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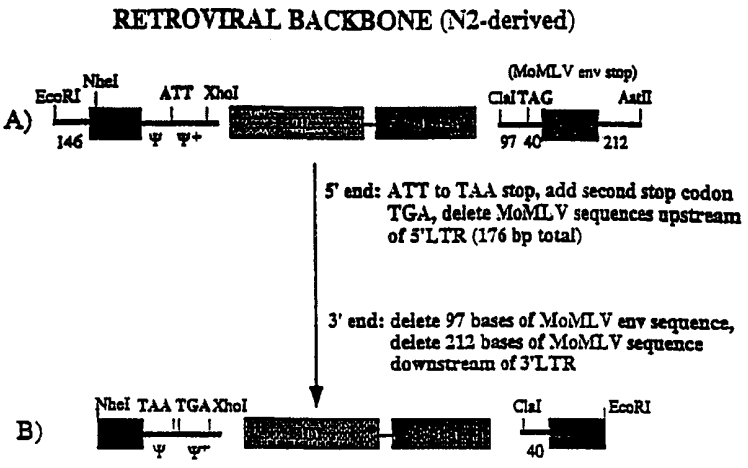
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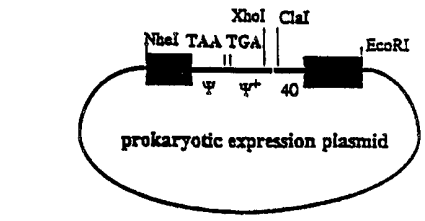
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(54) Title: COMPOSITIONS AND METHODS FOR TREATING ARTHRITIS UTILIZING GENE THERAPY

(57) Abstract

Methods are provided for treating or preventing arthritis, comprising the general step of administering to a mammal a therapeutically effective amount of a retroviral vector which directs the expression of an anti–arthritic agent, wherein the retroviral vector is administered at a titer of greater than 10^7 cfu/ml.





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COMPOSITIONS AND METHODS FOR TREATING ARTHRITIS UTILIZING GENE THERAPY

TECHNICAL FIELD

The present invention relates generally to pharmaceutical compositions and methods, and more specifically, to compositions and methods for treating arthritis utilizing gene therapy.

BACKGROUND OF THE INVENTION

Due in large part to the rapidly increasing number of aged individuals, arthritis has become a serious health problem worldwide. For example, one form of arthritis, rheumatoid arthritis, is a debilitating, chronic inflammatory disease affecting 1 to 2% of the world's population. This disease causes pain, swelling and destruction of multiple joints in the body. The disease can also result in damage to other organs such as the lungs and kidneys.

Briefly, it is generally believed that rheumatoid arthritis is an autoimmune disease and that many different stimuli can trigger an inappropriate immune response of an immunologically susceptible individual. Examples of potential stimuli include both infectious agents (*e.g.*, viruses) and endogenous host proteins (*e.g.*, collagen). In particular, the stimuli (*i.e.*, an antigen) is taken up by antigen-presenting cells (macrophages or dendritic cells in the synovial membrane), processed, and presented to T lymphocytes. This presentation initiates an immunological cascade mediated by numerous factors, ultimately resulting in the pain, swelling and cartilage destruction which are associated with rheumatoid arthritis.

Therapeutic regimens which are presently being utilized include both steroidal and non-steroidal anti-arthritic agents. For many patients with severe disease, anti-cancer drugs such as methotrexate have become the first line of therapy. Other drugs, such as cyclosporin and azathioprine (alone or in combination) have also been employed. Many of these agents however, are highly toxic, have significant side effects.

The present invention discloses novel compositions and methods for treating arthritis, and further provides other related advantages.

SUMMARY OF THE INVENTION

Briefly stated, the present invention provides compositions and methods for treating or preventing a variety of forms of arthritis, including for example, rheumatoid arthritis, osteoarthritis, and reactive forms of arthritis (*e.g.*, due to a disease process such as Lyme disease, see generally, Goldenberg, Textbook of Rheumatology, and Cooke and Dattwyler, *Ann. Rev. Med. 43*:93-103, 1992). Within one aspect of the present invention, methods are provided for treating or preventing arthritis comprising the general step of administering to a mammal a therapeutically effective amount of a recombinant retrovirus which directs the expression of an anti-arthritic agent, wherein the recombinant retrovirus is administered at a titer of greater than 10^7 cfu/ml, 10^8 cfu/ml, 10^9 cfu/ml, or, 10^{10} cfu/ml. A wide variety of mammals may be treated utilizing such techniques, including for example, humans, horses, dogs, cats, rats and mice.

The present invention also provides a variety of anti-arthritic agents that may be utilized, including for example, pro-drug converting enzymes, inhibitors of tumor necrosis factor, IL-1 Receptor antagonists and inhibitors of matrix metalloproteinase expression such as TIMP-1 or TIMP-2. Within certain embodiments of the invention, the recombinant retrovirus is administered intraarticularly.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth herein which describe in more detail certain procedures

or compositions (*e.g.*, plasmids, etc.), and are therefore incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a description of all modifications carried out on retroviral vector as shown in A), resulting in the cross-less retroviral vector shown in B). The cross-less retroviral backbone cloned into a prokaryotic vector is called pBA-5.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

"Retroviral vector" and "recombinant retroviral vector" refers to a nucleic acid construct which carries, and within certain embodiments, is capable of directing the expression of a nucleic acid molecule of interest. The retroviral vector must include at least one transcriptional promoter/enhancer or locus defining element(s), or other elements which control gene expression by other means such as alternate splicing, nuclear RNA export, post-translational modification of messenger, or post-transcriptional modification of protein. Such vector constructs must also include a packaging signal, long terminal repeats (LTRs) or portion thereof, and positive and negative strand primer binding sites appropriate to the retrovirus used (if these are not already present in the retroviral vector). Optionally, the recombinant retroviral vector may also include a signal which directs polyadenylation, selectable markers such as Neomycin resistance, TK, hygromycin resistance, phleomycin resistance histidinol resistance, or DHFR, as well as one or more restriction sites and a translation termination sequence. By way of example, such vectors typically include a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis, and a 3' LTR or a portion thereof.

"Recombinant retrovirus", "retroviral gene delivery vehicle" and "retroviral vector particle" as utilized within the present invention refers to a retrovirus

which carries at least one gene of interest. The retrovirus may also contain a selectable marker. The recombinant retrovirus is capable of reverse transcribing its genetic material into DNA and incorporating this genetic material into a host cell's DNA upon infection.

"Prodrug activating enzyme" refers to a compound that is capable of activating an otherwise inactive compound into an active compound. Representative examples of prodrug activating enzymes include herpes simplex thymidine kinase and cytosine deaminase.

"Anti-arthritic agent" refers to a molecule which is capable of (1) preventing or lessening the pathological and/or clinical symptoms associated with arthritis; (2) downregulating the white blood cell response which initiates the inflammatory cascade and results in pain, swelling and cartilage destruction; (3) inhibiting the proliferation of synoviocytes; (4) decreasing the production/activity of matrix metalloproteinases; or, (5) inhibiting blood vessel formation. Such molecules include, for example, proteins (*e.g.*, inhibitors of TNFα or the IL-1 Receptor), antisense and ribozyme nucleic acid sequences.

As noted above, the present invention provides compositions and methods for treating arthritis, comprising the general step of administering to a patient a therapeutically effective amount of a recombinant retrovirus which directs the expression of an anti-arthritic agent, wherein the recombinant retrovirus is administered at a titer of greater than 10^7 cfu/ml. That administration of such recombinant retroviruses can provide a significant therapeutic advantage is surprising, particularly in light of published results which suggest that such a method would be ineffective for the treatment or prevention of arthritis (*see* Nita et al., *Arthritis and Rheumatism 39*(5):820-828, 1996).

In order to further an understanding of the invention, a brief discussion is provided below of: (1) the preparation of recombinant retroviral vectors, packaging cell lines, producer cell lines and recombinant retroviruses; (2) anti-arthritic agents that can

be utilized within the context of the present invention; and (3) the preparation, formulation and administration of recombinant retroviruses.

PREPARATION OF RECOMBINANT RETROVIRAL VECTORS, PACKAGING CELLS, PRODUCER CELLS AND RECOMBINANT RETROVIRUSES

As noted above, the present invention provides recombinant retroviruses which are constructed to carry or express a selected nucleic acid molecule of interest. Briefly, numerous retroviral gene delivery vehicles may be utilized within the context of the present invention, including for example those described in EP 0,415,731; WO 90/07936; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 9311230; WO 9310218; Vile and Hart, *Cancer Res. 53*:3860-3864, 1993; Vile and Hart, *Cancer Res. 53*:962-967, 1993; Ram et al., *Cancer Res. 53*:83-88, 1993; Takamiya et al., *J. Neurosci. Res. 33*:493-503, 1992; Baba et al., *J. Neurosurg. 79*:729-735, 1993 (U.S. Patent No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO 91/02805). Particularly preferred recombinant retroviruses include those described in WO 91/02805, as well as lentiviral vectors based upon, for example, HIV (*see, e.g.*, PCT Application No. PCT/US98/14996; and Naldini et al., *Science 272*:263, 1996), FIV (*see, e.g.*, U.S. Application No. 60/086,825; and Poeschla et al., *Nature Med. 4*:354, 1998), and foamy viruses (*see, e.g.*, Russell and Miller, *J. Virol. 70*:217, 1996).

Retroviral gene delivery vehicles of the present invention may be readily constructed from a wide variety of retroviruses, including for example, B, C, and D type retroviruses as well as spumaviruses and lentiviruses (see RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985). Preferred retroviruses for the preparation or construction of retroviral gene delivery vehicles of the present invention include retroviruses selected from the group consisting of Avian Leukosis Virus, Bovine Leukemia Virus, Murine Leukemia Virus, Mink-Cell Focus-Inducing Virus, Murine Sarcoma Virus, Reticuloendotheliosis virus and Rous Sarcoma Virus. Particularly preferred Murine Leukemia Viruses include 4070A and 1504A (Hartley and Rowe, *J. Virol. 19*:19-25, 1976), Abelson (ATCC No. VR-999), Friend (ATCC No.

VR-245), Graffi, Gross (ATCC No. VR-590), Kirsten, Harvey Sarcoma Virus and Rauscher (ATCC No. VR-998), and Moloney Murine Leukemia Virus (ATCC No. VR-190). Such retroviruses may be readily obtained from depositories or collections such as the American Type Culture Collection ("ATCC"; Rockville, Maryland), or isolated from known sources using commonly available techniques.

Any of the above retroviruses may be readily utilized in order to assemble or construct recombinant retroviral vectors and recombinant retroviruses given the disclosure provided herein, and standard recombinant techniques (*e.g.*, Sambrook et al, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Kunkle, *PNAS 82*:488, 1985). In addition, within certain embodiments of the invention, portions of the recombinant retroviral vectors or viruses may be derived from different retroviruses. For example, within one embodiment of the invention, retroviral vector LTRs may be derived from a Murine Sarcoma Virus, a tRNA binding site from a Rous Sarcoma Virus, a packaging signal from a Murine Leukemia Virus, and an origin of second strand synthesis from an Avian Leukosis Virus.

In order to generate a recombinant retrovirus (and in particular, a recombinant retrovirus which is replication incompetent), it is generally preferred to construct a recombinant retroviral vector as discussed above which lacks certain elements such as gag/pol or env that are required for the production of infectious retrovirus. Such defective retroviral vectors can then be introduced into a cell (termed a "packaging cell") which contains those elements necessary for production of infectious recombinant retrovirus that are lacking in the retroviral vector. The result is to produce a recombinant retrovirus which is infectious, but which lacks those elements required to required to replicate.

As discussed in more detail below, a wide variety of retroviral vectors may be utilized within the present invention in order to prepare recombinant retroviruses. For example, within one aspect of the present invention retroviral vector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis and a 3' LTR, wherein the vector construct lacks gag/pol or env coding sequences. Briefly,

Long Terminal Repeats ("LTRs") are subdivided into three elements, designated U5, R and U3. These elements contain a variety of signals which are responsible for the biological activity of a retrovirus, including for example, promoter and enhancer elements which are located within U3. LTRs may be readily identified in the provirus due to their precise duplication at either end of the genome. As utilized herein, a 5' LTR should be understood to include a 5' promoter element and sufficient LTR sequence to allow reverse transcription and integration of the DNA form of the vector. The 3' LTR should be understood to include a polyadenylation signal, and sufficient LTR sequence to allow reverse transcription and integration of the DNA form of the vector.

The tRNA binding site and origin of second strand DNA synthesis are also important for a retrovirus to be biologically active, and may be readily identified by one of skill in the art. For example, retroviral tRNA binds to a tRNA binding site by Watson-Crick base pairing, and is carried with the retrovirus genome into a viral particle. The tRNA is then utilized as a primer for DNA synthesis by reverse transcriptase. The tRNA binding site may be readily identified based upon its location just downstream from the 5' LTR. Similarly, the origin of second strand DNA synthesis is, as its name implies, important for the second strand DNA synthesis of a retrovirus. This region, which is also referred to as the poly-purine tract, is located just upstream of the 3' LTR.

In addition to a 5' and 3' LTR, tRNA binding site, and origin of second strand DNA synthesis, certain preferred retroviral vector constructs which are provided herein also comprise a packaging signal, as well as one or more nucleic acid molecules (*e.g.,* heterologous sequences), each of which is discussed in more detail below.

Within one aspect of the invention, retroviral vector constructs are provided which lack both *gag/pol* and *env* coding sequences. As utilized herein, the phrase "lacks *gag/pol* or *env* coding sequences" should be understood to mean that the retroviral vector does not contain at least 20, preferably at least 15, more preferably at least 10, and most preferably at least 8 consecutive nucleotides which are found in

gag/pol or *env* genes, and in particular, within *gag/pol* or *env* expression cassettes that are used to construct packaging cell lines for the retroviral vector construct.

Packaging cell lines suitable for use with the above-described retroviral vector constructs may be readily prepared (see U.S. Serial No. 08/240,030, filed May 9, 1994; see also WO 92/05266 and WO 95/30763), and utilized to create producer cell lines (also termed vector cell lines or "VCLs") for the production of recombinant vector particles. Within particularly preferred embodiments of the present invention packaging cell lines are made from human (*e.g.*, HT1080 cells) or mink parent cell lines, thereby allowing production of recombinant retroviruses that are capable of surviving inactivation in human serum (*e.g.*, they are "complement resistant").

In addition, for those vectors which are derived from viruses that carry regulatory genes (such as lentiviral vectors and vectors derived from foamy or spuma-viruses), such regulatory genes can be inserted into either a packaging cell line or vector as desired or necessary.

Utilizing the methods of the present invention as disclosed herein, packaging cell lines that produce greater than recombinant retroviral particles at titers greater than 10^7 cfu/ml may readily be obtained. In addition, it should be noted that such titers are generally obtained from titer assays on HT1080 cells, which produce a three-fold lower titer than titers obtained on murine 3T3 cells.

ANTI-ARTHRITIC AGENTS

As noted above, a wide variety of anti-arthritic agents can be delivered (and expressed) by the recombinant retroviruses of the present invention, including not only proteins, but antisense and ribozymes sequences.

For example, within one embodiment of the invention the recombinant retrovirus directs the expression of a cytotoxic gene. Representative examples of cytotoxic genes include the genes which encode proteins such as ricin (Lamb et al., *Eur. J. Biochem. 148*:265-270, 1985), abrin (Wood et al., *Eur. J. Biochem. 198*:723-732, 1991; Evensen et al., *J. of Biol. Chem. 266*:6848-6852, 1991: Collins et al., *J. of Biol.*

Chem. 265:8665-8669, 1990; Chen et al., *Fed. of Eur. Biochem Soc. 309*:115-118, 1992), diphtheria toxin (Tweten et al., *J. Biol. Chem. 260*:10392-10394, 1985), cholera toxin (Mekalanos et al., *Nature 306*:551-557, 1983; Sanchez & Holmgren, *PNAS 86*:481-485, 1989), gelonin (Stirpe et al., *J. Biol. Chem. 255*:6947-6953, 1980), pokeweed (Irvin, *Pharmac. Ther. 21*:371-387, 1983), antiviral protein (Barbieri et al., *Biochem. J. 203*:55-59, 1982; Irvin et al., *Arch. Biochem. & Biophys. 200*:418-425, 1980; Irvin, *Arch. Biochem. & Biophys. 169*:522-528, 1975), tritin, Shigella toxin (Calderwood et al., *PNAS 84*:4364-4368, 1987; Jackson et al., *Microb. Path. 2*:147-153, 1987), and Pseudomonas exotoxin A (Carroll and Collier, *J. Biol. Chem. 262*:8707-8711, 1987).

Within other aspects of the invention, recombinant retroviruses are provided which direct the expression of a gene product that activates a compound with little or no cytotoxicity (*i.e.*, a "prodrug activating or converting enzyme") into a toxic product. Representative examples of such gene products include varicella zoster virus thymidine kinase (VZVTK), herpes simplex virus thymidine kinase (HSVTK) (Field et al., *J. Gen. Virol. 49*:115-124, 1980; Munir et al., *Protein Engineering 7*(1):83-89, 1994; Black and Loeb, *Biochem 32*(43):11618-11626, 1993), and *E. coli.* guanine phosphoribosyl transferase (*see* U.S. Patent Application Serial No. 08/155,944, entitled "Compositions and Methods for Utilizing Conditionally Lethal Genes," filed November 18, 1993; *see also* WO 93/10218 entitled "Vectors Including Foreign Genes and Negative Selection Markers", WO 93/01281 entitled "Cytosine Deaminase Negative Selection System for Gene Transfer Techniques and Therapies", WO 93/08843 entitled "Trapped Cells and Use Thereof as a Drug", WO 93/08844 entitled "Transformant Cells for the Prophylaxis or Treatment of Diseases Caused by Viruses, Particularly Pathogenic Retroviruses", and WO 90/07936 entitled "Recombinant Therapies for Infection and Hyperproliferative Disorders.")

Other gene products which may also be expressed by the recombinant retroviruses of the present invention include interleukin-1 antagonists such as Interleukin-1 Receptor Antagonist Protein or "IRAP" (see Genebank Accession Nos. X52015, X53296, X64532 and M63099, Eisenberg *et al., Nature 343*(6256):341-346,

1990, Carter *et al., Nature 344*(6267):633-638, 1990, Carrier *et al., Cytokine 4*(2):83-89, 1992, Eisenberg *et al., PNAS USA 88*(12):5232-5236, 1991), type II IL-1 receptors (*e.g.*, Genebank Accession Nos. X59770, McMahan *et al., EMBO, J 10*(10):2821-2832 (1991)), soluble or mutant type I IL-1 receptors (*see generally*, Genebank Accession Nos. M27492, X16896, Gubler, *Nucleic Acids Res. 17*(23):10114, 1989, Sims et al., PNAS USA 86(22):8946-8950, 1989, Dower *et al., J. Immunol. 142*:4314-4320, 1989); Poxvirus "decoy" receptors (naturally occurring proteins similar to the type II "decoy" receptor, which are used by the virus to help regulate local immune responses); anti-IL-1 antibodies which bind to and neutralize the biological activity of IL-1; and antisense or ribozyme molecules which target interleukin-1.

Other useful gene products include TNF antagonists such as sTNFR; sTNFR/Ig (*e.g.*, a construct of the extracellular portion of the TNF-R which is linked to an immunoglobulin constant region); anti-TNF antibodies (*e.g.*, antibodies which bind to and neutralize the biological activity of TNF); and antisense or ribozyme molecules which target TNF (*see generally* Nophar *et al., EMBO J. 9*(10):3269-3278, 1990, Gray *et al., PNAS USA 87*:7380-7384, 1990; Smith *et al., Science 248*(4958):1019-1023, 1990; Genebank Accession Nos. X55313, M60275, M37764 and M32315).

Other gene products which can also be utilized for treating or preventing arthritis include gene products which switch an immune response from helper 1 (inflammatory) to a helper 2 response (*e.g.*, IL-4 (Klein *et al., Immunogenetics 41*(1):57, 1995, Arai *et al., J. Immunol. 142*(1):274-282, 1989, Yokota *et al., PNAS USA 83*(16):5894-5898, 1986, Genebank Accession Nos. X81851, M23442 and M13982), IL-10 (Kube, *Immunogenetics 45*(1):82-82, 1996, Vieira *et al., PNAS USA 88*(44):1172-1176, 1991, Genebank Accession Nos. X78437 M57627 and U16720), and IL-13 (Dolganov *et al., Blood 87*(8):3316-3326, 1996, Smirnov *et al., Gene 155*(2):277-281, 1995, McKenzie *et al., J. Immunol. 150*:5436-5444, 1993, Genebank Accession Nos. L13209, U31120 and U10307)), as well as gene products which reverse synovial hyperproliferation or joint damage (*e.g.*, prodrugs as discussed above, or metalloprotease inhibitors such as TIMP-1 or -2).

Yet other gene products that may be particularly useful for treating certain forms of arthritis, such as osteoarthritis, include chrodrocyte growth factors and stimulators such as IGF-1 (EP 0155655, Tobin *et al., Mol. Endocrinol. 4*(12):1914-1920, 1990, Genebank Accession Nos. A29117, E00602, A04811, A04812, AA542914 AA456717 and M37484); TGF-β (JP 1986219395, EP 0542679, EP 0433225, Kim *et al., J. biol. Chem. 264*(1):402-408, 1989, Genebank Accession Nos. E00973 A18277 and A23751); and human growth hormone.

PREPARATION AND FORMULATION OF RECOMBINANT RETROVIRUSES

Recombinant retrovirus of the present invention is preferably concentrated, purified, and formulated so as to preserve the infectious capability of the virus upon reconstitution (see generally, U.S. Serial No. 08/153,342 and PCT Publication Nos. WO 95/16582 and WO 95/16852). More specifically, recombinant retrovirus of the present invention can be purified and concentrated as generally described in PCT publication No. WO 95/16582, and then preserved by adding a sufficient amount of a formulation buffer to the media containing the recombinant retrovirus, in order to form an aqueous suspension. Within certain embodiments of the invention, the formulation buffer is an aqueous solution that contains a saccharide, a high molecular weight structural additive, and a buffering component in water. (As utilized within the context of the present invention, a "buffering compound" or "buffering component" should be understood to refer to a substance that functions to maintain the aqueous suspension at a desired pH. The aqueous solution may also contain one or more amino acids.)

Within other embodiments, the recombinant retrovirus can be preserved prior to the addition of the formulation buffer. Briefly, the crude recombinant retrovirus is clarified by passing it through a filter, and then concentrated, such as by a cross flow concentrating system (Filtron Technology Corp., Nortborough, MA). Within one embodiment, DNase is added to the concentrate to digest exogenous DNA. The digest is then diafiltrated to remove excess media components and establish the recombinant

retrovirus in a more desirable buffered solution. The diafiltrate is then passed over a Sephadex S-500 gel column and a purified recombinant retrovirus is eluted. A sufficient amount of formulation buffer is added to this eluate to reach a desired final concentration of the constituents and to minimally dilute the recombinant retrovirus, and the aqueous suspension is then stored, preferably at -70°C or immediately dried. As noted above, the formulation buffer can be an aqueous solution that contains a saccharide, a high molecular weight structural additive, and a buffering component in water. The aqueous solution may also contain one or more amino acids.

The crude recombinant retrovirus can also be purified by ion exchange column chromatography. This method is described in more detail in U.S. Patent Application Serial No. 08/093,436. In general, the crude recombinant retrovirus is clarified by passing it through a filter, and the filtrate loaded onto a column containing a highly sulfonated cellulose matrix. The recombinant retrovirus is eluted from the column in purified form by using a high salt buffer. The high salt buffer is then exchanged for a more desirable buffer by passing the eluate over a molecular exclusion column. A sufficient amount of formulation buffer is then added, as discussed above, to the purified recombinant retrovirus and the aqueous suspension is either dried immediately or stored, preferably at -70°C.

The aqueous suspension in crude or purified form can be dried by lyophilization or evaporation at ambient temperature. Specifically, lyophilization involves the steps of cooling the aqueous suspension below the glass transition temperature or below the eutectic point temperature of the aqueous suspension, and removing water from the cooled suspension by sublimation to form a lyophilized retrovirus. Briefly, aliquots of the formulated recombinant retrovirus are placed into an Edwards Refrigerated Chamber (3 shelf RC3S unit) attached to a freeze dryer (Supermodulyo 12K). A multistep freeze drying procedure as described by Phillips et al. (Cryobiology 18:414, 1981) is used to lyophilize the formulated recombinant retrovirus, preferably from a temperature of -40°C to -45°C. The resulting composition contains less than 10% water by weight of the lyophilized retrovirus. Once lyophilized, the recombinant retrovirus is stable and may be stored at -20°C to 25°C.

ADMINISTRATION

As noted above, the recombinant retrovirus which directs the expression of one or more anti-arthritic agents is then administered in a therapeutically effective amount to a patient in order to treat or prevent the arthritis. As utilized within the context of the present invention, it should be understood that efficacious administration of the recombinant retroviruses described herein may be assessed in several ways, including: (1) by preventing or lessening the pathological and/or clinical symptoms associated with the arthritis; (2) by downregulating the inflammatory response which initiates the inflammatory cascade and results in pain, swelling and cartilage destruction; (3) by inhibiting the proliferation of synoviocytes; (4) by decreasing the production/activity of matrix metalloproteinases; and (5) by inhibiting blood vessel formation.

Briefly, a wide variety of assays may be utilized to determine whether a particular therapeutic regimen or anti-arthritic agent will prevent or lessen the pathological and/or clinical symptoms associated with arthritis, prior to use in humans. Examples include animal models such as adjuvant-induced arthritis (see Kaklamanis, *Clin. Rheumatol. 11*:41-47, 1992; Houri and O'Sullivan, *Curr. Opin. Rheumatol. 7*:201-205, 1995), rat streptococcal cell wall arthritis, collagen induced arthritis (Trentham et al., *J. Exp. Med. 146*:857, 1977; Stuart et al., *Ann. Rev. Immunol. 2*:199-218, 1984; and Wooley et al., *J. Exp. Med. 154*:688, 1981), and antigen-driven arthritis (Dumonde and Glynn, *Br. J. Exp. Pathol. 43*:373-383, 1962). Within such methods, disease progression may be monitored through measurements of inflammation (*e.g.*, volume of affected paws, or the diameter of affected joints), through radiologic evaluation, or by histological assessment of joint damage.

Other assays for determining efficacious agents and administration may likewise be readily determined prior to human clinical trials. For example, downregulation of the inflammatory response may be readily determined by assaying synovial fluid from animals which have been treated with the anti-arthritic agent (or recombinant retrovirus) (*e.g.*, by white cell counts or neutrophil activity). Similarly,

inhibition of synoviocyte proliferation may be readily determined by assaying the effect of the anti-arthritic agent or recombinant retroviruson on tritiated thymidine incorporation of synoviocytes, or by directly scoring pannus formation. Assessment of matrix metalloproteinase inhibition may be accomplished by measuring inhibition of collagenase activity utilizing commercially available kits (*e.g.*, available from Boehringer Mannheim). Inhibition of blood vessel formation may likewise be readily determined based upon standard assays, for example, the Chick Chorioallantoic Membrane or "CAM" assay (see the Examples below). The above assays may be readily practiced by one of ordinary skill in the art given the disclosure provided herein (see generally, Kelley et al., eds., Textbook of Rheumatology (4th edition), W.B. Saunders Company, Philadelphia, 1993).

Typically, one or more injections of the recombinant retrovirus is administered at a titer of greater than 10^7 cfu/ml, 10^8 cfu/ml, 10^9 cfu/ml, or, 10^{10} cfu/ml.

The recombinant retrovirus may be delivered at a variety of sites an locations (*e.g.*, intravenously) in order to systemically treat or prevent the arthritis, or locally (*e.g.*, intraarticularly by direct injection or under artheroscopic guidance) to selected joints. Within certain embodiments, prior to administration of the recombinant retrovirus or anti-arthritic agent, the synovial fluid may be aspirated from the joint, and the joint flushed or otherwise treated with one or more enzymes capable of breaking down extracellular matrix proteins. Representative examples of such enzymes include collagenases, elastase, hyaluronidase, dispase, papain, pronase, and trypsin.

Irrespective of the route or method of administration, it is generally preferred to administer a complement resistant recombinant retrovirus in order to help prevent or limit the amount of retrovirus that is degraded due to the action of complement. In addition, it is generally preferred that the recombinant retroviral preparation be 'clean', in that it should contain low levels of foreign antigens (*e.g.*, fetal calf serum) and low levels of endotoxin.

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

EXAMPLES

EXAMPLE 1

CONSTRUCTION OF RETROVIRAL VECTOR BACKBONES

This example describes the construction of retroviral vectors which have decreased sequence homology to the retroviral gag/pol and envelope expression constructs. In addition, two stop codons are introduced in the DNA sequence of the packaging signal sequence in order to increase the safety of these vectors. These modifications are generally described in Figure 1.

A. Substitution of Nonsense Codons in the Extended Packaging Sequence (Ψ+)

This example describes modification of the extended packaging signal (Ψ+) by PCR on the template KT-1 (*see* WO 95/16582) using primers that introduce two stop codons in the extended packaging signal sequence. In particular, the template pKT-1 contains the modification ATT at the normal ATG start site of *gag* (position 621-623 of SEQ ID NO: 1). Here the start site was further modified to the stop codon, TAA, and an additional stop codon TGA was added to replace the codon TTA at position 645-647 of SEQ ID NO: 1.

Briefly, two sets of PCR reactions were carried out on pKT1, each introducing one stop codon. The primers for the PCR were designed such that the two PCR products had overlapping regions and a splice-overlap extension PCR (SOE-PCR) was carried out with the two PCR products in order to combine the two introduced stop codons on one strand. The first set of oligonucleotides introducing the change from ATT to TAA were 5'-GGG-AGT-GGT-AAC-AGT-CTG-GCC-TTA-ATT-CTC-AG (SEQ ID NO: __) and 5'-CGG-TCG-ACC-TCG-AGA-ATT-AAT-TC (SEQ ID NO: __) and the second set of oligonucleotides introducing the change from TTA to TGA were 5'CTG-GGA-GAC-GTC-CCA-GGG-ACT-TC (SEQ ID NO: __) and 5'GGC-CAG-ACT-GTT-ACC-ACT-CCC-TGA-AGT-TTG-AC (SEQ ID NO: __). The flanking primers of the final 708 base pair PCR product introduced the *Aat*II and the *Xho*I sites, at the 5' and 3', respectively.

The ends of the 708 base pair product were blunted and phosphorylated and the product introduced into the *Sma*I and *Eco*RV digested vector pBluescript SK- (Stratagene, San Diego, Calif.). The resulting plasmid was designated pBA-2, and is shown diagrammatically in Figure 1.

B. Removal of Retroviral Sequences Upstream and Downstream from the 3' LTR and Upstream and within the 5' LTR

Retroviral envelope sequence was removed upstream of the 3' LTR between the *Cla*I site and the TAG stop codon of the envelope coding sequence. The DNA sequence was modified by PCR such that the TAG stop codon was replaced by a *Cla*I site and the 97 nucleotides upstream from this new *Cla*I site to the original *Cla*I site were deleted, as well as the 212 base pairs of retroviral sequence downstream of the 3' LTR.

Briefly, the following two oligonucleotides were used for the PCR: 5'-CATCGATAAA ATAAAAGATT TTATTTAGTC (SEQ ID NO: __) and 5'-CAAATGAAAG ACCCCCGCTG AC (SEQ ID NO: __) and the template was pKT1. The PCR product was cloned into pPCRII (Invitrogen, San Diego, Calif.) using the TA cloning kit (Invitrogen, San Diego, Calif.) and called pBA-1.

Subsequently, pBA-2 (described in section A above) was digested with *Xba*I and *Aat*II which deleted a part of the multiple cloning site and into this linearized vector the 780 base pair fragment from *Nhe*I to *Aat*II from pKT1 was cloned, resulting in the plasmid pBA-3. This plasmid pBA-3 combined the shortened 5' LTR with the above described packaging region including the two introduced stop codons.

Subsequently, pBA-1 was digested with *Cla*I and *Apa*I resulting in a 640 base pair fragment that was cloned into the *Cla*I and *Apa*I digested pBA-3 resulting in the plasmid pBA-4. This plasmid combines the above described 5' LTR and the packaging signal with the 3' LTR.

Subsequently, pBA-4 was digested with *Apa*I and *Eco*RI, ends blunted and religated in order to remove extraneous 3' polylinker sites, resulting in plasmid pBA-5a.

Subsequently, pBA-5a was cut with NotI (blunted) and *Eco*RI and introduced into *Sma*I and *Eco*RI digested pUC18 (GIBCO/BRL, Gaithersburg, MD) resulting in pBA-5b. This construct moved the retroviral vector from a pBluescript into an alternate pUC18 vector.

EXAMPLE 2

INSERTION OF GENES OF INTEREST INTO CROSS-LESS RETROVIRAL VECTOR BACKBONE pBA-5B

A. Construction of Recombinant Vector Containing HSV-TK

In order to insert a gene of interest such as HSV-TK into a retroviral vector backbone, the HSV-TK gene is first retrieved by digesting pBH-1 (PCT#UU 091-02805) with *Xho*I and *Eco*RI, resulting in a 1.2 kb fragment. The neomycin gene driven by the SV40 promoter was also retrieved by digesting pKT1 with *Eco*RI and BstBI resulting in a 1.3 kb fragment. Both fragments were cloned into a *Xho*I and *Cla*I digested pBA-5b resulting in a retroviral vector designated "pMO-TK".

B. Construction of Recombinant Vectors Containing Tissue Inhibitor of Metalloproteinase-I

The cloning of a human TIMP-1 gene into recombinant retroviral vectors can be accomplished by PCR amplification using two 38-mer oligonucleotides. The oligonucleotides contain 25 nt corresponding to the TIMP-1 gene template, plus additional 5'-flanking sequences comprising a *Pme* I recognition site and buffer sequence, as follows:

HTIMP-F:

(Sequence ID No.: __)

5'-TATATGTTTAAACCACCAGAGAACCCACCATGGCCCCC-3'

HTIMP-R:

(Sequence ID No.: __)

5'-ATATAGTTTAAACCCACTCCGGGCAGGATTCAGGCTAT-3'

PCR amplification using these primers, a TIMP-1 cDNA clone (Docherty et al., *Nature 318*:66, 1985) as template and Thermalase thermostable DNA polymerase, is performed in a reaction containing 1.5 mM $MgCl_2$ provided by the supplier, 5% DMSO, and Hot Start Wax beads according to the following protocol:

Temperature	Time (Min.)	No. Cycles
94	2.0	1
94	0.5	
50	0.5	5
72	2.0	
94	0.5	
55	0.5	25
72	2.0	
72	10.0	1

Following amplification, the approximately 700 bp TIMP-I gene amplicon is digested with *Pme* I (or *Xho* I and *Cla* I) and ligated into a retroviral vector backbone (pKT-1L, pKT-3BL, pKT-3BCL or pBA-5b) that have been digested with Pme I and treated with CIAP. Proper orientation of the insert sequence is determined by restriction endonuclease digests. Production of packaged vector particles is accomplished using the methods described below. Other related gene products from this group are readily cloned using these approaches and oligonucleotide primers which contain appropriate restriction sites, and are specific for their respective gene.

EXAMPLE 3

CONSTRUCTION OF VARIOUS *GAG/POL* EXPRESSION PLASMIDS WITH PARTIALLY OR COMPLETELY REDUCED SEQUENCE OVERLAP TO THE CROSS-LESS RETROVIRAL BACKBONE AND ENVELOPE

This example describes several modifications of the MoMLV gag/pol expression plasmid pSCV10 (PCT/US91/06852, WO 92/05266) resulting in decreased or eliminated sequence homology to the retroviral backbone and envelope expression constructs.

A. Creation of New Codons for the 5'Gag

This example describes the *gag/pol* expression plasmid cassette that contains wobbled non-coding sequences upstream from the gag start site, thereby reducing recombination potential between the *gag/pol* expression element and the extended packaging signal of a retroviral vector construct, and inhibiting co-packaging of the *gag/pol* expression element along with the retroviral vector. In order to construct such an expression cassette a 406 bp DNA fragment with a *ClaI* site at the 5' end (underlined) and a *NarI* site at the 3' end (underlined) was synthesized by Operon (Operon Technologies Inc, Alameda CA). The sequence of the 406 bp DNA fragment was verified and is provided in Table 1. The synthesized DNA was transferred to a shuttle plasmid as a *ClaI-NarI* fragment to create the plasmid pWOB.

Table 1

ATCGATACCATGGGGCAAACCGTGACTACCCCTCTGTCCCTCACACTGGGCC
ATTGGAAGGACGTGGAAAGAATTGCCCATAATCAAAGCGTGGACGTCAAAA
AACGCAGGTGGGTGACATTTTGTAGCGCCGAGTGGCCCACATTCAATGTTG
GCTGGCCTAGGGATGGAACTTTCAATCGCGATCTGATTACTCAAGTGAAAA
TTAAAGTGTTCAGCCCCGGACCCCACGGCCATCCCGATCAAGTTCCTTATAT
TGTCACATGGGAGGCTCTCGCTTTCGATCCACCACCTTGGGTGAAACCATTC
GTGCATCCCAAACCACCTCCACCCCTCCCACCCAGCGCTCCTAGCCTGCCCT

TGGAGCCCCCACGAAGCACACCACCCAGGAGCAGCTTGTACCCTGCTCTGA CCCCCAGCCTCGGCGCC (SEQ ID NO: __)

The *ClaI-NarI* fragment from pWOB was isolated by *ClaI-NarI* digest, and the 406 bp fragment cloned into the *ClaI-NarI* site of pSCV10 to create the plasmid pSCV10/wob (-NarI fragment) which resulted in the loss of the 481 bp *NarI-NarI* fragment just downstream of the wobbled *ClaI-NarI* fragment.

B. Creation of a 5' truncated *gag/pol* construct without MoMLV sequence upstream of the start codon in pSCV10

This example describes the *gag/pol* expression plasmid cassette that eliminated the MoMLV sequence upstream of the ATG start codon in order to prevent sequence overlap to the retroviral backbone.

Briefly, a new *ClaI* site followed by an ideal Kozak translational start sequence was introduced upstream of the start codon of the *gag/pol* construct pSCV10 by PCR using the forward primer 5' CGAATCGATA CCATGGGCCA GACTGTTACC AC (SEQ ID NO: __) (the *ClaI* site is underlined) and the reverse primer 5' CATTCTGCAG AGCAGAAGGT AAC (SEQ ID NO: __) containing a restriction site for *PstI* (underlined). The PCR fragment was digested with *ClaI* and *PstI* and the 131 bp fragment cloned into pSCV10 replacing the existing *ClaI-PstI* DNA fragment to create the plasmid pSCV10/5'truncated g/p.

C. Creation of a 5' truncated *gag/pol* construct without MoMLV sequence upstream of the start codon in pCI

This example describes the construction of the 5' truncated *gag/pol* construct analogous to that described under section B above in the pCI (Promega Corp, Madison, WI) vector backbone.

Briefly, fragments were prepared for a three-way ligation as follows: pCI was digested with *SmaI* and *NotI* and the 4 kb fragment was isolated. pSCV10 was digested with *XhoI* and *NotI* and the 4.7 kb fragment was isolated. pSCV10/5' truncated

g/p as described in section B was digested with *ClaI*, filled in with Klenow to blunt, then digested with *XhoI* and the 0.95 kb fragment was isolated. These three fragments were then ligated together to give the final plasmid pCI/5'truncated g/p.

D. Creation of a 5' truncated and wobbled *gag/pol* construct in pCI

This example describes the construction of the 5' truncated and wobbled *gag/pol* construct in the pCI vector where the 5' truncation as described in section C and the wobbled gag sequences as described in section A were combined.

Briefly, the wobbled *gag/pol* sequence (0.47 kb) was retrieved from plasmid pSCV10/wob (-NarI fragment) as described in section A above by digestion with *ClaI* and *XhoI*. This fragment was cloned into the *ClaI-XhoI* digested pBluescript SK- (Stratagene, San Diego, CA) to create pBluescript/wob (- NarI fragment). This plasmid was digested with *EcoRI* and *NarI* to retrieve the wobbled gag sequence and the *EcoRI-NarI* fragment cloned into *the EcoRI-NarI* digested pCI/5' truncated g/p described in section C above in order to create pCI/5'truncated wob g/p.

E. Creation of a 5' and 3' truncated *gag/pol* construct in pCI and pSCV 10

This example describes the construction of the 5' and 3' truncated *gag/pol* construct in the pCI vector where the 5' truncation as described in section C is combined with the following 3'truncation upstream of the stop codon eliminating the DNA sequence coding for the last 28 amino acids of the pol protein.

Briefly, the plasmid pCI/5'truncated g/p described in section C was linearized with the restriction enzyme *SmaI* which is located 84 bases upstream of the *gag/pol* termination codon in the open reading frame of *gag/pol*. The linearized plasmid was ligated to the oligonucleotide 5' TAAGCGGCCG CTTA (SEQ ID NO: __). This oligonucleotide is self-complementary and forms a palindromic duplex containing a TAA termination codon and a *NotI* restriction endonuclease site. After ligation of 100ng vector and 5μM oligo in the presence of T4 DNA ligase, the reaction was purified by GeneClean and digested with *SmaI* to recut any vector that did not contain an insert. The reaction was used to transform XL1 Blue *E. coli* (Stratagene, San

Diego, CA) and plasmid DNA from a correct clone was then digested with *NotI*. *NotI* cuts in the inserted oligonucleotide as well as just upstream of the SV40 termination/polyadenylation site of the pCI vector. The digested plasmids were purified by Geneclean and religated to recircularize. Bacteria were transformed and clones were identified which deleted the sequences between the *NotI* site introduced by the oligonucleotide and the *NotI* site in the pCI vector. These sequences include sequences encoding the last 28 amino acids of gag/pol as well as MoMLV sequences and vector sequences carried over from pSCV10. The resulting *gag/pol* construct was named pCI-GPM. The identically shortened *gag/pol* region was cloned by standard techniques into a pSCV10 background expression cassette. This expression plasmid was named pSCV10/5',3'tr.

F. Creation of a 5' and 3' truncated and wobbled *gag/pol* construct in pCI

This example describes the construction of the 5' and 3' truncated and wobbled *gag/pol* construct in the pCI vector combining the 5' truncation and wobbled *gag/pol* sequence from section D above with the 3'truncation described in section E above.

Briefly, pCI/5'truncated wob g/p was linearized with *SmaI* and all further steps leading to the 3'truncation of *gag/pol* were carried out as described in section E above, leading to the 5' and 3' truncated and wobbled *gag/pol* construct in pCI named pCI-WGPM.

EXAMPLE 4

CONSTRUCTION AND TESTING TITER POTENTIAL OF PCLS WITH VARIOUS COMBINATIONS OF *GAG/POL* AND *ENV* EXPRESSION CASSETTES RESULTING IN PCLS WITH VARIOUS DEGREES OF DNA SEQUENCE OVERLAP BETWEEN THE RETROVIRAL COMPONENTS: *GAG/POL*, *ENV* AND RETROVIRAL VECTOR

This example describes the production of PCLs with the *gag/pol* expression plasmid cassette pCI-WGPM described in Example 3 F and the *env* expression plasmid pCMV-b/envam (*see* WO 95/30763). PCLs with these *gag/pol* and *env* expression plasmids in conjunction with the retroviral vector derived from pBA-5b (Example 1) result in producer cell lines where sequence overlap between all three areas of homology is completely eliminated. The cell lines HT 1080 (ATCC #CCL 121) and D17 (ATCC #CCL 183) were used as parent cell lines to establish the PCLs.

Briefly, *gag/pol* plasmid pCI-WGPM was co-transfected together with a phleomycin^r expressing marker plasmid into HT 1080 and D17 cells, selected and dilution cloned as described above. HT 1080 and D17 derived clones were isolated and analyzed for intracellular p30 expression levels as described above. Results of the p30 Western are shown below in Table 2.

Table 2:

HT 1080 and D17 derived clones screened for intracellular p30 levels after introduction of *gag/pol* expression cassette pCI-WGPM

Gag/pol intermediates	**#clones screened for p30**	**#clones positive for p30 - (%)**	**p30 expression levels**
D-17g/p (pCI-WGPM)	82	36 (44%)	3-4 clones have p30 levels comparable to D17 4-15
HT-1080g/p (pCI-WGPM)	96	26 (27%)	3-4 clones have p30 levels that are comparable to HTSCV21

The 12 HT 1080 and 22 D17 gag/pol intermediates with the highest p30 expression levels were analyzed for titer potential as described above. The titer results for the HT 1080 gag/pol intermediates are shown below in Table 3.

Table 3:

Transient β-gal titers from transduced pools of HT 1080 gag/pol intermediates (pCI-WGPM)

Clone#	Transient β-gal titer (CFU/ml)	x-fold titer decrease (HTSCV21:HT 1080 gag/pol intermediate)
10	217	>9
12	28	>71
23	670	>3
29	565	>4
34	950	>2
35	398	>5
45	280	>7
52	670	>3
53	600	>3
71	590	>3
86	480	>4
87	55	>36
HTSCV21	>2000	

The Galactolight readout for HTSCV10 was out of the range with >2000, therefore the above shown decrease in titer potential is likely to be higher. The titer results for the D17 gag/pol intermediates are shown below in Table 4.

Table 4:

Transient β-gal titers from transduced pools of D17gag/pol (D17 g/p) intermediates and stable β-gal titers from transduced and G-418 selected pools of D17gag/pol intermediates

Clone#	Transient β-gal titer (CFU/ml)	x-fold decrease (D17 4-15:D17g/p inter.)	Stable β-gal titer (CFU/ml)	x-fold decrease (D17 4-15: D17g/p inter.)	Transient β-gal (CFU/ml)	x-fold decrease (D17 4-15:D17g/p inter.)
1	0	-				
3	40	>100				
6	20	>200				
14	10	>400				
21	10	>400				
22	1380	>3	800	>5	2.1E4	9
27	30	>133				
41	100	>40				
47	30	>133	730	>6	90	2111
48	30	>133				
49	500	>8	680	>6	9.4E3	20
50	140	>29				
51	10	>400				
56	600	>7	320	>13	1.8E3	105
57	230	>17			1.3E3	146
60	380	>11	580	>7	1.0E4	190
65	0	-				
66	470	>9	330	>12	1.1E3	172
70	30	>133				
73	320	>13	1.05E4	0		
76	40	>100				
79	20	>200				
D17 4-15	>4000*		>4000*		1.9E5	

*=out of range

The titer potential measured within the range indicates decreases in titer potential of 10-200 fold in most clones.

A total of 6 D17 and 4 HT 1080 gag/pol intermediates with the highest titer potential were co-transfected with the *env* expression plasmid pCMV-b/envam, pools selected and dilution cloned as described above. Several hundred HT 1080 and D17 derived PCL clones named HAIIwob and DAIIwob, respectively, were isolated and analyzed for titer potential.

Briefly, several rounds of titer potential analysis were carried out using various retroviral vectors. The DA or HA PCL (PCT #WO 92/05266) controls were included as a reference for high titer potential PCLs. In the first round, the PCL clones were transduced in 24-well plates with the β-gal coding retroviral vector DX/ND7 (WO 95/16852) at an moi of 5-10 in the presence of 8 μg/ml polybrene, transient supernatants harvested, filtered (0.45 μm), HT 1080 target cells transduced and transient β-gal titer determined using a standard Galactolight transfer of expression procedure described previously. In the second round, the same transduction assay as described for the first round was repeated with the top clones from round one using standardized PCL cell numbers. In the third round, the top clones from round two were used to transduce with several retroviral vectors, supernatant from transient and stable pools harvested, filtered, HT 1080 target cells transduced and titers determined.

Data on the titer potential analysis of the first and second round of screening is shown below in Table 5 on a small selection of representative DAII and HAII PCL clones.

Table 5:

Transient β-gal titer on VCL pools from transduced HAII and DAII PCLs determined by Galactolight readout.

Clone#	Transient β-gal titer (Galactolight, light units)	x-fold decrease in titer potential (DA:DAIIwob or HA:HAIIwob)
D-17 based PCLs called DAIIwob		
DAIIwob (pCI-WGPM#60):		
7	21	27
11	6	93
21	2	279
30	14	40
33	51	11
41	30	19
DA	558	
DAIIwob (pCI-WGPM#22):		
5	148	0
8	28	5
28	14	11
56	15	10
78	39	4
97	10	15
DA	153	
HT-1080 based PCLs called HAIIwob		
HAIIwob (pCI-WGPM)#34:		
4	8	128
7	9	114
35	7	147
43	4	257
53	5	205
65	9	114
66	10	103
77	19	54
79	6	171
80	4	257
95	4	257
105	2	500
115	9	114
118	6	171
HA	1026	

The best DAIIwob PCL clone shows a 4-fold reduction in titer but most clones show >10-fold reduction. The best HAIIwob PCL clone shows a 50-fold reduced titer potential and most HAIIwob clones have >100-fold reduced titer potential. In general, the DAII wob and HAIIwob PCL clones gave in average about a 5-50 fold lower titer potential when compared to DAII and HAII PCLs.

EXAMPLE 5

TRANSDUCTION OF PACKAGING CELL LINES HX AND DA WITH THE VECTOR CONSTRUCTS

To increase retroviral titers produced from packaging cells, it is preferable to transduce another packaging cell line with retroviral vectors transiently produced from another cell line. For example, DA (an amphotropic cell line derived from D 17 cells ATCC No. 183, WO 92/05266) cells are seeded at 5.0 x 10^5 cells/10 cm tissue culture dish in 10 ml DMEM and 10% FBS, 4 μg/ml polybrene (Sigma, St. Louis, MO) on day 1. On day 2, 3.0 ml, 1.0 ml and 0.2 ml of the freshly collected virus-containing HX media is added to the cells. The cells are incubated with the virus overnight at 37°C. In those instances when the retroviral also encodes a selectable marker, *e.g.*, neomycin resistance, on day 3, the media is removed and 1.0 ml DMEM, 10% FBS with 800 μg/ml G418 is added to the plate. Only cells that have been transduced with the vector and expressing the selectable marker will survive. In the case of neomycin resistance, G418 resistant pools can be generated over a period of a week. Typically, a pool of cells is then dilution cloned by removing the cells from the plate and counting the cell suspension, diluting the cells suspension down to 10 cells/ml and adding 0.1 ml to each well (1 cell/well) of a 96 well plate (Corning, Corning, NY). Cells are incubated for 14 days at 37°C, 10% CO_2. As many as twenty-four clones are selected and expanded up to 24 well plates, 6 well plates then 10 cm plates at which

time the clones are assayed for expression and the supernatant are collected and assayed for viral titer.

The titer of the individual clones is determined by infection of HT1080 cells, (ATCC No. CCL 121). On day 1, 5.0 x 10^5 HT1080 cells are plated on each well of a 6 well microtiter plate in 3.0 ml DMEM, 10% FBS and 4 μg/ml polybrene. On day 2, the supernatant from each clone is serially diluted 10 fold and used to infect the HT1080 cells in 1.0 ml aliquots. The media is replaced with fresh DMEM, 10% FBS media, and the cells incubated with the vector overnight at 37°C, 10% CO_2. On day 3, selection of transduced cells is performed (assuming the presence of a selectable marker in the recombinant vector) by replacing the media with fresh DMEM, 10% FBS media containing the appropriate selection agent, for instance, 800 μg/ml G418 in the case of neomycin resistance. Cells are incubated at 37°C, 10% CO_2 for 14 days at which time G418 resistant colonies are scored at each dilution to determine the viral titer of each clone as cfu/ml.

Using these procedures, cell lines are derived that produce greater than or equal to 1.0 x 10^6 cfu/ml in culture. In addition, as those in the art will appreciate, in those instances where selectable markers other than drug resistance, or when no selectable marker is encoded by the recombinant vector, other titer methods, such as antibody-based assays, PCR assays, etc., may be employed.

The packaging cell line HX is transduced with vector generated from the DA vector producing cell line in the same manner as described for transduction of the DA cells from HX supernatant.

Transduction of the DA or HX cells with vectors lacking a *neo* selectable marker was performed as described above. However, instead of adding G418 to the cells on day 3, the cells are cloned by limiting dilution. Titer is analyzed as described above.

EXAMPLE 6

DETERMINATION OF PROTEIN EXPRESSION

A. Western Blotting

i. Preparation of RIPA-Lysates

Cells transduced with the recombinant vectors of this invention are harvested with trypsin/EDTA and washed twice with cold PBS. The cells are lysed with 50 to 400 ml RIPA buffer (10 mM Tris-HCl pH 7.0; 1% (v/v) NP40; 0.1% (w/v) SDS; and 150 mM NaCl) and incubated for 15 minutes at room temperature. The lysate is centrifuged at full speed in an Eppendorf centrifuge for 5 minutes. The supernatant is removed and stored at -20°C. A Bradford protein assay is performed to determine the total protein concentration.

ii. SDS-Page

A sample of RIPA lysate containing a total protein concentration of 20 mg and a commercial molecular weight (MW) marker (Amersham, Chicago, IL) are mixed 1:1 with 2x sample buffer (4% (w/v) SDS, 50 mM Tris-HCl pH 7.0, 24% (v/v) glycerol, 0.1% (w/v) bromophenol blue and 0.05% b-mercaptoethanol) and heated to 65°C for 10 minutes. After heating the sample and MW marker are placed on ice. The slots of a precast 7.5% Tris HCl based minigel (BioRad, Hercules, CA) are rinsed with running buffer (3.0 gm Tris-HCl pH 8.6, 1.0 gm SDS and 14.4 gm glycine to 1.0 L) and the sample and MW marker are loaded onto the gel. Approximately 70 to 120V is applied to the gel until the marker reaches the bottom of the gel.

iii. Transfer

The protein bands are transferred from the gel to an Immobilon-P™ membrane (Millipore, Bedford, MA) by immersing the gel in CAPS buffer pH 11.0 with 5% (v/v) methanol for 5 minutes. The Hoefer HSI TTE transfer apparatus (Hoefer

Scientific Instruments, San Francisco, CA) is used to transfer proteins from the gel to the membrane. Approximately 70V is applied to the gel for 1.5 hours.

iv. Immunodetection

The Immobilon-P™ membrane is blocked for 30 minutes at room temperature with 0.5% BM blocking reagent (Boehringer Mannheim, Chicago, IL) containing 3% BSA, heated slowly in a microwave. The membrane is then probed with a primary mouse antibody that reacts specifically with the protein being detected at a 1:2000 dilution in BM blocking solution containing a 3% BSA and incubated for 1 hour at room temperature. The membrane is washed three times with 40 ml of PBST (0.2% Tween-20 in PBS) for 10 min and a secondary goat anti-mouse, HRP-labeled antibody (Jackson, Bar Harbor, MA) at a 1:20,000 dilution in 3% BSA solution (Sigma, St. Louis, MO). The membrane is then washed three times with 40 ml of PBST for 10 minutes. After washing, the membrane is submersed in ECL developing solution (Amersham, Chicago, IL) for 1 minute and then wrapped in plastic wrap and exposed to Hyperfilm (Amersham, Chicago, IL) for 5 seconds. The film is then developed and the protein bands are analyzed.

B. Indirect Immunofluorescence Staining and FACS Analysis

The indirect immunofluorescence staining and FACS analysis method of Amlot et al. (Lymphocytes: A Practical Approach, GGB Klaus (ed.), IRL Press (1987), pp 77-72) is used to detect cell surface expression of heterologous protein antigens from recombinant vector transduced cells. Fifty microliters of primary antibody stock, 62 µg/ml of antibody that specifically react with the protein to be detected IN FACS buffer PBSA containing 0.2% BSA and 0.2% NaN_3 are added to each well in the first column of a 96 well U-shaped microtiter plate. The antibody in this column is then serially diluted to the wells of the remaining columns. Approximately 50 µl of target cell suspension, 2.0 x 10^6 to 4.0 x 10^6 cells/ml, in FACS buffer are placed in each well (the antibody final concentrations are 31, 10, 3.1, 0.31, and 0.1 µg/ml) of the microtiter plate and incubated for 15 minutes at room temperature. Following incubation, 100 µl of

FACS buffer is added to each well and the microtiter plate is centrifuged at 2000 rpm for 1 minute. The supernatant is removed and 200 µl of fresh FACS buffer is added to each well. This process is repeated 3 additional times. After the final wash, the secondary antibody solution containing 85% FACS buffer, 10% normal mouse serum, and 5% FITC labeled anti-mouse antibody is added. The microtiter plate is washed four times with fresh FACS buffer. After the final wash, 150 µl from each well is transferred to a FACS tube (Falcon 2052 tubes) and 100 µl of FACS buffer is added. The fluorescent signal is detected by a FACScan counter.

C. Simple Indirect Immuno-peroxidase Staining on Frozen Sections

Frozen tissue sections are dried at room temperature for 30 minutes. The dried tissue sections are fixed with anhydrous acetone for 15 minutes at room temperature and allowed to air dry. The sections are rehydrated with PBS for several minutes. Tissue sections are then incubated in a humid chamber for 45 to 120 minutes at room temperature with 50 µl of primary antibody that specifically reacts with the protein to be detected (Marek et al., *Cancer 67*:1377, 1991). The tissue sections are then washed with PBS and allowed to soak in PBS for several minutes. Following soaking, the sections are incubated with 50 µl of HRP conjugated anti-mouse antibody or HRP-conjugated strept-avidin (DAKO, Carpenteria, CA) for 45 to 120 minutes at room temperature. The tissue sections are then washed with PBS and allowed to soak in PBS for several minutes. Following soaking, the sections are incubated with 60 to 100 µl substrate solution containing 0.01% H_2O_2 and 3,3 diaminodenzidine (DAB) for 8 minutes or for 20 minutes with substrate solution containing 100 µl of a-ethyl carbazole (60 mg of 3-amino-9-ethylcarbazole in 25 ml DMF) in 1 ml of acetate buffer (0.68 g sodium acetate in H_2O pH 5.2 adjusted to a volume of 250 mls) and 0.01% H_2O_2. The tissue sections are washed with water for 2-5 minutes and then stained with haemotoxylin for 15 seconds. The tissue sections are then washed with water for 10 seconds and mounted in a Crystal Mount™ (Biomeda, Alameda, CA) according to manufacturer's instructions.

EXAMPLE 7

DETERMINATION OF ACTIVITY OF THE EXPRESSED PROTEIN

A. Determination of Anti-Angiogenic Activity

Anti-angiogenic activity of various polypeptides useful in accordance with certain aspects of this invention may be assayed on the chorioallantoic membrane (CAM) as described by Takigawa et al., (*Biochem. Int. 14*:357, 1987). Briefly, B16 melanoma cells are inoculated subcutaneously into the loins of C57BL/6N mice. When the tumors reach approximately 1 cm in diameter, they are excised, cut into pieces of 2 mg and placed on sterile Whatman GF/B glass fiber filter disks (6 mm in diameter; Reeve-Angel, Clifton, NJ) to which 30 µl of transduced cells have been added. They are placed upside down on the CAM of 10-day-old chicken embryos through windows made in the egg shells on day 8 of inoculation. The embryos are killed 5 days later by injection of 10% formalin in PBS. The CAM is excised, fixed in 10% formalin in PBS inverted, and examined under a stereo-microscope. Angiogenesis is assayed by measuring the number and thickness of capillaries beneath the filter. A thick capillary, a middle sized capillary, a small capillary, and 5 minute capillaries are given 3, 2, 1 and 1 points, respectively, and the average number of points is defined as the angiogenic activity. The diameters of tumors on the filters are measured in three dimensions and the tumor size is calculated as (p/6) abc mm^3 (a, b, and c: length, width, and height, respectively). Low average number of points and decreased tumor size indicates anti-angiogenic activity.

B. Determination of the *Herpes* Simplex Virus Thymidine Kinase Activity

The sensitivity of HSVTK vector transduced cells to gancyclovir may be used to determine the activity of expressed HSVTK expressed in cells treated according to the disclosed methods. Briefly, cells that are transduced with pTK-3 are seeded into six plates at a density of 2.5×10^6 per plate. In addition, untransduced CT26 and CT26 b-gal (this cell line was transduced with a virus carrying the reporter gene b-

galactosidase from *E. coli.*), are also seeded into six plates as controls. Five plates of each cell type are treated twice per day for four consecutive days with medium containing ganciclovir concentrations of 100 μg/ml, 50 μg/ml, 25 μg/ml, 12.5 μg/ml, and 6.25 μg/ml. One plate of each cell type is left untreated. Following this treatment, the cells are removed from each dish using trypsin/EDTA, resuspended in DMEM with 10% FBS and counted. Similar protocols can be employed for other pro-drug activating enzymes.

EXAMPLE 8

FORMULATION OF THE RETROVIRAL VECTOR

Crude recombinant retrovirus is obtained from a Celligan bioreactor (New Brunswick, New Brunswick, NJ) containing DA cells transformed with the recombinant retrovirus (U.S.S.N. 07/395,932) bound to the beads of the bioreactor matrix. The cells release the recombinant retrovirus into the growth media that is passed over the cells in a continuous flow process. The media exiting the bioreactor is collected and passed initially through a 0.8 micron filter then through a 0.65 micron filter to clarify the crude recombinant retrovirus. The filtrate is concentrated utilizing a cross flow concentrating system (Filtron, Boston, MA). Approximately 50 units of DNase (Intergen, New York, NY) per ml of concentrate is added to digest exogenous DNA. The digest is diafiltrated using the same cross flow system to 150 mM NaCl, 25 mM tromethamine, pH 7.2. The diafiltrate is loaded onto a Sephadex S-500 gel column (Pharmacia, Piscataway, NJ), equilibrated in 50 mM NaCl, 25 mM tromethamine, pH 7.4. The purified recombinant retrovirus is eluted from the Sephadex S-500 gel column in 50 mM NaCl, 25 mM tromethamine, pH 7.4.

The formulation buffer containing lactose was prepared at a 2x concentrated stock solution. The formulation buffer contains 25 mM tromethamine, 70 mM NaCl, 2 mg/ml arginine, 10 mg/ml human serum albumin (HSA), and 100 mg/ml lactose in a final volume of 100 mls at a pH 7.4.

The purified recombinant retrovirus is formulated by adding one part 2x lactose formulation buffer to one part S-500 purified recombinant retrovirus. The formulated recombinant retrovirus can be stored at -70°C to -80°C or dried.

The formulated retrovirus is lyophilized in an Edwards Refrigerated Chamber (3 Shelf RC3S unit) attached to a Supermodulyo 12K freeze dryer (Edwards High Vacuum, Tonawanda, NY). When the freeze drying cycle is completed, the vials are stoppered under a vacuum following a slight nitrogen gas bleeding. Upon removal, vials are crimped with aluminum seals.

The lyophilized recombinant retrovirus is reconstituted with 1.0 ml water. The infectivity of the reconstituted recombinant retrovirus is determined by a titer activity assay. The assay is conducted on HT 1080 fibroblasts or 3T3 mouse fibroblast cell line (ATCC CCL 163). Specifically, 1.0×10^5 cells are plated onto 6 cm plates and incubated overnight at 37°C, 10% CO_2. Ten microliters of a dilution series of reconstituted recombinant retroviruses are added to the cells in the presence of 4 mg/mL polybrene (Sigma, St. Louis, MO) and incubated overnight at 37°C, 10% CO_2. Following incubation, cells that have been transduced with a recombinant vector which encodes the *neo* resistance gene are selected for neomycin resistance in G418 containing media and incubated for 5 days at 37°C, 10% CO_2. Following initial selection, the cells are re-fed with fresh media containing G418 and incubated for 5 to 6 days. After final selection, the cells are stained with Commassie blue for colony detection. The titer of the sample is determined from the number of colonies, the dilution and the volume used.

EXAMPLE 9

DETECTION OF REPLICATION COMPETENT RETROVIRUSES: CO-CULTIVATION OF PRODUCER LINES AND MDH MARKER RESCUE ASSAY

In order to detect the presence of replication competent retrovirus in a vector-producing cell line, producer cells are cocultivated with an equivalent number of

Mus dunni (NIH NIAID Bethesda, MD) cells. Briefly, small scale cocultivations are performed by mixing of 5.0 x 10^5 *Mus dunni* cells with 5.0 x 10^5 producer cells and seeding the mixture into 10 cm plates (10 ml standard culture media/plate, 4 μg/ml polybrene) at day 0. Every 3 to 4 days the cultures are split at a 1:10 ratio and 5.0 x 10^5 *Mus dunni* cells are added to each culture plate to effectively dilute out the producer cell line and provide maximum amplification of RCR. On day 14, culture supernatant is harvested, passed through a 0.45 μ cellulose-acetate filter, and tested in the MdH marker rescue assay. Large scale co-cultivations are performed by seeding a mixture of 1.0 x 10^8 *Mus dunni* cells and 1.0 x 10^8 producer cells into a total of twenty T-150 flasks (30 ml standard culture media/flask, 4 μg/ml polybrene). Cultures are split at a ratio of 1:10 on days 3, 6, and 13 and at a ratio of 1:20 on day 9. On day 15, the final supernatant is harvested, filtered and a portion of each is tested in the MdH marker rescue assay.

The MdH marker rescue cell line is cloned from a pool of *Mus dunni* cells transduced with LHL, a retroviral vector encoding the hygromycin B resistance gene (Palmer et al., *PNAS 84*: 1055-1059, 1987). The retroviral vector can be rescued from MdH cells upon infection of the cells with RCR. One ml of test sample is added to a well of a 6-well plate containing 1.0 x 10^5 MdH cells in 2 ml standard culture medium (DMEM with 10% FBS, 1% 200 mM L-glutamine, 1% non-essential amino acids) containing 4 μg/ml polybrene. Media is replaced after 24 hours with standard culture medium without polybrene. Two days later, the entire volume of MdH culture supernatant is passed through a 0.45 μ cellulose-acetate filter and transferred to a well of a 6-well plate containing 5.0 x 10^4 *Mus dunni* target cells in 2 ml standard culture medium containing polybrene. After 24 hours, supernatant is replaced with standard culture media containing 250 μg/ml of hygromycin B and subsequently replaced on days 2 and 5 with media containing 200 μg/ml of hygromycin B. Colonies resistant to hygromycin B appear and are visualized on day 9 post-selection by staining with 0.2% Coomassie blue.

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

SEQUENCE LISTING

(1) GENERAL INFORMATION: COMPOSITIONS AND METHODS FOR TREATING ARTHRITIS UTILIZING GENE THERAPY

(i) APPLICANTS: MCCORMACK, JAMES E.
CHADA, SUNIL
JOLLY, DOUGLAS J.

(ii) TITLE OF INVENTION:

(iii) NUMBER OF SEQUENCES:

(iv) CORRESPONDENCE ADDRESS:
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(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 94662-8097

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

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 33,963
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8332 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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

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CCCTCAGCAG TTTCTAGAGA ACCATCAGAT GTTTCCAGGG TGCCCCAAGG ACCTGAAATG 8160

ACCCTGTGCC TTATTTGAAC TAACCAATCA GTTCGCTTCT CGCTTCTGTT CGCGCGCTTC 8220

TGCTCCCCGA GCTCAATAAA AGAGCCCACA ACCCCTCACT CGGGGCGCCA GTCCTCCGAT 8280

TGACTGAGTC GCCCGGGTAC CCGTGTATCC AATAAACCCT CTTGCAGTTG CA 8332

CLAIMS

We claim:

1. A method for treating or preventing arthritis, comprising, administering to a mammal a therapeutically effective amount of a recombinant retrovirus which directs the expression of an anti-arthritic agent, wherein said recombinant retrovirus is administered at a titer of greater than 10^7 cfu/ml.

2. The method according to claim 1 wherein said arthritis is rheumatoid arthritis.

3. The method according to claim 1 wherein said arthritis is osteoarthritis.

4. The method according to claim 1 wherein said anti-arthritic agent is a pro-drug converting enzyme.

5. The method according to claim 1 wherein said anti-arthritic agent is an inhibitor of tumor necrosis factor.

6. The method according to claim 1 wherein said anti-arthritic agent is a IL-1 Receptor antagonist.

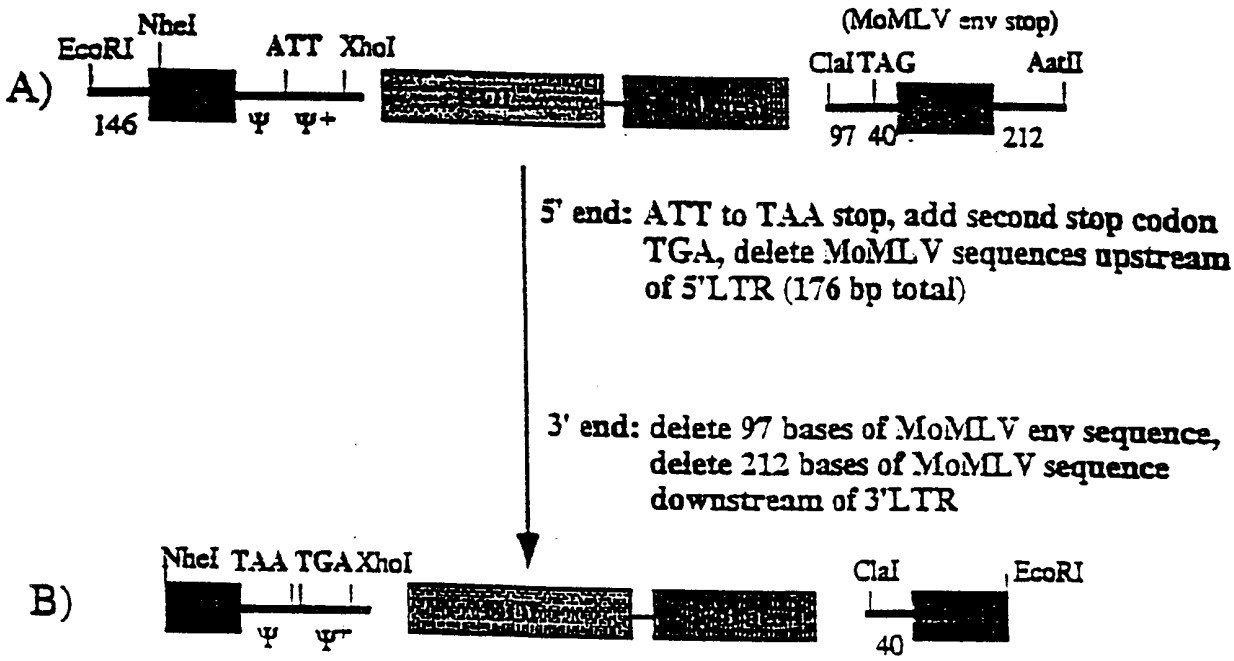
7. The method according to claim 1 wherein said anti-arthritic agent is an inhibitor of matrix metalloproteinase expression.

8. The method according to claim 7 wherein said inhibitor of matrix metalloproteinase expression is TIMP-1 or TIMP-2.

9. The method according to claim 1 wherein said recombinant retrovirus is administered intraarticularly.

FIGURE 1

RETROVIRAL BACKBONE (N2-derived)



CROSS-LESS RETROVIRAL BACKBONE: pBA-5

