell, a T cell, a cell of a tumor cell line or a primary tumor cell, a TIL, or any $CD3^+$, $CD4^+$, or $CD8^+$ cell. When the cell is from a cell line, preferably a tumor cell line, it may be a bladder cancer cell, a prostate cancer cell, a B lymphoma, or a cell of an embryonic kidney cell line.

The composition provided in the protein production method, as described above, may comprises cationic lipid and AAV material. The AAV material preferably comprises a plasmid, such as pMP6-IL2 or pACMV-IL2.

The above method for producing a protein can comprise a further step of integrating the genetic material of interest into the genetic material of the host cell. The step of expressing a protein can comprise expressing a lymphokine, such as IL-2, a lymphokine analog, the product of the MDR-I gene or a marker or reporter product such as J-gal or chloramphenicol-acetyl-transferase (CAT)

Another objective of the present invention is to provide methods for generating tumor antigen-specific CTL for use in adoptive immunotherapy. An approach taken by the present inventors involved the use of DC to express and present the desired tumor antigen either by direct loading of the DC with the antigen or by transfection of DC with genetic constructs which will result in expression of the antigen. Such transfections are preferably accomplished using the methods and compositions described herein, namely AAV plasmid DNA (which includes DNA encoding the TAA) complexed with cationic liposomes. The AAV plasmid/cationic liposome methods and compositions are also used to express other antigens on DC, for example, viral antigens, preferably HIV antigens which serve as the target of an effective antiviral T cell or antibody response.

The invention is directed, in one aspect, to the expression of nonendogenous peptides or proteins, in particular TAA or a viral antigen, in or on the surface of DC. The invention is further directed to the use of DC as APCs to generate CTL capable of killing tumor cells or virus-infected cells bearing the antigen. The antigens are

provided to the DC either by pulsing the cells with the desired peptide or by transfecting the cells with a vector capable of expressing the desired antigen whereby the DC can appropriately present the peptide on its surface.

Given applicants' discovery that antigen presentation by DC cells to CTL provokes a very effective response by CTL, it is believed that a variety of other methods for providing a given protein or peptide antigen to a DC cell can be used to generate CTL capable of killing tumor cells or virus infected cells which are bearing the antigen. The DC are preferably used to stimulate a potent reactive lymphocyte population, preferably CTL, in culture, and such lymphocytes are then administered to the subject to effect treatment of a condition such as a tumor or virus infection. Alternatively or additionally, as a direct form of immunotherapy, the DC expressing the desired antigen in immunogenic form are administered to a subject and used to elicit a CTL response or other protective immune response *in vivo*.

The present invention provides a non-immortalized DC transfected by a vector including a DNA sequence not native to the DC. Also provided is a DC or other APC transfected by a composition comprising a liposome (which comprises lipid material) and AAV material. The AAV material preferably comprises a plasmid which preferably includes a DNA sequence of interest encoding one or more of a tumor-specific antigen, a tumor-associated antigen, a microbial antigen, a cytokine, a cellular receptor, a reporter molecule or a selectable marker. A preferred plasmid is pMP6 in which is inserted the DNA sequence of interest.

This invention is further directed to a method for introducing a DNA sequence of interest into a DC or other APC comprising the steps of:

(a) providing a composition comprising liposome, preferably cationic lipid, AAV material, preferably a plasmid, and a DNA sequence of interest; and

(b) contacting the composition of step (a) with the cell, which cell comprises genetic material, such that the DNA sequence of interest is introduced into the cell.

A preferred plasmid is pMP6 in which is inserted the DNA sequence of interest.

In the above method, the DNA sequence of interest preferably encodes one or more of a tumor-specific or tumor-associated antigen, a microbial antigen, a cytokine, a cellular receptor, a reporter molecule or a selectable marker. The tumor-specific or tumor-associated antigen may be carcinoembryonic antigen, a breast tumor antigen, a colorectal tumor antigen, a gastric tumor antigen, a pancreatic tumor antigen, a lung tumor antigen, an ovarian tumor antigen, a bladder tumor antigen, a prostate tumor antigen, a melanoma antigen, a leukemia antigen or a lymphoma antigen. The microbial antigen may be any viral or bacterial antigen. Preferably viral antigens are human retrovirus or human DNA virus antigens, preferably Epstein-Barr viral antigen or an HIV-1 or HIV-2 antigen. The cytokine is preferably IL-2. The receptor may be nerve growth factor receptor. A preferred reporter molecule is bacterial chloramphenicol acetyl transferase.

In the foregoing method, the DNA sequence of interest may or may not integrate into the genetic material of the transfected cell.

The present invention provides a method for treating a subject having a disease or condition which is treatable by stimulating an immune response to a selected antigen in the subject, which method comprises the steps of:

(a) contacting DCs or other APCs of the subject *in vivo* with a composition comprising liposome, adeno-associated virus material and a DNA sequence of interest which encodes the selected antigen such that the DNA sequence is introduced into the cells; and

(b) allowing the antigen encoded by the DNA sequence to be expressed and to stimulate the immune response of the subject,

thereby treating the subject.

In a related embodiment, the method comprises steps of:

(a) contacting dendritic cells or other antigen-presenting cells, which cells are autologous or allogeneic to the subject, *ex vivo* with a composition comprising liposome,

adeno-associated virus material and a DNA sequence of interest which encodes the selected antigen such that the DNA sequence is introduced into the cells;

(b) allowing the antigen encoded by the DNA sequence to be expressed in the cells; and

(c) delivering the cells expressing the antigen to the subject to stimulate the immune response, thereby treating the subject.

In yet another related embodiment, the method comprises the steps of:

(a) contacting dendritic cells or other antigen-presenting cells, which cells are autologous or allogeneic to the subject, *ex vivo* with a composition comprising liposome, adeno-associated virus material and a DNA sequence of interest which encodes the selected antigen such that the DNA sequence is introduced into the cells;

(b) allowing the antigen encoded by the DNA sequence to be expressed in the cells;

(c) activating lymphocytes *ex vivo* by contacting them with the cells expressing the antigen such that the lymphocytes become cytotoxic or otherwise specifically immunoreactive to host cells bearing the antigen; and

(d) delivering the activated lymphocytes to the subject to mediate the immune response in the subject, thereby treating the subject.

In the foregoing treatment methods, the condition or disease to be treated may be neoplasia or an infection. Examples of neoplasia which are treated by these methods include breast cancer, colorectal cancer, gastric cancer, pancreatic cancer, lung cancer, ovarian cancer, bladder cancer, prostate cancer, melanoma, leukemia and lymphoma. Infections which may be treated by these methods include infection with a human retrovirus, preferably HIV-1 or HIV-2, HTLV-1, HTLV-2 and the like, or a human DNA virus, preferably Epstein-Barr virus or other herpesviruses.

In the foregoing treatment methods, the DNA sequence of interest preferably encodes one or more of a tumor-specific antigen, a tumor-associated antigen or a microbial antigen. Tumor-associated or tumor-specific antigens include

carcinoembryonic antigen, a breast tumor antigen, a colorectal tumor antigen, a gastric tumor antigen, a pancreatic tumor antigen, a lung tumor antigen, an ovarian tumor antigen, a bladder tumor antigen, a prostate tumor antigen, a melanoma antigen, a leukemia antigen or a lymphoma antigen. Microbial antigens include bacterial or viral antigens, preferably an HIV antigen, for example, an epitope of a protein encoded by the HIV *gag*, *pol*, *env* or *nef* gene.

In the foregoing treatment methods, the DNA sequence of interest may further encode a cytokine, a costimulatory factor or an antigen of an MHC class I molecule.

This invention is also directed to a method for producing a protein in a dendritic cell comprising:

(a) introducing a DNA sequence encoding the protein into a dendritic cell; and

(b) allowing the DNA sequence to be expressed,

thereby producing the protein. The introducing is preferably performed by transfecting the cell with a composition comprising liposome, adeno-associated virus material and the DNA sequence, as described herein.

Another aspect of the present invention is the provision of a method for eliciting an immune response to a tumor-associated antigen comprising providing a modified dendritic cell bearing a selected tumor-associated antigen and contacting a cytolytic T cell with said dendritic cell bearing said tumor-associated antigen. The contacting can occur *in vivo* or *in vitro*. The term "modified" refers to a dendritic cell that has been changed so that it bears a tumor-associated antigen that has been selected based on the nature of the tumor in a patient to be treated.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows plasmid maps of three plasmids used in the present studies. The plasmid pACMV-IL2 contained the CMV promoter, IL-2 cDNA and rat preproinsulin and SV40 polyadenylation sequences identical to pBC12CMV/IL2 plasmid. pACMV-IL2 also had AAV inverted terminal repeats (ITRs) at both ends. The plasmid pA1CMVIX-CAT was constructed with a CMV promoter and CAT gene inserted between the two AAV ITRs.

Figure 2 shows a detailed restriction map of the IL-2-expressing form of the pMP6 plasmid, named pMP6-IL2.

Figures 3a-3e depicts the nucleotide sequence of the pMP6-IL2 plasmid. Panels 3a-3e depict successive portions of the sequence. Portions of the pMP6-IL2 sequence which correspond to known DNA sequences are indicated; the corresponding sequence information is listed directly beneath sequence information for the pMP6-IL2 plasmid. Unmarked sequences are from linkers.

Figure 4a and 4b are graphs depicting the levels of gene expression induced by plasmid DNA:liposome complexes. Various IL-2 plasmid constructs complexed with liposomes were tested for their capability to induce gene expression (expressed as pg/ml/10^6 cells) in a rat bladder cell line and a rat prostate cell line (Figure 4a). In both cell lines, the AAV plasmid construct showed the highest level of expression. Figure 4b depicts the time-course of gene expression induced by AAV plasmid:liposome complexes. For comparison, the prostate cell line was transfected with the AAV plasmid (pACMV-IL2) and the corresponding control plasmid (pBC12/CMV-IL2) complexed with liposomes. Supernatants were collected at various time points and assayed for IL-2 levels using an ELISA. IL-2 levels were expressed as pg/ml/10^6 cells in 24 hrs of culture.

Figures 5a-5b compare AAV plasmid:liposome complex-mediated transfection to recombinant AAV transduction. To determine whether the levels of gene expression induced by AAV plasmid:liposome complexes were equivalent to rAAV transduction, The prostate cell line (Figure 5a) and bladder cell line (Figure 5b) were used to compare the transfection and transduction of IL-2 gene. IL-2 levels expressed as pg/ml/10^6 cells in 24 hrs of culture were assessed using an ELISA.

Figure 6 is a graph showing expression of the IL-2 gene after lipofection with AAV plasmid:liposome complexes of various primary tumor cells. One lung, one ovarian, and two breast tumor samples were isolated from fresh tumor biopsies. IL-2 levels (pg/ml/10^6 cells in 24 hrs of culture) were measured using an ELISA.

Figure 7a and 7b are graphs showing expression of IL-2 by transfected cells which were subjected to lethal irradiation. To determine the effect of irradiation on gene expression, the prostate cell line (Figure 7a) and primary breast tumor cells (Figure 7b) were transfected as described herein and assessed for expression of the IL-2 gene (as in the preceding Figures) following lethal irradiation. Supernatants were collected 24, 48, 72 and 96 hrs after irradiation and tested for IL-2 levels.

Figure 8 is a graph showing efficiency (on a per cell basis) of AAV:liposome transfection measured by *J-gal* gene expression. The prostate tumor cell line was transfected as described herein. The results are presented as percent fluorescent cells (positive for J-gal).

Figures 9a-9d present thin layer chromatograms (TLC) made from transfected T lymphocytes. Blood was obtained from two donors referred to as A or B, from which T cells were isolated for transfection. These T cells were transfected using AAV plasmid DNA:liposome complexes. T lymphocytes were fractionated into various subpopulations using AIS MicroCELLector® devices as follows: $CD3^+$ (Figure 9a), $CD5^+/8^+$ (Figure 9b), $CD4^+$ (Figure 9c) or $CD8^+$ (Figure 9d). The relevant cells were captured and cultured as described herein. Thereafter, 5-10 x 10^6 cells were plated and transfected with 50 μg AAV plasmid DNA and 50 or 100 nmoles of liposomes to obtain 1:1 or 1:2 DNA:liposome ratios. The cells were harvested 3 days after transfection. Normalized amounts of protein content from the extracts were assayed for CAT activity using a TLC assay.

Figure 10 shows TLCs of peripheral blood $CD34^+$ stem cells transfected with AAV plasmid:liposomes. The cells were harvested on day 3 and day 7 after transfection. Normalized amounts of protein from the extracts were assayed for CAT activity using TLC.

Figures 11a-11b shows results of enhanced chemiluminescence (ECL) Southern analysis of genomic DNA from clones transfected with AAV plasmid DNA:liposome complexes. In Figure 11a, samples were digested with BamHI and HindIII and probed with IL-2 DNA. For Figure 11b, samples were

digested with BamHI and probed with IL-2 DNA. All clones analyzed show presence of IL-2 gene, as demonstrated by the 0.685 kb bands. The lanes in Figures 11a and 11b represent the following. Lane 1: 1kb ladder; lane 2: plasmid cut with BamHI/HindIII (9a) and BamHI/PvuII (9b); lane 3: R33 untransfected; lanes 4-11: clones

Figures 12a-12b show results of Southern analysis (^{32}P) of clone 1A11 and 1B11. After Southern blotting, the filter depicted in Figure 12a was probed with a 0.68kb IL2 Bam HI/Hind III fragment of pACMV-IL2. In Figure 12a: lane 1: clone 1A11 cut with Bam HI/HindIII; lane 2: clone 1B11 cut with BamHI/HindIII; lane 3: clone R33 cut with Bam HI/HindIII; lane 4: clone 1A11 cut with BamHI; lane 5: clone 1B11 cut with BamHI; lane 6: clone R33 cut with BamHI; lane 7: clone 1A11 cut with HindIII; lane 8: clone 1B11 cut with HindIII; lane 9: clone R33 cut with HindIII; lane 10: empty; lane 11: pACMV-IL2 plasmid cut with BamHI/HindIII; lane 12: pACMV-IL2 plasmid cut with HindIII/PvuII; lane 13: pACMV-IL2 plasmid cut with BamHI/PvuII. In Figure 12b, the filter was probed with a 0.85 kb pvuII/HindIII (AAV ITR/CMV) fragment of the plasmid pACMV-IL2. Lane 1: clone 1A11 cut with SmaI; lane 2: clone 1B11 cut with SmaI; lane 3: clone R33 cut with SmaI; lane 4: clone 1A11 cut with PvuII/HindIII; lane 5: clone 1B11, cut with PvuII/HindIII; lane 6: clone R33, cut with PvuII/HindIII; lane 7: pACMV-IL2, cut with BamHI/HindIII; lane 8: pACMV-IL2, cut with HindIII/PvuII; lane 9: pACMV-IL2, cut with SmaI; lane 10: 1kb ladder

Figure 13 shows the results of T cell receptor (TCR) repertoire analysis using RNAase protection of breast cancer TIL expanded with: (a) autologous tumor, (b) IL-2 transduced tumor, and (c) IL-2 alone. "VJ" is a variable segment of the TCR J chain; "CJ" is the constant segment of the TCR J chain. A, B, and C on the abscissa represent three different human subjects.

Figure 14 depicts the proliferation of TIL infiltrating a breast tumor measured 5 days after IL-2 gene transfection.

Figure 15 depicts the efficiency of gene expression in breast cancer TIL transfected with the pMP6 plasmid containing the neomycin resistance gene and the Thy 1.2 gene

(pMP6/neo/Thy1.2) (instead of the IL-2 gene). The pMP6/neo/Thy1.2 plasmid was complexed to DDAB:DOPE liposomes. The liposcme compositions were the same compositions as those used for Figure 14.

Figure 16 shows a comparison of the levels and duration of transgene expression following transfections with various plasmid constructs. The prostate tumor cell line R3327 was transfected with standard plasmid pBC12/CMV-IL2 or the AAV plasmid pACMV-IL2 complexed to DDAB:DOPE liposomes. Supernatants were collected at various time points and assayed by ELISA for IL-2 levels (expressed as pg/ml/10^6 cells in 24 hrs of culture).

Figure 17 shows Southern blot analysis of chromosomal DNA from R3327 cells transfected with either the AAV plasmid pACMV-IL2 or the standard plasmid pBC12/CMV-IL2. The blot was probed with the 0.685 kb BamHI/HindIII fragment of the IL-2 gene. The lane marked "C" contained DNA from untransfected cells. The IL-2 insert is shown in the last lane marked "plasmid".

Figure 18 shows results of an intracellular assay of the transfection efficiency of the IL-2 gene in prostate tumor cell line R3327. The cells were transfected with the AAV IL-2 plasmid complexed with DDAB:DOPE liposomes (1:1 or 1:2 ratios). Transfected cells were stained at various time points for intracellular IL-2 protein. The results show percent positive cells expressing IL-2 protein. Untransfected cells were used as negative controls and the values of controls were subtracted from the values of transfected groups.

Figure 19 shows expression of IL-2 by irradiated prostate tumor cell line cells (Panel A) and by irradiated primary breast tumor (Panel B). Tumor cells were transfected and assessed for gene expression after lethal irradiation. Supernatants were collected 24, 48, 72 and 96 hrs after irradiation and tested for IL-2 levels (pg/ml/10^6 cells in 24 hr culture).

Figure 20 shows a schematic diagram describing isolation of the dendritic cells.

Figure 21 is a schematic diagram showing the transfection of dendritic cells with a mixture of liposomes and plasmid DNA.

Figure 22 shows the results of the analysis of dendritic cells lipofected with an AAV plasmid containing the chloramphenicol acetyl transferase (CAT) reporter gene.

Figure 23 is a graph showing the expression of nerve growth factor receptor (NGFR) by dendritic cells lipofected with an AAV plasmid containing the NGFR gene.

Figure 24 shows production of IL-2 with dendritic cells lipofected as in Figure 23.

Figure 25 shows stimulation of T cell-mediated cytotoxicity by dendritic cells transfected with pMP6Neo vectors containing EBNA 3b and 3c.

Figure 26 shows the CEA-specific cytotoxic activity of $CD8^+$ T cells from a healthy $HLA\text{-}A2^+$ donor which were stimulated by CEA peptide-pulsed DC. Effector:target ratios are 80:1, 40:1, 20:1, 10:1.

Figure 27 shows the phenotype of the cells used in Figure 26. Cells stimulated with CEA-pulsed DC ("CEA") or with cytokines alone ("Cytokines") were stained with antibodies to CD3, CD4, CD8, and CD56.

Figure 28 shows the CEA-specific cytotoxic activity of $CD8^+$ T cells from a pancreatic cancer patient which were stimulated by CEA peptide-pulsed DC. Effector:target ratios are 80:1, 40:1, 20:1, 10:1. The background cytotoxicity on empty T2 cells has been subtracted.

Figure 29 shows the CEA-specific cytotoxic activity of $CD8^+$ T cells from an $HLA\text{-}A2^+$ breast cancer patient which were stimulated by CEA peptide-pulsed DC. Cytotoxic activity is tested on two target cells. SW403 is an $HLA\text{-}A2^+CEA^+$ cell line. SW1417 is an $HLA\text{-}A2^-CEA^+$ cell line. Effector:target ratios: 70:1, 35:1, 18:1, 9:1.

Figure 30 shows the background cytotoxic activity of $CD8^+$ T cells from an $HLA\text{-}A2^+$ breast cancer patient which were cultured with 10 ng/ml interleukin-7 (IL-7) without DC. Target cells and effector:target ratios are the same as in Figure 29.

Figure 31 shows the phenotype of the cells used in Figures 29 and 11. Cells stimulated with CEA-pulsed DC ("CEA") or with cytokines alone ("Cytokines") were stained with antibodies to CD3, CD4, CD8, and CD56.

Figure 32 shows the results of a flow cytometric analysis of the expression of the CEA gene transfected into of MCF7 cells using an anti-CEA antibody. The left panel shows untransfected cells. The middle panel shows CAT transfected cells. The right panel shows CEA-transfected cells.

Figures 33-35 summarize the "secondary" response of a melanoma patient's lymphocytes to the MART-1 peptide.

Figure 33 shows MART-1-specific cytotoxicity on T2 target cells pulsed with MART-1 peptide (T2-MART) or empty T2 cells (T2) at effector:target ratios of 60:1, 30:1, 15:1, or 7.5:1.

Figure 34 shows MART-1-specific cytotoxicity on an $A2^+MART\text{-}1^+$ melanoma line (624Mel) or an $A2^+MART\text{-}1^-$ melanoma line (A375) at E:T ratios of 60, 30, 15, or 7.5.

Figure 35 characterizes the phenotypes of effector cells stimulated with MART-1-loaded DC (DC-MART) or IL-7 only on the day of the cytotoxicity assay. Cells were stained with antibodies to CD3, CD4, CD8, and CD56. Greater than 90% of the effector cells are $CD3^+CD8^+$.

Figures 36-38 summarize primary responses of lymphocytes from a healthy donor to the MART-1 peptide

Figure 36 shows MART-1-specific cytotoxicity on T2 cells pulsed with MART-1 peptide (T2-MART) or empty T2 cells (T2) at effector:target ratios of 60 or 30.

Figure 37 shows MART-1-specific cytotoxicity on an $A2^+MART\text{-}1^+$ cell line (624Mel) or an $A2^+MART\text{-}1^-$ cell line (Colo) at E:T ratios of 60 or 30.

Figure 38 characterizes the phenotypes of effector cells stimulated with MART-1-loaded DC (DC-MART) or control unpulsed DC on the day of the cytotoxicity assay. Cells were stained with antibodies to CD3, CD4, CD8, and CD56. About 70% of the effector cells are $CD3^+CD8^+$.

Figure 39 is a schematic illustration of the genetic map of canarypox vector vCP300 which expresses *nef*, *gag*, *gp120* and *pol* genes of HIV.

Figure 40 shows the cytotoxic activity of effector cells stimulated in primary culture by autologous DC cells infected with canarypox construct vCP300 against target cells expressing env, gag/pol or nef antigens of HIV. The responder:stimulator ratio in culture was 10:1 and the effector:target ratio was 40:1. Control groups included cells stimulated by DC infected with a canarypox virus construct lacking HIV genes (vCPpp) or cells stimulated by the mitogen phytohemagglutinin (PHA).

Figure 41 summarizes a flow cytometry analysis of the HIV antigens expressed on DC of 4 healthy donors infected in culture by the vCP300 vector. Cells were stained with serum from HIV^+ donors or pooled control serum.

Figure 42 shows a general schematic of the plasmids containing CAT, CEA or MART-1 and the relationship of the plasmid DNA to the mRNA produced in the reverse transcriptase-polymerase chain reaction analysis of the transfected dendritic cells.

Figure 43 shows an analysis of the mRNA present in dendritic cells following transfection with either CAT, CEA or MART-1.

Figure 44a shows the response of cytolytic T cells (CTL) to MART-1 peptide loaded and empty target cells following exposure of the CTL to dendritic cells expressing the MART-1 gene. Figure 44b shows the response of CTL to MART-1 peptide loaded and empty target cells following cytokine stimulation only.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The studies, disclosed for the first time herein, examined the transportation into cells of AAV plasmid DNA employing a system that did not involve viral transduction. Alternatively, a method in accordance with the present disclosure efficiently transfected several mammalian cell types by use of liposomes comprising AAV material. The successful transfection utilizes the elegant carrier system

of lipofection together with the proficient transduction capability of the AAV plasmid construct. Advantageously, cationic liposomes were used as a means to facilitate the entry of AAV plasmid DNA into cells in the absence of *rep* and capsid transcomplementation, recombinant virus or wild type AAV.

A lipofection method in accordance with the invention was evaluated to assess the efficiency of gene expression. The present results established the ability to transfect unmodified stem cells, unmodified primary lymphoid cells such as T cells, a variety of freshly isolated tumor cells, and cultured mammalian cell types, with high efficiency leading to both transient and sustained expression of the transfected DNA. The ability to efficiently transfect unmodified T cells, such as tumor infiltrating lymphocytes, unmodified stem cells, tumor cell line cells and primary tumor cells is disclosed for the first time in the art.

Cell and gene therapy of cancer primarily depends on the ability to isolate and manipulate, *ex vivo*, the effector cell populations utilizing an efficient vector system. The present inventors have used the combination of cationic liposomes with plasmids containing the inverted terminal repeats (ITRs) of adeno-associated virus (AAV) to express transgenes in a variety of primary cell types including monocyte-derived DC. This vector system demonstrated higher levels of expression when compared to the standard plasmid, although equal amounts of DNA was delivered to the cells. DC from peripheral blood mononuclear cell (PBMC) populations were used successfully to generate peptide-specific responses. In addition, using AAV plasmid DNA containing reporter genes, which DNA was complexed to cationic liposomes, the present inventors have achieved transgene expression in DC with an efficiency of about 10-30 %.

SOURCE MATERIALS AND METHODS

A. Cell Lines

A rat prostate cell line (R3327) and rat bladder cell line (MBT-2) were obtained from Dr. Eli Gilboa, Duke University. Both cell lines were maintained in RPMI-1640

medium supplemented with 5% fetal bovine serum (FBS). Cell line 293 is a human embryonic kidney cell line that was transformed by adenovirus type 5, and was obtained from the ATCC (Graham, F.L., *et al.*, *J. Gen. Virol.* (1977) *36*:59-72). 293 cells were grown in Dulbecco modified eagle medium supplemented with 10% FBS.

B. Cell preparation of Primary Tumor Cells

Primary lung, ovarian and three breast tumor cells were obtained from solid tumors of patients. The tumor samples were minced into small pieces and digested in 200 ml of AIM V medium (Gibco), supplemented with 450 u/ml collagenase IV (Sigma), 10.8 K units/ml DNase I (Sigma), and 2000u/ml hyaluronidase V (Sigma) (Topolian, S.L. *et al.*, *J. Immunol. Methods* (1987) *102*:127-141). After 1-2 hours of digestion, cells were homogenized with a glass homogenizer (Bellco) and washed three times in DPBS-CMF (Whittaker). Lymphocytes were separated from non-lymphoid cells by capture on an AIS MicroCELLector-CD5/8® device (Applied Immune Sciences, Santa Clara, CA). Nonadherent cells (mainly tumor cells) were removed and cultured in RPMI 1640 medium supplemented with 2mM L glutamine, 100u/ml penicillin-streptomycin, and 10% FBS. Tumor cells were cultured for 2 to 4 weeks prior to transfection.

C. Preparation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) from healthy control patients were isolated from buffy coats (Stanford University Blood Bank, Stanford, CA) using Lymphoprep (Nycomed, Norway).

T cells, T cell subsets, or $CD34^+$ cells were further isolated using AIS MicroCELLectors (Applied Immune Sciences, Santa Clara, CA), devices comprising surfaces having covalently attached specific binding proteins (such as monoclonal antibodies) attached thereto. Briefly, PBMCs were resuspended at 15 x 10^6 cells per ml in 0.5% Gamimmune (Miles, Inc., Elkhart, IN) and loaded onto washed CD3, CD4, CD8, CD5/8, or CD34 AIS MicroCELLectors®. After 1 hour, nonadherent cells were removed from the AIS MicroCELLectors®. Complete medium, RPMI 1640 (Whittaker) containing 10% fetal bovine serum, 2mM L-glutamine, and 100 u/ml

penicillin/streptomycin was added to the adherent cells in the AIS MicroCELLectors. After 2-3 days in a 5% CO_2, 370C humidified environment, adherent cells were removed and prepared for transfection.

D. Plasmid Preparation

A first plasmid used in the present studies, pACMV-IL2, contained the human interleukin-2 gene (IL-2) as IL-2 cDNA, and the immediate-early promoter-enhancer element of the cytomegalovirus (CMV) and rat preproinsulin and SV40 polyadenylation sequences, flanked by AAV ITRs at both ends. This plasmid was obtained from and is available from Dr. J. Rosenblatt, UCLA, Los Angeles, CA and is also designated pSSV9/CMV-IL2). A corresponding control plasmid pBC12/CMV-IL2, which was identical to pACMV-IL2 but which lacked the AAV terminal repeats, was also used (see Figure 1).

The plasmid pA1CMVIX-CAT contained the CMV immediate-early promoter enhancer sequences, an intron derived from pOG44 (Stratagene), the bacterial CAT gene, SV40 late polyadenylation signal flanked by AAV terminal repeats in a pBR322 backbone (see Figure 1).

The plasmids pATK-Jgal and pAADA-Jgal contained the *J-gal* gene linked to either the TK or ADA promoter, respectively, in an AAV plasmid backbone. These plasmids were provided by Dr. Eli Gilboa, Duke University, Durham, NC.)

Another plasmid used in the present studies was pMP6. As shown in Figure 2, this plasmid containing IL-2 DNA (and designated pMP6-IL2) is double stranded and circular. pMP6-IL2 has the human IL-2 gene under the control of a CMV promoter and a SV40 polyadenylation signal. Between the promoter and the coding sequences of IL-2, there is an intron (derived from pOG44) which is understood to enhance the expression of IL-2 or any other exogenous gene placed into the plasmid. The entire expression cassette is between the left and right terminal repeat sequences of AAV. The pMP6-IL2 plasmid also has a Bluescript backbone having a Col-El bacterial origin of replication and an ampicillin resistance gene which facilitates the propagation of this plasmid in *E. coli*.

Figure 3a-3e shows the DNA sequence of the pMP6-IL2 plasmid (SEQ ID NO:1) depicted as successive portions of the sequence. Portions of the pMP6-IL2 sequence which correspond to known DNA sequences are indicated; the corresponding sequence information is listed directly beneath sequence information for the pMP6-IL2 plasmid. Unmarked sequences are from linkers.

Standard plasmids containing the IL-2 gene, but that did not contain AAV components were also used. The standard plasmid constructs carried the IL-2 gene, with an adenosine deaminase (ADA), a thymidine kinase (TK) or the immediate-late cytomegalovirus (CMV) promoter (standard plasmids obtained from ATCC). Selected plasmids are described in Table 1, below.

All plasmids were isolated by alkaline lysis and ammonium acetate precipitation, followed by treatment with DNase-free RNase, phenol/chloroform/isoamyl alcohol extractions and ammonium acetate precipitation (Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc. 1993)).

Table 1

Selected Plasmids Used in Present Studies

Plasmid Name	Promoter	Genomic Elements
pACMV-IL2	CMV (immediate-early)	IL2, AAV
pBC12/CMV-IL2	CMV (immediate-early)	IL2
pA1CMVIX-CAT	CMV (immediate-early)	CAT, AAV
pADA-IL2	ADA	IL2
pTK-IL2	TK	IL2
pCMV-IL2	CMV (immediate-late)	IL2
pATC-Jgal	TK	Jgal, AAV
pAADA-Jgal	ADA	Jgal, AAV
pMP6-IL2	CMV (early)	IL2, AAV

E. Liposome Preparation

Small unilamellar liposomes were prepared from the cationic lipid dioctadecyl-dimethylammonium-bromide (DDAB) (Sigma) in combination with the neutral lipid dioleoyl-phosphatidyl-ethanolamine (DOPE) (Avanti Polar Lipids). Lipids were dissolved in chloroform. DDAB was mixed with DOPE in either a 1:1 or 1:2 molar ratio in a round-bottomed flask, and the lipid mixture was dried on a rotary

evaporator. The lipid film was rehydrated by adding sterile double distilled water to yield a final concentration of 1mM DDAB. This solution was sonicated in a bath sonicator (Laboratory Supplies, Hicksville, NY) until clear. The liposomes were stored at 40C under argon gas. For *in vivo* use of liposomes via intravenous administration, a DDAB:DOPE ratio of 1:4 to 1:5 is used. For intraperitoneal administration, a DDAB:DOPE ratio of 1:1 to 1:2 is used.

F. Preparation of recombinant AAV (rAAV) for transduction with viral infection

For the preparation of recombinant AAV stocks, cells of the 293 cell line were split and grown to approximately 30-50% confluence. Thereupon, the cells were infected with adenovirus type 5 at a multiplicity of infection of 1 to 5, and incubated at 37°C. After 2 to 4 hours, the infected cells were cotransfected with 10 μg of a plasmid comprising a gene of interest and 10 μg of the *rep* capsid complementation plasmid, pΔBal, per 100 mm tissue culture dish (0.5-1 x 10^7 cells). Calcium phosphate coprecipitation was used for transfection (Hermonat, P.L. *et al.*, *Proc. Natl. Acad. Sci. USA* (1984) *81*:6466-6470). At 12 to 18 hours after transfection, the medium was removed from the cells and replaced with 5 ml of DMEM medium containing 10% FBS.

At 48 to 72 hours after transfection, AAV was harvested according to the following procedure: Cells and medium were collected together, and frozen and thawed three times to lyse the cells. The suspension of cells and medium was then centrifuged to remove cellular debris, and the supernatant was incubated at 560C for 1 hour to inactivate adenovirus (Hermonat, P.L. *et al.*, *supra*; Tratschin, J.D. *et al.*, *Mol. Cell. Biol.* (1985) *5*:3251-3260). After heat inactivation, the virus-containing supernatant was filtered through cellulose acetate filters (1.2 Tm). Viral stocks were then stored at -20°C. One ml of AAV supernatant was used to transduce 10^6 cells.

G. Cellular Transfection ("Lipofection")

For primary tumor cells and the rat tumor cell lines (R3327 and M3T-2), 10^6 cells were plated in 2 ml serum-free

media per well of a 6 well dish. Thereafter, 5 μg AAV plasmid DNA was mixed with 5 nmoles DDAB as liposomes composed of DDAB and DOPE in a 1:2 molar ratio, respectively. Serum-free media (0.5 ml) was added to the AAV:liposome complex, which was then transferred to the cells. To effect lipofection, the cells were incubated at room temperature for 5 minutes, then FBS was added to the cells to yield a final concentration of 5%.

For T cells, 5-10 x 10^6 cells were plated in 1 ml of serum-free medium per well of a 6 well dish. 50 μg plasmid DNA was mixed with 50 nmoles of DDAB as liposomes composed of DDAB and DOPE in a 1:1 molar ratio. The transfections ("lipofections") were then performed as for tumor cells.

For stem cells, 1-2 x 10^6 cells were transfected with complexes comprising 10 μg plasmid DNA and 10 nmoles liposomes. The transfected cells were cultured with media containing stem cell factor, interleukin-3 (IL-3) and interleukin-1 (IL-1). On days 3 and 7, the cells were harvested and extracts were made.

H. IL-2 Assay

Cells were counted, and 1 x 10^6 cells were plated in 1 ml per well of a 24 well plate. The following day, supernatants were collected and assessed using a Quantikine® IL-2 ELISA kit (R&D Systems, Minneapolis, MN). IL-2 levels were defined as pg/ml supernatant.

I. J-galactosidase Assay

The FluoReporter® *lacZ* gene fusion detection kit from Molecular Probes (Eugene, OR) was used to quantitate lacZ J-D-galactosidase in single cells by measurement of the fluorescence of the enzyme hydrolysis product, fluorescein. The AAV/J-gal plasmids (pATK-Jgal and pAADA-Jgal) were used with this kit. Fluorescein is produced by enzymatic cleavage of fluorescein di-J-D-galactopyranoside (FDG) in cells that express the marker gene *lacZ* encoding J-D-galactosidase. The cells were analyzed by flow cytometry (FACScan®, Becton Dickinson. San Jose. CA)

RESULTS

A. Level of IL-2 gene expression by use of AAV plasmid:cationic liposome complex

To evaluate the gene transfer efficiency of AAV plasmids, the IL-2 gene transfer efficiencies of AAV plasmids were compared to the efficiencies of standard plasmid constructs. The standard plasmids carried the IL-2 gene, with an adenosine deaminase (ADA) promoter (pADA-IL2), a thymidine kinase (TK) promoter (pTKIL-2), or the immediate-late cytomegalovirus (CMV) promoter (pCMV-IL2). An AAV IL-2 study plasmid (pACMV-IL2) contained the CMV promoter (immediate early), with the IL-2 gene placed downstream of the promoter (Figure 1). As shown in Figure 1, the corresponding control plasmid, pBC12/CMV-IL2, was identical to pACMV-IL2, but lacked the AAV ITRs.

For comparison, five plasmids (pACMV-IL2, pBC12/CMVIL2, pADA-IL2, pTK-IL2, pCMV-IL2) containing the IL-2 gene were complexed with liposomes and tested for transfection efficiency on the two cultured tumor cell lines: the rat bladder (MBT-2) and the rat prostate (R3327) cell lines. The cell lines were transfected with 10 μg of plasmid DNA complexed to 10 nmoles of liposomes per 1 x 10^6 cells. Supernatants were collected on day 3 and tested for the levels of IL-2 using an IL-2 ELISA kit.

The AAV plasmid (pACMV-IL2) induced the highest levels of expression in both cell lines (Figure 4a). The IL-2 gene with an ADA promoter (pADA-IL2) induced the least amount of expression in both cell lines. As shown in Figure 4a, both TK and CMV (immediate-late promoter) IL-2 constructs induced comparable levels of IL-2 expression in both cell lines. However, the pBC12/CMV-IL2 plasmid, which contained CMV immediate-early promoter showed higher levels of gene expression in the prostate cell line when compared to the bladder cell line. Among the plasmids tested, the AAV IL-2 study plasmid induced the highest level of expression in both cell lines, with a significant level of increase observed in the prostate cell line.

The duration of expression induced by the corresponding control plasmid (pBC12/CMV-IL2) and the AAV IL-2 study plasmid (pACMV-IL2) in the prostate cell line R3327 were studied (Figure 4b). Expression was assessed up to 30 days in these cultures without any selection. The cells were seeded at 10^6/ml and supernatants were collected for analysis every 24 hours. The cells doubled every 48 hours in culture. The data in Figure 4b indicate that, in addition to the enhanced levels of expression, the duration of expression lasted 30 days post-transfection with AAV plasmid (pACMV-IL2). Notably, significant expression continued throughout the full duration of the time period of evaluation. As shown in Figure 4b, both plasmids induced maximum levels of expression between day 2 and day 7, by day 15 IL-2 levels declined and then were maintained at approximately 100 pg/ml only in the AAV plasmid transfected group. Similar, sustained levels of expression were observed in the bladder cell line, as well as with cells from primary lung, breast and ovarian tumor, when AAV plasmid:liposome complexes were used for transfection.

B. Comparison of AAV plasmid:liposome transfection "lipofection" and recombinant AAV transduction

The prostate and bladder cell lines were transfected and transduced, to determine whether optimal AAV:liposome transfection was comparable to optimal recombinant AAV transduction. For optimal transfection, 10 μg of AAV plasmid DNA was complexed to 10 nmoles of liposomes per 10^6 cells in 2 ml final volume. For maximal rAAV transduction, 2 ml of the viral supernatant was added to 10^6 cells in 1 ml of complete media. After 24 hrs, the cells were washed and resuspended in fresh complete media. Supernatant fluids were collected at various time points after transfection and transduction.

In the prostate line (Figure 5a), transfection induced higher levels of expression than AAV transduction under test conditions (2 ml of viral supernatant for 10^6 cells, versus 10μg DNA:10 nmoles of liposomes). Although results on day 3 through day 5 showed approximately 10-fold higher levels of

IL-2 with transfection, by day 20 comparable levels were observed in both transfected and transduced groups.

Transduction with recombinant AAV initially induced higher levels of IL-2 production in the bladder cell line, as compared to transfection using liposomes (Figure 5b). Similar to the prostate cell line, transduction of the bladder cell line also showed a decline in IL-2 levels by Day 20, although IL-2 levels from transfection increased during this period; comparable levels of IL-2 were produced through Day 33 in both transfected and transduced groups.

C. Transfection of primary tumor cells using AAV plasmid DNA:liposome complexes

In the foregoing experiments, significant transgene expression was demonstrated in cultured cell lines. In order to assess whether cationic liposome:AAV plasmid DNA complexes also mediated comparable transgene expression in freshly isolated primary tumor cells, cells of four different primary tumors were transfected with AAV IL-2 plasmids using liposomes. Tumor cells were cultured in RPMI-1640 media supplemented with 10% FBS for 2-3 weeks prior to the transfection. The cells were plated at 10^6 cells per ml and transfected with 10 μg DNA complexed with 10 nmoles liposomes. Supernatants were collected on days 2 and 3.

As shown in Figure 6, all four primary cell types produced significant levels of IL-2 after transfection. The highest level of expression was observed on day 3 during the 10 day study period (lung and one breast sample were studied for longer periods). IL-2 gene expression was followed in cells of the lung tumor and in cells of one of the breast tumors as long as 25 days after transfection in culture. The levels on day 15 were equivalent (100 pg/ml IL-2) in both cell lines, and in the cells derived from primary tumors.

D. Effect of lethal irradiation on transgene expression

To determine the effect of irradiation on gene expression, the prostate cell line (Figure 7a) and cells of a primary breast tumor (Figure 7b) were transfected and assessed for gene expression after lethal irradiation. Both cell types were transfected using optimal AAV

plasmid:liposome complexes. On the second day after transfection, an aliquot of each culture was subjected to 6000 rad using a ^{60}Co irradiator, whereby cellular division is abolished, and the aliquots were then kept in culture. One-half of each culture was maintained as a non-irradiated control. The aliquots were subjected to 6000 rad using a ^{60}Co irradiator, while the expression level of IL-2 was approximately 300-400 pg/ml. Supernatants were collected 24, 48, 72 and 96 hrs after irradiation, and tested for IL-2 levels.

As shown in Figures 7a-7b, lethal irradiation after transfection did not inhibit transgene expression. Neither the prostate cell line nor the primary tumor cells exhibited any change in IL-2 expression after irradiation. Thus, although cellular division was abolished, IL-2 secretion was not sensitive to irradiation. This is advantageous, since many gene therapy strategies involve gene delivery to primary T lymphocytes (which do not generally divide absent activation) and often cannot be transduced via viral infection.

E. Level of B-D-galactosidase gene expression by use of AAV plasmid:liposome complex

To demonstrate the expression levels on a per cell basis, the *J-gal* gene was used for transfection experiments. Each of two AAV J-gal plasmids (pATK-Jgal and pAADA-Jgal) were complexed with cationic liposomes and used for transfection of the prostate cell line. Ten μg pATK-Jgal or pAADA-Jgal plasmid DNA was complexed with 10 nmoles liposomes and the complexes used to transfect 10^6 cells in 2 ml volume. At various time points, approximately 5 x 10^5 cells were harvested and stained with fluorescent substrate FDG and analyzed by flow cytometry.

Maximum transgene expression was observed between day 7 and day 15 (Figure 8). Significant levels of J-gal activity were observed through Day 25. Flow cytometry analysis of J-gal$^+$ cells showed maximal levels of 10 to 50% transfection efficiency with both plasmid constructs. The levels declined

to 5 to 10% by day 25. The expression pattern and duration was similar to that of IL-2 expression as set forth above.

F. Transgene expression induced by AAV plasmid:liposome complex in freshly isolated peripheral blood T cell subpopulations

The effect of AAV plasmid:liposome complex in transfecting freshly isolated human peripheral blood T cell populations was examined. The gene for the CAT enzyme was used as the reporter gene in the pA1CMVIX-CAT plasmid (Figure 1). The pA1CMVIX-CAT constructs were made using the AAV backbone (pA1) with CMV immediate-early promoter enhancer sequences and CAT gene. Total and purified $CD4^+$ and $CD8^+$ subpopulations of T cells were used for transfections. Both total (CD3- or CD5/8-selected) and purified (CD4- or CD8-selected) subpopulations of T cells (Figure 9a-d), as well as $CD34^+$ stem cells (Figure 10; see Section G. below) showed significant levels of CAT gene expression.

Primary T cells freshly isolated from peripheral blood were tested for transgene expression using AAV plasmid DNA:liposome complexes. Results of thin layer chromatography (TLC) assays for CAT activity from $CD3^+$ T cells, CD5/8-selected T cells (total T cells), the $CD4^+$ T cells, and $CD8^+$ T cells are depicted in Figure 9a-9d, respectively.

T lymphocytes were fractionated as $CD3^+$, or $CD5/8^+$ or $CD4^+$ or $CD8^+$ populations using AIS MicroCELLector® devices. The relevant cells were captured and nonadherent cells were removed by washing. The adherent cells were removed from the devices after 2 days in culture with RPMI-1640 and 10% FBS. Five to 10 x 10^6 cells were plated and transfected with 50 μg AAV plasmid DNA and 50 or 100 nmoles liposomes to obtain 1:1 or 1:2 DNA:liposome ratios. The cells were harvested 3 days after transfection, the cell extracts were normalized by protein content and CAT activity was measured using a TLC assay. Blood was obtained from donors referred to as A or B. Peripheral blood of donor A or B was used to isolate the T cells, and for transfection.

As depicted in Figures 9a-9d, the lipid composition of the liposomes comprising AAV was varied, as was the ratio of

DNA to liposome. In the study of $CD3^+$ T cells (Figure 9a) cells from donor A were employed. For the studies of CD5/8 selected T cells (Figure 9b), $CD4^+$ T cells (Figure 9c), $CD8^+$ T cells (Figure 9d), and $CD34^+$ stem cells (Figure 10) were derived from two donors (A and B).

Table 2

Conditions Employed for Studies Depicted in Figure 9a

Condition	Parameters
1	pA1CMVIX-CAT + DDAB:DOPE (1:1), DNA:liposome ratio (1:1)
2	pA1CMVIX-CAT + DDAB:DOPE (1:1), DNA:liposome ratio (1:2)
3	pA1CMVIX-CAT + DDAB:DOPE (1:2), DNA:liposome ratio (1:1)
4	pA1CMVIX-CAT + DDAB:DOPE (1:2), DNA:liposome ratio (1:2)

Table 3

Conditions Employed for Studies Depicted In Figures 9b-d

Condition	Parameters
1	pA1CMVIX-CAT + DDAB:DOPE (1:1), DNA:liposome ratio (1:1)
2	pA1CMVIX-CAT + DDAB:DOPE (1:1), DNA:liposome ratio (1:2)
3	pA1CMVIX-CAT + DDAB chol (1:1), DNA:liposome ratio (1:1)
4	pA1CMVIX-CAT + DDAB chol (1:1), DNA:liposome ratio (1:2)

Table 4

Condition Employed for Studies Depicted in Figure 10

Condition	Parameters
1	pA1CMVIX-CAT + DDAB:DOPE (1:1), DNA:liposome ratio (1:1)
2	pA1CMVIX-CAT + DDAB:DOPE (1:1), DNA:liposome ratio (1:2)

For the studies depicted in Figure 9a-d, maximum levels of expression were observed on days 2 and 3 in both total and purified subpopulations. Significant levels of expression were detected up to day 14. The cells were harvested 3 days after transfection, and normalized protein content from each extract was analyzed for CAT activity. The same composition of liposome, and the DNA:liposome ratio induced similar levels of expression in all the populations.

G. Transgene expression induced by AAV plasmid:liposome complex in freshly isolated $CD34^+$ stem cells

The effect of AAV plasmid:liposome complex in transfecting freshly isolated human peripheral blood $CD34^+$ stem cells was examined. The CAT gene was used as the reporter gene in the pA1CMVIX-CAT plasmid (Figure 1). The pA1CMVIXCAT constructs were prepared as described above. The level of CAT expression as determined by TLC from $CD34^+$ stem cells is shown in Figure 10.

Freshly isolated $CD34^+$ peripheral blood stem cells were transfected with AAV CAT plasmid DNA:liposome complexes. $CD34^+$ cells were purified from peripheral blood using AIS CD34 MicroCELLectors® after removing essentially all the T cells using CD5/8 MicroCELLector® device. The stem cells were removed from the device and 0.5 - 1 x 10^6 cells were transfected with complexes comprising 10μg plasmid DNA and 10 nmoles liposomes. The transfected cells were cultured with medium containing stem cell factor, IL-3 and IL-1. On Day 3 and 7, the cells were harvested and extracts were made. Normalized protein content from the extract was assayed for CAT activity. As shown in Figure 10, there were significant levels of CAT gene expression in the $CD34^+$ peripheral blood stem cells.

H. Integration Studies

Figures 11a-11b illustrate enhanced chemiluminescence (ECL) Southern analyses of genomic DNA from stable clones (clones stable at least beyond day 30) that were transfected with AAV plasmid DNA:liposome complexes in accordance with the invention. Genomic DNA was isolated and analyzed using

the ECL direct nucleic acid labelling and detection system (Amersham). IL-2 probe was prepared from the 0.685 kb IL-2 gene from pACMV-IL2. After hybridization, the membrane was washed twice in 0.5x SSC/0.4% SDS at 550C for 10 minutes and twice in 2x SSC at room temperature for 5 minutes.

In Figure 11a, samples were digested with BamHI and HindIII and probed with IL-2. As shown in Figure 11a, all clones showed the presence of the IL-2 gene, as demonstrated by the 0.685 kb band in BamHI and HindIII digested genomic DNA.

For the results shown in Figure 11b, samples were digested with BamHI and probed with IL-2. Again, all clones showed IL-2 gene integration. As shown in Figure 11b, integration of IL-2 was demonstrated by the high molecular weight bands (between 1.6 and 2 kb), bands which is consistent with integration of the gene in conjunction with attached host genomic material obtained via digestion. The results in Figure 11b indicate that there was more than one integration site, since there were multiple high molecular weight bands in the BamHI digested genomic DNA. Furthermore, the integration site was shown to be in different locations in different clones, as demonstrated by the different size bands in the digested clones (Figure 11b).

Figures 12a-12b depict chromosomal DNA analyses, using a ^{32}P Southern assay, of two clones obtained from the present study. Nuclear DNA was isolated from the two IL-2 clones (1A11 and 1B11) using the Hirt fractionation protocol. As a negative control, total DNA was isolated from untransfected cells of the R3327 cell line. After restriction enzyme digestion, 10 μg of each sample, along with appropriate plasmid ccntrols, were loaded onto a 1% agarose gel, electrophoresed, denatured and transferred onto Hybond$^+$ membrane. The filters were hybridized overnight at 68°C with DNA fragments labelled with ^{32}P by random priming. The membranes were then washed twice at 68°C for 30 minutes each with 2x SSC, 0.1% SDS and 0.2x SSC, 0.1% SDS. Autoradiograms of these filters were exposed on x-ray film.

In the study shown in Figure 12a, the IL-2 gene was again used as the probe. After Southern blotting, the filter

was probed with a 0.685 kb IL2-BamHI/HindIII fragment of pACMV-IL2. The results indicate that the number of integrated copies of the IL-2 gene varied from clone to clone, as evidenced by the various densities of the 0.685 kb band in the digests of cells of the two clones (see description of this drawing, *supra*). Higher molecular weight bands were also demonstrated, consistent with integration of the IL-2 gene together with host genomic material obtained from the various digest protocols.

Figure 12b shows results of a study in which the filter was probed with a 0.85 kb PvuII/HindIII (AAV ITR/CMV) fragment of the plasmid pACMV-IL2. The presence of the 0.8 kb band in the SmaI and PvuII digested chromosomal DNA demonstrates the presence of the right AAV ITR. The presence of the 2.1 kb band in the SmaI and PvuII digested chromosomal DNA indicates the presence of the left AAV ITR in clone A.

EXAMPLES

A method in accordance with the invention utilizes liposomes comprising AAV viral material to deliver genes for cytokines, costimulatory molecules such as B7, and MHC class I antigens into a wide variety of cell types. For example, DNA encoding any of the foregoing proteins or peptides is delivered into primary tumor cells or tumor cell lines such that when this introduced DNA is expressed, the modified tumor cells serve as a tumor vaccine. Furthermore, DNA encoding any of the foregoing proteins or peptides is delivered into peripheral blood cells or bone marrow cells to treat hematologic or neoplastic diseases or conditions.

The present invention provides a method by which liposomes containing AAV viral material are used to deliver and express (a) genes encoding proteins or peptides, (b) anti-sense DNA or RNA oligonucleotides, or (c) RNA. Expression of such proteins/peptides, anti-sense oligonucleotides or RNA in a subject modulates the subject's immune response. The term "modulate" includes inducing, augmenting, suppressing or preventing the immune response.

Accordingly, HIV infection is treated by delivery of anti-sense oligonucleotides, RNA, or ribozymes that have been expressed in accordance with the invention.

Additionally, an anti-tumor immune response is modulated using peptides or RNA expressed in accordance with the invention to enhance reactivity to tumor-specific and/or tumor-associated antigens. Non-immunogenic or weakly immunogenic tumors are rendered immunogenic by delivery of DNA or RNA according to the invention such that the tumors induce a cytolytic T cell response or other forms of anti-tumor immunity *in vivo* and *in vitro*.

The method of this invention is used to deliver genes to primary lymphoid cells, including B lymphocytes or T lymphocytes. In another embodiment, genetic material is delivered to $CD34^+$ stem cells. The genes delivered to and expressed in the lymphoid cells or stem cells result in modified cells which are used to treat any of a variety of diseases and conditions including HIV infection, genetic defects, neoplasias, and autoimmunity. The conditions to be treated are those wherein expression of a gene of interest is desired, as is appreciated by one of ordinary skill in the art. For example, for treatment of a malignant neoplastic condition, the MDRI gene is delivered and expressed in cells of the tumor in accordance with the invention, resulting in a therapeutic effect.

In another example, $CD8^+$ cells are selected with AIS MicroCELLectors®. These $CD8^+$ cells are obtained, for example, from the peripheral blood of subjects infected with HIV or from tumor tissue of cancer patients. These T cells are then activated according to methods known in the art, such as by use of polyclonal T cell activating substances including IL2, phytohemagglutinin (PHA) and Concanavalin A (Con A). The activated $CD8^+$ T cells are grown in culture for about 20 days. Thereafter, the cells are transfected in accordance with the invention, with AAV:liposome complexes comprising IL-2 genomic material. The cells originally obtained from a given subject are returned to the subject after transfection according to this invention. As a result of the expressed IL-2 gene, the need for subsequent

administration of IL-2 to the patient to maintain cytotoxic T cell activity is reduced or eliminated, thereby reducing or eliminating the undesired side effects and possible lethal dose-related toxicity of IL-2 infusion.

A. Tumor Vaccination

Conventional tumor vaccination protocols employ nonproliferating neoplastic cells in which proliferation is prevented by exposure of the cells to radiation, chemical inhibitors or exposure to high pressure chambers. It is believed that the body is able to mount an effective antitumor response to neoplastic cells present in the body (apart from initial tumor masses or foci).

Past tumor vaccination trials have used genetically-modified tumor (GMT) in efforts to enhance tumor cell antigenicity in melanoma and renal cell cancer patients. These trials have relied upon *ex vivo* retroviral gene transfer, which suffers from the disadvantages that it is very complex and requires active cell division of the cells being transfected to achieve incorporation and expression of the delivered genes. Moreover, it can be very difficult to culture sufficient numbers of neoplastic cells *ex vivo* to provide a suitable quantity of therapeutic product.

In contrast to retroviral gene transfer, plasmid constructs possessing the terminal repeat elements of AAV at sites 3′ and 5′ to the gene to be transduced were expressed efficiently when introduced via nonviral liposome-mediated transfer. For example, as discussed in greater detail below, liposomes were used to deliver AAV plasmid, such as pMP6-IL2, that comprised cDNA encoding IL-2 into primary human tumor cells, such as melanoma cells. The primary tumor cells then expressed IL-2. Tumor cell lines were also effectively transfected.

The expression of IL-2 following AAV-liposome transfection has been durable and high level. The levels of cytokine secretion from cells genetically modified by AAV plasmid-liposome compositions has exceeded the levels obtained from retrovirally infected cells.

Accordingly, cells are genetically modified using a composition comprising AAV plasmid and liposomes; these

modified cells are utilized in therapeutic tumor vaccination regimens. Advantageously, such a gene modification method as disclosed herein successfully modified primary tumor cells. This is an important development because it obviates the need to first establish a tumor cell line from primary tumor cells in order to effect gene modification which process was required prior to the present invention. It is highly advantageous that tumor cells that are genetically modified to express IL-2, when infused into a patient in a therapeutic regimen, do not require co-administration of systemic IL-2 which is known to cause extremely serious side effects and possibly death.

B. Lipofection of Cells with Transgene DNA for Use in Therapeutic Administration

Systemic IL-2 is currently used to treat certain serious conditions such as cancer. Additionally, activated T cells become dependent on exogenous IL-2 for their growth and survival both *in vitro* and *in vivo*. When the IL-2 stimulus is withdrawn, the T cells undergo apoptosis (DNA fragmentation) and death within a few days. Systemically administered IL-2 is, however, known to cause severe side effects, including death. There is a need, therefore, to develop therapies which eliminate or decrease dependence on systemic IL-2 administration.

The studies presented below addressed the delivery into T cells of AAV plasmid DNA and transgene DNA using a novel system that does not involve viral transduction. More particularly, the present results show successful transfection utilizing the elegant carrier system of lipofection and the proficient transduction capability of AAV plasmid constructs.

An AAV plasmid containing a transgene and AAV terminal repeats was used as a DNA vector, and cationic liposomes were used as carrier molecules. (For a general discussion of transfection and expression in T lymphocytes, see, Philip *et al., Mol. Cell. Biol.* (1994) *14*:2411-2418.) In a preferred embodiment, the transgene encodes IL-2. AAV plasmid:liposome complexes induced levels of transgene expression comparable

to levels obtained by recombinant AAV transduction. Advantageously, the cationic liposomes facilitated the entry of AAV plasmid DNA into cells in the absence of *rep* and *capsid* transcomplementation, recombinant virus or wild-type AAV. The AAV plasmid DNA:liposome complexes efficiently transfected TILs. AAV plasmid DNA complexed with liposomes provided several-fold higher levels of expression than complexes with standard plasmids. Moreover, expression lasted for a period of 30 days without any selection.

The IL-2 gene expression system for T cells disclosed herein enables activated cells to produce sufficient endogenous IL-2 to support their maintenance *in vivo* thereby preventing apoptosis and obviating the need for systemic IL-2 administration.

In a controlled study, various T cell populations were transfected with an AAV plasmid, carrying IL-2 cDNA, complexed to liposomes. These cell populations were tested for their ability to maintain growth and proliferation without exogenous IL-2 *in vitro*.

For T cells, assays showed that when transfected with the IL-2 gene, primary and activated $CD8^+$ T cells proliferate to attain higher numbers of cells than "control" transfected with an irrelevant "control" gene or DNA sequence.

IL-2-transfected $CD8^+$ cells grew in culture without a need for exogenous IL-2, and apoptosis was significantly reduced. Southern blot analysis of such transfected T cells showed the presence of the IL-2-encoding plasmid for up to 25 days. These results demonstrated that transfer of the IL-2 gene into *ex vivo*- activated and expanded $CD8^+$ cells promoted the growth of such cells and prevented their apoptotic death, without the need for exogenous IL-2.

Cells that can be genetically modified according to the present invention include, but are not limited to: primary lung, ovarian and breast carcinoma cells, melanoma cells, autologous fibroblasts, transformed B cells, dendritic cells and cells of any desired cultured cell line. Such genetically modified cells can be used alone or in conjunction with tumor cells (unmodified or genetically modified) to stimulate TIL. Genetically modified cells have

be made to express tumor-associated antigens, including HER2, K-ras, mucins useful as stimulatory antigens for TILs or other T lymphocytes in culture. T cells, including non-MHC-restricted T cells are generated in response to mucin antigens, such as human mucin 1 (MUC1) present on the core protein of mucin and may be specific for a peptide epitope thereof appearing as a variable number of tandem repeats having the amino acid sequence PDTRPAPGSTAPPAHGVTSA (SEQ ID NO:2). MUC1 is expressed on adenocarcinomas of the breast, pancreas and ovary and in multiple myeloma. CTL specific for these antigens on various types of tumor cells have been found in patients with the foregoing cancers and can be found in lymph nodes or generated from blood lymphocytes (Barnd, D.L. *et al.*, *Proc. Natl. Acad. Sci. USA 86*:7159 (1989); Jerome, K.R. *et al.*, *Canc. Res. 51*:2908 (1991); Ioannides, C.G. *et al.*, *J. Immunol. 151*:3693-3703 (1993); Finn, O.J., *Biotherapy 4*:239 (1992); Jerome, K.R. *et al.*, *J. Immunol. 151*:1654-1662 (1993); Takahashi, T. *et al.*, *J. Immunol. 153*:2102-2109 (1994), which references are hereby incorporated by reference). Target cell can be transfected to express MUC1 (Jerome *et al.*, 1991, 1993, *supra*).

Moreover, known types of APCs, such as transformed B cells and dendritic cells, can be genetically modified to express tumor-associated antigenic peptides such as MAGE-1 and MART-1 for presentation to and stimulation of TILs in culture. The present invention as described herein enables the efficient transfection of T cells or neoplastic cells wherein the transfected DNA is expressed both transiently and for a sustained duration. The transfection described herein occurred in the absence of any recombinant virus (producible from *rep* and capsid particles in adenovirus-infected cells). The cells obtained by AAV transfection according to this invention are used to treat patients and achieve notable therapeutic benefit.

1. Reactant Preparation and Protocols

a. Plasmids

i. Plasmid pACMV-IL2

Plasmid pACMV-IL2 contains the human IL-2 gene as IL-2 cDNA, and the immediate-early promoter-enhancer element of the human cytomegalovirus (CMV), and rat preproinsulin and SV40 polyadenylation sequences, flanked by AAV ITRs at both ends. A corresponding control plasmid, pBC12/CMV-IL2, was identical to pACMV-IL2 but lacked the AAV terminal repeats. Figure 1 depicts plasmid maps of pACMV-IL2 and pBC12/CMV-IL2.

ii. Plasmid pMP6-IL2

As shown in Figure 2, plasmid pMP6-IL2 is a double stranded circular plasmid in which the human IL-2 gene is under the control of a CMV promoter and a SV40 polyadenylation signal. Between the promoter and the coding sequences of IL-2, there is an intron which enhances the expression of IL-2. The whole expression cassette is between the left and right terminal sequences of AAV. The pMP6-IL2 plasmid also has a Bluescript backbone; the backbone has a Col-E1 bacterial origin of replication and an ampicillin resistance gene which facilitates the propagation of this plasmid in *E. coli*.

Figures 3a-3e depict the DNA sequence of the pMP6-IL2 plasmid (shown in successive portions). In these Figures, portions of the pMP6-IL2 sequence which correspond to known DNA sequences are indicated; the corresponding sequence information is listed directly beneath sequence information for the pMP6-IL2 plasmid. Unmarked sequences are from linkers.

The pMP6-IL2 plasmids were purified by alkaline lysis and ammonium acetate precipitation. The concentration of nucleic acid was determined by absorption at 260nm.

iii. Plasmid pA1CMVIX-CAT

Plasmid pA1CMVIX-CAT contains the CMV promoter enhancer element, the intervening splice acceptor sequences, the bacterial CAT gene and the simian virus 540 late polyadenylation signal flanked by AAV terminal repeats in a pBR322 derivative. Plasmids were purified by alkaline lysis and ammonium acetate precipitation. Nucleic acid concentration was measured by absorption at 260 nm.

b. Liposome Preparation

i. Liposomes used with pACMV-IL2

Small unilamellar liposomes were prepared from the cationic lipid, dioctadecyl-dimethyl-ammonium-bromide (DDAB) (Sigma Chemical Co.), in combination with the neutral lipid, dioleoyl-phosphatidyl-ethanolamine (DOPE) (Avanti Polar Lipids). The lipids were dissolved in chloroform. DDAB was mixed with DOPE in a 1:1 molar ratio in a round-bottomed flask. The lipid mixture was dried on a rotary evaporator. The lipid film was rehydrated by adding sterile double distilled water to yield a final concentration of 1mM DDAB. This solution was sonicated in a bath sonicator until clear. The liposomes were stored at 4°C under argon gas.

ii. Liposomes used with pMP6-IL2

Liposomes were prepared by combining the cationic lipid DDAB with the neutral lipid DOPE in a 1:1 molar ratio; or by combining DDAB with cholesterol in a 1:0.6 molar ratio, and evaporating the lipids to dryness in a rotary evaporator. The lipids were resuspended in sterile deionized water to yield a concentration of 1mM DDAB and then sonicated to clarity in an ultrasonic bath. Liposomes were stored under argon gas at 4°C and were stable for at least 4 months.

iii. Liposomes used for TIL stimulation

Liposomes were prepared by combining the cationic lipid DDAB with either the neutral lipid DOPE or cholesterol in a 1:1 or 1:2 molar ratio and evaporating the lipids to dryness in a rotary evaporator. The lipids were resuspended in sterile deionized water to yield a concentration of 1mM DDAB. The solution was then sonicated to clarity in an ultrasonic bath. Liposomes were stored under argon at 4°C and were stable for at least 4 months.

c. Cell Preparation

i. Isolation of TILs

TIL cells were selected with AIS MicroCELLectors®. The source material was tumor tissue or lymphatic tissue samples taken from cancer patients. The T cells were then activated according to methods known in the art such as stimulation by IL-2. The activated cells were grown for 20 days.

ii. Isolation of T cells and Neoplastic Cells

Primary T cell populations were isolated from peripheral blood mononuclear cells, and TIL and tumor cells were isolated by use of AIS MicroCELLector® devices. The cells were prepared for transfection by standard methods.

1) Neoplastic Cells from a Solid Tissue

To obtain a cell population for transfection, cells were obtained from solid primary or metastatic lesions, or from lymphatic tissues. For example, biopsies of breast tumors were obtained from patients undergoing surgery with a pre-operative diagnosis of suspected refractory or recurrent breast cancer. These studies were also successfully performed with cells from ovarian tumors. The biopsy tissue cores were divided into fragments which were processed for routine pathology by light microscopy and immunohistochemical analysis.

Freshly excised tumors were cut into 0.5 cm cubes. Up to 10 tumor tissue cubes were transferred to a 25 ml spinner flask containing 25 ml of AIM V media (GIBCO). The flask was placed in an incubator at 37°C which contains 5% CO_2, and the flask was gently stirred at 100-120 RPM for 12-18h. After incubation, any tissue that was not disaggregated was filtered, and cells in suspension were pelleted by centrifugation. The pelleted breast cancer cells were placed into tissue culture flasks (Falcon) in AIM V medium and were maintained in humidified air containing 5% CO_2 at a temperature of 37°C.

After 48 hours of culture in the serum-free medium, adherent and non-adherent cells were separated by aspirating the non-adherent cells. The non-adherent cells were washed and then recultured in a fresh flask. During reculture, the adherent cells were grown to confluence, removed with 0.05%

trypsin and 0.02% EDTA, and passaged at high cell density into new flasks.

Alternatively, primary tumor cells of lung, ovarian and breast tumor origin were obtained from solid tumor samples and isolated as follows: The tumor was minced and subjected to enzymatic digestion for 2 hours. The tissue was homogenized and washed with PBS. Lymphocytes were separated from non-lymphoid cells by capture on AIS MicroCELLector-CD5/8® devices. The nonadherent population contained tumor cells which were cultured in RPMI 1640 + 10% FBS with L-glutamine and antibiotics.

2) Neoplastic Cells from a Fluid

As an alternate source of cells for transfection, autologous neoplastic cells were isolated from malignant ascites fluid or pleural effusions. Malignant ascitic or effusion fluid was centrifuged, and the cell pellet was resuspended in AIM V media. Cells were counted, and the lymphocyte population was depleted either by using a 2-step Ficoll gradient or by using AIS CELLector CD5/CD8® devices. The choice between these two methods was made based on the total cell number, as would be appreciated by one of ordinary skill in the art. Isolation of neoplastic cells from a fluid source is particularly important in malignancies such as ovarian and lung cancer which are known to result in pleural effusions. The T cell-depleted cell fraction was enriched in neoplastic cells.

All autologous neoplastic cells were characterized by light microscopy, flow cytometry and immunohistochemical staining to assess oncogene expression and to establish a proliferation index. For example, for studies of breast cancer, only cells with the morphology of breast cancer cells or that stained with breast cancer-specific antibodies were deemed autologous tumor cells and utilized as such.

3) Cell Lines

Cells of the murine B lymphoma cell line 38C13 were provided by Dr. Bernd Gansbacher (Memorial-Sloan Kettering Cancer Center); rat prostate cell line R3327 cells were provided by Dr. Eli Gilboa (Duke University); and, MDA-231 breast tumor cell line cells were obtained from the ATCC.

d. Cellular Transfection ("Lipofection")

i. Lipofection of TILs

For transfection of TILs, 5-10 x 10^6 cells were plated in 1ml of serum-free media per well of a 6-well dish. 50μg of plasmic DNA comprising IL-2 genomic material (*e.g.*, pMP6-IL2) was mixed with 50 nmoles of DDAB (as the liposomes composed of DDAB and DOPE in a 1:1 molar ratio). Serum-free medium (0.5 ml) was added to the AAV:liposome complex which was then added to the cells. To effect lipofection, the cells were incubated at room temperature for 5 minutes, and FBS was added to a final concentration of 5%.

The transfected TILs are returned to the patient from whom they originated. These cells provide the therapeutic benefits equal to or greater than conventional cytotoxic TILs administered in combination with IL-2. The need to systemically co-administer IL-2 to maintain cytotoxic T cell activity is reduced or eliminated which provides an advantage to the patient of avoiding adverse and potentially lethal toxicity.

ii. Lipofection of neoplastic cells

A culture of neoplastic cells such as breast cancer cells or ovarian tumor cells was transfected in the following manner: 10^6 tumor cells were transfected with 5μg plasmid DNA (*e.g.*, pMP6-IL2) mixed with 30 nmoles total lipid, wherein the lipid comprises liposomes composed of DDAB and DOPE in a 1:1 molar ratio. One ml of AIM V media was added to the liposome-DNA complex and the mixture incubated at room temperature for 30 minutes. This mixture was then added to the cells and incubated at 37°C for 24 hours after which the cells were lethally irradiated with 10,000 rads.

Alternatively, DNA-liposome complexes were formed by the following method: The desired amount of DNA was transferred to a sterile vial and 1 or 2 nmole DDAB per μg DNA was added with mixing. Then, 1ml serum free medium was added to the liposome-DNA complex. Cells to be transfected were plated in six well plates. Primary tumor and tumor cell lines were plated at 10^6 cells/well in 2 ml serum-free medium. The liposome-DNA complex was added to the cells and incubated for

5 min at room temperature. FBS was added to a final concentration of 10%.

e. Assay of Transgene Expression

i. Extracellular assays

Expression of the transgene was documented by assaying IL-2 production by the irradiated cells. IL-2 can be assayed by ELISA using methods well-known in the art.

Cell-free supernatants were collected and the IL-2 concentration determined by ELISA at various time points. For example, IL-2 assays were performed on 72 hour supernatants, in duplicate. Successful transfection of the IL-2 transgene was defined as IL-2 concentrations of >100 pg/72h/10^6 cells.

ii. Intracellular assays

Cells were harvested at various time points, washed with PBS and resuspended in cold 1% paraformaldehyde in PBS. After 10 minutes at 40C, cells were washed with cold saponin buffer (0.1% saponin, 10% FBS in PBS) and stained with mouse anti-human IL-2 antibody for 15 minutes at 40C. Cells were then washed with cold saponin buffer and stained with FITC-conjugated goat anti-mouse $F(ab')_2$ antibody for 15 min at 40C. Cells were washed with saponin, then PBS, and were analyzed by flow cytometry.

f. Southern Hybridization to Detect IL-2 DNA

Chromosomal DNA was isolated by Hirt fractionation. After restriction digestions, 5μg DNA per sample was electrophoresed, transferred to Hybond N+® nylon membrane and hybridized with the 0.685 kb IL-2 fragment.

g. Cytotoxicity Assay

Target cells were labelled with 100μCi 51Cr per 10^6 cells. 5000 target cells were plated in triplicate in 96 well microplates. Effector cells were added to yield a 20:1 effector:target ratio. After a 4 hr incubation, 100μl supernatant were collected from each well and the radioactivity counted in a K-counter.

h. Proliferation Assay

5 x 10^4 cells in 100 Tl AIM V media were plated in triplicate in 96 well microplates. Each well was pulsed with

1μCi ^{3}H-thymidine. Cells were harvested 24 hours later and the radioactivity determined in a scintillation counter.

i. TCR Analysis

TILs were frozen after various times in culture and after stimulation. TCR usage was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) using methodologies known in the art.

2. RESULTS

a. *Ex vivo* Activation of Tumor Specific CTL: Stimulation of TIL Cells During Culture

TILs were stimulated in culture for 5-7 days by autologous irradiated tumor cells or by IL-2-transfected, irradiated autologous tumor cells at a ratio of TILs/Stimulators of 50:1. The responses were compared to those of TILs cultured in AIM V medium supplemented with 600 IU/ml rIL-2. The stimulated TILs were analyzed for changes in phenotype, cytotoxic activity, proliferation and TCR repertoire. This simple and rapid method of stimulating TILs during culture is utilized for both *in vitro* and *in vivo* gene transfer protocols.

As an alternate means for stimulating TILs in culture, tumor-associated antigenic peptides may be added directly to the TIL cultures.

i. Stimulation of TIL with transfected tumor cells

Neoplastic cells were transfected, for example, with pMP6-IL2 to further increase their antigenicity. Transfected cells were irradiated and, 24 hrs later, the transfected cells were washed, harvested by trypsinization, pelleted by centrifugation and resuspended in culture medium. The transfected, irradiated neoplastic cells were then cultured with TIL.

Tumor-specific reactivity was retained by the TIL during culture and expansion.

Autologous tumor cells or HLA-matched allogeneic tumor cells were used to re-stimulate selected TILs during the expansion phase of culture. This is of significant value

because the specificity of T cells for their target is sometimes known to diminish during the course of expansion. Long term culture of TILs often results in polyclonal expansion, with a diminution of tumor-specificity by the expanded cell population. As shown in Figure 13, stimulation of expanded TIL with autologous tumor cells resulted in enhanced specificity as measured by TCR usage. The specificity of TILs stimulated with IL-2-transduced tumor cells was greater than that of TIL stimulated with unmodified tumor cells (assessed by TCR repertoire). The enhanced specificity of TILs stimulated by transfected tumor cells was particularly notable after 30 days of culture.

The results demonstrated that cationic liposomes complexed to an AAV plasmid efficiently transfected primary tumor cells as well as cultured tumor cell lines. Up to 50% of the transfected cells expressed IL-2 measured as intracellular IL-2 levels, and the duration of expression was up to 30 days. Irradiation of tumor cells after transfection did not alter transgene expression. TCR analysis demonstrated expansion of tumor-specific T cells from bulk expanded TIL under the stimulatory influence of gene-modified autologous tumor cells.

b. Proliferation of TIL After Transfection with the IL-2 Gene

Results in Figure 14 show the reactivity of breast TIL which had been isolated from pleural effusion using a AIS MicroCELLector CD8® device and cultured for three weeks in medium containing 600 IU/ml IL-2. Analogous experiments were performed with TILs from an ovarian tumor, with consistent results.

Approximately 10^7 TILs were transfected with various compositions comprising pMP6-IL2 DNA:liposome complexes. Two compositions of liposomes, "RPR DDAB" (Nattermann Phospholipid GmBH, Cologne, Germany) and "1100-28" (Applied Immune Sciences, Inc., Santa Clara, CA) were tested. The RPR DDAB liposomes had a DDAB:DOPE ratio of 1:1; the 1100-28 liposomes had a DDAB:DOPE ratio of 1:0.6. The transfected TIL cells were then cultured without exogenous IL2. Positive controls were cultured in the presence of 600IU/ml IL-2.

Five days after transfection, proliferation (or growth) of transfected cells and untransfected control cells were assessed using ^{3}H-thymidine incorporation. The counts from the positive control cells were set to be 100%. Percent growth (relative to the controls) ranged from 40-80% for the RPR DDAB liposome-transfected groups, and from 40-60% for the 1100-28 liposome group. Results presented in Figure 14 demonstrate that breast cancer TILs, when transfected with the IL-2 gene, did not require exogenous IL-2 to maintain proliferation *in vitro*.

c. Thy 1.2 Gene Expression in TIL (Figure 15)

Breast cancer TILs were transfected with pMP6 containing the neomycin-resistance gene and the murine Thy1.2 gene (pMP6/neo/Thy1.2). This was an alternate embodiment of the pMP6 plasmid containing the IL-2 gene. The pMP6/neo/Thy1.2 plasmid was complexed to the same DDAB:DOPE liposomes described above (in Figure 14). On day 2, the transfected cells were stained with anti-Thy1.2 antibodies conjugated to phycoerythrin (PE) (Pharmingen, San Diego, CA) and analyzed by flow cytometry. As depicted in Figure 15, the mouse T cell surface marker Thy1.2 was expressed efficiently in transfected human $CD8^+$ TILs.

d. Transgene Expression in Irradiated Human Melanoma Cells Following Transfection

Melanoma cells were successfully transfected with pMP6-IL2. For these transfections, lipid compositions in addition to DDAB:DOPE were utilized. These various lipid compositions successfully produced lipofection and subsequent cytokine expression.

Melanoma cells were isolated from metastatic foci using conventional enzymatic digestion methods known in the art. Cells were grown in DMEM supplemented with 5-10% fetal calf serum and maintained in culture for between 5 days and 8 years.

In preparation for lipofection, tumor cells were plated on 60 mm dishes at a density of 5×10^5 cells/dish. The next day, liposomes comprising 10-30 nmol of cationic lipid and 2-10μg DNA were admixed and transferred in serum-free medium

to the adherent monolayers. After 1-5 hours incubation, FCS was added to the medium.

Various liposome preparations were employed successfully, including: DMRIE:DOPE in a 1:1 molar ratio (Vical, San Diego CA); DOSPA:DOPE, 3:1 mass ratio (Gibco, Gaithersburg, MD) and DDAB:DOPE in a 1:2 molar ratio.

The transfected cells were exposed to lethal X-irradiation (5000 rads) 24 hours following lipofection. Culture supernatants were collected at 72 hours and the IL-2 levels therein were measured by ELISA using standard methods. The results appear in Table 5, below, which shows the highest level of IL-2 expression attained with each liposome preparation. High-level expression (>5000 pg/ml) was detected in nonproliferating viable cells up to 26 days following irradiation.

TABLE 5

Cell Line	IL-2 Levels (picograms/ml) LIPOSOMES		
	DMRIE:DOPE	DOSPA:DOPE	DDAB:DOPE
DM92	33,275	12,650	1,238
DM175	24,968	5,758	not tested
DM208	10,650	9,100	8,900
nw31s	26,022	35,150	31,769
DM336	24,967	not tested	15,775
DM336	not tested	18,730	8,713
DM377	5,504	3,546	not tested

These results demonstrate successful transfection of human melanoma cell lines by nonviral, liposome-mediated delivery of plasmid pMP6-IL2 which resulted in significant production of IL-2 (following lethal irradiation).

e. Extracellular Assays of Transgene Expression in a Prostate Tumor Cell Line

To compare the level and duration of transgene expression following transfections with different plasmid constructs, the prostate tumor cell line, R3327, was transfected with 10μg standard plasmid (pBC12/CMV-IL2) or 10μg AAV plasmid (pACMV-IL2) complexed to 10 nmole DDAB as DDAB:DOPE 1:2 liposomes.

Supernatants were collected at various time points and assayed for IL-2 levels by ELISA. Figure 10 shows IL-2 levels expressed as pg/ml/10^6 cells in 24 hrs of culture. Transfection with AAV plasmid produced IL-2 levels significantly higher than with standard plasmid. In addition, transfection with AAV plasmid caused production of IL-2 for at least 30 days, in contrast to only 7 days with a conventional IL-2 plasmid.

Figure 17 depicts a Southern blot analysis of chromosomal DNA from R3327 cells transfected with an AAV plasmid (pACMV-IL2) or the standard plasmid (pBC12/CMV-IL2). The blot was probed with the 0.685 kb BamHI/HindIII fragment of the IL-2 gene. Control (C) represents DNA from untransfected cells. The IL-2 insert is shown in the last lane.

f. Intracellular Assays of Transgene Expression in a Prostate Tumor Cell Line Transfected With AAV Plasmids

The prostate cell line R3327 was transfected with AAV IL-2 plasmid (such as pACMV-IL2) complexed with DDAB:DOPE liposomes; the liposomes in a 1:1 or 1:2 DDAB:DOPE composition ratio. The DNA:liposome ratio was 10 μg DNA:10 nmole DDAB in both groups.

Transfected cells were assessed at various time points for intracellular IL-2 protein using immunostaining in a modified flow cytometry procedure as described herein. The results are shown in Figure 18 (as percent positive cells expressing IL-2 protein). Untransfected cells were used as negative controls and the values of these controls were subtracted from the values of transfected groups.

g. Transgene Expression in Primary Tumor Cells

AAV plasmid-liposome complexes were employed to transfect various primary tumor cells. One lung, one ovarian, and two breast tumor samples were isolated from fresh tumor biopsies. Tumor cells were cultured in RPMI-1640 medium supplemented with 10% FBS for 2-3 weeks prior to the transfection.

The primary tumor cells were transfected with 10μg plasmid (such as pACMV-IL2) complexed to 10 nmoles of DDAB as DDAB:DOPE 1:1. Supernatants were collected on days 2 and 3 and the IL-2 levels measured by ELISA. The results are shown in Figure 6 (expressed as pg IL-2/ml/10^6 cells in 24 hours of culture.

h. Transgene Expression by Irradiated Primary Breast Tumor Cells and Prostate Tumor Line Cells

To determine the effect of irradiation on IL-2 gene expression, primary breast tumor cells and cells of a prostate cell line (R3327) were transfected with a composition comprising pACMV-IL2 and DDAB:DOPE liposome complexes, and assessed for gene expression after lethal irradiation. The results for the tumor cell line are shown in Figure 19A, and the results for the primary tumor cells is are in Figure 19B. On day 2, an aliquot of the cells was irradiated with 6000 rads using a ^{60}Co irradiator and then returned to culture. Supernatants were collected 24, 48, 72 and 96 hrs after irradiation and tested for IL-2 levels. As shown in Figure 19, lethal irradiation following transfection did not inhibit transgene expression, measured as pg IL-2/ml/10^6 cells in 24 hr culture.

IV. DISCUSSION

In the present studies, the AAV plasmid which contained a transgene and AAV terminal repeats was used as a DNA vector, and cationic liposomes were used as carrier molecules. The results demonstrated that the AAV plasmid DNA:liposome complexes efficiently transfected primary tumor cells, cultured cell lines, primary lymphoid cells, and $CD34^+$ stem cells. In the absence of any recombinant virus (producible from *rep* protein and *cap* capsid particles in adenovirus-infected cells), high levels of integration and sustained expression of a transgene was achieved by the elegant transfection process of this invention.

In addition to high levels of expression, the combination of AAV plasmid:liposomes disclosed herein induced long-term (up to 30 days) expression of genes (Figures 5a-5b), in contrast to the transient expression which typically follows liposome-mediated transfection. Notably, sustained expression was demonstrated in the AAV plasmid lipofected group, as well as in the recombinant AAV transduced group (Figures 5a-5b). Moreover, ten-fold higher levels of expression were observed with AAV plasmid as compared to standard plasmid transfection, as shown in Figures 4a-4b.

Under the test conditions disclosed herein, there was no difference in efficiency between optimal AAV transduction and maximal AAV plasmid:liposome transfection. Concerning the time-course of expression, cationic liposomes had previously been shown to mediate only transient expression of standard plasmid DNA in mammalian cell types (Felgner *et al.*, *supra*; Rose *et al.*, *supra*). Moreover, much lower efficiency of integration into the host genome was observed in prior art liposome-mediated transfection as compared to the results disclosed herein (Shaefer-Ridder, M. *et al.*, *Science* (1982) *212*:166-168). As shown herein, cationic liposomes complexed with AAV-plasmid DNA carrying the AAV terminal repeats increased the genomic DNA integration relative to the standard plasmid that lacked only the AAV ITRs. Liposomes comprising AAV plasmid material delivered the plasmid DNA in the absence of any specific cell surface receptors, and replaced the function of virus in gene delivery.

The foregoing disclosure demonstrates that virus vectors can be altogether replaced by liposomes, and efficient expression and integration attained by utilizing the present construct, including the viral elements responsible for both the efficiency and integration. Production of virus for infection is therefore avoided, virtually eliminating the possibility of a dangerous virus recombinational event. The end results were accomplished by use of an elegant transfection process combining AAV plasmid and cationic liposomes.

In a preferred embodiment, the combination of AAV plasmid and cationic liposomes not only transfected cultured cell lines efficiently, but also transfected primary tumor cells and fresh blood-derived cells including T cells and stem cells. These observations are noteworthy because most gene therapy strategies involve gene delivery to primary T lymphocytes or tumor cells. Up until now, these strategies have relied primarily upon transgene insertion into retrovirus or DNA virus vectors. A fundamental disadvantage of the retrovirus system is the inability to transfect non-dividing primary cells. The present invention shows that cationic liposomes comprising AAV material mediates transfection of dividing and non-dividing cell types. Accordingly, AAV plasmid:cationic liposomes provide a highly efficient transfection system for sustained, high-level gene expression.

Advantageously, plasmid DNA:liposome complexes can be delivered *in vivo*, such as by intravenous, intraperitoneal and aerosol administration, without any measurable toxicity (Philip, R. *et al.*, 1993, *supra*; Stribling, R. *et al.*, *Proc. Natl. Acad. Sci. USA* (1992) *89*:11277-11281; Zhu, N. *et al.*, *Science* (1993) *261*:209-211; Stewart, M.J. *et al.*, *Human Gene Therapy* (1992) *3*:267-275). In accordance with the invention, DNA concentration can be optimized to obtain maximum expression. Thus, gene transfer using liposomes comprising AAV material successfully transferred AAV and transgene material into a wide variety of cell types *ex vivo*, and can be used *in vivo* as well. The present invention therefore provides an immense advantage to any gene therapy protocol.

Moreover, various primary neoplastic cell types, neoplastic cell lines, and several T cell subpopulations were transfected with AAV plasmids using DNA:liposome complexes. As shown herein, cationic liposomes facilitated AAV plasmid transfections into cells. The ability to transfect primary tumor cells has great importance because such cells have generally been very difficult to transfect. In addition to the high level of gene expression effected by the present method, the use of AAV plasmid:liposomes induced long term

(>30 days) expression of transgenes. Moreover, when activated and naive T cells were transfected with IL-2 plasmids, the plasmids were detected in the cells a minimum of 25 days post-transfection under nonselective conditions. These achievements constitute an important step forward from the short term expression in the prior art using conventional liposome-mediated transfection with standard plasmids.

TILs transfected with a cytokine transgene were found to proliferate without a need for exogenous cytokine or growth factors. This capability is very advantageous because TILs prepared in accordance with this invention can be provided to patients without the need for simultaneous systemic infusions of IL-2.

IL-2 gene expression in transfected T cells prepared according to this invention altered the dependence of those T cells on exogenous IL-2 and the effects of IL-2 withdrawal. Notably, IL-2-transfected effector T cells produced sufficient endogenous IL-2 to maintain their growth and proliferation and to prevent apoptosis that normally occurs when exogenous IL-2 is withdrawn. The dependence on exogenous IL-2 was eliminated.

Primary breast, lung and ovarian tumor cells were transfectable using AAV plasmid DNA:liposome complexes of this invention. Transfected primary and cultured tumor cells expressed the transgene product even after lethal irradiation.

According to the present invention, tumor cells (both autologous and HLA-matched allogeneic) can be used to re-stimulate selected TILs during the expansion phase. Transfected tumor cells are used as potent immunogens in tumor vaccination protocols. For treatment, the transfected neoplastic cells are typically provided together with a pharmaceutical excipient, as is known in the art. Transfected neoplastic cells are also used to stimulate corresponding TIL cells during culture. Analysis of cell phenotype, cytotoxic activity and TCR usage demonstrated that TILs which initially show tumor specificity upon isolation,

but which generally lose this specificity when cultured in rIL-2, maintained and increased their tumor specificity when cultured in the presence of tumor cells, most preferably tumor cells transfected according to the present invention.

EXAMPLES OF USE OF TRANSFECTED OR ANTIGEN-LOADED DENDRITIC CELLS TO INDUCE ANTI-TUMOR AND ANTI-VIRAL IMMUNITY

Using the methods and compositions of the present invention, the inventors have employed peptide-loaded or AAV/liposome transfected DC to induce (1) primary CTL responses to tumor antigens and to viral antigens in lymphocytes from healthy donors and (2) secondary responses in lymphocytes of cancer patients or virus-infected subjects to either tumor or viral antigens. Based on these findings, it is feasible to generate tumor antigen-specific and viral antigen-specific CTL for adoptive immunotherapy in the patients having the corresponding tumor or virus infection.

TAA-specific as well as EBV-specific CTL were generated using peptide-loaded or antigen-expressing gene-modified DC from the peripheral blood of cancer patients or from EBV^+ normal individuals. PBMC-derived DC were either

(a) pulsed with MART-1 (a melanoma-specific antigen), CEA, or EBV peptide, which antigens are presented in the context of the HLA-A2 major histocompatibility glycoprotein; or

(b) transfected with MART-1, CEA, or EBNA constructs to express these proteins.

DC can similarly be pulsed ("loaded") or transfected with any other TAA for example proteins or peptide epitopes of MUC1 (or the MUC1 peptide PDTRPAPGSTAPPAHGVTSA, SEQ ID NO:2), K-ras, HER2 (*supra*), p53, Mage1, Mage3, pg100, tyrosinase, Mart 1 (Melan A), CEA, PSA, PSMA, Rage, Bage and Gage and other tumor associated antigens including, for example, the antigens identified in Storkus, W. and Lotze, M., Biologic

Therapy of Cancer: Principles and Practice, Second Edition, Section 3.2, "Tumor Antigens Recognized by Immune Cells," pp. 64-77, J.B. Lippincott Co. publishers (1995). A list of tumor associated antigens which may be used in the practice of the present invention is presented below in Table 6.

TABLE 6

Tumor-Associated Antigens and Peptide Epitopes

Source	TAA	Amino Acid Sequence
Adenovirus	E1A	p234-243; SGPSNTPPEI
HPV-16	E6/E7	mutiple putative
	E7	p49-57; RAHYNIVTF
	E7	p20-29; TDLYCYEQLN
	E7	p45-54; AEPDRAHYNI
	E7	p60-79; KCDSTLRLCVQSTHVIRTL
	E7	p85-94; GTLGIVCPIC
EBV	EBNA-2	p67-76; DTPLIPLTIF
	EBNA-2	p276-290; PRSPTVFYNIPPMPL
	EBNA-3A	p330-338; FLRGRAYGL
	EBNA-3C	p332-346; RGIKEHVIQNAFRKA
	EBNA-3C	p290-299; EENLLDFVRF
	EBNA-4/6	p416-424; IVTDFSVIK
p53	p53	p264-272; LLGRNSPEV
$p21^{ras}$	ras	p5-17; KLVVVGARGVGKS
	ras	p5-16; KLVVVGAVGVGK
	ras	p54-69; DILDTAGLEEYSAMRD
	ras	p60-67; GLEEYSAM
HER2/*neu*	neu	p971-980; ELVSEFSRMA
	neu	p42-56; HLDMLRHLYQGCQVV
	neu	p783-797; SRLLGICLTSTVQLV
Human Melanoma	MAGE1	p161-169; EADPTGHSY
	gp100	p457-466; LLDGTATLRL
	gp100	p280-288; YLEPGPVTA
	Tyrosinase	p1-9; MLLAVLYCL
	Tyrosinase	p368-376; YMNGTMSQV
	Tyrosinase	p368-376; YMNGTMSEV
	MART-1/Aa	p27-47; AAGIGILTVILGVLLLIGCWY

Such antigen-loaded or transfected DC were then employed *in vitro* to stimulate $CD8^+$ cells as described below. These DC are useful for treating subjects having primary tumors, recurring tumors or metastases *in vivo*, the cells of which express the relevant TAA either by direct administration or

by administration of T lymphocytes stimulated and activated *ex vivo* by such DC.

Isolation and Characterization of DC

Isolation of DC is performed, with some modifications, as described by Romani, N. *et al.*, *J. Exp. Med.* (1994) *180*:83-93. Peripheral blood mononuclear cells (PBMC) were isolated from healthy donor buffy coats by Lymphoprep gradient separation. PBMC were plated into 6 well plates at 15 x 10^6 cells per well and allowed to adhere to the plastic for 2-4 hours at 370C. Nonadherent cells were removed and the adherent PBMC were cultured in RPMI-10% FCS with granulocyte/macrophage colony stimulating factor (GM-CSF; 800 U/ml) and interleukin-4 (IL-4; 500 U/ml) for 5-7 days. Cells were harvested and dendritic cells were further purified by removing T cells using AIS CD5/8 MicroCELLectors®. Dendritic cells were 60-90% pure as determined by flow cytometric analysis.

As shown in Figure 20, DC have been isolated to 60-90% purity. The cells have characteristic DC markers and are more potent than PBMC in inducing responses in mixed leucocyte culture (MLC) and to tetanus toxoid (shown in Table 7, below).

Table 7

Characteristics of Isolated Dendritic Cells

Cell Surface Marker	Level of Expression
CD3, 14,15,16,19,20	--
HLA-A,B,C	++
HLA-DR	++
CD4	+
CD8	--
CD1a	+++
CD11c	+++
CD33	++
CD40	+++
CD45RO	+
CD54 (ICAM)	++
CD58 (LFA3)	++
CD80 (B7)	+

Property	
Concentration of Antigen for Tetanus Toxoid Response	$<10^{-11}M$[a]
Number of cells for 50% MLC Response	<1000[b]

[a] Compared to $>10^{-9}M$ for peripheral blood mononuclear cells

[b] Compared to 80,000 for peripheral blood mononuclear cells

DCs are also generated *in vitro* by culturing human $CD34^+$ progenitor or stem cells from peripheral blood, bone marrow or cord blood in the presence of GM-CSF and tumor necrosis factor-I, with the optional presence of IL-3 (Caux, C. *et al.*, *Nature* 360:258-261 (1992); Reid, C.D. *et al.*, *J. Immunol.* 149:2681-2688 (1992); Santiago-Schwarz, F. *et al.*, *J. Leukocyte Biol.* *52*:274 (1992), which references are incorporated by reference in their entirety). Such *in vitro*-generated DC have the capacity to capture and process native

antigens and can prime naive T cells (Caux, C. *et al.*, *J. Immunol.* *155*:5427-5435 (1995). DCs generated in this way are used to express tumor antigens, viral antigens, *etc.*, as has been described herein for DC isolated as such from peripheral blood.

Transfection of DC

Plasmids were purified by alkaline lysis and ammonium acetate precipitation. Nucleic acid concentration was measured by UV absorption at 260nM.

Liposomes were prepared by combining the cationic lipid dimethyldioctadecylammonium bromide (DDAB) with the neutral lipid dioleoylphosphatidylethanolamine (DOPE) alone or with both DOPE and cholesterol in the desired molar ratios as described above. After evaporating the lipids to dryness in a rotary evaporator, they were resuspended in sterile deionized water to yield a concentration of 1mM DDAB. The solution was then sonicated to clarity in an ultrasonic bath. Liposomes were stored under argon gas at 4°C.

To form the DNA-liposome complexes, the desired amount of DNA was transferred to a sterile vial and the desired amount of DDAB was added and mixed. 1 ml serum free medium was added to the liposome-DNA complex. Cells to be transfected were plated in 2 ml serum free medium in 100 mm dishes. The liposome-DNA complex was added to the cells and allowed to incubate for 10 minutes at room temperature. Medium containing serum, IL-4 and GM-CSF was added to reach the desired final volume.

To measure expression of CAT, cells were harvested 3 days post-transfection, and cell extracts were prepared and normalized according to protein content. Extracts were mixed with 200 nmoles acetyl coenzyme A and 0.3 TCi ^{14}C-chloramphenicol and incubated for 16 hours at 37°C. Acetylated and nonacetylated chloramphenicol species were extracted with cold ethyl acetate and resolved on silica TLC plates with 95:5 (vol/vol) chloroform/methanol solvent. The radiolabelled products were visualized by autoradiography.

For determination of intracellular IL-2, cells were treated with monensin 18 hours before immunostaining. After washing, cells were permeabilized with ethanol, blocked to

prevent nonspecific binding using IgG and immunostained with mouse anti-human IL-2 antibody followed by FITC-conjugated goat anti-mouse antibody. Cells were then analyzed by flow cytometry.

In the case of NGFR, three days after transfection with the NGFR gene, cells were analyzed for expression of NGFR by staining with an antibody specific for NGFR followed by flow cytometry.

In addition to providing an antigen for antigen presentation by lipofection, the antigen can be provided by "pulsing" DC with the desired protein or peptide. For example, DC have been isolated from the peripheral blood of an HLA-$A2^+$ melanoma patient, stripped of endogenous peptide associated with HLA-A2, and pulsed with an HLA-A2 restricted peptide, MART-1, a melanoma-associated antigen. (See, for example, Restifo, NP *et al.*, *Cancer Res* (1995) *55*:3149-3157).

The isolated dendritic cells have been transfected using the AAV plasmid:liposome gene delivery system described herein and in PCT publication WO 95/07995 (23 March 1995). Figure 21 provides an outline of this method. Cationic liposomes complexed to AAV plasmid DNA result in successful transfection of either dividing or nondividing cell types.

Using the vector system described herein, the genes encoding the CAT reporter gene as well as genes encoding NGFR and IL-2 were transfected or "lipofected" into the isolated dendritic cells. Production of the proteins encoded by the transfecting DNA was observed (Figures 22-24).

With reference to Figure 22, DC were lipofected with AAV plasmid containing the CAT gene complexed to DDAB:DOPE liposomes (lanes 1,2) or DDAB:CHOL:DOPE liposomes (lanes 3,4). Three days following transfection, CAT expression was analyzed as described. All three donors (A, B and C) showed unreacted chloramphenicol, as well as expression of both the monoacetylated position 1 and the monoacetylated position 3 forms of chloramphenicol. The mock-transfected DC (lane 5) showed only unreacted chloramphenicol. CAT expression was detected in as few as 1×10^6 dendritic cells.

In addition to the genes described above, the dendritic cells are preferably pulsed with genes encoding a tumor-

associated peptide such as EBV-associated peptides. For example, DC cells are pulsed with HLA-A2- or HLA-B8-restricted EBV peptides or with peptides derived from the oncogene HER2, carcinoembryonic antigens (CEA) (see below), MUC1 *supra*, K-ras and the like. Alternatively, DNA constructs containing the genes encoding these tumor-associated peptides are lipofected, as described herein, into DC. Expression efficiencies of 10-30% have been achieved.

As an example of the foregoing, the MCF7 cell line was transfected with the AAV plasmid containing the CAT gene complexed to DDAB:DOPE liposomes ("mock" transfected) or transfected with the AAV plasmid containing the CEA gene complexed to DDAB:DOPE liposomes. Three days following transfection CEA expression was analyzed by flow cytometric analysis of the cells stained with an antibody specific for CEA. The results are shown in Figure 32. CEA transfected cells show approximately 30% CEA expression compared with mock transfected cells (42% positive vs. 13 % positive).

Stimulation of CTL Activity by DC or Other Antigen Presenting Cells

The antigen presenting DC, prepared either by pulsing with peptide or by lipofection with the relevant gene are then used to elicit an immune response in the subject.

In one approach, CTL are isolated from the subject, for example, by harvesting $CD8^+$ cells using methods well-known in the art. The $CD8^+$ cells are then cultured with the antigen-presenting DC. Antigen-specific $CD8^+$ cells, stimulated by the DC, are expanded in culture to obtain numbers of cells which are capable of adoptively transferring immune reactivity to the subject, and are administered to the subject.

In another embodiment, the antigen-presenting DC are administered directly to the subject wherein they induce the subject's immune system to generate CTL specific for the tumor-antigens expressed on the DC. The CTL are then able to attack tumor cells bearing the relevant antigen *in vivo*.

Tumor cells themselves are used as APC in a similar manner employing the AAV-based vector/liposome system described herein. The AAV-based expression plasmid is

derived from AAV by replacing the viral *rep* and capsid genes with a heterologous gene. This differs from the non-AAV expression plasmid by the presence of two viral inverted terminal repeats (ITR) at either side of the promoter-gene construct. When compared to cells transfected with an identical plasmid lacking the ITRs, expression is elevated several fold. Efficient expression has been obtained in freshly isolated (uncultured) tumor cells from breast cancer, lung melanoma and ovarian tumors. For example, expression of IL-2 in such cells persisted for up to 35 days. Such IL-2 expressing cells were been used to stimulate a cytotoxic anti-tumor response in animal models for breast and ovarian cancer. When $CD8^+$ cells were modified to contain this expression system (for IL-2 result), they were resistant to apoptotic death.

Transformed DC have been used to generate antigen-specific human CTL. PBMC from a human subject infected with Epstein Barr virus were separated from buffy coat (EBV^+) cells. The dendritic cells in this population were grown by collecting the plastic-adherent cells and incubating them in GM-CSF and IL-4 for 5 days. Nonadherent PBMC were frozen for later use. The resulting dendritic cells were harvested and transfected with two vectors derived from pMP6Neo, one containing the EBV-induced nuclear antigenic peptide EBNA 3b and the other containing EBNA 3c. The transfected DC were incubated in complete medium (RPMI + 10% FBS) containing GM-CSF and IL-4 for 3 days; nontransfected DC were used as controls.

To test their ability to induce cytotoxic activity, the DC were harvested, irradiated, and co-cultured with autologous nonadherent PBMC (which had been frozen during the 5 days required to generate the DC and which were thawed immediately before culture. The nonadherent cells serve as the source of cytotoxic effector cells. The co-culture cells was incubated in AIM V medium (GIBCO/BRL) supplemented with IL-7 (10ng/ml) for 5 days before testing.

CTL activity was assessed using a ^{51}Cr release assay. The assay was conducted using autologous $CD4^+$ cells infected with either a vaccinia viral vector containing EBNA 3a, 3b,

and 3c, or with a control vaccinia vector. The assay used an effector:target ratio of 80:1. The results are shown in Figure 25.

DC transfected with pMP6NeoEBNA clearly stimulated specific cytotoxic effector cells. The stimulated effector cells showed about 40% specific cytotoxicity, while control effector cells (which had been co-cultured with control DC cells) had a background level of cytotoxicity (about 25%). Although the % cytotoxicity in such assays typically decreases with decreasing effector:target ratios, the cytotoxicity ratio, the present results levels of cytotoxicity (stimulated by transfected versus nontransfected DC) remained relatively constant.

GENERATION OF CEA-SPECIFIC CTL

Two strategies are used to generate CEA-specific CTL:

1) a peptide-based approach in which DC are pulsed with a CEA peptide (YLSGANLNL; Tsang, K.Y. *et al.*, *J. Natl.Canc. Inst.* 87:982-989 (1995)) that is recognized by CTL in the context of Class I HLA-A2 molecules; and
2) a gene-based approach in which DC are transfected with a CEA-encoding plasmid containing the ITRs of AAV complexed to cationic liposomes.

These systems and their utility in generating CEA-specific CTL is described below.

DC Isolation

DC were isolated using a modification of Romani *et al.* *(supra)*. Briefly, 1.5 x 10^8 PBMC from an HLA-$A2^+$ donor were allowed to adhere to a T150 flask for 2 hours at 37°C in RPMI- 10% FCS. After incubation, the nonadherent cells were removed, and the adherent cells were cultured in 30 ml of RPMI- 10% FCS medium containing 800 units/ml GM-CSF and 500 units/ml IL-4. After 6-7 days of culture the differentiated DC were harvested, stripped of endogenous peptides, loaded with CEA peptide (YLSGANLNL) and used to stimulate $CD8^+$ T cells.

Peptide Stripping and Loading

For peptide stripping, DC were washed once in a cold solution of 0.9% NaCl, 1% BSA solution, resuspended at 10^7

cells/ml in stripping buffer (0.13M L-ascorbic acid, 0.06M sodium phosphate monobasic, pH 3, 1% BSA, 3Tg/ml B2M, 10 μg/ml peptide) and incubated for 2 min on ice. The cells were then neutralized with 5 volumes of neutralizing buffer (0.15M sodium phosphate monobasic, pH 7.5, 1% BSA, 3 μg/ml B2M, 10 μg/ml peptide) and spun at 1500 rpm for 5 min. Finally, the cells were resuspended in peptide solution (PBS-CMF, 1% BSA, 30 μg/ml DNAase, and 40 μg/ml peptide) and incubated for 4 hr at room temperature. The cells were irradiated (3000 rad) and washed prior to being used for stimulation.

Generation of CTL

PBMC were separated from peripheral blood leukophoresis of HLA-A2$^+$ patients or buffy coats from healthy donors by Ficoll-Hypaque density gradient centrifugation. Responder lymphocyte precursors of peptide-specific CTL were prepared by capturing CD8$^+$ cells on AIS MicroCELLector® flasks. Captured CD8$^+$ cells were stimulated with irradiated DC loaded with CEA peptides at a stimulator:responder (S:R) ratio of 1:3. These co-culture cells were incubated in RPMI-10% FCS containing 10ng/ml IL-7. At days 10-12, the lymphocytes were restimulated with DC pulsed with CEA peptide (1:5 S:R ratio). Responder cells were restimulated weekly for a total of 3-4 stimulations at S:R ratios ranging between 1:5 and 1:15. As a control, CD8$^+$ captured cells were cultured with either (a) DC pulsed with an irrelevant peptide, (b) unpulsed DC or (c) IL-7 only.

Evaluation of Cytotoxicity

HLA-A2-restricted recognition of CEA by the CTL generated as above was assessed using a standard 4hr ^{51}Cr release cytotoxicity assay. Recognition of CEA peptide by CTL was assessed using T2 cells (a cell line defective in antigen processing which expresses "empty" HLA-A2 molecules until stabilized by the addition of peptide) preincubated for 2-4 hr with peptide at 40 μg/ml. In addition CEA specific cytotoxicity was evaluated using as targets the CEA-expressing cell lines SW403 (HLA-A2$^+$) and SW1417 (HLA-A2$^-$). Target cells were labeled overnight with 100 μCi ^{51}Cr, washed and mixed with effector cells at varying effector:target (E:T) ratios in U-bottom microtiter plates. After a 4 hr

incubation, supernatants were harvested, and the amount of ^{51}Cr released was measured in a scintillation counter. % specific cytotoxicity was calculated as follows:

$$\frac{(\text{cpm of test sample} - \text{cpm of spontaneous } ^{51}Cr \text{ release})}{(\text{cpm of maximal } ^{51}Cr \text{ release} - \text{cpm of spontaneous } ^{51}Cr \text{ release})} \times 100$$

RESULTS

CEA specific cytotoxicity by normal human T cells on T2 target cells pulsed with CEA peptide and "empty" T2 cells is shown in Figure 26. Here, autologous, normal $CD8^+$ captured responder cells had been stimulated with CEA-pulsed DC and cultured in 10ng/ml IL-7. Cytotoxicity was assayed after 3 rounds of restimulation with CEA peptide pulsed DC.

The phenotype of the above effector cells on the day of CTL assay is shown in Figure 27. The DC-CEA-stimulated and cytokine-stimulated T cells were stained with antibodies to CD3, CD4, CD8, and CD56. The large majority of cells in this effector cell population were $CD3^+8^+$.

The generation of CEA specific cytotoxicity by T cells from a pancreatic cancer patient on T2 target cells pulsed with CEA peptide is shown in Figure 28. Autologous $CD8^+$ captured responder cells were stimulated with CEA-pulsed DC and cultured in 10ng/ml IL-7. Cytotoxicity was assayed after 2 or 3 rounds of restimulation with CEA peptide pulsed DC at effector:target ratios of 80:1, 40:1, 20:1, 10:1.

T cells from an HLA-A-2^+ breast cancer patient were tested for stimulation of CEA-specific CTL by autologous DC pulsed with CEA (in the presence of 10ng/ml IL-7). The CTL generated were tested on two different target cells: an HLA-$A2^+CEA^+$ cell line (SW403) and an HLA-$A2^-CEA^+$ cell line (SW1417). The results are shown in Figure 29. The CTL activity appeared to be MHC restricted as the killing of HLA-$A2^-$ cells was markedly lower that killing of HLA-$A2^+$ cells. Furthermore, the generation of CTL was dependent on the presence of CEA-pulsed DC in the stimulatory phase, as little killing was observed with effector cells stimulated by cytokines alone (Figure 30).

The phenotype of the breast cancer patient's effector cells (on the day of CTL assay) is shown in Figure 31. The DC-CEA-stimulated and cytokine-stimulated T cells were stained with antibodies to CD3, CD4, CD8, and CD56. The large majority of cells in this effector cell populations were $CD3^+8^+$.

REVERSE TRANSCRIPTASE (RT) - POLYMERASE CHAIN REACTION (PCR) ANALYSIS OF TRANSFECTED DENDRITIC CELLS

This example shows successful gene transfer to the dendritic cells using AAV/liposome transfection. The data presented below indicates that the desired genes were successfully transferred to the dendritic cells since mRNA encoding the respective transfected genes was present in the cells.

Dendritic Cell Isolation

PBMCs from normal donor peripheral blood were isolated on Ficoll. 1.5×10^8 PBMCs were allowed to adhere to a T150 flask for 2-4 hours at 37°C in RPMI-10% FCS. After incubation, the nonadherent cells were removed and the adherent cells were cultured in 30 ml of RPMI-10% FCS medium containing 800 units/ml GM-CSF and 500 units/ml IL-4. After 7 days, the differentiated dendritic cells were harvested, washed and counted.

Transfection

3×10^6 cells in serum-free RPMI in 100 mm dishes were transfected for each condition. 30 μg of plasmid pMP6CAT, pMP6ACEA, or pMP6AMART1 DNA were complexed to 60 nmole total lipid as DDAB:DOPE 1:1 liposomes. After the cells were incubated with the complex, additional medium containing fetal calf serum, GM-CSF, and IL-4 was added to bring the final concentration to 10% FCS, 800 units/ml GM-CSF, and 500 units/ml IL-4. The cells were harvested 1, 2, or 3 days after transfection.

In addition to the dendritic cells, two tumor cell lines were also transfected following the protocol presented above for transfection of the dendritic cells. The tumor cells lines were MCF7 and CEM. MCF7 is a breast cancer tumor cell line and CEM is a lymphoblastoid cell line.

mRNA Isolation

mRNA was isolated and treated with DNase to remove contaminating plasmid DNA. Untransfected cells were treated similarly. A portion of mRNA was amplified by PCR without reverse transcriptase. The remaining mRNA was used to synthesize cDNA, which was then amplified by PCR. As a control, plasmid DNA was also amplified by PCR. PCR amplification was carried out using primers that flank the intron portion of the plasmid constructs. These primers will yield a full length product (same size as plasmid control) if the mRNA is unspliced, but a smaller product if the intron has been spliced out (see Figure 42). PCR products were visualized by gel electrophoresis and their size was determined.

Results

The results of the experiments are presented in Figure 43. Successful gene transfer to dendritic cells was confirmed by the presence of CAT, CEA, and MART-1 transgene mRNA. In all cases, a full-length product (CAT=710, CEA=774, MART-1=605) was detected, indicating that the mRNA from dendritic cells is unspliced. In contrast, mRNA analysis of tumor cell lines (MCF-7, CEM) transfected with the same constructs are positive for the smaller, spliced form. Untransfected cells and samples amplified without reverse transcriptase are negative for transgene mRNA.

Although the mRNA detected in transfected dendritic cells is unspliced, protein expression still occurs, as demonstrated by CAT protein assays and the ability to generate antigen specific CTL. It is believed that increasing the amount of spliced mRNA in dendritic cells may enhance protein expression.

GENERATION OF MELANOMA ANTIGEN-SPECIFIC CTL

A peptide-based approach was employed in which DC were pulsed with MART-1 ("melanoma antigen recognized by T cells"), a tumor-associated antigen that is specifically expressed on melanoma cells and normal melanocytes. An immunodominant MART-1 peptide (AAGIGILTV) is recognized by

CTL in the context of Class I HLA-A2 molecules (*J. Exp. Med.* *180*:347 (1994)) and was used in the present studies.

DC Isolation

DC were isolated essentially as described above in the studies of anti-CEA responses. After 6-7 days of culture the differentiated DC were harvested, stripped of endogenous peptides, loaded with MART-1 peptide (AAGIGILTV; *J. Exp. Med.* *180*:347 (1994)) and used to stimulate $CD8^+$ T cells.

Peptide Stripping and Loading:

This was accomplished as described above in the studies of anti-CEA responses.

Generation of CTL

CTL were generated as described above in the anti-CEA responses, except that patient donors were $HLA\text{-}A2^+$ melanoma patients. Here, the stimulator DC or T cell-depleted PBMC pulsed with MART-1 peptide (1:5 stimulator:responder ratio) and plated in 24-well plates at a density of 1.5-2 x 10^6 responder cells/well. Two days later, 20U/ml IL-2 was added. Responders were restimulated weekly for a total of 3-4 rounds of stimulation at stimulator:responder ratios ranging between 1:5 and 1:15. Here too, controls included $CD8^+$ captured cells cultured with either (a) DC pulsed with an irrelevant peptide, (b) unpulsed DC or (c) IL-7 only.

Evaluation of Cytotoxicity

HLA-A2-restricted recognition of MART-1 by the CTL generated as above was assessed in a standard 4 hr ^{51}Cr release assay described above for anti-CEA responses. Recognition of MART-1 peptide by CTL was assessed using T2 target cells preincubated for 2-4 hr with peptide at 40 μg/ml. In addition MART-1-specific cytotoxicity was evaluated using the following $HLA\text{-}A2^+$ cell lines as targets: 624mel ($MART\text{-}1^+$), A375 ($MART\text{-}1^-$), and/or Colo205 ($MART\text{-}1^-$). Labeling of target cells and the assay itself are described above for anti-CEA responses.

RESULTS

MART-1 specific cytotoxicity by T cells from a melanoma patient on T2 target cells pulsed with MART peptide (T2-MART) and "empty" T2 cells (T2) is shown in Figures 33-35. CD8-captured cells stimulated with irradiated DC loaded with

MART-1 peptides and cultured with IL-7, and subjected to 3 rounds of stimulation showed a specific cytotoxic response seen as the markedly higher killing of the MART-1-pulsed targets. Specific cytotoxicity against T2-MART (after subtraction of activity on T2) was 60%, 40% and 15% at E:T ratios of 60, 30, and 15, respectively (Figure 33).

Specificity of the cytotoxic response was also seen by the markedly higher killing of the MART-1^+ targets (624mel) compared to MART-1^- targets (A375) (Figure 34). Specific cytotoxicity against 624Mel (after substraction of activity on MART-1^- targets) was 25%, 15% and 5% at E:T ratios of 60, 30 and 15, respectively.

The phenotype of the above effector cells on the day of CTL assay is shown in Figure 35. The DC-MART-stimulated and IL-7-stimulated patient T cells were stained with antibodies to CD3, CD4, CD8, and CD56. Greater than 90% of the effector cells are $CD3^+CD8^+$.

Similar studies were done with peripheral blood T cells obtained from a normal, healthy donor (shown in Figures 36-38) which presumably reflect a primary response.

Specificity of the CTL response was demonstrated by the markedly higher killing of the MART-1-pulsed targets compared to unpulsed targets (Figure 36). Specific cytotoxicity against T2-MART (after subtraction of activity on T2) was 30% and 10% at E:T ratios of 60 and 30, respectively.

Further evidence for the specificity of the CTL response is shown in Figure 37, where killing of the MART-1^+ targets was markedly higher than killing of MART-1^- targets. Specific cytotoxicity against 624Mel (after substraction of activity on MART-1^- Colo targets) was 30% at an E:T ratio of 60.

The phenotype of the above effector cells on the day of CTL assay is shown in Figure 38. The DC-MART-stimulated and IL-7-stimulated normal T cells were stained with antibodies to CD3, CD4, CD8, and CD56. About 70% of the effector cells were $CD3^+CD8^+$.

USE OF A MART-1 GENE MODIFIED DENDRITIC CELL AS AN ANTIGEN PRESENTING CELL (APC)

This experiment demonstrated that transformed dendritic cells can express and present peptide antigens encoded by the tumor associated antigen (TAA) MART-1 gene and that these dendritic cells can stimulate $CD8^+$ cells specific for MART-1 resulting in killing by the $CD8^+$ cells of target cells which present the MART-1 antigen on their surface.

Dendritic Cell Isolation:

Dendritic cells (DC) were isolated using a modification of Romani et al. (J. Exp. Med., 180:83 (1994)). Briefly, 1.5 x 10^8 PBMC from an HLA $A2^+$ donor were allowed to adhere to a T150 flask for 2-4 hours at 37°C in RPMI-10% FCS. After incubation, the nonadherent cells were removed and the adherent cells were cultured in 30 ml of RPMI-10% FCS medium containing 800 units/ml GM-CSF and 500 units/ml IL-4. The cells were harvested 6 to 7 days after isolation.

Cell preparation

The harvested cells were resuspended in room temperature serum-free RPMI 1640 and the total number of cells (all cell types) was determined. The volume of the cells was then adjusted to yield 1-5 x 10^6 cells/ml.

Preparation of DNA:Liposome Complexes

Plasmid pMP6AMART1 contains the gene for the tumor associated antigen (TAA) gene MART-1, a melanoma antigen recognized by T cells cloned into pMP6. The MART-1 coding sequence was produced by PCR using primers generated from published sequences. The PCR product was digested with NheI and BamHI and cloned into NheI and BamHI digested pMP6 and filled in with T4 DNA polymerase, CIP and Klenow. For each 1 x 10^6 cells to be transfected, 10 μg of plasmid DNA at a concentration of 1 mg/ml in sterile H_2O were aliquoted into a sterile, round-bottom tube and combined with 10 nmoles of DDAB as DDAB:DOPE 1:1 liposomes at a concentration of 1 μmole DDAB/ml in sterile H_2O. The solution was mixed by gentle swirling and incubated at room temperature for 10 to 15 minutes. Serum-free RPMI 1640 was then added to the DNA:liposome complex. The volume of medium added equaled one-half of the volume of the cell suspension.

Liposome Transfection

The DNA:liposome complex was transferred to the cell suspension and the mixture was incubated at room temperature for 15 minutes. 2X dendritic cell medium (RPMI 1640 + 20% FCS + 1600 U/ml GM CSF + 1000 U/ml IL-4) was then added to the cells. The volume of medium added equaled the total volume of cells and complex. The cell suspension was then transferred to appropriate sized culture containers and placed in a 37°C, 5% CO_2 incubator.

Post-Transfection Assay

The day following transfection, the transfected dendritic cells (stimulators) were harvested, counted and mixed with autologous cytolytic T cells (responders) from a normal HLA-A2+ donor that had been captured on CD8 MicroCellector flasks. The stimulators were added to the responder cells at a ratio between 1:3 and 1:10 and 10 ng/ml IL7 was added to the culture. At 10 days post stimulation, another 10 ng/ml IL7 were added to the culture along with transfected dendritic cells. Two additional restimulations were performed weekly for a total of four stimulations. Additional IL7 is added between the weekly stimulations.

Five days after the final restimulation, the CTL were harvested and assayed for specific cytotoxicity against appropriate target cells by ^{51}Cr release.

Cytotoxicity Analysis:

HLA-A2 restricted MART-1 recognition by CTL was assessed by a standard 4-hour ^{51}Cr release cytotoxicity assay. The MART-1 peptide (AAGIGILTV) is recognized by CTL in the context of Class I HLA-A2 molecules (J. Exp. Med., 180:347 (1994)). Recognition of MART-1 peptide by CTL was assessed using T2 cells pulsed with MART-1 peptide (T2 + MART-1) and "empty" T2 cells (empty T2). T2 cells are a processing defective cell line which expresses empty A2 molecules until stabilized by the addition of peptide. The T2 cells pulsed with MART-1 were preincubated for 2-4 hours with MART-1 peptide at 40 mg/ml. Target cells were labeled overnight with 100 mCi ^{51}Cr; they were washed and mixed with effector cells at varying effector:target (E:T) ratios in U-bottom microtiter plates. After a 4 hour incubation, supernatants

were harvested, and the amount of ^{51}Cr released was measured by a beta counter. Percent specific cytotoxicity was calculated as follows: [(cpm of test sample - cpm of spontaneous ^{51}Cr release)/(cpm of maximal ^{51}Cr release - cpm of spontaneous ^{51}Cr release)] x 100.

Results

The results presented in Figure 44a show that T cells stimulated with gene modified dendritic cells expressing the tumor associated antigen gene MART-1 demonstrate significant cytotoxic response against MART-1 peptide loaded T2 cells (T2 + MART-1). There was some background killing of empty T2 cells at the high effector:target ratio. T cells stimulated with cytokine only (Figure 44b) were not cytotoxic against either empty or peptide loaded targets. The response observed was antigen specific. This data demonstrates that a single peptide response can be generated by stimulating with dendritic cells which express the whole gene product. It is believed that other HLA restricted responses can be generated using gene modified dendritic cells.

GENERATION OF HIV ANTIGEN-SPECIFIC CTL

A canarypox virus construct vCP300 (Virogenetics Corp., Troy, NY) which includes genes encoding HIV proteins (*gag*, *pol*, *gp120*, *nef*) is diagrammed in Figure 39. This vector was used to infect either PBMC depleted of $CD8^+$ and $CD4^+$ cells or DC to test whether these cells would express HIV peptides in immunogenic form capable of stimulating HIV-specific CTL. The vCP300 vector was mixed in polypropylene tubes with the cells being transfected at a multiplicity of infection (MOI) of 5 or 10 and incubated at 37°C in 5% CO_2 for one hour. For analysis of HIV gene expression, the infected cells were incubated for an additional 18 to 24 hours. Infected cells were used to stimulate cultures immediately. Any remaining cells were frozen for later use.

$CD8^+$ responder cells obtained from HIV^+ donors were cultured in AIM V media supplemented with 100 IU/ml rIL-2 (from Cetus). Stimulator cells were added on days 0 and 7 at a responder:stimulator ratio of 10:1.

Healthy PBMC obtained from buffy coats were cultured in AIM V medium supplemented with 5% human AB serum (Advanced

Biotechnologies Inc., Columbia, MD), 5.0 ng/ml rIL-7 (Genzyme Corp., Cambridge, MA). In addition, 100 IU/ml of rIL-2 was added 48 hours after the second stimulation on day 7. The cultures were stimulated as described above for $CD8^+$ cultures.

Mature dendritic cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 800 U/ml GM-CSF and 500 U/ml rIL-4 (both from Biosource International, Camarillo, CA).

Evaluation of Cytotoxicity

Autologous $CD4^+$ cells expressing selected HIV antigens were used as target cells in a ^{51}Cr release assay performed generally as described above. The target cells were prepared by infection with vaccinia virus vectors (Virogenetics Corp., Troy, NY) containing either (1) *env*, (2) *gag/pol*, or (3) *nef* proteins of HIV. Infection was accomplished by coincubating the $CD4^+$ cells and the vaccinia virus vector (MOI=10) for 1 hour at 37°C. The infected cells were then labeled with ^{51}Cr at a concentration of 100 μCi/ml per 10^6 cells overnight at 37°C in an atmosphere of 5% CO_2 in individual wells of a 24-well tissue culture plate.

Effector cells and target cells were mixed at E:T ratios ranging from 5 to 40, depending on cell numbers, in 96-well microplates and incubated in a standard 4 hour cytotoxicity assay. % Cytotoxicity was calculated as above.

Analysis of vCp300 Expression

Three populations of cells were infected with canarypox construct vCP300 as detailed above: (a) PBMC. (b) PBMC depleted of CD8- and CD4-bearing cells and (c) DC. These cells were incubated with either buffer (PBS-CMF), pooled human AB serum, or pooled serum from HIV^+ donors (New York State Health Dept., Troy, NY). As a second step, these cells were incubated with fluorescein isothiocyanate-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Stained cells were analyzed by flow cytometry (Becton Dickinson Immunocytometry Systems, San Jose, CA).

RESULTS

The results of these studies appear in Figures 40-41. PBMC stimulated with autologous DC infected with canarypox

construct vCP300 had cytotoxic activity against autologous target cells expressing HIV env, gag/pol or nef (Figure 40). Cytotoxicity activity ranged from 18% to 30% lysis. The activity was significantly higher than the background activity of PBMC stimulated in culture by DC infected with a control canarypox construct lacking any HIV genes (vCPpp). Similarly, PBMC stimulated by the mitogen PHA showed no lytic activity. Cytotoxicity was directed toward all of the HIV proteins present in the construct used to infect the antigen-presenting DC.

Flow cytometry was used to analyze expression of HIV proteins on DC isolated from 4 healthy donors and infected with vCP300. The results, shown in Figure 41, indicate that HIV proteins were detected by binding of anti-HIV antibodies (in the form of serum from HIV^+ donors) to infected DC. Control serum did not react with any of the infected DC preparations. The percent of cells expressing HIV antigens acquired through infection with the vCP300 vector ranged from 5% to 15%.

As used herein and in the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a formulation" includes mixtures of different formulations and reference to "the method of treatment" includes reference to equivalent steps and methods known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

The references cited above are all incorporated by reference herein, whether specifically incorporated or not.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation. It is to be understood that the phraseology or terminology employed herein is for the purpose of

description and not of limitation. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are described.

WHAT IS CLAIMED IS:

1. A non-immortalized dendritic cell transfected by a vector including a DNA sequence not native to said dendritic cell.

2. A dendritic cell or other antigen presenting cell transfected by a composition comprising a liposome which comprises lipid material and adeno-associated virus material.

3. A cell according to claim 2 which is a dendritic cell.

4. A cell according to claim 2 wherein said adeno-associated viral material comprises a plasmid

5. A cell according to claim 4 wherein said plasmid includes a DNA sequence of interest encoding one or more of a tumor-specific antigen, a tumor-associated antigen, a microbial antigen, a cytokine, a cellular receptor, a reporter molecule or a selectable marker.

6. A cell according to claim 5 wherein said plasmid is pMP6 in which is inserted said DNA sequence of interest.

7. A method for introducing a DNA sequence of interest into a dendritic cell or other antigen-presenting cell comprising the steps of:

(a) providing a composition comprising liposome, adeno-associated virus material and a DNA sequence of interest; and

(b) contacting the composition of step (a) with the cell, which cell comprises genetic material, such that the DNA sequence of interest is introduced into said cell.

8. The method of claim 7, wherein the liposome of step (a) comprises cationic lipid.

9. The method of claim 7, wherein the adeno-associated virus material comprises a plasmid.

10. The method of claim 9, wherein the plasmid is pMP6 which includes said DNA sequence of interest.

11. The method of claim 7, wherein the DNA sequence of interest encodes one or more of a tumor-specific or tumor-associated antigen, a microbial antigen, a cytokine, a cellular receptor, a reporter molecule or a selectable marker.

12. The method of claim 11, wherein

(a) said tumor-specific or tumor-associated antigen is carcinoembryonic antigen, a breast tumor antigen, a colorectal tumor antigen, a gastric tumor antigen, a pancreatic tumor antigen, a lung tumor antigen, an ovarian tumor antigen, a bladder tumor antigen, a prostate tumor antigen, a melanoma antigen, a leukemia antigen or a lymphoma antigen; or

(b) said microbial antigen is an Epstein-Barr viral antigen or an HIV antigen; or

(c) said cytokine is interleukin-2; or

(d) said receptor is nerve growth factor receptor; or

(e) said reporter molecule is bacterial chloramphenicol acetyl transferase.

13. The method of claim 7 wherein said DNA sequence of interests integrates into the genetic material of said cell.

14. A method for treating a subject having a disease or condition which is treatable by stimulating an immune response to a selected antigen in said subject, which method comprises the steps of:

(a) contacting dendritic cells or other antigen-presenting cells of said subject *in vivo* with a composition comprising liposome, adeno-associated virus material and a DNA sequence of interest which

encodes said selected antigen such that said DNA sequence is introduced into said cells; and

(b) allowing the antigen encoded by said DNA sequence to be expressed and to stimulate said immune response of said subject,

thereby treating said subject.

15. A method for treating a subject having a disease or condition which is treatable by stimulating an immune response to a selected antigen in said subject, which method comprises steps of:

(a) contacting dendritic cells or other antigen-presenting cells, which cells are autologous or allogeneic to said subject, *ex vivo* with a composition comprising liposome, adeno-associated virus material and a DNA sequence of interest which encodes said selected antigen such that said DNA sequence is introduced into said cells;

(b) allowing the antigen encoded by said DNA sequence to be expressed in said cells; and

(c) delivering said cells expressing said antigen to said subject to stimulate said immune response,

thereby treating said subject.

16. A method for treating a subject having a disease or condition which is treatable by stimulating an immune response to a selected antigen in said subject, which method comprises steps of:

(a) contacting dendritic cells or other antigen-presenting cells, which cells are autologous or allogeneic to said subject, *ex vivo* with a composition comprising liposome, adeno-associated virus material and a DNA sequence of interest which encodes said selected antigen such that said DNA sequence is introduced into said cells;

(b) allowing the antigen encoded by said DNA sequence to be expressed in said cells;

(c) activating lymphocytes *ex vivo* by contact with said cells expressing the antigen to become cytotoxic or

otherwise specifically immunoreactive to cells in the subject bearing the antigen;

(d) delivering said activated lymphocytes to said subject to mediate said immune response in said subject,

thereby treating said subject.

17. The method of claim 14, 15 or 16 wherein said disease or condition is neoplasia or an infection.

18. The method of claim 14, 15 or 16, wherein said DNA sequence of interest encodes one or more of a tumor-specific antigen, a tumor-associated antigen or a microbial antigen.

19. The method of claim 18 wherein said microbial antigen is an HIV antigen.

20. A method for producing a protein in a dendritic cell comprising:

(a) introducing a DNA sequence encoding said protein into a dendritic cell; and

(b) allowing said DNA sequence to be expressed,

thereby producing said protein.

21. The method of claim 20 wherein said introducing is performed by transfecting the cell with a composition comprising liposome, adeno-associated virus material and said DNA sequence.

22. A cell according to claim 5 wherein said tumor associated antigen is MART-1.

23. A cell according to claim 5 wherein said tumor associated antigen is CEA.

24. A cell according to claim 5 wherein said tumor associated antigen is MUC-1.

25. A cell according to claim 5 wherein said tumor associated antigen is EBNA.

26. A cell according to claim 5 wherein said tumor associated antigen is selected from the group consisting of: MART-1 (Melan A), CEA, MUC-1, p53, ras, her, Mage1, Mage3, pg100, tyrosinase, PSA, PSMA, Rage, Bage, Gage, E1A, E6, E7, EBNA2, EBNA-3A, EBNA-3C, EBNA-4, EBNA-6, neu and gp100.

27. A method for eliciting an immune response to a tumor-associated antigen comprising providing a modified dendritic cell bearing a selected tumor-associated antigen and contacting a cytolytic T cell with said dendritic cell bearing said tumor-associated antigen said contacting can occur *in vivo* or *in vitro*.

28. The method according to Claim 27 wherein said contacting occurs *in vivo*.

29. The method according to Claim 27 wherein said contacting occurs *in vitro*.

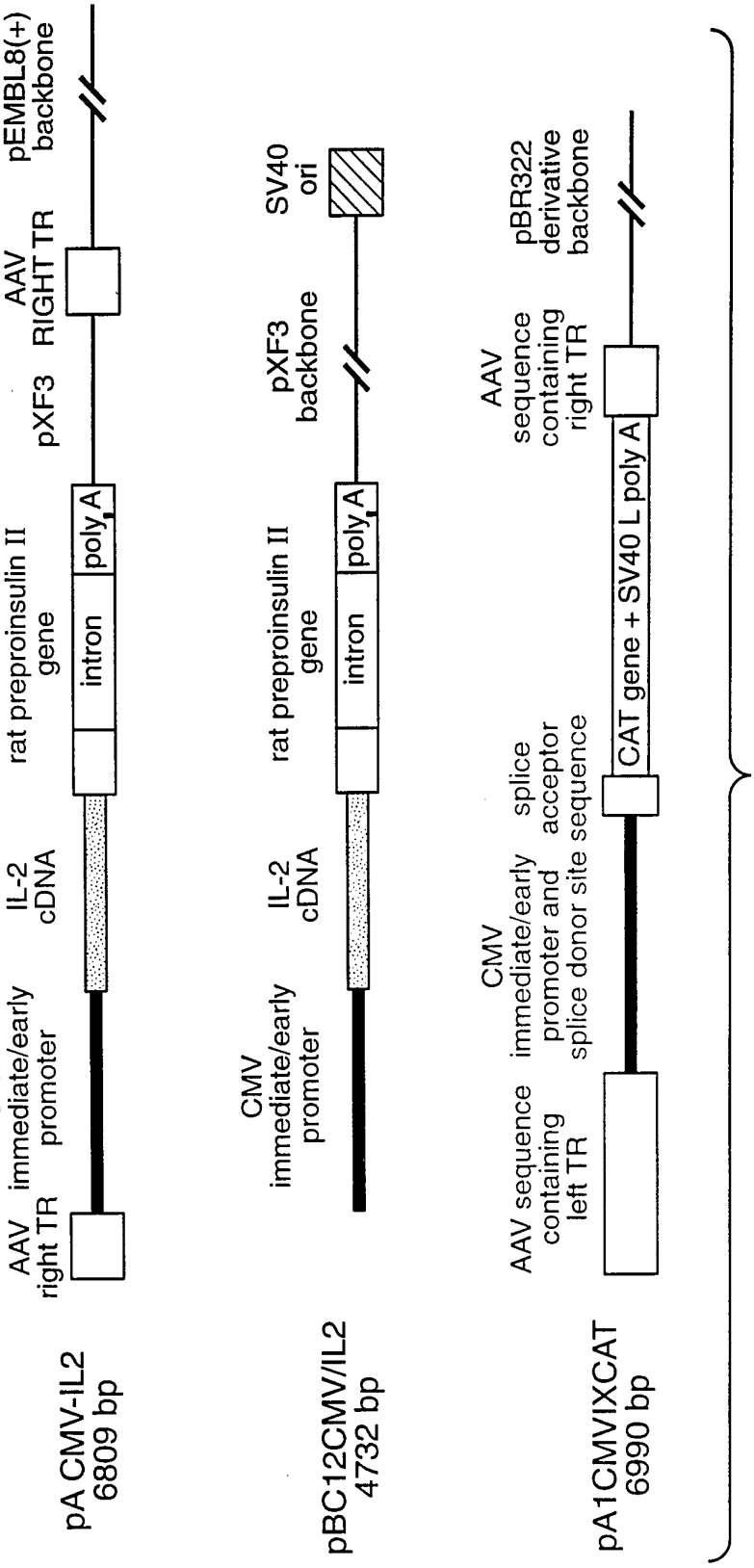
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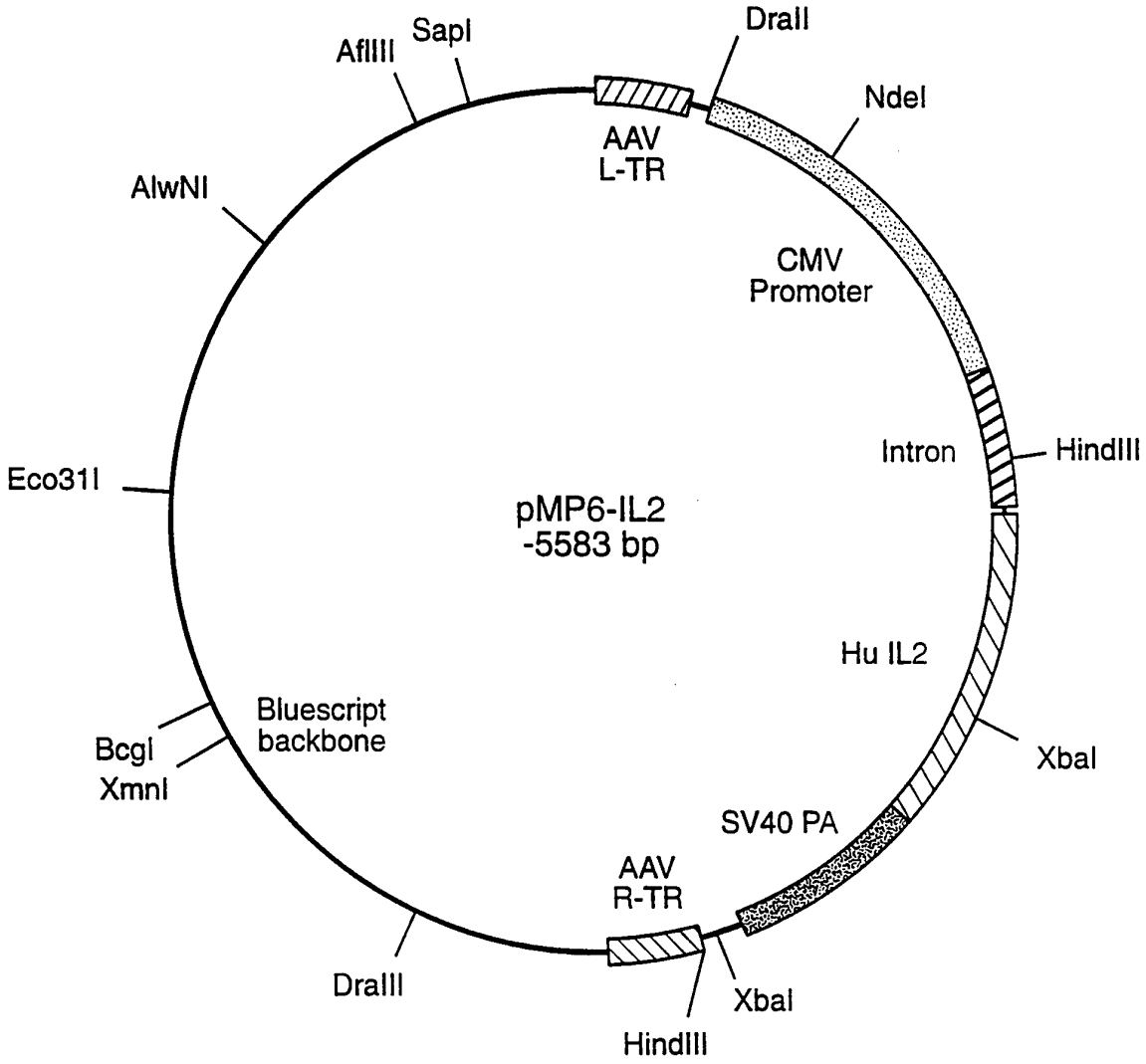
FIG._1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Hu IL2
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FIG._2

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Bluescript KS II +

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Left terminal region of AAV

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151 CGCAGAGAGG GAGTGGCCAA CTCCATCACT AGGGGTTCCT GGAGGGGTGG 200

201 AGTCGTGACG TGAATTACGT CATAGGGTTA GGGAGGTCCG CGCAATTAAC 250

251 CCTCACTAAA GGGAACAAAA GCTGGGTACC GGGCCCTTCG ATTCGCCCGA 300

301 CATTGATTAT TGACTAGTTA TTAATAGTAA TCAATTACGG GGTCATTAGT 350
CMV Promoter

351 TCATAGCCCA TATATGGAGT TCCGCGTTAC ATAACTTACG GTAAATGGCC 400

401 CGCCTGGCTG ACCGCCCAAC GACCCCCGCC CATTGACGTC AATAATGACG 450

451 TATGTTCCCA TAGTAACGCC AATAGGGACT TTCCATTGAC GTCAATGGGT 500

501 GGAGTATTTA CGGTAAACTG CCCACTTGGC AGTACATCAA GTGTATCATA 550

551 TGCCAAGTAC GCCCCCTATT GACGTCAATG ACGGTAAATG GCCCGCCTGG 600

601 CATTATGCCC AGTACATGAC CTTATGGGAC TTTCCTACTT GGCAGTACAT 650

651 CTACGTATTA GTCATCGCTA TTACCATGGT GATGCGGTTT TGGCAGTACA 700

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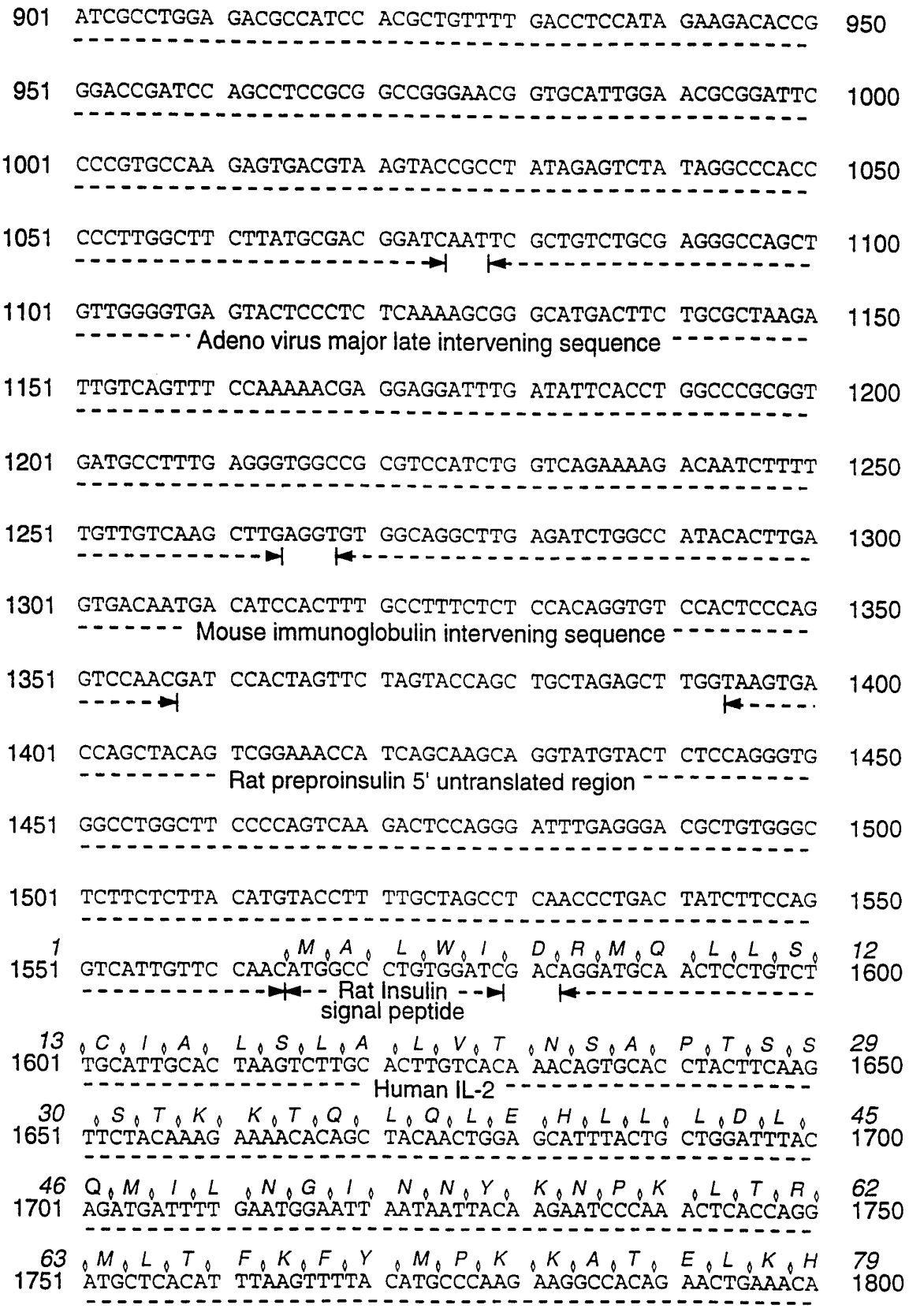
FIG._3A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1001 CCCGTGCCAA GAGTGACGTA AGTACCGCCT ATAGAGTCTA TAGGCCCACC 1050
1051 CCCTTGGCTT CTTATGCGAC GGATCAATTC GCTGTCTGCG AGGGCCAGCT 1100
1101 GTTGGGGTGA GTACTCCCTC TCAAAAGCGG GCATGACTTC TGCGCTAAGA 1150
Adeno virus major late intervening sequence
1151 TTGTCAGTTT CCAAAAACGA GGAGGATTTG ATATTCACCT GGCCCGCGGT 1200
1201 GATGCCTTTG AGGGTGGCCG CGTCCATCTG GTCAGAAAAG ACAATCTTTT 1250
1251 TGTTGTCAAG CTTGAGGTGT GGCAGGCTTG AGATCTGGCC ATACACTTGA 1300
1301 GTGACAATGA CATCCACTTT GCCTTTCTCT CCACAGGTGT CCACTCCCAG 1350
Mouse immunoglobulin intervening sequence
1351 GTCCAACGAT CCACTAGTTC TAGTACCAGC TGCTAGAGCT TGGTAAGTGA 1400
1401 CCAGCTACAG TCGGAAACCA TCAGCAAGCA GGTATGTACT CTCCAGGGTG 1450
Rat preproinsulin 5' untranslated region
1451 GGCCTGGCTT CCCCAGTCAA GACTCCAGGG ATTTGAGGGA CGCTGTGGGC 1500
1501 TCTTCTCTTA CATGTACCTT TTGCTAGCCT CAACCCTGAC TATCTTCCAG 1550
1 M A L W I D R M Q L L S 12
1551 GTCATTGTTC CAACATGGCC CTGTGGATCG ACAGGATGCA ACTCCTGTCT 1600
Rat Insulin signal peptide
13 C I A L S L A L V T N S A P T S S 29
1601 TGCATTGCAC TAAGTCTTGC ACTTGTCACA AACAGTGCAC CTACTTCAAG 1650
Human IL-2
30 S T K K T Q L Q L E H L L L D L 45
1651 TTCTACAAAG AAAACACAGC TACAACTGGA GCATTTACTG CTGGATTTAC 1700
46 Q M I L N G I N N Y K N P K L T R 62
1701 AGATGATTTT GAATGGAATT AATAATTACA AGAATCCCAA ACTCACCAGG 1750
63 M L T F K F Y M P K K A T E L K H 79
1751 ATGCTCACAT TTAAGTTTTA CATGCCCAAG AAGGCCACAG AACTGAAACA 1800

FIG._3B

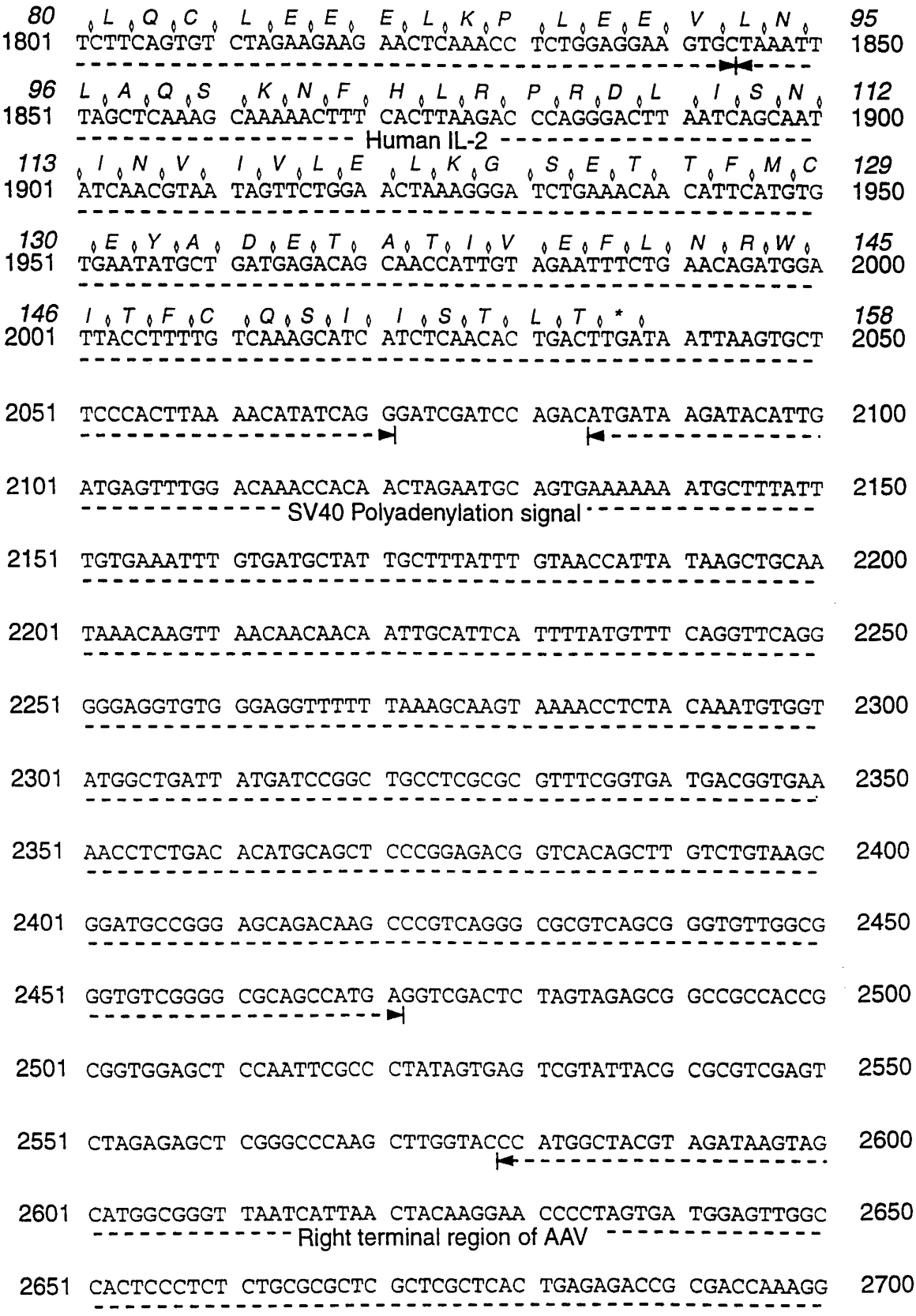

80 L Q C L E E E L K P L E E V L N 95
1801 TCTTCAGTGT CTAGAAGAAG AACTCAAACC TCTGGAGGAA GTGCTAAATT 1850
96 L A Q S K N F H L R P R D L I S N 112
1851 TAGCTCAAAG CAAAAACTTT CACTTAAGAC CCAGGGACTT AATCAGCAAT 1900
Human IL-2
113 I N V I V L E L K G S E T T F M C 129
1901 ATCAACGTAA TAGTTCTGGA ACTAAAGGGA TCTGAAACAA CATTCATGTG 1950
130 E Y A D E T A T I V E F L N R W 145
1951 TGAATATGCT GATGAGACAG CAACCATTGT AGAATTTCTG AACAGATGGA 2000
146 I T F C Q S I I S T L T * 158
2001 TTACCTTTTG TCAAAGCATC ATCTCAACAC TGACTTGATA ATTAAGTGCT 2050
2051 TCCCACTTAA AACATATCAG GGATCGATCC AGACATGATA AGATACATTG 2100
2101 ATGAGTTTGG ACAAACCACA ACTAGAATGC AGTGAAAAAA ATGCTTTATT 2150
SV40 Polyadenylation signal
2151 TGTGAAATTT GTGATGCTAT TGCTTTATTT GTAACCATTA TAAGCTGCAA 2200
2201 TAAACAAGTT AACAACAACA ATTGCATTCA TTTTATGTTT CAGGTTCAGG 2250
2251 GGGAGGTGTG GGAGGTTTTT TAAAGCAAGT AAAACCTCTA CAAATGTGGT 2300
2301 ATGGCTGATT ATGATCCGGC TGCCTCGCGC GTTTCGGTGA TGACGGTGAA 2350
2351 AACCTCTGAC ACATGCAGCT CCCGGAGACG GTCACAGCTT GTCTGTAAGC 2400
2401 GGATGCCGGG AGCAGACAAG CCCGTCAGGG CGCGTCAGCG GGTGTTGGCG 2450
2451 GGTGTCGGGG CGCAGCCATG AGGTCGACTC TAGTAGAGCG GCCGCCACCG 2500
2501 CGGTGGAGCT CCAATTCGCC CTATAGTGAG TCGTATTACG CGCGTCGAGT 2550
2551 CTAGAGAGCT CGGGCCCAAG CTTGGTACCC ATGGCTACGT AGATAAGTAG 2600
2601 CATGGCGGGT TAATCATTAA CTACAAGGAA CCCCTAGTGA TGGAGTTGGC 2650
Right terminal region of AAV
2651 CACTCCCTCT CTGCGCGCTC GCTCGCTCAC TGAGAGACCG CGACCAAAGG 2700

FIG._3C

2701 TCGCCCGACG CCCGGGCTTT GCCCGGGCGG CCTCAGTGAG CGAGCGAGCG 2750

2751 CGCAGAGAGG GACAGATCCA ATTCGCCCTA TAGTGAGTCG TATTACGCGC 2800
Bluescript KS II +

2801 GCTCACTGGC CGTCGTTTTA CAACGTCGTG ACTGGGAAAA CCCTGGCGTT 2850

2851 ACCCAACTTA ATCGCCTTGC AGCACATCCC CCTTTCGCCA GCTGGCGTAA 2900

2901 TAGCGAAGAG GCCCGCACCG ATCGCCCTTC CCAACAGTTG CGCAGCCTGA 2950

2951 ATGGCGAATG GGACGCGCCC TGTAGCGGCG CATTAAGCGC GGCGGGTGTG 3000

3001 GTGGTTACGC GCAGCGTGAC CGCTACACTT GCCAGCGCCC TAGCGCCCGC 3050

3051 TCCTTTCGCT TTCTTCCCTT CCTTTCTCGC CACGTTCGCC GGCTTTCCCC 3100

3101 GTCAAGCTCT AAATCGGGGG CTCCCTTTAG GGTTCCGATT TAGTGCTTTA 3150

3151 CGGCACCTCG ACCCCAAAAA ACTTGATTAG GGTGATGGTT CACGTAGTGG 3200

3201 GCCATCGCCC TGATAGACGG TTTTTCGCCC TTTGACGTTG GAGTCCACGT 3250

3251 TCTTTAATAG TGGACTCTTG TTCCAAACTG GAACAACACT CAACCCTATC 3300

3301 TCGGTCTATT CTTTTGATTT ATAAGGGATT TTGCCGATTT CGGCCTATTG 3350

3351 GTTAAAAAAT GAGCTGATTT AACAAAAATT TAACGCGAAT TTTAACAAAA 3400

3401 TATTAACGCT TACAATTTAG GTGGCACTTT TCGGGGAAAT GTGCGCGGAA 3450

3451 CCCCTATTTG TTTATTTTTC TAAATACATT CAAATATGTA TCCGCTCATG 3500

3501 AGACAATAAC CCTGATAAAT GCTTCAATAA TATTGAAAAA GGAAGAGTAT 3550

3551 GAGTATTCAA CATTTCCGTG TCGCCCTTAT TCCCTTTTTT GCGGCATTTT 3600

FIG._3D

```
3601  GCCTTCCTGT TTTTGCTCAC CCAGAAACGC TGGTGAAAGT AAAAGATGCT  3650
      ----------------------------------------------------

3651  GAAGATCAGT TGGGTGCACG AGTGGGTTAC ATCGAACTGG ATCTCAACAG  3700
      ----------------------------------------------------

3701  CGGTAAGATC CTTGAGAGTT TTCGCCCCGA AGAACGTTTT CCAATGATGA  3750
      ----------------------------------------------------

3751  GCACTTTTAA AGTTCTGCTA TGTGGCGCGG TATTATCCCG TATTGACGCC  3800
      ----------------------------------------------------

3801  GGGCAAGAGC AACTCGGTCG CCGCATACAC TATTCTCAGA ATGACTTGGT  3850
      ----------------------------------------------------

3851  TGAGTACTCA CCAGTCACAG AAAAGCATCT TACGGATGGC ATGACAGTAA  3900
      ----------------------------------------------------

3901  GAGAATTATG CAGTGCTGCC ATAACCATGA GTGATAACAC TGCGGCCAAC  3950
      ----------------------------------------------------

3951  TTACTTCTGA CAACGATCGG AGGACCGAAG GAGCTAACCG CTTTTTTGCA  4000
      ----------------------------------------------------

4001  CAACATGGGG GATCATGTAA CTCGCCTTGA TCGTTGGGAA CCGGAGCTGA  4050
      ----------------------------------------------------

4051  ATGAAGCCAT ACCAAACGAC GAGCGTGACA CCACGATGCC TGTAGCAATG  4100
      ----------------------------------------------------

4101  GCAACAACGT TGCGCAAACT ATTAACTGGC GAACTACTTA CTCTAGCTTC  4150
      ----------------------------------------------------

4151  CCGGCAACAA TTAATAGACT GGATGGAGGC GGATAAAGTT GCAGGACCAC  4200
      ----------------------------------------------------

4201  TTCTGCGCTC GGCCCTTCCG GCTGGCTGGT TTATTGCTGA TAAATCTGGA  4250
      ----------------------------------------------------

4251  GCCGGTGAGC GTGGGTCTCG CGGTATCATT GCAGCACTGG GGCCAGATGG  4300
      ----------------------------------------------------

4301  TAAGCCCTCC CGTATCGTAG TTATCTACAC GACGGGGAGT CAGGCAACTA  4350
      ----------------------------------------------------

4351  TGGATGAACG AAATAGACAG ATCGCTGAGA TAGGTGCCTC ACTGATTAAG  4400
      ----------------------------------------------------

4401  CATTGGTAAC TGTCAGACCA AGTTTACTCA TATATACTTT AGATTGATTT  4450
      ----------------------------------------------------

4451  AAAACTTCAT TTTTAATTTA AAAGGATCTA GGTGAAGATC CTTTTTGATA  4500
      ----------------------------------------------------
```

FIG._3E

4501 ATCTCATGAC CAAAATCCCT TAACGTGAGT TTTCGTTCCA CTGAGCGTCA 4550

4551 GACCCCGTAG AAAAGATCAA AGGATCTTCT TGAGATCCTT TTTTTCTGCG 4600

4601 CGTAATCTGC TGCTTGCAAA CAAAAAAACC ACCGCTACCA GCGGTGGTTT 4650

4651 GTTTGCCGGA TCAAGAGCTA CCAACTCTTT TTCCGAAGGT AACTGGCTTC 4700

4701 AGCAGAGCGC AGATACCAAA TACTGTCCTT CTAGTGTAGC CGTAGTTAGG 4750

4751 CCACCACTTC AAGAACTCTG TAGCACCGCC TACATACCTC GCTCTGCTAA 4800

4801 TCCTGTTACC AGTGGCTGCT GCCAGTGGCG ATAAGTCGTG TCTTACCGGG 4850

4851 TTGGACTCAA GACGATAGTT ACCGGATAAG GCGCAGCGGT CGGGCTGAAC 4900

4901 GGGGGGTTCG TGCACACAGC CCAGCTTGGA GCGAACGACC TACACCGAAC 4950

4951 TGAGATACCT ACAGCGTGAG CTATGAGAAA GCGCCACGCT TCCCGAAGGG 5000

5001 AGAAAGGCGG ACAGGTATCC GGTAAGCGGC AGGGTCGGAA CAGGAGAGCG 5050

5051 CACGAGGGAG CTTCCAGGGG GAAACGCCTG GTATCTTTAT AGTCCTGTCG 5100

5101 GGTTTCGCCA CCTCTGACTT GAGCGTCGAT TTTTGTGATG CTCGTCAGGG 5150

5151 GGGCGGAGCC TATGGAAAAA CGCCAGCAAC GCGGCCTTTT TACGGTTCCT 5200

5201 GGCCTTTTGC TGGCCTTTTG CTCACATGTT CTTTCCTGCG TTATCCCCTG 5250

5251 ATTCTGTGGA TAACCGTATT ACCGCCTTTG AGTGAGCTGA TACCGCTCGC 5300

5301 CGCAGCCGAA CGACCGAGCG CAGCGAGTCA GTGAGCGAGG AAGCGGAAGA 5350

5351 GCGCCCAATA CGCAAACCGC CTCTCCCCGC GCGTTGGCCG ATTCATTAAT 5400

FIG._3F

5401 GCAGCTGGCA CGACAGGTTT CCCGACTGGA AAGCGGGCAG TGAGCGCAAC 5450

5451 GCAATTAATG TGAGTTAGCT CACTCATTAG GCACCCCAGG CTTTACACTT 5500

5501 TATGCTTCCG GCTCGTATGT TGTGTGGAAT TGTGAGCGGA TAACAATTTC 5550
Bluescript KS II +

5551 ACACAGGAAA CAGCTATGAC CATGATTACG CCAAG 5585

FIG._3G

FIG._3

FIG._3A

FIG._3B

FIG._3C

FIG._3D

FIG._3E

FIG._3F

FIG._3G

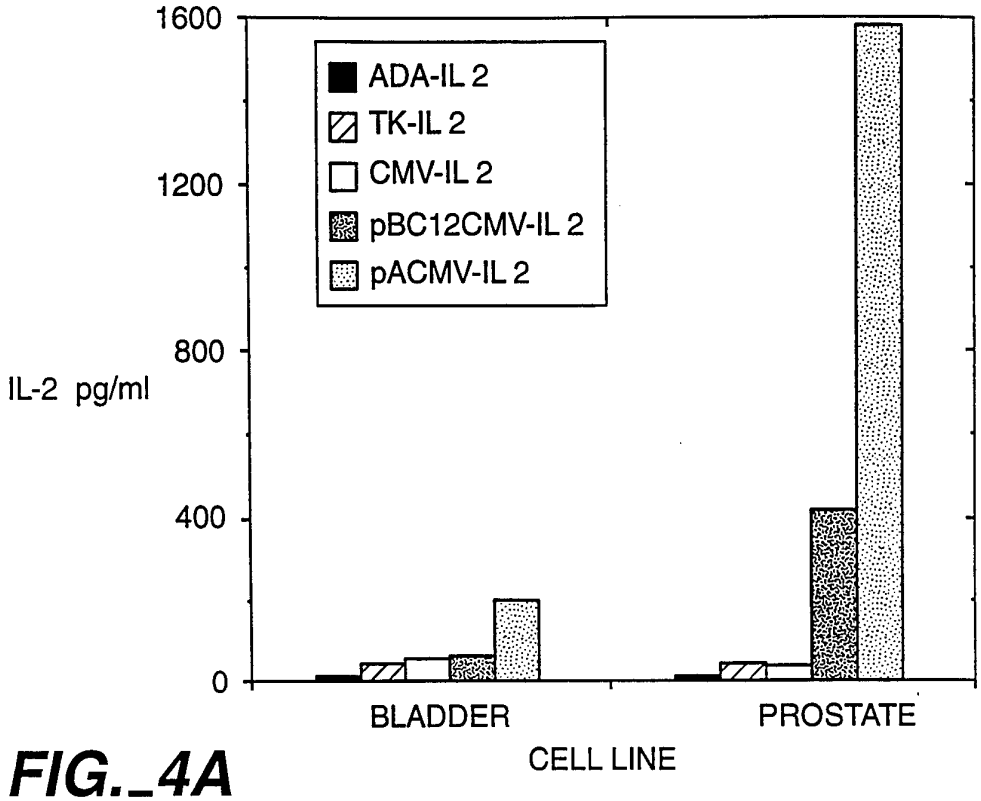

1600
1200
800
400
0
IL-2 pg/ml
ADA-IL 2
TK-IL 2
CMV-IL 2
pBC12CMV-IL 2
pACMV-IL 2
BLADDER
PROSTATE
CELL LINE

FIG._4A

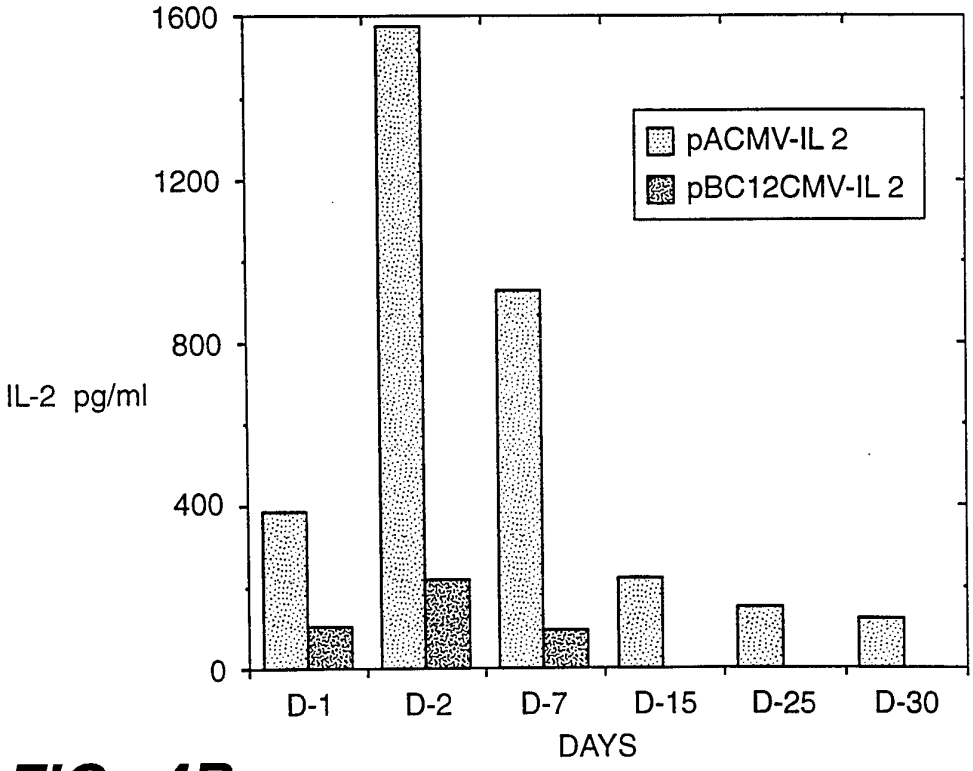

1600
1200
800
400
0
IL-2 pg/ml
pACMV-IL 2
pBC12CMV-IL 2
D-1
D-2
D-7
D-15
D-25
D-30
DAYS

FIG._4B

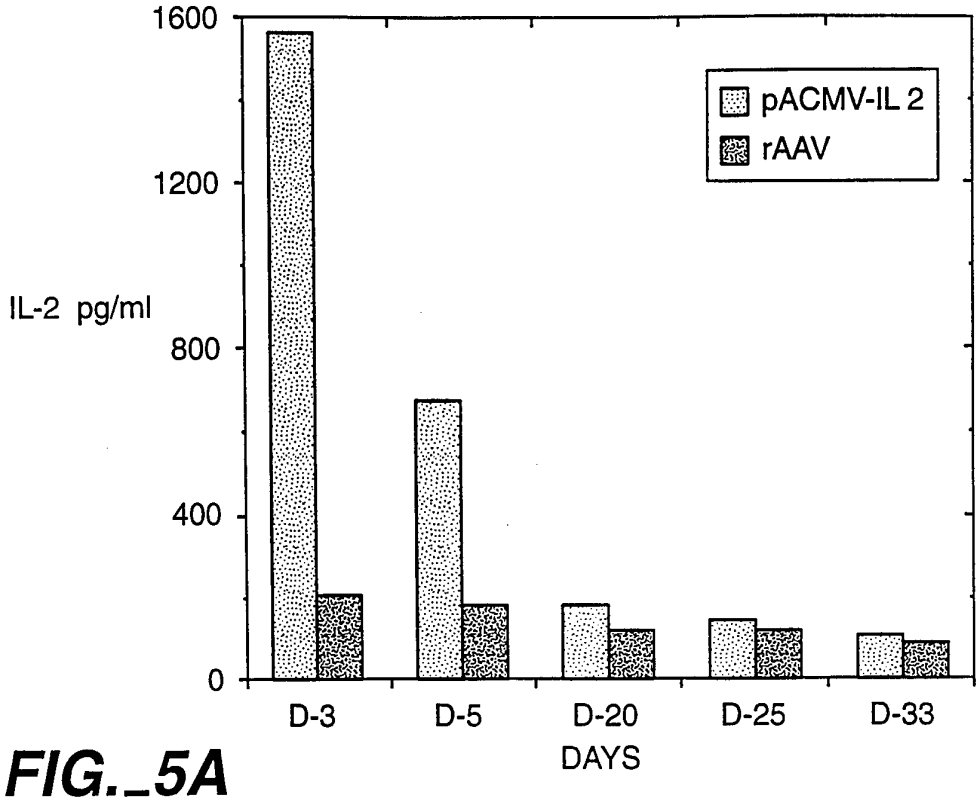

1600
1200
800
400
0
IL-2 pg/ml
pACMV-IL 2
rAAV
D-3
D-5
D-20
D-25
D-33
DAYS

FIG._5A

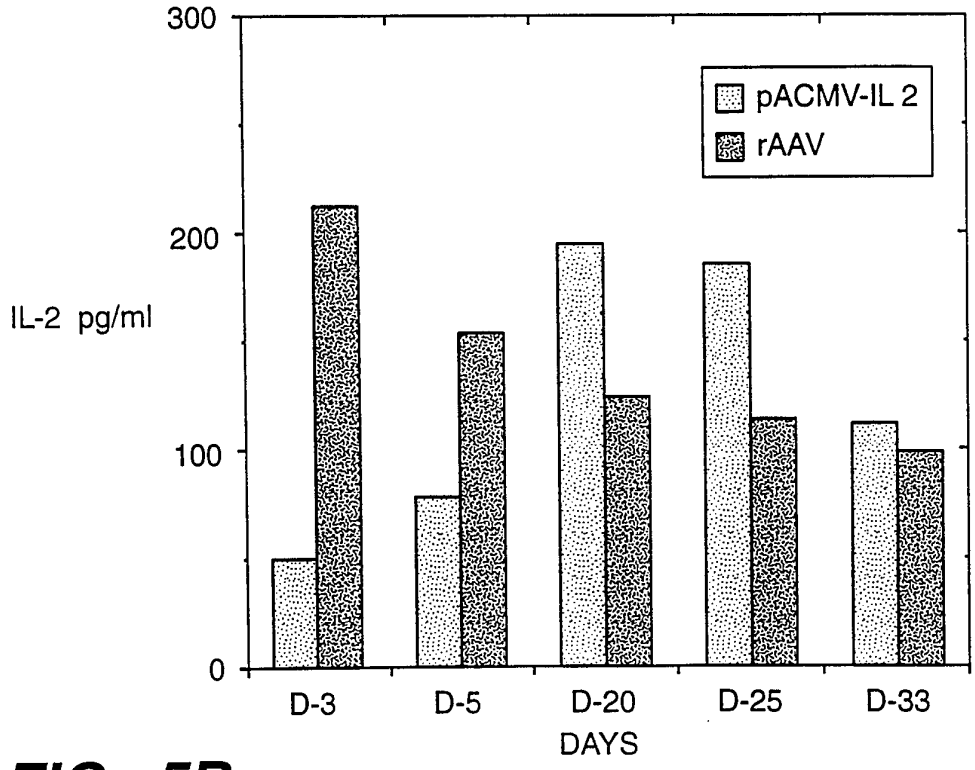

300
200
100
0
IL-2 pg/ml
pACMV-IL 2
rAAV
D-3
D-5
D-20
D-25
D-33
DAYS

FIG._5B

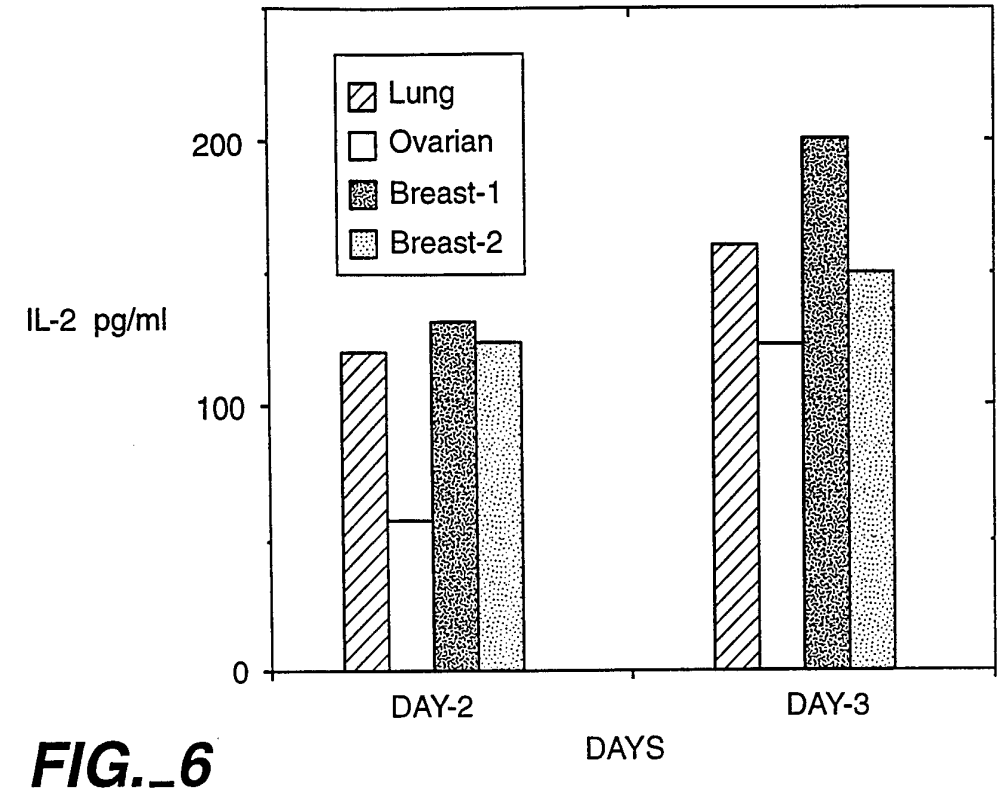

Lung
Ovarian
Breast-1
Breast-2
200
100
0
IL-2 pg/ml
DAY-2
DAY-3
DAYS

FIG._6

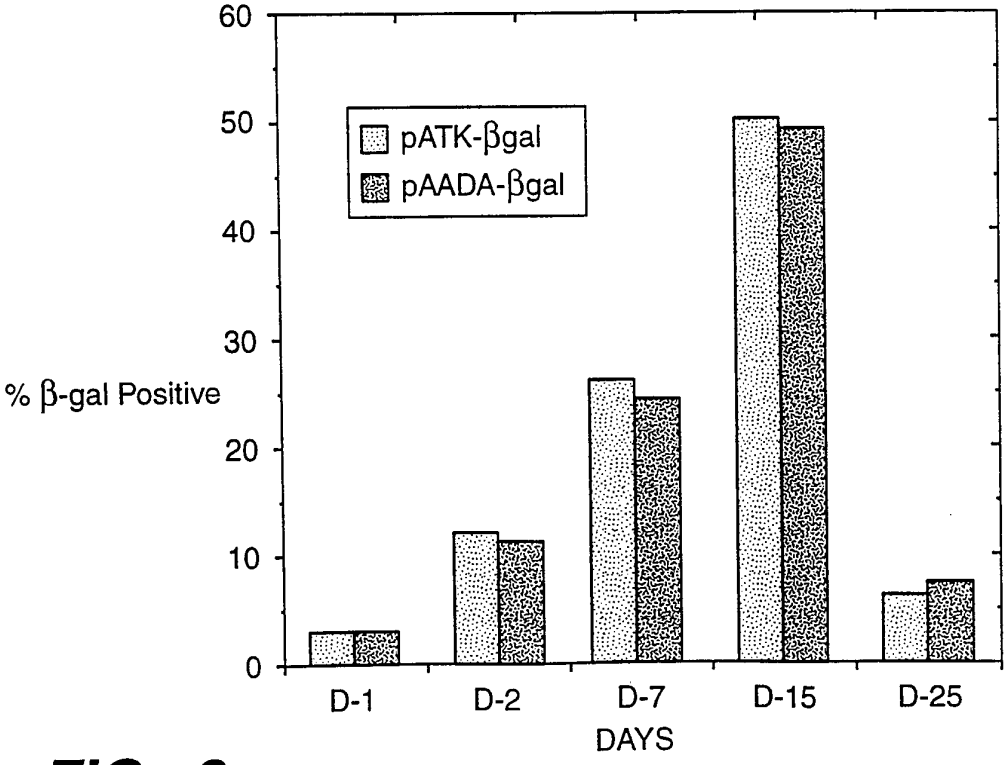

pATK-βgal
pAADA-βgal
60
50
40
30
20
10
0
% β-gal Positive
D-1
D-2
D-7
D-15
D-25
DAYS

FIG._8

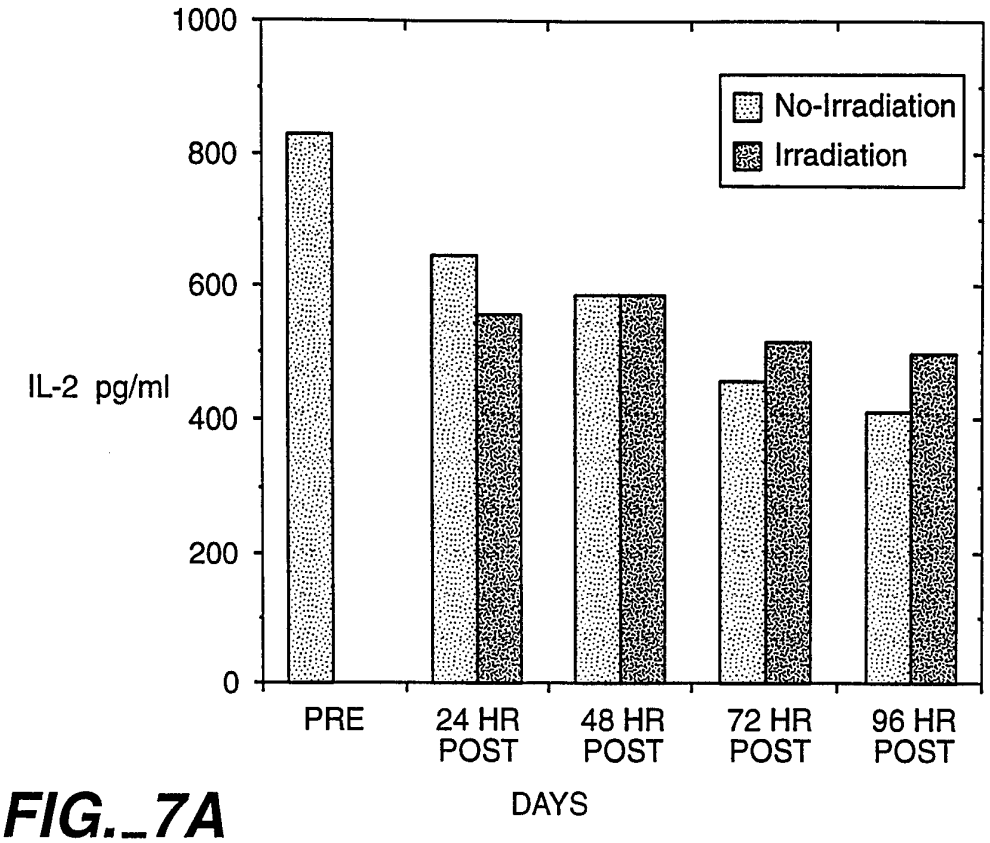

1000
800
600
400
200
0
IL-2 pg/ml
No-Irradiation
Irradiation
PRE
24 HR POST
48 HR POST
72 HR POST
96 HR POST
DAYS

FIG._7A

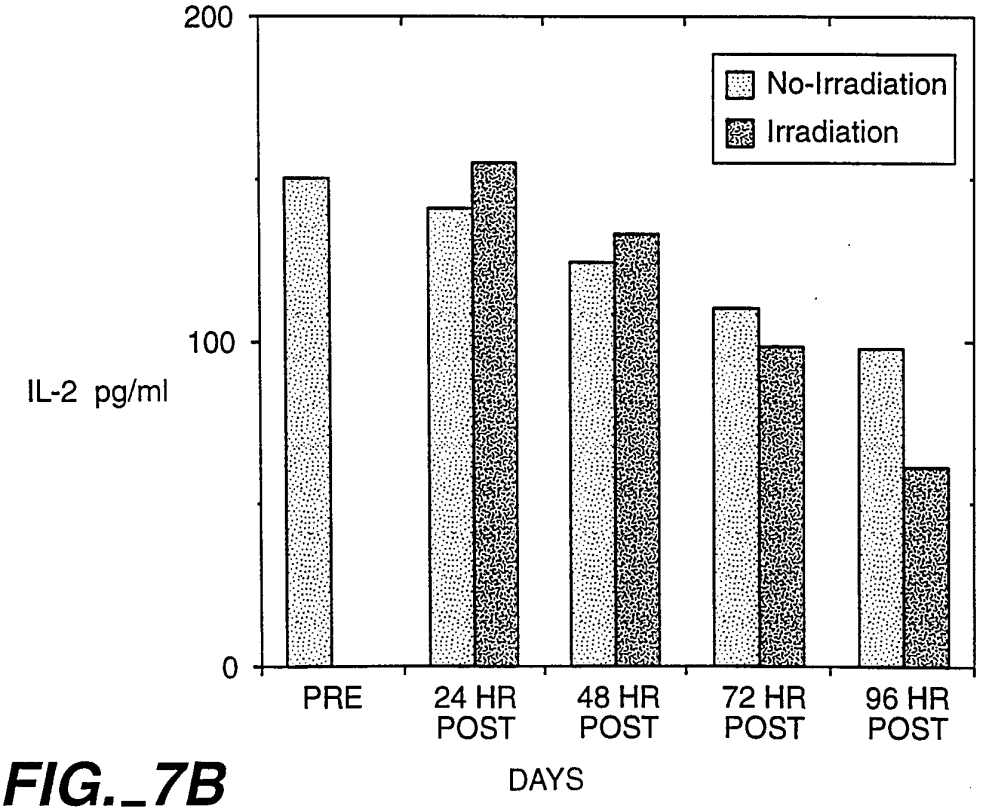
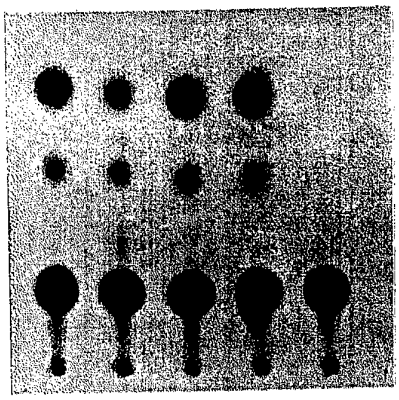

200
100
0
IL-2 pg/ml
No-Irradiation
Irradiation
PRE
24 HR POST
48 HR POST
72 HR POST
96 HR POST
DAYS

FIG._7B



d2

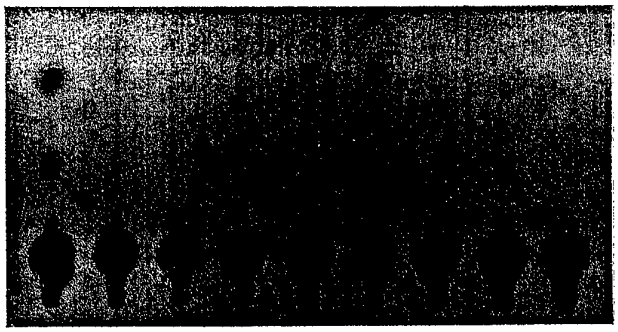
FIG._9A

①10 µg PACMVIXCAT + 10 nmole D as D:D 1:1

②10 µg PACMVIXCAT + 20 nmole D as D:D 1:1

③10 µg PACMVIXCAT + 10 nmole D as D:C 1:1

④10 µg PACMVIXCAT + 20 nmole D as D:C 1:1



CD 5/8

FIG._9B

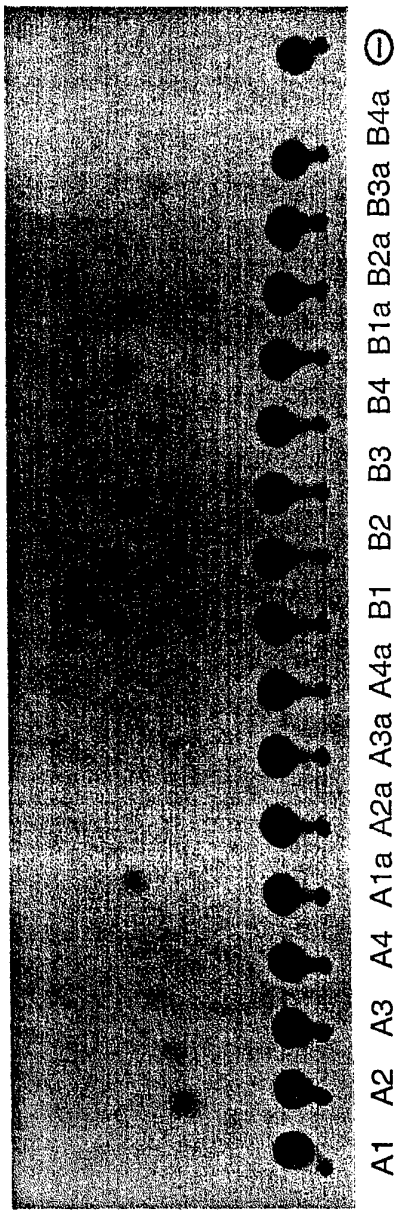

A1 A2 A3 A4 A1a A2a A3a A4a B1 B2 B3 B4 B1a B2a B3a B4a ⊖

FIG._9C

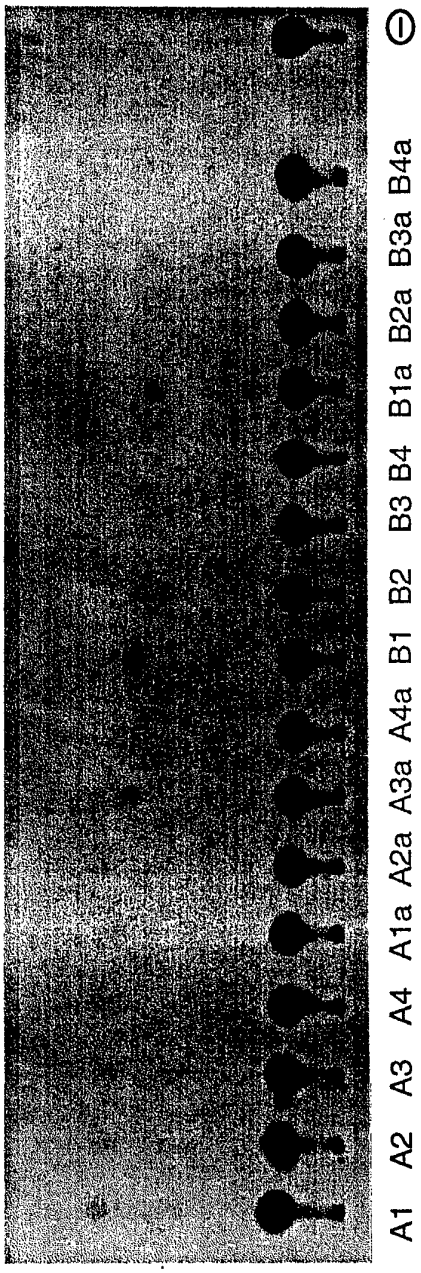
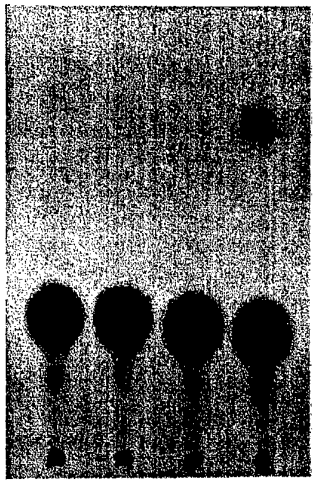

A1 A2 A3 A4 A1a A2a A3a A4a B1 B2 B3 B4 B1a B2a B3a B4a ⊖

FIG._9D

FIG._10


A B A B
d3 CD34 d7

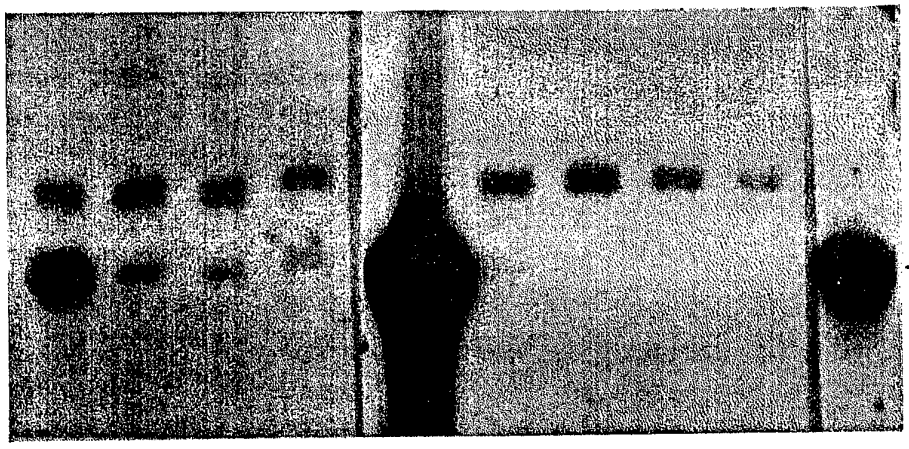

0.685kb
day 5 9 16 25 5 10 16 19 C plasmid
pA CMV-IL2
pBC12CMV-IL2

FIG._17

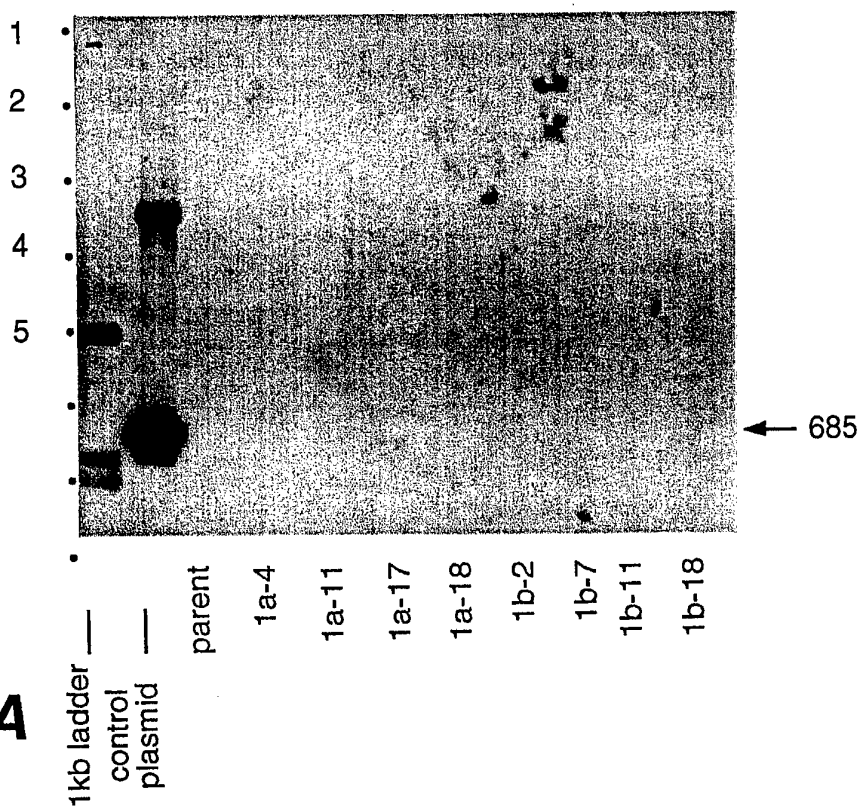


FIG._11A

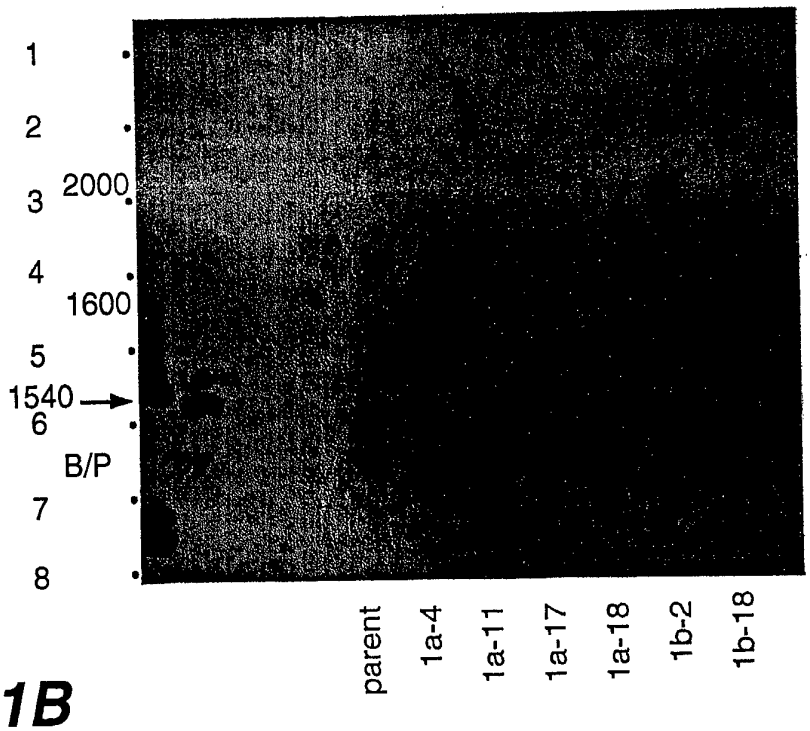
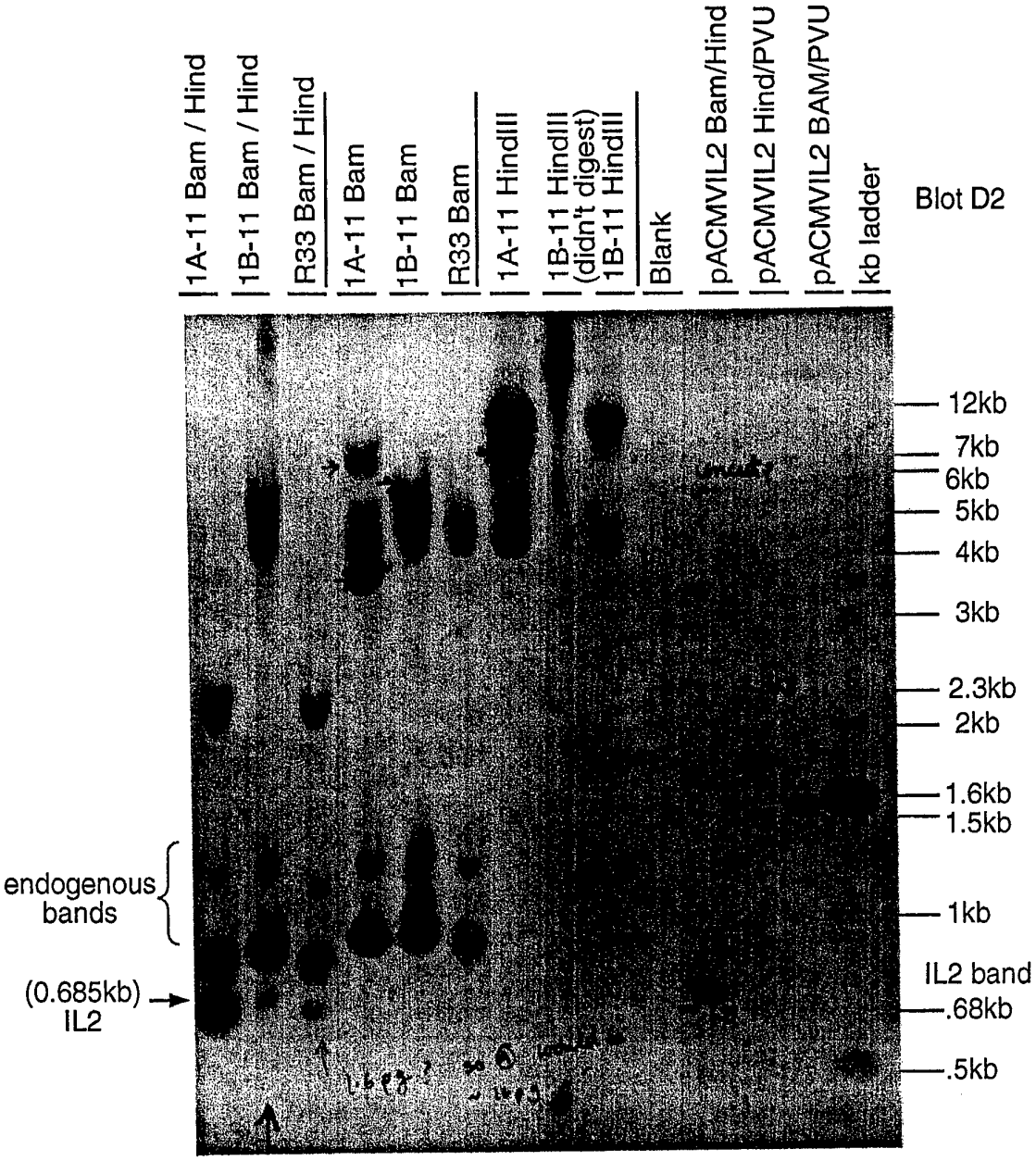


FIG._11B



7 hr exposure:

FIG._12A

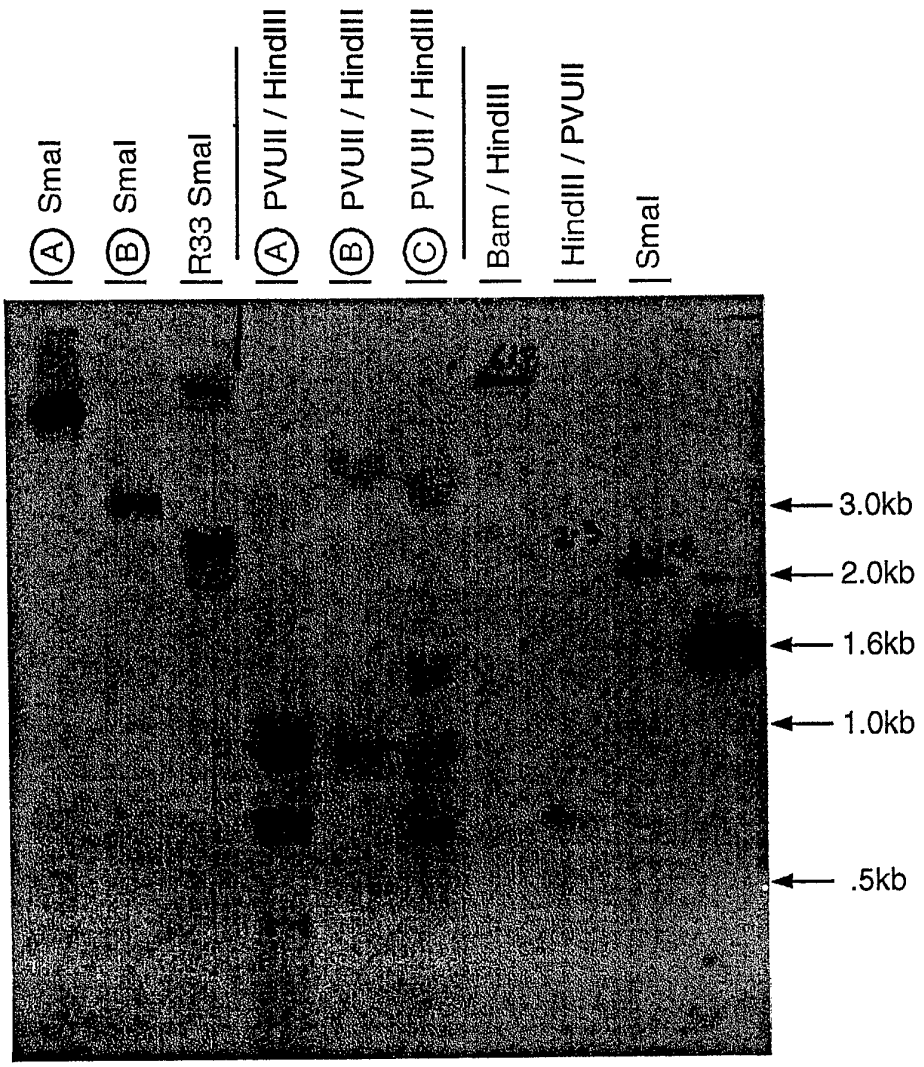

Ⓐ SmaI
Ⓑ SmaI
R33 SmaI
Ⓐ PVUII / HindIII
Ⓑ PVUII / HindIII
Ⓒ PVUII / HindIII
Bam / HindIII
HindIII / PVUII
SmaI
3.0kb
2.0kb
1.6kb
1.0kb
.5kb

FIG._12B

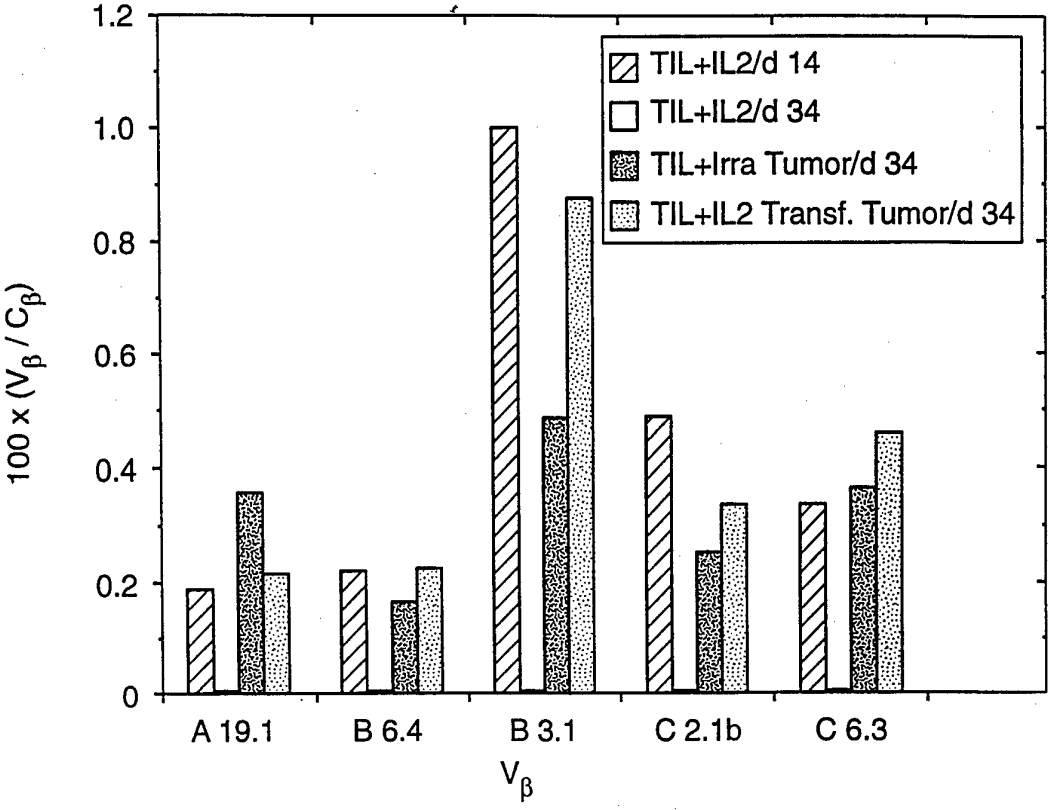

1.2
1.0
0.8
0.6
0.4
0.2
0
100 x (Vβ / Cβ)
TIL+IL2/d 14
TIL+IL2/d 34
TIL+Irra Tumor/d 34
TIL+IL2 Transf. Tumor/d 34
A 19.1
B 6.4
B 3.1
C 2.1b
C 6.3
Vβ

FIG._13

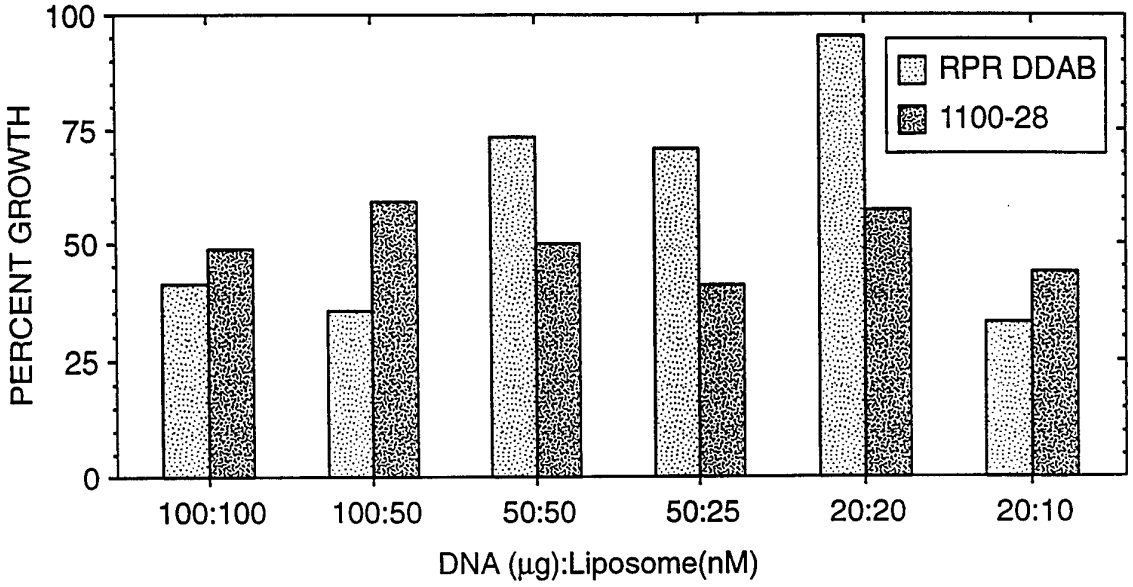

100
75
50
25
0
PERCENT GROWTH
RPR DDAB
1100-28
100:100
100:50
50:50
50:25
20:20
20:10
DNA (µg):Liposome(nM)

FIG._14

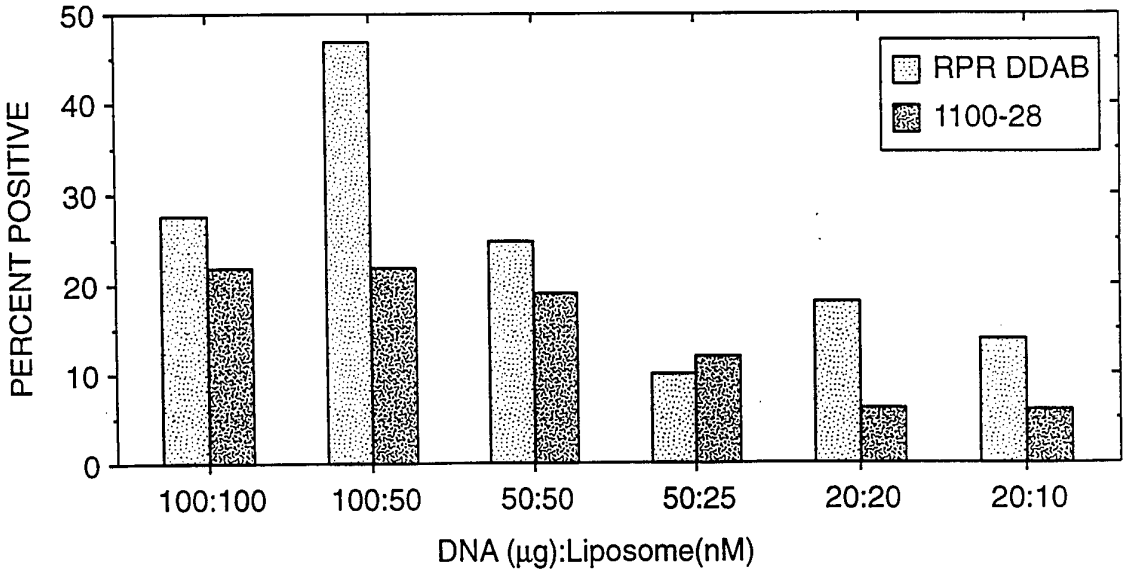

50
40
30
20
10
0
PERCENT POSITIVE
RPR DDAB
1100-28
100:100
100:50
50:50
50:25
20:20
20:10
DNA (µg):Liposome(nM)

FIG._15

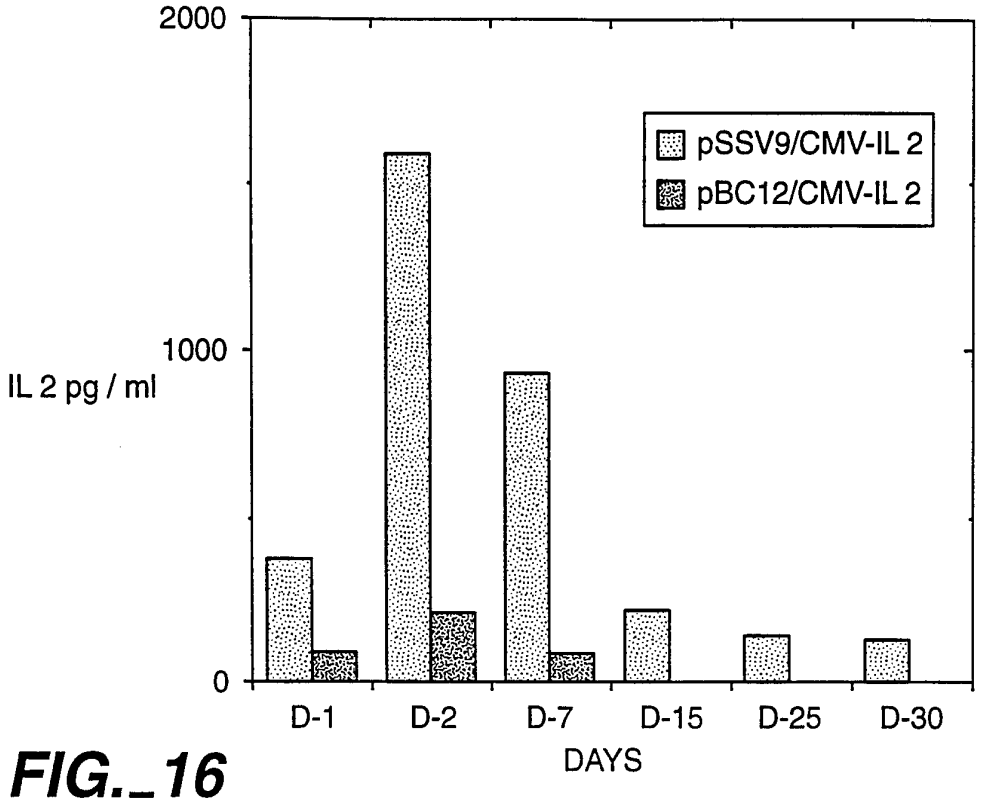

2000
1000
0
IL 2 pg / ml
pSSV9/CMV-IL 2
pBC12/CMV-IL 2
D-1
D-2
D-7
D-15
D-25
D-30
DAYS

FIG._16

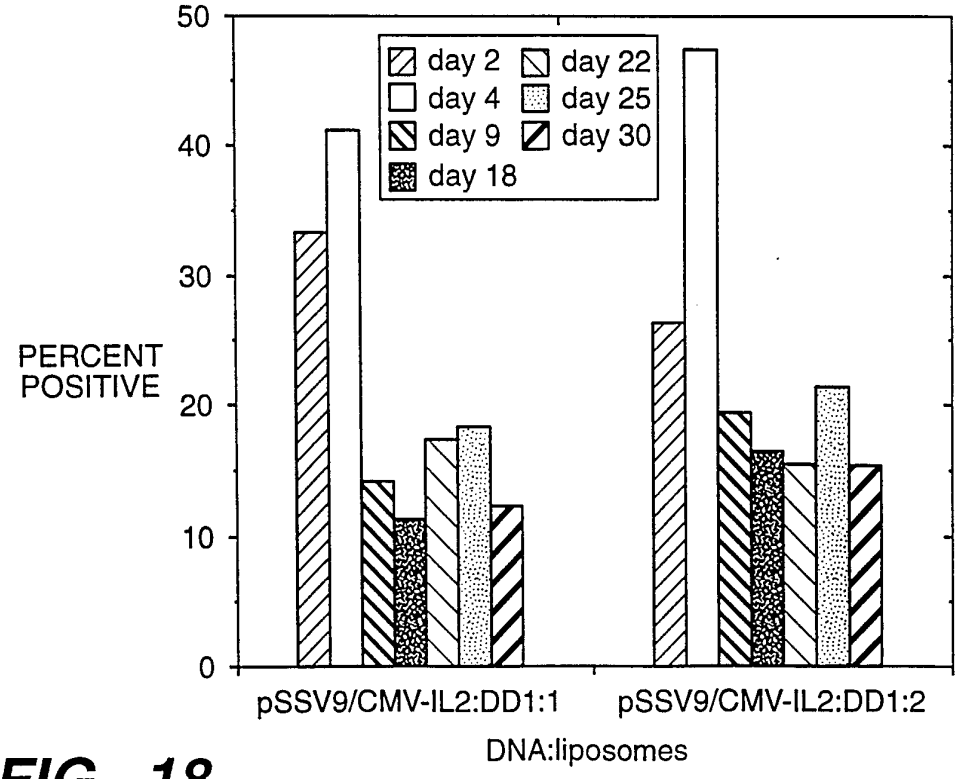

50
40
30
20
10
0
PERCENT POSITIVE
day 2
day 4
day 9
day 18
day 22
day 25
day 30
pSSV9/CMV-IL2:DD1:1
pSSV9/CMV-IL2:DD1:2
DNA:liposomes

FIG._18

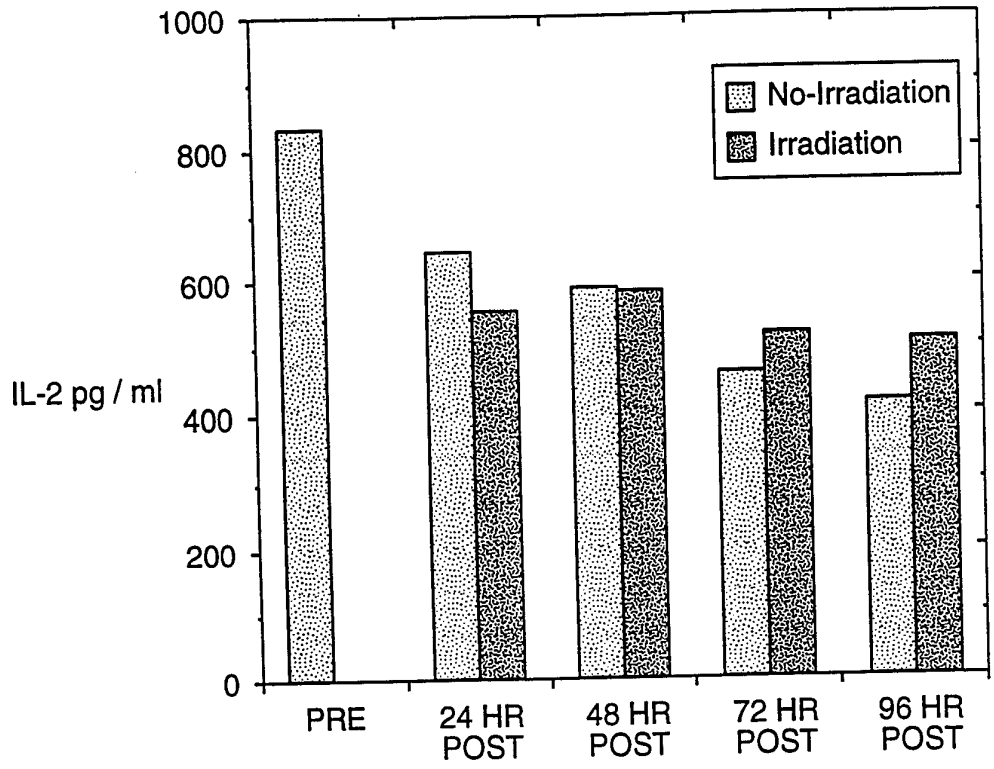


FIG._19A

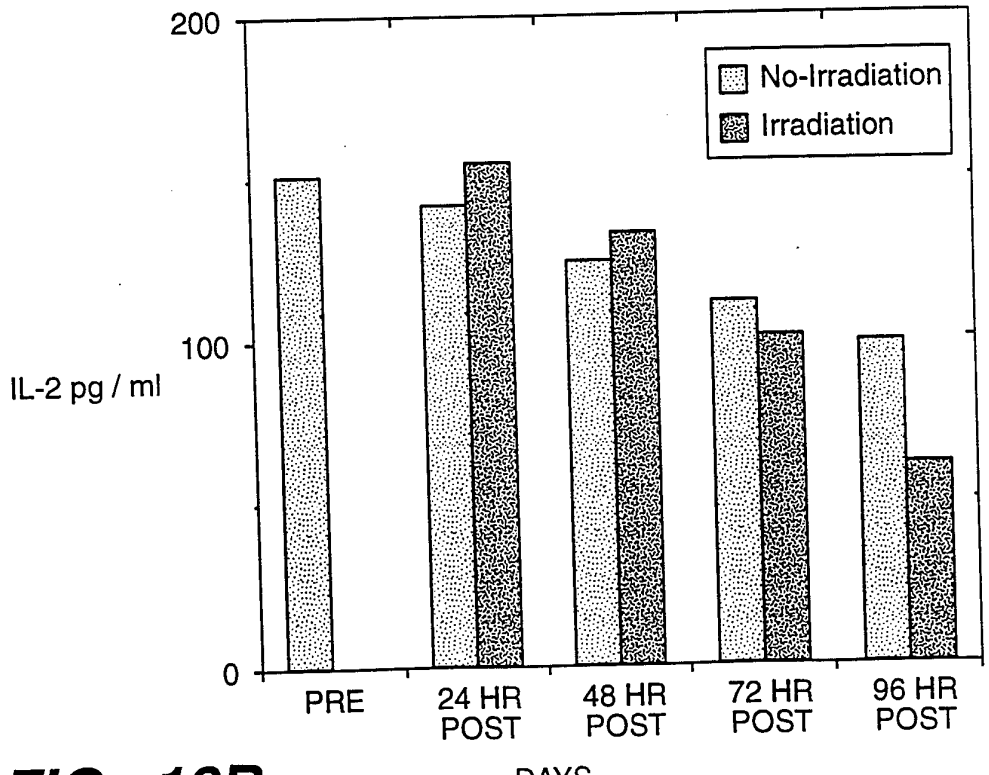


FIG._19B

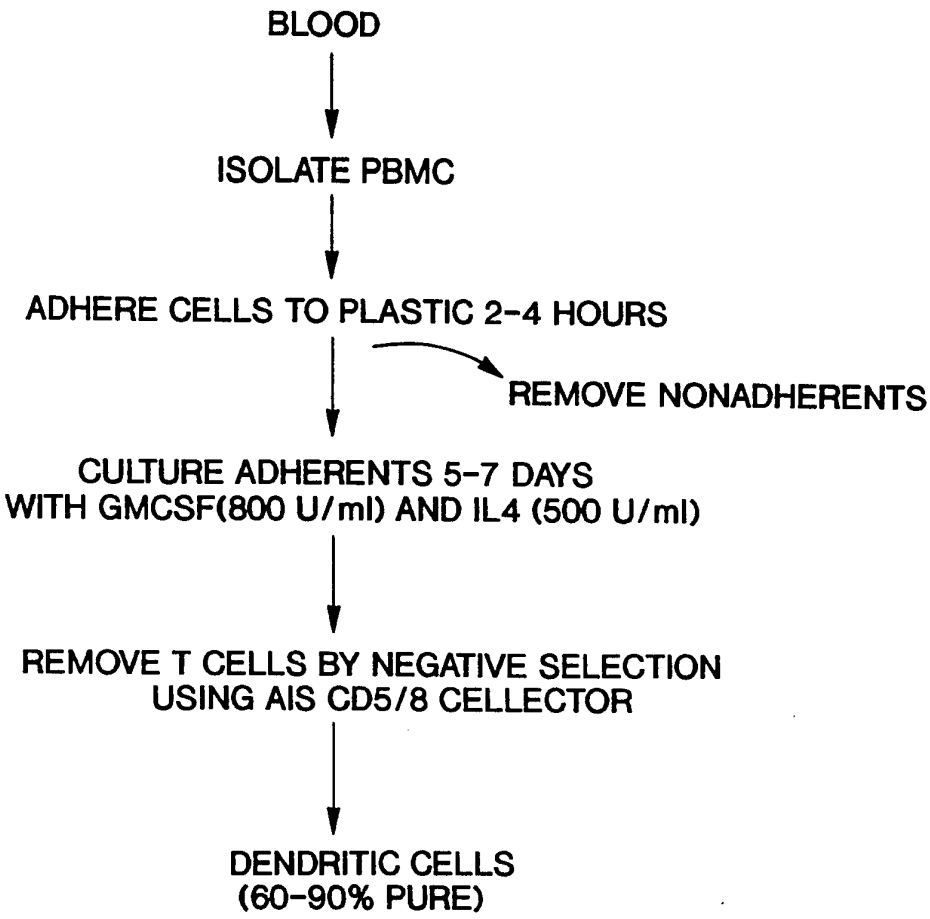

BLOOD
ISOLATE PBMC
ADHERE CELLS TO PLASTIC 2-4 HOURS
REMOVE NONADHERENTS
CULTURE ADHERENTS 5-7 DAYS
WITH GMCSF(800 U/ml) AND IL4 (500 U/ml)
REMOVE T CELLS BY NEGATIVE SELECTION
USING AIS CD5/8 CELLECTOR
DENDRITIC CELLS
(60-90% PURE)

FIG. 20

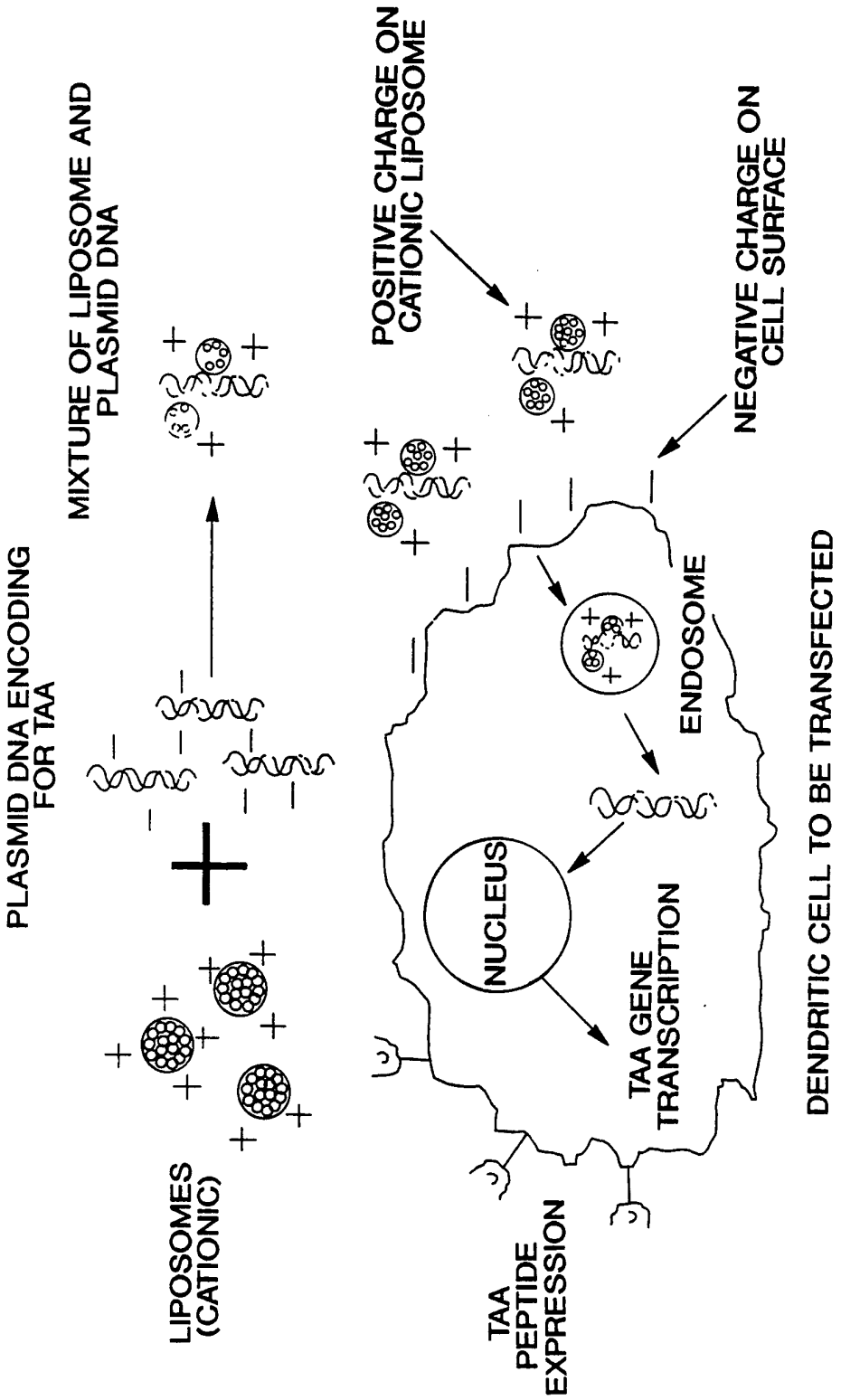
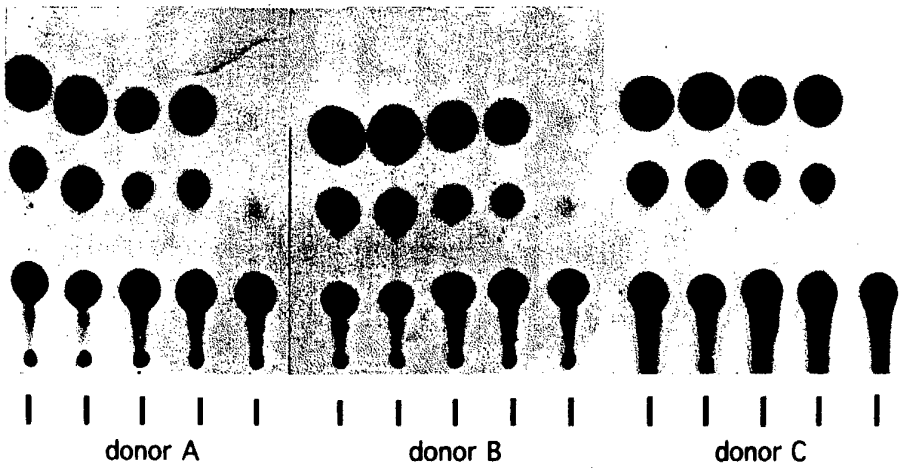

PLASMID DNA ENCODING FOR TAA
MIXTURE OF LIPOSOME AND PLASMID DNA
LIPOSOMES (CATIONIC)
POSITIVE CHARGE ON CATIONIC LIPOSOME
NEGATIVE CHARGE ON CELL SURFACE
ENDOSOME
NUCLEUS
TAA GENE TRANSCRIPTION
TAA PEPTIDE EXPRESSION
DENDRITIC CELL TO BE TRANSFECTED

FIG. 21



	donor A					donor B					donor C				
µgDNA	10	20	10	20	0	10	20	10	20	0	10	20	10	20	0
nmole liposomes	10	20	10	20	0	10	20	10	20	0	10	20	10	20	0
liposome composition	DD:D	1:1	DDCD	1:1:1		DD:D	1:1	DDCD	1:1:1		DD:D	1:1	DDCD	1:1:1	

FIG. 22a

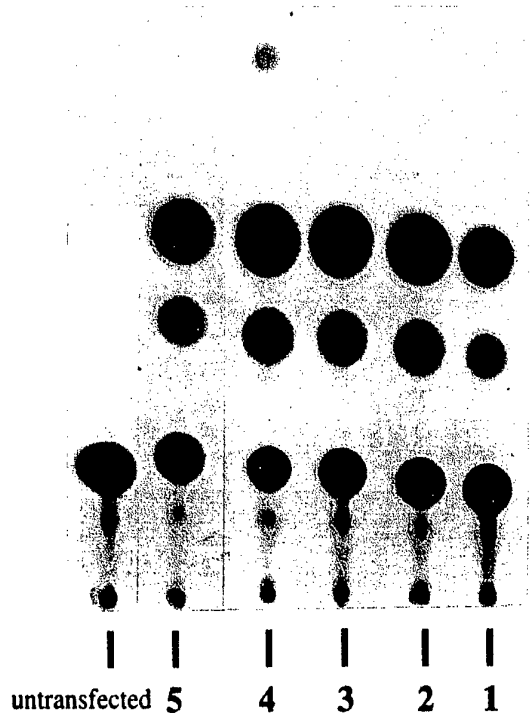


FIG. 22b

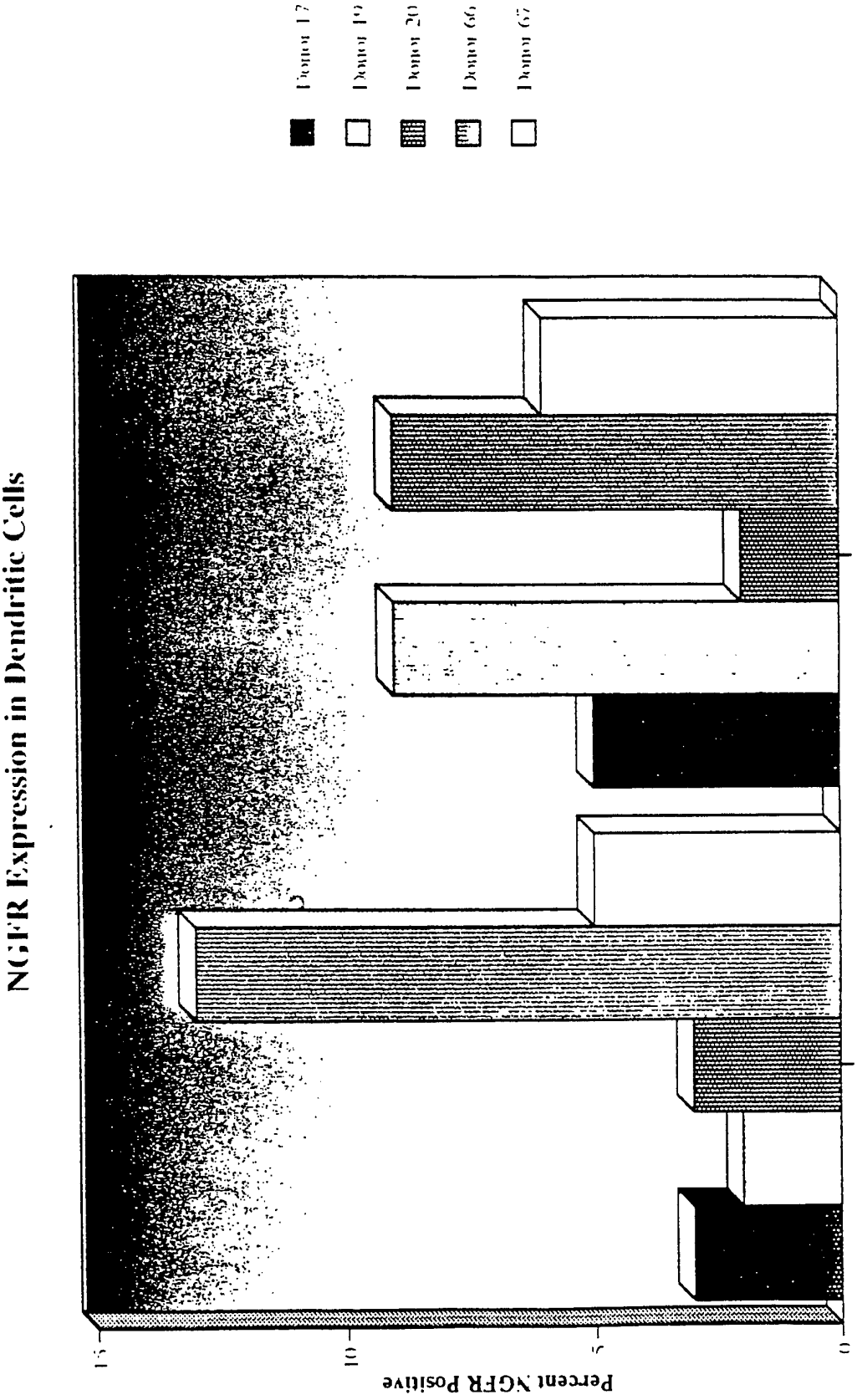

Donor 17
Donor 19
Donor 20
Donor 66
Donor 67
NGFR Expression in Dendritic Cells
Percent NGFR Positive
15
10
5
0
DDAB:Chol:DOPE
DDAB:DOPE
pMP6NGFR:Liposome

Figure 23

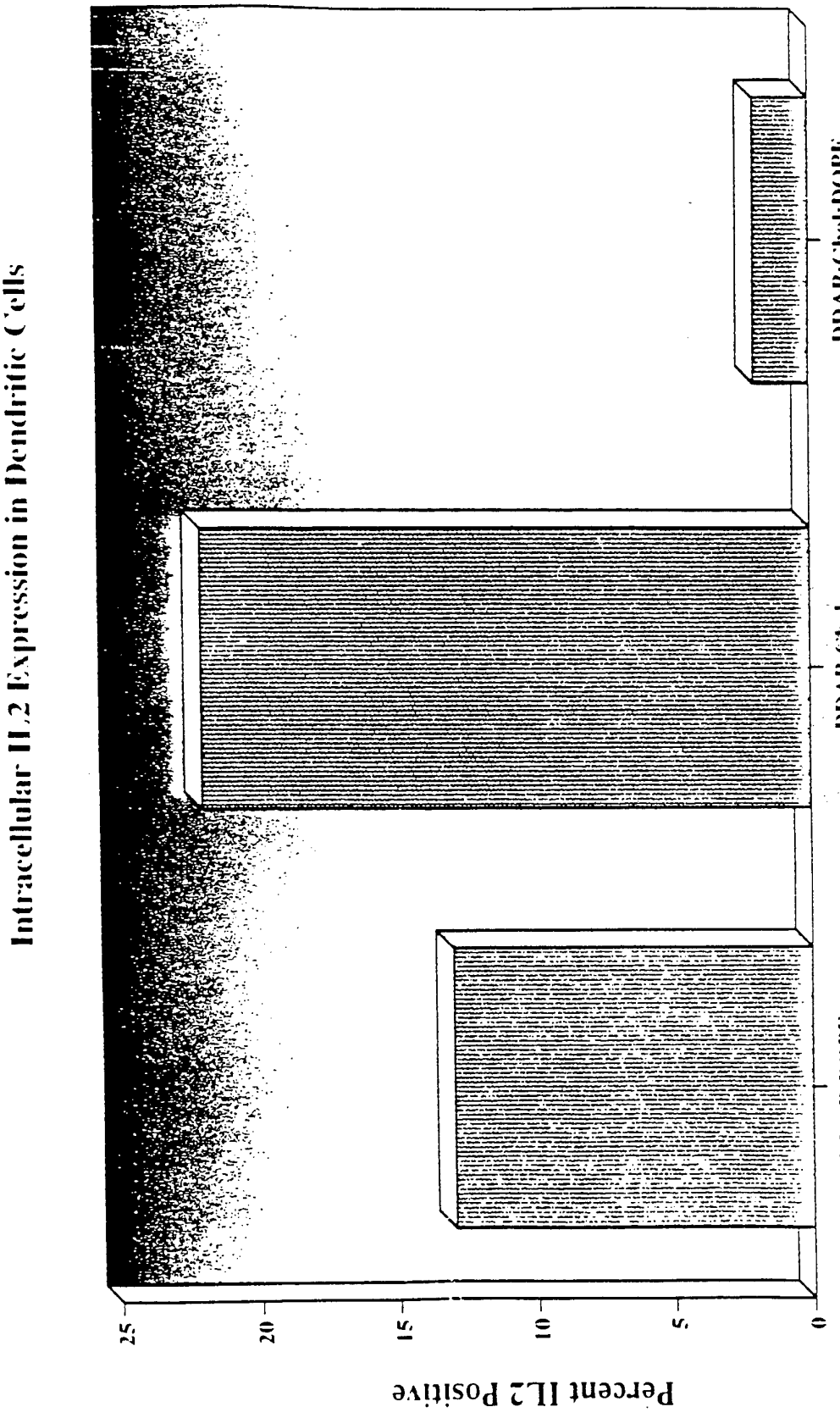

Intracellular IL2 Expression in Dendritic Cells
Percent IL2 Positive
0
5
10
15
20
25
DDAB:DOPE
DDAB:Chol
DDAB:Chol:DOPE
pMP6AIL2:Liposome

Figure 24

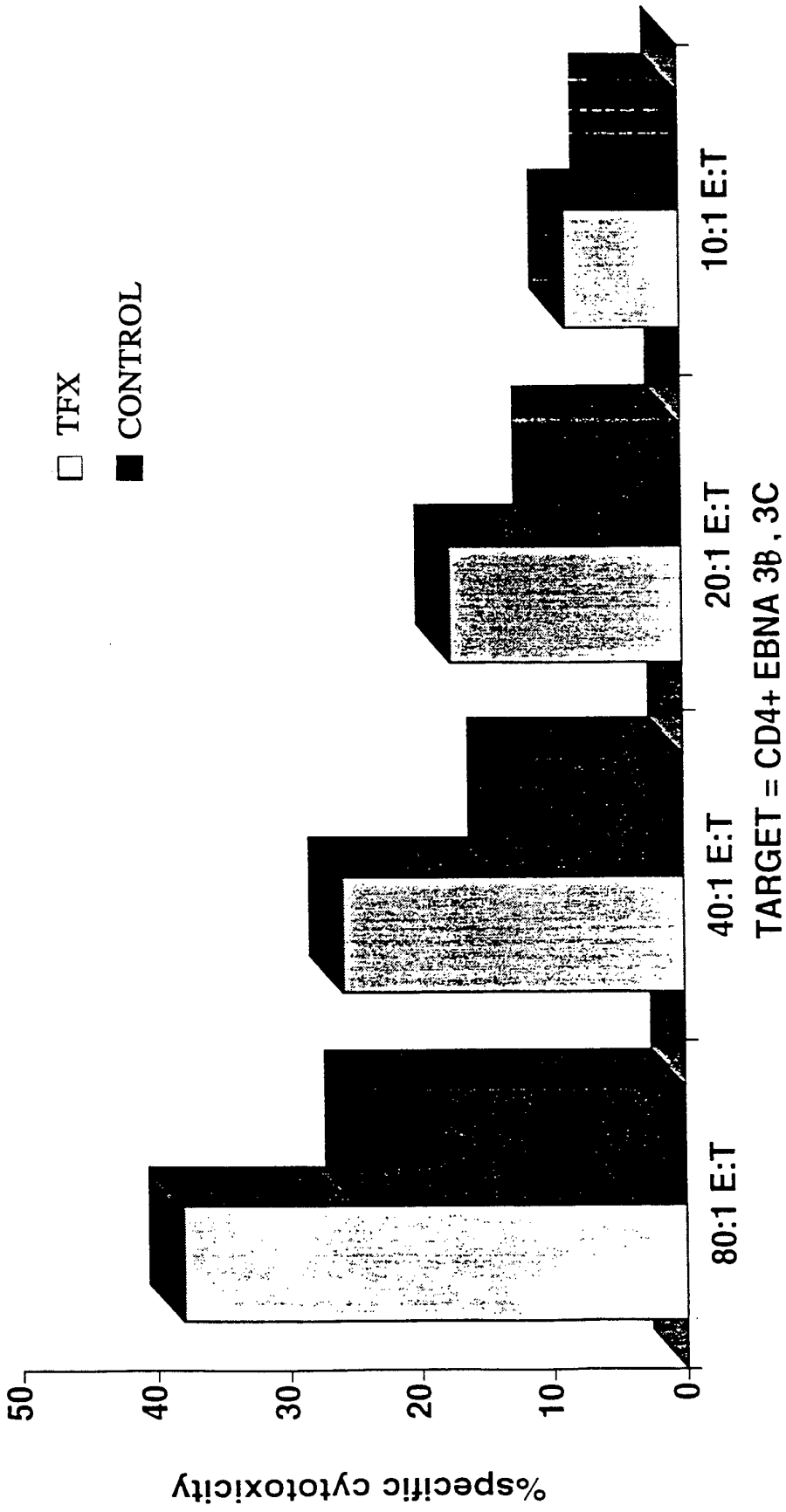

TFX
CONTROL
%specific cytotoxicity
0
10
20
30
40
50
80:1 E:T
40:1 E:T
20:1 E:T
10:1 E:T
TARGET = CD4+ EBNA 3B , 3C

Figure 25

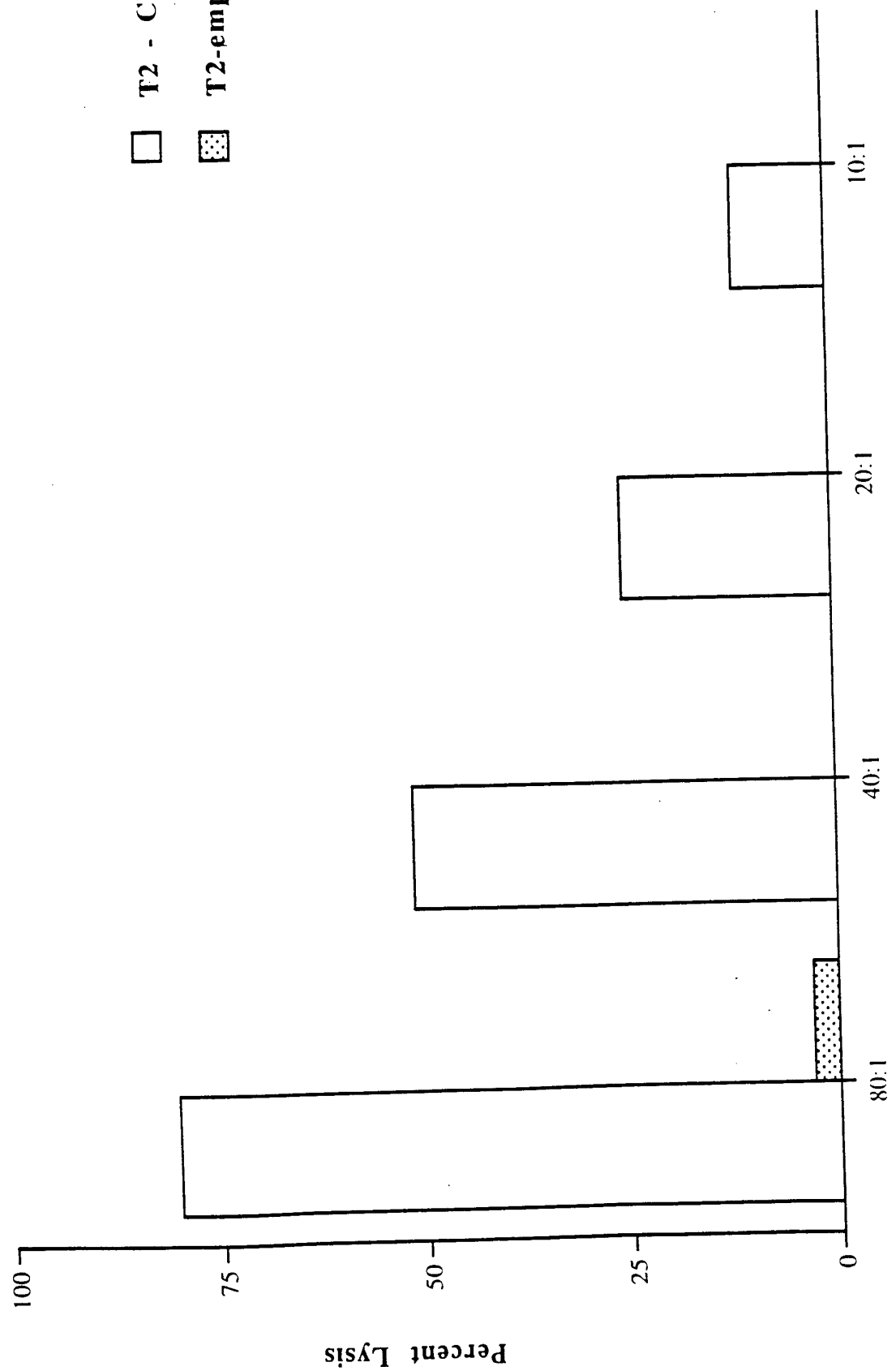

T2 - CEA
T2-empty
Percent Lysis
100
75
50
25
0
80:1
40:1
20:1
10:1
Effector:Target

Figure 26

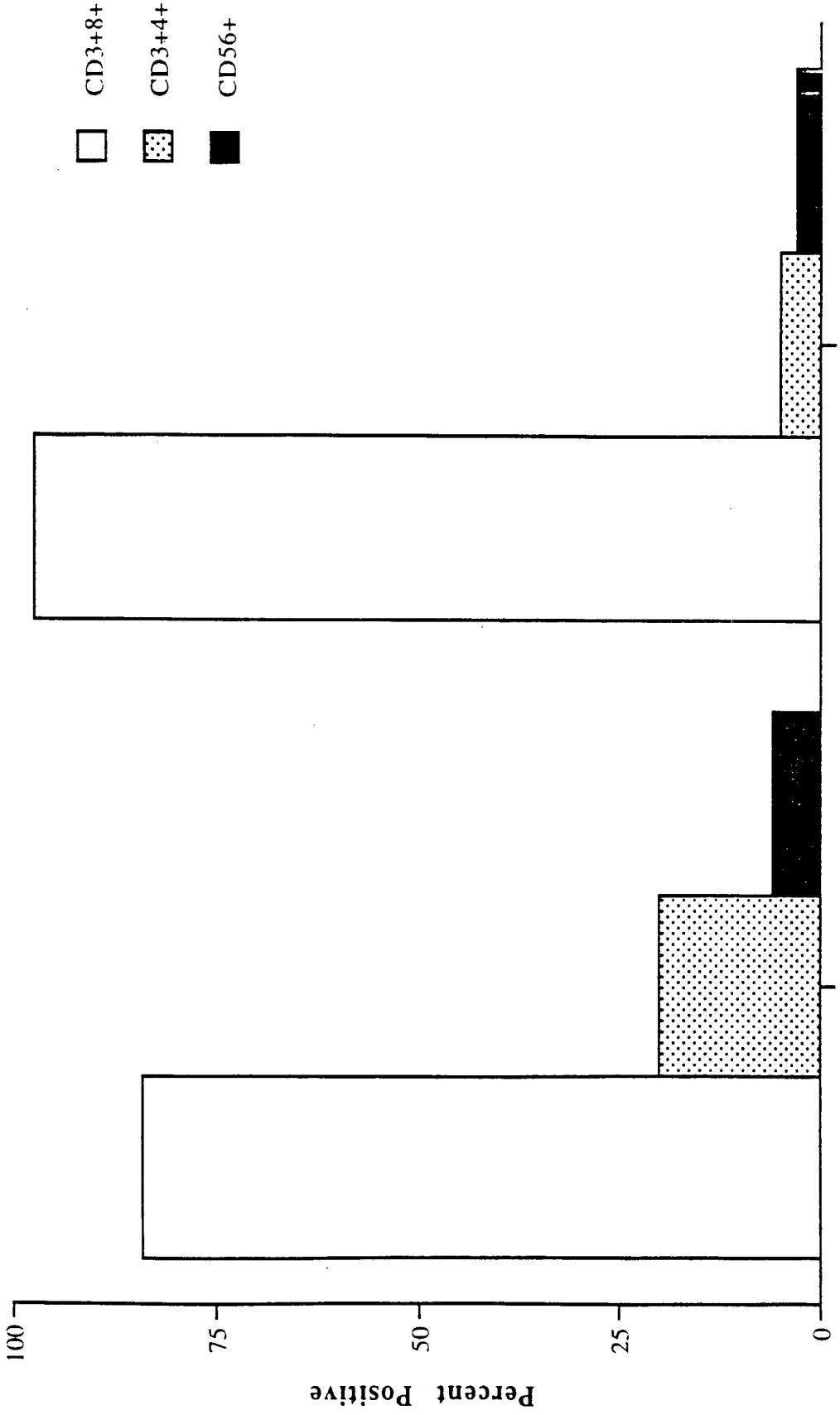

CD3+8+
CD3+4+
CD56+
Percent Positive
100
75
50
25
0
CEA
Cytokines
Effectors

Figure 27

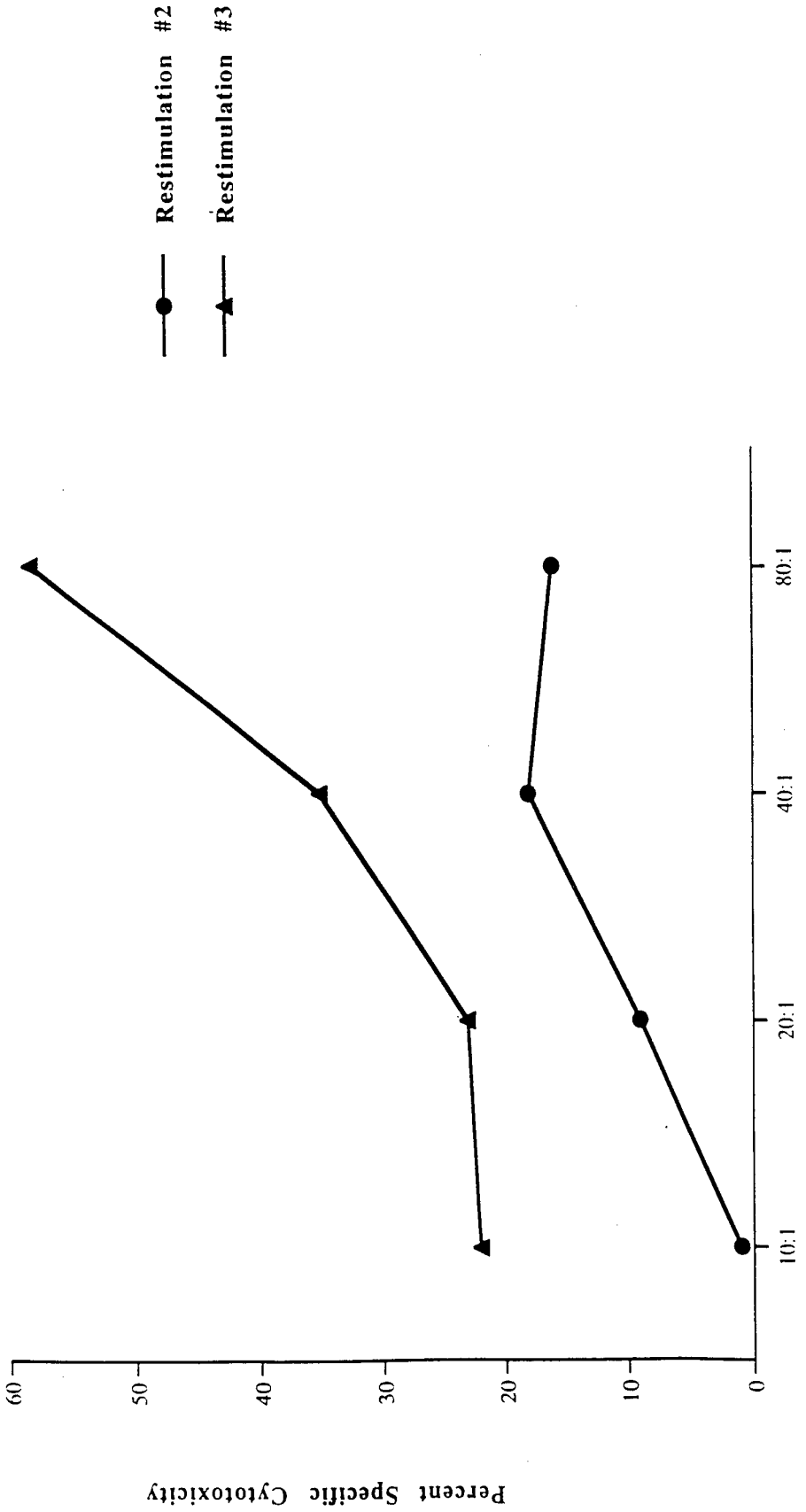

Restimulation #2
Restimulation #3
Percent Specific Cytotoxicity
0
10
20
30
40
50
60
10:1
20:1
40:1
80:1
Effector:Target

Figure 28

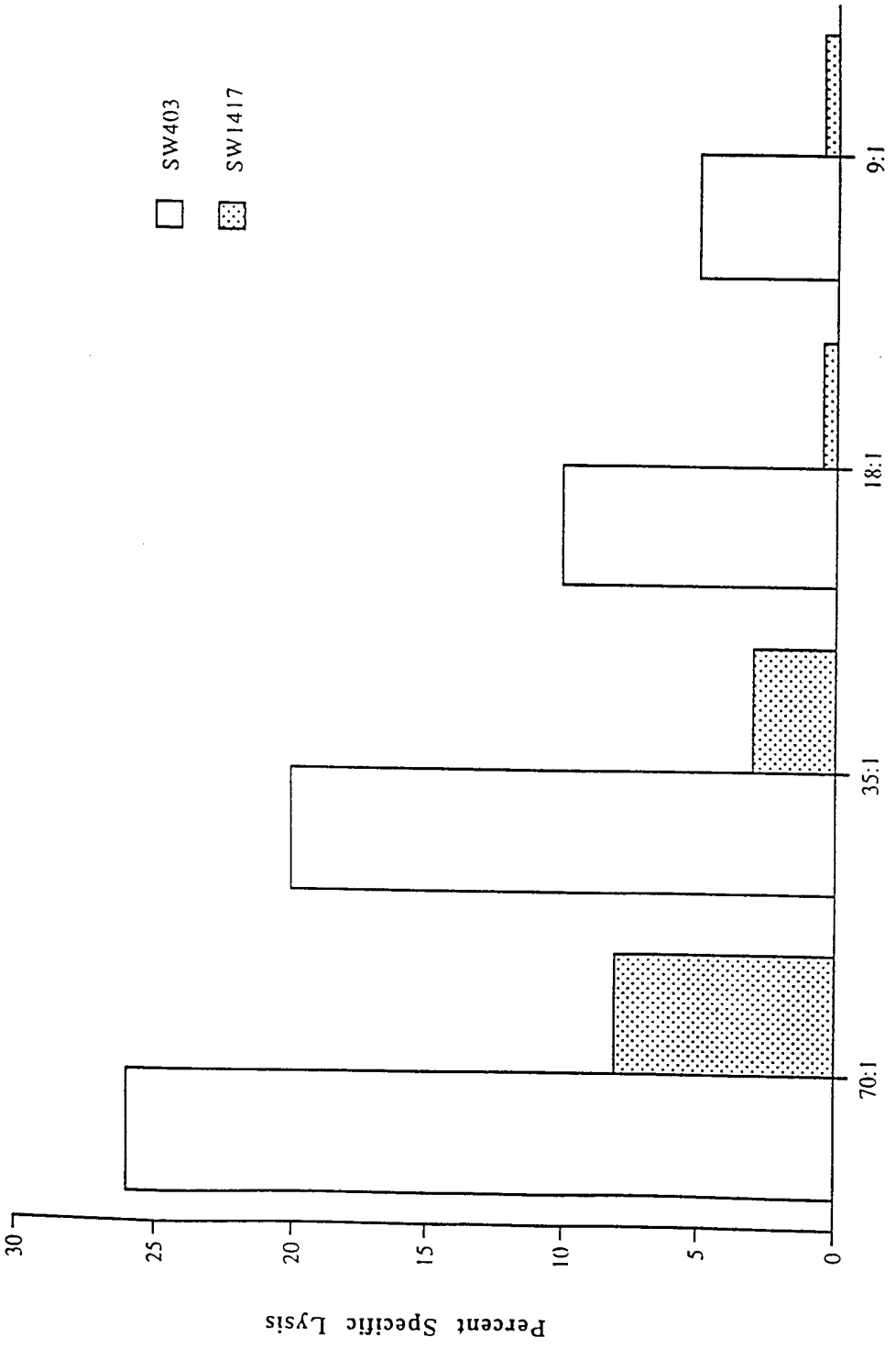

SW403
SW1417
Percent Specific Lysis
0
5
10
15
20
25
30
70:1
35:1
18:1
9:1
Effector:Target

Figure 29

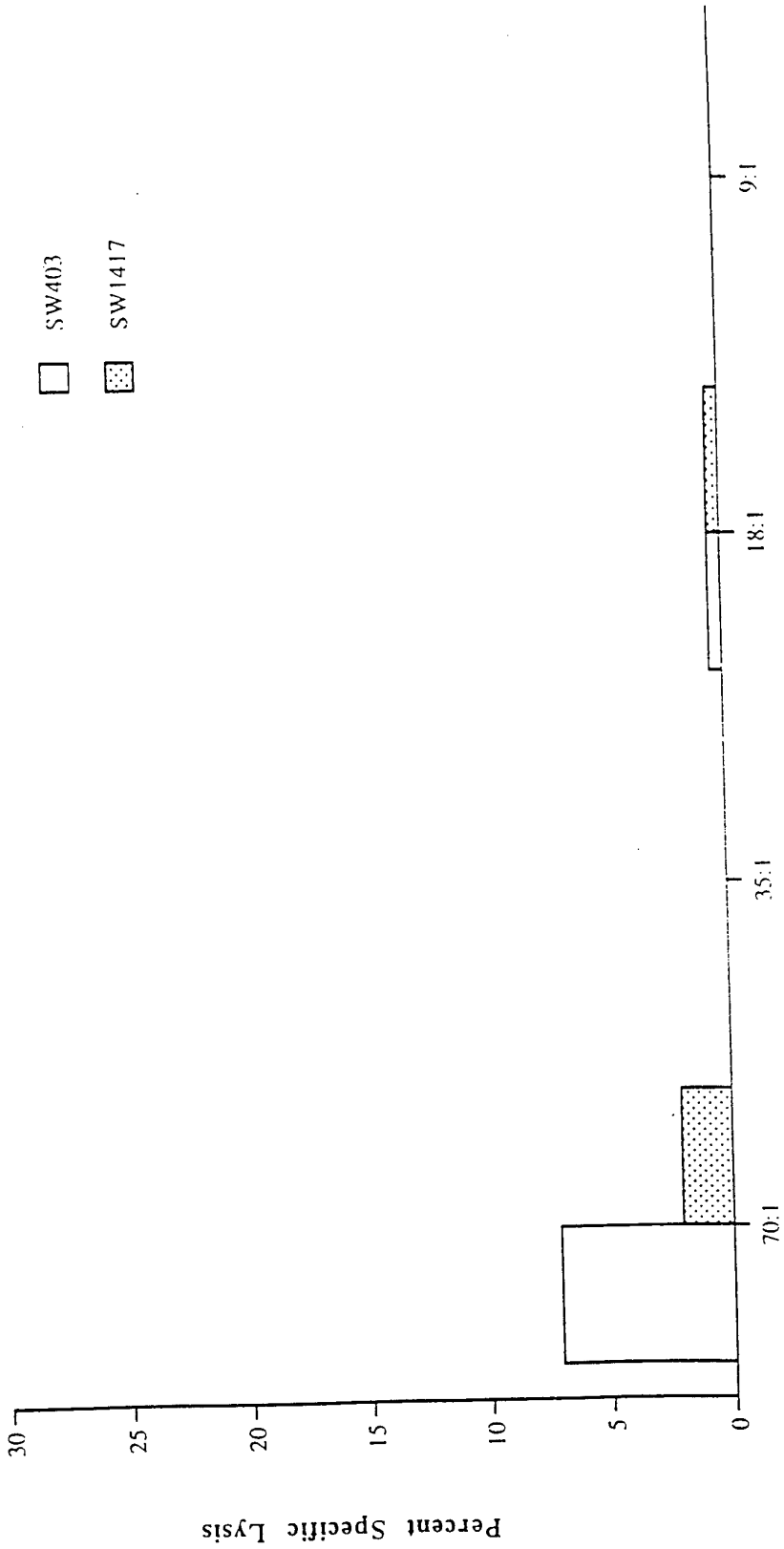

SW403
SW1417
Percent Specific Lysis
30
25
20
15
10
5
0
70:1
35:1
18:1
9:1
Effector:Target

Figure 30

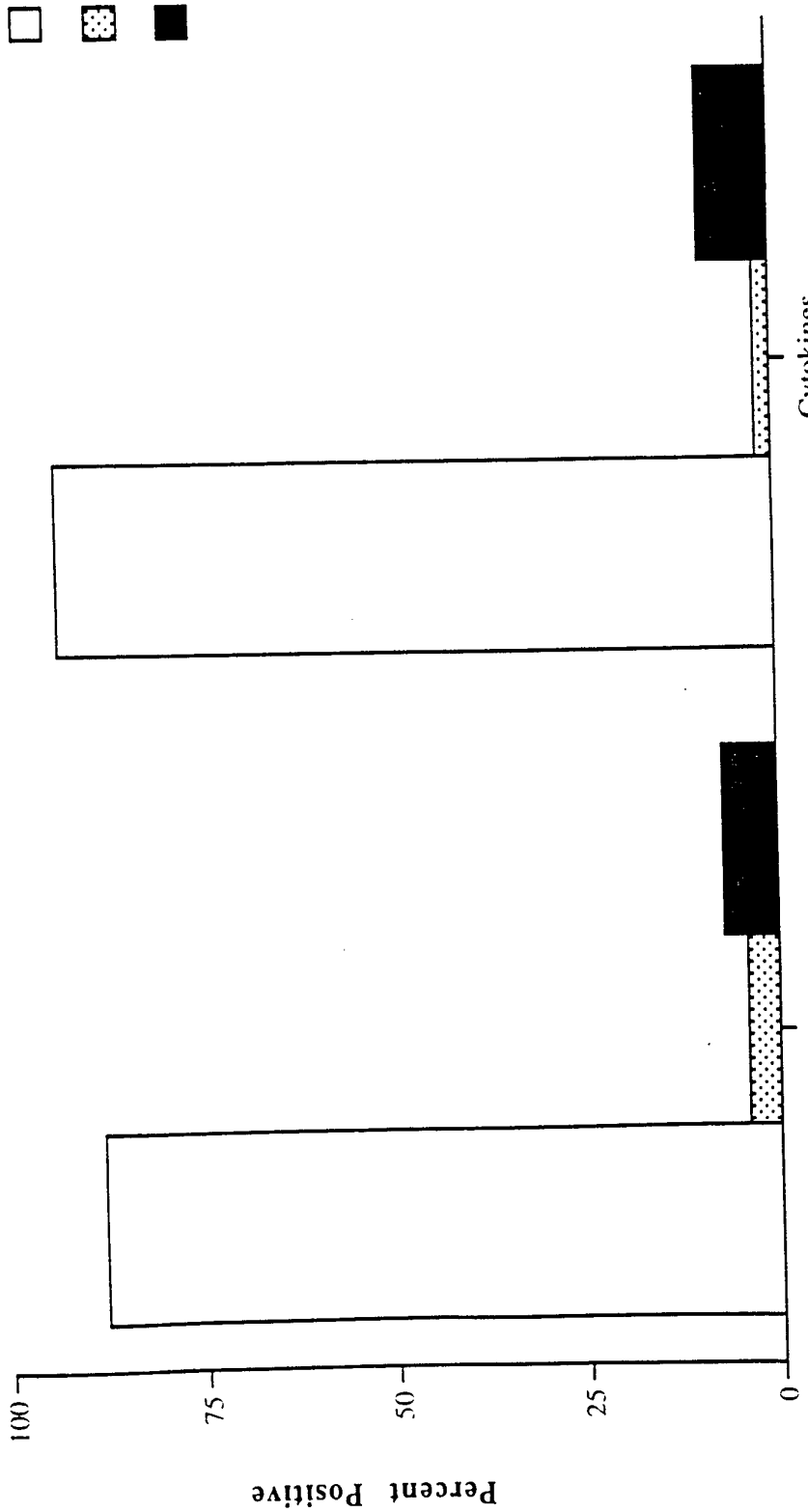

CD3+8+
CD3+4+
CD56+
Percent Positive
100
75
50
25
0
Cytokines
CEA
Effectors

Figure 31

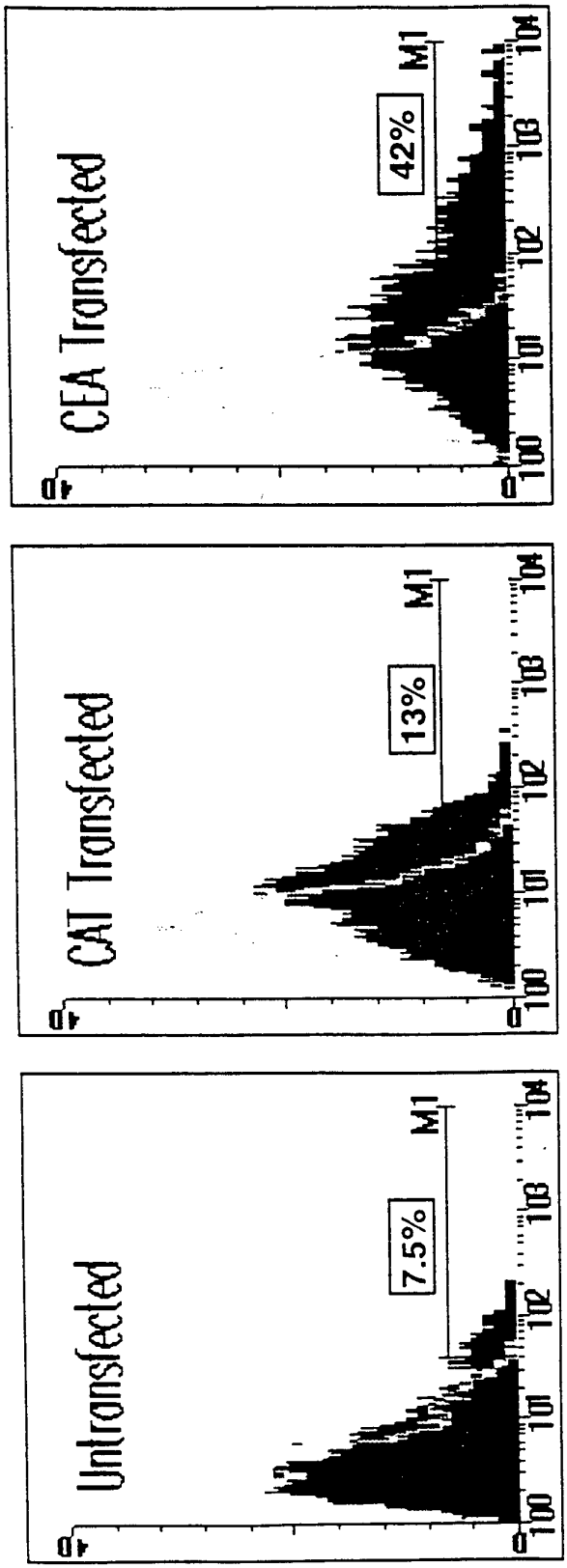

CEA GENE EXPRESSION IN MCF7
Untransfected
7.5%
M1
CAT Transfected
13%
M1
CEA Transfected
42%
M1
100
101
102
103
104

Figure 32

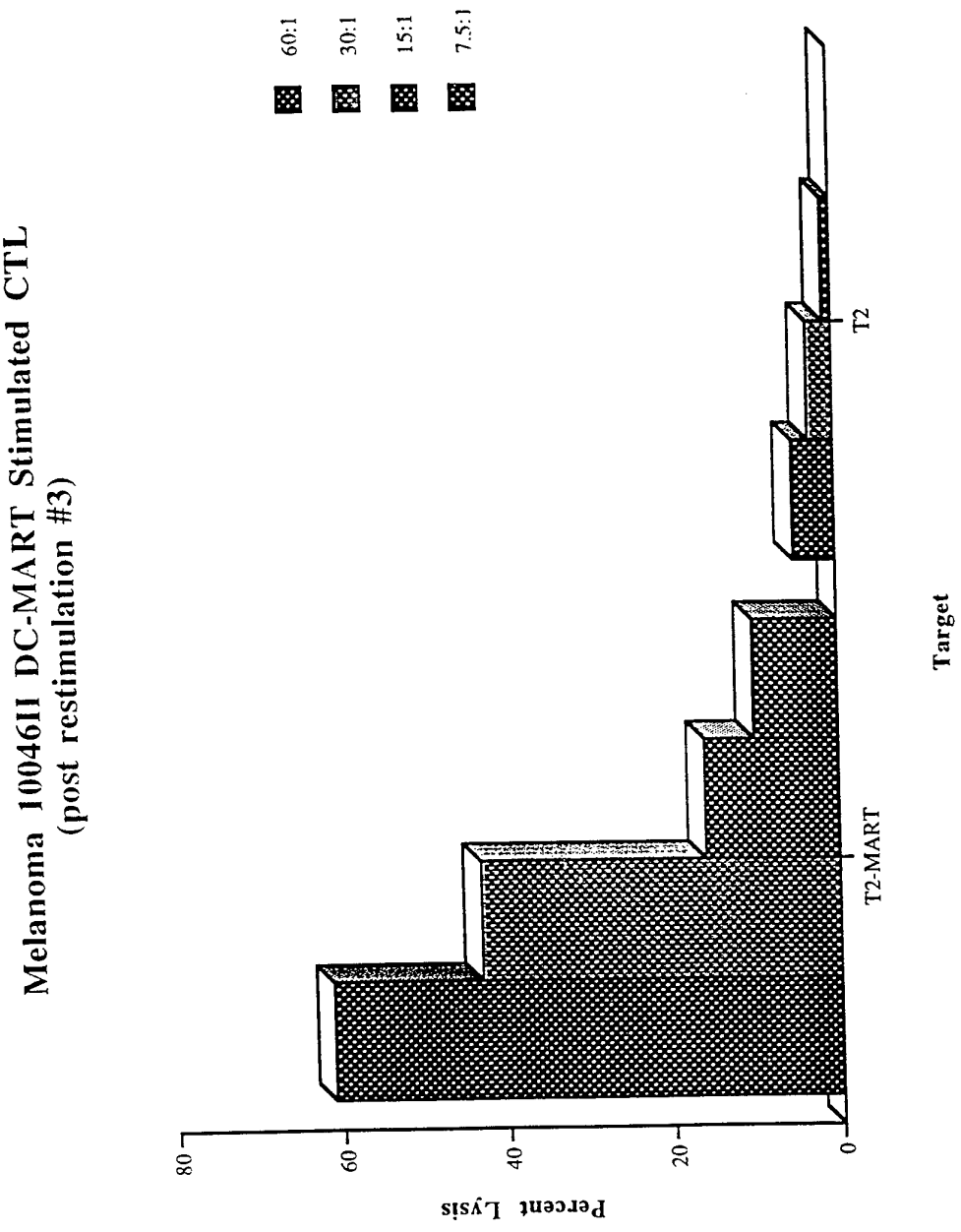
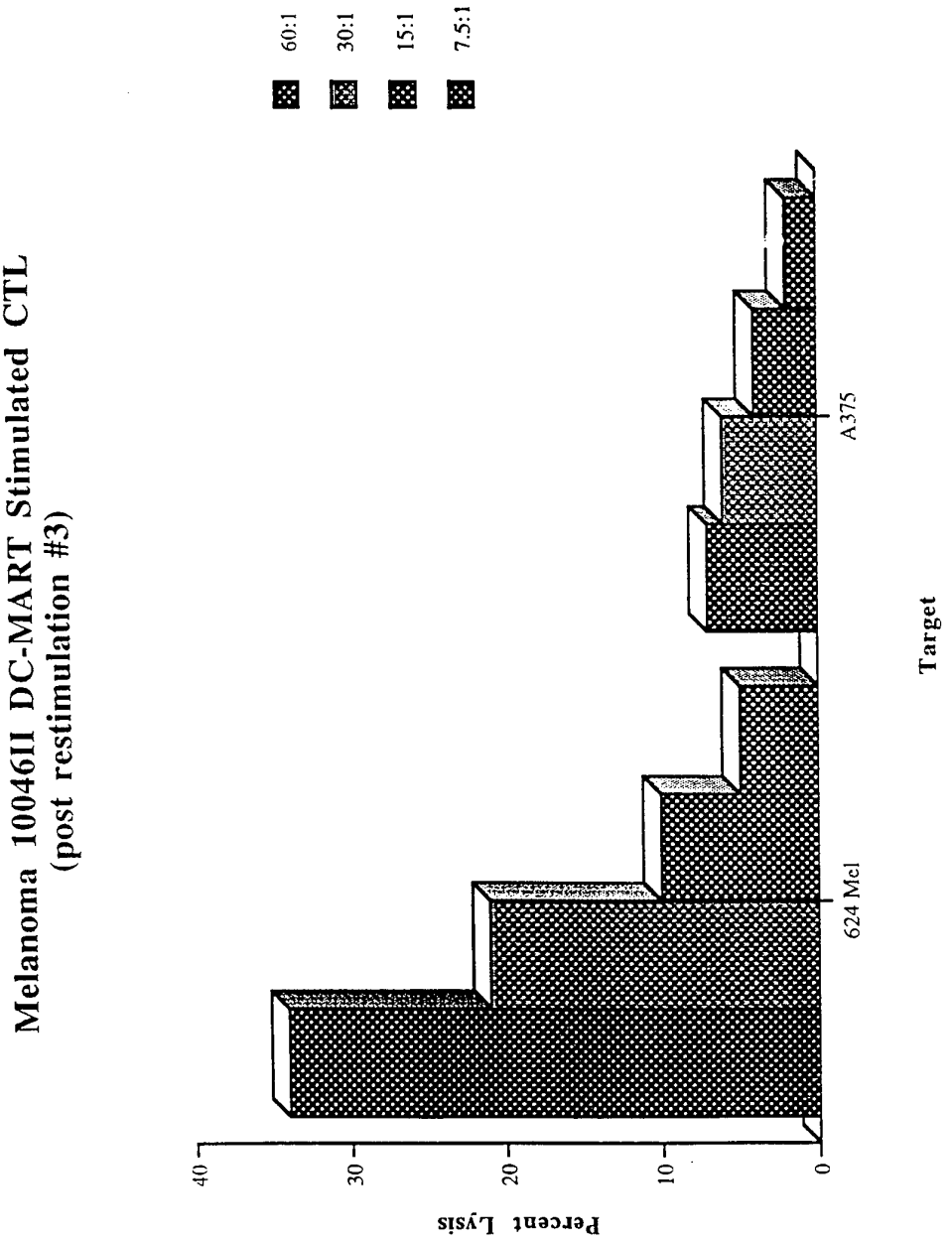

Melanoma 100461I DC-MART Stimulated CTL
(post restimulation #3)
60:1
30:1
15:1
7.5:1
Percent Lysis
0
20
40
60
80
T2-MART
T2
Target

Figure 33

Figure 34


Melanoma 10046II DC-MART Stimulated CTL
(post restimulation #3)
60:1
30:1
15:1
7.5:1
Percent Lysis
0
10
20
30
40
624 Mel
A375
Target

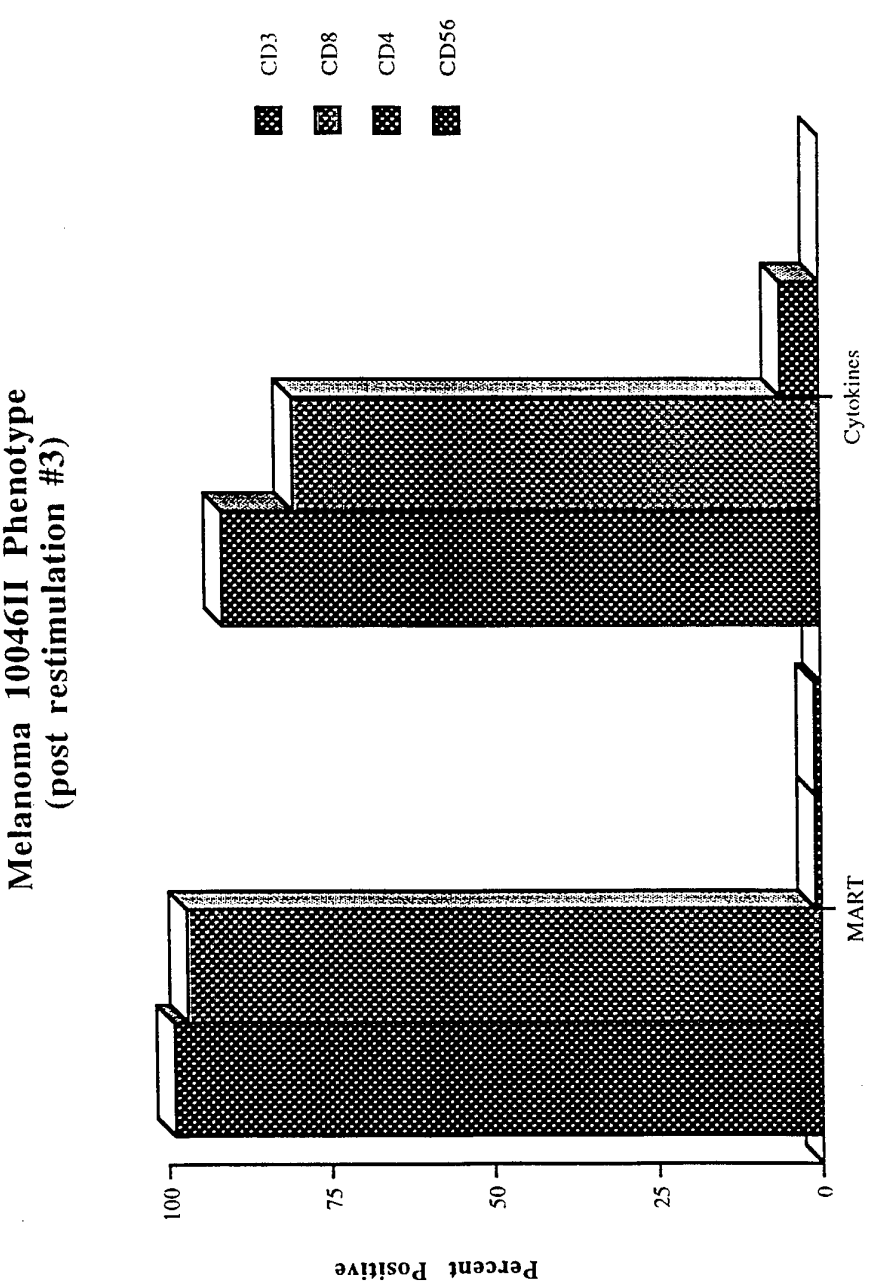

Melanoma 100461I Phenotype
(post restimulation #3)
CD3
CD8
CD4
CD56
Percent Positive
100
75
50
25
0
MART
Cytokines
Effectors

Figure 35

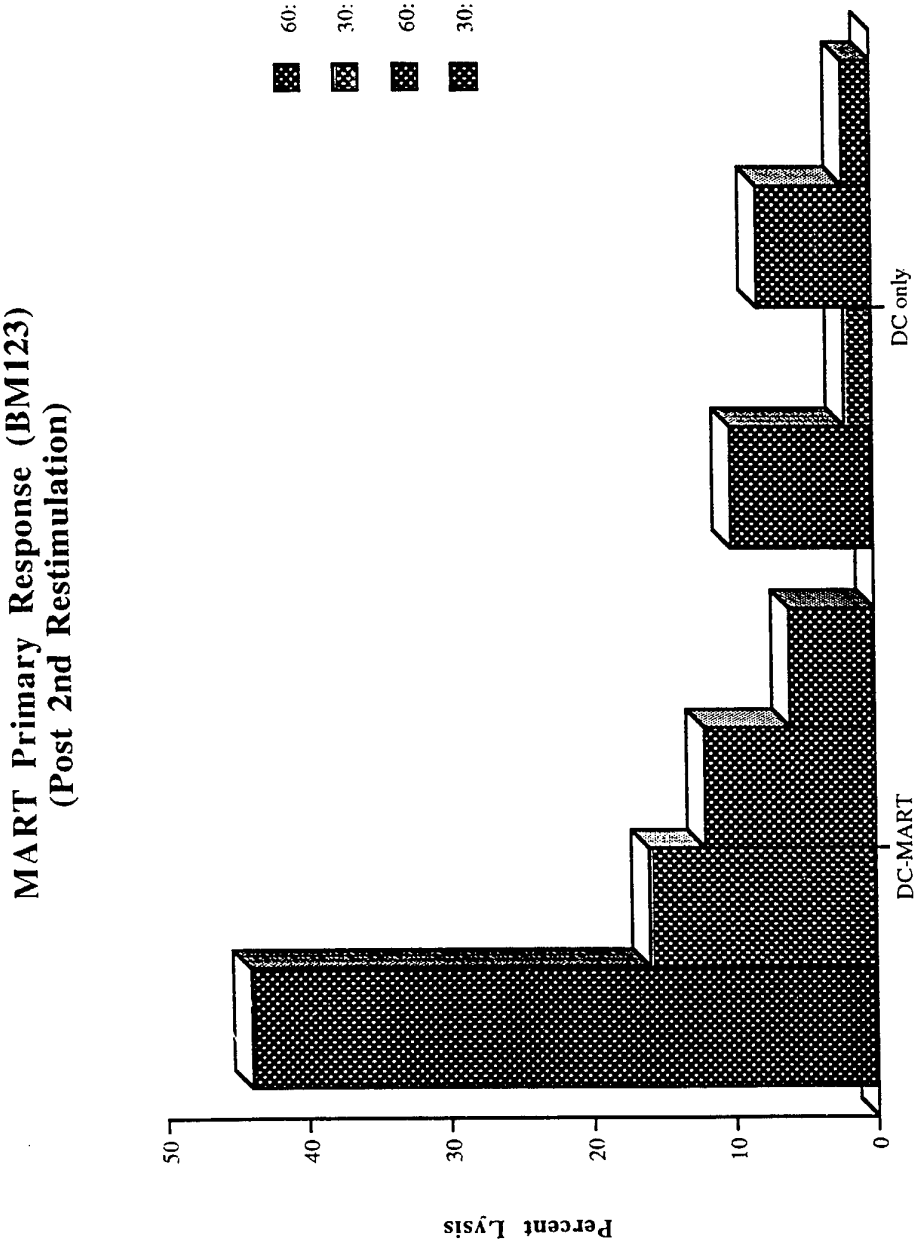

MART Primary Response (BM123)
(Post 2nd Restimulation)
60:1 T2-MART
30:1 T2-MART
60:1 T2
30:1 T2
Percent Lysis
50
40
30
20
10
0
DC-MART
DC only
Effectors

Figure 36

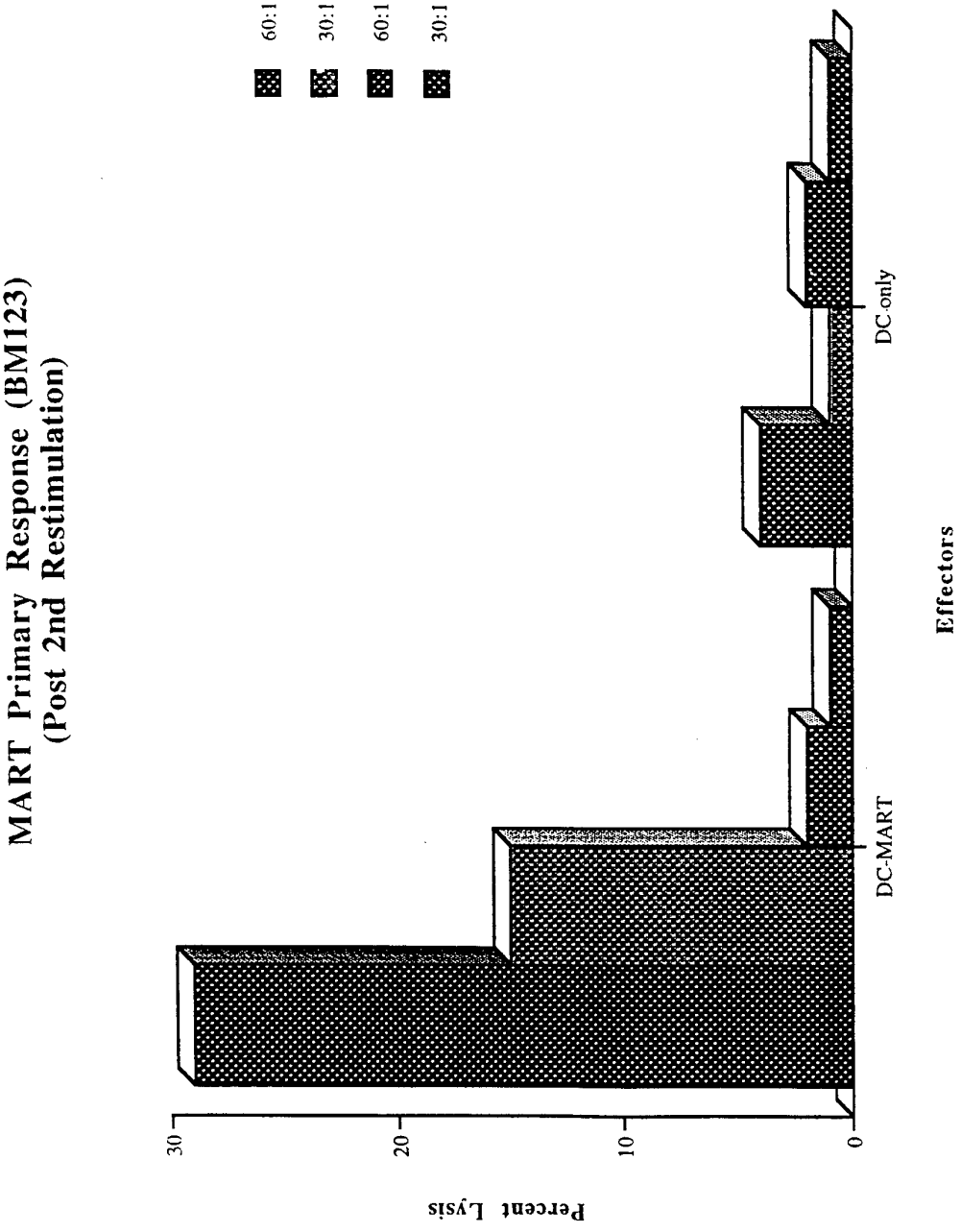

MART Primary Response (BM123)
(Post 2nd Restimulation)
60:1 624Mel
30:1 624Mel
60:1 Colo
30:1 Colo
Percent Lysis
0
10
20
30
DC-MART
DC-only
Effectors

Figure 37

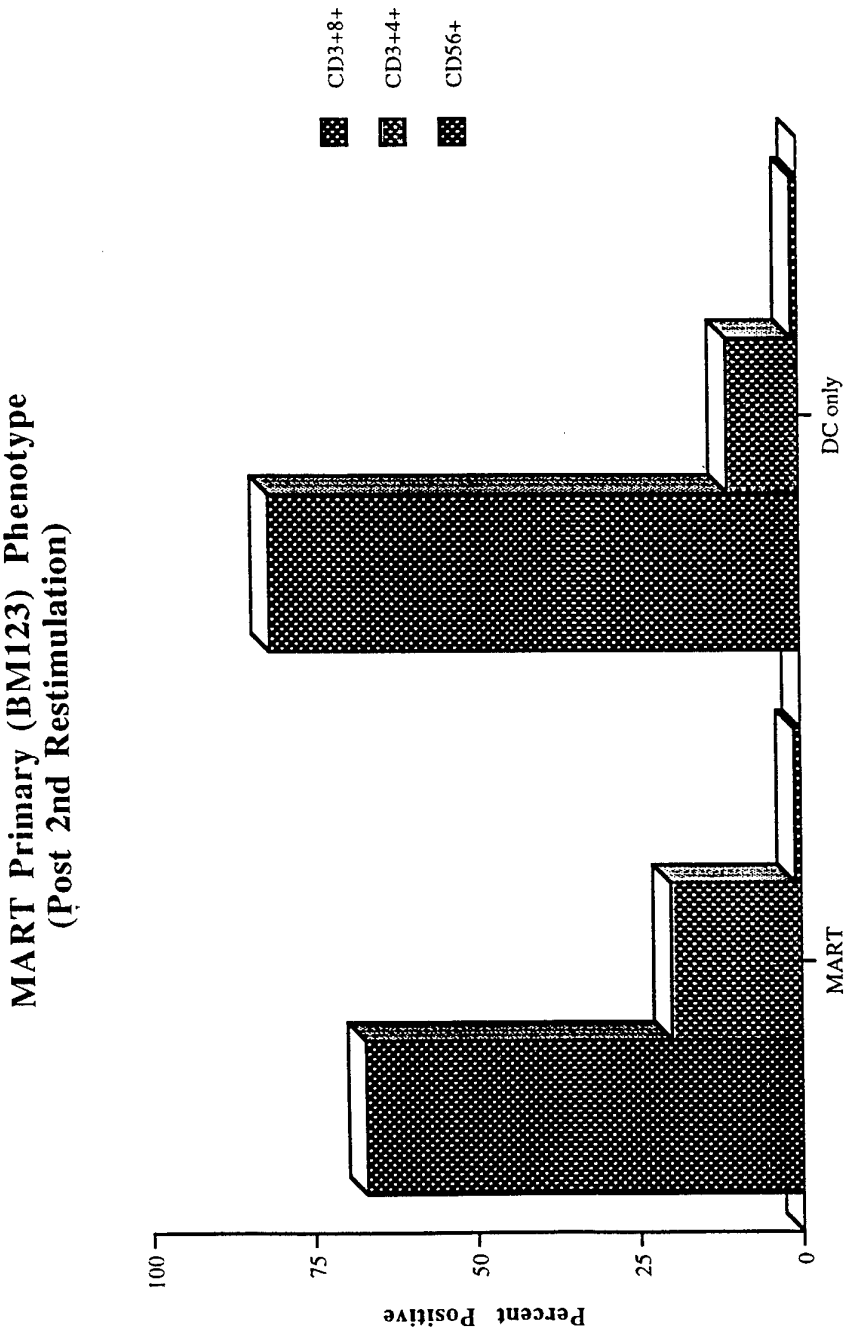

MART Primary (BM123) Phenotype
(Post 2nd Restimulation)
CD3+8+
CD3+4+
CD56+
Percent Positive
100
75
50
25
0
DC only
MART
Effectors

Figure 38

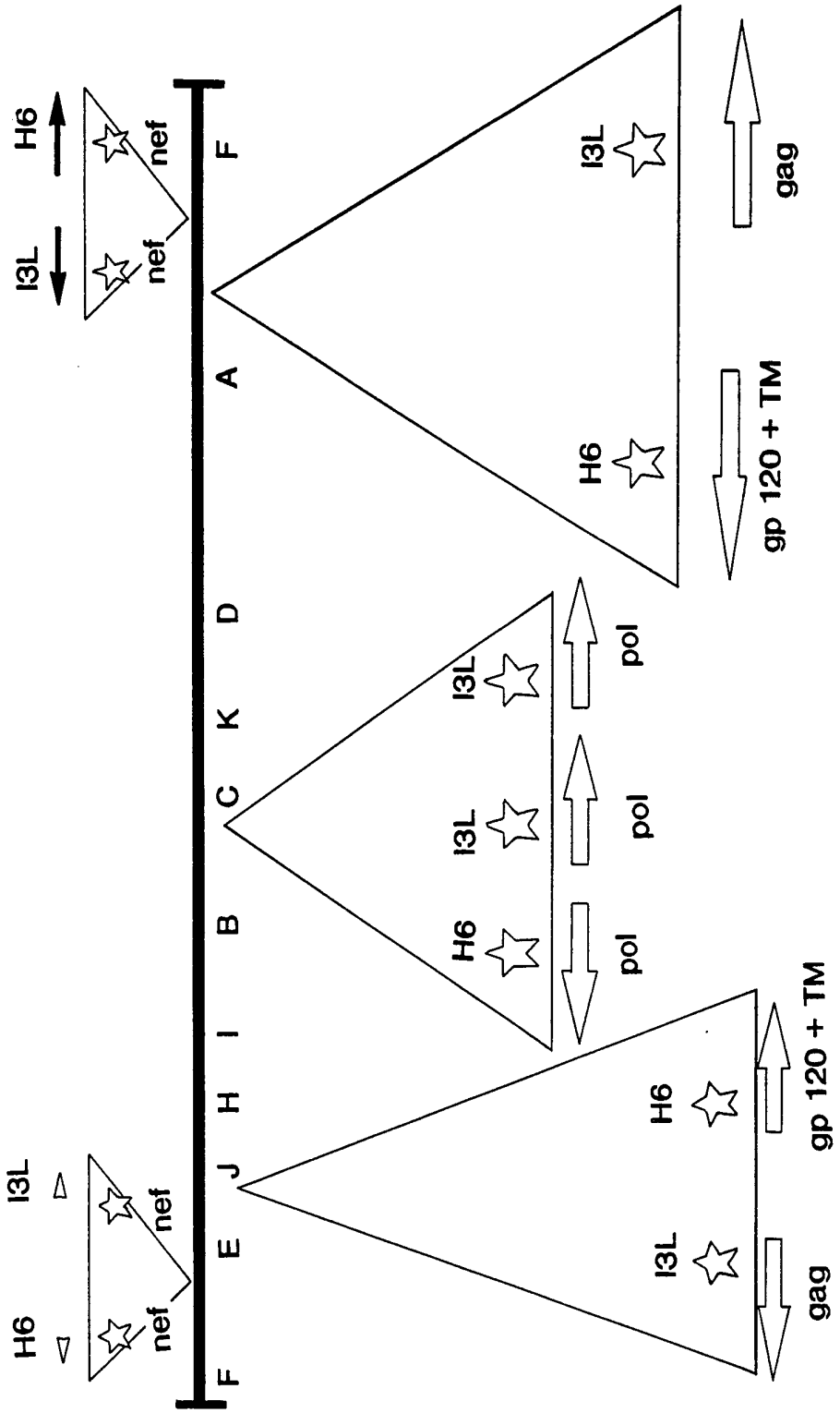

H6
I3L
nef
nef
F
A
I3L
gag
H6
gp 120 + TM
D
K
C
I3L
I3L
H6
pol
pol
pol
B
I
H
J
E
F
I3L
H6
nef
nef
H6
I3L
gp 120 + TM
gag

FIG. 39

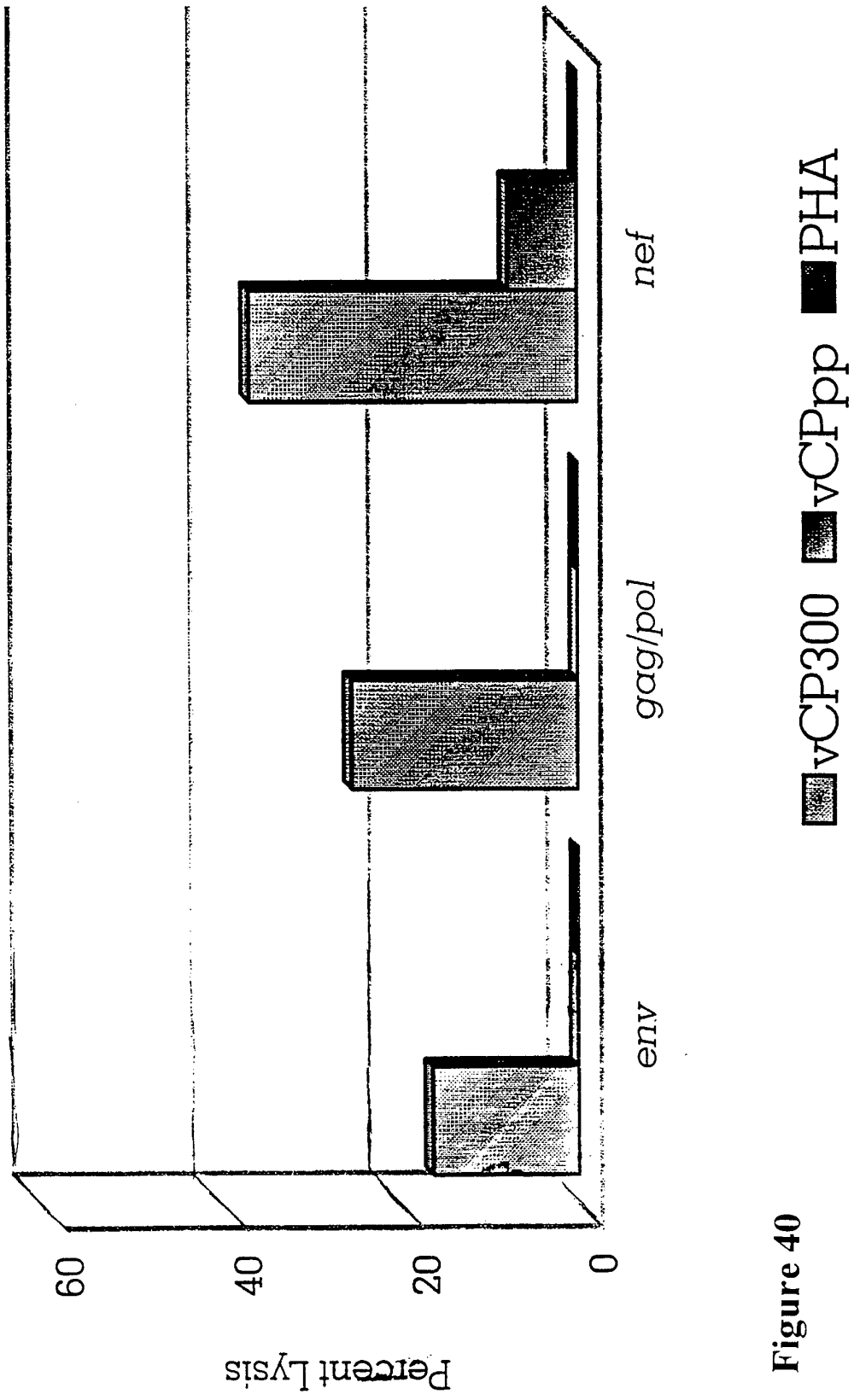


Figure 40

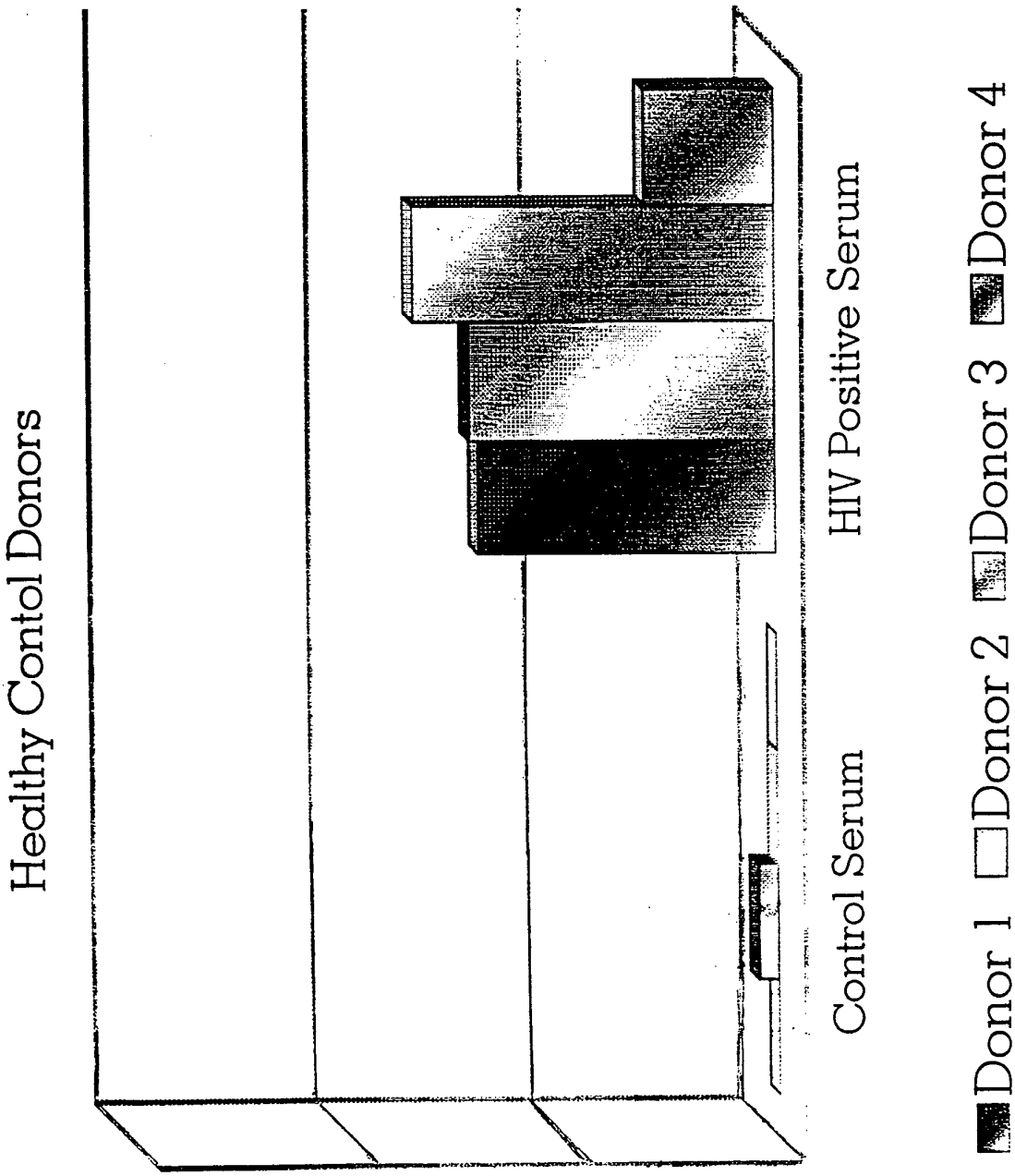

Healthy Contol Donors
HIV Positive Serum
Control Serum
Percent Expression
0
10
20
30
Donor 1
Donor 2
Donor 3
Donor 4

Figure 41

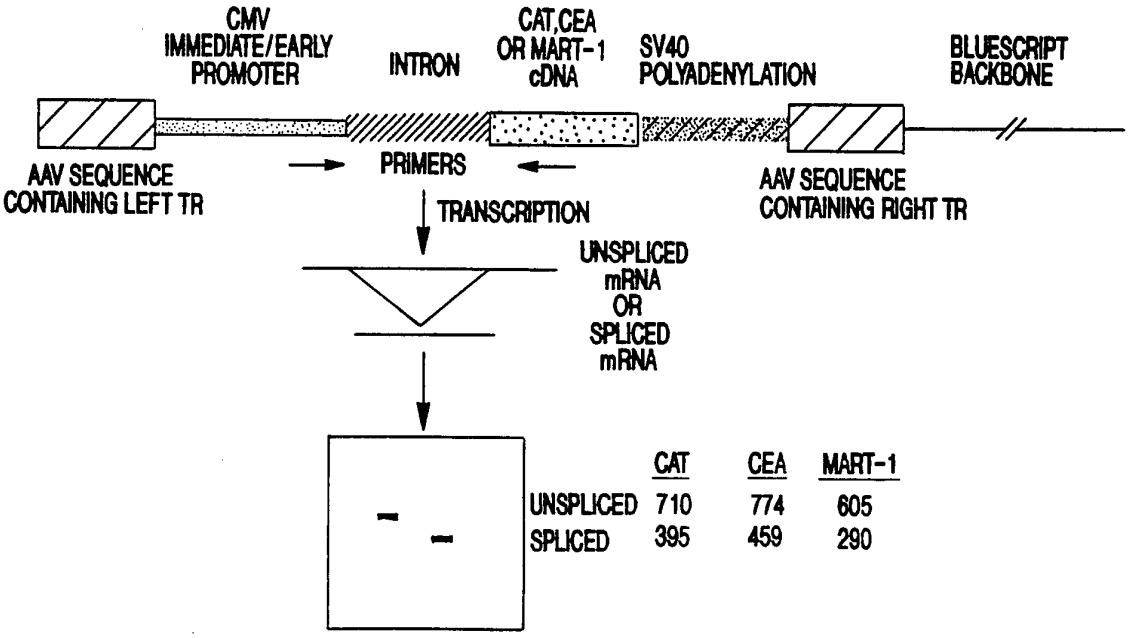

CMV
IMMEDIATE/EARLY
PROMOTER
INTRON
CAT,CEA
OR MART-1
cDNA
SV40
POLYADENYLATION
BLUESCRIPT
BACKBONE
AAV SEQUENCE
CONTAINING LEFT TR
PRIMERS
AAV SEQUENCE
CONTAINING RIGHT TR
TRANSCRIPTION
UNSPLICED
mRNA
OR
SPLICED
mRNA
CAT
CEA
MART-1
UNSPLICED 710 774 605
SPLICED 395 459 290

FIG. 42

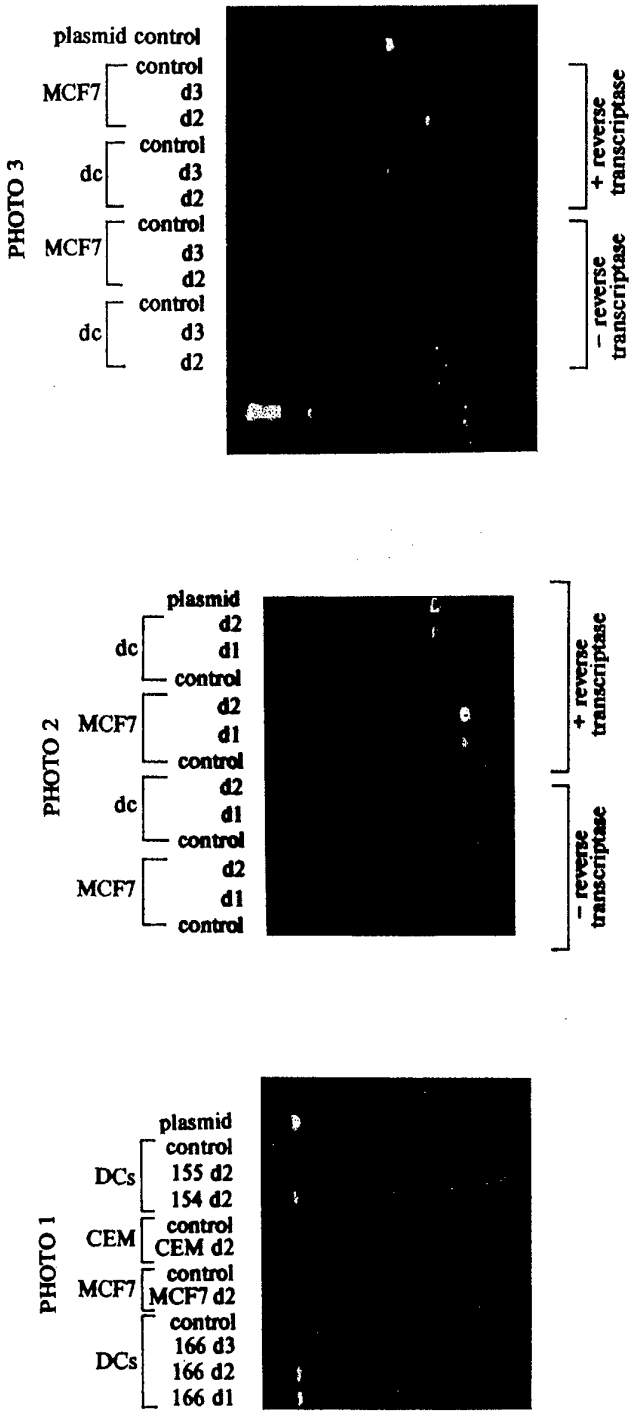


PHOTO 3
plasmid control
MCF7
control
d3
d2
dc
control
d3
d2
MCF7
control
d3
d2
dc
control
d3
d2
+ reverse transcriptase
– reverse transcriptase
PHOTO 2
plasmid
dc
d2
d1
control
MCF7
d2
d1
control
dc
d2
d1
control
MCF7
d2
d1
control
+ reverse transcriptase
– reverse transcriptase
PHOTO 1
plasmid
DCs
control
155 d2
154 d2
CEM
control
CEM d2
MCF7
control
MCF7 d2
DCs
control
166 d3
166 d2
166 d1

FIG. 43

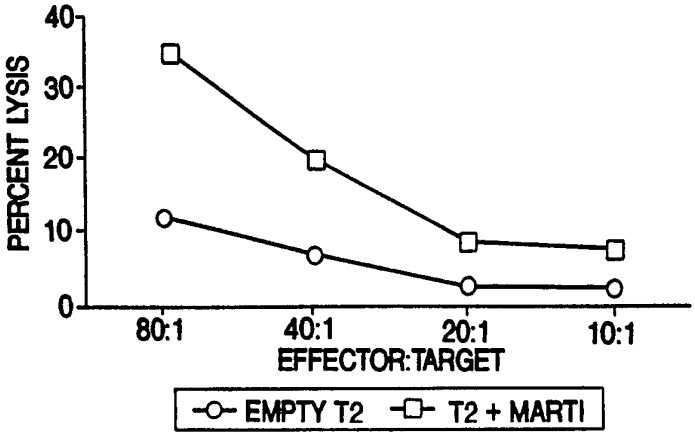


FIG. 44a

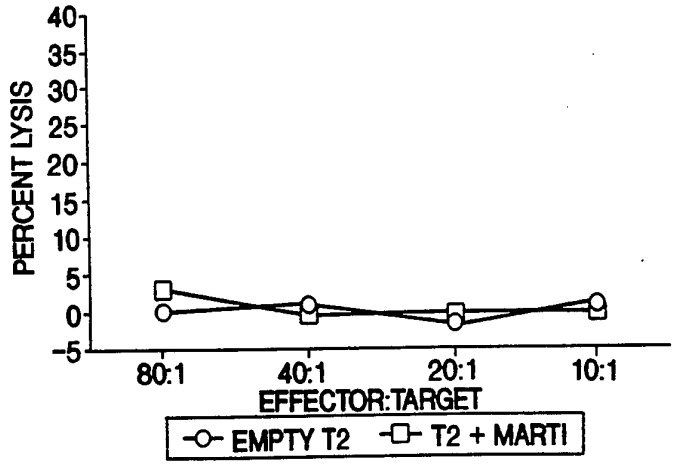


FIG. 44b

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/12012

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 48/00; C12N 5/00, 15/00
US CL :514/44; 435/320.1. 240.2, 172.3
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)

U.S. : 514/44; 435/320.1. 240.2, 172.3

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 practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE, CAPLUS, WPIDS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Trends in Genetics, Volume 10, Number 6, issued June 1994, FRIEDMANN, "Gene Therapy for Neurological Disorders", pages 210-214, see entire document.	1-29
Y	US, A, 5,126,132 (ROSENBERG) 30 June 1992, see entire document.	1-29
Y	Annals of Surgery, Volume 218, Number 4, issued 1993, ROSENBERG ET AL., "The Development of Gene Therapy for Cancer", pages 455-464, see entire document.	1-29
Y	WO, A, 93/24641 (THE UNITED STATES OF AMERICA, as represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES) 09 December 1993, see entire document.	1-29

[X] Further documents are listed in the continuation of Box C. [ ] See patent family 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 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
11 SEPTEMBER 1996	30 SEP 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ANDREW MILNE Telephone No. (703) 308-0196

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/12012

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 93/09239 (RESEARCH CORPORATION TECHNOLOGIES, INC.) 13 May 1993, see entire document.	1-29