METHODS FOR INHIBITION OF HIV

Methods of inhibiting HIV-1 using chimeric gene constructs are disclosed. The methods include administering constructs containing a gene encoding the S1 subunit of pertussis toxin (S1 gene) operably linked to an HIV long terminal repeat (LTR) region.
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METHODS FOR INHIBITION OF HIV

FIELD OF THE INVENTION

The present invention relates to a genetic therapy method for the inhibition of human immunodeficiency virus (HIV).

REFERENCES


BACKGROUND OF THE INVENTION

Acquired Immune Deficiency Syndrome (AIDS) is caused by the human immunodeficiency virus (HIV). It is estimated that 1 to 1.5 million Americans are infected with the HIV type I virus (HIV-I). Of these, approximately 30% will progress to AIDS in 5 or 6 years. There have been approximately 360,000 AIDS patients, of whom approximately 250,000 have died. Health Care for AIDS patients in 1992 was estimated to have cost nearly 10 billion dollars. An effective therapy has not been identified to date.

A number AIDS-related symptoms and complications are correlated with the viral load imposed on the patient's immune system by HIV (Fauci; Weiss; Verhofstede, et al.). Therapeutics which are effective to reduce this viral load may therefore alleviate the severity of some pathological manifestations of HIV.

SUMMARY OF THE INVENTION

In one aspect, the present invention includes a method of inhibiting the production of infectious retroviral virions in a cell infected by the retrovirus. The method includes providing a retrovirus-infected cell containing a chimeric gene which contains a DNA sequence encoding the S1 subunit of pertussis toxin (S1 gene) operably linked to a retroviral long terminal repeat (LTR) region, and growing the cell. The growing is carried out under conditions where expression of the chimeric gene is induced. Expression of the S1 subunit by the chimeric gene in the cell inhibits the production of infectious retroviral virions by the cell. In one embodiment, the method inhibits the production of infectious Human
Immunodeficiency Virus (HIV) virions (e.g., HIV-1 virions), the cell is infected with HIV (e.g., HIV-1) and the LTR region is an HIV LTR region (e.g., an HIV-1 LTR region).

The DNA sequence encoding the S1 subunit may code for an amino acid sequence identical or homologous to any of sequences SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6, or fragments thereof. The chimeric gene may further include an element effective to reduce, inhibit or eliminate undesired expression of the S1 subunit of PT in cells not infected by retrovirus. One example of such an element is a DNA fragment containing a head-to-tail trimer (A-trimer) of an SV40 polyadenylation sequence. The element is preferably inserted upstream of the LTR region in the chimeric gene. The infected cell may be any cell susceptible to infection by HIV, such as a monocyte, macrophage or CD4+ T lymphocyte.

In another aspect, the present invention provides a chimeric gene, which includes a retroviral LTR region operably linked to a DNA sequence encoding the S1 subunit of pertussis toxin. In one embodiment, the LTR region is an HIV LTR region (e.g., an HIV-1 LTR region). The chimeric gene may further include an element effective to reduce, inhibit or eliminate undesired expression of the S1 subunit of PT in cells not infected by retrovirus, such as the A-trimer described above. The DNA sequence encoding the S1 subunit may code for an amino acid sequence identical or homologous to any of sequences SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6, or fragments thereof.

In a related aspect, the present invention includes a retroviral expression vector, containing a chimeric gene as described above.

In yet another aspect, the invention includes a method of reducing the HIV viral load in an HIV-infected subject. The method includes (i) isolating CD4+ lymphocytes from the subject, (ii) transforming the lymphocytes with a chimeric gene comprising an HIV LTR region operably linked to a DNA sequence encoding the S1 subunit of pertussis toxin (PT), and (iii) introducing lymphocytes carrying the chimeric gene into the subject. In the subject, the lymphocytes express the S1 subunit of PT, inhibiting production of infectious HIV virions. This inhibition reduces viral load in the HIV-infected subject, and inhibits the spread of infection in the individual.

In a related aspect, the present invention includes a method of reducing HIV-1 viral load in a subject harboring HIV-1-infected cells. The method includes administering to the subject, a retroviral expression vector containing a chimeric gene comprising an HIV-1 LTR region operably linked to a DNA sequence encoding the S1 subunit of pertussis toxin (PT), under conditions which promote transfection of the vector into said infected cells. Infected
cells carrying the vector express the S1 subunit, which inhibits HIV production and results in a reduced viral load in the HIV-1-infected subject.

The present invention also includes methods of treating HIV infections in HIV-infected subjects. The methods contain the steps outlined above in methods for reduction of viral load in HIV-infected subjects.

Also contemplated by the present invention are methods of treatment of an HIV infection and of reducing viral load in an HIV-infected subject which combine any of the methods summarized above with other anti-HIV therapies, such as treatment with AZT, HIV protease inhibitor and the like.

These and other objects and features of the invention will become more fully apparent when the following detailed description is read in conjunction with the accompanying drawings.

**BRIEF DESCRIPTION OF THE FIGURES**

Figure 1A shows an alignment of the amino acid sequences of HIV gp41 and G_{α} in the region defined by amino acids 589 through 595, and 12 through 17, of the respective proteins.

Figure 1B shows an alignment of the amino acid sequences of HIV gp41 and G_{α} in the region defined by amino acids 597 through 611, and 36 through 50, of the respective proteins.

Figure 2 shows a schematic diagram of an expression cassette containing an HIV-1 long terminal repeat (LTR), a sequence encoding the S-1 subunit of pertussis toxin (PT S1) and an SV40 polyadenylation signal.

**BRIEF DESCRIPTION OF THE SEQUENCES**

SEQ ID NO:1 is the nucleotide sequence of the *Bordetella pertussis* gene for toxin subunit S1 (EMBL accession number X16347).

SEQ ID NO:2 is the predicted amino acid sequence from SEQ ID NO:1.

SEQ ID NO:3 is the nucleotide sequence of the *B. pertussis* gene for toxin subunit S1 (GenBank accession number M13223).

SEQ ID NO:4 is the predicted amino acid sequence from SEQ ID NO:3.

SEQ ID NO:5 is the nucleotide sequence of the *B. pertussis* gene for toxin subunit S1 (EMBL accession number A13359).

SEQ ID NO:6 is the predicted amino acid sequence from SEQ ID NO:5.
**Detailed Description of the Invention**

I. **Definitions**

The term "operably linked", as used herein, denotes a relationship between a regulatory region (typically a promoter element, but may include an enhancer element) and the coding region of a gene, whereby the transcription of the coding region is under the control of the regulatory region.

"Chimeric gene" refers to a polynucleotide containing heterologous DNA sequences, such as promoter and enhancer elements from one source operably linked to a gene, encoding a desired gene product, from a second source. For example, a construct containing a human immunodeficiency virus 1 (HIV-1) long terminal repeat (LTR) region operably linked to a gene encoding the S1 subunit of *pertussis* toxin comprises an exemplary chimeric gene.

"Treating" a disease refers to administering a therapeutic substance effective to reduce the symptoms of the disease and/or lessen the severity of the disease.

"Treating" an infection, such as a viral infection, in an individual refers to administering a therapeutic substance effective to reduce the symptoms of the infection, reduce the viral burden, or reduce the pathogenicity of the infectious agent in the individual.

"Upstream" with respect to location along a polynucleotide refers to a location that is 5' to the reference location.

II. **Overview of Invention**

Methods and compositions of the present invention, useful in treating infections caused by the human immunodeficiency virus (HIV), employ chimeric gene constructs, such as retroviral expression vectors, containing the gene for the S-1 subunit of *pertussis* toxin (PT) under the control of the HIV long terminal repeat (LTR). The HIV LTR region serves to initiate transcription of downstream genes in response to the transactivating protein (Tat), which is produced by HIV upon infection of a host cell. Accordingly, the infection of a cell harboring an HIV LTR/S1 PT construct by HIV results in the production of the S1 subunit of PT.

The S1 subunit may have several effects in the HIV-infected host cell, depending on its level of expression. High levels of expression of S1 result in death and lysis of the host cell. The effects are typically mediated through ADP (adenosine diphosphate)-ribosylation of a target protein. One such target is Gαs, an endogenous inhibitory mammalian guanine nucleotide binding protein (G-protein).
Because the S-1 PT recognition site on Gα is similar to a region of HIV gp41, the S1 subunit of PT may also ADP ribosylate HIV gp41. HIV having ADP ribosylated gp41 has a markedly reduced infectivity and cytopathogenicity. The combined action of killing virus-infected cells, and inhibition of existing virions by ADP ribosylation of HIV gp41, is effective to inhibit HIV and reduce viral burden in infected individuals.

III. Human Immunodeficiency Virus

HIV belongs to a large, diverse family of viruses known as "retroviruses". It has been identified in and isolated from the CD4+ lymphocytes (T-4 lymphocytes) of AIDS patients. The envelope protein of HIV-1 is composed of a glycoprotein (gp160) that serves to attach the virus to CD4 receptors on the host cells, as well as facilitating the fusion of the virus with the host cell.

The HIV gp160 glycoprotein is cleaved between the arginine at position 518 (arg518) and the alanine at position 519 (ala519) of the env gene product to produce an N-terminal gp120 glycoprotein and a smaller transmembrane C-terminal gp41 glycoprotein. The HIV env gp1 glycoprotein consists of 345 amino acids (from ala519 to the leucine at position 863 (leu863)), and has six potential glycosylation sites (Bergeron, et al., 1992). The cleavage of the gp160 glycoprotein into the gp120 and the gp41 glycoproteins is essential for viral infectivity (Lee, et al., 1992). Based on a number of studies, it is now believed that the transmembrane protein HIV gp41 is the mediator of viral fusion (Yamada, et al., 1991; Kure, et al., 1990; Tas, et al., 1988; Orloff, et al., 1991; Robinson, Jr., et al., 1991; Robinson, Jr., et al., 1990; Tyler, et al., 1990; Nardi, et al., 1989; Ruegg, et al., 1989; Eisenberg and Wesson, 1990; Narvanen, et al., 1988a; Bell, et al., 1992).

IV. G Proteins

The G proteins are heterotrimers, with subunits designated α, β and γ in order of decreasing mass. The α subunits clearly differ among the members of the G protein family and serve to define the individual G protein. Common β and γ subunits are probably shared among some α subunits to form the specific oligomers.

G-protein mediated transmembrane signaling pathways involve the interaction of membrane receptor proteins, G proteins and effector proteins. The receptor proteins typically respond to extracellular stimuli, such as biogenic amines, proteins, polypeptide hormones, autacoids and/or neurotransmitters (Stryer, 1988; Linder and Gilman, 1992).
The G proteins couple the activation state of a receptor to the activation or inactivation of an effector protein.

G proteins are regulated cyclically by association of (guanosine triphosphate) GTP with the α subunit, hydrolysis of GTP to guanine diphosphate (GDP) and phosphate (P), and dissociation of GDP from the G protein. The binding of GTP typically activates the G protein, resulting in corresponding regulation of the activity of the appropriate effector protein (Stryer, 1988; Linder and Gilman, 1992).

Hydrolysis of GTP to GDP initiates deactivation of the G protein. The dissociation of GDP from the G protein is apparently the rate limiting step of this process, and dissociation of GDP is accelerated by interaction between the G protein and the receptor protein. Considerable evidence exists that a cycle of dissociation and association of G protein subunits is superimposed on this regulatory GTPase cycle.

There exist several distinct families of α subunits, one of which, termed Gα, inhibits adenylate cyclase and stimulates certain potassium channels. The Gα oligomers are composed of approximately 354 amino acids and have a calculated molecular weight of about 40,400.

V. Pertussis Toxin

Bordetella pertussis produces a substance which activates an insulin-secreting response in mammals (Sumi and Ui, 1975). A protein secreted by B. pertussis was purified and termed Islet-Activating Protein (IAP; Yajima, et al., 1978a; Yajima, et al., 1978b). Previous studies have shown that B. pertussis produces adjuvant active substances including LPF (lymphocytosis promoting factor), HSF (histamine sensitizing factor), ADP-ribosyltransferase and NAD-glycohydrolase (Ui, 1986). These activities are now attributed to IAP, and it is generally acknowledged that IAP is the B. pertussis toxin, or pertussis toxin (Ui, 1986).

IAP is a 77-kD multimeric protein comprised of six subunits which associate through non-covalent interactions (Tamura, et al., 1982). The bioactivity of IAP activity resides on the largest subunit, which has been designated the A (active) protomer, or S1 subunit (Katada, et al., 1983). The remaining 5 subunits, which bind specifically to the cell membrane and deliver the A protomer into the cell, have been collectively designated the B (binding) oligomer (Tamura, et al., 1983).

The S1 subunit is an enzyme which acts as an ADP-ribosyltransferase, transferring the ADP-ribose moiety of NAD to the cysteine residual groups of proteins (Katada and Ui,
1982a; Katada and Ui, 1982b). As is described in more detail below, one substrate (target protein) for this enzyme is the 41-kD guanine nucleotide binding protein G\(\alpha\) discussed above (Katada and Ui, 1982a; Katada and Ui, 1982b; Bokoch, et al., 1983; Hsia, et al., 1983; Ohta, et al., 1990; Center, et al., 1989), which is selectively ADP-ribosylated by IAP (Katada and Ui, 1982a; Katada and Ui, 1982b; Bokoch, et al., 1983; Hsia, et al., 1983; Ohta, et al., 1990; Center, et al., 1989). IAP has also been shown to ADP ribosylate another type of G protein, G\(\alpha\), which is thought to be involved in the regulation of neuronal potassium and calcium channels, as well as certain types of phospholipase C activation.

It is recognized that the active subunits of at least two other toxins, cholera toxin and E. coli heat labile toxin, possesses ADP-ribosyltransferase activity, and accordingly, may be used in place of the S1 subunit of pertussis toxin in the practice of the present invention.

VI. The Action of Pertussis Toxin on G\(\alpha\) and gp41


Amino acid sequence alignments performed in support of the present invention and shown in Figures 1A and 1B reveal homologies between a region of the HIV env gp41 glycoprotein (the GTPase region) and the human 41kd G\(\alpha\) protein. The HIV env gp41 glycoprotein is similar to G\(\alpha\), but lacks two of the three required phosphorylation sites in a GTP-binding hydrophobic pocket (Eisenberg and Wesson, 1990; Narvanen, et al., 1988a; Bell, et al., 1992; Narvanen, et al., 1988b). The HIV env gp41 retains human G\(\alpha\) serine-44, deletes G\(\alpha\) serine-47 and mutates threonine-48 to leucine, a non phosphorylatable amino acid residue. However, the similarity of the HIV gp41 GTP-hydrophobic pocket to
that of \( \Gamma_{\alpha} \) renders HIV gp41 susceptible to ADP ribosylation by pertussis toxin, which disrupts viral production.

The specificity of the S1 subunit used in methods and compositions of the present invention may be altered by site-directed mutagenesis, such that, for example, a modified S1 subunit exhibits a substrate preference for HIV gp41 over \( \Gamma_{\alpha} \). In this regard, according to the teachings of the present invention, a single S1 subunit amino acid substitution of lysine (K) for arginine (R) at the 57 position (i.e., K-57 for R-57) can result in specific ADP-ribosylation of cysteine (C-253) on HIV env gp41 without concurrent ADP-ribosylation of \( \Gamma_{\alpha} \) and \( \Gamma_{\alpha}\). Accordingly, vectors employing a construct encoding such a modified S1 subunit may be particularly useful for inhibiting HIV replication in infected cells with a lower overall toxicity than wild-type S1.

Due to the relatively high degree of homology between HIV gp41, and the env gp41 of other retroviruses, methods of the present invention may be applied to the inhibition of these other retroviruses as well. The LTR region incorporated into therapeutic constructs directed against a particular virus preferably corresponds to a portion of the LTR region of that virus. Accordingly, such constructs preferably include PT S1 operably linked to the appropriate LTR region. Effective constructs express PT S1 in cells transformed with the constructs and infected by the selected retrovirus.

**VII. Therapeutic Constructs**

According to the present invention, vector constructs containing HIV-1 long terminal repeat (LTR) control elements and a gene encoding the S-1 subunit of *pertussis* toxin (PT) may be used to inhibit the production of infectious HIV in target cells carrying the vector and expressing the S1 subunit.

The HIV LTR has been demonstrated to markedly enhance viral expression in cells already infected with the HIV virus. This enhanced expression is stimulated by trans-acting regulatory factors which act on elements in the long terminal repeat (LTR) sequence of HIV. Heterologous genes placed under control of the HIV LTR sequences are therefore preferentially expressed in HIV-infected cells.

1989) and post-transcriptional levels (Cullen, 1986; Feinberg, et al., 1986; Wright, et al., 1986; Braddock, et al., 1989; Edery, et al., 1989) and can stimulate expression of heterologous genes placed 3' to the TAR region (Tong-Starken, et al., 1987; Felber and Pavlaskis, 1988).

The construction of specific retroviral vectors (pLTR-S1PT1, pLTR-S1PT2 and pLTR-S1PT3) containing the HIV-LTR and pertussis toxin S-1 sequences is described in Example 1. Vectors pLTR-S1PT1 and pLTR-S1PT2 are plasmid constructs, which may be used to transfect mammalian cells, such as peripheral blood mononuclear cells (PBMCs), including monocytes, macrophages and CD4+ T-cells, as well as other cells susceptible to infection by HIV. Transfected cells may then be selected for clones that have stably incorporated the constructs. The plasmid constructs may be transfected into cells using any of several methods known to those skilled in the art, including, for example, calcium phosphate precipitation or electroporation as described in Example 1.

pLTR-S1PT3 is a retroviral expression vector that may be used to transfect proliferating PBMCs, such as CD4+ T-cells, either in vitro or in vivo. Retroviral vectors typically have a very high efficiency of transfection, with certain vectors being capable of stably transducing close to 100% of the target cells. Target cells are transfected with virions containing pLTR-S1PT3 by co-incubating the cells with viral stocks produced as described in Example 1, or by co-cultivation with the appropriate packaging cell line containing the desired vector.

The constructs described in Example 1 have an HIV-1 long terminal repeat (HIV-1 LTR) region placed upstream of a DNA fragment containing the coding sequence for pertussis toxin (PT) S1 subunit. Other elements may be introduced into the constructs to improve the efficiency and/or selectivity of expression. For example, a fragment containing the SV40 small t intron and poly(A) signals is typically inserted downstream of the PT-S1 DNA. The constructs may also contain an element or elements designed to reduce or prevent spurious or undesired expression of the PT-S1 gene in the absence of activation of the HIV-1 LTR by the Tat protein. One example of such an element is the A-trimer (Maxwell, et al., 1989), which is inserted upstream of the LTR in pLTR-S1PT2. The A-trimer is a head-to-tail trimer of an SV40 BclI/BamHI DNA fragment specifying polyadenylation of RNA transcripts. It has been shown to prevent spurious expression of chimeric genes resulting from transcriptional initiation in prokaryotic plasmid sequences in transfected mammalian cells (Maxwell, et al., 1989).
Additional elements, such as ones that modify the expression strength of the HIV LTR, may also be introduced. Such elements may be useful for modulating the level of PT S1 expression in HIV-infected cells.

5 VIII. *In Vitro Assays*

The anti-HIV efficacy of vector constructs produced in accordance with the present invention may be assessed using any of several *in vitro* HIV assays known to those skilled in the art. In such assays, one sample of cells is typically transfected with a vector construct containing a region of the HIV LTR operably linked to a gene encoding the S-1 subunit of *pertussis* toxin, and another sample of cells is transfected with a mock construct or left untransfected. The cells are then infected with HIV, and the amount of HIV contained in the samples following a suitable incubation period is assayed using an HIV assay. Effective constructs are those that reduce the amount of HIV in samples of cells transfected with those constructs.

10 Two exemplary HIV assays (measurement of HIV p24 protein and syncytium formation) are described herein. Example 2 describes experiments designed to measure the amount of HIV using a p24 antigen assay. Cells transfected with pLTR-S1PT vectors are infected with HIV-1 by co-cultivation with an equal number of virus-infected cells or by incubation with HIV-1 viral stocks. The cells are then assayed for the expression of the HIV-1 p24 antigen using the HIV-1 p24 Antigen Quantitation Panel from Abbott Laboratories (North Chicago, IL).

Example 3 describes a syncytium formation assay. Infection of a monolayer of cells by a syncytium-inducing (SI) isolate of HIV typically results in the formation of syncytia (cells with five or more nuclei). Substances which inhibit viral infection and/or replication also typically inhibit the formation of syncytia. Accordingly, the inhibition of syncytia formation can be used as an assay for compounds effective to inhibit HIV. In Example 3, control and pLTR-S1PT-transfected CD4+ cells are infected with HIV-1 as described in Example 1. The cultures are then fixed and examined microscopically for syncytia formation (cells with five or more nuclei).

30 Other methods known to those skilled in the art may be used to assess the level of HIV expression in the cells. For example, quantitative or semi-quantitative PCR (Mullis; Mullis, *et al.*.) may be used to assess the relative amounts of proviral HIV DNA or viral HIV RNA in a sample (Piatak, *et al.*, Verhofstede, *et al.*, Loussert-Ajaka, *et al.*). The proviral HIV DNA level in PBMCs has been found to be highly correlated with the viral load in the
plasma of HIV-infected individuals (Verhofstede, et al.), and a measure of the proviral HIV DNA may therefore be used as a measure of the viral load.

IX. In Vivo Assays

The efficacy of constructs of the present invention to inhibit HIV replication may also be assayed in suitable in vivo models of HIV infection, such as the SCID-Hu mice model (Namikawa, et al., 1988; McCune, et al., 1991; Mosier, et al., 1993; Aldrovandi, et al., 1993) described in Example 4. In this model, mice homozygous for the severe combined immunodeficiency defect (SCID; Bosma, et al., 1983) are transplanted with human fetal thymus and liver (Thy/Liv). The transplantation results in the development of a co-joint human organ which allows normal maturation of human thymocytes.

Five to six months post-implantation, the implants are infected with HIV, and the effects of therapies designed to inhibit HIV may be assayed, for example, by analyzing thymocyte subset distributions using flow cytometry. Example 4 describes such an analysis using anti-CD4 and anti-CD8 monoclonal antibodies to determine the percentage of CD4/CD8 double positive, CD4+/CD8- and CD4-/CD8+ single positive, and CD4/CD8 double negative cells. Untreated animals infected with HIV typically show significant reductions of CD4+ cells relative to uninfected (control) animals. Infected animals treated with an effective therapy, such as a vector construct of the present invention, have CD4+ cell populations more similar to those of the control animals than those of untreated infected animals.

X. Clinical Results Using Pertussis Toxin

Various forms of pertussis toxin have been utilized in clinical settings for nearly 50 years, beginning with vaccines against pertussis (whooping cough) employing inactivated forms of the bacterium Bordetella pertussis. The success of the early vaccines was due, in large part, to the antigenic nature of PT, which was later shown to confer protection against pertussis (Anderson, et al.; Sato, et al.). The toxicity of the pertussis toxin molecule, however, posed some difficulties, and post-synthesis processes designed to inactivate the toxin often significantly reduced its immunogenicity (Linggood, et al.). Recent studies of recombinant PT S1 mutants with reduced cytopathogenicity and conserved protective epitopes suggest that it is possible to separate the toxicity of the molecule (ADP ribosylation of G,α and/or G,α) from the antigenicity (Burnette, et al.; Pizza, et al.).
Early clinical studies using complete, active IAP multimers to treat diabetes showed some promising therapeutic results (Toyota, et al., 1978, 1980), but were not actively pursued due to problems related to IAP's antigenicity and allergenicity. In order to overcome the neutralization of injected IAP by circulating anti-IAP antibodies, injection of large amounts or repeated administration appeared necessary, thereby increasing the risk of allergic and/or anaphylactic reactions. Methods to reduce the antigenicity of IAP interfere with the target cell-receptor binding that allows S1 subunit to enter the cells and ADP-ribosylate the target proteins, decreasing the utility of the toxin as a therapeutic.

The present invention reduces or eliminates the antigenicity problems of IAP described above. Because the active subunit of IAP (S1 subunit) is expressed inside the target cells, it is not detected by the circulating T-cells. The small amounts of S1 PT that may escape following lysis of infected cells are not expected to elevate plasma concentrations of the toxin to the levels that result in clinically significant immune responses.

Since the methods of the present invention utilize only the S1 subunit of PT, they are considerably safer than applications using the complete protein. While S1 is the active subunit, it does not easily penetrate cells in the absence of the B oligomer. This means that any PT S1 released by lysed cells would be considerably less toxic than a similar molar amount of the complete multimeric protein.

The expression of PT S1 from constructs of the present invention is preferably minimal to non-existent in the absence of Tat or HIV infection. One way to reduce such undesired expression is by incorporating, into the constructs, elements which act to silence the HIV LTR promoter in the absence of the Tat protein. One example of such an element is the A trimer (Maxwell, et al., 1989), discussed in Example 1.

Further, elements which allow the expression of PT S1 to be up- or down-regulated in the presence of HIV infection may also be introduced into vector constructs of the present invention, to provide a finer degree of control over S1 expression.

XI. Delivery of Constructs to Cells and Tissues

Any of a variety of methods known to those skilled in the art may be used to introduce HIV-LTR/PT chimeric genes of the present invention into selected target cells. For example, CD4+ positive cells may be isolated from an HIV-positive individual and transfected in vitro with a HIV-LTR/PT construct of the present invention (e.g., as described in Example 1). The cells may then be infused back into the subject. These cells
now provide normal CD4+ immune functions. If the cell becomes infected by HIV, expression of the Tat protein induces expression of pT S1. This blocks the further production of infectious HIV virions in the cell, reducing the viral load in patients treated with the methods and/or compositions of the present invention.

Therapeutic protection may also be obtained by isolating and transforming a population of hematopoietic stem cells with vector constructs of the present invention using methods known to those skilled in the art. One such isolation method is described by Peault and Uchida. In the method, a mixture of hematopoietic cells is isolated from a hematopoietic source, such as bone marrow or spleen, and is enriched for pluripotent human stem cells using a fluorescence activated cell sorter and monoclonal antibody F84.1, which recognizes a stem cell marker. The cells are then transformed with a construct made according to the guidance herein (e.g., pLTR-S1PT1, pLTR-S1PT1 or pLTR-S1PT1), and implanted back into the individual in need of treatment. Other methods for isolating human stem cells have also been described (e.g., Ccivin, Tsukamoto, et al.).

Alternatively, replication-defective virions containing hybrid vectors (the chimeric genes along with selected viral sequences) of the present invention may be injected directly into selected organ systems (e.g., thymus) or into the bloodstream, to infect CD4+ cells or other cell types susceptible to HIV infection (e.g., monocytes and macrophages). The virions used to transfect host cells are preferably replication-defective, such that the virus is not able to replicate in the host cells.

The virions may be produced by co-infection of cultured host cells with a helper virus. Following co-infection, the virions are isolated (e.g., by cesium chloride centrifugation) and any remaining helper virus is inactivated (e.g., by heating). The resulting mature virions contain a chimeric gene of the present invention and may be used to infect host cells in the absence of helper virus.

High titers of replication-defective recombinant virus, free of helper virus, may also be produced in packaging cell lines containing those components for which the virus is defective (Miller). Methods for manipulating viral vectors are also known in the art (e.g., Grunhaus and Horowitz; Hertz and Gerard; Rosenfeld, et al., 1991, 1992.).

The following examples illustrate but in no way are intended to limit the present invention.
MATERIALS AND METHODS

Unless otherwise indicated, restriction enzymes and DNA modifying enzymes are obtained from New England Biolabs (Beverly, MA) or Boehringer Mannheim (Indianapolis, IN). Other chemicals were purchased from Sigma (St. Louis, MO) or United States Biochemical (Cleveland, OH).

General recombinant manipulations (Sambrook, *et al.*; Ausubel, *et al.*) and immunological procedures (Harlow, *et al.*) are carried out by standard procedures.

EXAMPLE 1

**Production of CD4+ T Cells Containing HIV-1 LTR/Pertussis Toxin Vector Constructs**

Vector pLTR-S1PT1 is constructed as follows. The HIV-1 long terminal repeat (HIV-1 LTR) region (nucleotides -1068 to +83, where +1 is the transcriptional start; Wright, *et al.*, 1986), which includes the Tat-responsive element, TAR, as well as other regulatory sequences, is ligated as a BamHI/HindIII fragment to the 5’ end of a DNA fragment containing the coding sequence for *pertussis* toxin S1 subunit (the sequence between nucleotides 507 and 1314 of SEQ ID NO:1). The resulting fragment is in turn ligated to the 5’ end of a ~850 bp BglII/BamHI fragment containing the SV40 small t intron and poly(A) signals (obtained from pSV2-327-β-globin; Maxwell, *et al.*, 1986). This cassette, schematized in Fig. 2, is inserted into the EcoRI site of pBR322 (Ausbelen, *et al.*, 1988). The pLTR-S1PT1 plasmid is grown in bacteria, purified, and introduced into CD4+ cells using a calcium phosphate precipitation method (Graham and Van der Eb, 1973; Ausubel, *et al.*, 1988; Sambrook, *et al.*, 1989; Israel and Honigman, 1991).

Vector pLTR-S1PT2 is constructed as follows. An HIV-1 LTR region (nucleotides -167 to +80; Jones, *et al.*, 1988) is isolated as a Xhol-HindIII fragment, blunted by filling in with Klenow DNA polymerase and cloned into pBR327. The PT-S1 DNA fragment and SV40 small t intron and poly(A) signals (described above) are cloned immediately downstream of the HIV-1 LTR. An A-trimer, isolated as a HindIII fragment from pUC.A.1.5 (Maxwell, *et al.*, 1989), is cloned upstream of the HIV-LTR. The A-trimer is a head-to-tail trimer of an SV40 BclI/BamHI DNA fragment specifying polyadenylation of RNA transcripts.

Several cell lines are transfected with pLTR-S1PT2 using electroporation (Maxwell and Maxwell, 1988; BioRad "GENE PULSER", BioRad Laboratories, Hercules, CA) as follows. HeLa, Jurkat, EL-4 and NIH 3T3 cells are grown in Opti-MEM medium (Gibco-BRL, Gaithersburg, MD) with 3.8% fetal bovine serum in Falcon T-75 flasks, and are
harvested and resuspended in Opti-MEM with 10% fetal bovine serum for the
electroporation pulse. HeLa cells are grown to about 80% confluence and are suspended at
2-4X10^7 cell/ml. Jurkat and EL-4 cells (human and murine T cell lines, respectively) are
grown to about 1X10^6 cells/ml and are suspended at 0.5-1X10^6 cells/ml. NIH 3T3 cells are
grown to about 80% confluence and are suspended at 5X10^6 cells/ml. Pulses are performed
in 0.1 ml volumes in Biorad cuvettes. Gene Pulser settings are between 220 and 290 volts,
with a capacitance of 250 μ farads, yielding time constants of 25-30 msec.

Stably transfected cells are produced by co-transfecting pLTR-S1PT2 with pSV2neo
(Southern and Berg) using electroporation as above and are selected using 400 μg/ml G418
(Gibco), added fresh every 3-5 days for approximately two weeks. G418-resistant cells are
either cloned or maintained as a pooled population. Cells transfected with a vector
containing a reporter construct in place of the S1PT gene are assayed for expression of the
reporter following treatment with Tat or infection by HIV. Reporter expression is
measured 12-24 hours after transfection. Reporter expression may also be used to test HIV
LTR promoter modifications which resulted in up- or down-regulated expression upon
activation of the LTR promoter.

Vector pLTR-S1PT3 is constructed by replacing the structural gene for
chloramphenicol acetyltransferase (CAT) in the vector pGVL3CATs (Felber, et al., 1989)
with the DNA fragment encoding the S1 subunit of pertussis toxin described above. The
vector pGVL3CATs, which is derived from pGV1 (Jhappan, et al., 1986), contains HIV-1
LTR (nucleotides -524 to +80), the CAT gene and SV40 sequences including poly(A)
signals and the small t splice region. The vector also contains a pBR322 origin of
replication (ori), and an SV40 ori and early promoter ligated to the neo gene (which confers
neomycin resistance in bacteria and G418 resistance in animal cells).

Viral stocks of pLTR-S1PT3 are produced using packaging cell line Y2 (Mann, et
al., 1983), which contains a packaging-defective murine sarcoma virus (MSV). Forty-eight
hours after transfection with pLTR-S1PT3, the medium of the Y2 cells containing
recombinant retrovirus is collected and used to infect YAM cells, which contain a
packaging-defective MSV retrovirus carrying an amphotropic env coat to enable infection of
human cells (Cone and Mulligan, 1984). The infected YAM cells are treated with G418,
and resistant YAM colonies containing integrated recombinant pLTR-S1PT3 proviruses are
tested for virus production on HeLa cells as described by Cone and Mulligan (1984).
Several independent G418-resistant colonies of YAM cells that generate 10^2 to 10^4
infectious viral particles per milliliter are identified. These clones are used for viral production and infection of CD4+ cells and cell lines.

CD4+ T-cells and cell lines are transfected with pLTR-S1PT3 virions by co-incubating the cells with viral stocks produced as above or by co-cultivation with the appropriate packaging cell line containing the pLTR-S1PT3 vector using standard methods (Ausubel, et al., 1988). Suitable CD4+ cell lines include H9, U937 and Molt4 (Felber, et al., 1989).

**EXAMPLE 2**

**HIV-1 p24 Assay**

CD4+ cells transfected with pLTR-S1PT vectors as described above, as well as corresponding control cells, are infected with HIV-1 by co-cultivation with an equal number of virus-infected cells or by incubation with HIV-1 viral stocks. The infected cultures of transfected and control cells are incubated for 6-48 hours at 37°C, and compared for the expression of the HIV-1 p24 antigen using the HIV-1 p24 Antigen Quantitation Panel from Abbott Laboratories (North Chicago, IL) according to the manufacturer’s instructions. Lower levels of HIV-1 p24 antigen in cells carrying a pLTR-S1PT construct (relative to normal cells) suggest that the pLTR-S1PT construct is effective to inhibit HIV production.

**EXAMPLE 3**

**Syncytium Formation Assay**

Syncytium inhibition assays are conducted according to the methods of Verhofstede, et al., (1994). Control and pLTR-S1PT-transfected CD4+ cells, such as MT2 cells, are infected with HIV-1 as described above and grown in 28 mm² flat wells of 96-well Nunc Microtest plates (Fisher Scientific, Pittsburgh, PA) for 6 to 24 hours at 37°C. The cultures are then fixed, and the cells examined for syncytia (cells with five or more nuclei). Reduction of the number of syncytia observed in cells carrying a pLTR-S1PT vector relative to control cultures suggests that the pLTR-S1PT construct is effective to inhibit HIV production.

**EXAMPLE 4**

**SCID-Hu Mouse Assay**

The efficacy of pLTR-S1PT constructs on HIV-1 infection *in vivo* is assessed using the SCID-hu mouse model for HIV infection (Aldrovandi, et al., 1993). Mice homozygous
for the severe combined immunodeficiency defect (SCID; Bosma, et al., 1983) are
transplanted with human fetal thymus and liver (Thy/Liv) as described by Aldrovandi, et

Five to six months post-implantation, the implants are injected with ~50 µl of heat-
inactivated HIV-1 (control) or 1000 infectious units (IU) of either HIV-1_{R-CSF} strain
(Koyanagi, et al., 1987), HIV-1_{NL4-3} strain (Adachi, et al., 1986) or a pool of HIV clinical isolates. The group injected with live HIV is split into two sets, one of which is injected with pLTR-S1PT3 virus stock.

Further, uninfected mice can be injected with the pLTR-S1PT3 construct to examine
in vivo effects of the construct alone. Also, the pLTR-reporter construct, described above,
can be injected into infected and uninfected mice as an in vivo control demonstrating levels of expression. When using the reporter gene, cell samples are typically isolated and
directly assayed for the presence of reporter.

The thymocyte subset distribution of the implants is analyzed using flow cytometry
10 - 20 days after inoculation with HIV, using anti-CD4 and anti-CD8 monoclonal
antibodies (Becton-Dickinson, San Jose, CA) directly conjugated to phycoerythrin or FITC, respectively.

Thy/Liv implants from mice injected with heat-inactivated HIV typically have a
subset distribution pattern similar to that of normal human thymus (about 80% of cells are
CD8+/CD4+ and most of the remaining cells are CD8-/CD4+). Implants from mice
injected with live HIV-1, but not with pLTR-S1PT3, are expected to be depleted of both
cell populations. In contrast, implants from mice injected with both live HIV-1 and pLTR-
S1PT3 are expected to have a subset distribution more similar to that observed in the
control (heat-inactivated HIV) injected animals.

While the invention has been described with reference to specific methods and
embodiments, it is appreciated that various modifications and changes may be made without
departing from the invention.
21

SEQUENCE LISTING

(i) APPLICANT: Panther Scientific, Inc.

(ii) TITLE OF INVENTION: Methods for Inhibition of HIV

(iii) NUMBER OF SEQUENCES: 6

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

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT
(B) FILING DATE: 21-MAY-1996
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/452,598
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(viii) ATTORNEY/AGENT INFORMATION:
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(C) REFERENCE/DOCKET NUMBER: 6215-0001.41

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1316 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: Bordetella pertussis gene for toxin
subunit S1 - X16347

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 507..1316
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GAATTGTCG CTCGCCCCGT GTTCGCGCTG ATGGCCCCCC AAGGAACC GA CA CCCAAGATA

ATGCCTCTGC TCACGCGCA CATCAAGAG AGGCCTGCGT CCAAGCCGGT CTGCAGGCGC

TTGCCGCCA AAGGGCCAC CGGCCTACAT GCCACGCACG ATCAGACCCG CGGTCTCATC

GCAGACGGA TACCGCCCTG GCCGCGGCTG TGGCGCGAAA CGCGCCGCAA GCTGAGTAG

CAGCGCAGGC CTCAAGCGGG CCACTCCCCGT CGCGCGGGCACA CCACTCGCGCA TACGGTGGG

CAGCGAGCA CGGCATGGC TGCGATACGG ATGGTACAGA CCGCGGAAAA CCCGACCGCG

CCGCTACTG CAATCAGCA CGCGATGACA GCTCTCTCGG CGGACAATGC CGCGATGGA

CGGTCACGG TCCGGACCGGT GCTGACCCCC CTGGCAGCGTT GTGATCCGATA AATAGGGCAC

CATCAAAACGC GAGAGGGAAG GACGGG ATG GTG ACT CGG GCA ATT CGC CAA
Met Arg Cys Thr Arg Ala Ile Arg Gln

ACC GCA AGA ACA GGC TGG CTG AGC TGG TGG GCC ATT CTT GCC GTC TGC
Thr Ala Arg Thr Gly Trp Leu Thr Trp Leu Ala Ile Leu Ala Val Thr

10 15 20 25

GCG CCC GTG ACT TCG CCG GCA TGG GCC GAC GAT CCT CCC GCC ACC GTA
Ala Pro Val Thr Ser Pro Ala Trp Ala Asp Asp Pro Pro Ala Thr Val

30 35 40

TAC CGC CAT GAC TCC GCG CCG CCC GAG GAC GTT TTC CAG AAC GGA TTC
Tyr Arg Tyr Asp Ser Arg Pro Glu Val Phe Gly Asn Gly Phe

45 50 55

AGC GCG TGG GGA AAC AAG GAC GAT GTG CTC GAA CAT CTG ACC GGA CTG
Thr Ala Trp Gly Asn Asn Asp Val Leu Glu His Ala Thr Gly Arg

60 65 70

TCC TGC CAG GTC GGC AGC AGC AAC AGC GCT TTC GTC TCC ACC AGC AGC
Ser Gln Val Gly Ser Ser Asn Ser Ala Phe Val Ser Thr Ser

75 80 85

AGC CGG CGC TAT ACC GAG GTC TAT CTC GAA CAT CGC ATG CAG GAA CGG
Ser Arg Arg Tyr Thr Glu Val Tyr Leu Glu His Arg Met Glu Glu Ala

90 95 100 105

GTC GAG GCC GAA CGG GCC GGC AGG GCC ACC GGC CAC TTC ATC GCC TAC
Val Glu Ala Glu Arg Arg Gln Arg Glu His Phe Ile Gly Tyr

110 115 120

ATC TAC GAA GTC CGC GCC GAC AAC AAT TTC TAC GGC GCC CAC AGC TCG
Ile Tyr Glu Val Arg Ala Asp Asn Phe Tyr Gly Ala Ala Ser Ser

125 130 135

TAC TCC GAA TAC GTC GAC ACT TAT GGC GAC AAT GCC GCC CGT ATC CTC
Tyr Phe Glu Tyr Val Asp Thr Tyr Gly Asp Ala Gly Arg Ile Leu

140 145 150

GCC GGC GCG GTG GCC ACC TAC CAG AGC GAA TAT CTG GCA CAC CGG GCC
Ala Gly Ala Leu Ala Thr Tyr Glu Tyr Leu Ala His Arg Arg

155 160 165

ATT CGG CCC GAA AAC ATC CGC AGG GTA ACG CGG GTC TAT CAC AAG GCC
Ile Pro Pro Glu Asn Ile Arg Arg Val Thr Arg Val Tyr His Asn Gly

170 175 180 185
(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 269 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

Met Arg Cys Thr Arg Ala Ile Arg Gln Thr Ala Arg Thr Gly Trp Leu
1       5
Thr Trp Leu Ala Ile Leu Ala Val Thr Ala Pro Val Thr Ser Pro Ala
20      25
Trp Ala Asp Asp Pro Pro Ala Thr Val Tyr Arg Tyr Asp Ser Arg Pro
35      40
Pro Glu Asp Val Phe Gln Asn Gly Phe Thr Ala Trp Gly Asn Asn Asp
50      55
Asn Val Leu Glu His Leu Thr Gly Arg Ser Cys Gln Val Gly Ser Ser
65      70
Asn Ser Ala Phe Val Ser Thr Ser Ser Ser Arg Arg Tyr Thr Glu Val
85      90
Tyr Leu Glu His Arg Met Gln Glu Ala Val Glu Ala Arg Ala Gly
100     105
Arg Gly Thr Gly His Phe Ile Gly Tyr Ile Tyr Glu Val Arg Ala Asp
115     120
Asn Asn Phe Tyr Gly Ala Ala Ser Ser Tyr Phe Glu Tyr Val Asp Thr
130     135
Tyr Gly Asp Asn Ala Gly Arg Ile Leu Ala Gly Ala Leu Ala Thr Tyr
145     150
Gln Ser Glu Tyr Leu Ala His Arg Arg Ile Pro Pro Glu Asn Ile Arg
    165  170
Arg Val Thr Arg Val Tyr His Asn Gly Ile Thr Gly Glu Thr Thr
    180  185  190
Thr Glu Tyr Ser Asn Ala Arg Tyr Val Ser Gln Gln Thr Arg Ala Asn
    195  200  205
Pro Asn Pro Tyr Thr Ser Arg Arg Ser Val Ala Ser Ile Val Gly Thr
    210  215  220
Leu Val Arg Met Ala Pro Val Val Gly Ala Cys Met Ala Arg Gln Ala
    225  230  235  240
Glu Ser Ser Glu Ala Met Ala Ala Trp Ser Glu Arg Ala Gly Glu Ala
    245  250  255
Met Val Leu Val Tyr Tyr Glu Ser Ile Ala Tyr Ser Phe
    260  265

(2) INFORMATION FOR SEQ ID NO:3:

  (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 1500 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: double
      (D) TOPOLOGY: unknown

  (ii) MOLECULE TYPE: DNA (genomic)

  (iii) HYPOTHETICAL: NO

  (iv) ANTI-SENSE: NO

  (vi) ORIGINAL SOURCE:
      (C) INDIVIDUAL ISOLATE: Bordetella pertussis gene for toxin
          subunit S1 - M13223

  (ix) FEATURE:
      (A) NAME/KEY: CDS
      (B) LOCATION: 507..1316

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

GAATTGTGCG CCTGCCCTTG GTTCGGCTGC ATGGCCCCCA AGGGAACCGA CCCCAGATA
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ATCGTCTCTGC TCAACCGGCA CATCAACGAG GCCTGCAGT CCAAGCCGCT GTCTAGACCC
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55
GCAGACGAGA TCCACCGCTG GGCGGCGGTG GTGCGGAAAA CGGCGGCACG GCTGAAAGTAG
CAGGCCGACG CTCAACACGG CATCCCCGGT CGGCGCGCGA CCAATCCCCGA TACGTGGTTG
CAGGCCGCCA GGGGATCGGG TGGAGATCGG TGCTACAAAA CCCCTGGAGTTC TTGGGATACAT
60
CCGCTACTGG CAATCCAAAC GCGCAATGAC GGCTCCCTGCG GCGAAACTGGC GCGCATAGTA
CCGGTACCGG TGCCGCACTG GTCGATCCC TGATCCGCTGAA AAATAGGGAC
CATCAAACGG CAGAGGGGAA GAGGGG ATG CGT TGC ACT CGG GCA ATT CGC CAA
65
Met Arg Cys Thr Arg Ala Ile Arg Gln
25

ACC GCA AGA ACA GCC TGG CTG ACG TGG CTG GCG ATT CTT GCC GTC ACG
Thr Ala Arg Thr Gly Trp Leu Thr Trp Leu Ala Ile Leu Ala Val Thr
10  15  20  25

5

GGC CCC GTG ACT TCG CCG GCA TGG GCC GAC GAT CCT CCC GCC ACC GTG
Ala Pro Val Thr Ser Pro Ala Trp Ala Asp Asp Pro Pro Ala Thr Val
30  35  40

10

TAC CGC TAT GAC TCC CGC CGG CCG TAG GAC GTT TTC CAG AAC GGA TTC
Tyr Arg Tyr Asp Ser Arg Pro Pro Glu Val Phe Gln Asn Gly Phe
45  50  55

15

ACG GCC TGG GGA AAC AAC GAC AAC TGT CTC GAC CAT CTC ACC AGA CGT
Thr Ala Trp Gly Asn Asn Asp Asn Val Leu Asp His Leu Thr Gly Arg
60  65  70

20

TCC TGC CAG GTG GCC AGC AGC AAC AGC CCT TGC TCC ACC AGC AGC
Ser Cys Gln Val Gly Ser Ser Ser Ser Ala Phe Val Ser Thr Ser Ser Ser
75  80  85

25

AGC CGG CGC TAT ACC GAG TAT TAT CTC GAA CAT CGC ATG CAG GAA GCC
Ser Arg Arg Tyr Thr Glu Val Tyr Leu Glu His Arg Met Gln Glu Ala
90  95 100 105

GTC GAG GCC GAA GCC GCC GCC AGG GCC ACC GCC CAC TTC ATC GCC TAC
Val Glu Ala Glu Arg Ala Gly Arg Gly Thr Gly His Phe Ile Gly Tyr
110 115 120

30

ATC TAC GAA GTC CGC GCC GAC AAC AAT TTC TAC GCC GCC GCC AGC TCG
Ile Tyr Glu Val Arg Ala Asp Asn Phe Tyr Gly Ala Ala Ser Ser
125 130 135

35

TAC TTC GAA TAC GTG GAC ACT TAT GCC GAC AAT GCC GCC CGT ATC CTC
Tyr Phe Glu Tyr Val Asp Thr Tyr Gly Asp Ala Gly Arg Ile Leu
140 145 150

GCC GCC CGG CTG GCC ACC TAC CAG AGC GAA TAT CTG GCA CAC CGG CGC
Ala Gly Ala Leu Ala Thr Tyr Gln Ser Glu Tyr Leu Ala His Arg
155 160 165

40

ATT CCG CCC GAA AAC ATC CGC AGG GTA ACG CGG GTG TAT CAC AAC GGC
Ile Pro Pro Glu Asn Ile Arg Arg Val Thr Arg Val Tyr His Asn Gly
170 175 180 185

45

ATC ACC GGC GAG ACC AGC AGG GAG TAT CCC AAC GCT CGC TAC TGT
Ile Thr Gly Glu Thr Gln Ser Asn Ala Arg Tyr Val
190 195 200

AGC CAG CAG ACT CGC GCC AAT CCC AAC CCC TAC ACA TCG CAA AGG TCC
Ser Glu Gln Thr Arg Ala Asn Pro Asn Pro Tyr Thr Ser Arg Arg Ser
205 210 215

GTA GCG TCG ATC GTC GCC ACA TTG GTG CGC ATG GCC CGG GTG ATA GCC
Val Ala Ser Ile Val Gly Thr Leu Val Arg Met Ala Pro Val Ile Gly
220 225 230

50

GCT TGC ATG GCG CGG CAG GCC AAC AAG CGC TGC AAG GCC ATG GCA GCC TGG
Ala Cys Met Ala Arg Gln Ala Glu Ser Ser Glu Ala Met Ala Ala Trp
235 240 245

55

TCC GAA CGC GCC GGC GAG CGG ATG GTT CTC GTG TAC GAA AGC ATC
Ser Glu Arg Ala Gly Glu Ala Met Val Leu Val Tyr Tyr Glu Ser Ile
250 255 260 265

60

GGT TCG TTC TAGACCTGGG CCAGCCCCGC CCAAAGGCGG TGATTTGAACA
Ala Tyr Ser Phe
270

WO 96/37235
PCT/US96/07518

581

629

677

725

773

821

869

917

965

1013

1061

1109

1157

1205

1253

1301

1353
GCATGCGGAT CGACCGCAAG AGGCTCTGCC ATCTCCTGTC CGTTCTGCCG TTG66CCCTCC 1413
TGATGCTCA C66GCGCCGG GC66TCACGC CAG66CATCGT CATTTCGCGG CAGGAACAGA 1473
TTACCCAGCA TGG66GCCCCC TATGGAC 1500

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 269 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

Met Arg Cys Thr Arg Ala Ile Arg Gln Thr Ala Arg Thr Gly Trp Leu 1
Thr Trp Leu Ala Ile Leu Ala Val Thr Ala Pro Val Thr Ser Pro Ala 20
Trp Ala Asp Asp Pro Pro Ala Thr Val Tyr Arg Tyr Asp Ser Arg Pro 35
Pro Glu Asp Val Phe Gln Asn Gly Phe Thr Ala Trp Gly Asn Asn Asp 50
Asn Val Leu Asp His Leu Thr Gly Arg Ser Cys Gln Val Gly Ser Ser 65
Asn Ser Ala Phe Val Ser Thr Ser Ser Ser Arg Tyr Thr Glu Val 85
Tyr Leu Glu His Arg Met Gln Glu Ala Val Glu Ala Glu Arg Ala Gly 100
Arg Gly Thr Gly His Phe Ile Gly Tyr Ile Tyr Glu Val Arg Ala Asp 115
Asn Asn Phe Tyr Gly Ala Ala Ser Ser Tyr Phe Glu Tyr Val Asp Thr 130
Tyr Gly Asp Asn Ala Gly Arg Ile Leu Ala Gly Ala Leu Ala Thr Tyr 145
Gln Ser Glu Tyr Leu Ala His Arg Arg Ile Pro Pro Glu Asn Ile Arg 165
Arg Val Thr Arg Val Tyr His Asn Gly Ile Thr Gly Thr Thr Ser 180
Thr Glu Tyr Ser Asn Ala Arg Tyr Val Ser Gln Gln Thr Arg Ala Asn 195
Pro Asn Pro Tyr Thr Ser Arg Arg Ser Val Ala Ser Ile Val Gly Thr 210
Leu Val Arg Met Ala Pro Val Ile Gly Ala Cys Met Ala Arg Gln Ala 225
Glu Ser Ser Glu Ala Met Ala Trp Ser Glu Arg Ala Gly Glu Ala 245
Met Val Leu Val Tyr Thr Glu Ser Ile Ala Tyr Ser Phe 260

265
(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1500 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: Bordetella pertussis gene for toxin subunit S1 - A13359

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 507..1316

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

GAATTCGTCG CCTGCCCCCTG GTTGCCGGTC ATGGCCCCCA AGGGAAACGAA CCCCAGATA
ATCGTCCTGC TCAACCGCCA CATCAACGAG GCCGCTGCAGT CCAAGCCGGT GTGCGAGGCC
TTTGCGCCCC AAGGGCCAC GCCGTCATC GCCAGCCGGG ATCAGACCCCG CGGCTTCATC
GCAGACCGA TGACGCGCTG GCCGGCGGTC GTGCGCGAAAA CCGGGCGCAA GCTGAGTAG
35 CAGGCGCCCG CTCAACCCGCG CCATCCCCGT CCGGCGGGCA CCATCCGCA TAGGTGTGG
CAACCGCCA ACGGTATGCG TGCAATTCG TGCTAACA AA CCGTACGTT TCCGTTACAT
 CCCGCTACTG CAATTCAGCA CGGCTTGAC GCCTCTTGGG CGCAAAGTGC CGCGATGGTA
40 CGGTCACCG CGGAGCCTCC GTGCACCCCC CTGCCATGCT GTGATCCGTA AATAGGCAC
CATCAAAACG CAGAGGGAAA GACGGG ATG CTG TGC ACT CGG GCA ATT CCG CAA
ACC GCA AGA ACA GGC TGG CTG ACG TGG CTG GCG ATT CTT GCC GTC ACG
50 Thr Ala Arg Thr Gly Trp Leu Thr Trp Leu Ala Ile Leu Ala Val Thr
15 15 20 25

GCG CCC GTG ACT TCG CGC ACG TGG GCC GAC GAT CCT CCC GCC ACC GTA
60 Ala Pro Val Thr Ser Pro Ala Trp Ala Asp Asp Pro Pro Ala Thr Val
30 35 40

TAC CGC TAT GAC TCC CGC CGG CGG GAG GAC GTT TTC CAG AAC GGA TTC
65 Tyr Arg Tyr Asp Ser Arg Pro Pro Glu Val Phe Gln Asn Gly Phe
45 50 55

ACG GCG TGG GGA AAC AAC GAC AAT GTG CTC GAA CAT CTG ACC GGA CGT
70 Thr Ala Trp Gly Asn Asn Asp Asn Val Leu Glu His Leu Thr Gly Arg
60 65 70

TCC TGC CAG GTC GGC AGC AGC AAC AGC CCT GTT TTC TCC ACC AGC AGC
75 Ser Cys Gln Val Gly Ser Ser Asn Ser Ala Phe Val Ser Thr Ser Ser
80 85
AGC CGG GCC TAT ACC GAG GTC TAT CTC GAA CAT CGC ATG CAG GAA GCG
Ser Arg Arg Tyr Thr Glu Val Tyr Leu Glu His Arg Met Gln Glu Ala
90 95 100 105

GTC GAG GCC GAA CGC GCC GCC AGG GCC ACC GCC CAC TTC TAC GCC TAC
Val Glu Ala Glu Arg Ala Gly Arg Gly Thr Gly His Phe Ile Gly Tyr
110 115 120

ATC TAC GAA GTC CGC GCC GAC AAC AAT TTC TAC GCC GGC GCC AGG TCG
Ile Tyr Glu Val Arg Ala Asp Asn Asn Phe Tyr Gly Ala Ala Ser Ser
125 130 135

TAC TTC GAA TAC GTC GAC ACT TAT GGC GAC AAT GCC GGC CGT ATC CTC
Tyr Phe Glu Tyr Val Asp Thr Tyr Gly Asp Asn Ala Gly Arg Ile Leu
140 145 150

GCC GCC CGG CCG GCC ACC TAC CAG AGC GAA TAT CTG GCA CAC CGG GCC
Ala Gly Ala Leu Ala Thr Glu Thr Val Ser Tyr Glu Leu Ala His Arg
155 160 165

ATT CGG CCC GAA AAC ATC CGC AGG GTA ACG CGG GTC TAT CAC AAC GGC
Ile Pro Pro Glu Asn Ile Arg Arg Val Tyr Val Arg Tyr His Asn Gly
170 175 180 185

ATC ACC GGC GAG ACC ACC AGC GAG TAT GCC ACC GCC TAC GCC TAC
Ile Thr Gly Thr Thr Thr Thr Thr Glu Thr Thr Thr Thr Thr Thr Thr
190 195 200

AGC CAG CAG ACT CGC GCC AAT CCC AAC CCC TAC ACA TCG CGA AGG TCC
Ser Gln Glu Thr Arg Ala Asn Pro Asn Pro Tyr Thr Ser Arg Arg Ser
205 210 215

GTA CGG TCG ATC GTC GGC ACA TTG GTG CGC ATG GCG CCG GTG GTG GCC
Val Ala Ser Ile Val Gly Thr Leu Val Arg Met Ala Pro Val Val Gly
220 225 230

GCT TGC ATG CGC CGG CAG GCC GAA AGC GCC CCC GAG GCC ATG GCA GCC TGG
Ala Cys Met Ala Arg Gln Ala Glu Ser Ser Glu Ala Met Ala Trp
235 240 245

TCC GAA CGC GCC GGC GAG CGG ATG GCT GTC GTC TAC GAA AGC ATC
Ser Glu Arg Ala Gly Ala Met Val Leu Tyr Tyr Glu Ser Ile
250 255 260 265

GCC TAT TCG TTC TAGACCTGGC CGACGCCCGC CCAAATCGCG TAAATAGAACA
Ala Tyr Ser Phe
270

GCATGCGCAT CGACGCGCAAG AGCGTCTGCC ATCTCTTGTC GTGTCTGCCG TTGCGGCTCC
1473

TCGATATTTCA CGGGCGCGCG GCCGCCAGCG CAGGCGATCGT CATCGCGCG CAGGACAGA
1473

TTACCGAGCG TGGGAGGCGA TATAGG
1500

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 269 amino acids
   (B) TYPE: amino acid
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Arg Cys Thr Arg Ala Ile Arg Gln Thr Ala Arg Thr Gly Trp Leu

1  5

Thr Trp Leu Ala Ile Leu Ala Val Thr Ala Pro Val Thr Ser Pro Ala

20  25  30

Trp Ala Asp Asp Pro Pro Ala Thr Val Tyr Arg Tyr Asp Ser Arg Pro

35  40  45

Pro Glu Asp Val Phe Gln Asn Gly Phe Thr Ala Trp Gly Asn Asn Asp

50  55  60

Asn Val Leu Glu His Leu Thr Gly Arg Ser Cys Gln Val Gly Ser Ser

65  70  75  80

Asn Ser Ala Phe Val Ser Thr Ser Ser Ser Arg Arg Gly Thr Gly Val

85  90  95

Tyr Leu Glu His Arg Met Gln Glu Ala Val Glu Ala Glu Arg Ala Gly

100 105 110

Arg Gly Thr Gly His Phe Ile Gly Tyr Ile Tyr Glu Val Arg Ala Asp

115 120 125

Asn Asn Phe Tyr Gly Ala Ala Ser Ser Tyr Phe Glu Tyr Val Asp Thr

130 135 140

Tyr Gly Asp Asn Ala Gly Arg Ile Leu Ala Gly Ala Leu Ala Thr Tyr

145 150 155 160

Gln Ser Glu Tyr Leu Ala His Arg Arg Ile Pro Pro Glu Asn Ile Arg

165 170 175

Arg Val Thr Arg Val Tyr His Asn Gly Ile Thr Gly Glu Thr Thr Thr

180 185 190

Thr Glu Tyr Ser Asn Ala Arg Tyr Val Ser Glu Glu Thr Arg Ala Asn

195 200 205

Pro Asn Pro Tyr Thr Ser Arg Arg Ser Val Ala Ser Ile Val Gly Thr

210 215 220

Leu Val Arg Met Ala Pro Val Val Gly Ala Cys Met Ala Arg Gln Ala

225 230 235 240

Glu Ser Ser Glu Ala Met Ala Ala Trp Ser Glu Arg Ala Gly Glu Ala

245 250 255

50

Met Val Leu Val Tyr Tyr Glu Ser Ile Ala Tyr Ser Phe

260 265
IT IS CLAIMED:

1. A method of inhibiting the production of infectious Human Immunodeficiency Virus 1 (HIV-1) virions in an HIV-1-infected cell, comprising
   providing an HIV-1-infected cell containing a chimeric gene containing a DNA sequence encoding the S1 subunit of pertussis toxin (S1 gene) operably linked to an HIV-1 long terminal repeat (LTR) region, and
   growing said cell, where said growing is carried out under conditions where expression of said chimeric gene is induced,
   wherein expression of the S1 subunit inhibits production of infectious HIV-1 virions.

2. A method of claim 1, where the DNA sequence encodes an S1 subunit that contains an amino acid sequence selected from the group consisting of sequences represented as SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6.

3. A method of claim 1 or 2, where said chimeric gene further includes, upstream of the LTR region, a DNA fragment comprising a head-to-tail trimer of SV40 polyadenylation signal sequences.

4. A method of any of claims 1-3, where said cell is a CD4+ lymphocyte.

5. A method of any of claims 1-3, where said cell is a monocyte cell.

6. A method of any of claims 1-3, where said cell is a macrophage cell.

7. A chimeric gene, comprising an HIV-1 LTR region operably linked to a DNA sequence encoding the S1 subunit of pertussis toxin.

8. A chimeric gene of claim 7, further including, upstream of the LTR region, a DNA fragment comprising a head-to-tail trimer of SV40 polyadenylation signal sequences.

9. A chimeric gene of claim 7 or 8, where the DNA sequence encodes an S1 subunit containing an amino acid sequence selected from the group consisting of sequences represented as SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6.
10. A retroviral expression vector, comprising a chimeric gene of any of claims 7-9.

11. A method of reducing HIV-1 viral load in an HIV-1-infected subject, comprising
   isolating CD4+ lymphocytes from the subject,
   transforming the lymphocytes with a chimeric gene comprising an HIV-1 LTR region operably linked to a DNA sequence encoding the S1 subunit of pertussis toxin (PT), and introducing lymphocytes carrying the chimeric gene into the subject,
   wherein said lymphocytes express the S1 subunit of PT, infectious HIV-1 production is inhibited and said inhibition results in a reduced viral load in the HIV-1-infected subject.

12. A method of reducing HIV-1 viral load in a subject harboring HIV-1-infected cells, comprising
   administering to the subject, a retroviral expression vector containing a chimeric gene comprising an HIV-1 LTR region operably linked to a DNA sequence encoding the S1 subunit of pertussis toxin (PT), under conditions which promote transfection of the vector into said infected cells,
   wherein infected cells carrying the vector express the S1 subunit, which inhibits HIV production and results in a reduced viral load in the HIV-1-infected subject.
**Fig. 1A**

\[
\begin{align*}
g_{41} & \quad (589) \quad A V E R y l K \quad (595) \\
G_{1a} & \quad (12) \quad A V E R - s K \quad (17)
\end{align*}
\]

**Fig. 1B**

\[
\begin{align*}
g_{41} & \quad (597) \quad q q L L G \quad i \quad w \quad G \quad c \quad S \quad G \quad K \quad - \quad l \quad I \quad c \quad (611) \\
G_{1a} & \quad (36) \quad l l L L G \quad - \quad a \quad G \quad e \quad S \quad G \quad K \quad s \quad t \quad I \quad v \quad (50)
\end{align*}
\]

**Fig. 2**

BamHI  HindIII  Bgl II  BamHI

\[
\begin{align*}
\text{HIV-1-LTR} & \quad - \quad \text{PT SI} & \quad - \quad \text{SV40polyA}
\end{align*}
\]
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC(6) : A61K 48/00; C12N 15/00
US CL : 514/44; 424/93.21; 435/320.1
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; 424/93.21; 435/320.1

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

Electronic database consulted during the international search (name of database and, where practicable, search terms used)
APS, MEDLINE, EMBASE, BIOSIS, CAPLUS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>EP 0,232,229 A2 (SCLAVO S.p.A.) 12 August 1987, see entire document.</td>
<td>1-12</td>
</tr>
</tbody>
</table>

[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
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Date of the actual completion of the international search: 24 JUNE 1996

Date of mailing of the international search report: 02 JUL 1996

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Relevant to claim No. 1-12