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INTRACELLULAR IMMUNIZATION

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(57) Claim

1. In a method for conducting gene therapy wherein a recombinant gene is introduced into cells of a mammal, the improvement comprising using a recombinant gene encoding an antibody that is selectively specific for an intracellular antigen associated with a disease.

10. A method for preventing or halting the progress of a disease comprising administering to the subject a recombinant gene in an infectious vector, the gene encoding an antibody that is selectively specific for an intracellular antigen associated with the intracellular pathogen.

19. A method for preventing or halting the progression of a disease in a subject caused by an intracellular pathogen comprising introducing into cells ex vivo a recombinant gene encoding an antibody that is selectively specific for an antigen associated with the pathogen to form immunized cells, and introducing the immunized cells into the subject.

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(54) Title: INTRACELLULAR IMMUNIZATION

(57) Abstract

A method for conducting gene therapy is provided. The therapy involves using a recombinant gene that encodes an antibody that binds an antigen associated with a disease. The invention is in particular useful in protecting cells with "immunity" against intracellular pathogens. Novel vectors and cell lines also are provided.

INTRACELLULAR IMMUNIZATION

Field of the Invention

This invention relates to an immunological approach using gene therapy to treat infectious disease.

Background of the Invention

Advances in medicine and public health have eradicated or significantly reduced the incidence of serious illness or death caused by many pathogens. Nevertheless, infectious diseases still are responsible for many serious health problems. some of the more problematic agents of these diseases are: viruses; mutated-resistant bacterial strains; agents that reside beyond the reach of conventional therapeutics due to, for example, a barrier such as the blood brain barrier; and readily mutating strains.

One serious health risk has resulted from a relatively new pathogen, the human immunodeficiency virus (HIV). This virus has had devastating effects, particularly in that it opens the door to infection by a variety of opportunistic pathogens (e.g. hepatitis and tuberculosis) that usually do not pose serious health risks in HIV negative individuals. Despite billions of dollars in research, an effective treatment for HIV infection has not been discovered to date.

One common approach to treating infectious disease is the use of vaccines, which stimulate the host's immune system to be in a ready state for recognizing and destroying the pathogen. Vaccines contain immunogens that are incapable of producing the disease state, but capable of producing

immunity against the pathogen. Vaccines have been very successful in protecting against infection by some pathogens, but ineffective in protecting against infection by others.

Another approach is passive immunization, which involves supplying systemically to a host antibodies that can bind the pathogen. The utility of this approach was greatly increased with the development of humanized antibodies and single-chain antibodies, both of which do not provoke an immune response by the host.

The foregoing treatments are limited in that the most active site for many diseases is within the cell, beyond the reach of antibodies. In addition, synthetic antibodies have a relatively short life, during which they are subject to serious proteolytic and other degradation.

A current experimental approach for treating infectious disease is to intracellularly express in a host a mutant form of viral protein that can strongly interfere with the replication of the wild-type virus. In cultured cells, this strategy has been successfully implemented to produce cell lines with acquired resistance to Herpes Simplex Virus (HSV) and Human Immunodeficiency Virus (HIV). Different approaches to this intracellular binding have been developed for human viral infections, including: (1) transdominant-negative mutant inhibitors; (2) specific target gene ribozymes; (3) anti-sense oligonucleotides; (4) viral receptors and receptor analogs; (5) suicide constructs; (6) virus specific inhibitory molecules; and (7) molecular decoys. To date, most of the reports of these experiments did not show completely satisfactory results.

The present invention overcomes the limitations of this prior art and vastly expands the therapeutic potential of antibodies.

Summary of the Invention

The present invention involves treating diseases by intracellular immunization. Antibody genes are delivered to cells in vectors. They "immunize" the host cells by enabling the intracellular expression of antibodies or modified antibody binding domains which are specific for important disease related antigens. These antibodies bind the antigens, thereby halting, inhibiting or retarding the development or progression of the disease. The invention can provide immunity before or after the development of the disease as well as treatment to control its severity.

According to one aspect of the invention, an improved method for conducting gene therapy is provided. The improvement involves using a recombinant gene encoding an antibody that is selectively specific for an intracellular antigen associated with an intracellular pathogen. Because intracellular expression of the antibody is desired, the recombinant genes of the invention preferably are prepared so as to be free of a signal sequence. In addition, the recombinant genes can be provided with localization sequences, such as a nuclear localization sequence, so that the antibodies can be targeted to desired compartments. The preferred recombinant genes encode single chain antibodies that are selectively specific for intracellular viral antigens and that are part of an infectious agent that is replication-deficient. In accordance with other aspects of the invention, the recombinant genes encode single or multiple binding domains from one or more antibodies.

In one particularly preferred embodiment, the antibody gene is under the control of a pathogen promoter such as the HTLV-1 LTR promoter which is expression dependent upon the presence of HIV-1 tat protein, so that intracellular expression of the antibody will not occur until the cell is also infected by a pathogen that can initiate the regulatory effects of that promoter.

According to another aspect of the invention, a method for treating a subject having a disease caused by an

intracellular pathogen is provided. A recombinant gene in an infectious vector is administered to the subject, the gene encoding an antibody that is selectively specific for an intracellular antigen associated with the pathogen.

According to still another aspect of the invention, an ex vivo treatment is provided. Cells may be isolated from a subject or derived from another source. A recombinant gene is introduced into the cells, the gene encoding an antibody that is selectively specific for an antigen associated with an intracellular pathogen, to form immunized cells. The immunized cells then are introduced into the subject.

Still another aspect of the invention involves a method for inhibiting replication of an intracellular pathogen in a cell by causing to be introduced into the cell a recombinant gene encoding an antibody that is selectively specific for an antigen associated with an intracellular pathogen.

In all of the foregoing methods, the recombinant gene can be as described above.

The invention also includes vectors containing the recombinant genes of the invention and cell lines transduced or transfected with such genes.

These and other aspects of the invention will be described in greater detail below.

Brief Description of the Drawings

Figure 1 is the vector pT7H3-10.

Figure 2 is the vector p4ZABVKRIDO.

Figure 3 is the vector pSCCribo.

Figure 4 is a photograph of a gel.

Figure 5 is the vector pLXSNCAT.

Figure 6 is the vector pLX-GAL.

Figure 7 is the vector pET19vHLc8.

Figure 8 is the vector p9CESAR.

Figure 9 is a graph showing the effect of sFv-anti-rev production on the levels of soluble p24.

Figure 10 is a graph showing the effect of sFv-anti-rev production on syncytia formation.

Figure 11 is a graph showing the effect of anti-rev sFv expression on different clinically isolated HIV-1 strains.

Figure 12 is a bar graph showing Human Anti-tat Fab binding domains.

Figure 13 is a bar graph showing Human Anti-tat Fab Binding to cysteine rich domain of tat and effect of reduction thereon.

Figure 14 is a bar graph showing Human Anti-rev binding domains.

Detailed Description of the Invention

The invention provides antibody-based intracellular immunity against intracellular pathogens. Recombinant antibody genes are introduced into cells. The recombinant antibody genes encode antibodies that are selectively specific for antigens associated with the pathogen. The antibodies are expressed intracellularly and the pathogen-associated antigens are present intracellularly. The antibodies bind to the antigens and interfere with the replication of the pathogen, thereby providing the "immunity" or treatment.

The invention may be used prophylactically or therapeutically. When used prophylactically, the invention is applied to a subject that is at risk of being infected by an intracellular pathogen. When used therapeutically, the invention is applied to a subject that is known to have or that is suspected of having an infection by an intracellular pathogen.

As used herein, subject means animal. Preferred subjects are mammals, fowl and fish. Most preferred are humans, primates, dogs, cats, horses, cows, sheep, goats, pigs, rodents, chickens and turkeys.

An "intracellular pathogen" means a disease-causing organism which resides, during only part of its life cycle, within a host cell. such pathogens include certain viruses, bacteria, fungi and protozoans. Examples include: Human

Immunodeficiency Virus including, without limitation, HIV-1 and HIV-2; human T cell leukemia virus (including, without limitation, HTLV-I and HTLV-II); herpes virus including, without limitation, Herpes simplex type 1 (HSV-1) and type 2, Herpes zoster; cytomegalovirus (CMV); Epstein-Barr virus (EBV); papillomavirus; hepatitis (including, without limitation hepatitis A, B, C, D and E); creutzfeldt-jacob virus; feline leukemia virus; influenza virus; variola; rubeola; mumps virus; mycobacteria including, without limitation, M. tuberculosis and M. leprae; candida including, without limitation, candida albicans and candida tropicalis, mycoplasma, toxoplasma gondii; trypanosoma cruzi; organisms of the genus leishmani; and organisms of the genus plasmodium.

Recombinant genes encoding antibodies with a particular binding specificity are used in the methods and products of the invention. A recombinant gene as used herein is an isolated protein-coding sequence operably linked to a promoter, whereby the protein is capable of being produced when the recombinant gene is introduced into a cell. The coding region can encode a full length gene product or a subfragment thereof, or a novel mutated or fusion sequence as described in greater detail below. The protein coding sequence may be a sequence endogenous to the target cell, although according to the preferred embodiments it typically will not be a sequence endogenous to the target cell. If it is an endogenous sequence, then it is not normally expressed intracellularly within the cell or, if expressed, not at biologically significant levels. The promoter, with which the coding sequence is operably associated, may or may not be one that normally is associated with the coding sequence.

The promoters useful in constructing the recombinant genes of the invention may be constitutive or inducible. A constitutive promoter is expressed under all conditions of cell growth. Exemplary constitutive promoters include the promoters for the following genes: hypoxanthine phosphoribosyl transferase (HPRT), adenosine deaminase,

pyruvate kinase, the β-action promoter and others. In addition, many viral promoters function constitutively in eukaryotic cells. These include: the early and late promoters of SV40; the long terminal repeats (LTRs) of Moloney Leukemia Virus and other retroviruses, and the thymidine kinase promoter of Herpes Simplex Virus. Others are known to those of ordinary skill in the art.

Inducible promoters are expressed in the presence of an inducing agent. For example, the metallothionein promoter is induced to promote (increase) transcription in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the art.

The recombinant genes of the invention are prepared synthetically or, preferably, from isolated nucleic acids. A nucleic acid is "isolated" when purified away from other cellular constitutives, i.e., other cellular nucleic acids or proteins, by standard techniques known to those by ordinary skill in the art.

The recombinant genes of the invention can be derived from sequencing information or cell lines publicly available or may be derived from antibody producing cell lines or isolated antibody producing lymphocytes prepared according to a variety of methods. One such method involves the formation of monoclonal antibody producing hybridomas. Generally, an animal is immunized with an antigen. A fused cell hybrid then is formed between the antibody-producing cells from the immunized animal and an immortalizing cell line such as a myeloma. Alternatively, cell lines can be produced by directly immortalizing antibody-producing human lymphocytes with Epstein-Barr virus (EBV).

The recombinant genes of the invention encode antibodies that are selectively specific for intracellular antigens associated with intracellular pathogens. An antibody that is "selectively specific" for an intracellular antigen binds to that antigen, but does not bind to any appreciable degree to native intracellular constituents of the host cell. Antibodies as used herein means any portion

of an antibody that retains the variable region binding specificity, including whole antibody, Fab portions, chimeric antibodies or fragments thereof including humanized and human antibodies and single chain antibodies. Single or multiple binding domains from one or more antibodies may be combined to form a chimeric antibody having the specificity of the binding domains of each antibody.

The antibodies should be selected such that they interfere with replication of the pathogen upon binding to the antigen. Antibodies that selectively bind to antigens or elements that are conserved, that are critical to regulation or that are critical to replication are preferred. For example, for HIV-1, the antibodies can be selected to have specificity for important enzymes or regulatory proteins such as HIV-1 integrase, Tat, Rev and RT. For Herpes Simplex Virus (HSV), antibodies with specificity for HSV-1 IE gene transactivator VP16 and ICP4 can be used. For Hepatitis B (HBV), antibodies with specificity for HBV polymerase can be used. It should be understood that the foregoing are merely examples of antigens against which antibodies may be directed, and other appropriate antigens well known to or easily identified by those of ordinary skill in the art can be selected depending upon the particular pathogen of interest. Antigens can be derived from virtually any pathogen associated source, including parts, extracts or isolates of pathogens. Recombinant antigens also are useful according to the invention. Many such antigens are available in various forms from commercial sources or from depositories such as the ATCC, Rockville, MD.

One method for selecting the antigens is DNase shotgun cleavage. This method is based upon the observation that bovine pancreatic DNA I causes double strand scission of DNA in the presence of Mn^{++}. Since cleavage is random and can be controlled by varying the enzyme concentration, temperature and/or incubation time, this method is very useful in the initial step in the generation of representative libraries having virtually any insert size

range. Small random-size specific DNAs can be inserted into a vector, such as the pTOPE-T vector (Novagen, Madison, WI; U.S. Pat. No. 4,952,496, the entire disclosure of which is incorporated herein by reference), for expression of fusion proteins. These bacterial expression libraries will represent substantially the epitope domain of the specific antigen.

This bacterial expression system is suitable for human antibody epitope screening. The fusion partner can ensure a high level of expression and help protect the target sequence from proteolytic degradation. Desired clones may be identified by direct screening on colony lift filters. Reagents and specific protocols are available in kits, including the Colony Finder™ Immunoscreening Kit sold by Novagen.

Because intracellular expression is desired, the recombinant genes of the invention preferably are prepared so as to be free of a signal sequence. "Free of a signal sequence" means a deletion, mutation or modification of the signal sequence which ordinarily directs antibodies to the secretory compartments. for example, the hydrophobic amino acid core of the signal sequence for secretion can be substituted with hydrophilic residues by site directed mutagenesis. See Biocca, S. Et al., "Expression and Targeting of Intracellular Antibodies in Mammalian Cells," European Molecular Biology Organization (EMBO) Journal 1: 101 (1990).

The antibodies also can be targeted to desired compartments. For example, the antibodies can be targeted to the nucleus using the nuclear localization sequence PKKKRKV of the large T antigen of SV40 virus. Id.

The preferred recombinant genes encode single chain FV antibodies (sFv). The sFv antibody is described in U.S. patent 4,946,778 to Genex Corporation, issued August 7, 1990, the entire disclosure of which is incorporated herein by reference. sFv antibodies incorporate the complete antigen-binding Fv domain of an antibody into a single polypeptide by

joining the light and heavy variable domains (vL and vH with a linker peptide. sFv antibodies having specificity for haptens, proteins, receptors and tumor antigens have been shown to have binding affinities equivalent to those of the monoclonal antibodies from which they were derived. sFv antibodies are preferred because of their small size and their reported lack of immunogenicity.

The recombinant genes of this invention will preferably be free of a signal sequence and will encode an appropriate targeting sequence as desired.

The recombinant genes encoding the sFv antibodies are prepared according to methods well known to those of ordinary skill in the art. See e.g. U.S. Patent 4,946,778. Briefly, hybridomas or immortalized B-cells making monoclonal antibodies to the antigens of interest are produced. Heavy and light chain cDNAs then are isolated and characterized, for example, by making DNA libraries from the foregoing immortalized cells and screening these libraries with probes for heavy and light chain clones. The heavy and light chain clones then are studied to determine the sequence of the variable domains.

The variable domains of the heavy and light chain are joined by a linker. To design a suitable linker, it is preferred to first define the extent of the variable domains. Kabat et al. defined the variable domain as extending from residue 1 to residue 107 for the lamba light chain, to residue 108 for kappa light chains and to 113 for heavy chains. (Kabat, E.A., "Sequencing of Protein of Immunologic Interest", U.S. Department of Health and Human Services, U.S. Government Printing Office, 1987) The 59 linker described in U.S. Patent no. 4,704,692 (incorporated herein by reference) can be used to join the domains. This linker was designed using a computer program that matched the ends of the variable domain with all possible structural fragments found in the protein DATABank. It should be understood that the design of a suitable linker is within the knowledge of those of ordinary skill in the art. (See e.g. U.S. Patent

4,946,778; Methods: A Companion to Methods in Enzymology Vol. 2, No. 2, April 1991, pp 97-105).

The sFv antibody may be constructed by joining either vL as the N-terminal domain followed by the linker and vH. A preferred linker for constructing a vH-linker-vL sFv antibody is the single linker designed by Huston, et al., a (gly-gly-gly-ser) 3 linker designed to bridge the 3.5 nm gap between C terminals of vH and the N terminals of vL, without exhibiting any propensity for ordered secondary structure (Huston, J.S. et al., Proc. Natl. Acad. Sci. USA 85 pp 5879-5883, 1988). Minor modifications of this linker design appear to have little effect upon the in vivo performance of an sFv antibody.

The sFv gene then can be engineered to encode an identification signal such as the Tat nuclear translocational signal. Because there exist specific antibodies to this signal, anti-idiotype antibody will not be necessary for immunostaining to determine sFv expression and intracellular location.

The sFv recombinant gene may be placed in a cassette that provides for efficient introduction into a cell and subsequent selection, for example, by G418 or gpt selection. After selection, cells can be evaluated for DNA, RNA and protein expression using DNA-PCR, RT-PCR and radioimmune precipitation, as well as immunostaining.

The recombinant genes of the invention are introduced into cells using vectors. Almost any delivery vector can be used, although the vector selected will depend upon the particular disease being treated, the particular form of treatment, whether the treated cells are replicating cells and other factors known to those of ordinary skill in the art.

Genetic material can be introduced into a cell by, for example, transfection or transduction. Transfection refers to the acquisition by a cell of new genetic material by incorporation of added DNA. Transfection can occur by physical or chemical methods. Many transfection techniques

are known to those of ordinary skill in the art including: calcium phosphate DNA co-precipitation; DEAE-dextran DNA transfection; electroporation and cationic liposome-mediated transfection. Transduction refers to the process of transferring nucleic acid into a cell using a DNA or RNA virus.

The treatment of cells may be in vivo or ex vivo. For ex vivo treatment, cells are isolated from an animal (preferably a human), transformed (i.e. transduced or transfected in vitro) with a vector containing a recombinant gene of the invention, and then administered to a recipient. Procedures for removing cells from animals are well known to those of ordinary skill in the art. In addition to cells, tissue or the whole or parts of organs may be removed, treated ex vivo and then returned to the patient. Thus, cells, tissue or organs may be cultured, bathed, perfused and the like under conditions for introducing the recombinant genes of the invention into the desired cells. The preferred treatment is ex vivo and the preferred cells for ex vivo treatment are stem cells.

For in vivo treatment, cells of an animal, preferably a mammal and most preferably a human, are transformed in vivo with a vector containing a recombinant gene of the invention. The in vivo treatment may involve systemic treatment with a vector such as intravenously, local internal treatment with a vector such as by perfusion, topical treatment with a vector and the like. When performing in vivo therapy, the preferred vectors are based on noncytopathic eukaryotic viruses in which nonessential or complimentable genes have been replaced with the gene of interest. Such noncytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have recently been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e. capable of directing synthesis of the desired proteins,

but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell line with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, M. "Gene Transfer and Expression, a Laboratory Manual", W.H. Freeman Co., New York (1990) and Murry, E.J. e.d. "Methods in Molecular Biology", Vol. 7, Humana Press, Inc., Cliffton, New Jersey (1991).

The most preferred virus for certain applications is the adeno-associated virus, a double-stranded DNA virus. The adeno-associated virus can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as: heat and lipid solvent stability, high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Recent reports indicate that the adeno-associated virus can also function in an extrachromosomal fashion.

Recombinant genomes that are between 50% and 110% of wild-type adeno-associated virus size can be easily packaged. Thus, a vector such as d13-94 can accommodate an insertion of 4.7kb in length. A modified sFv will be approximately 1 to 1.5kb in length, and therefore the adeno-associated virus may be an ideal delivery system.

In one preferred embodiment, an anti-HIV-1 sFv (pAVsFv-Integ) can be constructed by removing all endogenous coding sequences (bases 190-4034) from an infectious molecular clone of an adeno-associated virus (pAV1 from ATCC, Rockville, MD.). The RSV long terminal repeat (LTR) driven

sFv and the Neo gene under the control of the SV40 early promoter will be inserted.

Transgenic animals also may be produced according to the invention. A "transgenic animal" is an animal having cells that contain DNA which has been artificially inserted into a cell, which DNA becomes part of the genome of the animal which develops from that cell. Preferred transgenic animals are primates, mice, rats, cows, pigs, horses, goats, sheep, dogs and cats.

A variety of methods are available for the production of transgenic animals associated with this invention. DNA can be injected into the pronucleus of a fertilized egg before fusion of the male and female pronuclei, or injected into the nucleus of an embryonic cell (e.g., the nucleus of a two-cell embryo) following the initiation of cell division (Brinster et al, Proc. Natl. Acad. Sci. USA 82: 4438-4442 (1985)). Embryos can be infected with viruses, especially retroviruses, modified to carry the nucleotide sequences of the invention which encode intracellularly expressed antibodies.

Pluripotent stem cells derived from the inner cell mass of the embryo and stabilized in culture can be manipulated in culture to incorporate nucleotide sequences of the invention. A transgenic animal can be produced from such cells through implantation into a blastocyst that is implanted into a foster mother and allowed to come to term.

Animals suitable for transgenic experiments can be obtained from standard commercial sources such as Charles River (Wilmington, MA), Taconic (Germantown, NY), Harlan Sprague Dawley (Indianapolis, IN), etc.

The procedures for manipulation of the rodent embryo and for microinjection of DNA into the pronucleus of the zygote are well known to those of ordinary skill in the art (Hogan et al., supra). Microinjection procedures for fish, amphibian eggs and birds are detailed in Houdebine and Chourrout, Experientia 47: 897-905 (1991). Other procedures for introduction of DNA into tissues of animals are described

in U.S. Patent No. 4,945,050 (Sandford et al., July 30, 1990).

By way of example only, to prepare a transgenic mouse, female mice are induced to superovulate. Females are placed with males, and the mated females are sacrificed by CO_2 asphyxiation or cervical dislocation and embryos are recovered from excised oviducts. Surrounding cumulus cells are removed. Pronuclear embryos are then washed and stored until the time of injection. Randomly cycling adult female mice are paired with vasectomized males. Recipient females are mated at the same time as donor females. Embryos then are transferred surgically.

The procedure for generating transgenic rats is similar to that of mice. See Hammer et al., Cell 63: 1099-1112 (1990).

Methods for the culturing of embryonic stem (ES) cells and the subsequent production of transgenic animals by the introduction of DNA into ES cells using methods such as electroporation, calcium phosphate/DNA precipitation and direct injection also are well known to those of ordinary skill in the art. See, for example, Teratocarcinomas and Embryonic Stem Cells, A Practical Approach, E.J. Robertson, ed., IRL Press (1987).

In cases involving random gene integration, a clone containing the sequence(s) of the invention is co-transfected with a gene encoding resistance. Alternatively, the gene encoding neomycin resistance is physically linked to the sequence(s) of the invention. Transfection and isolation of desired clones are carried out by any one of several methods well known to those of ordinary skill in the art (E.J. Robertson, supra).

DNA molecules introduced in o ES cells can also be integrated into the chromosome through the process of homologous recombination. Capecchi, Science 244: 1288-1292 (1989). Methods for positive selection of the recombination event (i.e., neo resistance) and dual positive-negative selection (i.e. neo resistance and gancyclovir resistance)

and the subsequent identification of the desired clones by PCR have been described by Capecchi, supra and Joyner et al., Nature 338: 153-156 (1989), the teachings of which are incorporated herein. The final phase of the procedure is to inject targeted ES cells into blastocysts and to transfer the blastocysts into pseudopregnant females. The resulting chimeric animals are bred and the offspring are analyzed by Southern blotting to identify individuals that carry the transgene.

Procedures for the production of non-rodent mammals and other animals have been discussed by others. See Houdebine and Chourrout, supra; Pursel et al., Science 244: 1281-1288 (1989); and Simms et al., Bio/Technology 6: 179-183 (1988).

The following examples are illustrative and are not meant to be limiting of the invention.

Examples

Example 1: RNA Isolation, cDNA Synthesis and Amplification of Vh and Vl

RNA was prepared from 5 x 10^7 hybridoma cells. The total RNA was used for first strand cDNA synthesis using 17 bp poly-T mixed with either the Hv or vL 3' primer at 42°C for 1 hour in 50 µl reaction mixture containing 100 µg of RNA and AMV reverse transcriptase 100 Units, with a standard buffer system. For amplification of vL and vH, 5 µl of cDNA was subjected to 35 cycles of PCR using reagents, as per the manufacturer's instructions (Gene Amp. Perkin-Elmer/Cetus), in two separate tubes with 1 uM each with either vL-5' or vH-5' primer (obtained from Novagen, Inc., Madison, WI). Each PCR cycle consisted of denaturation at 94°C for 1 minute annealing at 50°C for 90 sec, and polymerization at 72°C for 2 minutes, and finally a 10 minute extension. The amplified vL and vH fragments were purified on 1.5% low-melting agarose with the Promega PCR magic purification kit (Madison, WI).

Taq polymerase-amplified PCR products were directly ligated into a modified pT7Blue(R) vector (Novagen), pT7H3-

10, Fig. 1, which carries extra T's at the 5' end. After transformation into the Novablue E. coli strain (Novagen), recombinants were selected on X-gal plates. For each of the cDNA, 40 colonies were picked up for mini preparation of plasmid for further enzymatic digestion to check the size of the insert. Plasmids were further prepared for DNA sequencing. All of the plasmids were sequenced on a 373A ABI automatic DNA sequencer (ABI, Foster City, CA). Finally, the plasmids were confirmed using the USB Sequenase Kit (United States Biochemical, Cleveland, OH).

The foregoing was applied to a murine hybridoma making a monoclonal antibody against HIV-$1_{(IIIB)}$ rev.

For the vL chain, the original insert DNA sequence was confirmed to be a mutant endogenous K chain by computer homology searching. Because sp0/2 myeloma cells have endogenous K chain expression, the Complimentarity Determining Region (CDR) sequence specific for endogenous K chains is used for K chain PCR recombinant plasmid screening to eliminate the contamination of this K chain from the recombinant plasmids. Only less than 5% of the plasmid do not contain this K chain and those plasmids are DNA sequenced.

For each of the cDNA fragments, at least 3 different colonies are sequenced to confirm sequence. Specific primer targeting-CDR derived from those cDNA sequences are designed to repeat RT-PCR for each of the parent hybridomas to confirm the sequence.

Specific protocols to eliminate aberrant endogenous K chains permit quickly obtaining larger numbers of different Ig K genes.

Two methods have been developed to eliminate endogenous ABVK chains. The first method is to eliminate ABKV RNA background by cleaving ABKV RNA directly with the ribozyme RNA system. This reduces the ABKV RNA RT-PCR background and enhances the specific Ig light chain RNA signal for cloning. Specifically;

A 62bpABKV ribozyme DNA fragment was synthesized by PCR and inserted into vector pGEM4Z at the HindIII-BamII site to form the plasmid p4ZABVKRIBO (Fig. 2). After the plasmid is linearized by BamHI digestion, the specific ABKV ribozyme can be synthesized with T7 RNA polymerase in vitro as follows:

Heat using 2 ug of the linearized plasmid for 3 minutes at 75°C and then cool on ice. Add the following reagents:

4 ul 5x transcription buffer
1 ul RNase inhibitor (40u/ul)
2 ul 100mM dithiothreitol (DTT)
4 ul 250 uM NTP
1 ul RNA polymerase
Add DEPC-H_2O to final volume of 20 ul.

Incubate 37°C for 1 hour. After transcription, the reaction mixture is treated with 1 ul of RQ1 DNase (5u/ul) at 37°C for 39 minutes.

This is followed by phenol/chloroform extraction and ethanol precipitation. The specific Ribozyme RNA can be resuspended in diethylpyrocarbonate (DEPC) treated water and stored at -70°C.

Total or polyA RNA, which is extracted from the hybridomas and resuspended in 5 ul DEPC water, is mixed with 4 ul ABKV ribozyme RNA. This mixture is heated to 75°C for 5 minutes, quickly cooled down on ice and resuspended in 4x RT buffer (200mM tris HC, pH 8.3, 200 mM KCl, 40mM MgCl2, 2mM spermidine, 40 mM DTT. It is then incubated at 37°C for 30-60 minutes. 5 ul of the mixture can be used for the standard PT-PCR for the Ig light chain.

A second method for eliminating the endogenous ABVK chain is as follows:

Manipulation of RNA for extended time periods or in multiple step processes may cause dramatic RNA degradation and affect the efficiency of RT-PCR. To further improve on the above ABVK ribozyme system, we have inserted this 62 bp ABVK ribozyme fragment into a new plasmid pSCCribo which may

be transfected into the packaging cell line PA317 to produce an infectious but replication deficient virus (Fig. 3). The supernatants containing the virus can be used to introduce the ABVK ribozyme into any hybridoma cell line with high efficiency.

Typically, 10 ml of cell free viral supernatants (10^6 - 10^7 cpu/ml) is used to infect 5-8 x 10^6 hybridoma cells in a 20 ml total volume with 8 ug/ml polybrene at 37°C in CO_2 incubator for 24 hours. Then cells are washed twice with serum free medium and fresh medium added, with 10% FCS, and 500 ug/ml G418. This selection carried out for 1 week. RNA can then be extracted directly from hybridoma cells due to the high level of expression of the CAT-ABVK ribozyme RNA (CAT RNA is very stable in the cells). The CAT-ABVK ribozyme can specifically target the endogenous ABVK RNA resulting in cleavage. This dramatically reduces the ABVK RNA background and enhances the antigen specific hybridoma 1g light chain for RT-PCR (See Fig. 4, Gel).

An alternative way to obtain vL fragments is to use commercially available filamentous phage vector systems. The vector systems can concurrently produce free Fab fragments and Fab displayed on the surface of bacteriophage via a vHC_{H1}-pIII fusion protein. When expressed in a supo (non-suppressor) strain of E. coli, free Fab can be produced. Antibody Fab fragments are secreted into culture medium at high concentration, because vH and vL are found to accumulate in the periplasmic space.

Example 2: E. Coli bacteriophage expression system for Fab

The bacteriophage expression is carried out as specified in Barbas and Lerner, Methods: A Companion to Methods in Enzymology 2: 119-124 (1991). Briefly, RT-PCR DNA encoding the Fd is inserted into a phage vector and transformed into host bacteria. RT-PCR light chain DNA fragments from the same hybridoma are then inserted into the pComb3 vector. Following bacterial transformation, the combinatorial libraries are treated to prepare phagemid.

Solid phase selection (panning) of the Fab against the antigen of interest proceeds as follows: Microtiter wells were coated with 9.5 μg of purified E. coli recombinant antigen (such as HIV-1-RT, Tat or Rev), overnight at 4°C. Wells are blocked with Bovine Serum Albumin (3% BSA in PBS) for one hour at 37°C, incubated with phage libraries (typically $>10^{11}$ colony-forming phage per well), washed and eluted. The selected phage are then allowed to infect E. coli XL-IBlue cells and used to prepare a new phage stock by infection with the helper phage VCSM 13 (both from Stratagene, La Jolla, CA). For repanning against the same antigen (up to 4 more cycles) this procedure was repeated three or four times.

The culture of phagemid containing XL-IBlue cells from the last panning against antigen is split in two and one half is packaged with CSM13 helper phage, the other half used to prepare phagemid DNA. Phagemid DNA is digested with restriction enzymes to excise the geneIII coding for the phage cap protein allowing the Fabs to be expressed in soluble form. The religated DNA is retransformed into XL-IBlue cells and clone supernatants screened for Fab production by ELISA using microtiter wells coated with 0.1ug of antigen, followed by clone supernatant, then goat anti-human $F(ab)_2$ conjugated to alkaline phosphatase, then alkaline phosphatase substrate.

Positive clones are then tested for specificity against a umber of different antigens (viral and human) by ELISA and phagemid DNA prepared from each clone.

The above methods have been applied to production of human viral neutralizing Fab to HIV-L, Respiratory Syncytial Virus (RSV), CMV, HSV I and II viruses (Burton et al., PNAS 88: 10134 (1991); Barbas et al., PNAS 89: 10164 (1992); Williamson et al., PNAS 90: 1993.

Example 3: Retrovirus construction using CAT and in vitro expression of CAT

In order to demonstrate that antivirus sFv can function at different levels in different types of cells, U1 and ACH2 cells were selected to test LXSN expression function. The U1 cell line, a U937-derived HIV-1 infected clone, has been used as a model for viral latency, and the effects of monocyte-specific cytokines on the induction of HIV-1 expression were studied in this model system. ACH2 is derived from an infected T-lymphocyte line which has one copy of provirus integration while U1 cells have two proviral copies. Both cell lines produce very low levels of HIV-1 P24 expression and act as an HIV-1 latency state. With different stimulation, such as PMA or TNF-α, both of these cells will increase HIV-1 p24 by more than 1000-fold in 48 hours, and will produce infectious functional virus. Those cell lines provide good cell line model systems which not only represent both T-lymphocyte and macrophage type cells but also represent most of the HIV-1 infected cellular populations.

A 734 bp CAT fragment was inserted into the pLXSN vector (MuLV retrovector). This pLXSNCAT plasmid (Fig. 5) was transfected into the packaging cell line PA 317 and selected in G418 (1 mg/ml). 1 x 10^6 PA317 cells were plated in 100mm dishes in 10 ml Dulbecco's Modified Eagles Medium (DMEM) + 10% Fetal Calf Serum (FCS) one day before transfection. Three hours before transfection, the 10 ml of medium was replaced with 10 ml of fresh prewarmed medium. 20 μg pLXSNCAT was transfected into pA317 cells. After an additional 48 hours, the cultured medium containing the virus was collected and passed through a 0.45 um filter to prepare cell free virus. After determining the infectious titer of the virus (cfu/ml), the medium was mixed with 3 x 10^6 U1 or ACH2 cells with 8 ug/ml polybrene to help increase efficiency and incubated for 12-16 hours. Cells were then washed twice with serum free medium and resuspended in 10 ml RPMI 1640 with 10% FCS for further culture. From this test, we can detect CAT activity after 48 hours transfection without non-

specific stimulation of HIV replication (i.e. maintained at the same level of p24 as prior to superinfection).

In order to test actual infection efficiency, the 3.2 kb of E. coli betagalactosidase was inserted into the same vector, pLXSN, to construct the pLX-GAL (pLXNLacZ-13) plasmid (Fig. 6). The same protocol as above was used (s for transfection of U1 and ACH2 cells) to produce virus carrying the b-galactosidase.

After transduction into U1 and ACH2 cells, cells were stained with X-gal substrate so that cells carrying the plasmid became blue due to expression of beta-galactosidase. By counting the blue cells under the microscope, U1 and ACH2 efficiency may be measured (usually more than 70% cells/per infection).

To test long term expression level following transduction, with the CAT expression virus, both U1 and ACH2 cell lines were maintained in G418 selection for more than 6 months. Data shows that in ACH2 cells, CAT activity is maintained at the same level of expression for the long term. In U1 cells, CAT expression in most cases is maintained only for 2 weeks and then completely shuts off. Further tests show that after PMA stimulation, CAT activity remains at the same level but HIV-1 expression occurs as in the parent line. These data show that this DNA delivery model system can be used for delivering sFv.

Example 4: Plasmid vector construction using sFv with anti-REV activity and expression in Hela-T4s

A single chain sFv anti-rev antibody was constructed consisting of variable domains of the heavy (vH) and light (vL) chains of a murine monoclonal antibody against HIV-1_{IIIB}) rev (the "parent antibody").

Protocols for constructing the vH and vL regions are as follows:

After sequencing the anti-rev vH and vL cDNA, the CDR region was compared by computer with the published Ig protein sequences. The full length sequence was then designed. First two synthesized oligonucleotides were used

to create a linker DNA fragment with ApaI-BgIII sites. This was then cloned into pT7/Blue (R) vector in order to determine the DNA sequence. vH and vL were then reamplified with two new pairs of oligonucleotides with suitable enzyme sites at both ends, cloned into pT7/Blue (R). After verifying the DNA sequence, the linker DNA, N-GGGGSGGGGSGGGGS-C (Sequence I.D. number 2), was inserted into the ApaI and the BgIII site to connect the vH and vL DNA sequence to make a full length sFv fragment. Following digestion with NdeI-BamHI, the full length sFv DNA sequence was inserted into the E. coli expression vector, pET19b to construct plasmid pET19bHLc8 (Fig. 7). Transformation into E. coli BL21 (DE3) allowed the expression of the sFv protein. A 10 histidine (HIS) amino acid domain was located on the end terminal of the sFv protein. The His-Tag sequence binds to the divalent cation (Ni^{2+}) immobilized on a His binding metal chelation resin allowing purification by Ni^{2+} affinity chromatography.

The DNA sequence of the sFv anti-rev was determined to be as follows (Sequence I.D. Number 1):

ATGGGCCATC ATCATCATCA TCATCATCAT CATCATAGCA
GCGGCCATAT CGACGACGAC GACAACCATA TGTTGGTGCT
GACGTTCTGG ATTCCTGCTT CCAGCAGTGA TGTTGTGATG
GCCCAAACTC CACTCTCCCT GCCTGTCAGT CTTGGACATC
AAGCCTCCAT CTCTTGCATA TCTAGTCAGA GCCTTGTACA
CAGTAATGGA AACACCTATT TACATTGGTA CCTGCAGAAG
CCAGGCCAGT CTCCAAAGCT CCTGATCTAC AAAGCTTCCA
ACCGATTTTC TGGGGTCCCA GACAGGTTCA GTGGCAGTGG
ATCAGGGACA GATTTCACAC TCAAGATCAG CAGAGTGGAG
GCTGAGGATC TCCCAGTTTA TTTCTGCTCT CAAAGTACAC
ATTTTCCGTG GACGTTCGGT GGAGGCACCA AGCTGGAAAT
CAAACGGGCT GATGGGCCCG GTGGGGGCGG TTCGGGTGGC
GGGGGCTCGG GCGGGGGTGG CTCAGAGCTC GGCAGATCTG
ATGTGCAGCT GGTGGACTCT GGGGGAGGGT TAGTGCAGCC
TGGAGGGTCC CGGAAACTCT CCTGTGCAGG CTCTGGATTC
ACTTTGACTA GGTTTGGAAT GCACTGGGTT CGGCAGGCTC
CAGAGAAGGG GCTGGACTGG GTCGCATACA TTAGTAGTGG
GAGTAGTACC CTCCACTATG CAGACACAGT GAAGGGCCGA
TTCACCATCT CCAGACACAA TCCCAAGAAC ACCCTGTTCC
TGCAAATGAA ACTACCCTCA CTATGCTATG CACTACTGGG
GTCAAGGAAC CTCAGTCACC GTCTCCTCAG CCAAAACGAC
ACCCCCACCC GTCTATCCTG A

Rev is one of the essential regulatory proteins of Human Immunodeficiency Virus. It is a 19kD phosphorprotein

localized primarily in the nucleolus/nucleus, and acts by binding to Rev Responsive Element (RRE) and promoting the nuclear export, stabilization and utilization of the viral mRNA's containing RRE.

The binding affinity of the sFv anti-rev produced in E. coli was then determined by using an ELISA (Enzyme Linked Immunoassay) utilizing recombinant rev conjugated with biotin. The binding affinity was approximately 10^{-} which was comparable to the affinity of the present antibody.

The binding efficiency was determined as follows:

Purified E. coli derived sFv anti-Rev was diluted in Phosphate Buffered Saline (PBS) solution at 200ug/ml. ELISA plate wells were coated with 200 ul per well of this solution, overnight at 4°C. The same concentration of BSA/PBS was used for coating control wells. Wells were washed once with PBS and blocked by the addition of 10% BSA/PBS, 200 ul/well. After blocking for 1 hour at 37°C wells were washed three times with 0.5% Tween 20/PBS. 100ul of biotin conjugated-Rev dilutions (serial 5 fold 50 ug/ml to 16 ng/ml) were added to the wells and the plates incubated for two hours at 37°C. Wells were washed 3 times with 0.5% Tween 20/PBS. 100 ul of avadin-labelled with Horseradish Peroxidase (HRP) was added to wells and incubated for 15 minutes at 37°C. Washing was repeated 3 times as above. 100 ul of color substrate solution was added to each well. After incubation at room temperature for 30 minutes the reaction wa stopped by the addition of 100 ul of 4M sulphuric acid. Optical density was then read at 495m mm.

Calculation of the native Rev protein was performed as follows:

Rev. MW=13019.855 (117 amino acids)

E. coli derived Rev has an additional 12 aa leading sequence (Sequence I.D. number 3).

Leading sequence: MRAKLLGIVLTT=1485.4

Actual molecular weight of the E. coli derived Rev is = 14505.25

Therefore 14505 ug/ml = 1uM/ml= 10^{-}M.

The data from ELISA indicates binding of Rev to HLc8 protein between 2 ug and 4 ug per ml. Therefore binding affinity is approximately 7.25 x 10 $^{-9}$M.

The effect of sFv-anti-rev production on the levels of soluble p24 expressed is demonstrated in the graph in Fig. 8.

In combination, the results of syncytia formation and p24 production show that the expression of sFv anti-rev resulted in a decrease in HIV expression of approximately 80% as compared with the Hela-T4 control. This proves that sFv antibodies can be expressed intracellularly to inhibit HIV.

The sFv was then cloned into a plasmid vector (pREP$_4$, Invitrogen, San Diego, CA) which allows for expression of the sFv in mammalian cells. The sFv gene was inserted into XhoIBamHI site on the vector. It was driven by the RSV-LTR promoter. The HIV-Tat nuclear translocation signal DNA was cloned by PCR. The HIV Tat cDNA was amplified with two oligo primers. It was then ligated into pT7 Blue(R) vector and sequenced. The amino acid sequence of the signal is: N-GRKKRRQRRRAHQN-C (Sequence I.D. number 4). The corresponding DNA sequence is: 5' GGC AGG AAG AAG CGG AGA CAG CGA CGA AGA GCT CAT CAG AAC AGT CAG ACT 3' (Sequence I.D. number 5).

It was inserted into the pETHLC8 SacI-BgIII site. Then the XhoI BamHI fragment was inserted into pP9 pREP9 (Neo resistant) vector to form plasmid p9CESAR (Fig. 8). Hela cells expressing CD4 (Hela-T4's) were then transfected with the pREP$_4$-sFv construct which also contained tk driven neomycin resistant gene as a marker. After transfection, the Hela-T4's were incubated with neomycin (G418) to enrich the population of sFv expressing cells.

sFv expressing cells and non-transfected Hela-T4's (as a control) were then infected with a high titer of HIV-1$_{(HXB2}$, vigorously washed and incubated for 10 days to determine the effect of sFv anti-rev production on HIV infection. This effect was measured in terms of (a) syncytia formation and (b) the levels of soluble p24 antigen.

The effect of sFv-anti-rev production on syncytia is demonstrated in the graph shown in Fig. 10.

Due to the high rate of mutation of the HIV-1 genome it is important that therapies be effective on different clinically isolated strains. Figure 11 shows that the sFv specifically binds a highly conserved Rev domain. The HeLa T4 cells expressed sFv resistance to all of the tested clinically isolated strains of HIV-1.

The invention can clearly be translated to other diseases caused by other pathogens and diseases associated with the elevated expression of proteins, such as cancers.

Example 5: Human Lymphocyte RNA Preparation

Five milliliters of bone marrow was removed by aspiration from an long term asymptomatic HIV-1 positive donor. Immediately, 10 ml of 3M guanidium isothiocyanate containing 71ul of 2-mercaptoethanol was added and then RNA was prepared by standard methods.

Example 6: Phagemid Library Construction

Total RNA (typically 10 μg) was reverse-transcribed as described by Burton, et al. *Proc. Natl. Acad. Sci., U.S.A.*, **88**, 10134-10137 (1991), incorporated by reference herein in its entirety and γ1 (Fd region) and κ chains were amplified by PCR. The resulting γ1 heavychain DNA was cut with an excess of the restriction enzymes Xho I and Spe I and typically about 350 ng was ligated with 2 μg of Xho I/Spe I-linearized pComb3 vector (isolated by agarose gel electrophoresis) in a total volume of 150 μl with 10 units of ligase (BRL) at 16°C overnight. Following ligation, DNA was precipitated at -20°C for 2 hr by the addition of 2 μl of 2% (wt/vol) glycogen, 15 μl of 3 M sodium acetate (pH 5.2), and 330 μl of ethanol. DNA was pelleted by microcentrifugation at 4°C for 15 min. The DNA pellet was washed with cold 70% ethanol and dried under vacuum. The pellet was resuspended in 10 μl of water and transformed by electroporation into 300 μl of *Escherichia coli* XL1-Blue. After transformation, 3 ml

of SOC medium (20mM glucose pH 7.0, 2% bacto-tryptone, 0.5% yeast extract, 0.05% $NaCl_2$, 2.5mM KCl) was added and the culture was shaken at 220 rpm for 1 hr at 37°C after which 10 ml of SB (super broth; 30 g of tryptone, 20 g of yeast extract, and 10 g of Mops per liter, pH 7) containing carbenicillin (20 μg/ml) and tetracycline (10 μg/ml) was added. At this point, samples (20, 1, and 0.1 μl) were withdrawn for plating to determine the library size. Typically the library had about 10^7 members. The culture was grown for an additional hour at 37°C while shaking at 300 rpm. This culture was added to 100 ml of SB containing carbenicillin (50 μg/ml) and tetracycline (10 μg/ml) and was grown overnight. Phagemid DNA containing the heavy-chain library was prepared from this overnight culture. To determine the insert frequency of this ligation, 10 colonies from the plates used to titer the library were picked and grown. DNA was prepared and then digested with Xho I and Spe.

For the cloning of the light chain, phagemid DNA (pcomb3) (10 μg) was digested as described above except that the restriction enzymes Sac I and Xba I were used. The resulting linearized vector was treated with phosphatase and purified by agarose gel electrophoresis. The desired fragment, 4.7 kilobases long, was excised from the gel. Ligation of this vector with prepared light-chain PCR DNA proceeded as described above for the heavy chain. After transformation, 3 ml of SOC medium was added and the culture was shaken at 220 rpm for 1 hour at 37°C. Then 10 ml of SB containing carbenicillin (20 μg/ml) and tetracycline (10 μg/ml) was added (samples were removed for titering as described above for the heavy-chain cloning) and the culture was shaken at 300 rpm for an additional hour. This culture was added to 100 ml of SB containing carbenicillin (50 μg/ml) and tetracycline (10 μg/ml) and then shaken for 1 hr. Helper phage VCS-M13 (10^{12} plaque-forming units) was added and the culture was shaken for an additional 2 hours. After this time, kanamycin (70 μg/ml) was added and the culture was

incubated at 37°C overnight. The supernatant was cleared by centrifugation (4000 rpm for 15 minutes in a JA-10 rotor) at 4°C. Phage were precipitated by addition of 4% (wt/vol) polyethylene glycol 8000 and 3% (wt/vol) NaCl followed by incubation on ice for 30 minutes and centrifugation. Phage pellets were resuspended in 2 ml of phosphate-buffered saline (PBS: 50 mM phosphate, pH 7.2/150 mM NaCl) and microcentrifuged for 3 minutes to pellet debris. Supernatants were transferred to fresh tubes and stored at -20°C.

Example 7: Titering of Colony-Forming Units.

Phagemids that have been packaged into virions are capable of infecting male *E. coli* to form colonies on selective plates. Phage (packaged phagemid) was diluted in SB (dilutions: 10^{-3}, 10^{-6}, and 10^{-8}) and 1 µl was used to infect 50 µl of fresh *E. coli* XL1-Blue culture (OD_{600}=1) grown in SB containing tetracycline (10 µg/ml). Phage and cells were incubated at room temperature for 15 minutes and then directly plated on LB/carbenicillin plates.

Example 8: Panning of the Combinatorial Library to Select Antigen Binders

Four wells of a microtiter plate (Costar 3690) were coated overnight at 4°C with 25 µl of recombinantly produced rev or tat protein (40 µg/ml in 0.1 M bicarbonate buffer, pH 8.6). The wells were washed twice with water and blocked by completely filling the well with 1% (wt/vol) bovine serum albumin (BSA) in PBS and incubating the plate at 37°C for 1 hour. Blocking solution was shaken out, 50 µl of the phage library (typically 10^{11} colony-forming units) was added to each well, and the plate was incubated at 37°C for 2 hours. Phage were removed and the plate was washed once with water. Each well was then washed 10 times with 50 mM Tris-HCl, pH 7.5/150 mM NaCl/0.5% Tween 20 over a period of 1 hour at room temperature. The plate was washed once more with distilled water and adherent phage were eluted by the addition of 50 µl of elution buffer (0.1 M HCl, adjusted to pH 2.2 with solid

glycine and containing 0.1% BSA) to each well and incubation at room temperature for 10 minutes. The elution buffer was pipetted up and down several times, removed, and neutralized with 3 μl of 2 M Tris base per 50 μl of elution buffer used. Eluted phage were used to infect 2 ml of fresh *E. coli* XL1-Blue cells (OD_{600}=1) for 15 minutes at room temperature after which 10 ml of SB containing carbenicillin (20 μg/ml) and tetracycline (10μg/ml) was added. Samples (20, 1, and 0.1 μl) were removed for plating to determine the number of phage (packaged phagemids) that were eluted from the plate. The culture was shaken for 1 hour at 37°C and then added to 100ml of SB containing carbenicillin (50μg/ml) and tetracycline (10 μg/ml) and shaken for 1 hour. Helper phage VCS-M13 (10^{12} plaque-forming units) were added and the culture shaken for an additional 2 hours. Then kanamycin (70 μg/ml) was added and the culture was incubated at 37°C overnight. Phage preparation and further panning were repeated four times as described above.

Example 9: Preparation of Soluble Fab Fragments.

Phagemid DNA from positive clones was isolated and digested with *Spe I* and *Nhe I*. Digestion with these enzymes produces compatible cohesive ends. The 4.7-kilobase DNA fragment lacking the gene III (cap protein) portion was gel-purified (0.6% agarose) and self-ligated.

Transformation of *E. coli* XL1-Blue afforded the isolation of recombinants lacking the gene III (cap protein) fragment. Clones were examined for removal of the gene III fragment by *Xho I/Xba I* digestion, which yielded a 1.6-kilobase fragment. Clones were grown in 15 ml of SB containing carbenicillin (50μg/ml) and 20 mM $MgCl_2$ at 37°C until OD_{600} of 0.2 was achieved.

Isopropyl β-D-thiogalactopyranoside (1mM) was added and the culture was incubated overnight at 37°C. Cells were pelleted by centrifugation at 4000 rpm for 15 minutes in a JA-10 rotor (Beckman J2-21) at 4°C. Cells were resuspended in 3 ml of PBS containing 0.2 mM phenylmethylsulfonyl

fluoride and lysed by sonication on ice (2-4 min, 50% duty). The debris was pelleted by centrifugation at 14,000 rpm in a JA-20 rotor at 4°C for 15 minutes. The supernatant was used directly for ELISA analysis and was stored at -20°C.

Example 10: ELISA Analysis of Human anti-rev and anti-tat Fab Supernatants

ELISA wells were coated with rev and tat proteins exactly as above, washed five times with water, blocked in 100 μl of 1% BSA/PBS for 1 hour at 37°C, and then incubated with 25 μl Fab supernatants for 1 hour at 37°C. After 10 washes with water, 25 μl of a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-human IgG $F(ab')_2$ (Pierce) was added and incubated for 1 hour at 37°C. Following 10 washes with water, 50 μl of *p*-nitrophenyl phosphate substrate was added and color development was monitored at 405 nm. Positive clones gave A_{405} values >1 (mostly >1.5) after 10 minutes, whereas negative clones gave values of 0.1-0.2.

Three Fab producing clones were isolated against HIV-1 rev and 4 Fab producing clones were isolated against HIV-1 tat. Results are shown in Table 1.

TABLE 1

Properties of Human Monoclonal Fab Derived from an Asymptomatic, 10 yr HIV-1 Positive Donor

ANTIGEN	CLONE	BINDING CONSTANT	YIELD/ LITER (μg)	TITER
HIV-1 rev	rev 9(Fd)	8×10^{-7}M	20	1/1
	rev 9/12LC			
	rev 9/16LC	6×10^{-8}M	12	1/4
HIV-1 rev	rev 16	6×10^{-7}M	67	1/2
HIV-1 rev	rev 20	6×10^{-7}M	39	1/2
HIV-1 tat	tat 31	4.2×10^{-7}M	94	1/8
HIV-1 tat	tat 16	1.7×10^{-6}M	66	1/1
HIV-1 tat	tat 104	3.2×10^{-7}M	84	1/8
HIV-1 tat	tat 107	3.0×10^{-7}M	12	1/1

Example 11: Sequencing

Nucleic acid sequencing was carried out on double-stranded DNA with Sequenase 1.0 (United States Biochemical). Amino acid sequences were determined and are set forth in Table 2.

TABLE 2
HUMAN ANTI-HIV Fab AMINO ACID SEQUENCES

CLONE	SEQ ID NO.	FR1	CDR1	FR2
Heavy Chain VH Sequences				
rev9(VH3)	6	LLESGGGVVQPGRSLRLSCAASGFIFS	TYGIY	WVPQAPGKGLEWVA
rev16(VH3)	7	LLESGGGLAQPGGSLRLSCAASGFTFS	SYEMN	WVRQPPGKGLEWVS
rev20(VH3)	8	LLESGGGLAQTGGSLRLSCAASGFTFS	SYEMN	WVRQPPGKGLEWVS
tat104(VH3)	9	LLESGGGGVVQPGGSLRLSCEASGFSLI	NTAMH	WVRQAPXKGPEWVS
Light Chain VL Sequences				
rev16/20 (VL5-FR1, CDR1, CL)	10	AELQPPSVSAAPGQKVIISC	SGSSSHT/ /GQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAXSS	
tat16(VL1)	11	AELQPPSVSAAPGQKVTISC	SGSTSNIGNRHVS	WYQQLPGTXPKLLIY
tat31(VL1)	12	AELQPPSVSAAPGQSVTISC	SGSSSNIGNYNVX	WYQQTPGSAPKTLIY
tat104(VL4)	13	GELQDPVVSVALGQTVRMTC	QGDSLRYHYAN	WYQQKPGQAPILVIK
tat107(VL4)	14	AELQDPVVSVALGQTVRITC	QGDSLRXYHAN	WYQQKPGKAPIFVIY
Light Chain CL Sequences				
rev16/20	15	/GQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAXSSYLSLTPEQWKSHKSYXCQVTHEGSTVEKTVXPTECS		
tat16	16	QPKXAPSVTLFPPSSEELQANKXTLVCLISDFFPGXXXVXWKXDSXPXKGGVETTXPP		
tat31	17	QPKXAPSVTLFPPSSEELQANKXTLVCLISDFYPGAXTVXWKADSSPVKAGVENTTPSIXMQQQVSGPGGI		
tat104	18	QPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKGGVETTTPSNQSNNKFAASRYLSLTPEQWKSHRSYSCQV		
tat107	19	QPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVXVAWKADSSPVKVGVXXTTPSXHXINMFAGSXYLSLTPEQWXSHRKLQLPGQRRMXGAPXR		

HUMAN ANTI-HIV Fab AMINO ACID SEQUENCES				
CLONE	**CDR2**	**FR3**	**CDR3**	**FR4**
Heavy Chain VH Sequences				
rev9(VH3)	IISHDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTXVYYCAR	EGVHKXFDH	WGQGTLVTVSSASTKGPSV
rev16(VH3)	YISSGSDTIYYADSVKG	RFIISRDNAKNTLYLQMNNLRGEDTAVYYCAR	DPRRWTQLWIPPDY	WGQGTLVSVSSASTKGPSV
rev20(VH3)	YISSGSDTIYYADSVKG	RFIISRDNAKNTLYLQMNNLRGEDTAVYYCAR	DPRRWTQLWIPPDY	WGQGTLVSVSSASTKGPSV
tat104(VH3)	VSSYDGREKYYTDSVKG	RFSISRDDSTNMLYLQMNSVKIDDTAVYYCAR	TNRAYCSGVRCHDGLDV	WGQGTMVTVSSASTKGPSV
Light Chain VL Sequences				
rev16/20 (VL5-FR1, CDR1, CL)	YLSLTPEQWKSHKSYXCQVTHEGSTVEKTVXPTECS			
tat16(VL1)	ENNIRPS	GIPDRFSASKSGTSATLDITGLQTGDEADYYC	GTWDSSLSTGHWV	FGGGTKLTVLS
tat31(VL1)	DTHKRPS	GISERFSGSKSGTSATLGITGLQTGDEADYYC	GTWDTSLNSAL	FGGGTKLTXLG
tat104(VL4)	SKNNRPS	GIPDRFSGSSSGNTASLTITGAQAEDEADYYC	DSRDTSGNHPRVL	FGGGTKLTVLG
tat107(VL4)	GKNNRPS	GIPDRFSGSSSGNTASLTITGAQAEDEADYYC	NSRDSSSNHVV	FGGGTKLTVLG
Light Chain CL Sequences				
rev16/20				
tat16				
tat31				
tat104				
tat107				

Example 12: Epitope Mapping

ELISA assays were performed as described above using defined peptides of the tat and rev proteins set forth in Tables 3 and 4, respectively. The anti-tat Fab bound to the cysteine rich tat functional domain as shown in Figure 13. Reduction of the antibody reduced binding of the Fab to the functional domain as shown in Figure 14.

Binding of anti-rev Fd and Fab is shown in Figure 15. The anti-rev Fd rev9 bound to the sequence immediately adjacent to the basic nucleolar localization domain. Anti-rev Fab rev16 and rev20 were found to be identical and binding was evident to the region immediately adjacent to the activation domain.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Intracellular Immunization

(iii) NUMBER OF SEQUENCES: 19

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Woodcock Washburn Kurtz Mackiewicz and Norris
(B) STREET: One Liberty Place - 46th Floor
(C) CITY: Philadelphia
(D) STATE: PA
(E) COUNTRY: U.S.A.
(F) ZIP: 19103

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

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/099,870
(B) FILING DATE: 30-JUL-1993
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Ralph, Rebecca L.
(B) REGISTRATION NUMBER: 35,152
(C) REFERENCE/DOCKET NUMBER: TJU-

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 215-546-8396
(B) TELEFAX: 215-568-3439

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 861 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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

ATGGGCCATC ATCATCATCA TCATCATCAT CATCATAGCA GCGGCCATAT CGACGACGAC 60

GACAACCATA TGTTGGTGCT GACGTTCTGG ATTCCTGCTT CCAGCAGTGA TGTTGTGATG 120

GCCCAAACTC CACTCTCCCT GCCTGTCAGT CTTGGACATC AAGCCTCCAT CTCTTGCATA 180

TCTAGTCAGA GCCTTGTACA CAGTAATGGA AACACCTATT TACATTGGTA CCTGCAGAAG 240

CCAGGCCAGT CTCCAAAGCT CCTGATCTAC AAAGCTTCCA ACCGATTTTC TGGGGTCCCA 300

GACAGGTTCA GTGGCAGTGG ATCAGGGACA GATTTCACAC TCAAGATCAG CAGAGTGGAG 360

GCTGAGGATC TCCCAGTTTA TTTCTGCTCT CAAAGTACAC ATTTTCCGTG GACGTTCGGT 420

GGAGGCACCA AGCTGGAAAT CAAACGGGCT GATGGGCCCG GTGGGGGCGG TTCGGGTGGC 480

GGGGGCTCGG GCGGGGGTGG CTCAGAGCTC GGCAGATCTG ATGTGCAGCT GGTGGACTCT 540

GGGGGAGGGT TAGTGCAGCC TGGAGGGTCC CGGAAACTCT CCTGTGCAGG CTCTGGATTC 600

ACTTTGACTA GGTTTGGAAT GCACTGGGTT CGGCAGGCTC CAGAGAAGGG GCTGGACTGG 660

GTCGCATACA TTAGTAGTGG GAGTAGTACC CTCCACTATG CAGACACAGT GAAGGGCCGA 720

TTCACCATCT CCAGACACAA TCCCAAGAAC ACCCTGTTCC TGCAAATGAA ACTACCCTCA 780

CTATGCTATG CACTACTGGG GTCAAGGAAC CTCAGTCACC GTCTCCTCAG CCAAAACGAC 840

ACCCCCACCC GTCTATCCTG A 861

(2) INFORMATION FOR SEQ ID NO:3:

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

(ii) MOLECULE TYPE: peptide

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

Met Arg Ala Lys Leu Leu Gly Ile Val Leu Thr Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

Gly Arg Lys Lys Arg Arg Gln Arg Arg Arg Ala His Gln Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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

GGCAGGAAGA AGCGGAGACA GCGACGAAGA GCTCATCAGA ACAGTCAGAC T 51

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

Leu Leu Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg Ser Leu Arg
1 5 10 15

Leu Ser Cys Ala Ala Ser Gly Phe Ile Phe Ser Thr Tyr Gly Ile Tyr
20 25 30

Trp Val Pro Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Ile Ile
35 40 45

Ser His Asp Gly Ser Asn Lys Tyr Tyr Ala Asp Ser Val Lys Gly Arg
50 55 60

Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr Leu Gln Met
65 70 75 80

Asn Ser Leu Arg Ala Glu Asp Thr Xaa Val Tyr Tyr Cys Ala Arg Glu
85 90 95

Gly Val His Lys Xaa Phe Asp His Trp Gly Gln Gly Thr Leu Val Thr
100 105 110

Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val
115 120

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 128 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

```
Leu Leu Glu Ser Gly Gly Gly Leu Ala Gln Pro Gly Gly Ser Leu Arg
1               5                  10                  15

Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr Glu Met Asn
            20                  25                  30

Trp Val Arg Gln Pro Pro Gly Lys Gly Leu Glu Trp Val Ser Tyr Ile
        35                  40                  45

Ser Ser Gly Ser Asp Thr Ile Tyr Tyr Ala Asp Ser Val Lys Gly Arg
    50                  55                  60

Phe Ile Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr Leu Gln Met
65                  70                  75                  80

Asn Asn Leu Arg Gly Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Asp
                85                  90                  95

Pro Arg Arg Trp Thr Gln Leu Trp Ile Pro Pro Asp Tyr Trp Gly Gln
            100                 105                 110

Gly Thr Leu Val Ser Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val
        115                 120                 125
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 128 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

```
Leu Leu Glu Ser Gly Gly Gly Leu Ala Gln Thr Gly Gly Ser Leu Arg
1               5                  10                  15

Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr Glu Met Asn
            20                  25                  30

Trp Val Arg Gln Pro Pro Gly Lys Gly Leu Glu Trp Val Ser Tyr Ile
        35                  40                  45

Ser Ser Gly Ser Asp Thr Ile Tyr Tyr Ala Asp Ser Val Lys Gly Arg
    50                  55                  60

Phe Ile Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr Leu Gln Met
65                  70                  75                  80

Asn Asn Leu Arg Gly Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Asp
                85                  90                  95

Pro Arg Arg Trp Thr Gln Leu Trp Ile Pro Pro Asp Tyr Trp Gly Gln
            100                 105                 110

Gly Thr Leu Val Ser Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val
        115                 120                 125
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 132 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

Leu Leu Glu Ser Gly Gly Gly Gly Val Val Gln Pro Gly Gly Ser Leu
1 5 10 15

Arg Leu Ser Cys Glu Ala Ser Gly Phe Ser Leu Ile Asn Thr Ala Met
20 25 30

His Trp Val Arg Gln Ala Pro Xaa Lys Gly Pro Glu Trp Val Ser Val
35 40 45

Ser Ser Tyr Asp Gly Arg Glu Lys Tyr Tyr Thr Asp Ser Val Lys Gly
50 55 60

Arg Phe Ser Ile Ser Arg Asp Asp Ser Thr Asn Met Leu Tyr Leu Gln
65 70 75 80

Met Asn Ser Val Lys Ile Asp Asp Thr Ala Val Tyr Tyr Cys Ala Arg
85 90 95

Thr Asn Arg Ala Tyr Cys Ser Gly Val Arg Cys His Asp Gly Leu Asp
100 105 110

Val Trp Gly Gln Gly Thr Met Val Thr Val Ser Ser Ala Ser Thr Lys
115 120 125

Gly Pro Ser Val
130

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 133 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

Ala Glu Leu Gln Pro Pro Ser Val Ser Ala Ala Pro Gly Gln Lys Val
1 5 10 15

Ile Ile Ser Cys Ser Gly Ser Ser Ser His Thr Gly Gln Pro Lys Ala
20 25 30

Ala Pro Ser Val Thr Leu Phe Pro Pro Ser Ser Glu Glu Leu Gln Ala
35 40 45

Asn Lys Ala Thr Leu Val Cys Leu Ile Ser Asp Phe Tyr Pro Gly Ala
50 55 60

Val Thr Val Ala Trp Lys Ala Asp Ser Ser Pro Val Lys Ala Gly Val
65 70 75 80

Glu Thr Thr Thr Pro Ser Lys Gln Ser Asn Asn Lys Tyr Ala Xaa Ser
85 90 95

Ser Tyr Leu Ser Leu Thr Pro Glu Gln Trp Lys Ser His Lys Ser Tyr
100 105 110

Xaa Cys Gln Val Thr His Glu Gly Ser Thr Val Glu Lys Thr Val Xaa
115 120 125

Pro Thr Glu Cys Ser
130

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 111 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

Ala Glu Leu Gln Pro Pro Ser Val Ser Ala Ala Pro Gly Gln Lys Val
1 5 10 15

Thr Ile Ser Cys Ser Gly Ser Thr Ser Asn Ile Gly Asn Arg His Val
20 25 30

Ser Trp Tyr Gln Gln Leu Pro Gly Thr Xaa Pro Lys Leu Leu Ile Tyr
35 40 45

Glu Asn Asn Ile Arg Pro Ser Gly Ile Pro Asp Arg Phe Ser Ala Ser
50 55 60

Lys Ser Gly Thr Ser Ala Thr Leu Asp Ile Thr Gly Leu Gln Thr Gly
65 70 75 80

Asp Glu Ala Asp Tyr Tyr Cys Gly Thr Trp Asp Ser Ser Leu Ser Thr
85 90 95

Gly His Trp Val Phe Gly Gly Gly Thr Lys Leu Thr Val Leu Ser
100 105 110

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

Ala Glu Leu Gln Pro Pro Ser Val Ser Ala Ala Pro Gly Gln Ser Val
1 5 10 15

Thr Ile Ser Cys Ser Gly Ser Ser Ser Asn Ile Gly Asn Tyr Asn Val
20 25 30

Xaa Trp Tyr Gln Gln Thr Pro Gly Ser Ala Pro Lys Thr Leu Ile Tyr
35 40 45

Asp Thr His Lys Arg Pro Ser Gly Ile Ser Glu Arg Phe Ser Gly Ser
50 55 60

Lys Ser Gly Thr Ser Ala Thr Leu Gly Ile Thr Gly Leu Gln Thr Gly
65 70 75 80

Asp Glu Ala Asp Tyr Tyr Cys Gly Thr Trp Asp Thr Ser Leu Asn Ser
85 90 95

Ala Leu Phe Gly Gly Gly Thr Lys Leu Thr Xaa Leu Gly
100 105

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

Gly Glu Leu Gln Asp Pro Val Val Ser Val Ala Leu Gly Gln Thr Val
1 5 10 15

Arg Met Thr Cys Gln Gly Asp Ser Leu Arg Tyr His Tyr Ala Asn Trp
20 25 30

Tyr Gln Gln Lys Pro Gly Gln Ala Pro Ile Leu Val Ile Lys Ser Lys
35 40 45

Asn Asn Arg Pro Ser Gly Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser
50 55 60

Gly Asn Thr Ala Ser Leu Thr Ile Thr Gly Ala Gln Ala Glu Asp Glu
65 70 75 80

Ala Asp Tyr Tyr Cys Asp Ser Arg Asp Thr Ser Gly Asn His Pro Arg
85 90 95

Val Leu Phe Gly Gly Gly Thr Lys Leu Thr Val Leu Gly
100 105

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

Ala Glu Leu Gln Asp Pro Val Val Ser Val Ala Leu Gly Gln Thr Val
1 5 10 15

Arg Ile Thr Cys Gln Gly Asp Ser Leu Arg Xaa Tyr His Ala Asn Trp
20 25 30

Tyr Gln Gln Lys Pro Gly Lys Ala Pro Ile Phe Val Ile Tyr Gly Lys
35 40 45

Asn Asn Arg Pro Ser Gly Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser
50 55 60

Gly Asn Thr Ala Ser Leu Thr Ile Thr Gly Ala Gln Ala Glu Asp Glu
65 70 75 80

Ala Asp Tyr Tyr Cys Asn Ser Arg Asp Ser Ser Ser Asn His Val Val
85 90 95

Phe Gly Gly Gly Thr Lys Leu Thr Val Leu Gly
100 105

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 106 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

Gly Gln Pro Lys Ala Ala Pro Ser Val Thr Leu Phe Pro Pro Ser Ser
1 5 10 15

Glu Glu Leu Gln Ala Asn Lys Ala Thr Leu Val Cys Leu Ile Ser Asp
20 25 30

Phe Tyr Pro Gly Ala Val Thr Val Ala Trp Lys Ala Asp Ser Ser Pro
35 40 45

Val Lys Ala Gly Val Glu Thr Thr Thr Pro Ser Lys Gln Ser Asn Asn
50 55 60

Lys Tyr Ala Xaa Ser Ser Tyr Leu Ser Leu Thr Pro Glu Gln Trp Lys
65 70 75 80

Ser His Lys Ser Tyr Xaa Cys Gln Val Thr His Glu Gly Ser Thr Val
85 90 95

Glu Lys Thr Val Xaa Pro Thr Glu Cys Ser
100 105

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

Gln Pro Lys Xaa Ala Pro Ser Val Thr Leu Phe Pro Pro Ser Ser Glu
1 5 10 15

Glu Leu Gln Ala Asn Lys Xaa Thr Leu Val Cys Leu Ile Ser Asp Phe
20 25 30

Phe Pro Gly Xaa Xaa Xaa Val Xaa Trp Lys Xaa Asp Ser Xaa Pro Xaa
35 40 45

Lys Gly Gly Val Glu Thr Thr Xaa Pro Pro
50 55

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

Gln Pro Lys Xaa Ala Pro Ser Val Thr Leu Phe Pro Pro Ser Ser Glu
1 5 10 15

Glu Leu Gln Ala Asn Lys Xaa Thr Leu Val Cys Leu Ile Ser Asp Phe
20 25 30

Tyr Pro Gly Ala Xaa Thr Val Xaa Trp Lys Ala Asp Ser Ser Pro Val
35 40 45

Lys Ala Gly Val Glu Asn Thr Thr Pro Ser Ile Xaa Met Gln Gln Gln
50 55 60

Val Ser Gly Pro Gly Gly Ile
65 70

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 88 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

Gln Pro Lys Ala Ala Pro Ser Val Thr Leu Phe Pro Pro Ser Ser Glu
1 5 10 15

Glu Leu Gln Ala Asn Lys Ala Thr Leu Val Cys Leu Ile Ser Asp Phe
20 25 30

Tyr Pro Gly Ala Val Thr Val Ala Trp Lys Ala Asp Ser Ser Pro Val
35 40 45

Lys Gly Gly Val Glu Thr Thr Thr Pro Ser Asn Gln Ser Asn Asn Lys
50 55 60

Phe Ala Ala Ser Arg Tyr Leu Ser Leu Thr Pro Glu Gln Trp Lys Ser
65 70 75 80

His Arg Ser Tyr Ser Cys Gln Val
85

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 98 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

Gln Pro Lys Ala Ala Pro Ser Val Thr Leu Phe Pro Pro Ser Ser Glu
1 5 10 15

Glu Leu Gln Ala Asn Lys Ala Thr Leu Val Cys Leu Ile Ser Asp Phe
20 25 30

Tyr Pro Gly Ala Val Xaa Val Ala Trp Lys Ala Asp Ser Ser Pro Val
35 40 45

Lys Val Gly Val Xaa Xaa Thr Thr Pro Ser Xaa His Xaa Ile Asn Met
50 55 60

Phe Ala Gly Ser Xaa Tyr Leu Ser Leu Thr Pro Glu Gln Trp Xaa Ser
65 70 75 80

His Arg Lys Leu Gln Leu Pro Gly Gln Arg Arg Met Xaa Gly Ala Pro
85 90 95

Xaa Arg

What is claimed is:

1. In a method for conducting gene therapy wherein a recombinant gene is introduced into cells of a mammal, the improvement comprising using a recombinant gene encoding an antibody that is selectively specific for an intracellular antigen associated with a disease.

2. The improvement of claim 1 wherein the recombinant gene is free of a secretion sequence for said antibody.

3. The improvement of claim 1 wherein the recombinant gene encodes a single chain antibody.

4. The improvement of claim 1 wherein the recombinant gene encodes a single binding domain.

5. The improvement of claim 1 wherein the recombinant gene encodes a multiple binding domain.

6. The improvement of claim 1 wherein the recombinant gene includes an intracellular localization signal.

7. The improvement of claim 1 wherein the recombinant gene encodes an antibody that is selectively specific for an intracellular viral antigen.

8. The improvement of claim 1 wherein the recombinant gene encodes an antibody that is selectively specific for an intracellular antigen associated with the human immunodeficiency virus.

9. The improvement of claim 1 wherein the recombinant gene is part of an infectious agent that is replication-defective.

10. A method for preventing or halting the progress of a disease comprising administering to the subject a recombinant gene in an infectious vector, the gene encoding an antibody that is selectively specific for an intracellular antigen associated with the intracellular pathogen.

11. A method as claimed in claim 10 further characterized by administering a gene that is free of a secretion sequence for said antibody.

12. A method as claimed in claim 10 wherein the antibody is selectively specific for a viral antigen.

13. A method as claimed in claim 10 wherein the recombinant gene includes an intracellular localization signal.

14. A method as claimed in claim 10 wherein the infectious vector is replication-defective.

15. A method as claimed in claim 10 wherein the antibody is a single chain antibody.

16. A method as claimed in claim 10 wherein the antibody is a single chain antibody that is selectively specific for a human immunodeficiency virus antigen.

17. A method as claimed in claim 10 wherein the antibody comprises a single binding domain.

18. A method as claimed in claim 10 wherein the antibody comprises a multiple binding domain.

19. A method for preventing or halting the progression of a disease in a subject caused by an intracellular pathogen comprising introducing into cells ex vivo a recombinant gene encoding an antibody that is selectively specific for an antigen associated with the pathogen to form immunized cells, and introducing the immunized cells into the subject.

20. A method as claimed in claim 19 wherein the cells are isolated from the subject prior to forming the immunized cells.

21. A method for inhibiting replication of an intracellular pathogen in a cell, comprising causing to be introduced into the cell a recombinant gene encoding an antibody that is selectively specific for an intracellular antigen associated with the pathogen.

22. A method as claimed in claim 21 wherein the recombinant gene is part of an infectious agent and wherein the recombinant gene is introduced into the cell by contacting the cell with the infectious agent.

23. A method as claimed in claim 21 wherein the recombinant gene is free of a secretion sequence for said antibody.

24. A method as claimed in claim 21 wherein the recombinant gene encodes a single chain antibody.

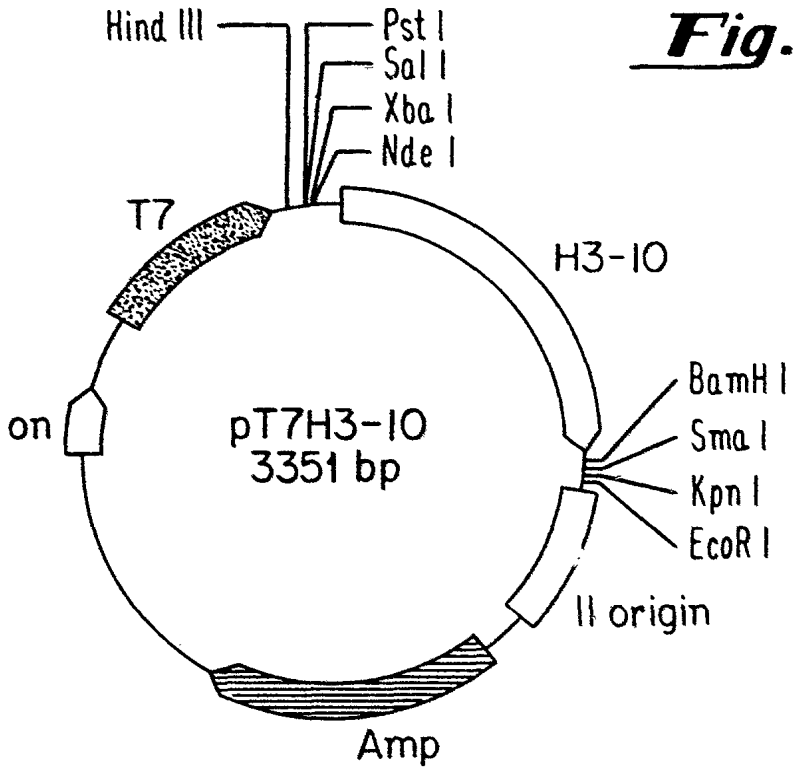
25. A method as claimed in claim 21 wherein the recombinant gene encodes a single binding domain.

26. A method as claimed in claim 21 wherein the recombinant gene encodes a multiple binding domain.

27. A method as claimed in claim 22 wherein the infectious agent is replication defective.

28. A method as claimed in claim 21 wherein the recombinant gene includes an intracellular localization sequence.

29. A method as claimed in claim 21 wherein the pathogen is a human immunodeficiency virus.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Xba I
Nde I
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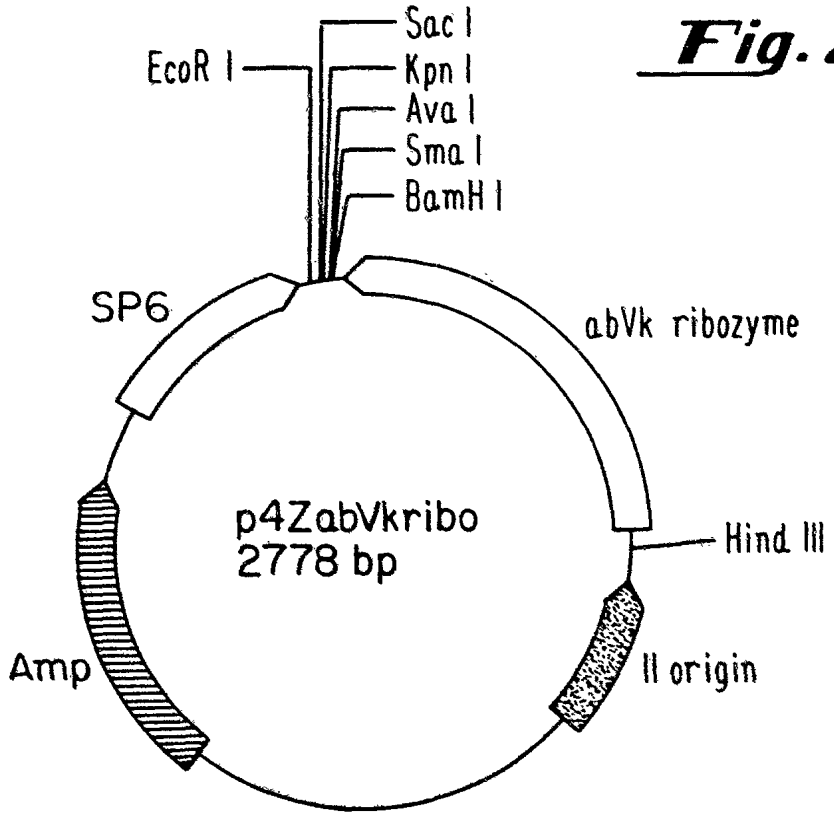
II origin
Amp

Fig. 1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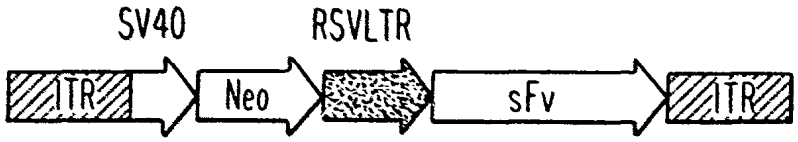
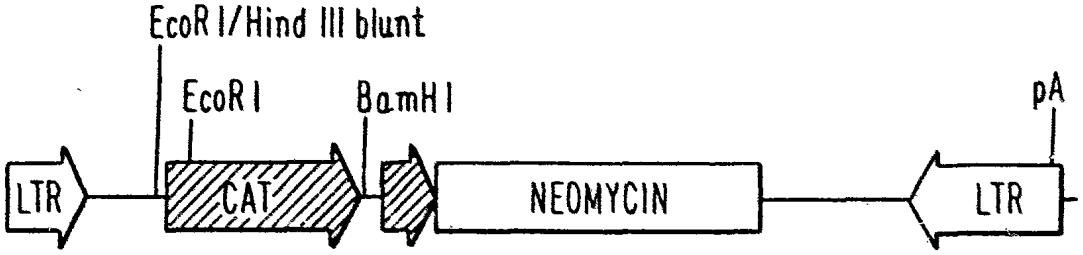
Amp
II origin

Fig. 2



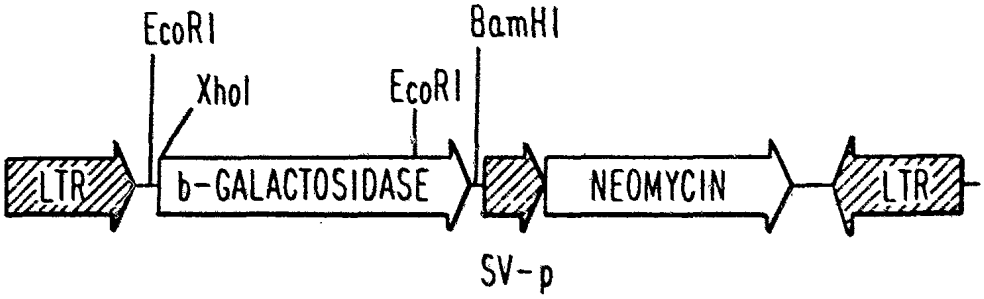
CONSTRUCT OF AAV-sFv

Fig. 3



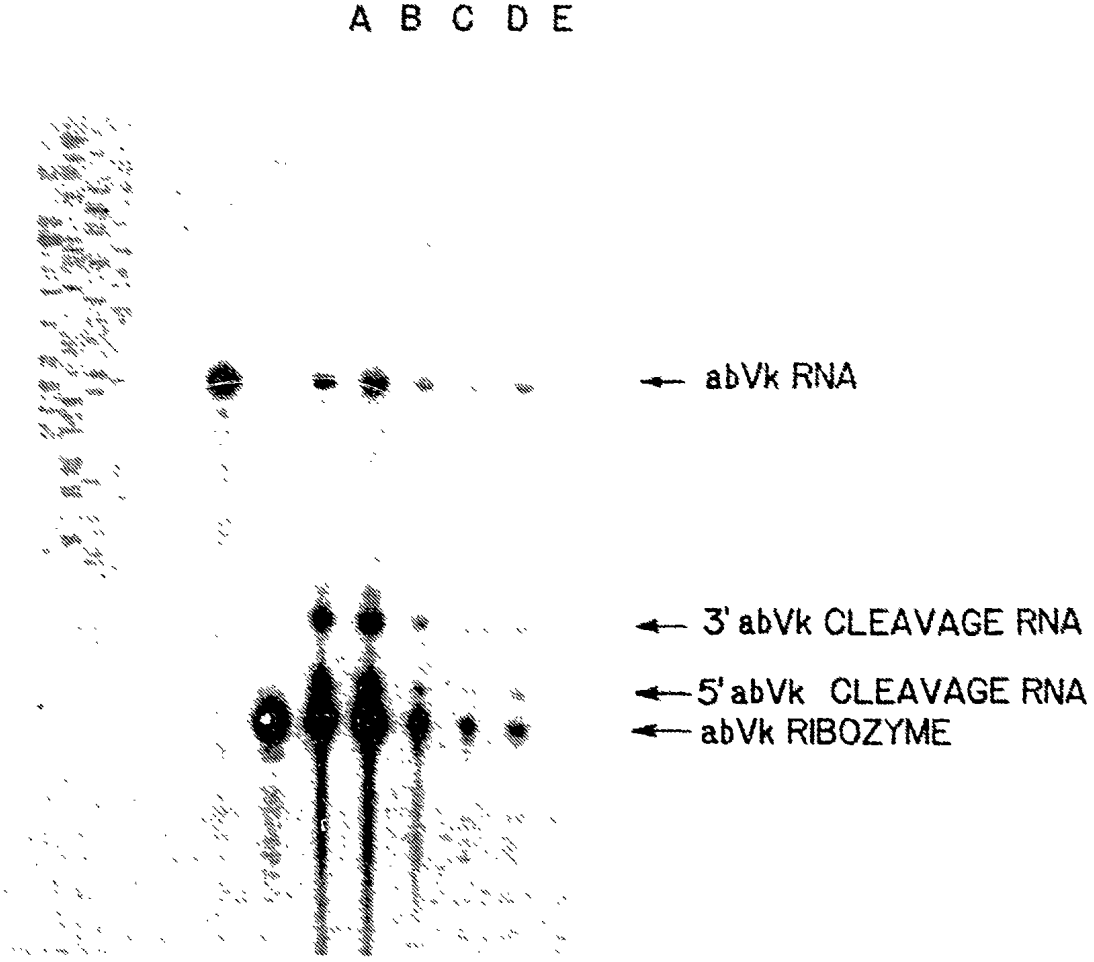
SV PROMOTOR

Fig. 5



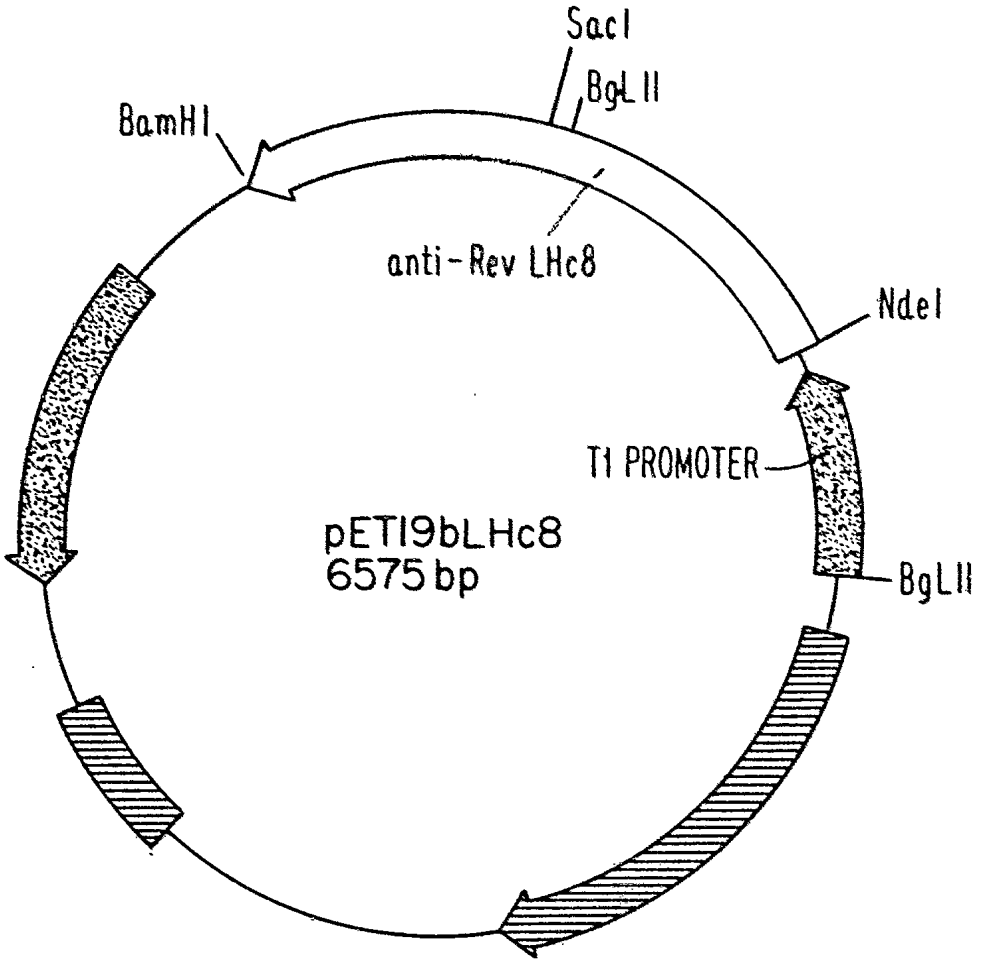
SV-p

Fig. 6



A: REACTION IN 5 x RT BUFFER
B: REACTION IN 4 x RT BUFFER
C: REACTION IN 3 x RT BUFFER
D: REACTION IN 2 x RT BUFFER
E: REACTION IN 1 x RT BUFFER

FIG. 4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anti-Rev LHc8
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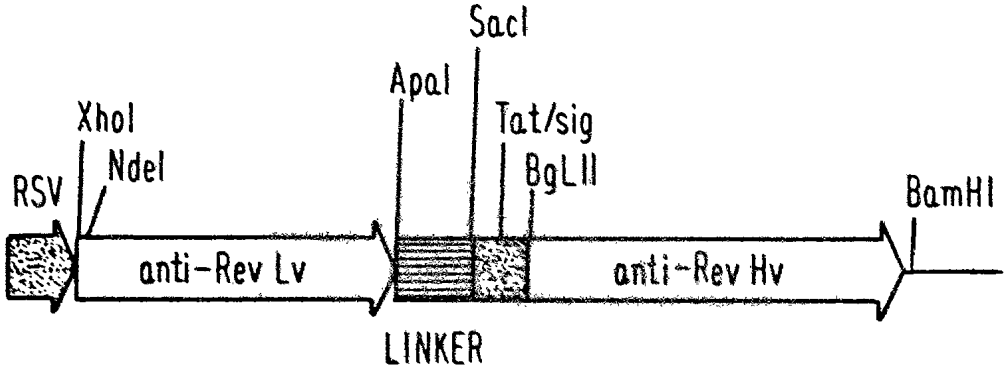
BglII

Fig. 7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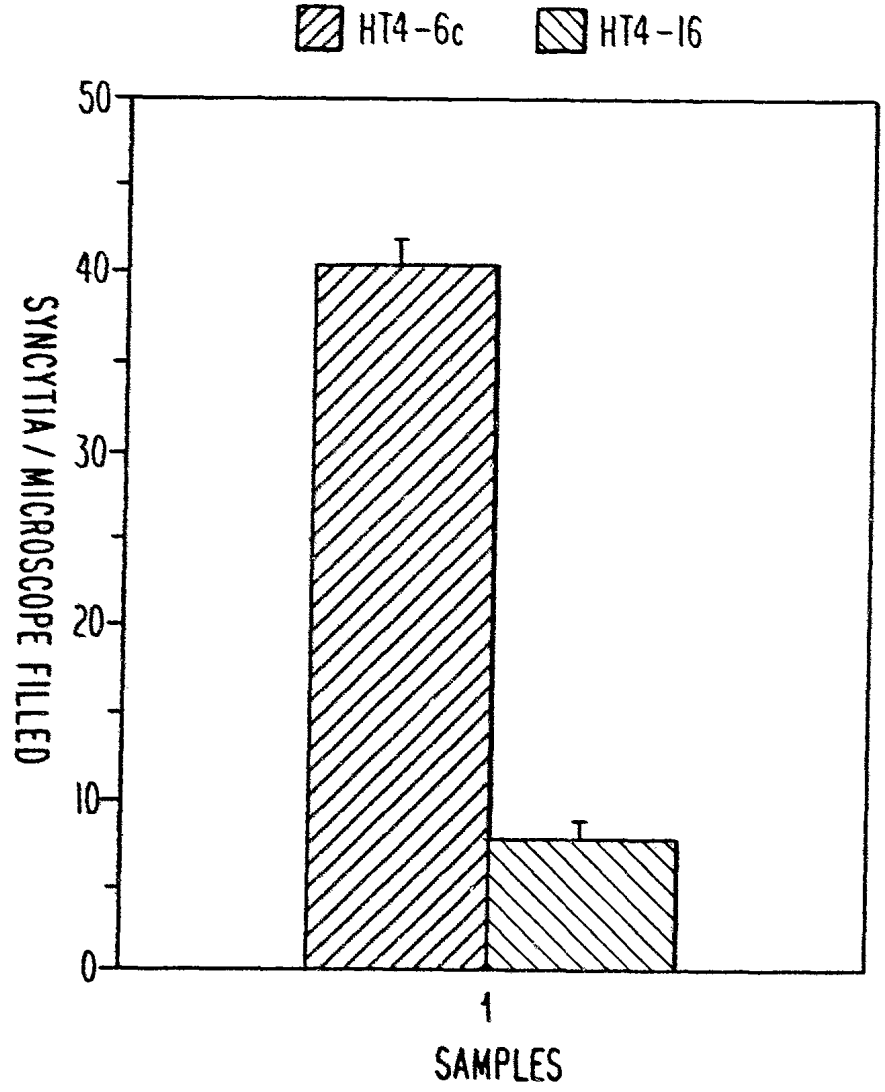
ApaI
XhoI
NdeI
Tat/sig
BglII
RSV
BamHI
anti-Rev Lv
anti-Rev Hv
LINKER

Fig. 8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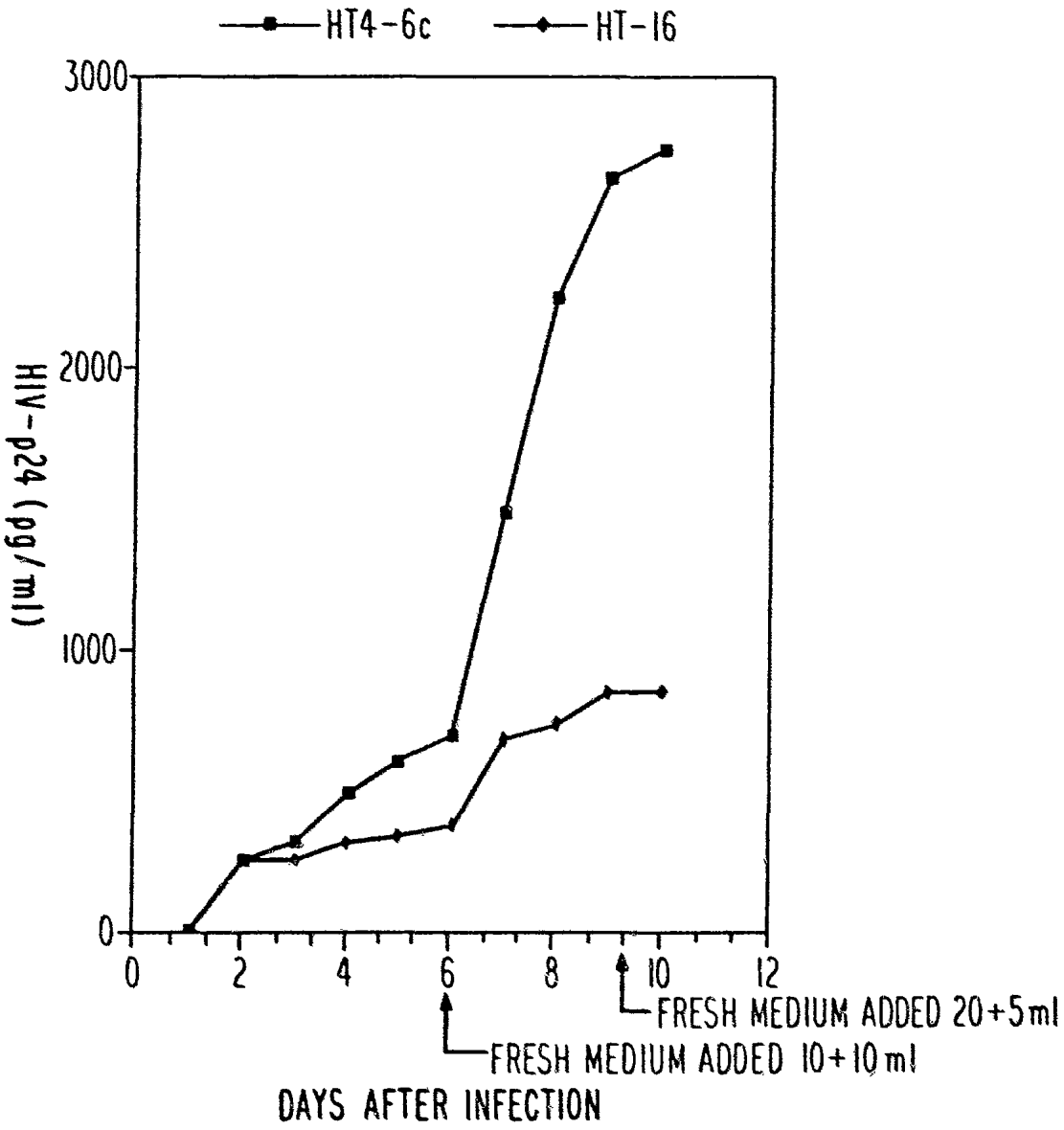
40
30
20
10
0
SYNCYTIA / MICROSCOPE FILLED
1
SAMPLES

Fig. 9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HT-16
3000
2000
1000
0
HIV-p24 (pg/ml)
0
2
4
6
8
10
12
FRESH MEDIUM ADDED 20+5ml
FRESH MEDIUM ADDED 10+10 ml
DAYS AFTER INFECTION

Fig. 10

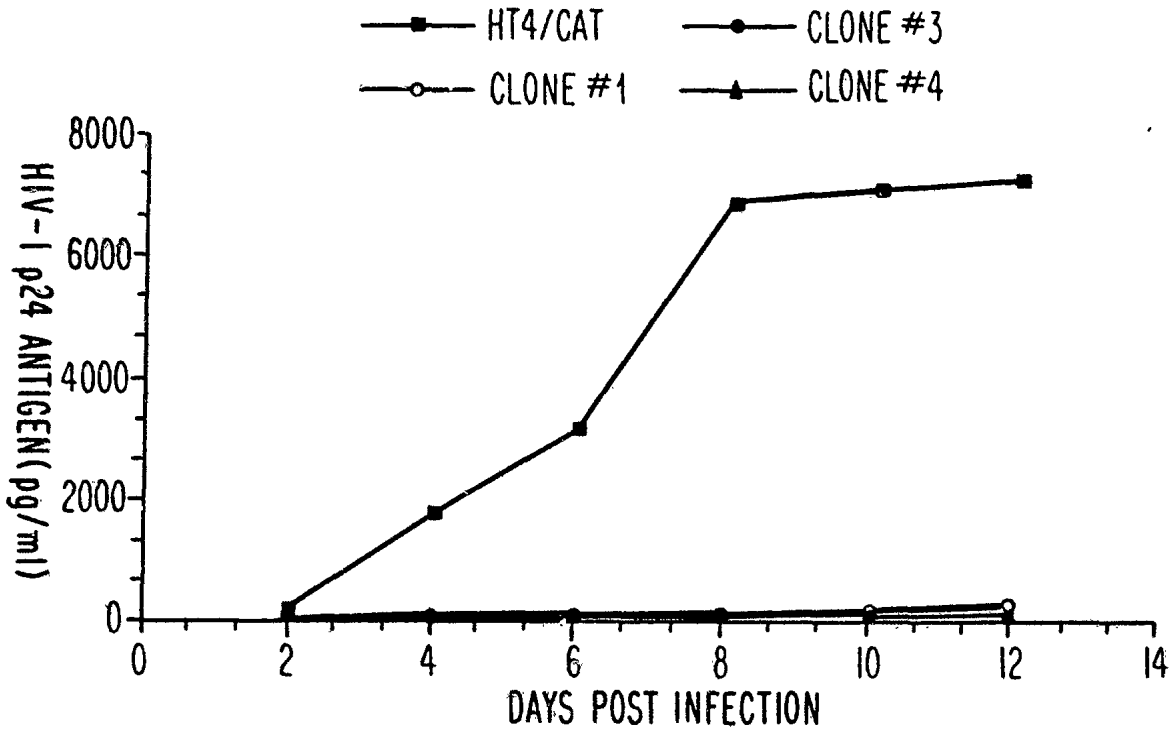


Fig. IIa

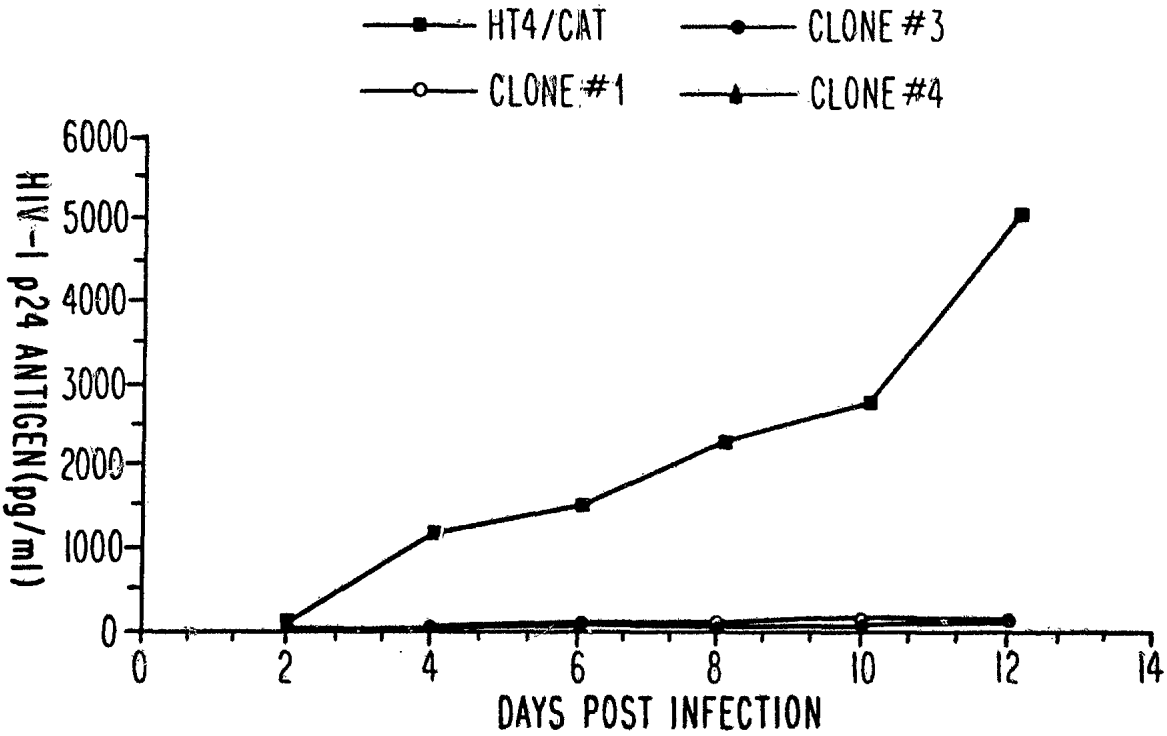


Fig. IIb

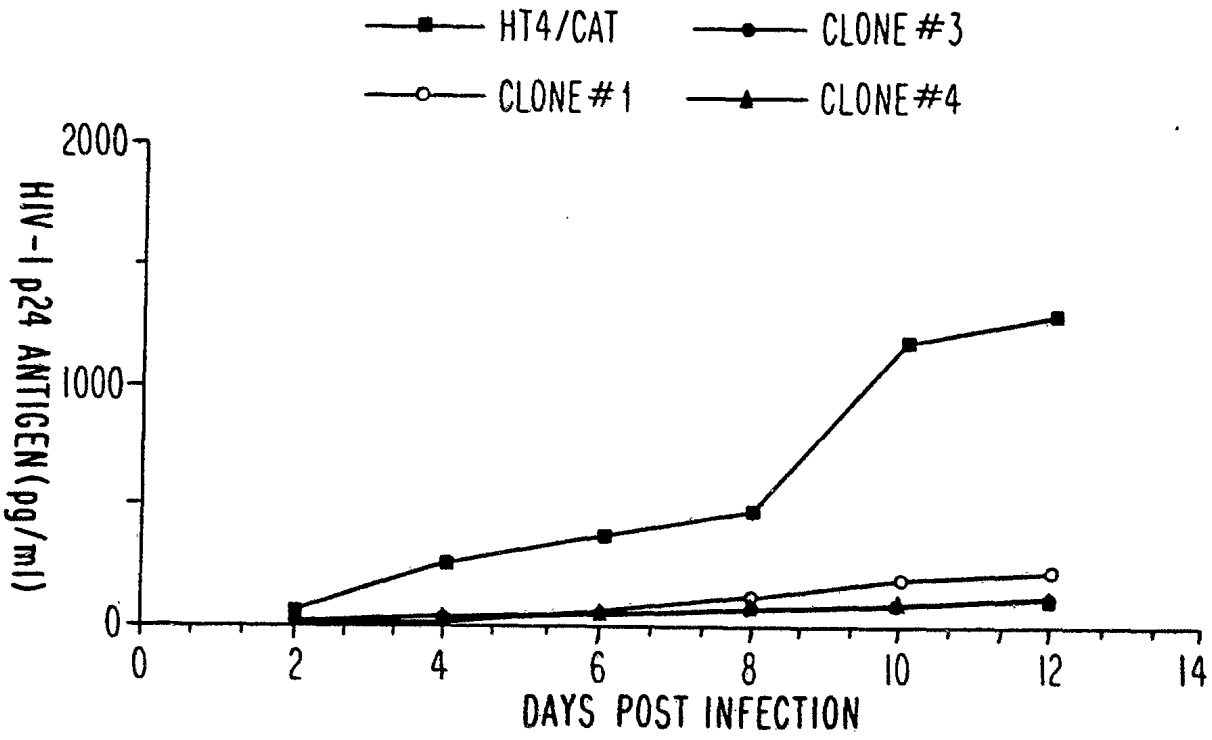


Fig. IIc

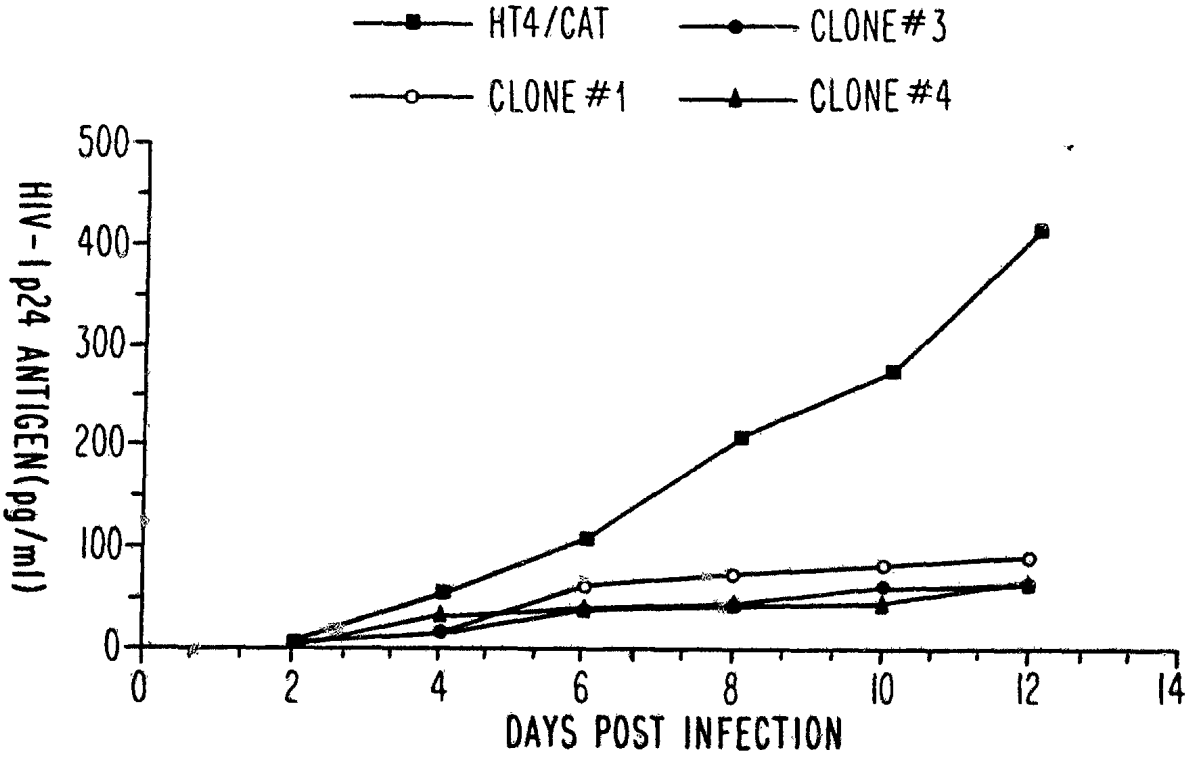


Fig. IId

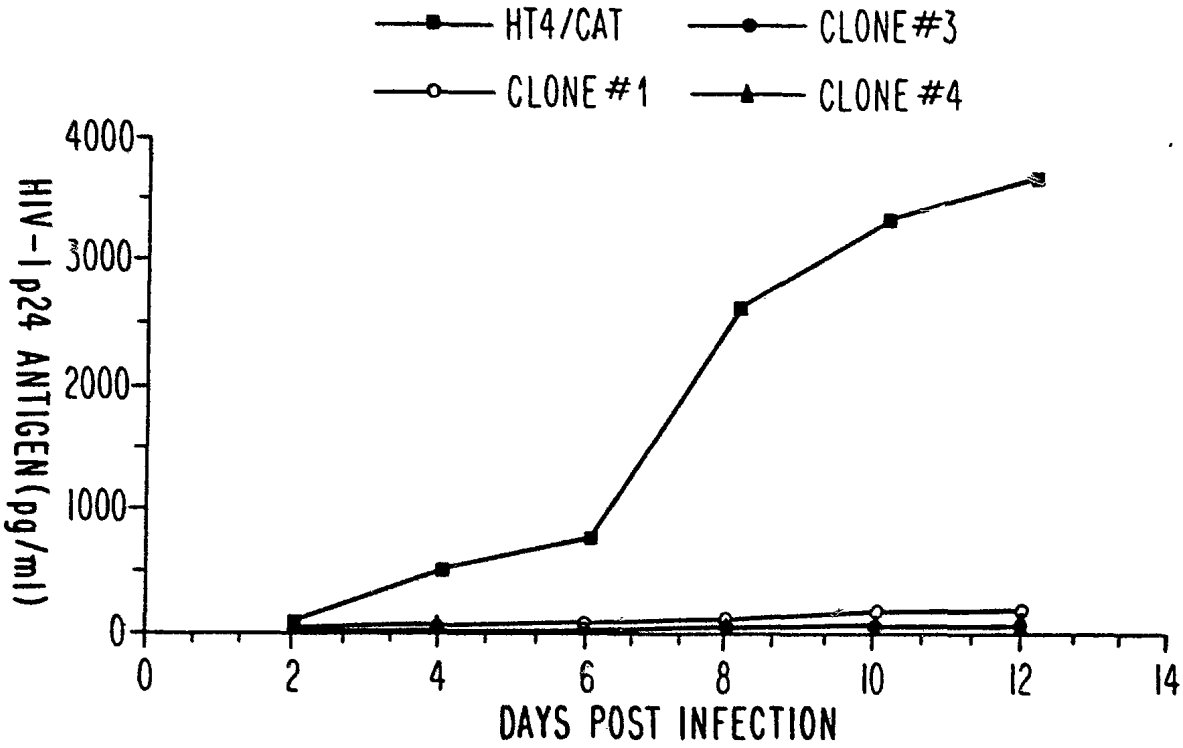


Fig. IIe

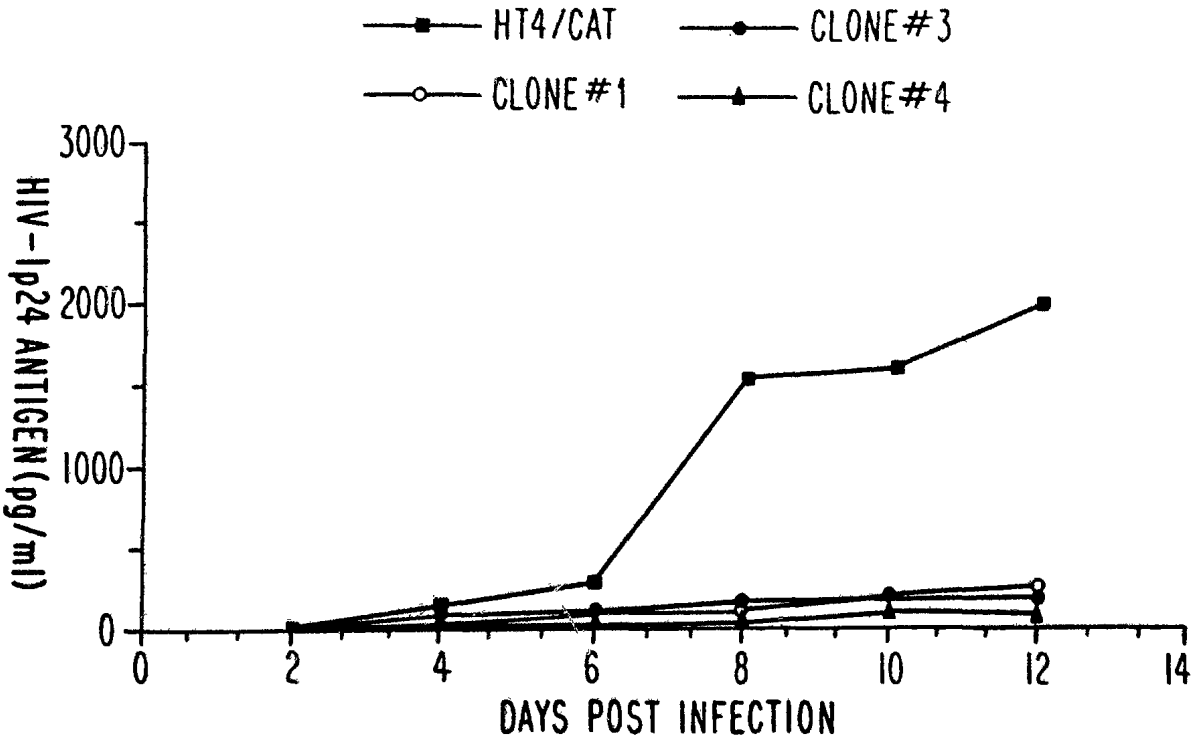
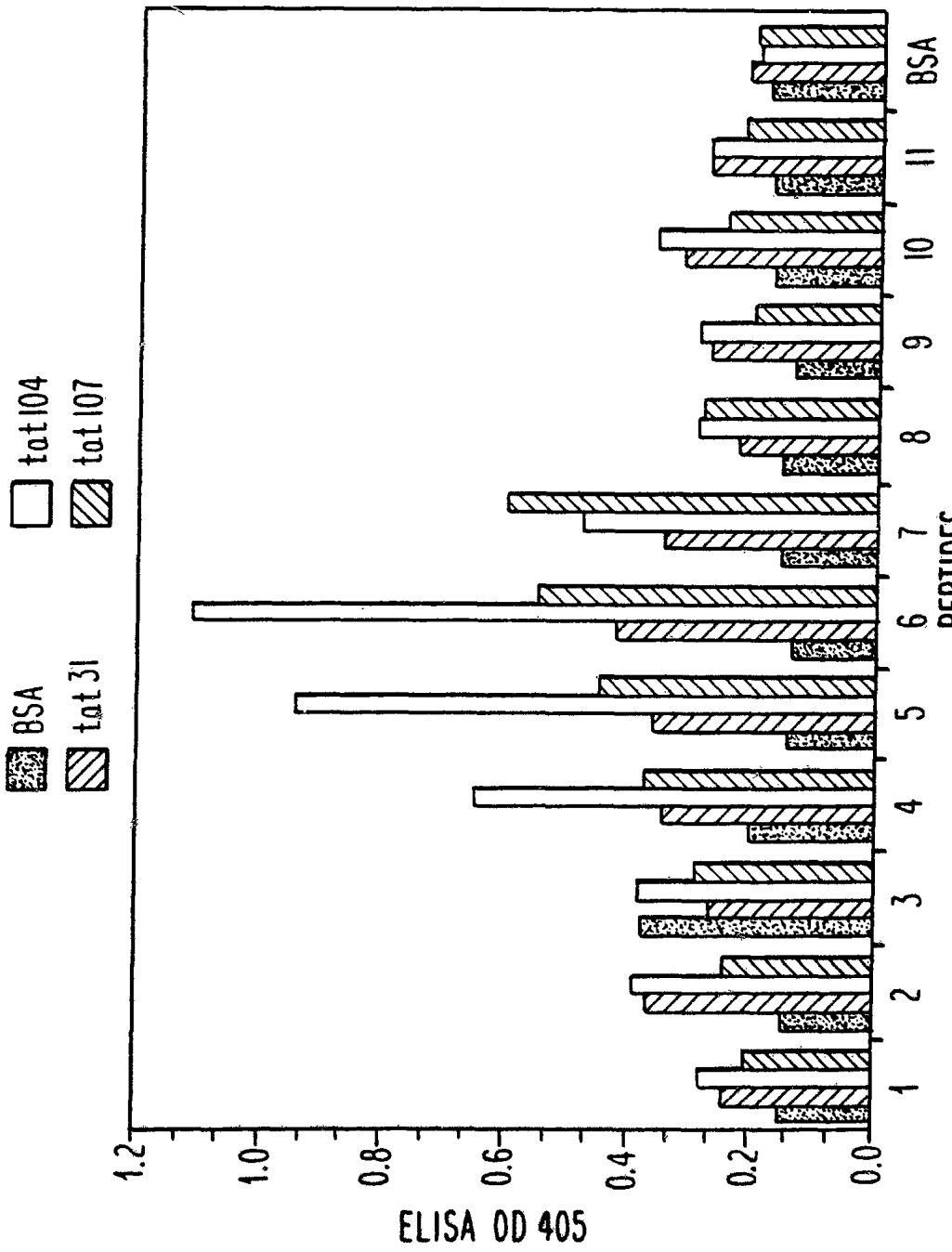


Fig. IIf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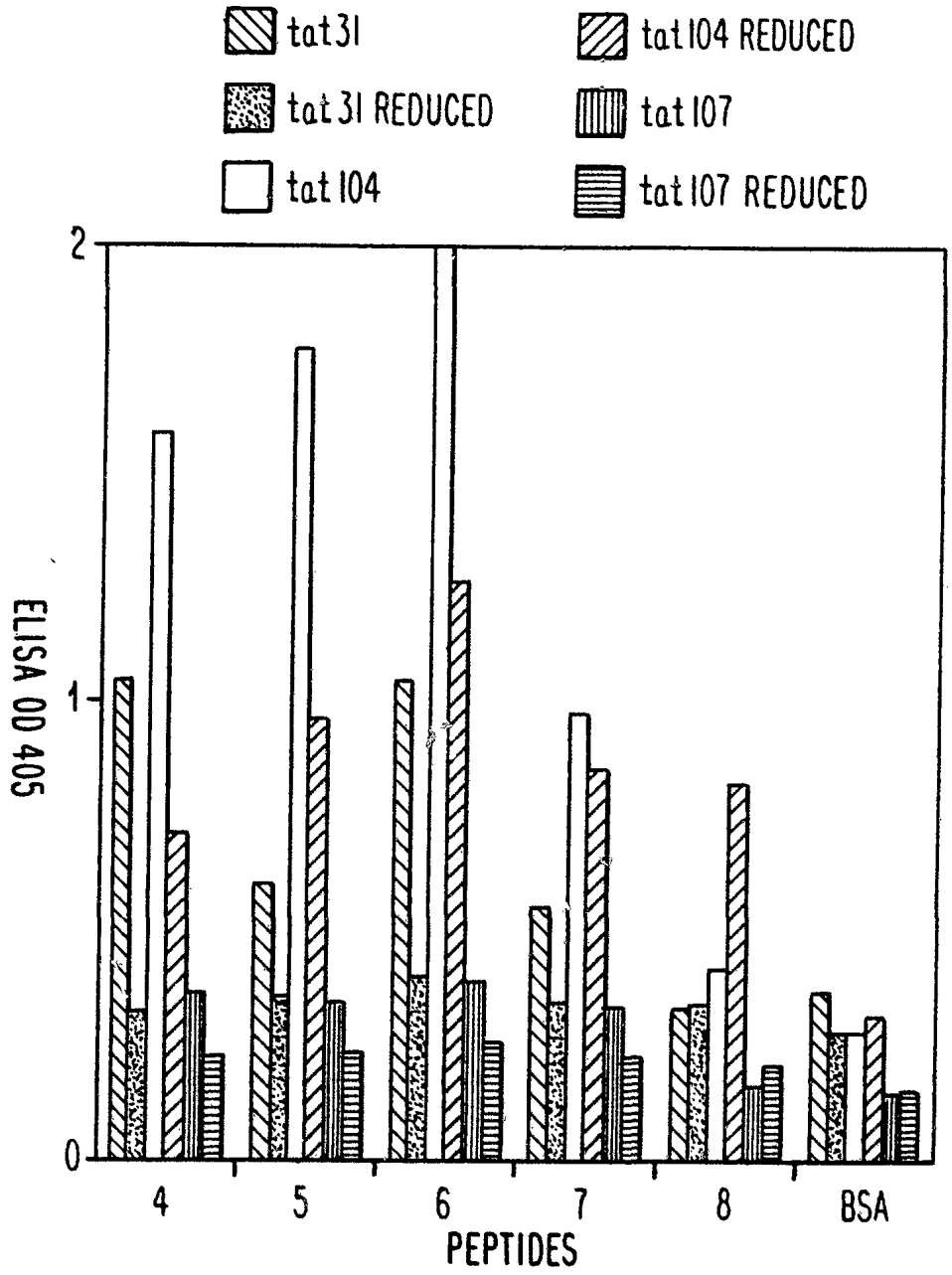
BSA
PEPTIDES

Fig. 12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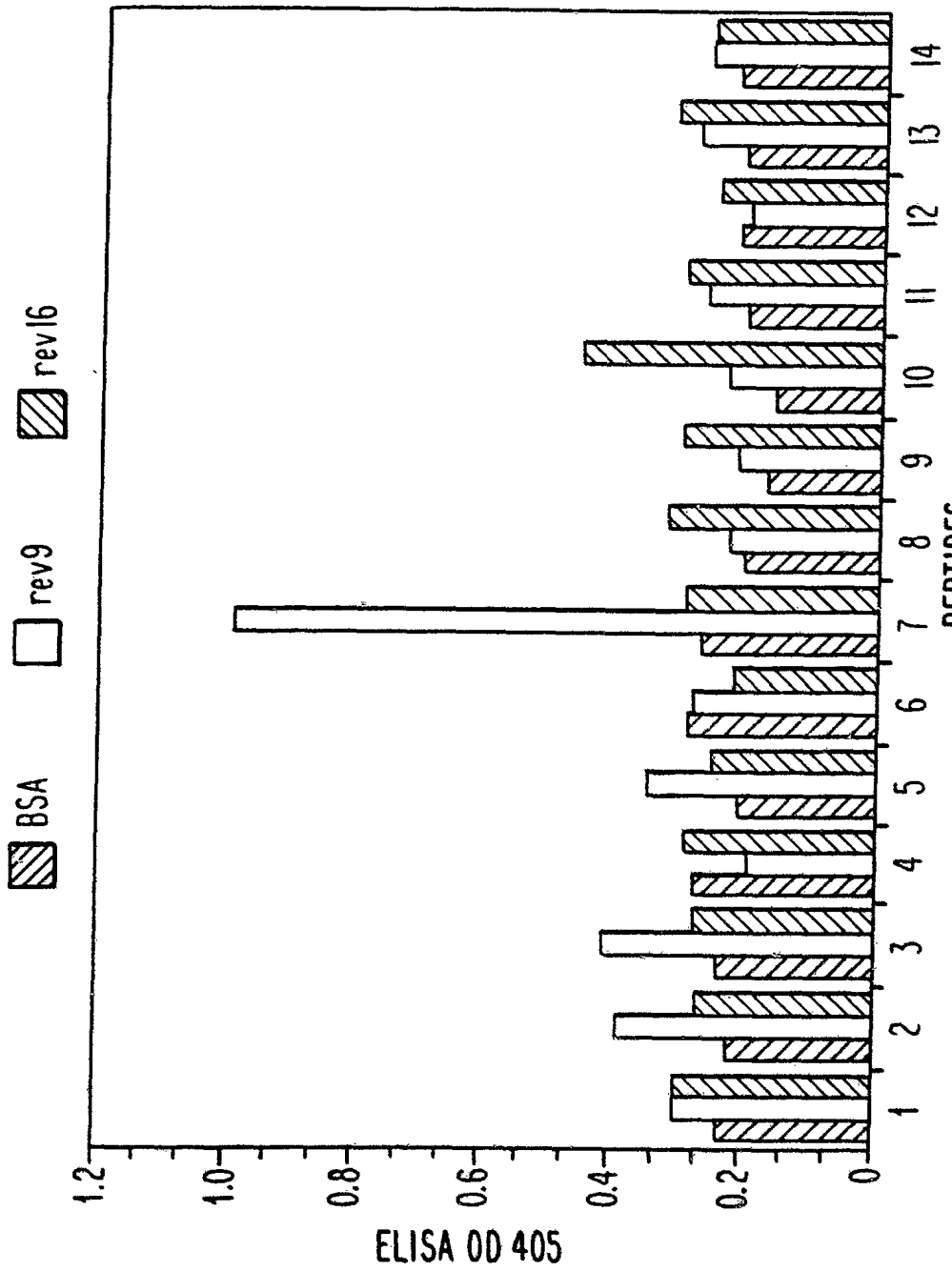
BSA
PEPTIDES

Fig. 13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13
14
PEPTIDES

Fig. 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/08448

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 48/00; C12N 5/10, 15/13, 15/85, 15/86
US CL :424/93.1, 93.2, 93.21; 514/44

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. : 424/93.1, 93.2, 93.21; 514/44

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)

DIALOG DATABASES: BIOSIS PREVIEWS, MEDLINE, AIDSLINE, WORLD PATENT INDEX, CA SEARCH; APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Medical Hypotheses, Volume 32, issued 1990, Faraji-Shadan et al., "A Putative Approach for Gene Therapy Against Human Immunodeficiency Virus (HIV)," pages 81-84, see entire article.	1-29
Y	Nature, Volume 335, issued 29 September 1988, Baltimore, "Gene Therapy: Intracellular Immunization," pages 395-396, see entire article.	1-29
Y	The EMBO Journal, Volume 9, Number 1, issued 1990, Biocca et al., "Expression and targeting of intracellular antibodies in mammalian cells," pages 101-108, see entire article.	1-29

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"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
"A"	document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier document published on or after the international filing date	"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
"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)	"&"	document member of the same patent family
"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		

Date of the actual completion of the international search 03 OCTOBER 1994	Date of mailing of the international search report 07 NOV 1994
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 JOHNNY F. RAILEY II, PH.D. Telephone No. (703) 308-0196

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