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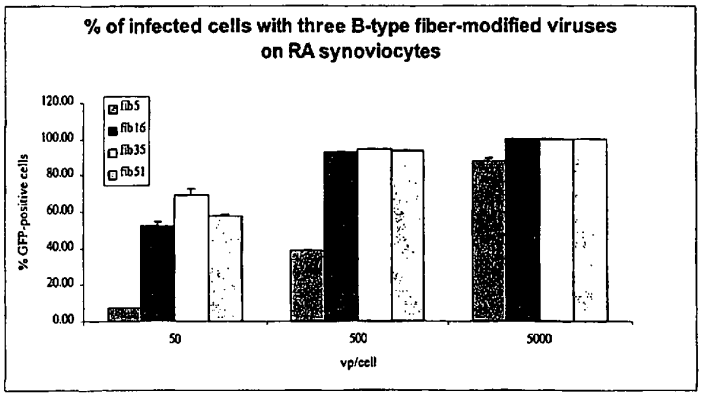

(57) Abstract

The invention provides a nucleic acid delivery vehicle with or having been provided with at least a tissue tropism for fibroblast–like or macrophage–like cells, preferably synoviocytes. In one aspect said nucleic acid delivery vehicle is a virus capsid or a functional part, derivative and/or analogue thereof. Preferably said virus capsid is an adenovirus capsid. Preferably said adenovirus is a subgroup B adenovirus, preferably adenovirus 16. Preferably said tissue tropism is provided by at least a tissue tropism determining part of an adenovirus fiber protein or a functional derivative and/or analogue thereof. The invention further presents methods for the treatment of diseases, preferably joint related diseases.

Title: Means and methods for fibroblast-like or macrophage-like cell transduction.

The invention lies in the field of recombinant DNA technology. More particularly, the invention relates to novel means and methods for transferring nucleic acid into fibroblast-like or macrophage-like cells, preferably synoviocytes. The invention further relates to means and methods for the treatment of diseases by at least transferring nucleic acid into fibroblast-like or macrophage-like cells, preferably synoviocytes for instance for the treatment of rheumatoid arthritis.

Efficient delivery of foreign genetic material to fibroblast-like or macrophage-like cells, especially synoviocytes, has proven to be a difficult goal to achieve. Even with the currently developed viral vectors that are, in general, very effective in delivering foreign genetic material to cells, fibroblast-like or macrophage-like cells have been difficult to provide with foreign genetic material. The relative inefficient transduction of these cells, especially of synoviocytes, has hampered the development of therapeutic approaches based on nucleic acid transfer to these cells.

As a result of the inefficient delivery of nucleic acid into for example synoviocytes, therapeutic approaches based on nucleic acid transfer involving these cells, have focused on strategies in which a low transduction efficiency could at least in part be tolerated. For instance by relying on delivering nucleic acid encoding proteins with so-called bystander effect, i.e. the expression in a transduced cell of which affects the function of untransduced cells in the vicinity of transduced cells. Non-limiting examples of proteins with bystander effect are for instance excreted factors and/or suicide gene expression such as herpes simplex virus (HSV) thymidine kinase (TK) expression which in the

presence of ganciclovir leads to production of toxic metabolites. The herpes simplex virus TK-gene encodes a protein capable of metabolising the relatively non-toxic anti-viral drug ganciclovir (GCV) into a mono-phosphorylated product. Subsequent phosphorylation by mammalian kinases results in a tri-phosphorylated nucleoside analogue (GCV-PPP) that inhibits DNA-polymerase and kills cells, probably through apoptosis (Vincent et al, 1996).

Although the use of a bystander effect may indeed in part reduce the requirement for efficient transduction of fibroblast-like or macrophage-like cells, a more efficient method of transferring genetic material nevertheless is still desirable for economic reasons and for safety reasons. Safety aspects include for instance the relative sensitivity of liver cells toward toxicity of HSV-TK based cell kill. When cells other than liver cells form the target population for suicide by HSK-TK, liver cell transduction should be prevented as much as possible. Unintended liver cell transduction can occur for instance through leakage of a nucleic acid delivery vehicle from the site of delivery into the blood stream from where it is transported to the liver. This leakage is among others dependent on the amount of nucleic acid delivery vehicle used. Thus when for instance synoviocytes form the target cells, a certain amount of nucleic acid delivery vehicle will be needed for obtaining a desired level of transduction. When less nucleic acid delivery vehicle is used, leakage of nucleic acid delivery vehicle is less of a problem.

Non-limiting examples in which nucleic acid transfer to fibroblast-like or macrophage-like cells would be beneficial are chronic erosive joint diseases like rheumatoid arthritis, ankylosing spondylitis and juvenile chronic arthritis. A favourable target cell for nucleic acid transfer in these diseases is the synoviocyte. However, with the current

methods the efficiency of transduction of such cells leaves much to be desired.

In a diarthrodial movable joint, smooth articulation is ensured by the unique macromolecular structure of articular cartilage, which covers the end of the bones. The articular cartilages move against each other within a cavity, the joint space, which is lined by a tissue known as the synovium. The synovium consists of macrophage-like type A cells and fibroblast-like type B cells and is underlain by a sparsely cellular subsynovium which, depending on anatomical localisation, may be fibrous, adipose or areolar in nature. The fibroblast-like synoviocytes (FLS) are distinguishable from normal fibroblast cells in the subintimal synovium by differential gene expression patterns. FLS have been shown to express high levels of uridine diphosphoglucose dehydrogenase (UDPGD), high levels of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) as well as CD44 (hyaluronic acid receptor), fibronectin receptor and β_3-integrins. Sublining fibroblasts or fibroblasts from other sources do not, or at a lower level, express these markers (reviewed by J.C.W. Edwards, 1995; G.S. Firestein, 1996).

Rheumatoid arthritis is characterised by massive hyperplasia of the synovium and presence of inflammatory cells (lymphocytes, macrophages and mast cells) in and around the synovial tissue. Both the FLS and the type A macrophage-like cells play an important role in the destructive aspects of the disease. The type A cells constitute the majority of the cells in normal intima and hyperplastic RA tissue. The highly invasive FLS exhibits histological features usually associated with immature tumour like fibroblasts (Qu et al, 1994; Firestein 1996). Proliferation of these synovial cells leads to pannus tissue which invades and overgrows the cartilage, leading to bone destruction (Zvaifler and Firestein, 1994). Removal of the diseased synovium is beneficial by decreasing inflammation and by preventing

destruction of the proliferating pannus in adjacent structures (Thompson et al, 1973). Specific removal of this proliferating pannus tissue by a simple, non-destructive local procedure, suitable for all joints and rather specific for cells that are proliferating, is a valuable treatment for RA (Nakamura et al, 1997; Cruz-Esteban and Wilke, 1995).

Gene therapy is a promising treatment modality for rheumatoid arthritis (RA). Nucleic acid transfer to rheumatoid synovial tissue may result either in the production of mediators that inhibit inflammation or hyperplasia or may result in toxic substances that destroy specifically the synovium. The first clinical trials in humans were based on *ex-vivo* transduction of synoviocytes with IL1-RA, in order to inhibit inflammation (Evans, 1996).

The present invention was made in the course of the manipulation of adenovirus vectors. In the following section therefore adenoviruses will be discussed.

Adenoviruses

Adenoviruses contain a linear double-stranded DNA molecule of approximately 36000 base pairs. It contains identical Inverted Terminal Repeats (ITR) of approximately 90-140 base pairs with the exact length depending on the serotype. The viral origins of replication are within the ITRs exactly at the genome ends. The transcription units are divided in early and late regions. Shortly after infection the E1A and E1B proteins are expressed and function in transactivation of cellular and adenoviral genes. The early regions E2A and E2B encode proteins (DNA binding protein, pre-terminal protein and polymerase) required for the replication of the adenoviral genome (reviewed in van der Vliet, 1995). The early region E4 encodes several proteins

with pleiotropic functions e.g. transactivation of the E2 early promoter, facilitating transport and accumulation of viral mRNAs in the late phase of infection and increasing nuclear stability of major late pre-mRNAs (reviewed in Leppard, 1997). The early region 3 encodes proteins that are involved in modulation of the immune response of the host (Wold *et al*, 1995). The late region is transcribed from one single promoter (major late promoter) and is activated at the onset of DNA replication. Complex splicing and poly-adenylation mechanisms give rise to more than 12 RNA species coding for core proteins, capsid proteins (penton, hexon, fiber and associated proteins), viral protease and proteins necessary for the assembly of the capsid and shut-down of host protein translation(Imperiale, M.J., Akusjnarvi, G. and Leppard, K.N. (1995) Post-transcriptional control of adenovirus gene expression. In: The molecular repertoire of adenoviruses I. P139-171. W. Doerfler and P. Bohm (eds), Springer-Verlag Berlin Heidelberg).

Interaction between virus and host cell

The interaction of the virus with the host cell has mainly been investigated with the serotype C viruses Ad2 and Ad5. Binding occurs via interaction of the knob region of the protruding fiber with a cellular receptor. The receptor for Ad2 and Ad5 and probably more adenoviruses is known as the `Coxsackievirus and Adenovirus Receptor' or CAR protein (Bergelson *et al*, 1997). Internalisation is mediated through interaction of the RGD sequence present in the penton base with cellular integrins (Wickham *et al*, 1993). This may not be true for all serotypes, for example serotype 40 and 41 do not contain a RGD sequence in their penton base sequence (Kidd et al, 1993).

The fiber protein

The initial step for successful infection is binding of adenovirus to its target cell, a process mediated through fiber protein. The fiber protein has a trimeric structure (Stouten et al, 1992) with different lengths depending on the virus serotype (Signas et al, 1985; Kidd et al, 1993). Different serotypes have polypeptides with structurally similar N and C termini, but different middle stem regions. The first 30 amino acids at the N terminus are involved in anchoring of the fiber to the penton base (Chroboczek et al, 1995), especially the conserved FNPVYP region in the tail (Arnberg et al, 1997). The C-terminus, or knob, is responsible for initial interaction with the cellular adenovirus receptor. After this initial binding, secondary binding between the capsid penton base and cell-surface integrins leads to internalisation of viral particles in coated pits and endocytosis (Morgan et al, 1969; Svensson and Persson, 1984; Varga et al, 1991; Greber et al, 1993; Wickham et al, 1993). Integrins are αβ-heterodimers of which at least 14 α-subunits and 8 ß-subunits have been identified (Hynes, 1992). The array of integrins expressed in cells is complex and will vary between cell types and cellular environment. Although the knob contains some conserved regions between serotypes, knob proteins show a high degree of variability, indicating that different adenovirus receptors exist.

Adenoviral serotypes

At present, six different subgroups of human adenoviruses have been proposed, which in total encompass approximately 50 distinct adenovirus serotypes. Besides

these human adenoviruses, many animal adenoviruses have been identified (see e.g. Ishibashi and Yasue, 1984). A serotype is defined on the basis of its immunological distinctiveness as determined by quantitative neutralisation with animal antisera (horse, rabbit). If neutralisation shows a certain degree of cross-reactivity between two viruses, distinctiveness of serotype is assumed if A) the hemagglutinins are unrelated, as shown by lack of cross-reaction on hemagglutination-inhibition, or B) substantial biophysical/biochemical differences in DNA exist (Francki et al, 1991). The serotypes identified last (42-49) were isolated for the first time from HIV infected patients (Hierholzer et al, 1988; Schnurr et al, 1993). For reasons not well understood, most of such immuno-compromised patients shed adenoviruses that were never isolated from immuno-competent individuals (Hierholzer et al, 1988, 1992; Khoo et al, 1995).

Besides differences towards the sensitivity against neutralising antibodies of different adenovirus serotypes, adenoviruses in subgroup C such as Ad2 and Ad5 bind to different receptors as compared to adenoviruses from subgroup B such as Ad3 and Ad7 (Defer et al, 1990; Gall *et al*, 1996). Likewise, it was demonstrated that receptor specificity could be altered by exchanging the Ad3 knob protein with the Ad 5 knob protein, and vice versa (Krasnykh et al, 1996; Stevenson et al, 1995, 1997). Serotypes 2, 4, 5 and 7 all have a natural affiliation towards lung epithelia and other respiratory tissues. In contrast, serotypes 40 and 41 have a natural affiliation towards the gastrointestinal tract. These serotypes differ in at least capsid proteins (penton-base, hexon), proteins responsible for cell binding

(fiber protein), and proteins involved in adenovirus replication. It is unknown to what extend the capsid proteins determine the differences in tropism found between the serotypes. It may very well be that post-infection mechanisms determine cell type specificity of adenoviruses. It has been shown that adenoviruses from serotypes A (Ad12 and Ad31),C (Ad2 and Ad5),D (Ad9 and Ad15),E (Ad4) and F (Ad41) all are able to bind labelled, soluble CAR (sCAR) protein when immobilised on nitro-cellulose. Furthermore, binding of adenoviruses from these serotypes to Ramos cells, that express high levels of CAR but lack integrins (Roelvink *et al*, 1996), could be efficiently blocked by addition of sCAR to viruses prior to infection (Roelvink *et al*, 1998). However, the fact that (at least some) members of these subgroups are able to bind CAR does not exclude that these viruses have different infection efficiencies in various cell types. For example subgroup D serotypes have relatively short fiber shafts compared to subgroup A and C viruses. It has been postulated that the tropism of subgroup D viruses is to a large extend determined by the penton base binding to integrins (Roelvink *et al*, 1996; Roelvink *et al*, 1998). Another example is provided by Zabner *et al*, 1998 who have tested 14 different serotypes on infection of human ciliated airway epithelia (CAE) and found that serotype 17 (subgroup D) was bound and internalised more efficiently then all other viruses, including other members of subgroup D. Similar experiments using serotypes from subgroup A-F in primary foetal rat cells showed that adenoviruses from subgroup A and B were inefficient whereas viruses from subgroup D were most efficient (Law *et al*, 1998). Also in this case viruses within one subgroup displayed different efficiencies. The importance of fiber binding for the

improved infection of Ad17 in CAE was shown by Armentano *et al* (WO 98/22609) who made a recombinant LacZ Ad2 virus with a fiber gene from Ad17 and showed that the chimaeric virus infected CAE more efficiently than LacZ Ad2 viruses with Ad2 fibers.

Thus despite their shared ability to bind CAR, differences in the length of the fiber, knob sequence and other capsid proteins like penton base may determine the efficiency by which an adenovirus infects a certain target cell. Of interest is that Ad5 and Ad2 fibers bind to fibronectin III and MHC class 1 α2 derived peptides, while Ad3 fibers do not. This suggests that adenoviruses are able to use cellular receptors other than CAR (Hong *et al*, 1997). Serotypes 40 and 41 (subgroup F) are known to carry two fiber proteins differing in the length of the shaft. The long shafted 41L fiber is shown to bind CAR whereas the short shafted 41S is not capable of binding CAR (Roelvink *et al*, 1998). The receptor for the short fiber is not known.

Adenoviral nucleic acid delivery vectors

Most adenoviral nucleic acid delivery vectors currently used in gene therapy are derived from the serotype C adenoviruses Ad2 or Ad5. The vectors have a deletion in the E1 region, where novel genetic information can be introduced. The E1 deletion renders the recombinant virus replication defective. It has been demonstrated extensively that recombinant adenovirus, in particular serotype 5 is suitable for efficient transfer of genes *in vivo* to the liver, the airway epithelium and solid tumours in animal models and human xenografts in immuno-deficient mice (Bout 1996, 1997; Blaese *et al*, 1995).

Nucleic acid transfer vectors derived from adenoviruses (adenoviral vectors) have a number of features that make them particularly useful for nucleic acid transfer:

1) the biology of the adenoviruses is well characterised,
2) the adenovirus is not associated with severe human pathology,
3) the adenovirus is extremely efficient in introducing its DNA into the host cell,
4) the adenovirus can infect a wide variety of cells and has a broad host-range,
5) the adenovirus can be produced at high titers in large quantities,
6) and the adenovirus can be rendered replication defective by deletion of the early-region 1 (E1) of the viral genome (Brody and Crystal, 1994).

However, there are still a number of drawbacks associated with the use of adenoviral vectors:

1) Adenoviruses, especially the well investigated serotypes Ad2 and Ad5 usually elicit an immune response by the host into which they are introduced,
2) it is currently not feasible to target the virus to certain cells and tissues,
3) Some cell types are not easily transduced by the current generation of adenovirus vectors.
4) the serotypes Ad2 or Ad5, are not ideally suited for delivering additional genetic material to organs other than the liver. The liver can be particularly well transduced with vectors derived from Ad2 or Ad5. Administration of these vectors via the bloodstream leads to a significant delivery of the vectors to the cells of the liver. In therapies were cell types other than liver cells need to be

transduced some means of liver exclusion must be applied to prevent uptake of the vector by these cells. Current methods rely on the physical separation of the vector from the liver cells, most of these methods rely on localising the vector and/or the target organ via surgery, balloon angioplasty or direct injection into an organ or a bone structure via for instance needles. Liver exclusion is also being practised through delivery of the vector to compartments in the body that are essentially isolated from the bloodstream thereby preventing transport of the vector to the liver. Although these methods mostly succeed in avoiding gross delivery of the vector to the liver, most of the methods are crude and still have considerable leakage and/or have poor target tissue penetration characteristics. In some cases inadvertent delivery of the vector to liver cells can be toxic to the patient. For instance, delivery of a herpes simplex virus (HSV) thymidine kinase (TK) gene for the subsequent killing of dividing cancer cells through administration of ganciclovir is not without risk when also a significant amount of liver cells are transduced by the vector. Significant delivery and subsequent expression of the HSV-TK gene to liver cells is associated with severe toxicity. Thus there is a discrete need for an inherently safe vector provided with the property of a reduced transduction efficiency of liver cells.

The present invention provides novel means for the introduction of genetic material into fibroblast-like or macrophage-like cells, preferably synoviocytes. The present invention further provides means and methods for at least in part preventing delivery of nucleic acid into liver cells. The present invention further provides means and methods for

the treatment of disease by at least in part specifically transferring nucleic acid into synoviocytes.

It is an object of the current invention to provide materials and methods to overcome at least part of the limitations of nucleic delivery vehicles.

In one aspect, the invention provides new adenoviruses, derived in whole or in part from serotypes different from Ad5. Specific genes of serotypes with preferred characteristics may be combined in a chimaeric vector to give rise to a vector that is better suited for specific applications. Preferred characteristics include, but are not limited to, improved infection of a specific target cell, reduced infection of non-target cells, improved stability of the virus, reduced uptake in antigen presenting cells (APC), or increased uptake in APC, reduced toxicity to target cells, reduced neutralisation in humans or animals, reduced or increased CTL response in humans or animals, better and/or prolonged transgene expression, increased penetration capacity in tissues, improved yields in packaging cell lines, etc.

One aspect of the present invention facilitates the combination of the low immunogenicity of some adenoviruses with the characteristics of other adenoviruses. Such adenoviruses may be favourable for gene therapy approaches. Such characteristics may be a high specificity for certain host cells, a good replication machinery for certain cells, a high rate of infection in certain host cells, low infection efficiency in non-target cells, high or low efficiency of APC infection, etc.

The invention thus may provide chimaeric adenoviruses having the useful properties of at least two adenoviruses of different serotypes. Typically, two or more requirements from the above non-exhaustive list are required to obtain an adenovirus capable of efficiently transferring genetic material to a host cell. In one aspect the present invention therefore provides adenovirus derived vectors which can be used as cassettes to insert different adenoviral genes from different adenoviral serotypes at the required sites. This way one can obtain a vector capable of producing a chimaeric adenovirus, whereby of course also a gene of interest can be inserted (for instance at the site of E1 of the original adenovirus). In this manner the chimaeric adenovirus to be produced can be adapted to the requirements and needs of certain hosts in need of gene therapy for certain disorders. To enable this virus production, a packaging cell will generally be needed in order to produce sufficient amount of safe chimaeric adenoviruses.

In one of its aspects the present invention provides adenoviral vectors comprising at least a part of a fiber protein of an adenovirus from subgroup B, in particular of serotypes 11, 16, 35 and/or 51. Said fiber protein may be the native fiber protein of the adenoviral vector or may be derived from a serotype different from the serotype the adenoviral vector is based on. In the latter case the adenoviral vector according to the invention is a chimaeric adenovirus displaying at least a part of the fiber protein derived from subgroup B adenoviruses that part comprising at least the receptor binding sequence. Typically such a virus will be produced using a vector (typically a plasmid, a cosmid or baculovirus vector). Such vectors are also subject of the present invention. A preferred vector is a vector

that can be used to make a chimaeric recombinant virus specifically adapted to the host to be treated and the disorder to be treated.

The present invention also provides a chimaeric adenovirus based on adenovirus type 5 but having at least a part of the fiber sequence from adenovirus type 16, whereby the part of the fiber of Ad16 at least comprises a part of the fiber protein that is involved in binding a host cell.

The present invention also provides chimaeric adenoviral vectors that show improved infection as compared to adenoviruses from other subgroups in specific host cells for example, but not limited to, fibroblast-like or macrophage-like cells, preferably synoviocytes of human or animal origin. An important feature of part of the present invention is a means to produce the chimaeric virus. Typically, one does not want an adenovirus batch to be administered to the host cell, which contains replication competent adenovirus. In general therefore it is desired to omit a number of genes (but at least one) from the adenoviral genome on the vector encoding the chimaeric virus and to supply these genes in the genome of the cell in which the vector is brought to produce chimaeric adenovirus. Such a cell is usually called a packaging cell. The invention thus also provides a packaging cell for producing a chimaeric adenovirus according to the invention, comprising in trans all elements necessary for adenovirus production not present on the adenoviral vector according to the invention. Typically vector and packaging cell have to be adapted to one another in that they have all the necessary elements, but that they do not have overlapping elements which lead to replication competent virus by recombination. Thus the invention also provides a kit of parts comprising a

packaging cell according to the invention and a recombinant vector according the invention whereby there is essentially no sequence overlap leading to recombination resulting in the production of replication competent adenovirus between said cell and said vector.

For certain applications for example when the therapy is aimed at eradication of tumour cells, the adenoviral vector according to the invention may be replication competent or capable of replicating under certain conditions for example in specific cell types like tumour cells.

It is within the scope of the invention to insert more genes, or a functional part of these genes from the same or other serotypes into the adenoviral vector replacing the corresponding native sequences. Thus for example replacement of (a functional part of the) fiber sequences with corresponding sequences of other serotypes may be combined with for example replacements of (a functional part of) other capsid genes like penton base or hexon with corresponding sequences of said serotype or of other distinct serotypes. Persons skilled in the art understand that other combinations not limited to said genes are possible and are within the scope of the invention. A chimaeric adenoviral vector according to the invention may originate from at least two different serotypes. This may provide the vector with preferred characteristics such as improved infection of target cells and/or less infection of non-target cells, improved stability of the virus, reduced immunogenicity in humans or animals (e.g. reduced uptake in APC, reduced neutralisation in the host and/or reduced cytotoxic T-lymphocyte (CTL) response), increased penetration of tissue, better longevity of transgene expression, etc. In this aspect it is preferred to use

capsid genes e.g. penton and/or hexon genes from less immunogenic serotypes as defined by the absence or the presence of low amounts of neutralising antibodies in the vast majority of hosts. It is also preferred to use fiber and/or penton sequences from serotypes that show improved binding and internalisation in the target cells. Furthermore it is preferred to delete from the viral vector those genes which lead to expression of adenoviral genes in the target cells. In this aspect a vector deleted of all adenoviral genes is also preferred. Furthermore it is preferred that the promoter driving the gene of interest to be expressed in the target cells is a cell type specific promoter.

In order to be able to precisely adapt the viral vector and provide the chimaeric virus with the desired properties at will, it is preferred that a library of adenoviral genes is provided whereby the genes to be exchanged are located on plasmid- or cosmid-based adenoviral constructs whereby the genes or the sequences to be exchanged are flanked by restriction sites. The preferred genes or sequences can be selected from the library and inserted in the adenoviral constructs that are used to generate the viruses. Typically such a method comprises a number of restriction and ligation steps and transfection of a packaging cell. The adenoviral vector can be transfected in one piece, or as two or more overlapping fragments, whereby viruses are generated by homologous recombination. For example the adenoviral vector may be built up from two or more overlapping sequences for insertion or replacements of a gene of interest in for example the E1 region, for insertion or replacements in penton and/or hexon sequences, and for insertions or replacements into fiber sequences. In one aspect the

invention provides a method for producing chimaeric adenoviruses having one or more desired properties like a desired host range and diminished antigenicity, comprising providing one or more vectors according to the invention having the desired insertion sites, inserting into said vectors at least a functional part of a fiber protein derived from an adenovirus serotype having the desired host range and/or inserting a functional part of a capsid protein derived from an adenovirus serotype having relatively low antigenicity and transfecting said vectors in a packaging cell according to the invention and allowing for production of chimaeric viral particles. Of course other combinations of other viral genes originating from different serotypes can also be inserted as disclosed herein before. Chimaeric viruses having only one non-native sequence in addition to an insertion or replacement of a gene of interest in the E1 region, are also within the scope of the invention.

An immunogenic response to adenovirus that typically occurs, is the production of neutralising antibodies by the host. This is typically a reason for selecting a capsid protein like penton, hexon and/or fiber of a less immunogenic serotype.

Of course it may not be necessary to make chimaeric adenoviruses which have complete proteins from different serotypes. It is well within the skill of the art to produce chimaeric proteins. For instance in the case of fiber proteins, it is very well possible to have the base of one serotype and the shaft and the knob from another serotype. In this manner it becomes possible to have the parts of the protein responsible for assembly of viral particles originate from one serotype, thereby enhancing the production of intact viral particles. Thus the invention also provides a chimaeric

adenovirus according to the invention, wherein the hexon, penton, fiber and/or other capsid proteins are chimaeric proteins originating from different adenovirus serotypes. Besides generating chimaeric adenoviruses by swapping entire wild type capsid (protein) genes etc. or parts thereof, it is also within the scope of the present invention to insert capsid (protein) genes etc. carrying non-adenoviral sequences or mutations such as point mutations, deletions, insertions, etc. Such chimaeric adenoviruses can be easily screened for preferred characteristics such as temperature stability, assembly, anchoring, redirected infection, altered immune response etc. Again other chimaeric combinations can also be produced and are within the scope of the present invention.

It has been demonstrated in mice and rats that upon in vivo systemic delivery of recombinant adenovirus of common used serotypes for gene therapy purposes, more than 90% of the virus is trapped in the liver (Herz et al, 1993; Kass-Eisler et al, 1994; Huard et al, 1995). It is also known that human hepatocytes are efficiently transduced by adenovirus serotype 5 vectors (Castell, J.V., Hernandez, D. Gomez-Foix, A.M., Guillen, I, Donato, T. and Gomez-Lechon, M.J. (1997). Adenovirus-mediated gene transfer into human hepatocytes: analysis of the biochemical functionality of transduced cells. Gene Ther. 4(5), p455-464). Thus in vivo gene therapy by systemic delivery of Ad2 or Ad5 based vectors is seriously hampered by the efficient uptake of the viruses in the liver leading to unwanted toxicity and less virus being available for transduction of the target cells. Therefore, alteration of the adenovirus serotype 5 host cell range to be able to target other organs in vivo is a major interest of the invention.

To obtain re-directed infection of recombinant adenovirus serotype 5, several approaches have been or still are under investigation. Wickham et al have altered the RGD (Arg, Gly, Asp) motif in the penton base which is believed to be responsible for the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin binding to the penton base. They have replaced this RGD motif by another peptide motif which is specific for the $\alpha_4\beta_1$ receptor. In this way targeting the adenovirus to a specific target cell could be accomplished (Wickham et al, 1995). Krasnykh et al (1998) have made use of the HI loop available in the knob. This loop is, based on X-ray crystallography, located on the outside of the knob trimeric structure and therefore is thought not to contribute to the intramolecular interactions in the knob. Insertion of a FLAG coding sequence into the HI loop resulted in fiber proteins that were able to trimerise and it was further shown that viruses containing the FLAG sequence in the knob region could be made. Although interactions of the FLAG-containing knob with CAR are not changed, insertion of ligands in the HI loop may lead to retargeting of infection. Although successful introduction of changes in the adenovirus serotype 5 fiber and penton-base have been reported, the complex structure of knob and the limited knowledge of the precise amino acids interacting with CAR render such targeting approaches laborious and difficult. The use of antibodies binding to CAR and to a specific cellular receptor has also been described (Wickham et al, 1996; Rogers et al, 1997). This approach is however limited by the availability of a specific antibody and by the complexity of the gene therapy product.

To overcome the limitations described above we used pre-existing adenovirus fibers, penton base proteins, hexon

proteins or other capsid proteins derived from other adenovirus serotypes. By generating chimaeric adenovirus serotype 5 libraries containing structural proteins of alternative adenovirus serotypes, we have developed a technology, which enables rapid screening for a recombinant adenoviral vector with preferred characteristics.

It is an object of the present invention to provide methods for the generation of chimaeric capsids which can be targeted to specific cell types *in vitro* as well as *in vivo*, and thus have an altered tropism for certain cell types. It is a further object of the present invention to provide methods and means by which an adenovirus or an adenovirus capsid can be used as a protein or nucleic acid delivery vehicle to a specific cell type or tissue.

The generation of chimaeric adenoviruses based on adenovirus serotype 5 with modified late genes is described. For this purpose, three plasmids, which together contain the complete adenovirus serotype 5 genome, were constructed. From one of these plasmids part of the DNA encoding the adenovirus serotype 5 fiber protein was removed and replaced by linker DNA sequences that facilitate easy cloning. This plasmid subsequently served as template for the insertion of DNA encoding fiber protein derived from different adenovirus serotypes. The DNA sequences derived from the different serotypes were obtained using the polymerase chain reaction technique in combination with (degenerate) oligonucleotides. At the former E1 location in the genome of adenovirus serotype 5, any gene of interest can be cloned. A single transfection procedure of the three plasmids together results in the formation of a recombinant chimaeric adenovirus. Alternatively, cloning of sequences obtained

from a library of genes can be such that the chimaeric adenoviral vector is built up from one or more fragments. For example one construct contains at least the left ITR and sequences necessary for packaging of the virus, an expression cassette for the gene of interest and sequences overlapping with a second construct, wherein a second construct comprises all sequences necessary for replication and virus formation not present in the packaging cell as well as non-native sequences providing the preferred characteristics. This new technology of libraries consisting of chimaeric adenoviruses thus allows for a rapid screening for improved recombinant adenoviral vectors for *in vitro* and *in vivo* gene therapy purposes.

The use of adenovirus type 5 for in vivo gene therapy is limited by the apparent inability to infect certain cell types efficiently e.g. fibroblast-like or macrophage-like cells, preferably synoviocytes and the preference of infection of certain organs e.g. liver and spleen. Specifically this has consequences for treatment of rheumatoid arthritis (RA). Adenovirus-mediated delivery of for instance herpes simplex virus thymidine kinase into synoviocytes has been proposed as a possible treatment for RA. However, efficient delivery of said gene is required.

In one embodiment this invention describes adenoviral vectors that are, amongst others, especially suited for nucleic acid delivery to fibroblast-like or macrophage-like cells, most especially to synoviocytes. This feature is of particular importance for the treatment of diseases related to joints, particularly for the treatment of rheumatoid arthritis. The adenoviral vectors preferably are derived

from subgroup B adenoviruses or contain at least a functional part of the fiber protein from an adenovirus from subgroup B comprising at least the cell-binding moiety of said fiber protein.

In a further preferred embodiment the adenoviral vectors are chimaeric vectors based on adenovirus type 5 and contain at least a functional part of the fiber protein from adenovirus type 16.

In another embodiment this invention provides adenoviral vectors or chimaeric adenoviral vectors that escape at least in part the liver following systemic administration. Preferably said adenoviral vectors are derived from subgroup B, in particular serotype 16 or contain at least the cell-binding moiety of the fiber protein derived from said adenovirus.
It is to be understood that in all embodiments adenoviral vectors and/or particles may be derived solely from one serotype having the desired properties or that an adenoviral vector and/or particle comprises sequences and/or protein or functional parts, derivatives and/or analogues thereof, of two or more adenovirus serotypes.

In another aspect this invention describes chimaeric adenoviruses and methods to generate viruses that have an altered tropism different from that of adenovirus serotype 5. For example, viruses based on adenovirus serotype 5 but displaying any adenovirus fiber existing in nature. This chimaeric adenovirus serotype 5 is able to infect certain cell types more efficiently, or less efficiently *in vitro* and *in vivo* than the adenovirus serotype 5. Such cells include but are not limited to endothelial cells, smooth muscle cells, dendritic cells, neuronal cells, glial cells,

synovical cells, lung epithelial cells, hemopoietic stem cells, monocytic/macrophage cells, tumour cells, skeletal muscle cells, mesothelial cells, synoviocytes, etc.

In another aspect the invention describes the construction and use of libraries consisting of distinct parts of adenovirus serotype 5 in which one or more genes or sequences have been replaced with DNA derived from alternative human or animal serotypes. This set of constructs, in total encompassing the complete adenovirus genome, allows for the construction of unique chimaeric adenoviruses customised for a certain disease, group of patients or even a single individual.

In all aspects of the invention the chimaeric adenoviruses may, or may not, contain deletions in the E1 region and insertions of heterologous genes linked either or not to a promoter. Furthermore, chimaeric adenoviruses may, or may not, contain deletions in the E3 region and insertions of heterologous genes linked to a promoter. Furthermore, chimaeric adenoviruses may, or may not, contain deletions in the E2 and/or E4 region and insertions of heterologous genes linked to a promoter. In the latter case E2 and/or E4 complementing cell lines are required to generate recombinant adenoviruses. In fact any functional nucleic acid in the genome of the viral vector can be taken out and supplied in trans. Thus, in the extreme situation, chimaeric viruses do not contain any adenoviral genes in their genome and are by definition minimal adenoviral vectors. In this case required adenoviral functions are supplied in trans using stable cell lines and/or transient expression of these genes. A method for producing minimal adenoviral vectors is described in WO97/00326 and is taken

as reference herein. In another case Ad/AAV chimaeric molecules are packaged into the adenovirus capsids of the invention. A method for producing Ad/AAV chimaeric vectors is described in EP 97204085.1 and is taken as reference herein. In principle any nucleic acid may be provided with the adenovirus capsids of the invention.

In one embodiment the invention provides a nucleic acid delivery vehicle comprising or having been provided with at least a tissue tropism for fibroblast-like or macrophage-like cells, preferably synoviocytes. In another embodiment the invention provides a nucleic acid delivery vehicle comprising an at least in part reduced or having at least in part been deprived of a tissue tropism for at least liver cells. Preferably, said nucleic acid delivery vehicle is provided with a tissue tropism for at least fibroblast-like or macrophage-like cells, preferably synoviocytes and at least in part deprived of a tissue tropism for at least liver cells. In a preferred embodiment of the invention said nucleic acid delivery vehicle is provided with a tissue tropism for at least fibroblast-like or macrophage-like cells, preferably synoviocytes and/or at least in part deprived of a tissue tropism for at least liver cells using a fiber protein derived from a subgroup B adenovirus, typically of serotypes 11, 16, 35 and/or 51 and preferably of adenovirus 16. In a preferred aspect of the invention said nucleic acid delivery vehicle comprises a virus capsid or a functional part, derivative and/or analogue thereof. Preferably said virus capsid comprises a virus capsid derived in whole or in part from an adenovirus of subgroup B, preferably from adenovirus 16, or it comprises proteins, or functional parts, derivatives or analogues thereof, from an adenovirus of subgroup B, preferably of adenovirus 16. In preferred embodiment of the invention said virus capsid comprises proteins, or functional parts, derivatives or

analogues thereof, from at least two different viruses, preferably adenoviruses. In a preferred embodiment of the invention at least one of said viruses is an adenovirus of subgroup B, preferably adenovirus 16.

In a preferred embodiment of the invention said nucleic acid delivery vehicle comprises an adenovirus fiber protein or parts thereof. Said fiber protein is preferably derived from an adenovirus of subgroup B, preferably of adenovirus 16. Said nucleic acid delivery vehicle may further comprise other fiber proteins, or parts thereof, from other adenoviruses. Said nucleic acid delivery vehicle may or may not comprise other adenovirus proteins. Nucleic acid may be linked directly to said fiber protein, or parts thereof, but may also be linked indirectly. Examples of indirect linkages include but are not limited to, packaging of nucleic acid into adenovirus capsids or packaging of nucleic acid into liposomes, wherein a fiber protein, or a part thereof, is incorporated into an adenovirus capsid or linked to a liposome. Direct linkage of nucleic acid to a fiber protein, or a part thereof, may be performed when said fiber protein, is not part of a complex or when said fiber protein is part of complex such as an adenovirus capsid.

In one embodiment, the invention provides a nucleic acid delivery vehicle comprising an adenovirus fiber protein wherein said fiber protein comprises at least a tissue determining part of an adenovirus of subgroup B adenovirus, preferably of adenovirus 16. Adenovirus fiber protein comprises at least three functional domains. One domain, the base, is responsible for anchoring a fiber to a penton base of an adenovirus capsid. Another domain, the knob, is responsible for receptor recognition whereas the shaft domain functions as a spacer separating the base from the knob. The different domains may also have other function. For instance, the shaft is presumably also involved in target cell specificity. Each of the domains mentioned above alone or in

combination, may be used to define a part of a fiber. However, parts may also be identified in another way. For instance the knob domain comprises of a receptor binding part and a shaft binding part. The base domain comprises of a penton base binding part and a shaft binding part. Moreover, the shaft comprises of repeated stretches of amino acids. Each of these repeated stretches may be a part or used in combination with one or more other parts to form a tissue determining part of a fiber protein. Preferably said tissue determining part of a fiber protein comprises at least the knob domain of said fiber protein, or a functional part, derivative and/or analogue thereof.

A tissue tropism determining part of a fiber protein may be a single part of a fiber protein or a combination of parts derived from at least one fiber protein, wherein said tissue tropism determining part, either alone or in combination with a virus capsid, determines the efficiency with which a nucleic acid delivery vehicle can transduce a given cell or cell type, preferably but not necessarily in a positive way. With a tissue tropism for liver cells is meant a tissue tropism for cells residing in the liver, preferably liver parenchyma cells.

A tissue tropism for a certain tissue may be provided by increasing the efficiency with which cells of said tissue are transduced, alternatively, a tissue tropism for a certain tissue may be provided by decreasing the efficiency with which other cells than the cells of said tissue are transduced.

Fiber proteins possess tissue tropism determining properties. The most well described part of fiber protein involved in tissue tropism is the knob domain. However, the shaft domain of the fiber protein also possesses tissue tropism determining properties. However, not all of the tissue tropism determining properties of an adenovirus capsid are incorporated into a fiber protein.

In a preferred embodiment of the invention, a fiber protein derived from a subgroup B adenovirus, typically ad 11, 16, 35 and/or 51, preferably adenovirus 16 or a functional part, derivative and/or analogue thereof, is combined with at least one non-fiber capsid proteins from an adenovirus of subgroup C, preferably of adenovirus 5.

In one aspect of the invention is provided a nucleic acid delivery vehicle comprising at least part of a nucleic acid derived from an adenovirus. In a preferred embodiment said adenovirus nucleic acid comprises at least one nucleic acid encoding a fiber protein comprising at least a tissue tropism determining part of a subgroup B adenovirus fiber protein, preferably of adenovirus 16. In a preferred aspect said adenovirus comprises nucleic acid from at least two different adenoviruses. In a preferred aspect said adenovirus comprises nucleic acid from at least two different adenoviruses wherein at least part of said nucleic acid encodes a fiber protein comprising at least a tissue tropism determining part of a subgroup B adenovirus fiber protein, preferably of adenovirus 16.

In a preferred embodiment of the invention adenovirus nucleic acid is modified such that the capacity of said adenovirus nucleic acid to replicate in a target cell has been reduced or disabled. This may be achieved among others, through inactivating or deleting genes encoding early region 1 proteins.

In another preferred embodiment said adenovirus nucleic acid is modified such that the capacity of a host immune system to mount an immune response against adenovirus proteins encoded by said adenovirus nucleic acid has been reduced or disabled. This may among others, be achieved through deletion of genes encoding proteins of early region 2 and/or early region 4. Alternatively, genes encoding early region 3 proteins, may be deleted, or on the contrary, considering the anti-immune system function of some of the

proteins encoded by the genes in early region 3, the expression of early region 3 proteins may be enhanced for some purposes. Also, adenovirus nucleic acid may be altered by a combination of two or more of the alterations of adenovirus nucleic acid mentioned above. It is clear that when nucleic acid encoding essential functions are deleted from adenovirus nucleic acid, said essential functions must be complemented in the cell that is going to produce adenovirus nucleic acid, adenovirus vector, vehicle or chimaeric capsid. Adenovirus nucleic acid may also be modified such that the capacity of a host immune system to mount an immune response against adenovirus proteins encoded by said adenovirus nucleic acid has been reduced or disabled, in other ways then mentioned above, for instance through exchanging capsid proteins, or parts thereof, by capsid proteins, or parts thereof, from other serotypes for which humans or animals do not have, or have low levels of, neutralising antibodies. Another example is the exchange of nucleic acid encoding capsid proteins with nucleic acid encoding capsid proteins from other serotypes. Also capsid proteins, or parts thereof, may be exchanged for other capsid proteins, or parts thereof, for which individuals are not capable of, or have a low capacity of, raising an immune response against.

An adenovirus nucleic acid may be altered further or instead of one or more of the alterations mentioned above, by inactivating or deleting genes encoding adenovirus late proteins such as but not-limited to, hexon, penton, fiber and/or protein IX.

In a preferred embodiment of the invention all genes encoding adenovirus proteins are deleted from said adenovirus nucleic acid, turning said nucleic acid into a minimal adenovirus vector.

In another preferred embodiment of the invention said adenovirus nucleic acid is an Ad/AAV chimaeric vector,

wherein at least the integration means of an adeno-associated virus (AAV) are incorporated into said adenovirus nucleic acid.

In a preferred embodiment of the invention, a vector or a nucleic acid, which may be one and the same or not, according to the invention further comprises at least one non-adenovirus nucleic acid. Preferably, at least one of said non-adenovirus nucleic acid is a nucleic acid encoding the following protein or a functional part, derivative and/or analogue thereof: an apolipoprotein, a nitric oxide synthase, a herpes simplex virus thymidine kinase, an interleukin-3, an interleukin-1RA, an interleukin-1α, an (anti)angiogenesis protein such as angiostatin or endostatin, an anti-proliferation protein, a vascular endothelial growth factor (VGEF), a basic fibroblast growth factor (bFGF),a hypoxia inducible factor 1α (HIF-1α), a PAI-1, a smooth muscle cell anti-migration protein, an erythropoietin, a CD40, a FasL, an interleukin-12, an interleukin-10, an interleukin-4, an interleukin-13, an excreted single chain antibody to CD4, CD5, CD7, CD52, interleukin-2, interleukin-1, interleukin-6, tumour necrosis factor (TNF), etc. or an excreted single chain antibody to a T-cell receptor on the auto-reactive T-cells, a dominant negative mutant of promyelocytic leukemia (PML) to inhibit the immune response, an antagonist of inflammation promoting cytokines such as for example interleukin-1RA(receptor antagonist) and soluble receptors like soluble interleukin 1 receptor I (IL-1RI), soluble interleukin 1 receptor II (sIL-1RII), soluble tumour necrosis factor receptor I (sTNFRI) and II (sTNFRII), a growth and/or immune response inhibiting protein such as a protein encoded by a the genes Bcl3, cactus or IκBα, β or γ, an apoptosis inducing protein like the VP3 protein of chicken anemia virus or a protein encoded by a suicide gene like cytosine deaminase, nitroreductase and linamerase. Gene delivery vehicles according to the invention comprising nucleic acid encoding one or more of said proteins or a functional parts,

derivatives and/or analogues thereof can be used to kill or inhibit growth of synoviocytes and/or T-cells in the affected joints.

In another aspect, the invention provides a cell for the production of a nucleic acid delivery vehicle comprising or provided with at least a tissue tropism for fibroblast-like or macrophage-like cells, preferably synoviocytes. In another aspect, the invention provides a cell for the production of a nucleic acid delivery vehicle comprising a reduced tissue tropism for liver cells or having at least in part been deprived of a tissue tropism for liver cells. In another aspect, the invention provides a cell for the production of a nucleic acid delivery vehicle comprising or provided with at least a tissue tropism for fibroblast-like or macrophage-like cells, preferably synoviocytes and comprising a reduced tissue tropism for liver cells or having at least in part been deprived of a tissue tropism for liver cells. In a preferred embodiment of the invention said cell is an adenovirus packaging cell, wherein an adenovirus nucleic acid is packaged into an adenovirus capsid. In one aspect of an adenovirus packaging cell of the invention all functions required for the replication and packaging of an adenovirus nucleic acid, except for the proteins encoded by early region 1, are provided by proteins and/or RNA encoded by said adenovirus nucleic acid. Early region 1 encoded proteins in this aspect of the invention may be encoded by genes incorporated into the cells genomic DNA. In a preferred embodiment of the invention said cell is PER.C6 (ECACC deposit number 96022940). In general, when gene products required for the replication and packaging of adenovirus nucleic acid into adenovirus capsid are not provided by a adenovirus nucleic acid, they are provided by the packaging cell, either by transient transfection, or through stable transformation of said packaging cell. However, an adenovirus product provided by the packaging cell may also, in addition, be provided by a nucleic acid present on said adenovirus

nucleic acid. For instance fiber protein may be provided by the packaging cell, for instance through transient transfection, and may be encoded by adenovirus nucleic acid. This feature can among others be used to generate adenovirus capsids comprising of fiber proteins with two different tissue tropisms for instance through the use of fiber proteins from two different viruses.

Nucleic acid delivery vehicles of the invention are useful for the treatment diseases, preferably joint related diseases such as rheumatoid arthritis, ankylosing spondylitis and juvenile chronic arthritis. Non-limiting examples of proteins or functional parts, derivatives and/or analogues thereof, of which expression in for instance synoviocytes ameliorates at least in part symptoms of diseases are, an apolipoprotein, a nitric oxide synthase, a herpes simplex virus thymidine kinase, an interleukin-3, an interleukin-1RA, an interleukin-1α, an (anti)angiogenesis protein such as angiostatin or endostatin, an anti-proliferation protein, a vascular endothelial growth factor (VGEF), a basic fibroblast growth factor (bFGF),a hypoxia inducible factor 1α (HIF-1α), a PAI-1, a smooth muscle cell anti-migration protein, an erythropoietin, a CD40, a FasL, an interleukin-12, an interleukin-10, an interleukin-4, an interleukin-13, an excreted single chain antibody to CD4, CD5, CD7, CD52, interleukin-2, interleukin-1, interleukin-6, tumour necrosis factor (TNF), etc. or an excreted single chain antibody to a T-cell receptor on the auto-reactive T-cells, a dominant negative mutant of promyelocytic leukemia (PML) to inhibit the immune response, an antagonist of inflammation promoting cytokines such as for example interleukin-1RA(receptor antagonist) and soluble receptors like soluble interleukin 1 receptor I (IL-1RI), soluble interleukin 1 receptor II (sIL-1RII), soluble tumour necrosis factor receptor I (sTNFRI) and II (sTNFRII), a growth and/or immune response inhibiting protein such as a protein encoded by a the genes Bcl3, cactus or IκBα, β or γ, an apoptosis inducing protein like the VP3

protein of chicken anemia virus or a protein encoded by a suicide gene like cytosine deaminase, nitroreductase and linamerase.

Nucleic acid delivery vehicles of the invention may be used as a pharmaceutical for the treatment of diseases. Alternatively, nucleic acid delivery vehicles of the invention may be used for the preparation of a medicament for the treatment of diseases.

In one aspect the invention provides an adenovirus capsid with or provided with a tissue tropism for fibroblast-like or macrophage-like cells, preferably synoviocytes wherein said capsid preferably comprises proteins from at least two different adenoviruses and wherein at least a tissue tropism determining part of a fiber protein is derived from a subgroup B adenovirus, preferably of adenovirus 16. In another aspect the invention provides an adenovirus capsid with a reduced or having at least in part been deprived of a tissue tropism for liver cells wherein said capsid preferably comprises proteins from at least two different adenoviruses and wherein at least a tissue tropism determining part of a fiber protein is derived from a subgroup B adenovirus, preferably of adenovirus 16.

In one embodiment the invention comprises the use of an adenovirus capsid of the invention, for the delivery of nucleic acid to fibroblast-like or macrophage-like cells, preferably synoviocytes. In another embodiment the invention comprises the use of an adenovirus capsid of the invention, for at least in part preventing delivery of nucleic acid to liver cells.

In another embodiment the invention provides adenovirus for the treatment rheumatoid arthritis or disease treatable by nucleic acid delivery to fibroblast-like or macrophage-like cells, preferably synoviocytes.

In yet another embodiment the invention provides adenovirus capsids as part of a pharmaceutical for the treatment of diseases. In yet another embodiment the

invention provides adenovirus capsids for the preparation of a medicament for the treatment of diseases.

In another aspect of the invention is provided construct pBr/Ad.BamRΔFib, comprising adenovirus 5 sequences 21562-31094 and 32794-35938.

In another aspect of the invention is provided construct pBr/AdBamRfib16, comprising adenovirus 5 sequences 21562-31094 and 32794-35938, further comprising an adenovirus 16 nucleic acid encoding fiber protein.

In yet another aspect of the invention is provided construct pBr/AdBamR.pac/fib16, comprising adenovirus 5 sequences 21562-31094 and 32794-35938, further comprising an adenovirus 16 nucleic acid encoding fiber protein, and further comprising a unique PacI-site in the proximity of the adenovirus 5 right terminal repeat, in the non-adenovirus sequence backbone of said construct.

In another aspect of the invention is provided construct pWE/Ad.AflIIrITRfib16 comprising Ad5 sequence 3534-31094 and 32794-35938, further comprising an adenovirus 16 nucleic acid encoding fiber protein.

In another aspect of the invention is provided construct pWE/Ad.AflIIrITRDE2Afib16 comprising Ad5 sequences 3534-22443 and 24033-31094 and 32794-35938, further comprising an adenovirus 16 nucleic acid encoding fiber protein.

In the numbering of the sequences mentioned above, the number is depicted until and not until plus.

In a preferred embodiment of the invention said constructs are used for the generation of a nucleic acid delivery vehicle or an adenovirus capsid with a tissue tropism for fibroblast-like or macrophage-like cells, preferably synoviocytes.

In another aspect the invention provides a library of adenovirus vectors, or nucleic acid delivery vehicles which may be one and the same or not, comprising a large selection

of non-adenovirus nucleic acids. In another aspect of the invention, adenovirus genes encoding capsid proteins are used to generate a library of adenovirus capsids comprising of proteins derived from at least two different adenoviruses, said adenoviruses preferably being derived from two different serotypes, wherein preferably one serotype is an adenovirus of subgroup B. In a particularly preferred embodiment of the invention a library of adenovirus capsids is generated comprising proteins from at least two different adenoviruses and wherein at least a tissue tropism determining part of fiber protein is derived from an adenovirus of subgroup B, preferably of adenovirus 16.

In one embodiment the invention provides a subgroup B adenovirus capsid comprising a nucleic acid encoding at least one non-adenovirus proteinaceous molecule or RNA molecule. Preferably said subgroup B adenovirus nucleic acid further comprises subgroup B adenovirus nucleic acid. More preferably said subgroup B adenovirus nucleic acid has been deprived of the capacity to express E1-region encoded proteins. Most preferably said subgroup B adenovirus is adenovirus 16.

In another aspect the invention provides a method for at least in part removing synovium from a joint in an individual comprising administering to said joint a nucleic acid delivery vehicle comprising nucleic acid encoding at least herpes simplex virus thymidine kinase or a functional part, derivative and/or analogue thereof and administering to said individual ganciclovir or a functional part, derivative and/or analogue thereof. Preferably, said gene delivery vehicle is vehicle of the invention.

A fiber protein of adenovirus 16 preferably comprises at least part of the sequence given in figure 7. However within the scope of the present invention other sequences may be used for instance obtained through using codon degeneracy.

Alternatively, a fiber sequence may comprise amino-acid substitutions or insertions or deletions compared to the sequence depicted in figure 7, as long as the desired tissue tropism determining property is not significantly altered. Amino-acid substitutions may be within the same polarity group or without.

A transduced cell is a cell provided with nucleic acid. Said cell may have been provided with nucleic acid through any means. Similarly, to measure transduction of a cell means to measure nucleic acid transfer into said cell. Said transfer may have occurred through any means capable of transferring nucleic into a cell.

EXAMPLES

Example 1: Generation of adenovirus serotype 5 based viruses with chimaeric fiber proteins

Generation of adenovirus template clones lacking DNA encoding for fiber. The fiber coding sequence of adenovirus serotype 5 is located between nucleotides 31042 and 32787. To remove the adenovirus serotype 5 DNA encoding fiber we started with construct pBr/Ad.Bam-rITR (Figure 1; ECACC deposit P97082122). From this construct first a NdeI site was removed. For this purpose, pBr322 plasmid DNA was digested with NdeI after which protruding ends were filled using Klenow enzyme. This pBr322 plasmid was then re-ligated, digested with NdeI and transformed into *E. coli* DH5α. The obtained pBr/ΔNdeI plasmid was digested with ScaI and SalI and the resulting 3198 bp vector fragment was ligated to the 15349 bp ScaI-SalI fragment derived from pBr/Ad.BamrITR, resulting in plasmid pBr/Ad.Bam-rITRΔNdeI which hence contained a unique NdeI site. Next a PCR was performed with

oligonucleotides "NY-up" and "NY-down" (Figure 2). During amplification, both a NdeI and a NsiI restriction site were introduced to facilitate cloning of the amplified fiber DNAs. Amplification consisted of 25 cycles of each 45 sec. at 94°C, 1 min. at 60°C, and 45 sec. at 72°C. The PCR reaction contained 25 pmol of oligonucleotides NY-up or NY-down, 2mM dNTP, PCR buffer with 1.5 mM $MgCl_2$, and 1 unit of Elongase heat stable polymerase (Gibco, The Netherlands). One-tenth of the PCR product was run on an agarose gel which demonstrated that the expected DNA fragment of ± 2200 bp was amplified. This PCR fragment was subsequently purified using Geneclean kit system.(Bio101 Inc.) Then, both the construct pBr/Ad.BamrITRΔNdeI as well as the PCR product were digested with restriction enzymes NdeI and SbfI. The PCR fragment was subsequently cloned using T4 ligase enzyme into the NdeI and SbfI sites thus generating pBr/Ad.BamRΔFib (Figure 3).

Amplification of fiber sequences from adenovirus serotypes.

To enable amplification of the DNAs encoding fiber protein derived from alternative serotypes degenerate oligonucleotides were synthesised. For this purpose, first known DNA sequences encoding for fiber protein of alternative serotypes were aligned to identify conserved regions in both the tail region as well as the knob region of the fiber protein. From the alignment, which contained the nucleotide sequence of 19 different serotypes representing all 6 subgroups, (degenerate) oligonucleotides were synthesised (see Table I). Also shown in table I is the combination of oligonucleotides used to amplify the DNA encoding fiber protein of a specific serotype. The amplification reaction (50 µl) contained 2 mM dNTPs, 25 pmol of each oligonucleotide, standard 1x PCR buffer, 1,5 mM $MgCl_2$, and 1

Unit Pwo heat stable polymerase (Boehringer Mannheim) per reaction. The cycler program contained 20 cycles, each consisting of 30 sec. 94°C, 60 sec. 60-64°C, and 120 sec. 72°C. One-tenth of the PCR product was run on an agarose gel to demonstrate that a DNA fragment was amplified. Of each different template, two independent PCR reactions were performed.

Generation of chimaeric adenoviral DNA constructs

All amplified fiber DNAs as well as the vector (pBr/Ad.BamRΔFib) were digested with NdeI and NsiI. The digested DNAs were subsequently run on a agarose gel after which the fragments were isolated from the gel and purified using the Geneclean kit (Bio101 Inc). The PCR fragments were then cloned into the NdeI and NsiI sites of pBr/AdBamRΔFib, thus generating pBr/AdBamRFibXX (where XX stands for the serotype number of which the fiber DNA was isolated). The inserts generated by PCR were sequenced to confirm correct amplification. The obtained sequences of the different fiber genes are shown in Figure 4.

Generation of recombinant adenovirus chimaeric for fiber protein.

To enable efficient generation of chimaeric viruses an AvrII fragment from the pBr/AdBamRFib16, pBr/AdBamRFib28, pBr/AdBamRFib40-L constructs was subcloned into the vector pBr/Ad.Bam-rITR.pac#8 (ECACC deposit #P97082121) replacing the corresponding sequences in this vector. pBr/Ad.Bam-rITR.pac#8 has the same adenoviral insert as pBr/Ad.Bam-rITR but has a PacI site near the rITR that enables the ITR to be separated from the vector sequences. The construct pWE/Ad.AflII-Eco was generated as follows. pWE.pac was

digested with ClaI and the 5 prime protruding ends were filled in with klenow enzyme. The DNA was then digested with PacI and isolate from agarose gel. pWE/AflIIrITR was digested with EcoRI and after treatment with klenow enzyme digested with PacI. The large 24 kb. fragment containing the adenoviral sequences was isolated from agarose gel and ligated to the ClaI digested and blunted pWE.pac vector. Use was made of the ligation express kit from Clontech. After transformation of XL10-gold cells from Stratagene, clones were identified that contained the expected construct. pWE/Ad.AlfII-Eco contains Ad5 sequences from basepairs 3534-27336. Three constructs, pClipsal-Luc (Figure 5) digested with SalI, pWE/Ad.AflII-Eco digested with PacI and EcoRI and pBr/AdBamR.pac/fibXX digested with BamHI and PacI were transfected into adenovirus producer cells (PER.C6, Fallaux *et al*, 1998). Figure 6 schematically depicts the method and fragments used to generate the chimaeric viruses. Only pBr/Ad.BamRfib12 was used without subcloning in the PacI containing vector and therefore was not liberated from vector sequences using PacI but was digested with ClaI which leaves approximately 160 bp of vector sequences attached to the right ITR. Furthermore, the pBr/Ad.BamRfib12 and pBr/Ad.BamRfib28 contain an internal BamHI site in the fiber sequences and were therefor digested with SalI which cuts in the vector sequences flanking the BamHI site. For transfection, 2 µg of pCLIPsal-Luc, and 4 µg of both pWE/Ad.AflII-Eco and pBr/AdBamR.pac/fibXX were diluted in serum free DMEM to 100 µl total volume. To this DNA suspension 100 µl 2.5x diluted lipofectamine (Gibco) in serum-free medium was added. After 30 minutes at room temperature the DNA-lipofectamine complex solution was added to 2.5 ml of serum-free DMEM which was subsequently added to

a T25 cm^2 tissue culture flask. This flask contained PER.C6 cells that were seeded 24-hours prior to transfection at a density of $1x10^6$ cells/flask. Two hours later, the DNA-lipofectamine complex containing medium was diluted once by the addition of 2.5 ml DMEM supplemented with 20% foetal calf serum. Again 24 hours later the medium was replaced by fresh DMEM supplemented with 10% foetal calf serum. Cells were cultured for 6-8 days, subsequently harvested, and freeze/thawed 3 times. Cellular debris was removed by centrifugation for 5 minutes at 3000 rpm room temperature. Of the supernatant (12.5 ml) 3-5 ml was used to infect again PER.C6 cells (T80 cm^2 tissue culture flasks). This re-infection results in full cytopathogenic effect (CPE) after 5-6 days after which the adenovirus is harvested as described above.

Production of chimaeric adenoviruses

10 ml of the above crude cell lysate was used to inoculate a 1 litre fermentor which contained 1 - 1.5 x 10^6 PER.C6 cells/ml growing in suspension. Three days after inoculation, the cells were harvested and pelleted by centrifugating for 10 min at 1750 rpm at room temperature (RT). Adenovirus present in the pelleted cells was subsequently extracted and purified using the following downstream processing protocol. The pellet was dissolved in 50 ml 10 mM $NaPO_4^-$ and frozen at -20°C. After thawing at 37°C, 5.6 ml deoxycholate (5% w/v) was added. The solution was mixed and incubated for 15 minutes at 37°C to completely lyse the cells. After homogenising the solution, 1875 µl 1M $MgCl_2$ and 5 ml glycerol was added. After the addition of 375 µl DNase (10 mg/ml) the solution was incubated for 30 minutes at 37°C. Cell debris was removed by centrifugation at 1880xg

for 30 minutes at RT without brake. The supernatant was subsequently purified from proteins by extraction with freon (3x). The cleared supernatant was loaded on a 1M Tris/HCl buffered caesiumchloride blockgradient (range: 1.2/1.4 gr/ml) and centrifuged at 21000 rpm for 2.5 hours at 10°C. The virus band is isolated after which a second purification using a 1M Tris/HCl buffered continues gradient of 1.33 gr/ml of caesiumchloride was performed. The virus was then centrifuged for 17 hours at 55000 rpm at 10°C. The virus band is isolated and sucrose (50 % w/v) is added to a final concentration of 1%. Excess caesiumchloride is removed by dialysis (three times 1 hr at RT) in dialysis slides (Slide-a-lizer, cut off 10000 kDa, Pierce, USA) against 1.5 ltr PBS supplemented with $CaCl_2$ (0.9 mM), $MgCl_2$ (0.5mM) and an increasing concentration of sucrose (1, 2, 5%). After dialysis, the virus is removed from the slide-a-lizer after which it is aliquoted in portions of 25 and 100 µl upon which the virus is stored at -85°C.

To determine the number of virus particles per ml, 50 µl of the virus batch is run on an high pressure liquid chromatograph (HPLC) as described by Shabram et al (1997) using a 300-600 mM NaCl gradient. The virus titer of the chimaeric virus was found to be in the same range as the Ad5.Clip.Luc virus batch (Ad5.Clip.Luc: $2.2x10^{11}$ vp/ml; Ad5.Luc-fib16: $3.1x10^{12}$ vp/ml).

EXAMPLE 2:

Biodistribution of chimaeric viruses after intravenous tail vein injection of rats.

To investigate the biodistribution of the chimaeric adenovirus Ad5.Luc-fib16 in comparison to Ad5 based luciferase viruses, $1x10^{10}$ particles of each of the virus batches were diluted to 1 ml with PBS and the virus was injected in the tail vein of adult male Wag/Rij rats (3 rats/virus). Forty-eight hours after the administration of the virus, the rats were sacrificed after which the liver, spleen, lung, kidney, heart and brain were dissected. These organs were subsequently mixed with 1 ml of lysis buffer (1% Triton X-100 in PBS) and minced for 30 seconds to obtain a protein lysate. The protein lysate was tested for luciferase activity and the protein concentration was determined. The results, shown in Table II, demonstrate that the adenovirus serotype 5 is targeted for a large part to the liver and to the spleen, whereas the Ad5.Luc-fib16 chimearic virus is not. This experiment shows that it is possible to circumvent the uptake of adenoviruses by the liver by making use of fibers of other serotypes.

EXAMPLE 3:

Production of fiber chimeric adenovirus

Another batch of Ad5.Luc-fib16 was made by using 10 ml crude extract to inoculate a 1 litre fermentor which contained 1 - 1.5 x 10^6 cells/ ml PER.C6 that were specifically adapted to grow in suspension. Three days after inoculation, the cells were harvested and pelleted by centrifuging for 10 min at 1750 rpm at room temperature. The chimeric adenovirus present in the pelleted cells was subsequently extracted and purified using the following

downstream processing protocol. The pellet was dissolved in 50 ml 10 mM $NaPO_4$ and frozen at -20°C. After thawing at 37°C, 5.6 ml deoxycholate (5% w/v) was added after which the solution was homogenated. The solution was subsequently incubated for 15 minutes at 37°C to completely crack the cells. After homogenising the solution, 1875 µl (1M) $MgCl_2$ was added and 5 ml 100% glycerol. After the addition of 375 µl DNase (10 mg/ ml) the solution was incubated for 30 minutes at 37°C. Cell debris was removed by centrifugation at 1880xg for 30 minutes at room temperature without the brake on. The supernatant was subsequently purified from proteins by loading on 10 ml of freon. Upon centrifugation for 15 minutes at 2000 rpm without brake at room temperature three bands are visible of which the upper band represents the adenovirus. This band was isolated by pipetting after which it was loaded on a Tris/HCl (1M) buffered caesium chloride block gradient (range: 1.2 to 1.4 gr./ml). Upon centrifugation at 21000 rpm for 2.5 hours at 10°C the virus was purified from remaining protein and cell debris since the virus, in contrast to the other components, does not migrate into the 1.4 gr./ ml caesium chloride solution. The virus band is isolated after which a second purification using a Tris/ HCl (1M) buffered continues gradient of 1.33 gr. /ml of caesium chloride is performed. After virus loading on top of this gradient, the virus is centrifuged for 17 hours at 55.000 rpm at 10°C. Subsequently, the virus band is isolated and after the addition of 30 µl of sucrose (50 w/v) excess caesium chloride is removed by three rounds of dialysis, each round comprising of 1 hour. For dialysis the virus is transferred to dialysis slides (Slide-a-lizer, cut off 10.000 kDa, Pierce, USA). The buffers used for dialysis are PBS which are supplemented with an increasing concentration of sucrose (round 1 to 3: 30 ml, 60 ml, and 150 ml sucrose (50% w/v)/ 1.5 litre PBS, all supplemented with 7.5 ml 2% (w/v) $CaMgCl_2$). After dialysis, the virus is removed from the slide-a-lizer after which it is aliquoted in portions of 25

and 100 µl upon which the Ad5.Luc-fib16 virus is stored at -85°C.

To determine the number of virus particles per millilitre, 50 µl of the virus batch is run on a high-pressure liquid chromatograph (HPLC). The adenovirus is bound to the column (anion exchange) after which it is eluted using a NaCl gradient (range 300-600 mM). By determination of the area under the virus peak the number of virus particles can be calculated. To determine the number of infectious units (IU) per ml present in a virus batch, titrations are performed on 911 cells. For this purpose, $4x10^4$ 911 cells are seeded per well of 96-well plates in rows B, D, and F in a total volume of 100 µl per well. Three hours after seeding the cells are attached to the plastic support after which the medium can be removed. To the cells a volume of 200 µl is added, in duplicate, containing different dilutions of virus (range: 10^2 times diluted to $2x10^9$). By screening for CPE the highest virus dilution which still renders CPE after 14 days is considered to contain at least one infectious unit. Using this observation, together with the calculated amount of virus volume present in these wells renders the number of infectious units per ml of a given virus batch.

EXAMPLE 4:
Chimeric viruses display differences in synoviocyte cell transduction

Infection of human synoviocytes

In a first set of experiments 50.000 synoviocytes (derived from 1 individual) were seeded in each well of a 24-wells plate in a volume of 1 ml per well. Twenty-four hours after seeding, the cells were washed with PBS after which 200 µl of DMEM supplemented with 2% FCS was added to the cells. This medium contained various amounts of virus (a

multiplicity of infection (MOI) of 50, 250, 1250, 2500, 5000, and 10000 vp/cell was used). Viruses were either Ad5.Clip.Luc or Ad5.Luc-fib16. Two hours after addition of virus the medium was replaced by normal medium thus removing the non-bound virus (each infection in duplicate). Again forty-eight hours later cells were washed and lysed by the addition of 100 µl lysis buffer after which luciferase transgene expression was monitored. In figure 8, results are shown of the luciferase transgene expression per microgram protein after infection of synoviocytes. These results show that the fiber 16 chimeric adenovirus infects synoviocytes significantly better, based on transgene expression, as compared to the control Adenovirus serotype 5. The fold increase of the fiber 16 chimeric adenovirus over the control adenovirus serotype 5 ranged, depending on the MOI used, from 2.4x (MOI 50) to 1052x (MOI 10000). Identical experiments demonstrated on average (n=4) at least a factor 100 difference in transgene expression between the adenovirus serotype 5 and the fiber 16 chimeric adenovirus.

In a second set of experiments, an equal number of virus particles was added to different concentrations of synoviocytes. This experiment was performed since it is possible that the efficiency of infection of these cells is dependent on the confluency of the synoviocyte cell layer. A highly confluent cell layer may mimic the *in vivo* situation better. For this purpose, synoviocytes were seeded at concentrations of 12.500, 25.000, 50.000, and 100.000 cells per well of 24-well plates (in duplicate). Twenty-four hours later these cells were infected as described above with medium containing 2.5×10^8 virus particles. The result of the luciferase transgene expression determined 48 hours after a two hours infection procedure (see figure 9) shows that the fiber 16 chimeric adenovirus renders a ±1000 fold higher expression of luciferase and thus is clearly better suited to infect synoviocytes also when cells are 100% confluent.

In a third set of experiments we determined the differences in the level of luciferase transgene expression versus the time of virus exposure. This experiment was performed to demonstrate that the binding kinetics of the fiber 16 chimeric adenovirus is different from that of the adenovirus serotype 5 control virus. For this purpose 15.000 synoviocytes were seeded in 24-well plates in a volume of 1 ml. Twenty-four hours later cells were infected (in triplicate) with an MOI of 50, 500, or 5.000 vp/cell. infection was allowed to proceed either for two hours or for 20 hours. The results, shown in figure 10, demonstrate that binding kinetics and characteristics of the fiber 16 chimeric adenovirus is distinct from that of the control adenovirus serotype 5 and that the fiber 16 chimeric adenovirus infects synoviocytes much more efficient as compared to the control adenovirus serotype 5 virus.

From the above described results it is clear that the fiber 16 chimeric virus is better suited to infect synoviocytes as compared to the adenovirus serotype 5. Since it is known that adenovirus serotype 5 requires the coxacki adenovirus receptor (CAR) and the integrins $\alpha_v\beta3$ and $\alpha_v\beta5$ for entry we monitored expression of these molecules on synoviocytes using flow cytometry. For this purpose $1x10^5$ synoviocytes were transferred to tubes designed specifically for flow cytometry. Cells were washed once with PBS/ 0.5% BSA after which the cells were pelleted by centrifugation for 5 minutes at 1750 rpm at room temperature. Subsequently, 10 µl of a 100 times diluted $\alpha_v\beta3$ antibody (Mab 1961, Brunswick chemie, Amsterdam, The Netherlands), a 100 times diluted antibody $\alpha_v\beta5$ (antibody (Mab 1976, Brunswick chemie, Amsterdam, The Netherlands), or 2000 times diluted CAR antibody (a gift from Dr. Bergelson, Harvard Medical School, Boston, USA (Hsu et al, 1988) was added to the cell pellet after which the cells were incubated for 30 minutes at 4°C in a dark environment. After this incubation, cells were washed

twice with PBS/0.5% BSA and again pelleted by centrifugation for 5 minutes at 1750 rpm room temperature. To label the cells, 10 µl of rat-anti-mouse IgG1 labelled with phycoerythrine (PE) was added to the cell pellet upon which the cells were incubated for 30 minutes at 4°C in a dark environment. Finally, the cells were washed twice with PBS/0.5% BSA and analysed on a flow cytometer. The results of this experiment are shown in table III.

These flow cytometric results demonstrate that synoviocytes do not express detectable levels of CAR, which may be at least one of the reasons that these cells are difficult to transduce with the adenovirus serotype 5.

As a control for the experiments performed on synoviocytes, A549 and PER.C6 cells were infected. These cell lines can be readily infected by adenovirus serotype 5. This experiment is performed to investigate whether the observed differences on the synoviocytes can indeed be attributed to differences in cell binding or that the differences are caused by differences in virus particle per infectious unit ratio. For this purpose, 10^5 A549 cells were seeded in 24-well plates in a volume of 200 µl. Two hours after seeding the medium was replaced by medium containing different amounts of particles of either Ad5.Luc-fib16 or Ad5.Clip.Luc (MOI = 0, 5, 10, 25, 100, 500). Twenty-four hours after the addition of virus, the cells were washed once with PBS after which the cells were lysed by the addition of 100 µl lysis buffer to each well (1% Triton X-100 in PBS) after which transgene expression (luciferase activity) and the protein concentration was determined. Subsequently, the luciferase activity per µg protein was calculated. These data, shown in table IV demonstrate that when using a identical amount of virus particles, differences in transgene expression observed in relevant cell types is due to differences in binding and/

or internalisation of the virus and not to the amount of virus used.

A similar experiment was performed on PER.C6 cells using adenovirus serotype 5 and the fiber chimera fiber 16. For this purpose, 10^5 PER.C6 cells, were seeded in 24-wells plates in a total volume of 100 µl. Three hours after seeding, the medium was replaced by medium containing 10^6 particles of either Ad5.Clip.Luc or Ad5.Luc-fib16 (MOI = 10). Twenty-four hours after addition of the virus, cells were washed once with PBS after which 100 µl lysis buffer was added to the attached cells. The lysate was subsequently used to determine transgene expression (luciferase activity) and the protein concentration. The results, shown in table V, again demonstrate that the differences in infection efficiency as observed on synoviocytes, in favour of the fiber 16 chimeric adenovirus, are differences related to binding efficiency rather than to the amount of virus used.

Example 5

Treatment of RA with herpes simplex virus thymidine kinase

MATERIALS AND METHODS

Recombinant adenoviral vectors:

The adenoviral vectors used in this study contain the recombinant gene inserted into the E1 region of an Ad type 5 mutant. The cytomegalovirus promoter (CMV) and the major late promoter (mlp) were used to drive gene expression in the constructs harbouring the lacZ and luciferase marker genes. Mlp was used to drive gene expression in the Ad harbouring the TK gene. Virus concentrations were determined by titration of the virus. Ad were tested to contain no replication competent wild-type Ad or E1a recombination. The adenoviral vectors IG.Ad.CMV.lacZ, IG.Ad.mlp.lacZ, IG.Ad.CMV.luc, IG.Ad.mlp.luc and IG.Ad.mlp-I. TK and their

production have been previously described in detail (Imler et al; Vincent et al, 1996).

Synovial fibroblast culture.

Human synovium was obtained from patients with RA defined by ARA-criteria 1987 (Arnett et al, 1988) at the time of joint replacement surgery. Synovial tissue was collected in sterile Phosphate Buffered Saline (PBS). Fat and connective tissue were discarded and tissue was incubated with 0.5 mg collagenase/ml for 2 h at 37 EC. Cells were washed and seeded in 75-cm^2 flasks in 10 ml of Iscoves Modified Dulbeco's Medium (IMDM) 17 % fetal calf serum (FCS). Medium was refreshed twice a week. Confluent cultures of adherent synoviocytes were passaged at a 1:2 ratio in 75-cm^2 flasks. The cells were detached from the flasks with 1.5 ml 0.25 % trypsin-EDTA dissolved in PBS at room temperature.

Infections:

The day prior to infections, synovial cells were plated at a density of 100,000 per 25-cm^2 bottle in reporter gene experiments or 5,000 per well (24 wells plate) in TK experiments. Cells were cultured in respectively 10 or 1 ml of IMDM 17 % FCS. In the procedure of infection of synoviocytes, medium was replaced by the appropriate dose of modulated virus in IMDM 17 % FCS.

LacZ in-vitro experiments:

After 2 days of incubation the number of synoviocytes were counted in a negative control and in a sample incubated with virus concentration multiplicity of infection (MOI) 100. Remaining samples were washed with PBS, fixed briefly with glutaraldehyde 0.25%, washed with PBS (2 x) and stained by immersion in 5 mM $K_4Fe(CN)_6$, 5mM $K_3Fe(CN)_6$, 2mM $MgCl_2$ in PBS containing 0.5 mg/ml of X-gal stain (5-bromo-4-chloro-3-indolyl-8-D-galactopyranoside; Sigma Chemical Co., St.Louis, USA). After four to six hours samples were washed twice and the reaction was stopped by glutaraldehyde 0.25% Percentage of infected cells was assessed by light microscopy after counting at least 300 cells (magnification 10x40).

Luciferase in-vitro experiments:

After 3 days of incubation with Ad.luc synoviocyte counts were made comparable to lacZ experiment. Remaining samples were washed with PBS and trypsinised briefly. Synoviocytes were lysed using 200 :l lysis buffer. Samples of 20 :l were analysed by luminometric methods.

TK in-vitro experiments:

One day after incubation with Ad.TK medium was replaced by IMDM 40 % Normal Human Serum (NHS). In half of the cultures 10 µg GCV (9-[1,3-dihydrate-2-propoxy]methyl]guanine, Roche Nederland BV, the Netherlands) was added per ml medium. Medium plus or minus GCV was refreshed on day 3. Cells counts were made 5 days after virus infection.

In the TK-bystander killing experiment one 75-cm^2 flask with synoviocytes was trypsinised and divided over three flasks. Two flasks were infected with respectively IG.Ad.mlpI.TK or IG.Ad.CMV.TK. One day later infected and non-infected cells were mixed according to scheme (see figure 14). Medium was replaced by IMDM 40 % NHS plus or minus GCV. Cell counts were made after 7 days.

Animals and intra-articular injections:

All animal protocols were approved by the Medical Ethical Committee and performed according to institutional guidelines. 8 Adult rhesus monkeys (Macaca mulatta) suffering from CIA (Bakker, 1992) were used for these experiments and held under D2 containment. Before handling, monkeys were anaesthetised with a single intramuscular dose of approximately 1 ml of 85-90 % ketamine [100 :l/kg, 10 mg/ml] (ASP Pharma BV Oudewater, The Netherlands) and 10-15 % vetranquil. If an animal was experiencing severe pain it was given twice a day 0.06 mg Burprenorfine (Temgesic-R, Schering-Plough BV, Amstelveen, the Netherlands).

Before intra-articular punction the area surrounding knees was shaved and rinsed with iodine. Using sterile technique, respectively 1 ml or 0.1 ml of purified recombinant virus suspended in PBS was injected according to scheme into the

intra-articular space of the knee or proximal interphalangeal joint (pip). Beginning forty-eight hours after injection of the virus, monkey 7 and 8 received 10 mg/kg GCV infused in half an hour, daily for fourteen days. Animals were killed by intracordial punction and bleeding. For summary of rhesus monkeys experiments see table VI.

Parameters:

Animals were monitored daily for general health, which included recording of behaviour, appetite and stool consistency. Evaluation of biochemical parameters was performed on a number of days after virus administration (see table VI). For this purpose, animals were sedated as described above, body weight and rectal temperature were measured and venous blood samples were collected [clotted and sodium ethylenediamine tetra-acetic acid (EDTA)-treated blood]. Analysis of the blood serum included electrolytes (Na, K, Cl and bicarbonate); kidney function (urea, creatinine) and liver function [alkaline phosphatase, asparagine-aminotransferase (ASAT), alanine-aminotransferase (ALAT); lactate dehydrogenate (LDH) and total bilirubin]; total protein and albumin; and haematological parameters (red and white blood cell counts, differential count, platelet count, erythrocyte sedimentation rate (ESR). In monkey 5-8 venous blood was drawn in clot tubes and analysed for the presence of antibodies against Ad by complement fixation assay, according to routine procedures at the department of infectious diseases and immunology (SSDZ Delft, The Netherlands).

Faeces, urine and pharyngeal swabs were collected on different sampling days (see table VI) and stored frozen. Analysis consisted of culturing extracts on 293 cells (growth of wild-type and recombinant virus) and hep-2 cells (growth of wild-type virus)(Bout et al, 1994).

A complete post-mortem necropsy and histopathological examination of aorta, axillary lymphnodes, bladder, colon, duodenum, hart, inguinal lymphnodes, lung, liver, lymphnodes

of the lung hilus, spleen, left kidney, oesophagus, pancreas, thyroid gland, skeleton muscle, bone marrow, thymus, trachea, cervix/vagina and ovary or prostate and testis were performed. Samples of these tissues were fixed in 10 % phosphate buffered formalin for routine histopathological analysis.

In addition in monkey 1-5 snap frozen samples of axillary lymphnodes, hart, inguinal lymphnodes, liver, spleen, left kidney, lung, bladder, oesophagus, bone marrow and synovium injected joints and non-injected control joints were taken for luciferase assay (Sawchuk, 1996). Joints were opened, coloured with X-gal staining solution (Roessler et al, 1993; Bout et al, 1993) and post-fixed in formalin for at least 72 hours. Joints were cut using a diamond saw, subsequently pieces were imbedded in plastic and 6 : slices were cut using a microtome. Slices were stained with haematoxylin and eosin according to standard procedures at the pathological laboratory of Leiden University Hospital, The Netherlands.

RESULTS

IN-VITRO:

Possibility of gene transfer to synoviocytes.

Synoviocytes were infected with modified Ad using different reportergenes and different promoters. Two days after infection of synoviocytes with IG.Ad.CMV.lacZ at MOI 100, 67 % cells were positive for X-gal, as evidenced by a microscopically visible blue colour of the cells. In synovial cell cultures a doses response relation was observed between the amount of virus added and gene expression of the reporter gene, both after infection with IG.Ad.CMV.lacZ and IG.Ad.CMV.luc (see table XI and table XII). When incubation time was prolonged to five days, 100 % of synoviocytes stained blue. Gene expression after infection with Ad constructs driven by the CMV promoter is higher than by Ad constructs driven by the mlp-promoter. This difference is more prominent using lacZ as a reporter gene (± 100 x) than using luciferase as a reporter gene (± 10 x). Two days after infection with IG.Ad.mlp.lacZ at MOI 100, less than 1 % was positive for lacZ. However, clear gene expression in a dose dependent fashion was observed if the luciferase reporter gene was used (table XII).

Toxicity of gene transfer to synoviocytes.

To assess possible toxicity of high doses Ad for synoviocytes, synoviocytes were cultured without virus or incubated with Ad.lacZ, Ad.luc or Ad.TK at MOI 100. Cell counts of synoviocyte cultures after infection with modified Ad at MOI 100 showed no significant differences compared to non-infected cultures (table VII).Students t-test for paired samples $p > 0.2$.

Efficacy of cell-killing.

Synoviocytes incubated with IG.Ad.mlp.TK were cultured with or without 10 :µg/ml GCV. 99 percent cell killing was observed after infection of synoviocytes with IG.Ad.CMV.TK and incubation with GCV, infection with IG.Ad.mlp.TK led to

80 % cell killing (see figure 11). After mixing 25 % transduced with 75 % untransduced synoviocytes, bystander killing was assessed. Both in IG.Ad.CMV.TK and IG.Ad.mlp.TK experiments extensive cell killing was observed (see figure 12).

IN-VIVO:

Possibility and specificity of gene transfer to inflamed synovial tissue *in-vivo*:

36 joints (10 knees and 26 pip's) of 8 different monkeys were injected with different amounts of IG.Ad.lacZ, IG.Ad.luc or IG.Ad.mlp-I.TK. In the biodistribution experiments, a CMV promoter was chosen to allow maximum sensitivity in detection of reporter gene product.

Histological examination of articular and peri-articular tissues obtained 2-3 days after infection with IG.Ad.CMV.lacZ showed lacZ expressing cells present in synovial villi as well as in the synovial tissue covering tendons, bone, articular cartilage and subsynovial adipose tissue (figure 13). The cells expressing lacZ activity were synoviocytes as evidenced by typical location and morphologic appearance. The percentage of infected cells ranged from 5 to 70 %. Joints injected with PBS and non-injected joints did not show any lacZ positive cells. No infection of cartilage, bone, fat or muscle tissue was observed. If the less efficient mlp promoter was used (in monkey 5 and in pip 2 monkey 2) no lacZ positive cells could be found.

Dose response after gene transfer to synoviocytes in-vivo.

In monkey 4 and 5 increasing amounts of modified Ad were injected in consecutive pip-joints in the monkeys. In the pip-joints of monkey 4, injected with IG.Ad.CMV.lacZ, an obvient dose-response relation was observed in percentage of lacZ expressing cells (table VIII). In monkey 5, injected with IG.Ad.mlp.lacZ, no lacZ positive cells were observed in the synovium.

Toxicity of intra-articular (i.a.) administration of Ad harbouring a reportergene:

Biodistribution:

To asses toxicity of the procedure, biodistribution of the virus was determined using Ad harbouring the luciferase reportergene. Luciferase-activity, measured by luminometric methods, indicates infection of the organ by Ad. Monkey 1-5 were injected by Ad.CMV.luc or IG.Ad.mlp.luc and were sacrificed 2-3 days after virus administration. Specimens of synovial tissue were harvested. From the same biopsies histological confirmation was obtained to judge if the sample contained relevant tissue. In monkey 4 the samples contained mainly connective tissue and no synovial tissue. Samples of above mentioned organs and joints were analysed using the luciferase assay. Samples obtained from a non-treated monkey were used as a control. Except for one sample (cervix) and two non-virus injected joints that had slightly elevated luciferase counts, only IG.Ad.luc injected joints were positive in the luciferase assay (table IX).

To assess shedding of the virus excreta were cultured during the first 3 days of the experiment. In the faeces (day 0-3) of monkey 5 Ad could be cultured both on 293- and hep-2 cells. The throat swab of this monkey was positive on 293 cells on day 1. In the other monkeys faeces, urine and throat swabs were negative in the Ad culture assay.

Clinical behaviour.

During the experiment monkey 3 and 5 suffered from a severe arthritis, which made climbing difficult and led to diminished appetite and weight loss. One monkey that suffered from severe arthritis had a slightly elevated body temperature up to 40 °C. Analyses on blood samples indicated increase in CRP, thrombocytosis, hypalbuminaemia and anaemia related to the presence of arthritis symptoms. A small increase in LDH-levels was observed (table X).

Histopathological analysis showed synovitis, moderate chronic pleuritis, necrotizing dermatitis, mild-chronic enteritis and inguinal and axillary lymphadenopathy in all monkeys. In order to analyse local inflammation induced by the procedure of i.a. administration of Ad, non-injected, saline-injected and Ad-injected joints were compared by routine histopathological analysis. No significant differences were observed in synovial hyperplasia or lymphocyte infiltration.

Toxicity of i.a. administration of Ad harbouring the suicide gene TK.

During the TK-experiments, monkeys were closely observed to detect any toxicity of the procedure. The behaviour of the monkeys, clinical observations, biochemical parameters and histopathological analysis did only show abnormalities as has been reported before in monkeys with CIA. No additional toxicity was seen in suicide gene treated groups as compared to reporter gene treated groups. Histopathological analysis revealed no differences except for multifocal mid-zonal and peripheral infiltrations with lymphocytes and plasma cells in the liver of monkey 6 with single hepatocellular necrosis. Effectivity of suicide gene transfer to inflamed synovial tissue can be seen as local toxicity of the procedure. Histopathological analysis of the injected joint revealed no differences in synovial hyperplasia or lymphocyte infiltration compared to control joints. Joint circumference diminished 1 cm in knees injected with IG.Ad.mlp-I.TK followed by GCV and 1 to 1.5 cm in non-injected knees. In monkey 6, 7 and 8 who were terminated 14 to 18 days after intra-articular injection, a turn in antibody titer from negative to positive was observed after day 5-7. No viruses were cultured from the excreta.

EXAMPLE 6:

Plasmid-based system for rapid RCA-free generation of recombinant adenoviral vectors.

Construction of adenovirus clones

1. pBr/Ad.Bam-rITR (ECACC deposit P97082122)

In order to facilitate blunt end cloning of the ITR sequences, wild-type human adenovirus type 5 (Ad5) DNA was treated with Klenow enzyme in the presence of excess dNTPs. After inactivation of the Klenow enzyme and purification by phenol/chloroform extraction followed by ethanol precipitation, the DNA was digested with BamHI. This DNA preparation was used without further purification in a ligation reaction with pBr322 derived vector DNA prepared as follows: pBr322 DNA was digested with EcoRV and BamHI, dephosphorylated by treatment with TSAP enzyme (Life Technologies) and purified on LMP agarose gel (SeaPlaque GTG). After transformation into competent E.coli DH5α (Life Techn.) and analysis of ampiciline resistant colonies, one clone was selected that showed a digestion pattern as expected for an insert extending from the BamHI site in Ad5 to the right ITR.

Sequence analysis of the cloning border at the right ITR revealed that the most 3' G residue of the ITR was missing, the remainder of the ITR was found to be correct. Said missing G residue is complemented by the other ITR during replication.

2. pBr/Ad.Sal-rITR (ECACC deposit P97082119)

pBr/Ad.Bam-rITR was digested with BamHI and SalI. The vector fragment including the adenovirus insert was isolated in LMP agarose (SeaPlaque GTG) and ligated to a 4.8 kb SalI-BamHI fragment obtained from wt Ad5 DNA and purified with the Geneclean II kit (Bio 101, Inc.). One clone was chosen and the integrity of the Ad5 sequences was determined by

restriction enzyme analysis. Clone pBr/Ad.Sal-rITR contains adeno type 5 sequences from the SalI site at bp 16746 up to and including the rITR (missing the most 3' G residue).

3. pBr/Ad.Cla-Bam (ECACC deposit P97082117)
wt Adeno type 5 DNA was digested with ClaI and BamHI, and the 20.6 kb fragment was isolated from gel by electro-elution. pBr322 was digested with the same enzymes and purified from agarose gel by Geneclean. Both fragments were ligated and transformed into competent DH5α. The resulting clone pBr/Ad.Cla-Bam was analysed by restriction enzyme digestion and shown to contain an insert with adenovirus sequences from bp 919 to 21566.

4. pBr/Ad.AflII-Bam (ECACC deposit P97082114)
Clone pBr/Ad.Cla-Bam was linearised with EcoRI (in pBr322) and partially digested with AflII. After heat inactivation of AflII for 20' at 65 °C the fragment ends were filled in with Klenow enzyme. The DNA was then ligated to a blunt double stranded oligo linker containing a PacI site (5'-AATTGTCTTAATTAACCGCTTAA-3'). This linker was made by annealing the following two oligonucleotides: 5'-AATTGTCTTAATTAACCGC-3' and 5'-AATTGCGGTTAATTAAGAC-3', followed by blunting with Klenow enzyme. After precipitation of the ligated DNA to change buffer, the ligations were digested with an excess PacI enzyme to remove concatameres of the oligo. The 22016 bp partial fragment containing Ad5 sequences from bp 3534 up to 21566 and the vector sequences, was isolated in LMP agarose (SeaPlaque GTG), religated and transformed into competent DH5α. One clone that was found to contain the PacI site and that had retained the large adeno fragment was selected and sequenced at the 5' end to verify correct insertion of the PacI linker in the (lost) AflII site.

5. pBr/Ad.Bam-rITRpac#2 (ECACC deposit P97082120) and pBr/Ad.Bam-rITR#8 (ECACC deposit P97082121)

To allow insertion of a PacI site near the ITR of Ad5 in clone pBr/Ad.Bam-rITR about 190 nucleotides were removed between the ClaI site in the pBr322 backbone and the start of the ITR sequences. This was done as follows: pBr/Ad.Bam-rITR was digested with ClaI and treated with nuclease Bal31 for varying lengths of time (2', 5', 10' and 15'). The extend of nucleotide removal was followed by separate reactions on pBr322 DNA (also digested at the ClaI site), using identical buffers and conditions. Bal31 enzyme was inactivated by incubation at 75 °C for 10', the DNA was precipitated and resuspended in a smaller volume TE buffer. To ensure blunt ends, DNAs were further treated with T4 DNA polymerase in the presence of excess dNTPs. After digestion of the (control) pBr322 DNA with SalI, satisfactory degradation (~150 bp) was observed in the samples treated for 10' or 15'. The 10' or 15' treated pBr/Ad.Bam-rITR samples were then ligated to the above described blunted PacI linkers (See pBr/Ad.AflII-Bam). Ligations were purified by precipitation, digested with excess PacI and separated from the linkers on an LMP agarose gel. After religation, DNAs were transformed into competent DH5α and colonies analysed. Ten clones were selected that showed a deletion of approximately the desired length and these were further analysed by T-track sequencing (T7 sequencing kit, Pharmacia Biotech). Two clones were found with the PacI linker inserted just downstream of the rITR. After digestion with PacI, clone #2 has 28 bp and clone #8 has 27 bp attached to the ITR.

pWE/Ad.AflII-rITR (ECACC deposit P97082116)

Cosmid vector pWE15 (Clontech) was used to clone larger Ad5 inserts. First, a linker containing a unique PacI site was inserted in the EcoRI sites of pWE15 creating pWE.pac. To this end, the double stranded PacI oligo as described for pBr/Ad.AflII-BamHI was used but now with its EcoRI protruding

ends. The following fragments were then isolated by electro-elution from agarose gel: pWE.pac digested with PacI, pBr/AflII-Bam digested with PacI and BamHI and pBr/Ad.Bam-rITR#2 digested with BamHI and PacI. These fragments were ligated together and packaged using λ phage packaging extracts (Stratagene) according to the manufacturer's protocol. After infection into host bacteria, colonies were grown on plates and analysed for presence of the complete insert. pWE/Ad.AflII-rITR contains all adenovirus type 5 sequences from bp 3534 (AflII site) up to and including the right ITR (missing the most 3' G residue).

pWE/Ad.AflII-EcoRI

pWE.pac was digested with ClaI and 5' protruding ends were filled using Klenow enzyme. The DNA was then digested with PacI and isolated from agarose gel. pWE/AflII-rITR was digested with EcoRI and after treatment with Klenow enzyme digested with PacI. The large 24 kb fragment containing the adenoviral sequences was isolated from agarose gel and ligated to the ClaI-digested and blunted pWE.pac vector using the Ligation Express™ kit from Clontech. After transformation of Ultracompetent XL10-Gold cells from Stratagene, clones were identified that contained the expected insert. pWE/AflII-EcoRI contains Ad5 sequences from bp 3534-27336.

Generation of adapter plasmids and recombinant adenoviruses.

Generation of the adapter plasmid pMLPI.TK

Adapter plasmid pMLPTK (patent application EP 95202213) was modified as follows: SV40 polyA sequences were amplified with primer SV40-1 (introduces a BamHI site) and SV40-2 (introduces a BglII site). In addition, Ad5 sequences present in this construct (from nt. 2496 to nt. 2779; Ad5 sequences nt. 3511 to 3794) were amplified with primers Ad5-1 (introduces a BglII site) and Ad5-2.

SV40-1: 5'-GGGGGATCCGAACTTGTTTATTGCAGC-3'
SV40-2: 5'-GGGAGATCTAGACATGATAAGATAC-3'
Ad5-1: 5'-GGGAGATCTGTACTGAAATGTGTGGGC-3'
Ad5-2: 5'-GGAGGCTGCAGTCTCCAACGGCGT-3'
Both PCR fragments were digested with BglII and ligated. The ligation product was amplified with primers SV40-1 and Ad5-2 and digested with BamHI and AflII. The digested fragment was then ligated into pMLP.TK predigested with the same enzymes. The resulting construct, named pMLPI.TK, contains a deletion in adenovirus E1 sequences from nt. 459 to nt. 3510.

Generation of pAd5/L420.HSA, pAd5/Clip and pAd5/Clipsal

pMLPI.TK was used to make a new vector in which nucleic acid molecules comprising specific promoter and gene sequences can be easily exchanged.
First, a PCR fragment was generated from pZipΔMo+PyF101(N-) template DNA (described in PCT/NL96/00195) with the following primers: LTR-1: 5'-CTG TAC GTA CCA GTG CAC TGG CCT AGG CAT GGA AAA ATA CAT AAC TG-3' and LTR-2: 5'-GCG GAT CCT TCG AAC CAT GGT AAG CTT GGT ACC GCT AGC GTT AAC CGG GCG ACT CAG TCA ATC G-3'. Pwo DNA polymerase (Boehringer Mannheim) was used according to manufacturers protocol with the following temperature cycles: once 5' at 95°C; 3' at 55°C; and 1' at 72°C, and 30 cycles of 1' at 95°C, 1' at 60°C, 1' at 72°C, followed by once 10' at 72°C. The PCR product was then digested with BamHI and ligated into pMLP10 (Levrero et al., 1991; Gene 101, 195-202) digested with PvuII and BamHI, thereby generating vector pLTR10. This vector contains adenoviral sequences from bp 1 up to bp 454 followed by a promoter consisting of a part of the Mo-MuLV LTR having its wild-type enhancer sequences replaced by the enhancer from a mutant polyoma virus (PyF101). The promoter fragment was designated L420. Sequencing confirmed correct amplification of the LTR fragment however the most 5' bases in the PCR fragment were missing so that the PvuII site was not

restored. Next, the coding region of the murine HSA gene was inserted. pLTR10 was digested with BstBI followed by Klenow treatment and digestion with NcoI. The HSA gene was obtained by PCR amplification on pUC18-HSA (Kay et al., 1990; J. Immunol. 145, 1952-1959) using the following primers: HSA1, 5'-GCG CCA CCA TGG GCA GAG CGA TGG TGG C-3' and HSA2, 5'-GTT AGA TCT AAG CTT GTC GAC ATC GAT CTA CTA ACA GTA GAG ATG TAG AA-3'. The 269 bp amplified fragment was subcloned in a shuttle vector using the NcoI and BglII sites. Sequencing confirmed incorporation of the correct coding sequence of the HSA gene, but with an extra TAG insertion directly following the TAG stop codon. The coding region of the HSA gene, including the TAG duplication was then excised as a NcoI(sticky)-SalI(blunt) fragment and cloned into the 3.5 kb NcoI(sticky)/BstBI(blunt) fragment from pLTR10, resulting in pLTR-HSA10.

Finally, pLTR-HSA10 was digested with EcoRI and BamHI after which the fragment containing the left ITR, packaging signal, L420 promoter and HSA gene was inserted into vector pMLPI.TK digested with the same enzymes and thereby replacing the promoter and gene sequences. This resulted in the new adapter plasmid pAd5/L420-HSA that contains convenient recognition sites for various restriction enzymes around the promoter and gene sequences. SnaBI and AvrII can be combined with HpaI, NheI, KpnI, HindIII to exchange promoter sequences, while the latter sites can be combined with the ClaI or BamHI sites 3' from HSA coding region to replace genes in this construct.

Another adapter plasmid that was designed to allow easy exchange of nucleic acid molecules was made by replacing the promoter, gene and polyA sequences in pAd5/L420-HSA with the CMV promoter, a multiple cloning site, an intron and a polyA signal. For this purpose, pAd5/L420-HSA was digested with AvrII and BglII followed by treatment with Klenow to obtain blunt ends. The 5.1 kb fragment with pBr322 vector and adenoviral sequences was isolated and ligated to a blunt 1570 bp fragment from pcDNA1/amp (Invitrogen) obtained by

digestion with HhaI and AvrII followed by treatment with T4 DNA polymerase. This adapter plasmid was named pAd5/Clip. To enable removal of vector sequences from the adenoviral fragment pAd5/Clip was partially digested with EcoRI and the linear fragment was isolated. An oligo of the sequence 5' TTAAGTCGAC-3' was annealed to itself resulting in a linker with a SalI site and EcoRI overhang. The linker was ligated to the partially digested pAd5/Clip vector and clones were selected that had the linker inserted in the EcoRI site 23 bp upstream of the left adenovirus ITR in pAd5/Clip resulting in pAd5/Clipsal.

Generation of pAd5ClipLacZ, pAd5Clip.Luc, pAd5Clip.TK and pAd5Clipsal.Luc

The adapter plasmid pAd5/Clip.LacZ was generated as follows: The E.coli LacZ gene was amplified from the plasmid pMLP.nlsLacZ (EP 95-202 213) by PCR with the primers 5'GGGGTGGCCAGGGTACCTCTAGGCTTTTGCAA and 5'GGGGGGATCCATAAACAAGTTCAGAATCC. The PCR reaction was performed Ex Taq (Takara) according to the suppliers protocol at the following amplification program: 5 minutes 94°C, 1 cycle; 45 seconds 94°C and 30 seconds 60°C and 2 minutes 72°C, 5 cycles; 45 seconds 94°C and 30 seconds 65°C and 2 minutes 72°C, 25 cycles; 10 minutes 72; 45 seconds 94°C and 30 seconds 60°C and 2 minutes 72°C, 5 cycles, I cycle. The PCR product was subsequently digested with Kpn1 and BamHI and the digested DNA fragment was ligated into KpnI/BamHI digested pcDNA3 (Invitrogen), giving rise to pcDNA3.nlsLacZ. Next, the plasmid pAd5/Clip was digested with SpeI. The large fragment containing part of the 5' part CMV promoter and the adenoviral sequences was isolated. The plasmid pcDNA3.nlsLacZ was digested with SpeI and the fragment containing the 3'part of the CMV promoter and the lacZ gene was isolated. Subsequently, the fragments were ligated, giving rise to pAd/Clip.LacZ. The reconstitution of the CMV promoter was confirmed by restriction digestion.

The adapter plasmid pAd5/Clip.Luc was generated as follows: The plasmid pCMV.Luc (EP 95-202 213) was digested with HindIII and BamHI. The DNA fragment containing the luciferase gene was isolated. The adapter plasmid pAd5/Clip was digested with HindIII and BamHI, and the large fragment was isolated. Next, the isolated DNA fragments were ligated, giving rise to pAd5/Clip.Luc. The adapter pClipsal.Luc was generated in the same way but using the adapter pClipsal digested with HIII and BamHI as vector fragment. Likewise, the TK containing HIII-BamHI fragment from pCMV.TK (EP 95-202 213) was inserted in pClipsal to generate pAd5/Clip.TK. The presence of the SalI site just upstream of the left ITR enables liberation of vector sequences from the adeno insert. Removal of these vector sequences enhances frequency of vector generation during homologous recombination in PER.C6.

Generation of pWE/Ad.AflII-rITRfib16

To enable convenient generation of recombinant adenoviruses with a Ad5/Ad16 chimeric fiber we cloned the chimeric fiber gene in the place of the Ad5 fiber in the cosmid clone pWE/Ad.AflII-rITR. The pBr/AdBamRpac.fib16 constructs and the pBr/Ad.AflII-BamHI construct were digested with BamHI and PacI to free it from the pBr plasmid. They were isolated from gel and cleaned by using agarase (Boehringer). The pWE.pac construct was digested with PacI to linearize it and cleaned by phenol/ chloroform. A three-point ligation was used in which the BamHI sites of the pBr/AdBamRpac.fib16 constructs and the pBr/Ad.AflII-BamHI construct are ligated together and the pWE.pac construct is ligated at the PacI sites. The ligation mix consists out of the three constructs, T4 ligase, 5mM ATP and ligation buffer without PEG. 1-4 µl of the ligation mixture, containing 0.1-1.0 µg of ligated DNA, is added to the packaging extract. Separately, 1 µl of the positive wild-type lambda DNA control was packaged. The tubes were spinned quickly and incubated at RT for maximum 2 hrs. Respectively 500 µl SM buffer (5.8 g

NaCl, 2.0 g $MgSO_4 \cdot 7H_2O$, 50 ml 1M Tris-HCl (PH 7.5), 5 ml 2% (w/v) gelatine and deionised water up to 1 litre) and 20 µl of chloroform was added to the packaging mixture to stop the reaction. The tube was spinned briefly to sediment the debris. The supernatant, which contains the phage, can now be stored at 4°C up to 1 month.

A bacterial glycerol stock of DH5α strain and VCS257 strain were streaked onto LB agar plates and incubated O/N at 37°C. The next day 10 ml LB medium supplemented with 10 mM $MgSO_4$ and 0.2% (w/v) maltose was inoculated with a single colony of each bacteria strain. This was grown at 37°C until an OD_{600} value of maximum 1.0 is reached. The bacteria were then pelleted at 500 x g for 10 minutes. The pellet is resuspended into 5 ml of sterile 10 mM $MgSO_4$ and diluted in 10 mM $MgSO_4$ till an OD_{600} value of approximately 0.5 is reached.

Of the positive wild-type lambda phage control a 10^{-2} and a 10^{-4} dilution was made in SM buffer. Of the other final packaged reactions a 10^{-1} and a 10^{-2} dilution was made in SM buffer. Out of the 10^{-4} dilution of the positive control 10 µl was added to 200 µl of VCS257 host cells (OD_{600} 0.5). This is incubated for 15 minutes at 37°C, 3 ml of LB top agar (0.7% agarose in LB medium) (50°C) is added and immediately plated on a prewarmed LB agar plate. Out of the 10^{-1} and a 10^{-2} dilution of the other final packaged reactions 25 µl was added to 25µl of DH5α host cells (OD_{600} 0.5). This is incubated for 30 minutes at RT. Respectively 200 µl LB medium is added and an incubation for 1 hr at 37°C followed. The mixture is spinned down shortly, the bacteria pellet is resuspended in 100 µl LB medium and plated on LB agar plates with the required amount of ampicillin. The plates are incubated O/N at 37°C. Eventually the colonies are grown and the required DNA is tested by restriction digestion. pWE/Ad.AflII-rITRfib16 contains all adenovirus type 5 sequences except for the fiber coding region 3' from the NdeI site present in Ad5 fiber, these sequences are replaced by

fiber sequences from Ad16 leaving the open reading frame intact.

Generation of recombinant viruses with fiber modifications

The adapter plasmids pAd5/Clip.TK, pAd5/Clip.LacZ or pAd5/Clip.Luc were digested with SalI to liberate the homologous adenovirus sequences and the left ITR from the vector. pWE/Ad.AflII-rITRfib16 was digested with PacI. DNA was then purified using phenol/chloroform extraction and EtOH precipitation and redissolved in sterile transfection qualified water. Four μgr of each construct was transfected into PER.C6 cells in a T25 flask seeded one day before with $2.5x10^6$ cells. At the occurrence of full CPE 6-8 days later cells were harvested in the medium and amplified by infection of 3 ml 3x freeze-thawed cell lysate on fresh PER.C6 cells. At full CPE cells were harvested by freeze-thawing and virus was purified by two rounds of plaque purification on PER.C6 cells. In all cases plaques were positive for transgene expression and one was picked to generate seed stocks for production.

Example 7

Infection of synoviocytes with recombinant adenoviral vectors in non-human primates suffering from collagen induced arthritis.

The transducibility of arthritic synovium by chimeric adenoviruses carrying the LacZ gene from E.coli, which codes for the enzyme β-galactosidase, was tested in vivo in a non-human primate model for RA. The rhesus monkey Maccaca mulatta was injected 10 times subcutaneously with, in total, 5 mg Bovine Collagen type II (10 mg/ml) emulsified in an equal volume of Complete Freunds Adjuvant. The animal developed a full blown collagen induced arthritis (CIA) within a period of 8 weeks. Subsequently, the left knee was injected intra-articularly with $1*10^{11}$ virus particles (vp) IG.Ad.CLIP.LacZ. The right knee was injected with $1*10^{11}$ vp

IG.Ad.ClipLacZ.fib16. The vectors were administered in a total volume of 1 ml diluens (PBS supplemented with 5% sucrose). The site of entry was medially, just below the midpoint of the patella. The needle was introduced in a line towards the suprapatellar pouch. After passing the joint capsule, the vector was injected into the joint cavity. Thereafter, the syringe and needle were removed from the joint. At day 3 post infection, the animal was sacrificed. The left elbow was injected with 1 ml diluens only and served as a negative control. The right elbow was left untreated. The knee joints and elbows were isolated and fixed in phosphate buffered 2% paraformaldehyde/0.25% glutaraldehyde for 3 hours and washed 3 times with PBS, incubated over night in X-Gal solution (5 mM $K_4Fe(CN)_6$, 5mM $K_3Fe(CN)_6$, 2mM $MgCl_2$ and 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-8-D-galactopyranoside) and extensively washed with PBS. The hyperplastic synovial lining of the knee joint stained blue with IG.Ad.CLIP.LacZ. However, the knee IG.Ad.ClipLacZ.fib16 injected with stained blue more intensely, showing that recombinant chimeric adenoviruses carrying the fiber of Ad16 infects hyperplastic synovium more efficiently than recombinant adenoviruses carrying fiber of Ad5. Detailed analysis of the transduced tissue confirmed that the number of positive nuclei in the pannus tissue of the IG.Ad.ClipLacZ.fib16 treated joint was significantly higher than the number of positive nuclei found in the IG.Ad.CLIP.LacZ treated joints. Stained nuclei were found several cell layers deep in the pannus tissue. No staining was found in chondrocytes of the cartilage layer or in the diluens or non-injected injected joint. These results show that hyperplastic synovium can be transduced more efficiently with chimeric recombinant IG.Ad.ClipLacZ.fib16 vectors in vivo, as compared to IG.Ad.CLIP.LacZ vectors. Moreover, the results show that the diseased tissue (hyperplastic synovium), but not the chondrocytes (benign cells that are required for cartilage regeneration) were at

least preferably transduced by the recombinant adenoviral vectors.

Example 8

Dose dependent transduction of synoviocytes with recombinant adenoviral vectors in non-human primates suffering from collagen induced arthritis.

The transducibility of arthritic synovium by chimeric adenoviruses carrying the LacZ gene was tested in a dose escalation study in vivo in the non-human primate model for RA as described above. A monkey suffering from CIA was treated with increasing doses of IG.Ad.CLIP.LacZ or IG.Ad.ClipLacZ.fib16 given intra-articularly in the proximal interphalangeal (pip) in a total volume of 0.1 ml. Pip 2 to 5 were injected with increasing vector doses, ranging from $1x10^7$ to $1x10^{10}$ vp, in the left or right hand, for IG.Ad.CLIP.LacZ or IG.Ad.ClipLacZ.fib16 respectively. As a control, 0.1 ml diluens was injected in pip 1 of both hands. After sacrifice on day 5, the pip joints of the hands were fixed in 2% paraformaldehyde / 0.25 % and stained with X-GAL to monitor lacZ expression, as described above. A positive correlation was observed between injected dose of LacZ Adenoviruses and the number of lacZ expressing cells in the synovial tissue. Moreover, the IG.Ad.ClipLacZ.fib16 vector treated joints contained more LacZ positive cells than the IG.Ad.CLIP.LacZ treated joints at the same vector dose, confirming that hyperplastic synovium is transduced more efficiently by chimeric recombinant IG.Ad.ClipLacZ.fib16 vectors than by IG.Ad.CLIP.LacZ vectors in a relevant model for rheumatoid arthritis. Microscopy of the injected joints confirmed that the cells expressing lacZ activity were synoviocytes, as evidenced by typical location and morphologic appearance.

Example 9

Killing of diseased synovium from patients suffering from RA infected with IG.Ad.CLIP.TK and IG.Ad.CLIP.TK.fib16 followed by treatment with Ganciclovir in vitro.

Synovium was isolated from patients suffering from RA as discussed above. The day prior to infection, 10^4 synovium cells were plated on a tissue culture dish. The next day, eight dishes with synovial cells were infected with either IG.Ad.CLIP.TK or IG.Ad.CLIP.TK.fib16 at an increasing m.o.i. of 1, 10, 100 and 1000 vp/cell or mock treated. Four hours post infection, the infection medium was replaced by IMDM containing 40% normal human serum supplemented with or without 10 µg/ml Ganciclovir. At day 0, 5, 7 and 10 cells were counted. IG.Ad.CLIP.TK.fib infected cells were killed in medium containing Ganciclovir more efficiently, especially at lower m.o.i.'s, than IG.Ad.CLIP.TK infected cells, as determined by the decrease in the total cell numbers in these dishes. Neither the mock treated cells, nor the infected cells in medium without Ganciclovir were killed, showing that killing was caused by the combination of Ad.TK vectors and Ganciclovir. Thus hyperplastic synovium from patients suffering from RA is sensitive to infection with recombinant IG.Ad vectors expressing TK in combination with treatment with the pro-drug Ganciclovir. Moreover, killing of synoviocytes following IG.Ad.CLIP.TK.fib16 infection was more efficient than killing after IG.Ad.CLIP.TK infection in the presence of Ganciclovir.

Next, the bystander effect of the treatment was addressed. To that end, synovial cells were infected with IG.Ad.CLIP.TK at an M.O.I. of 100 as described above. The next day, the infected cells were trypsinized and mixed with non-infected synovial cells from the same patient at the ratio of 1:4 (25%) or 1:2 (50%). As controls, non-infected (0%) and non-mixed (100%) cells were included in the experiment. The following 7 days, the cells were cultured in IMDM supplemented with 40% normal human serum and 10µg/ml

Ganciclovir and the total amount of cells per dish was determined. The synovial cells infected (100%) with IG.Ad.CLIP.TK were killed. Moreover, the mixed cell populations in which only a percentage of the cells (50% and 25% respectively) was infected with IG.Ad.CLIP.TK were killed too. This shows that human synovium cells that are infected with recombinant IG.Ad vectors expressing the TK gene have a substantial bystander effect following GCV treatment.

Example 10

Killing of hyperplastic synovium after intra-articular injection of IG.Ad.CLIP.TK and IG.Ad.CLIP.TK.fib16 followed by Ganciclovir treatment in non-human primates suffering from collagen induced arthritis.

A rhesus monkey was injected 10 times subcutaneously with, in total, 5 mg Bovine Collagen type II (10 mg/ml) emulsified in an equal volume of Complete Freunds Adjuvant to induce CIA. The animal developed a full-blown arthritis within a period of 8 weeks. Subsequently, the left knee was injected intra-articularly with $1x10^{11}$ vp IG.Ad.CLIP.TK in a total volume of 1 ml diluens. The right knee was injected with $1x10^{11}$ vp IG.Ad.CLIP.TK.fib16 in a total volume of 1 ml diluens. The sites of entry were medially, just below the midpoint of the patella. The needle was introduced in a line towards the suprapatellar pouch. After the joint capsule was passed, the substances were injected into the knee joint. Thereafter, syringes and needles were removed from the joints. The left elbow was injected with 1 ml diluens and served as a negative control. From day 2 to day 15 the monkey was treated daily intravenously with Ganciclovir, 10 mg/kg/day in 25 ml sterile water given in approximately 30 minutes. After sacrifice on day 18 the knees and elbows of the monkey were taken out for histopathological analysis. Synovial biopsies of the knees were snap-frozen in liquid nitrogen and stored at < - 60 °C.

Cleaving of genomic DNA during apoptosis yields double-stranded low molecular weight nuclear DNA fragments (mono-

and oligonucleosomes) as well as single strand breaks ("nicks") in high molecular weight DNA. TUNEL (TdT-mediated dUTP nick end labeling) is a method for enzymatic in situ labeling of apoptosis induced DNA strand breaks. Strand breaks in the DNA can be identified by labeling the free 3'-OH termini of DNA fragments with modified nucleotides in an enzymatic reaction. Terminal deoxynucleotidyl transferase (TdT), which catalyses polymerization of nucleotides to the free 3'-OH termini of fragmented DNA, is used as the polymerase. Incorporated fluorescein-12-dUTP is detected by anti-fluorescein antibody Fab fragments from sheep, conjugated with horseradish peroxidase (POD). The procedure is extensively described by the supplier (Promega). After substrate reaction, the labelled fragmented genomic DNA were visualised under the light microscope.

The synovial tissue from the elbow that was injected with diluens showed background tissue staining, indicating that some apoptosis has taken place in diseased synovium. In the negative control (no TdT enzyme was added) no staining could be observed. The positive control (a sample that was treated with DNase to induce DNA strand breaks before the TUNEL assay was started) showed staining in all parts of the tissue. The sample from the IG.Ad.CLIP.TK injected joint showed more stained cells than the synovial tissue sample of the diluens treated joint, indicating that more cells went into apoptosis due to the IG.Ad.CLIP.TK-GCV treatment. Most stained cells were found in the joint injected with IG.Ad.CLIP.TK.fib16. The staining was present both in the synovial membrane and in the subsynovial tissue, suggesting that the treatment is efficacious throughout the whole tissue sample. These data show that treatment with recAd vectors expressing the TK gene followed by GCV treatment is a feasible method to perform non-surgical synovectomy in arthritic joints. In addition, these data show that recombinant adenoviral vectors containing fiber 16 are superior in transducing transgenes to synovial tissue.

Example 11

Comparison of infection of Ad5.luc and Ad5.fib16.luc on RA synoviocytes

In each experiment, a total of 15,000 RA synoviocytes was seeded per well in 12-well microtiter dishes. Cells were infected with Ad5.luc (batch nr. IC020-032) or Ad5.fib16.luc (batch nr. B204-130C) at various m.o.i.'s, and incubated overnight. Luciferase activity was measured after 72 hrs. Data are summarized in table XIII; the graphic representation is in Figure 14.

Example 12

Comparison of infection of Ad5.lacZ and Ad5.fib16.lacZ on RA synoviocytes

In each experiment, a total of 15,000 RA synoviocytes was seeded per well in 12-well microtiter dishes. Cells were infected with Ad5.lacZ (batch nr. B269-186) or Ad5.fib16.lacZ (batch nrs. B204-120A and B204-120B) at various m.o.i.'s, and incubated overnight. % of lacZ-positive cells was determined after 72 hrs. Data are summarized in table XIV; the graphic representation is in Figures 15.

Example 13

Comparison of infection of Ad5.GFP and Ad5.fib16.GFP on RA synoviocytes

In each experiment, a total of 15,000 RA synoviocytes was seeded per well in 12-well microtiter dishes. Cells were infected with Ad5.GFP (batch nr. B204-103 and B204-130D) or Ad5.fib16.GFP (batch nrs. B204-103 and IC054-024B) at various

m.o.i.'s, and incubated overnight. GFP activity was measured after 72 hrs, and % of GFP-positive cells was determined. Data are summarized in tables XVa and XVb; the graphic representation is in Figures 16A and B.

Example 14

Comparison of infection of panel of fiber-modified viruses on RA synoviocytes

A panel of chimeric adenoviruses was tested for its infectivity on RA synoviocytes. The following chimeric adenoviruses were produced : Ad5.fib5,11,16,24,28,33,35,45 and 47, each carrying a luciferase transgene. In each experiment, a total of 15,000 RA synoviocytes was seeded per well in 12-well microtiter dishes. Cells were infected the chimeric adenoviruses at various m.o.i.'s, and incubated overnight. Luciferase activity was measured after 72 hrs. Data are summarized in table XVI; the graphic representation is in Figure 17.

Example 15

Comparison of infection of three B-type fiber-modified viruses on RA synoviocytes

Three chimeric adenoviruses, each carrying a B-type fiber were tested for its infectivity on RA synoviocytes, in comparison to Ad5. The following chimeric adenoviruses were produced : Ad5.fib16,35 and 51, each carrying a GFP transgene. In each experiment, a total of 50,000 RA synoviocytes was seeded per well in 12-well microtiter dishes. Cells were infected the chimeric adenoviruses at various m.o.i.'s , and incubated overnight. GFP activity was measured after 72 hrs, and % of GFP-positive cells was

determined. Data are summarized in tables XVIIa and b; the graphic representation is in Figures 18a and b.

Example 16

Comparison of infection of three B-type fiber-modified viruses on RA synoviocytes

Three chimeric adenoviruses, each carrying a B-type fiber were tested for its infectivity on RA synoviocytes, in comparison to Ad5. The following chimeric adenoviruses were produced : Ad5.fib11,16 and 35, each carrying a luciferase transgene. In each experiment, a total of 15,000 RA synoviocytes was seeded per well in 12-well microtiter dishes. Cells were infected the chimeric adenoviruses at various m.o.i.'s , and incubated overnight. Luciferase activity was measured after 72 hrs. Data are summarized in table XVIII; the graphic representation is in Figure 19.

Example 17

Comparison of infection of RA synoviocytes with Ad5 and Ad5.fib16 in different patients

Synoviocyte cells were obtained from 6 different patients suffering from rheumatoid arthritis. Synoviocytes were infected with Ad5.lacZ or Ad5.fib16.lacZ during 2 or 20 hours, and stained for lacZ expression after 48 hours. Numbers of blue cells were counted under the microscope. Data are summarized in table XIX, plotted in Figure 20.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1: Schematic drawing of the pBr/Ad.Bam-rITR construct.

Figure 2: Schematic drawing of the strategy used to delete the fiber gene from the pBr/Ad.Bam-rITR construct.

Figure 3: Schematic drawing of construct pBr/Ad.BamRΔfib.

Figure 4: Sequence chimaeric fiber Ad5/16.

Figure 5: Schematic drawing of the construct pClipsal-Luc.

Figure 6: Schematic drawing of the method to generate chimaeric adenoviruses using three overlapping fragments. Early (E) and late regions (L) are indicated. L5 is the fiber coding sequence.

Figure 7: Sequences including the gene encoding adenovirus 16 fiber protein as published in Genbank and sequences including a gene encoding a fiber from an adenovirus 16 variant as isolated in the present invention, wherein the sequences of the fiber protein are from the NdeI-site. Figure 7A nucleotide sequence comparison. Figure 7B amino-acid comparison.

Figure 8 : Infection of synoviocytes using different amounts of virus particles per cell (MOI) and two different adenoviruses: Ad5=Ad5.Clip.Luc; Ad5/16= Ad5.Luc-fib16. Luciferase transgene expression, 48 hours after a 2 hours infection procedure is depicted as relative light units (=RLU) per microgram whole cell lysate. Error bars represent standard error of the mean (SEM).

Figure 9: Infection of synoviocytes using different concentrations of cells. Luciferase transgene expression, 48 hours after a 2 hours infection procedure is depicted as relative light units (=RLU) per microgram total protein. Error bars represent SEM. The actual MOI differed between the cell concentrations and ranged from 20.000 virus particles per cell (cell density 12.500) to 2.500 virus particles per cell (cell density 100.000).

Figure 10: Infection of synoviocytes using different virus exposure periods. Luciferase transgene expression, 48 hours

after either a 2 hours or a 20 hours virus exposure is depicted as relative light units (=RLU) per microgram protein. Error bars represent standard deviations.

Figure 11: Synoviocytes were incubated with IG.Ad.CMV.TK or IG.Ad.mlp-I.TK. Cells were cultured with or without GCV.

Figure 12: Bystander killing was assessed in cultures containing both TK-infected and non-TK-infected synoviocytes in a proportion 0/100, 50/50, 25/75 and 0/100. Cells were cultured with or without GCV.

Figure 13 A + B: X-gal expression in synovial tissue 3 days after intra-articular injection of IG.Ad.CMV.lacZ in the knee. 13A: macroscopy. 13B: direct LacZ staining of synovial tissue counterstained with Mayers Hamalanlosung.

Figure 14: Comparison of infection of Ad5.luc and Ad5.fib16.luc on RA synoviocytes

Figure 15 % lacZ positive cells with Ad5.lacZ and Ad5.fib16.lacZ in RA synoviocytes

Figure 16A Infection efficiency of Ad5.GFP and Ad5fib16.GFP in RA synoviocytes

Figure 16B GFP production in RA synoviocytes infected with Ad5.GFP vs. Ad5fib16.GFP

Figure 17 Infectivity of panel of chimeric adenoviruses on RA synoviocytes

Figure 18A % of infected cells with three B-type fiber-modified viruses on RA synoviocytes

Figure 18B: GFP production with three B-type fiber-modified viruses on RA synoviocytes

Figure 19 Comparison of three B-type fiber modified adenoviruses for infectivity on RA synoviocytes

Figure 20: Comparison of infectivity Ad5.lacZ vs. Ad5.fib16.lac6 in RA synoviocytes from 6 different patients

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that that prior art forms part of the common general knowledge in Australia.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

TABLES

Table I: Oligonucleotides and degenerate oligonucleotides used for the amplification of DNA encoding fiber protein derived from alternative adenovirus serotypes. (Bold letters represent NdeI restriction site (A-E), NsiI restriction site (1-7, 8), or PacI restriction site (7).

Serotype	Tail oligonucleotide	Knob oligonucleotide
4	A	1
8	B	2
9	B	2
12	E	3
16	C	4
19p	B	2
28	B	2
32	B	2
36	B	2
37	B	2
40-1	D	5
40-2	D	6
41-s	D	5
41-1	D	7
49	B	2
50	B	2
51	C	8

A: 5'- CCC GTG TAT CCA **TAT** GAT GCA GAC AAC GAC CGA CC- 3'

B: 5'- CCC GTC TAC CCA **TAT** GGC TAC GCG CGG- 3'

C: 5'- CCK GTS TAC CCA **TAT** GAA GAT GAA AGC- 3'

D: 5'- CCC GTC TAC CCA **TAT** GAC ACC TYC TCA ACT C- 3'

E: 5'- CCC GTT TAC CCA **TAT** GAC CCA TTT GAC ACA TCA GAC- 3'

1: 5''- CCG **ATG CAT** TTA TTG TTG GGC TAT ATA GGA - 3'

2: 5'- CCG **ATG CAT** TYA TTC TTG GGC RAT ATA GGA - 3'

3: 5'- CCG **ATG CAT** TTA TTC TTG GGR AAT GTA WGA AAA GGA - 3'

4: 5'- CCG **ATG CAT** TCA GTC ATC TTC TCT GAT ATA - 3'

5: 5'- CCG **ATG CAT** TTA TTG TTC AGT TAT GTA GCA - 3'

6: 5'- GCC **ATG CAT** TTA TTG TTC TGT TAC ATA AGA - 3'

7: 5' - CCG **TTA ATT AAG** CCC TTA TTG TTC TGT TAC ATA AGA A - 3'

8: 5'- CCG **ATG CAT** TCA GTC ATC YTC TWT AAT ATA - 3'

Table II:

Biodistribution of chimaeric adenovirus upon intravenous tail vein injection. Values represent luciferase activity expressed as relative light units/µg protein. Values in the brain are considered background.

Organ	Ad5.Clip.Luc	Ad5.Luc-fib16
Liver	740045	8844
Spleen	105432	3442
Lung	428	334
Kidney	254	190
Heart	474	276
Brain	291	294

Table III: Expression of CAR and integrins on the cell surface of synoviocytes. Values represent percentages of cells that express CAR or either one of the integrins at levels above background. Synoviocytes incubated only with the secondary, rat-anti-mouse IgG1-PE labelled antibody served as a background control.

Cells	$\alpha_v\beta3$	$\alpha_v\beta5$	CAR
synoviocytes	27.2%	35.4%	0%
PER.C6	7.8%	16.8%	99.6%

Table IV: Determination of transgene expression (luciferase activity) per µg of total cellular protein after infection of A549 cells

MOI (VP/Cell)	Control Ad5	Fiber 16
0	0	0
5	1025	661
10	1982	1704
25	4840	3274
100	21875	13432
500	203834	93163

Table V: Determination of transgene expression (luciferase activity) per µg of total cellular protein after infection of PER.C6 cells

Virus particles/ ml	Control Ad5	Ad5fiber16
10	24800	9300

Table VI: Summary of rhesus monkeys experiments.

monkey name	date of birth	sex	weight (kg)	virus	dose (pfu's)	joint	sacrifice (day)	blood sampling (day)	excreta (day)
1: BB 112	01-01-93	M	3.000	IG.Ad.CMV.lacZ IG.Ad.CMV.luc	5 x 10^9 1 x 10^9	knee L knee L	2	0	-
2: 9163	25-09-91	F	3.900	IG.Ad.CMV.lacZ IG.Ad.CMV.luc	5 x 10^9 1 x 10^9	knee L knee L	2	0	-
3: 9179	19-12-91	F	3.500	IG.Ad.CMV.lacZ IG.Ad.mlp.lacZ IG.Ad.CMV.luc	5 x 10^8 1.5 x 10^9 1 x 10^8	pip 4 R F pip 2 LH pip 2 LH, 4 RF	2	0	-
4: Q 079	01-12-93	F	2.600	IG.Ad.CMV.lacZ IG.Ad.CMV.luc	3.2 x 10^{5-9} 2.2 x 10^{1-8}	pip 2,3,4,5 FF + HH and knee L+R pip 2,3,4,5 FF + HH and knee L+R **	3	-3,0,3	-
5: 94074	05-09-94	F	2.900	IG.Ad.mlp.lacZ IG.Ad.mlp.luc	1 x 10^{6-10} 1 x 10^{10}	pip 2,3,4,5 LH+RH and knee L knee R	3	-7,0,1,2,3	-7,0,1,2,3
6: Z 02	01-01-92	F	4.900	IG.Ad.CMV.luc IG.Ad.mlp-I.TK	1 x 10^8 1 x 10^{11}	knee L+R knee L+R	15	0,3,7,15	0,15
7: 94044		M	2.600	IG.Ad.mlp-I.TK	5.8 x 10^{10}	knee L	18	-7,0,2,7, 10,14,18	-7,0,1,2, 3,4,5
8: 94048		M	2.850	IG.Ad.mlp-I.TK	5.8 x 10^{10}	knee L	18	-7,0,2,7, 10,14,18	-7,0,1,2, 3,4,5

M = male, F = female; LH = left hand, RF = right foot, HH = both hands, FF = both feet; ** = in all joint on left side 10 % triamcinolonhexacetonide 20 mg/ml is added.

Table VII: Survival of synoviocytes after infection with modified Ad. Negative controls were carried out in duplos in 3 of 5 experiments.

patient	number of cells t = 0 (x 1000)	experiment	cell count on day	neg. control (cells x 1000)	CMV MOI 100 (cells x 1000)	mip MOI 100 (cells x 1000)
RA-1-a	200	IG.Ad.lacZ	2	76	187	201
RA-1-b	100	IG.Ad.lacZ	2	90 - 160	118	194
RA-2	100	IG.Ad.lacZ	2	76	56	56
RA-3-a	100	IG.Ad.luc	3	42 - 38	49	38
RA-3-b	100	IG.Ad.luc	3	35 - 31	31	52

Table VIII: Lac Z data monkey 4.

joint:	left hand **	left foot **	right hand	right foot	concentrations injected Ad.lacZ
pip 2	-	-	-	-	3.2×10^5
pip 3	-	+	-	-	3.2×10^6
pip 4	-	+	++	-	3.2×10^7
pip 5	++	+++	+++	++	3.2×10^8
knee	left:	++++	right:	++++	3.2×10^9

Virus concentrations in plague forming units.

** =10 % triamcinolonhexacetonide (20 mg/ml) in injection.

- : no blue cells

+ : 1-10 blue cells in synovial lining

++ : 1-5% blue cells in synovial lining

+++ : 5-50% blue cells in synovial lining

++++ : > 50 % blue cells in synovial lining

Table IX: Luciferase counts in organs as a measure of virus spread after intra-articular injection of Ad.CMV.luc.

monkey :	1-4 mean (range)	5 mean (range)	6	control
conc. injected Ad.	2,2 - 10 10^8 IG.Ad.CMV.luc	10^{10} IG.Ad.mlp.luc	10^8 IG.Ad.CMV.luc	-
termination day:	2-3	3	15	
Ad.luc-injected joints	**553,491** (47,773-1,000,460)	**368**	69 (66-71)	-
non Ad-injected joints	390 (63 - **2515**)	93	-	-
hart	86 (82 - 114)	147 (105-223)	60	142
liver	99 (81 - 128)	115	49	126
spleen	99 (65 - 165)	98	57	101
testis	82	female	55	female
cervix	227 (90 - **457**)	101	male	115
prostate	120	female	-	female
ovary	90 (76 - 111)	107	male	92
bone marrow	60 (54 - 69)	110	-	-
blood t=0	68 (62 - 71)	165 (86-349)	-	-
blood section	56 (50 - 62)	91 (86-96)	-	-
draining lymphnode		96		
non-draining lymphnodes		112 (105-118)		
kidney		110		
lung		102		
oesophagus		78		
bladder		100		

Table X: Lactate Dehydrogenate levels in U/l.

t (days)	first:	0	2	3	4	5	7	10	14	15	18
monkey 4	t-4: 358	216		763							
monkey 6	t-30: 1050	6340	6835		5939						
monkey 5		760		627			1182			686	
monkey 7	t-38: 999	5185	4387	4679		6547	4740	5586	6128		5226
monkey 8	t-38: 958	5994	4881	10140	8000	8230	2952	7259	3800		4053

LDH levels in monkey 4 (CMV.lacZ/CMV.luc), 5 (mlp.lacZ / mlp.luc), 6 (mlp.TK), 7 and 8 (mlp.TK + GCV). LDH-levels in monkey 6, 7 and 8 are determined with an other test than monkey 4 and 5.

Table XI: Percentage of lacZ-expressing synoviocytes 2 days after infection with IG.Ad.CMV.lacZ or IG.Ad.mlp.lacZ

MOI	0	0.1	1	10	100
IG.Ad.CMV. lacz (SD)	0	<1% (0%)	2.5% (1.5%)	12.9% (6.3%)	53.4% (14.9%)
IG.ad.MLP. lacZ (SD)	0	0	0	<1% (0%)	<1% (0%)

Table XII: Light counts as a measure of luciferase-expression in synoviocytes 3 days after infection with IG.Ad.CMV.luc or IG.Ad.mlp.luc

MOI	0	0.1	1	10	100
IG.Ad.MLP. luc	0	53 (5)	$9.6\ 10^2$ ($2.0\ 10^2$)	$7.5\ 10^3$ ($2.6\ 10^3$)	$1.7\ 10^3$ ($2.3\ 10^4$)
IG.Ad.CMV. luc (SD)	0	$2.9\ 10^2$ (18)	$2.1\ 10^3$ ($6.0\ 10^2$)	$7.4\ 10^4$ ($4.4\ 10^4$)	$1.1\ 10^6$ ($9.5\ 10^4$)

Table XIII: Comparison of RA synoviocyte infection of Ad5.luc and Ad5.fib16.luc (average luciferase activity (n=3))

Virus	0 vp/cell	50 vp/cell	500 vp/cell	5000 vp/cell
Ad5.luc	63	243	559	189519
Ad5.fib16.luc	62	715484	7.86E+07	1.02E+09

Table XIV: % lacZ positive cells with Ad5.lacZ and Ad5.fib16.lacZ in RA synoviocytes

	50 vp/cell	500 vp/cell	5000 vp/cell
Ad5.lacZ	2.17	7.33	35.50
Ad5.Fib16 .lacZ(120 A)	28.00	67.50	100.00
Ad5.Fib16.lacZ (130 B)	33.83	74.54	100.00

Table XVa: comparison of infection efficiency Ad5.fib5.GFP and Ad5.fib16.GFP in RA Synoviocytes (% of GFP-positive cells)

	Fib5 (103)	Fib5 (130D)	Fib16 (103)	Fib16 (IC)
50 vp/cell	4.81	5.66	35.05	52.07
500 vp/cell	26.75	24.66	85.87	90.21
5000 vp/cell	75.56	72.53	100.00	100.00

Table XVb: GFP production in RA synoviocytes infected with Ad5.GFP vs. Ad5.fib16.GFP

	Fib5 (103)	Fib5 (130D)	Fib16 (103)	Fib16 (IC)
50 vp/cell	1.02	1.04	1.61	11.92
500 vp/cell	1.36	1.31	586.34	1540.93
5000 vp/cell	217.85	184.96	8058.42	7773.65

Table XVI: infectivity of panel of chimeric adenoviruses on RA synoviocytes (Luciferase activity/well)

	0 vp/cell	50 vp/cell	500 vp/cell	5000 vp/cell
virus				
Ad5. Fib 5	31	6348	32612	497488
Ad5.Fib 11	26	10775	524221	33831033
Ad5.Fib 16	32	17937	821418	38760900
Ad5.Fib 24	29	220	1237	52601
Ad5.Fib 28	24	106	1798	15199
Ad5.Fib 33	31	163	1865	92049
Ad5.Fib 35	27	2319	103286	7812200
Ad5.Fib 45	29	95	1304	28373
Ad5.Fib 47	29	145	1901	54053

Table XVIIa: % of infected cells with three B-type fiber-modified viruses on RA synoviocytes

	Ad5	Ad5.fib16	Ad5.fib35	Ad5.fib51
50 vp/cell	7.32	52.31	69.17	57.84
500 vp/cell	38.83	92.60	94.49	93.28
5000 vp/cell	87.91	100.00	100.00	100.00

Table XVIIb: GFP production with three B-type fiber-modified viruses on RA synoviocytes

	Ad5	Ad5.fib16	Ad5.fib35	Ad5.fib51
50 vp/cell	1.76	42.29	262.84	78.59
500 vp/cell	5.77	3126.39	8012.87	3929.21
5000 vp/cell	1386.49	9646.62	9646.62	9646.62

Table XVIII: comparison of three B-type fiber modified adenoviruses for infectivity on RA synoviocytes (luc-production)

virus	0 vp/cell	50 vp/cell	500 vp/cell	5000 vp/cell
Ad5	31	2073	67365	848476
Ad5.Fib 11	35	16926	1094044	69718467
Ad5.Fib 16	38	58366	3371600	164933133
Ad5.Fib 35	35	3321	129236	10440833

Table XIX: comparison of Ad5.lacZ vs. Ad5.fib16.lacZ in 6 patients (# of lacZ-positive cells)

	P1	P2	P3	P4	P5	P6
Ad5-2hrs	3	1	28	26	14	16
Ad5-20hrs	26	32	101	39	86	34
Ad5.fib16-2hrs	267	209	365	325	713	259
Ad5.fib16-20hrs	2565	1762	5124	3258	6158	2923

5

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A recombinant adenoviral vector derived from an adenovirus of subgroup C, said adenoviral vector comprising a chimaeric fiber protein wherein at least the knob domain is replaced with at least the knob domain of a fiber protein of an adenovirus selected from the group consisting of: adenovirus serotype 11, 16, 35 and 51.

2. A recombinant adenoviral vector according to claim 1, wherein said adenovirus of subgroup C, is adenovirus serotype 5.

3. A recombinant adenoviral vector according to claim 1 or 2, wherein said vector comprises an adenoviral nucleic acid derived from at least two different adenoviruses.

4. A recombinant adenoviral vector according to claim 3, wherein said nucleic acid encodes for a chimaeric fiber protein wherein at least the knob domain is replaced with at least the knob domain of a fiber protein of an adenovirus selected from the group consisting of: adenovirus serotype 11, 16, 35 and 51, and wherein the remaining part of the chimaeric fiber protein is of an adenovirus of subgroup C.

5. A recombinant adenoviral vector according to claim 4, wherein the remaining part of the chimaeric fiber protein is of adenovirus serotype 5.

6. A recombinant adenoviral vector according to any one of claims 3 to 5, wherein said nucleic acid is a modified nucleic acid such that the capacity of said adenovirus nucleic acid to replicate in a target cell has been reduced or disabled.

7. A recombinant adenoviral vector according to claim 6, wherein the modification comprises a deletion of at least part of the E1-region.

8. A recombinant adenoviral vector according to any one of claims 1 to 7, further comprising at least one nucleic acid of interest.

9. An adenoviral nucleic acid encoding a recombinant adenoviral vector according to any one of claims 1 to 8, wherein said adenoviral nucleic acid comprises a deletion in the E1-region rendering said adenoviral vector replication-defective.

10. A cell comprising an adenoviral nucleic acid according to claim 9, wherein said cell comprises another nucleic acid, which complements for the deletion of the E1-region in said adenoviral nucleic acid.

11. A cell according to claim 10, wherein said cell is a PER.C6 cell as represented by cells deposited under number ECACC 96022940, or a derivative thereof.

12. A method for the production of a recombinant adenoviral vector according to any one of claims 1 to 8, comprising culturing a cell according to claim 10 or 11 in a suitable medium and harvesting said recombinant adenoviral vector.

13. A recombinant adenoviral vector according to any one of claims 1 to 8 for use as a pharmaceutical.

14. Use of a recombinant adenoviral vector according to any one of claims 1 to 8 in the production of a medicament for the treatment of rheumatoid arthritis.

15. Use of a recombinant adenoviral vector according to claim 8 for the delivery of said nucleic acid of interest to fibroblast-like or macrophage-like cells.

16. Use according to claim 15, wherein said fibroblast-like or macrophage-like cells are synoviocytes.

17. Construct pBr/Ad.BamRΔFib, comprising adenovirus 5 sequences 21562-31094 and 32794-35938.

18. Construct pBr/AdBamRFib16, comprising adenovirus 5

sequences 21562-31094 and 32794-35938, further comprising an adenovirus 16 nucleic acid encoding at least part of a fiber protein of adenovirus 16.

19. Construct pBr/Ad.BamR.pac/fib16, comprising adenovirus 5 sequences 21562-31094 and 32794-35938, further comprising an adenovirus 16 nucleic acid encoding at least part of a fiber protein of adenovirus 16, and further comprising a unique PacI-site in the proximity of the adenovirus 5 right terminal repeat, in the non-adenovirus sequence backbone of said construct.

20. Construct pWE/Ad.AflIIrTTRfib16, comprising adenovirus 5 sequences 3534-31094 and 32794-35938, further comprising an adenovirus 16 nucleic acid encoding at least part of a fiber protein of adenovirus 16.

21. Construct pWE/Ad.AflIIrTTRDE2Afib16, comprising adenovirus 5 sequences 3534-22443, 24033-31094 and 32794-35938, further comprising an adenovirus 16 nucleic acid encoding at least part of a fiber protein of adenovirus 16.

22. The use of a construct according to any one of claims 17 to 21 for the generation of a vehicle according to any one of claims 1 to 8.

23. A fibroblast-like or macrophage-like cell comprising a nucleic acid delivered to said cell through a recombinant adenoviral vector according to any one of claims 1 to 8.

24. A fibroblast-like or macrophage-like cell according to claim 23, which is a synoviocyte.

25. A recombinant adenoviral vector according to any one of claims 1 to 9 or 13, a cell according to any one of claims 10 to 11, a method according to claim 12, a use according to any one of claims 14 to 16 or 22, a construct according to any one of claims 17 to 21 or a fibroblast-like or macrophage-like cell according to any one of claims 23 to 24

substantially as hereinbefore described with reference to the Figures and/or Examples.

DATED this 28th day of June, 2004
CRUCELL HOLLAND B.V.
by DAVIES COLLISON CAVE
Patent Attorneys for the Applicant(s)

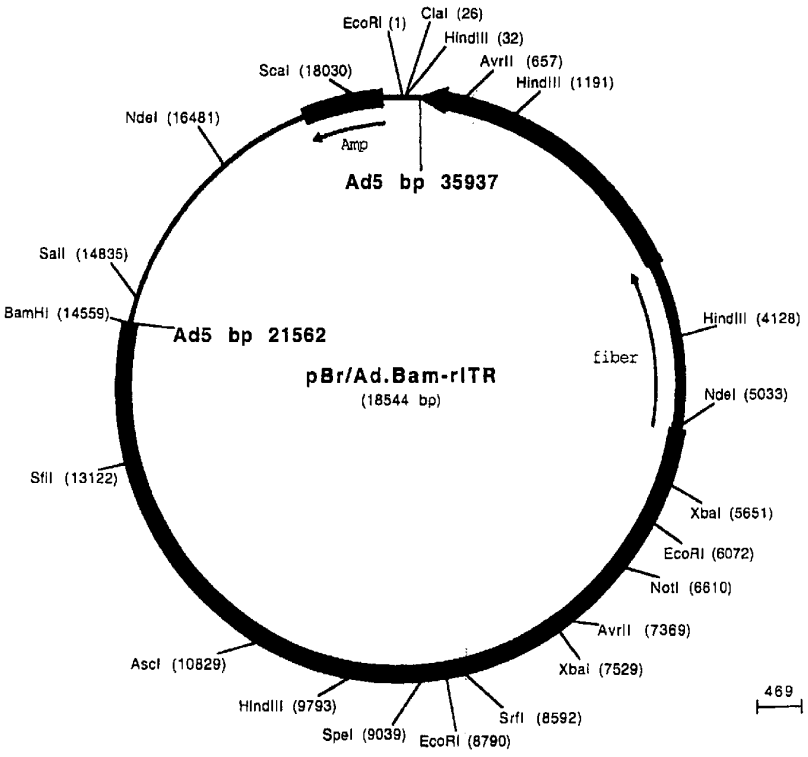
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Fig. 1

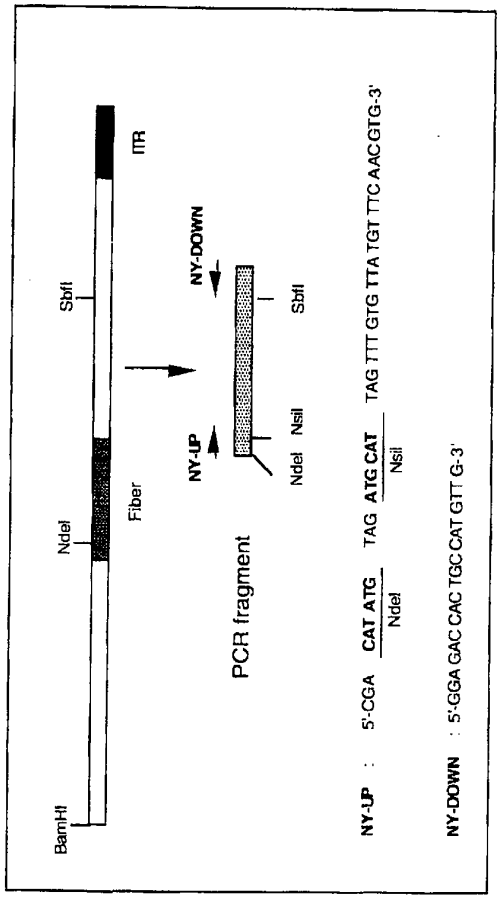
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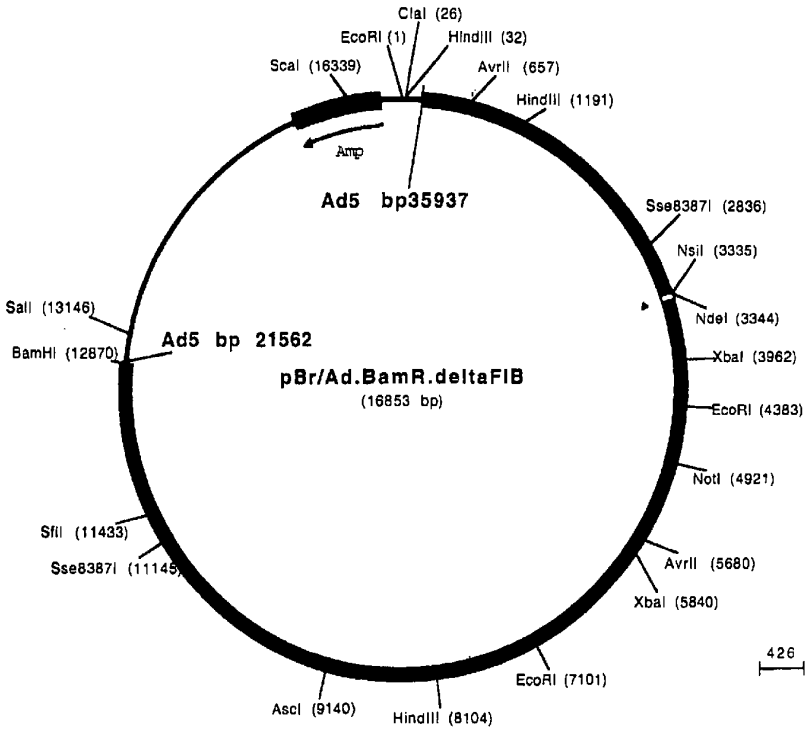
Fig. 2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XbaI (5840)
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EcoRI (7101)
AscI (9140)
HindIII (8104)

Fig. 3

Sequence of Ad5/fib16 chimeric fiber

ATGAAGCGCGCAAGACCGTCTGAAGATACCTTCAACCCCGTGTATCCATATGAAGATGAAAGCAGCT
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TAAAGACAACCAAAACATGGCTACTGGAACCATAACCAGTGCCAAAGGCTTCATGCCCAGCACCACC
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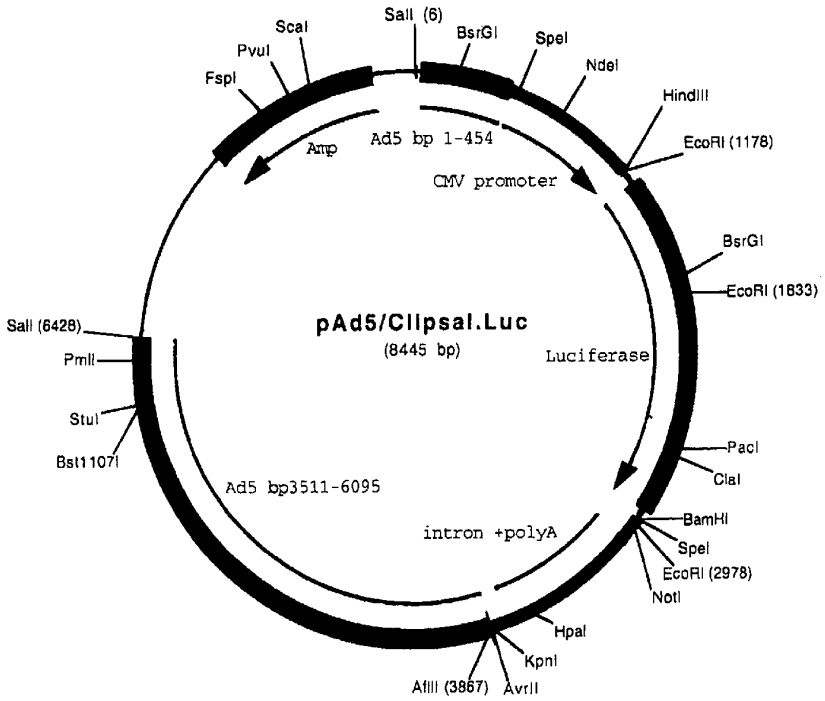
Fig. 4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EcoRI (1833)
SalI (6428)
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(8445 bp)
PmlI
Luciferase
StuI
PacI
Bst1107I
ClaI
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BamHI
intron +polyA
SpeI
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AflII (3867)
AvrII

Fig. 5

FIG. 6: Generation of (chimaeric) adenoviruses

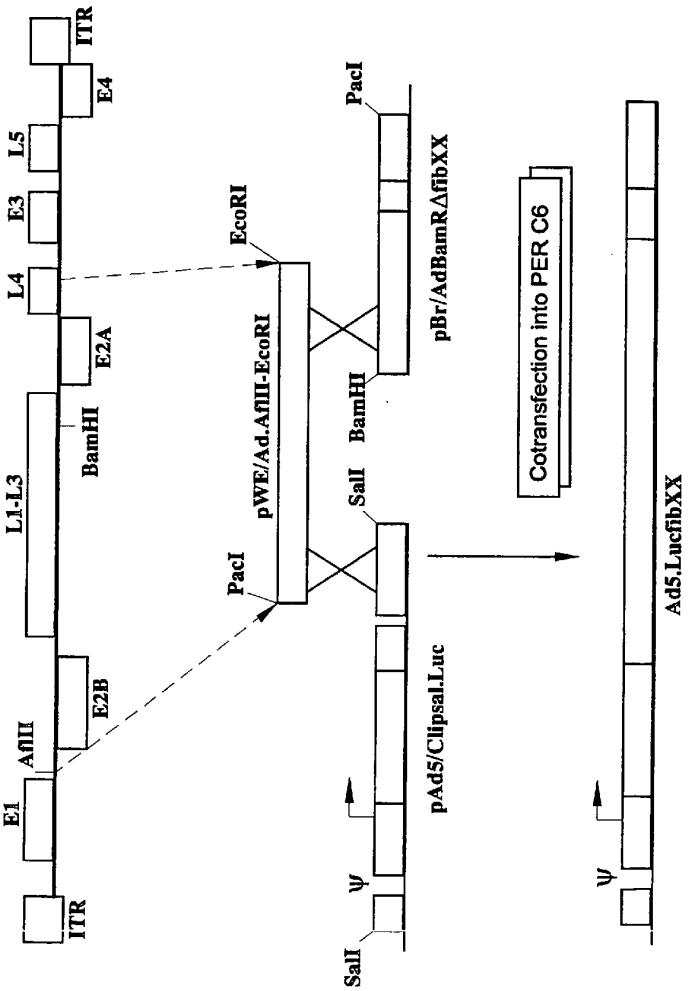

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Fig. 7A

Alignment Report of Untitled, using Clustal method with Weighted residue weight table. Page
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761  AAAACATGGCTACTGGAACCATAACCAGTGCCAAAGGCTT Ad5/fib16.seq

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801  CATGCCCAGCACCACCGCCTATCCATTTATAACATACGCC Ad5/fib16.seq

829  ACTGAGACCCTAAATGAAGATTACATTTATGGAGAGTGTT Ad16 genbank.se
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949  GCCTATGCTATGAATTTTTCATGGTCTCTAAATGCAGAGG Ad16 genbank.se
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989  AAGCCCCGGAAACTACCGAAGTCACTCTCATTACCTCCCC Ad16 genbank.se
1001 AAGCCCCGGAAACTACCGAAGTCACTCTCATTACCTCCCC Ad5/fib16.seq

1029 CTTCTTTTTTTCTTATATCAGAGAAGATGACTGA       Ad16 genbank.se
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Fig. 7A Contd.

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1   M-KRARPSEDTFNPVYPYEDESSSQHPFIN Ad16A fib protein

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30  PGFISSNGFAQSPDGVLTLKCVNPLTTASG Ad16A fib protein

60  PLQLKVGSSLTVDTIDGSLEENITAAAPLT Ad16 fiber protein GenBank
60  PLQLKVGSSLTVDTIDGSLEENITAEAPLT Ad16A fib protein

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180 DSPDCKLTLVLVKNGGLINGYITLMGASEY Ad16A fib protein

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210 TNTLFKNNQVTIDVNLAFDNTGQIITYLSS Ad16A fib protein

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240 LKSNLNFKDNQNMATGTITSAKGFMPSTTA Ad16A fib protein

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270 YPFITYATETLNEDYIYGECYYKSTNGTLF Ad16A fib protein

300 PLKVTVTLNRRMLASGMAYAMNFSWSLNAE Ad16 fiber protein GenBank
300 PLKVTVTLNRRMLASGMAYAMNFSWSLNAE Ad16A fib protein

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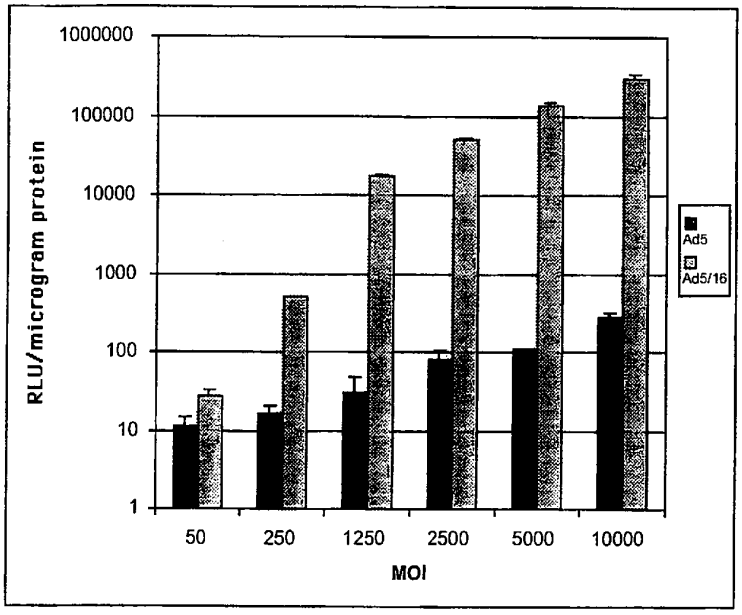
Fig. 7B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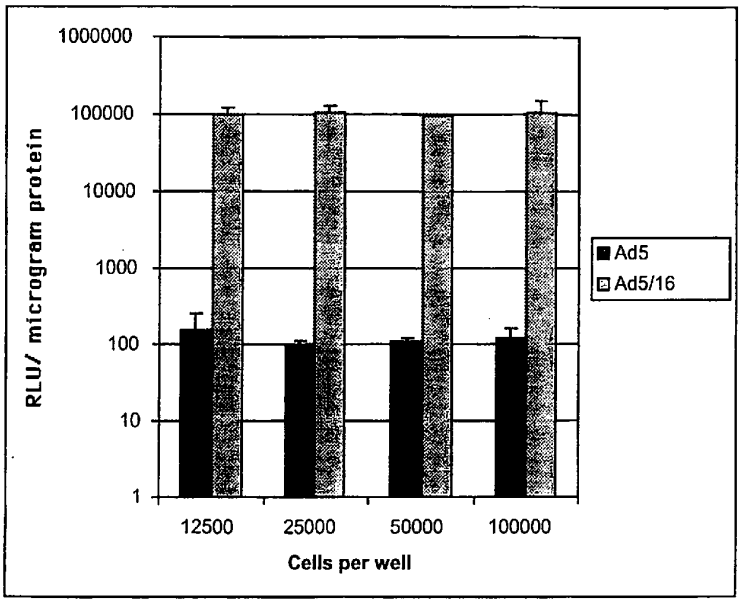
Ad5
Ad5/16

Fig. 8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50000
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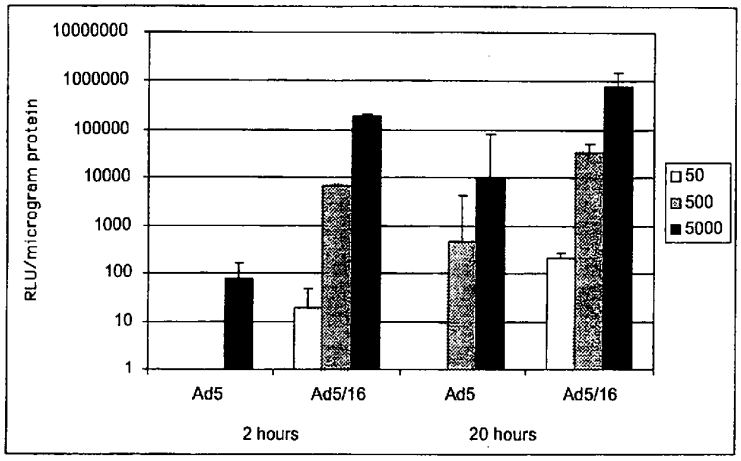
Cells per well
Ad5
Ad5/16

Fig. 9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500
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Ad5
Ad5/16
Ad5
Ad5/16
2 hours
20 hours

Fig. 10

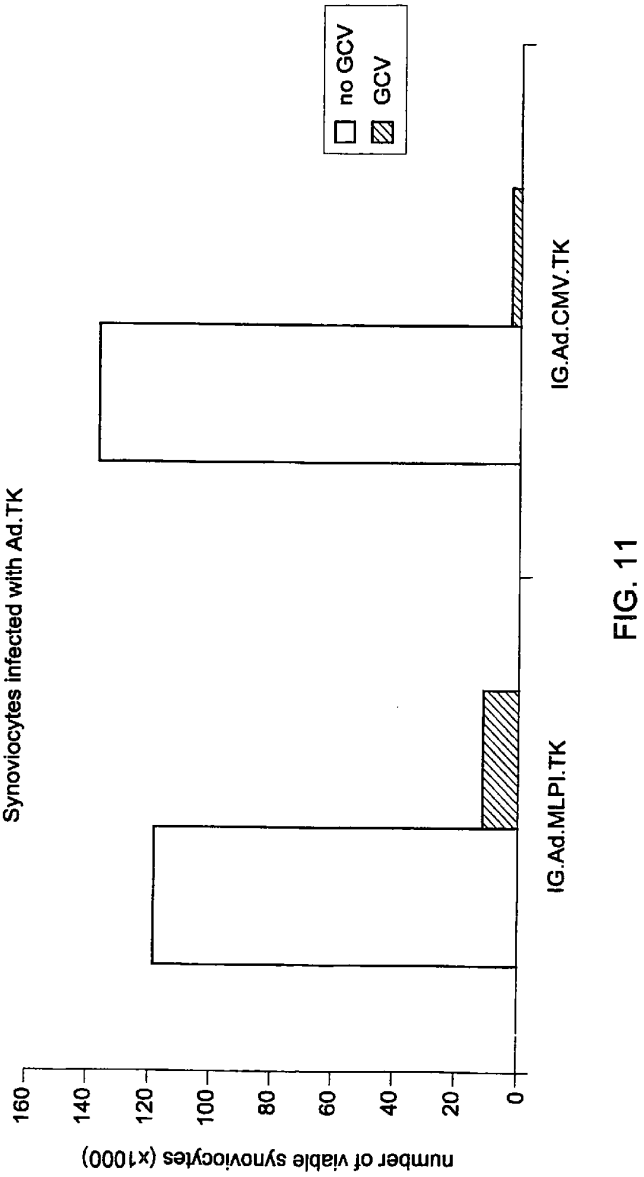
no GCV
GCV
IG.Ad.CMV.TK
IG.Ad.MLPI.TK
Synoviocytes infected with Ad.TK
number of viable synoviocytes (x1000)
160
140
120
100
80
60
40
20
0

FIG. 11

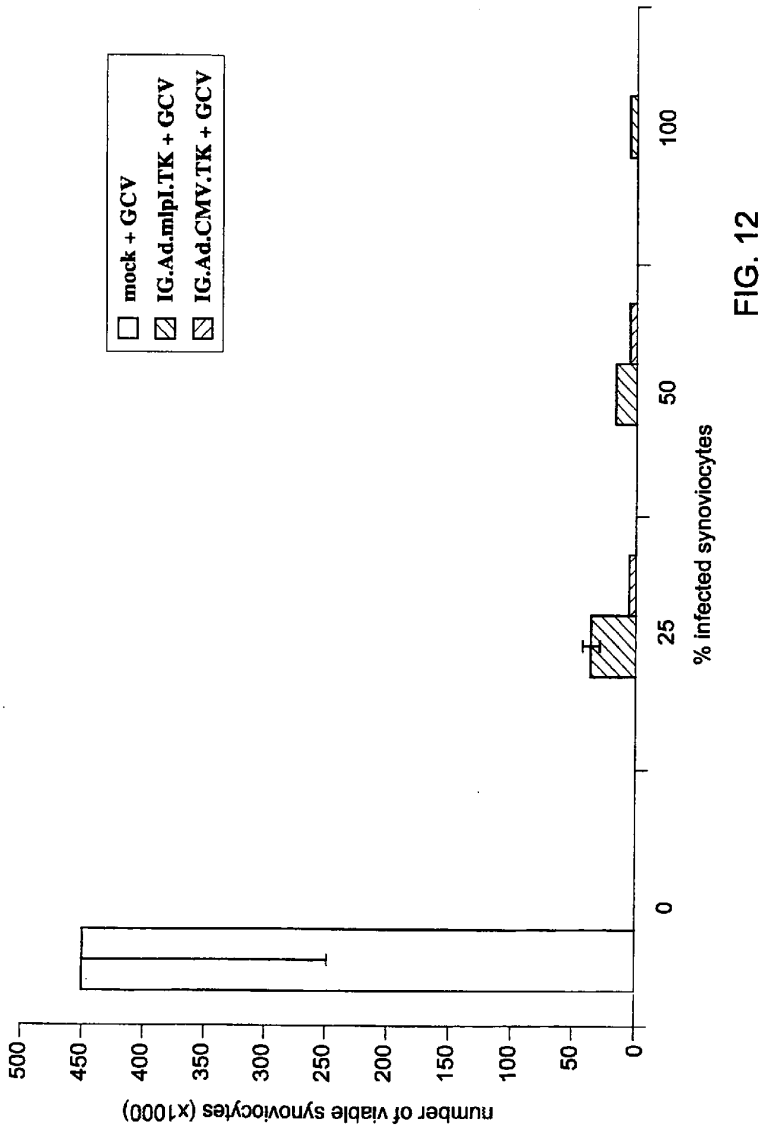
mock + GCV
IG.Ad.mlpI.TK + GCV
IG.Ad.CMV.TK + GCV
number of viable synoviocytes (x1000)
500
450
400
350
300
250
200
150
100
50
0
0
25
50
100
% infected synoviocytes

FIG. 12

Fig. 13A

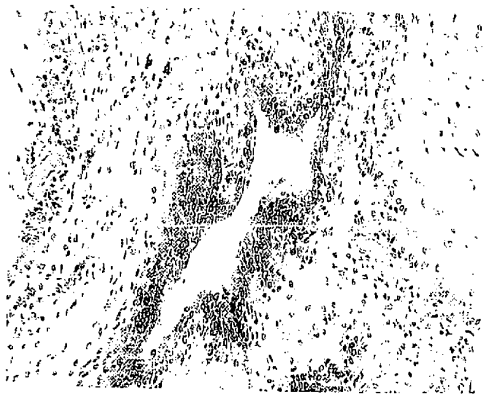

Fig. 13B

Figure 14: Comparison of infection of Ad5.luc and Ad5.fib16.luc on RA synoviocytes

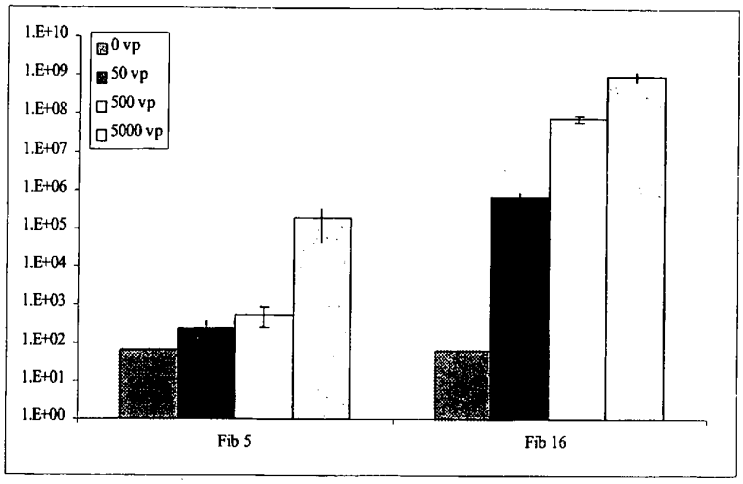

Fig. 14

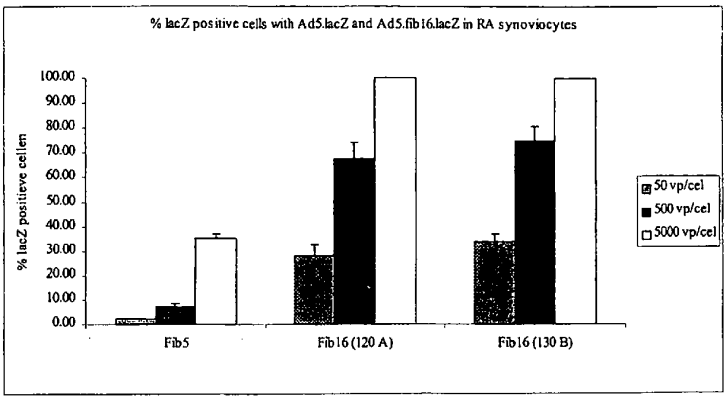
% lacZ positive cells with Ad5.lacZ and Ad5.fib16.lacZ in RA synoviocytes
% lacZ positieve cellen
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Fib5
Fib16 (120 A)
Fib16 (130 B)
50 vp/cel
500 vp/cel
5000 vp/cel

Fig. 15

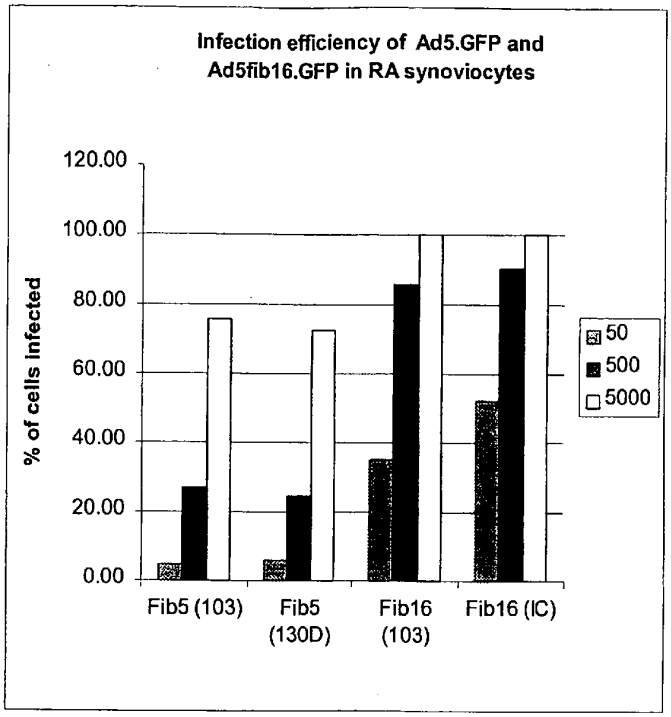
Infection efficiency of Ad5.GFP and Ad5fib16.GFP in RA synoviocytes
% of cells infected
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Fib5 (103)
Fib5 (130D)
Fib16 (103)
Fib16 (IC)
50
500
5000

Fig. 16A

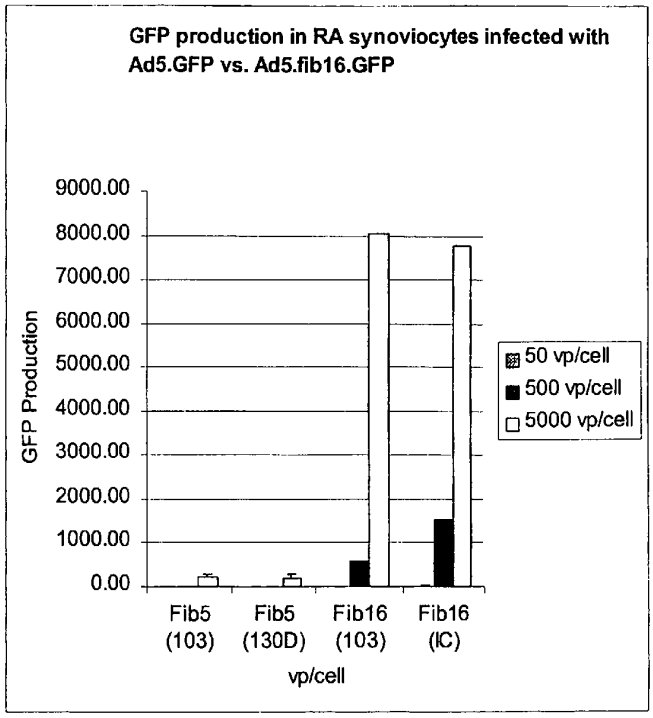
GFP production in RA synoviocytes infected with Ad5.GFP vs. Ad5.fib16.GFP
GFP Production
9000.00
8000.00
7000.00
6000.00
5000.00
4000.00
3000.00
2000.00
1000.00
0.00
Fib5 (103)
Fib5 (130D)
Fib16 (103)
Fib16 (IC)
vp/cell
50 vp/cell
500 vp/cell
5000 vp/cell

Fig. 16B

infectivity of panel of chimeric adenoviruses on RA synoviocytes

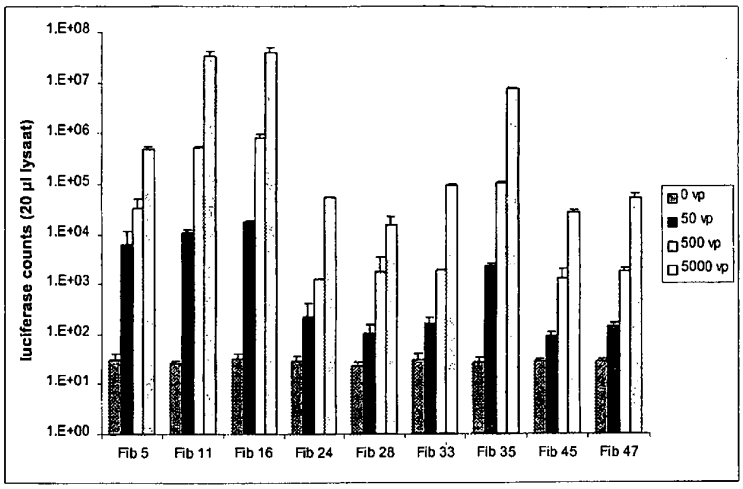

Fig. 17

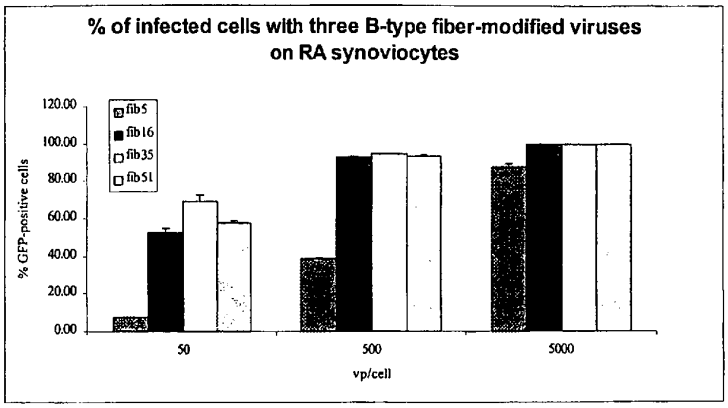
% of infected cells with three B-type fiber-modified viruses on RA synoviocytes
% GFP-positive cells
120.00
100.00
80.00
60.00
40.00
20.00
0.00
fib5
fib16
fib35
fib51
50
500
5000
vp/cell

Fig. 18A

GFP production with three B-type fiber-modified viruses on RA synoviocytes

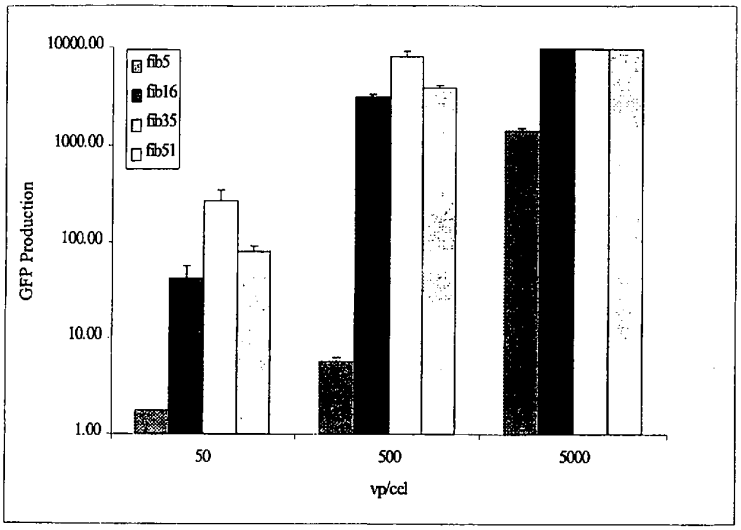

Fig. 18B

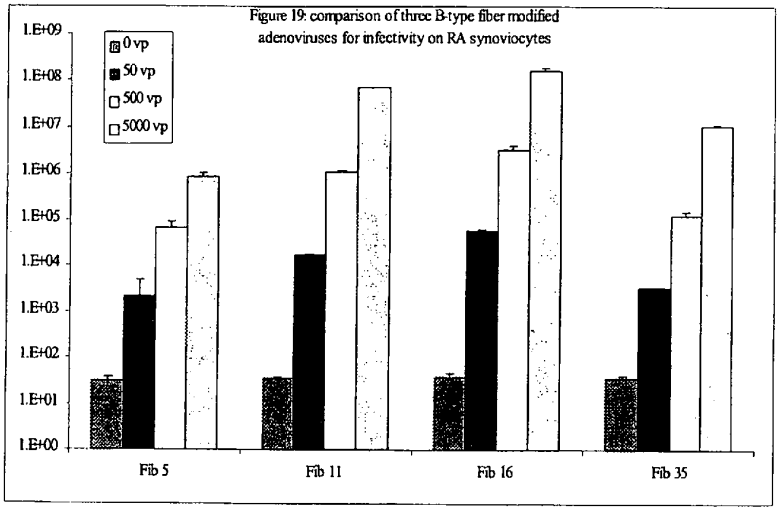
Figure 19: comparison of three B-type fiber modified adenoviruses for infectivity on RA synoviocytes
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
0 vp
50 vp
500 vp
5000 vp
Fib 5
Fib 11
Fib 16
Fib 35

Fig. 19

Figure 20: comparison of infectivity Ad5.lacZ vs. Ad5.fib16.lac6 in RA synoviocytes from 6 different patients

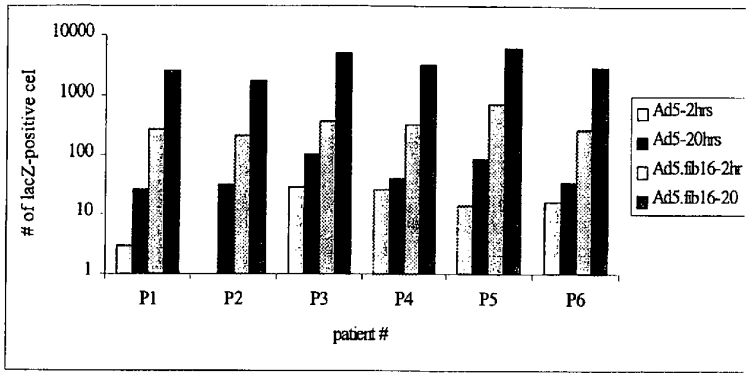

Fig. 20