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(54) Title: COMBINATION THERAPIES WITH FTC FOR THE TREATMENT OF HEPATITIS B VIRUS INFECTION

(57) Abstract: A method, use and composition for the treatment of a host infected with hepatitis B is provided that includes administering β-L-FTC or its pharmaceutically acceptable salt or prodrug (shown below) in combination or alternation with an immunomodulator, or in particular, an immunostimulating agent to achieve minimal or no detectable viral load in the host, which may be a human.

COMBINATION THERAPIES WITH FTC FOR THE TREATMENT OF HEPATITIS B VIRUS INFECTION

FIELD OF THE INVENTION

5 The present invention describes methods and compositions for the treatment of a host infected with hepatitis B virus that includes the co-administration of FTC with an immunomodulatory and in particular immunostimulatory agent.

BACKGROUND OF THE INVENTION

Despite the existence of efficient vaccines, hepatitis B virus (HBV) infection 10 remains a major public health problem worldwide with 400 million chronic carriers. These infected patients are exposed to a risk of developing liver cirrhosis and hepatocellular carcinoma (Lee, W. M. 1997, *N. Engl. J. Med.* 337:1733–1745). Currently, there are believed to be approximately 1.25 million chronic hepatitis B carriers just in the United States, with 200,000 people newly infected each year by 15 contact with blood or body fluids.

Hepatitis B virus ("HBV") is second only to tobacco as a cause of human cancer. The mechanism by which HBV induces cancer is unknown, although it is postulated that it may directly trigger tumor development, or indirectly trigger tumor development through chronic inflammation, cirrhosis and cell regeneration associated with the 20 infection.

The number of humans infected with HBV has reached epidemic levels. After a two to six month incubation period in which the host is unaware of the infection, HBV

infection can lead to acute hepatitis and liver damage, which results in abdominal pain, jaundice, and elevated blood levels of certain enzymes. HBV can cause fulminant hepatitis, a rapidly progressive, often fatal form of the disease in which massive sections of the liver are destroyed. Patients typically recover from acute viral hepatitis. In some
5 patients, however, high levels of viral antigen persist in the blood for an extended, or indefinite, period, causing a chronic infection. Chronic infections can lead to chronic persistent hepatitis. Patients infected with chronic persistent HBV are most common in developing countries. Chronic persistent hepatitis can cause fatigue, cirrhosis of the liver and hepatocellular carcinoma, a primary liver cancer. In western industrialized
10 countries, high risk groups for HBV infection include those in contact with HBV carriers or their blood samples. The epidemiology of HBV is in fact very similar to that of acquired immunodeficiency syndrome, in fact, HBV infection is common among patients with AIDS or HIV-associated infections. However, HBV is more contagious than HIV.

15 To date, only three drugs have been approved by the FDA for the treatment of chronic HBV infection: interferon alpha, 3TC and adefovir dipivoxil.

FDA Approved Drugs for HBV:

Drug Name	Drug Class	Company	FDA Status
Intron A (interferon α-2b)	interferon	Schering-Plough	FDA-approved
3TC (lamivudine; Epivir-HBV)	nucleoside analogue	GlaxoSmithKline	FDA-approved
Adefovir dipivoxil	nucleotide analogue	Gilead Sciences	FDA-approved

Interferon alpha

20 Interferon is a protein made naturally by the body to modulate the immune system and to regulate other cell functions. A manufactured form of interferon is used to treat hepatitis B. This treatment involves the administration of interferon by injection for about four months.

Not all patients respond to interferon, and sometimes re-treatment is necessary. In clinical studies, only 45% of patients who were treated for hepatitis B with Intron A (Interferon alfa-2b, recombinant, Schering Corporation) for Injection had no evidence of the hepatitis B virus in their blood over time. In addition, most patients have difficulty 5 tolerating interferon treatment, which causes severe flu-like symptoms, weight loss, and lack of energy and stamina.

3TC

The (-)-enantiomer of BCH-189 (2',3'-dideoxy-3'-thiacytidine), also known as 10 3TC (Epivir, lamivudine) is an antiviral drug that is active against both HIV and HBV. It belongs to the class of drugs called nucleoside analog reverse transcriptase inhibitors (NRTI), which work by inhibiting the polymerase enzyme that HIV and HBV need to replicate. In December 1998, the U.S. Food and Drug Administration (FDA) approved Epivir HBV for the treatment of hepatitis B virus infection.

15 Although 3TC efficiently inhibits HBV replication, the slow kinetics of viral elimination during 3TC therapy (Nowak, M., S. Bonhoeffer, et al. 1996. *Proc. Natl. Acad. Sci. USA* 93:4398–4402) and the spontaneous viral genome variability lead to the emergence of drug-resistant mutants which carry mutations affecting the reverse transcriptase (RT) domain (Mason, W. S., J. Cullen, et al. 1998. *Virology* 245:18–32. 20 Nafa, S., S. Ahmed, et al. 2000. *Hepatology* 32:1078–1088; Melegari, M., P. P. Scaglioni, and J. R. Wands. 1998 *Hepatology* 27:628–633.; Seigneres, B., C. Pichoud, et al. 2000. *J. Infect. Dis.* 181:1221–1233). Approximately 50% of treated patients develop viral resistance after 3 years of treatment with 3TC (Leung, N. W., C. L. Lai, et al. 2001. *Hepatology* 33:1527–1532). Resistance to nucleoside analogs is associated with 25 substitutions in the nucleic acid sequence of the polymerase gene causing changes in the amino acid sequence of the HBV RT, notably in the YMDD motif within the catalytic site. The most common polymerase variant is the rtL180M-plus-M204V change (according to the recent genotype-independent nomenclature for HBV drug-resistant variants) (Stuyver, L. J., S. A. Locarnini, et al. 2001. *Hepatology* 33:751–757) that 30 associates a mutation in the catalytic site (rtM204V) with a compensatory mutation in the B domain of the RT (rtL180M) which provides a higher replication capacity to the catalytic site variant (Allen, M. I., M. Deslauriers, et al. 1998. *Hepatology* 27:1670–

1677. Chayama, K., Y. Suzuki, et al. 1998. *Hepatology* 27:1711–1716. Melegari, M., P. P. Scaglioni, and J. R. Wands. 1998. *Hepatology* 27:628–633. Ono, S. K., N. Kato, et al. 2001. *J. Clin. Investig.* 107:449–455. Seigneres, B., S. Aguesse-Germon, et al. 2001. *J. Hepatol.* 34:114–122).

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Adefovir dipivoxil (Hepsera)

On September 20, 2002, the U.S. Food and Drug Administration approved adefovir dipivoxil for the treatment of chronic hepatitis B. HEPSERA™ is the tradename for adefovir dipivoxil, a diester prodrug of adefovir. Adefovir is an acyclic 10 nucleotide analogue of adenosine monophosphate that inhibits the hepatitis B virus (HBV) DNA polymerase by competing with the natural substrate deoxyadenosine triphosphate and by causing DNA chain termination after its incorporation into viral DNA. The chemical name of adefovir dipivoxil is 9-[2-[bis[(pivaloyloxy)methoxy]phosphinyl]methoxy]-ethyl]adenine. Adefovir is 15 phosphorylated to the active metabolite, adefovir diphosphate, by cellular kinases. See, for example, U.S. Patent Nos. 5,641,763 and 5,142,051, entitled, N-phosphonylmethoxyalkyl derivatives of pyrimidine and purine bases and a therapeutical composition therefrom with antiviral activity.

20 **(-)-FTC**

β-L-FTC ((β-L-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane, Emtriva; emtricitabine) is approved for the treatment of HIV and currently in human clinical trials for the treatment of hepatitis B virus infection. The compound show strong activity against hepatitis B virus in duck models (Aguesse-Germon, S., S.-H, Liu, *et al.* 25 Antimicrob. Agents Chemother. **1998**, *42*, 369-376; Seignéres, B., C. Pichoud, *et al.* **2000**) and woodchucks. In a woodchuck model of HBV, FTC was found to inhibit viral replication but not induce viral clearance. (Cullen, J. M., S. L. Smith, *et al.* Antimicrob. Agents Chemother. **1997**, *41*, 2076-2082; Korba, B. E., R. F. Schinazi, P., *et al.* Antimicrob. Agents Chemother. **2000**, *44*, 1757-60).

Immunomodulatory Agents

Certain immunomodulatory agents, such as immunostimulatory agents, have shown promise against viral diseases. In certain animal models of HBV infection, such as the woodchuck model and in transgenic mice, it has been demonstrated that the intrahepatic expression of immunomodulatory agents can induce an inhibition of viral replication via a non-cytolytic pathyway. Specific immunomodulatory agents that were tested included such as TH1 cytokines including IFN gamma, TNF alpha, and Interleukine 12 (Guidotti, L. G., P. Borrow, A. Brown, H. McClary, R. Koch, and F. V. Chisari "Noncytopathic clearance of lymphocytic choriomeningitis virus from the hepatocyte" J Exp Med. 1999, *189*, 1555-1564; Guo,J. T., H. Zhou, C. Liu, C. Aldrich, J. Saputelli, T. Whitaker, M. I. Barrasa, W. S. Mason, and C. Seeger "Apoptosis and regeneration of hepatocytes during recover from transient hepadnavirus infections" J Virol. 2000, *74*, 1495-1505).

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a synthetic, non-interferon-inducing, broad spectrum antiviral nucleoside analog sold under the trade name Virazole (The Merck Index, 11th edition, Editor: Budavari, S., Merck & Co., Inc., Rahway, NJ, p1304, 1989). U.S. Patent No. 3,798,209 and RE29,835 disclose and claim ribavirin. Ribavirin is structurally similar to guanosine, and has *in vitro* activity against several DNA and RNA viruses including *Flaviviridae* (Gary L. Davis Gastroenterology 118:S104-S114, 2000). Ribavirin has been shown to exhibit activity against hepatitis A, B and C. Ribavirin alone is not effective in reducing viral RNA levels, but rather it acts an an immunomodulator by altering the T-cell balance in the immune system (Hultgren *et al. J. Gen. Virol.* 1998 Oct;79 (Pt 10):2381-91).

A main class of immunomodulators are interferons. Types of interferons include: interferon alpha, pegylated interferon alpha, interferon alpha-2a, interferon alpha-2b, pegylated interferon alpha-2a, pegylated interferon alpha-2b ROFERON®-A (interferon alpha-2a, Roche), PEGASYS® (pegylated interferon alpha-2a, Roche), INTRON®A (Interferon alpha-2b, Schering Corporation), PEG-INTRON® (pegylated Interferon alpha-2b, Schering Corporation), interferon beta, interferon gamma, interferon tau, interferon omega, consensus interferon, INFERGEN (interferon alphacon-1) by InterMune, OMNIFERON (natural interferon) by Viragen, ALBUFERON by Human Genome Sciences, REBIF (interferon beta-1a) by Ares-Serono, Omega Interferon by

BioMedicine, Oral Interferon Alpha by Amarillo Biosciences, interferon gamma-1b by InterMune, SuperFeron (natural human multi-subtype IFN-alpha, Genetrol, Inc.), and HuFeron (human IFN-beta, Genetrol, Inc.).

Other types of immunomodulators include hepatitis B immune globulin (Nabi-HB intravenous, Nabi Pharmaceuticals), ZADAXIN™ (thymosin alpha 1, SCV-07 (SciClone Pharmaceuticals), Theradigm (Epimmune), anti-hepatitis B hyperimmune product (Cangene Corp), RC-529 (Corixa/Rhein Biochem), HYB2055 (Hybridon), ViroKine (human antiviral proteins, Genetrol, Inc.), Levovirin (Ribapharm, Inc.), interleukin-2 (IL-2), tumor necrosis factor-alpha, interleukin 1-beta, interleukin-12 (IL-12), Granulocyte-macrophage colony-stimulating factor (GM-CSF), polyadenylic-polyuridylic acid, thymosin alpha, Ampligen® (Hemispherx BioPharma), Polyadenur™ (Poly A:Poly U RNA, Hemispherx BioPharms), Oragen™ (Hemispherx BioPharms), Hepatitis B Virus (HBV)-specific Cytotoxic T Lymphocytes (CTL) (CellExSys, Inc.), therapeutic hepatitis b vaccine (Epimmune), PJ Hep B DNA prophylactic vaccine (Powderject Pharmaceuticals), interleukin 4, interleukin 6, interleukin 7, and granulocyte colony stimulating factor.

Immunostimulatory Sequences

ImmunoStimulatory Sequences (ISS) are short nucleic acid, typically DNA, sequences that enhance the ability of the immune system to fight disease and control chronic inflammation. Certain ISS have been shown to strengthen insufficient immune responses against foreign pathogens, such as viruses.

U.S. Application No. 2002/0098199, published July 25, 2002, discloses immunostimulatory sequences for the treatment of HBV and HCV.

U.S. Patent No. 6,225,292, assigned to The Regents of the University of California and Dynavax Technologies Corp., discloses oligonucleotides which inhibit the immunostimulatory activity of ISS-ODN (immunostimulatory sequence oligodeoxynucleotides) as well as methods for their identification and use. The disclosed oligonucleotides of are useful in controlling therapeutically intended ISS-ODN adjuvant activity as well as undesired ISS-ODN activity exerted by recombinant expression vectors, such as those used for gene therapy and gene immunization. The

oligonucleotides also have anti-inflammatory activity useful in reducing inflammation in response to infection of a host with ISS-ODN containing microbes, in controlling autoimmune disease and in boosting host Th2 type immune responses to an antigen. The patent also encompasses pharmaceutically useful conjugates of the oligonucleotides of
5 the invention (including conjugate partners such as antigens and antibodies).

U.S. Patent No. 6,589,940, assigned to Dynavax Technologies Corp., discloses immunostimulatory oligonucleotide compositions. These oligonucleotides comprise an immunostimulatory octanucleotide sequence. These oligonucleotides can be administered in conjunction with an immunostimulatory peptide or antigen. Methods for
10 modulating an immune response upon administration of the oligonucleotide are also disclosed. In addition, an in vitro screening method to identify oligonucleotides with immunostimulatory activity is provided.

U.S. Patent No. 6,562,798, assigned to Dynavax Technologies Corp., discloses immunomodulatory oligonucleotide compositions, including immunostimulatory
15 hexanucleotide sequence comprising a modified cytosine. These oligonucleotides can be administered in conjunction with an immunomodulatory peptide or antigen. Methods of modulating an immune response upon administration of the oligonucleotide comprising a modified immunostimulatory sequence are also disclosed.

PCT Publication No. WO 03/014316, assigned to Dynavax Technologies Corp.,
20 discloses compositions and methods for immunomodulation of individuals. Immunomodulation is accomplished by administration of immunomodulatory polynucleotide/microcarrier (IMO/MC) complexes comprising 3-6mer immunomodulatory oligonucleotides. The IMO/MC complexes may be covalently or non-covalently bound. Also disclosed are immunomodulatory compositions comprising
25 a 3-6m er IMO encapsulated in an MC.

PCT Publication No. WO 03/000922, assigned to Dynavax Technologies Corp., discloses immunomodulatory compounds and methods for immunomodulation of individuals using the immunomodulatory compounds.

PCT Publication No. WO 02/052002, assigned to Dynavax Technologies Corp.,
30 discloses immunomodulatory polynucleotides and methods for immunomodulation of individuals using the immunomodulatory polynucleotides.

PCT Publication Nos. WO 01/68144 and WO0168143 are assigned to Dynavax Technologies Corp., disclose compositions and methods for immunomodulation of individuals. Immunomodulation is accomplished by administration of immunomodulatory polynucleotide/microcarrier (IMP/MC) complexes. The IMP/MC complexes may be covalently or non-covalently bound, and feature a polynucleotide comprising at least one immunostimulatory sequence bound to a biodegradable microcarrier or nanocarrier.

PCT Publication No. WO 01/68117, assigned to Dynavax Technologies Corp., discloses methods for the treatment of papillomavirus infections. A polynucleotide comprising an immunstimulatory sequence is administered to an individual who has been exposed to or infected by papillomavirus. The polynucleotide is not administered with papillomavirus antigen. Administration of the polynucleotide results in amelioration of symptoms of papillomavirus infection.

PCT Publication No. WO 01/68078, assigned to Dynavax Technologies Corp., discloses methods for the treatment of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections. A polynucleotide comprising an immunostimulatory sequence is administered to an individual who has been exposed to or infected by HBV and/or HCV. The polynucleotide is not administered with a HCV or HBV antigen. Administration of the polynucleotide results in amelioration of symptoms of HBV and/or HCV infection.

PCT Publication No. WO 01/68077, assigned to Dynavax Technologies Corp., discloses methods of suppression, prevention, and/or treatment of infection by viruses. A polynucleotide comprising an immunostimulatory sequence (an "ISS") is administered to an individual who is at risk of being exposed to, has been exposed to or is infected with a virus. The ISS-containing polynucleotide is administered without any antigens of the virus. Administration of the ISS-containing polynucleotide results in reduced incidence and/or severity of one or more symptoms of virus infection.

PCT Publication No. WO 01/12223, assigned to Dynavax Technologies Corp., discloses methods of modulating an immune response to a second antigen which entail administration of a first antigen and an immunostimulatory polynucleotide. Modulation of the immune response is generally manifested as stimulation of a Th1 response.

PCT Publication No. WO 00/21556, assigned to Dynavax Technologies Corp., discloses anti-viral immunomodulatory compositions comprising immunostimulatory polynucleotides and HIV antigens, such as gp120. Methods for modulating an immune response upon administration of the oligonucleotide and antigen compositions are also
5 disclosed.

PCT Publication No. WO 00/16804, assigned to Dynavax Technologies Corp., discloses methods of treating IgE-associated disorders and compositions for use therein. The methods are particularly useful in treatment of allergies and allergy-related disorders. The methods generally comprise administering an IgE inhibitor (such as anti-
10 IgE antibody) and an antigen and/or immunostimulatory polynucleotide sequence (ISS). These combination methods offer significant advantages, such as allowing more aggressive therapy while reducing unwanted side effects, such as anaphylaxis.

PCT Publication No. WO 99/62923, assigned to Dynavax Technologies Corp., discloses oligonucleotides comprise an immunostimulatory hexanucleotide sequence
15 comprising a modified cytosine. These oligonucleotides can be administered in conjunction with an immunomodulatory peptide or antigen. Methods of modulating an immune response upon administration of the oligonucleotide comprising a modified immunostimulatory sequence are also disclosed.

PCT Publication No. WO 98/55495, assigned to Dynavax Technologies Corp.,
20 discloses immunostimulatory oligonucleotide composition including immunostimulatory octanucleotide sequence. These oligonucleotides can be administered in conjunction with an immunostimulatory peptide or antigen. Methods for modulating an immune response upon administration of the oligonucleotide are also disclosed. In addition, an in vitro screening method to identify oligonucleotides with immunostimulatory activity is also
25 disclosed.

PCT Publication No. WO 03/015711, assigned to Coley Pharmaceutical Group, Inc., discloses a class of immunostimulatory nucleic acids having at least two functionally and structurally defined domains. This class of combination motif immunostimulatory nucleic acids activates an immune response and is useful for treating
30 a variety of immune related disorders such as cancer, infectious disease, and allergic

disorders. The nucleic acids also stimulate activation of natural killer cells and production of type 1 interferon.

U.S. Patent No. 6,406,705, assigned to Coley Pharmaceutical Group, Inc., discloses methods and products utilizing a synergistic combination of immunostimulatory oligonucleotides having at least one unmethylated CpG dinucleotide (CpG ODN) and a non-nucleic acid adjuvant. Such combinations of adjuvants may be used with an antigen or alone. Methods and products utilizing immunostimulatory oligonucleotides having at least one unmethylated CpG dinucleotide (CpG ODN) for induction of cellular immunity in infants are also disclosed.

U.S. Patent No. 6,339,068, assigned to Coley Pharmaceutical Group, Inc., discloses DNA vaccine vectors that can be improved by removal of CpG-N motifs and optional addition of CpG-S motifs. In addition, for high and long-lasting levels of expression, the optimized vector should include a promoter/enhancer that is not down-regulated by the cytokines induced by the immunostimulatory CpG motifs. Vectors and methods of use for immunostimulation are provided herein. The invention also provides improved gene therapy vectors by determining the CpG-N and CpG-S motifs present in the construct, removing stimulatory CpG (CpG-S) motifs and/or inserting neutralizing CpG (CpG-N) motifs, thereby producing a nucleic acid construct providing enhanced expression of the therapeutic polypeptide.

U.S. Patent No. 6,239,116, assigned to Coley Pharmaceutical Group, Inc., discloses nucleic acid sequences containing unmethylated CpG dinucleotides that modulate an immune response including stimulating a Th1 pattern of immune activation, cytokine production, NK lytic activity, and B cell proliferation are disclosed. The sequences are also useful a synthetic adjuvant.

U.S. Patent No. 6,207,646, assigned to Coley Pharmaceutical Group, Inc., discloses Nucleic acids containing unmethylated CpG dinucleotides and therapeutic utilities based on their ability to stimulate an immune response and to redirect a Th2 response to a Th1 response in a subject are disclosed.

U.S. Patent No. 6,194,388, assigned to Coley Pharmaceutical Group, Inc., discloses oligonucleotides containing unmethylated CpG dinucleotides and therapeutic utilities based on their ability to stimulate an immune response in a subject are disclosed.

Also disclosed are therapies for treating diseases associated with immune system activation that are initiated by unmethylated CpG dinucleotides in a subject comprising administering to the subject oligonucleotides that do not contain unmethylated CpG sequences (i.e. methylated CpG sequences or no CpG sequence) to outcompete
5 unmethylated CpG nucleic acids for binding. Further disclosed are methylated CpG containing dinucleotides for use antisense therapies or as in vivo hybridization probes, and immunoinhibitory oligonucleotides for use as antiviral therapeutics.

U.S. Publication No. 20030091599, assigned to Coley Pharmaceutical Group, Inc., discloses methods and products utilizing a synergistic combination of
10 immunostimulatory oligonucleotides having at least one unmethylated CpG dinucleotide (CpG ODN) and a non-nucleic acid adjuvant. Such combinations of adjuvants may be used with an antigen or alone. The publication also relates to methods and products utilizing immunostimulatory oligonucleotides having at least one unmethylated CpG dinucleotide (CpG ODN) for induction of cellular immunity in infants.

15 PCT Publication No. WO 03/031573, assigned to Coley Pharmaceutical Group, Inc., discloses compositions and methods are provided to identify, characterize, and optimize immunostimulatory compounds, their agonists and antagonists, working through TLR3.

PCT Publication No. WO 03/012061, assigned to Coley Pharmaceutical Group,
20 Inc., discloses methods and compositions relating to a dentritic cell expression database.

PCT Publication No. WO 02/069369, assigned to Coley Pharmaceutical Group, Inc., discloses immunostimulatory compositions described as CpG-like nucleic acids are provided, including nucleic acids having immunostimulatory characteristics of CpG nucleic acid, despite certain substitutions of C, G, or C and G of the CpG dinucleotide.
25 The substitutions can include, among others, exchange of methylated C for C, inosine for G, and ZpY for CpG, where Z is Cytosine or dSpacer and Y is inosine, 2-aminopurine, nebularine, or dSpacer. Also disclosed are methods for inducing an immune response in a subject using the CpG-like nucleic acids. The methods are useful in the treatment of a subject that has or is at risk of developing an infectious disease, allergy, asthma, cancer,
30 anemia, thrombocytopenia, or neutropenia.

PCT Publication No. WO 01/95935, assigned to Coley Pharmaceutical Group, Inc., discloses methods and products for inducing an immune response using immunostimulatory nucleic acids. In particular the immunostimulatory nucleic acids preferentially induce a Th2 immune response. The invention is useful for treating and preventing disorders associated with a Th1 immune response or for creating a Th2 environment for treating disorders that are sensitive to Th2 immune responses.

PCT Publication No. WO 01/22990, assigned to Coley Pharmaceutical Group, Inc., discloses methods and compositions for extending the clinical utility of IFN-'alpha' in the treatment of a variety of viral and proliferative disorders. Also disclosed are methods which increase the efficacy of IFN-alpha treatment and reduce IFN-alpha treatment-related side effects. In addition, methods are provides for supporting the survival and for activating natural interferon producing cells (IPCs) *in vitro* without exogenous IL-3 or GM-CSF.

PCT Publication No. WO 01/22972, assigned to Coley Pharmaceutical Group, Inc., discloses immunostimulatory nucleic acid compositions and methods of using the compositions. The T-rich nucleic acids contain poly T sequences and/or have greater than 25 % T nucleotide residues. The TG nucleic acids have TG dinucleotides. The C-rich nucleic acids have at least one poly-C region and/or greater than 50 % c nucleotides. These immunostimulatory nucleic acids function in a similar manner to nucleic acids containing CpG motifs. The invention also encompasses preferred CpG nucleic acids.

Combination Therapy

In order to better control viral replication and delay the emergence of virus-resistant mutants, it is desired to develop antiviral strategies based on combination therapy (Zoulim, F. 2001. *Antivir. Chem. Chemother.* 12(Suppl. 1):131–142), which may also prevent the selection of cross-resistant mutants, as was shown with human immunodeficiency virus (Chow, Y. K., M. S. Hirsch, et al. 1993. *Nature* 361:650–654; Snyder, S., D. Z. D'Argenio, et al. 2000. *Antimicrob. Agents Chemother.* 44:1051–1058.Villahermosa, M. L., et al. .1997. *Biochemistry* 36:13223–13231). A few studies have focused on examination of the antiviral effect of the combination of polymerase inhibitors for the therapy of chronic hepadnavirus infection, using several experimental

models (Korba, et al. 2000. *Antivir. Res.* 45:19–32; Colledge, D., S. Locarnini, and T. Shaw. 1997. *Hepatology* 26:216–225; Colledge, et al. 2000. *Antimicrob. Agents Chemother.* 44:551–560). The development of combination therapy for hepatitis B infection is one current necessary strategy underway to further reduce the morbidity and mortality of chronic hepatitis B infection.

The effects of pyrimidine and purine nucleoside analog combinations in the duck hepatitis B virus infection model are demonstrated in Seigneres et al., *Antimicrobial Agents and Chemotherapy*, 47(6):1842-1852 (2003). Combinations of amdoxovir (DAPD), emtricitabine (-FTC) and clevudine (L-FMAU) have an enhanced antiviral effect.

Treatments for hepatitis B infection are also described in Lok and McMahon, AASLD Practice Guidelines, pp. 1225-1241 (2001), including treatment with interferons. Eastern woodchucks chronically infected with the woodchuck hepatitis virus (WHV) were used as a model of HBV infection to study the antiviral effect of 1-(2-fluoro-5-methyl-β-L-arabinofuranosyl)-uracil (L-FMAU) and WHV surface antigen vaccine. The humoral and cellular immunity associated with the combination of L-FMAU and vaccine resembled that observed in self-limited WHV infection (Menne *et al.*, *J. Virology*, 76(11):5305-5314 (2002)).

Jacquard et al. published a study of the multidrug treatment of chronic hepatitis B using L-FMAU and FTC, along with adenovirus delivery of recombinant interferon gamma. (Jacquard et al., "Evaluation and Combination of Clevudine and Emtricitabine with Adenovirus Mediated Delivery of Interferon Gamma in the Woodchuck Model of GBV Infection", Poster presented at the Boston AASLD meeting, Nov. 2-5, 2002. See also the Asilomar meeting "The Molecular Biology of Hepatitis B Virus" Sept 29 - Oct 3, 2002.) This study suggested that interferon did not enhance the effect of L-FMAU and FTC, however, the study was limited to woodchuck hepatitis virus in woodchucks, and not human hepatitis B in humans.

U.S. Patent No. 5,808,040 to the University of Georgia Research Foundation and Yale University discloses that L-FMAU can be administered in combination with FTC, 3TC, carbovir, acyclovir, interferon, AZT, DDI (2',3'-dideoxyinosine), DDC (2',3'-dideoxycytidine), L-DDC, L-F-DDC, and D4T.

International Patent Application No. PCT/US99/25673, published as WO 00/25797 discloses combinations and methods using such combinations for treating HBV infection and related conditions in humans. In particular, the compositions comprise a synergistically effective amount of β-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane (FTC) and one of penciclovir (2-amino-1,9-dihydro-9-[4-hydroxy-3-(hydroxymethyl)butyl]-6H-purin-6-one, also referred to as "PCV"), famciclovir, or 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA, also referred to below as Bis-POM-PMEA or BP-PMEA). Alternatively, the compositions comprise a synergistically effective amount of 2'-fluoro-5-methyl-β-L-arabinofuranolyluridine (L-FMAU) and one of Penciclovir, 9-[2-(phosphono-methoxy)ethyl]adenine (PMEA) or a β-D-1,3-dioxolane nucleoside, such as DAPD. Alternatively, the compositions comprise a synergistically effective amount of a β-D-1,3-dioxolane nucleoside, such as DAPD and PMEA.

PCT Publication No. WO 98/23285 discloses a method for the treatment or prophylaxis of hepatitis B virus infections in a human or animal patient which comprises administering to the patient effective or prophylactic amounts of penciclovir (or a bioprecursor thereof such as famciclovir) and alpha-interferon.

U.S. Patent No. 5,990,093 and International Patent Publication Nos. WO 95/07086 and WO 96/40164, assigned to Emory University, disclose methods to treat HBV comprising administering β-L-2',3'-dideoxyadenosine in combination or alternation with 2-hydroxy-methyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane; 2-hydroxymethyl-5-(cytosin-1-yl)-1,3-oxathiolane; 2'-fluoro-5-iodo-arabinosyluracil (FIAU); 2'-fluoro-5-ethyl-arabinosyluracil (FEAU), carbovir, or interferon.

U.S. Patent Nos. 5,703,058, 5,905,070, and 6,232,300 and International Patent Publication No. WO 96/22778 disclose (5-carboximido or 5-fluoro)-(2',3'-unsaturated or 3'-modified) pyrimidine nucleosides for the treatment of HIV or HBV. In particular, the patents and international patent publication disclose combination therapies for the treatment of HIV or HBV comprising a (5-carboximido or 5-fluoro)-(2',3'-unsaturated or 3'-modified) pyrimidine nucleoside in combination with one or more of 2-hydroxy-methyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane, 2-hydroxymethyl-5-(cytosin-1-yl)-1,3-oxathiolane; 9->4-(hydroxymethyl)-2-cyclopenten-1-yl-guanine (carbovir), 9-(2-hydroxy-ethoxy)methyl-guanine (acyclovir), interferon, 3'-deoxy-3'-azidothymidine (AZT), 2',3'-

dideoxyinosine (DDI), 2',3'-dideoxycytidine (DDC), (-)-2'-fluoro-5-methyl-.beta.-L-ARAuridine (L-(-)-FMAU) and 2',3'-didehydro-2',3'-dideoxythymidine (D4T).

While advances have been made in the treatment of hepatitis B and in combination therapies, it is still not known which combinations are effective to optimally substantially reduce or eliminate the human hepatitis B viral load in the host. It has been observed that while certain hepatitis B agents and combinations can be augmented with additional therapies, in other cases, additional therapies provide no added benefit and can actually be detrimental to the patient's well being. The health care provider, therefore, needs information on beneficial and advantageous combinations of hepatitis B therapies so that he or she can proceed with optimal treatment of the patient.

In light of the fact that hepatitis B virus has reached epidemic levels worldwide, and has severe and often tragic effects on the infected patient, there remains a strong need to provide new effective pharmaceutical regimens, which treat humans infected with the virus while exhibiting low toxicity to the host.

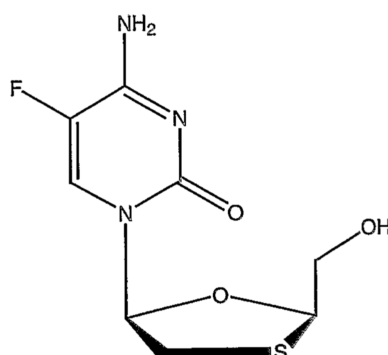
Therefore, it is an object of the present invention to provide compositions and methods for the treatment of human patients or other hosts infected with HBV which exhibit high efficacy in reducing viral load, low toxicity, or may not encourage the development of resistant viral strains.

It is another object of the present invention to provide a method and composition to substantially reduce or eliminate the human hepatitis B viral load in the host.

SUMMARY OF THE INVENTION

A method, use and composition for the treatment of a host infected with hepatitis B is provided that includes administering β-L-FTC or its pharmaceutically acceptable salt or prodrug (shown below) in combination or alternation with an immunomodulator, or in particular, an immunostimulating agent to achieve minimal or no detectable viral load in the host, which may be a human. The immunomodulator in combination with β-L-FTC may help stimulate the non-cytolytic TH1 response to treat and/or clear viral

infection. One non-limiting illustration of such combination therapy includes combination or alternation therapy with interferon, for example, alpha, beta, omega, tau or consensus interferon or an immunostimulatory seequence. Alternatively, β-L-FTC or its pharmaceutically acceptable salt or prodrug and an immunomodulator can be further combined with at least one other anti-HBV agent to achieve minimal or no detectable viral load in the patient.



β-L-FTC

In a preferred embodiment, the β-L-FTC is at least 90% free of its opposite β-D-FTC enantiomer. In a more preferred embodiment, β-L-FTC is at least 95%, 98% or 99% free of its opposite β-D-FTC enantiomer.

Therefore, in one embodiment, compositions, methods and uses for the treatment and/or prophylaxis of an HBV infection in a host, preferably a mammal, and even more preferably a human, are provided comprising administering effective amounts of β-L-FTC in combination and/or alternation with an effective amount of an immunomodulator, such as interferon or an immunostimulatory sequence to treat the residual infected hepatocytes and reduce viral load. In one embodiment, the immunomodulator is administered in the form of a protein. In an alternate embodiment, the immunomodulator is administered in the form of a nucleic acid (which can be random, palindromic, encoding or other), gene or gene fragment thereof.

In an alternate embodiment, compositions and methods for the treatment and/or prophylaxis of an HBV infection in a host, preferably a mammal, and even more preferably a human, are provided comprising administering effective amounts of B-L-FTC in combination and/or alternation with an effective amount of an immunomodulator, including but not limited to a TH1 cytokine, as well as in

combination and/or alternation with at least one additional anti HBV agent, including but not limited to another nucleoside analog to treat the residual infected hepatocytes.

In one embodiment, immunomodulators can be immunostitulatory (ISS) molecules. The ISS can be a nucleic acid sequence comprising an unmethylated CG sequence. In a further embodiment, the ISS can include the sequence 5'-T, C, G-3'. In alternate embodiments, the ISS can be the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3' or 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, C-3'. In still further embodiments, the ISS can be selected from the following group, including, but not limited to: 5'-AACGTTCC-3', 5'-AACGTTCG-3', 5'-GACGTTCC-3' and 5'-GACGTTCG-3', 5'-TGACTGTGAACGTTCGAGATGA-3'.

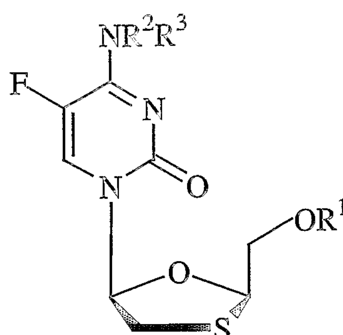
In another embodiment, the immunomodulator can be a cytokine, adjuvant or antibody. In another embodiment, the immunomodulator can be an interferon. In a further embodiment, the immunomodulator selected from the following group, including, but not limited to: interferon alpha-2a, interferon alpha-2b, ROFERON®-A (interferon alpha-2a, Roche), PEGASYS® (pegylated interferon alpha-2a, Roche), INTRON®A (Interferon alpha-2b, Schering Corporation), PEG-INTRON® (pegylated Interferon alpha-2b, Schering Corporation), interferon alpha, interferon beta, interferon gamma, interferon tau, interferon omega, INFERGEN (interferon alphacon-1) by InterMune, OMNIFERON (natural interferon) by Viragen, ALBUFERON by Human Genome Sciences, REBIF (interferon beta-1a) by Ares-Serono, Omega Interferon by BioMedicine, Oral Interferon Alpha by Amarillo Biosciences, interferon gamma- 1b by InterMune, SuperFeron (natural human multi-subtype IFN-alpha, Genetrol, Inc.), and HuFeron (human IFN-beta, Genetrol, Inc.), Ribavirin (Ribapharm, Inc.), hepatitis B immune globulin (Nabi-HB intravenous, Nabi Pharmaceuticals), ZADAXIN™ (thymosin alpha 1, SCV-07 (SciClone Pharmaceuticals), Theradigm (Epimmune), anti-hepatitis B hyperimmune product (Cangene Corp), RC-529 (Corixa/Rhein Biochem), HYB2055 (Hybridon), ViroKine (human antiviral proteins, Genetrol, Inc.), Levovirin (Ribapharm, Inc.), interleukin-2 (IL-2), tumor necrosis factor-alpha, interleukin 1-beta, interleukin-12 (IL-12), Granulocyte-macrophage colony-stimulating factor (GM-CSF), polyadenylic-polyuridylic acid, thymosin alpha, Ampligen® (Hemispherx BioPharma), Polyadenur™ (Poly A:Poly U RNA, Hemispherx BioPharms), Oragen™ (Hemispherx BioPharms), Hepatitis B Virus (HBV)-specific Cytotoxic T Lymphocytes (CTL)

(CellExSys, Inc.), therapeutic hepatitis b vaccine (Epimmune), PJ Hep B DNA prophylactic vaccine (Powderject Pharmaceuticals), interleukin 4, interleukin 6, interleukin 7, and granulocyte colony stimulating factor.

In one embodiment of the invention, an immunomodulator protein is delivered in the form of the protein itself, or its pharmaceutically acceptable salt or prodrug. In an alternate embodiment, the immunomodulator is delivered in the form of a nucleic acid (including a cDNA), nucleic acid, gene or gene fragment that expresses the immunomodulator protein. In one particular embodiment of the present invention, the immunomodulator is delivered in the form of a nucleic acid, gene or gene fragment thereof, and the delivery is mediated by a vector including but not limited to a viral vector including an adenovirus. In one particular embodiment of the invention, the immunomodulator is interferon (such as interferon gamma), and its delivery is in the form of a nucleic acid, gene or gene fragment that is mediated by an adenovirus.

In an alternate embodiment, the immunomodulator can be an HBV vaccine. In one embodiment, the vaccine can be a recombinant HBV vaccine. In another embodiment, the HBV vaccine includes is not limited to: Engerix-B® (GlaxoSmithKline), Recombivax HB® (Merck), Hepatitis B Vaccine (Recombinant), PJ Hep B DNA therapeutic vaccine (Powderject Pharmaceuticals), Hepavax-Gene® (DNA recombinant hepatitis B vaccine , Berna Biotech group), Gen H-B Vax™ (Chiron Corporation), Hepatavax-B® (Merck & Co.), Hevac B® (Pasteur), KGC® (Korea Green Cross), TGP 943™ (Takeda Chem, Japan), Gen Hevac B® (Pasteur, France), Bio-Hep-B™/Sci-B-Vac™ (Bio-Technology General, Israel), AG-3™, Hepagene™, Hepacare™, (Medeva, UK, Evans UK).

In another embodiment, compositions and methods are provided for the treatment and/or prophylaxis of an HBV infection in a host, preferably a mammal, and even more preferably a human, are provided comprising administering an effective amount of β-L-FTC of the formula:



or its pharmaceutically acceptable salt or prodrug, wherein

R^1 is H, alkyl, aryl, acyl, phosphate, including monophosphate, diphosphate or
5 triphosphate or a stabilized phosphate moiety, including a phospholipid, or an ether-lipid,

R^2 and R^3 are independently hydrogen, alkyl, acyl or cycloalkyl (such as cyclopropyl), optionally in a pharmaceutically acceptable carrier

in combination and/or alternation with an immunomodulator.

In general, during alternation therapy, an effective dosage of each agent is
10 administered serially, whereas in combination therapy, effective dosages of two or more agents are administered together. The dosages will depend on such factors as absorption, bio-distribution, metabolism and excretion rates for each drug as well as other factors known to those of skill in the art. It is to be noted that dosage values will also vary with the severity of the condition to be alleviated. It is to be further understood that for any
15 particular subject, specific dosage regimens and schedules should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions. Examples of suitable dosage ranges can be found in the scientific literature and in the *Physicians Desk Reference*. Many examples of suitable dosage ranges for other compounds described
20 herein are also found in public literature or can be identified using known procedures. These dosage ranges can be modified as desired to achieve a desired result.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is an illustration of a non-limiting example of an adenoviral vector comprising woodchuck interferon gamma suitable for delivery. Coding regions are depicted and defined.

5 **Figure 2** is an illustration of a non-limiting schematic of treatment regimes of 1-(2-fluoro-5-methyl-β-L-arabinosyl) uracil (L-FMAU), and 4-amino-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxothiolan-5-yl]2 (*1H*)-pyrimidinone (*2R-cis*) (B-L-FTC), with or without injections of an adenovirus vector for expressing woodchuck interferon gamma (Ad IFN) or an adenovirus vector expressing Green Fluorescent Protein (Ad GFP), given 10 to representative woodchucks.

Figure 3 shows four graphs depicting the amount of viral DNA (copies/ml) of woodchuck hepatitis B virus (WHV) over time (days) before and after injection of woodchucks with different doses (pfu) of adenovirus vector for expressing woodchuck interferon gamma (Ad IFN). Arrows indicate time of inoculation and time of biopsy. 15 On the left Y axes, amount of viral plus strand DNA is shown, measured by endogenous polymerase assay (EPA, dpm/ml) as described in Example 3, plotted against time.

Figure 4 shows five graphs depicting the amount of viral DNA (copies/ml) of woodchuck hepatitis B virus (WHV) over time (days) before and after injection of woodchucks with $3x10^9$ pfu of the complete adenovirus vector for expressing 20 woodchuck interferon gamma (Ad IFN GFP). Arrows indicate time of inoculation and time of biopsy. On the left Y axes, amount of viral plus strand DNA is shown, measured by endogenous polymerase assay (EPA, dpm/ml) as described in Example 3, plotted against time. The data are described in more detail in Example 5.

Figure 5 is three graphs depicting the amount of viral DNA (copies/ml) of 25 woodchuck hepatitis B virus (WHV) over time (days) before and after injection of woodchucks with $3x10^9$ pfu of adenovirus vector for expressing Green Fluorescent Protein (Ad GFP) (top two graphs) or an untreated control (bottom graph). Arrows indicate time of inoculation and time of biopsy. On the left Y axes, amount of viral plus strand DNA is shown, measured by endogenous polymerase assay (EPA, dpm/ml) as

described in Example 3, plotted against time. The data are described in more detail in Example 5.

Figure 6 is a copy of southern blots analyzing viral DNA found in liver biopsies of woodchucks infected with woodchuck hepatitis B virus (WHV), taken at 1 month before treatment (M-1) and day 5 post-injection (D5) from woodchucks injected with the complete adenovirus vector for expressing woodchuck interferon gamma (Ad IFN GFP), adenovirus vector for expressing Green Fluorescent Protein (Ad GFP), or not treated. Bottom blots are of covalently closed circular viral form of DNA (CCC) DNA. Numbers below the graphs demonstrate the ratio of DNA at D5/M-1 in percent.

Figure 7 shows graphs of viral concentration (copies/ml) of woodchuck hepatitis B virus over time (days) during and after treatment of animals with 1-(2-fluoro-5-methyl-_-L-arabinosyl) uracil (L-FMAU), and 4-amino-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxothiolan-5-yl]2 (1*H*)-pyrimidinone (2*R-cis*) (B-L-FTC), with or without injections of an adenovirus vector for expressing woodchuck interferon gamma (Ad IFN) as described in Example 6. The results were obtained using dot blot assays described in Example 3.

Figure 8 shows graphs of viral concentration (copies/ml) of woodchuck hepatitis B virus over time (days) during and after treatment of animals with injections of adenovirus vector for expressing woodchuck interferon gamma (Ad IFN) or adenovirus vector for expressing Green Fluorescent Protein (Ad GFP) as described in Example 6. The results were obtained using dot blot assays described in Example 3. Control woodchucks treated with Ad IFN alone showed no significant variation in viremia.

Figure 9 is a graph of viral concentration (copies/ml) of woodchuck hepatitis B virus over time (days) in an untreated animal as described in Example 6. The results were obtained using dot blot assays described in Example 3.

Figure 10 is three graphs of viral load (copies/ml) of woodchuck hepatitis B virus over time (days) during and after treatment of animals with 1-(2-fluoro-5-methyl-_-L-arabinosyl) uracil (L-FMAU), and 4-amino-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxothiolan-5-yl]2 (1*H*)-pyrimidinone (2*R-cis*) (B-L-FTC), with or without injections of an adenovirus vector for expressing woodchuck interferon gamma (IFN), measured using real-time PCR as described in Example 3.

Figure 11 is a copy of southern blots analyzing viral DNA found in liver biopsies of woodchucks infected with woodchuck hepatitis B virus (WHV), taken just prior to therapy (T0), at 1 month into treatment (M1) and at two months into treatment (M2) from woodchucks treated with 1-(2-fluoro-5-methyl-_-L-arabinosyl) uracil (L-FMAU), 4-amino-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxothiolan-5-yl]2 (1*H*)-pyrimidinone (2*R*-*cis*) (B-L-FTC), adenovirus vector for expressing woodchuck interferon gamma (Ad IFN), adenovirus vector for expressing Green Fluorescent Protein (Ad GFP), or no treatment. Bottom blots are of covalently closed circular viral form of DNA (CCC) DNA. Numbers below the graphs demonstrate the ratio of DNA at D5/M-1 in percent..

Figure 12 are graphs depicting the percent of woodchuck hepatitis B virus (WHV) viral DNA and covalently closed circular viral form of DNA (CCC) DNA in animals treated with 1-(2-fluoro-5-methyl-_-L-arabinosyl) uracil (L-FMAU), 4-amino-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxothiolan-5-yl]2 (1*H*)-pyrimidinone (2*R*-*cis*) (B-L-FTC), or an adenovirus vector for expressing woodchuck interferon gamma (IFN), indicating the depletion of virus. M0 indicates samples taken just prior to therapy, M1 indicates samples taken at 1 month into treatment, and M2 indicates samples taken at two months into treatment.

Figure 13 are graphs depicting the percent of woodchuck hepatitis B virus (WHV) viral DNA and covalently closed circular viral form of DNA (CCC) DNA in animals treated with 1-(2-fluoro-5-methyl-_-L-arabinosyl) uracil (L-FMAU), and 4-amino-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxothiolan-5-yl]2 (1*H*)-pyrimidinone (2*R*-*cis*) (B-L-FTC), indicating the depletion of virus. T0 indicates samples taken just prior to therapy, M1 indicates samples taken at 1 month into treatment, and M2 indicates samples taken at two months into treatment..

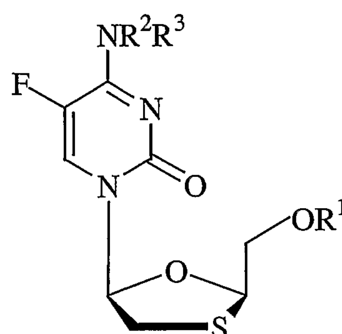
Figure 14 are graphs depicting the percent of woodchuck hepatitis B virus (WHV) viral DNA and covalently closed circular viral form of DNA (CCC) DNA in animals treated with injections of adenovirus vector for expressing woodchuck interferon gamma (Ad IFN), adenovirus vector for expressing Green Fluorescent Protein (Ad GFP), or untreated. T0 indicates samples taken just prior to therapy, M1 indicates samples taken at 1 month into treatment, and M2 indicates samples taken at two months into treatment.

Figure 15 are graphs depicting the amount of inflammatory activity (Metavir score) as described in Example 2, after treatment with with 1-(2-fluoro-5-methyl-_-L-arabinosyl) uracil (L-FMAU), 4-amino-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxothiolan-5-yl]2 (1*H*)-pyrimidinone (2*R-cis*) (B-L-FTC), injections of adenovirus vector for expressing woodchuck interferon gamma (Ad IFN), adenovirus vector for expressing Green Fluorescent Protein (Ad GFP), or untreated controls.

DETAILED DESCRIPTION OF THE INVENTION

Administration of the combination of an immunomodulator with B-L-FTC or its pharmaceutically acceptable salt or prodrug can substantially reduce or eliminate the human hepatitis B viral load in a host, and in particular a human. The immunomodulator may help stimulate the non-cytolytic TH1 response to treat and/or clear viral infection. In one embodiment, the immunomodulator is administered in the form of a protein. In an alternate embodiment of the invention, the immunomodulator is administered in the form of a nucleic acid, gene or gene fragment thereof. Alternatively, B-L-FTC and an immunomodulator can be further combined with at least one other anti-HBV agent. The additional anti-HBV agent(s) can have a different mechanism of action than B-L-FTC and/or that exhibit synergy with B-L-FTC.

In another embodiment, compositions and methods are provided for the treatment and/or prophylaxis of an HBV infection in a host, preferably a mammal, and even more preferably a human, are provided comprising administering an effective amount of a nucleoside of the formula:



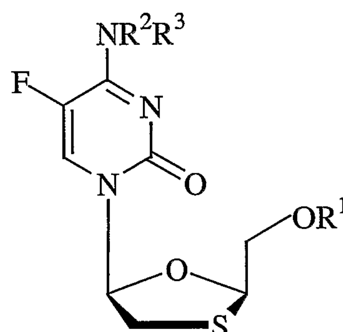
or its pharmaceutically acceptable salt or prodrug, wherein

R^1 is H, alkyl, aryl, acyl, phosphate, including monophosphate, diphosphate or triphosphate or a stabilized phosphate moiety, including a phospholipid, or an ether-lipid,

R^2 and R^3 are independently hydrogen, alkyl, acyl or cycloalkyl (such as cyclopropyl),
5 optionally in a pharmaceutically acceptable carrier

in combination and/or alternation with an immunomodulator.

In another embodiment, compositions and methods are provided for the treatment and/or prophylaxis of an HBV infection in a host, preferably a mammal, and even more preferably a human, are provided comprising administering an effective amount of a
10 nucleoside of the formula:



or its pharmaceutically acceptable salt or prodrug, wherein

R^1 is H, alkyl, aryl, acyl, phosphate, including monophosphate, diphosphate or triphosphate or a stabilized phosphate moiety, including a phospholipid, or an ether-lipid,

15 R^2 and R^3 are independently hydrogen, alkyl or cycloalkyl (such as cyclopropyl), optionally in a pharmaceutically acceptable carrier

in combination and/or alternation with an immunomodulator; and further in combination and/or alternation with at least one additional anti-HBV agent.

I. Definitions

The term alkyl, as used herein, unless otherwise specified, refers to a saturated straight, branched, or cyclic, primary, secondary or tertiary hydrocarbon of typically C_1 to C_{10}, and specifically includes methyl, trifluoromethyl, CCl_3, $CFCl_2$, CF_2Cl, ethyl, CH_2CF_3, CF_2CF_3, propyl, isopropyl, cyclopropyl, butyl, isobutyl, *t*-butyl, pentyl, cyclopentyl, isopentyl, neopentyl, hexyl, isohexyl, cyclohexyl, cyclohexylmethyl, 3-methylpentyl, 2,2-dimethylbutyl, and 2,3-dimethylbutyl. The term includes both substituted and unsubstituted alkyl groups. Moieties with which the alkyl group can be substituted include, but are not limited to the group consisting of halogen (fluoro, chloro, bromo or iodo), hydroxyl, amino, alkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfonic acid, sulfate, phosphonic acid, phosphate, or phosphonate, either unprotected, or protected as necessary, as known to those skilled in the art, for example, as taught in Greene, *et al.*, Protective Groups in Organic Synthesis, John Wiley and Sons, Second Edition, 1991, hereby incorporated by reference.

The term lower alkyl, as used herein, and unless otherwise specified, refers to a C_1 to C_4 saturated straight, branched, or if appropriate, a cyclic (for example, cyclopropyl) alkyl group, including both substituted and unsubstituted forms. Unless otherwise specifically stated in this application, when alkyl is a suitable moiety, lower alkyl is preferred. Similarly, when alkyl or lower alkyl is a suitable moiety, unsubstituted alkyl or lower alkyl is preferred.

The term aryl, as used herein, and unless otherwise specified, refers to phenyl, biphenyl, or naphthyl, and preferably phenyl. The term includes both substituted and unsubstituted moieties. The aryl group can be substituted with one or more moieties including but not limited to the group consisting of halogen (fluoro, chloro, bromo or iodo), hydroxyl, amino, alkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfonic acid, sulfate, phosphonic acid, phosphate, or phosphonate, either unprotected, or protected as necessary, as known to those skilled in the art, for example, as taught in Greene, *et al.*, Protective Groups in Organic Synthesis, John Wiley and Sons, Second Edition, 1991.

The term acyl refers to a carboxylic acid ester in which the non-carbonyl moiety of the ester group is selected from straight, branched, or cyclic alkyl or lower alkyl,

alkoxyalkyl including methoxymethyl, aralkyl including benzyl, aryloxyalkyl such as phenoxymethyl, aryl including phenyl optionally substituted with halogen (e.g., F, Cl, Br or I), C_1 to C_4 alkyl or C_1 to C_4 alkoxy, sulfonate esters such as alkyl or aralkyl sulphonyl including methanesulfonyl, the mono, di or triphosphate ester, trityl or monomethoxytrityl, substituted benzyl, trialkylsilyl (e.g. dimethyl-t-butylsilyl) or diphenylmethylsilyl. Aryl groups in the esters optimally comprise a phenyl group. The term "lower acyl" refers to an acyl group in which the non-carbonyl moiety is lower alkyl.

The term "substantially pure" is used throughout the specification to describe a compound which includes approximately 90% or greater, typically at least 95%, 96%, 97%, 98%, or 99% of a single enantiomer of that compound. When a nucleoside of a particular configuration (D or L) is referred to in this specification, it is presumed that the nucleoside is in substantially pure form unless otherwise stated.

The term "immunomodulator," as used herein, refers to an agent that regulates either directly or indirectly an immune response. Non-limiting examples of immunomodulators include TH1 cytokines, and in particular, interferon, interferon-α, purified interferon-α, interferon-α2a, interferon-α2b, interferon-β, interferon-γ, consensus interferon, pegylated interferon, pegylated interferon-α, granulocyte macrophage colony-stimulating factor, interleukin, interleukin-2, and interleukin-12.

The term "host," as used herein, refers to a unicellular or multicellular organism in which the virus can replicate, including cell lines and animals, and preferably a human. Alternatively, the host can carry a part of the viral genome, whose replication or function can be altered by the compounds of the present invention. The term host specifically refers to infected cells, cells transfected with all or part of the viral genome and animals, in particular, primates (including chimpanzees) and humans. In most animal applications of the present invention, the host is a human patient. Veterinary applications, in certain indications, however, are clearly anticipated by the present invention.

II. Pharmaceutically Acceptable Salts and Prodrugs

Pharmaceutically acceptable prodrugs refer to a compound that is metabolized, for example hydrolyzed or oxidized, in the host to form the compound of the present

invention. Typical examples of prodrugs include compounds that have biologically labile protecting groups on a functional moiety of the active compound. Prodrugs include compounds that can be oxidized, reduced, aminated, deaminated, hydroxylated, dehydroxylated, hydrolyzed, dehydrolyzed, alkylated, dealkylated, acylated, deacylated, phosphorylated, dephosphorylated to produce the active compound. Any of the compounds which are described herein for use in combination and/or alternation therapy can be administered for example as an acylated prodrug, wherein the term acyl in one embodiment refers to a carboxylic acid ester in which the non-carbonyl moiety of the ester group is selected from straight, branched, or cyclic alkyl or lower alkyl, alkoxyalkyl including methoxymethyl, aralkyl including benzyl, aryloxyalkyl such as phenoxymethyl, aryl including phenyl optionally substituted with halogen, C_1 to C_4 alkyl or C_1 to C_4 alkoxy, sulfonate esters such as alkyl or aralkyl sulphonyl including methanesulfonyl, the mono, di or triphosphate ester, trityl or monomethoxytrityl, substituted benzyl, trialkylsilyl (e.g. dimethyl-t-butylsilyl).

In cases where any of the compounds as disclosed herein are sufficiently basic or acidic to form stable nontoxic acid or base salts, administration of the compound as a pharmaceutically acceptable salt may be appropriate. Examples of pharmaceutically acceptable salts are organic acid addition salts formed with acids, which form a physiological acceptable anion, for example, tosylate, methanesulfonate, acetate, citrate, malonate, tartarate, succinate, benzoate, ascorbate, α-ketoglutarate and α-glycerophosphate. Suitable inorganic salts may also be formed, including, sulfate, nitrate, bicarbonate and carbonate salts.

Pharmaceutically acceptable salts may be obtained using standard procedures well known in the art, for example by reacting a sufficiently basic compound such as an amine with a suitable acid affording a physiologically acceptable anion. Alkali metal (for example, sodium, potassium or lithium) or alkaline earth metal (for example calcium) salts of carboxylic acids can also be made.

Any of the nucleosides described herein can be administered as a nucleotide prodrug to increase the activity, bioavailability, stability or otherwise alter the properties of the nucleoside. A number of nucleotide prodrug ligands are known. In general, alkylation, acylation or other lipophilic modification of the hydroxyl group of the compound or of the mono, di or triphosphate of the nucleoside will increase the stability

of the nucleotide. Examples of substituent groups that can replace one or more hydrogens on the phosphate moiety are alkyl, aryl, steroids, carbohydrates, including sugars, 1,2-diacylglycerol and alcohols. Many are described in R. Jones and N. Bischofberger, *Antiviral Research*, 27 (1995) 1-17. Any of these can be used in
5 combination with the disclosed nucleosides to achieve a desired effect.

The active nucleoside or other hydroxyl containing compound can also be provided as an ether lipid (and particularly a 5'-ether lipid or a 5'-phosphoether lipid for a nucleoside), as disclosed in the following references, which are incorporated by reference herein: Kucera, L.S., N. Iyer, E. Leake, A. Raben, Modest E.K., D.L.W., and
10 C. Piantadosi. 1990. "Novel membrane-interactive ether lipid analogs that inhibit infectious HIV-1 production and induce defective virus formation." *AIDS Res. Hum. Retro Viruses*. **6**:491-501; Piantadosi, C., J. Marasco C.J., S.L. Morris-Natschke, K.L. Meyer, F. Gumus, J.R. Surles, K.S. Ishaq, L.S. Kucera, N. Iyer, C.A. Wallen, S. Piantadosi, and E.J. Modest. 1991. "Synthesis and evaluation of novel ether lipid
15 nucleoside conjugates for anti-HIV activity." *J. Med. Chem.* **34**:1408.1414; Hosteller, K.Y., D.D. Richman, D.A. Carson, L.M. Stuhmiller, G.M. T. van Wijk, and H. van den Bosch. 1992. "Greatly enhanced inhibition of human immunodeficiency virus type 1 replication in CEM and HT4-6C cells by 3'-deoxythymidine diphosphate dimyristoylglycerol, a lipid prodrug of 3,-deoxythymidine." *Antimicrob. Agents*
20 *Chemother*. **36**:2025.2029; Hostetler, K.Y., L.M. Stuhmiller, H.B. Lenting, H. van den Bosch, and D.D. Richman, 1990. "Synthesis and antiretroviral activity of phospholipid analogs of azidothymidine and other antiviral nucleosides." *J. Biol. Chem.* 265:61127.

Nonlimiting examples of U.S. patents that disclose suitable lipophilic substituents that can be covalently incorporated into the nucleoside or other hydroxyl or amine
25 containing compound, preferably at the 5'-OH position of the nucleoside or lipophilic preparations, include U.S. Patent Nos. 5,149,794 (Sep. 22, 1992, Yatvin et al.); 5,194,654 (Mar. 16, 1993, Hostetler et al., 5,223,263 (June 29, 1993, Hostetler et al.); 5,256,641 (Oct. 26, 1993, Yatvin et al.); 5,411,947 (May 2, 1995, Hostetler et al.); 5,463,092 (Oct. 31, 1995, Hostetler et al.); 5,543,389 (Aug. 6, 1996, Yatvin et al.);
30 5,543,390 (Aug. 6, 1996, Yatvin et al.); 5,543,391 (Aug. 6, 1996, Yatvin et al.); and 5,554,728 (Sep. 10, 1996; Basava et al.), all of which are incorporated herein by reference. Foreign patent applications that disclose lipophilic substituents that can be

attached to the nucleosides of the present invention, or lipophilic preparations, include WO 89/02733, W0 90/00555, W0 91/16920, W0 91/18914, W0 93/00910, W0 94/26273, W0 96/15132, EP 0 350 287, EP 93917054.4, and W0 91/19721.

Nonlimiting examples of nucleotide prodrugs are described in the following references: Ho, D.H.W. (1973) "Distribution of Kinase and deaminase of 1β-D-arabinofuranosylcytosine in tissues of man and muse." *Cancer Res.* 33, 2816-2820; Holy, A. (1993) Isopolar phosphorous-modified nucleotide analogues," In: De Clercq (Ed.), Advances in Antiviral Drug Design, Vol. I, JAI Press, pp. 179-231; Hong, C.I., Nechaev, A., and West, C.R. (1979a) "Synthesis and antitumor activity of 1-β-D-arabino-furanosylcytosine conjugates of cortisol and cortisone." *Bicohem. Biophys. Rs. Commun.* **88**, 1223-1229; Hong, C.I., Nechaev, A., Kirisits, A.J. Buchheit, D.J. and West, C.R. (1980) "Nucleoside conjugates as potential antitumor agents. 3. Synthesis and antitumor activity of 1-(β-D-arabinofuranosyl) cytosine conjugates of corticosteriods and selected lipophilic alcohols." *J. Med. Chem.* **28**, 171-177; Hosteller, K.Y., Stuhmiller, L.M., Lenting, H.B.M. van den Bosch, H. and Richman *J. Biol. Chem.* **265**, 6112-6117; Hosteller, K.Y., Carson, D.A. and Richman, D.D. (1991); "Phosphatidylazidothymidine: mechanism of antiretroviral action in CEM cells." *J. Biol Chem.* **266**, 11714-11717; Hosteller, K.Y., Korba, B. Sridhar, C., Gardener, M. (1994a) "Antiviral activity of phosphatidyl-dideoxycytidine in hepatitis B-infected cells and enhanced hepatic uptake in mice." *Antiviral Res.* **24**, 59-67; Hosteller, K.Y., Richman, D.D., Sridhar. C.N. Felgner, P.L. Felgner, J., Ricci, J., Gardener, M.F. Selleseth, D.W. and Ellis, M.N. (1994b) "Phosphatidylazidothymidine and phosphatidyl-ddC: Assessment of uptake in mouse lymphoid tissues and antiviral activities in human immunodeficiency virus-infected cells and in rauscher leukemia virus-infected mice." *Antimicrobial Agents Chemother.* **38**, 2792-2797; Hunston, R.N., Jones, A.A. McGuigan, C., Walker, R.T., Balzarini, J., and DeClercq, E. (1984) "Synthesis and biological properties of some cyclic phosphotriesters derived from 2'-deoxy-5-fluorouridine." *J. Med. Chem.* **27**, 440-444; Ji, Y.H., Moog, C., Schmitt, G., Bischoff, P. and Luu, B. (1990); "Monophosphoric acid esters of 7-β-hydroxycholesterol and of pyrimidine nucleoside as potential antitumor agents: synthesis and preliminary evaluation of antitumor activity." *J. Med. Chem.* **33** 2264-2270; Jones, A.S., McGuigan, C., Walker, R.T., Balzarini, J. and DeClercq, E. (1984) "Synthesis, properties, and

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II. Pharmaceutical Compositions

Humans or other hosts suffering from effects caused by HBV or which have been exposed to HBV can be treated by administering to the patient an effective amount of B-L-FTC, in combination and/or alternation with an immunomodulator, or a pharmaceutically acceptable salt or prodrug, including an ester thereof in the presence of a pharmaceutically acceptable carrier or diluent, optionally in combination and/or alternation with at least one additional anti-HBV agent or a pharmaceutically acceptable salt or ester thereof in the presence of a pharmaceutically acceptable carrier or diluent. The active materials can be administered by any appropriate route, for example, orally, parenterally, enterally, intravenously, intradermally, subcutaneously, topically, nasally, rectally, in liquid, or solid form. In general, since B-L-FTC is a small molecule and the

immunomodulator is a biologic, the two active compounds will be administered separately as is routine for such substances. For example, in one embodiment, B-L-FTC is delivered orally and the immunomodulator is delivered through a vein or artery. Methods for delivering biologics, for example, interfereons, are known in the art

5 The active compounds are included in the pharm aceutically acceptable carrier or diluent in an amount sufficient to deliver to a patient a therapeutically effective amount of compound to inhibit viral replication *in vivo*, especially HBV replication, without causing serious toxic effects in the treated patient. By "inhibitory amount" is meant an amount of active ingredient sufficient to exert an inhibitory effect as measured by, for
10 example, an assay such as the ones described herein.

B-L-FTC is conveniently administered in unit any suitable dosage form, including but not limited to one containing 7 to 3000 mg, preferably 70 to 1400 mg of active ingredient per unit dosage form. An oral dosage of 50 to 1000 mg is usually convenient.

15 Ideally, at least one of the active ingredients, though preferably the combination of active ingredients, should be administered to achieve peak plasma concentrations of the active compound of from about 0.2 to 70 or 100 mM, preferably about 1.0 to 10 mM. This may be achieved, for example, by the intravenous injection of a 0.1 to 10 % solution of the active ingredient, optionally in saline, or administered as a bolus of the active
20 ingredient.

The concentration of active compound in the drug composition will depend on absorption, distribution, metabolism and excretion rates of the drug as well as other factors known to those of skill in the art. It is to be noted that dosage values will also vary with the severity of the condition to be alleviated. It is to be further understood that
25 for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition. The active ingredient may be administered
30 at once, or may be divided into a number of smaller doses to be administered at varying intervals of time.

A preferred mode of administration of B-L FTC is oral. Oral compositions will generally include an inert diluent or an edible carrier. They may be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, 5 troches, or capsules. Pharmaceutically compatible bind agents, and/or adjuvant materials can be included as part of the composition.

The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a 10 disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring. When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier such as a fatty oil. In addition, 15 dosage unit forms can contain various other materials which modify the physical form of the dosage unit, for example, coatings of sugar, shellac, or other enteric agents.

The compounds can be administered as a component of an elixir, suspension, syrup, wafer, chewing gum or the like. A syrup may contain, in addition to the active compounds, sucrose as a sweetening agent and certain preservatives, dyes and colorings 20 and flavors.

The compounds or their pharmaceutically acceptable derivative or salts thereof can also be mixed with other active materials that do not impair the desired action, or with materials that supplement the desired action, such as antibiotics, anti-fungals, anti-inflammatories, protease inhibitors, or other nucleoside or non-nucleoside antiviral 25 agents, as discussed in more detail above. Solutions or suspensions used for parental, intradermal, subcutaneous, or topical application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium 30 bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium

chloride or dextrose. The parental preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

If administered intravenously, preferred carriers are physiological saline or phosphate buffered saline (PBS).

5 If administered by nasal aerosol or inhalation, these compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other solubilizing or dispersing agents known in the art.

10 If rectally administered in the form of suppositories, these compositions may be prepared by mixing the drug with a suitable non-initiating excipient, such as cocoa butter, synthetic glyceride esters of polyethylene glycols, which are solid at ordinary temperatures, but liquefy and/or dissolve in the rectal cavity to release the drug.

In a preferred embodiment, the active compounds are prepared with carriers that 15 will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and micro-encapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. 20 The materials can also be obtained commercially from Alza Corporation.

Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) are also preferred as pharmaceutically acceptable carriers. These may be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811 (which is incorporated 25 herein by reference in its entirety). For example, liposome formulations may be prepared by dissolving appropriate lipid(s) (such as stearoyl phosphatidyl ethanolamine, stearoyl phosphatidyl choline, arachadoyl phosphatidyl choline, and cholesterol) in an inorganic solvent that is then evaporated, leaving behind a thin film of dried lipid on the surface of the container. An aqueous solution of the active compound or its monophosphate, 30 diphosphate, and/or triphosphate derivatives is then introduced into the container. The

container is then swirled by hand to free lipid material from the sides of the container and to disperse lipid aggregates, thereby forming the liposomal suspension.

III. Anti-HBV Agents Used in Combination and/or Alternation

The compositions of the present invention include B-L-FTC, in combination and/or alternation with an immunomodulator, optionally in combination and/or alternation with at least one additional anti-HBV agent. The additional anti-HBV agents may have a synergistic effect with B-L-FTC and/or have a different mechanism of action than B-L-FTC In one embodiment of the present invention, the anti-HBV agents can be selected from the following group, including, but not limited to: 3TC, DAPD (Amdoxovir, Gilead Sciences), DXG, famciclovir, penciclovir, BMS-200475, adefovir dipivoxil (bis pom PMEA); lobucavir; tenofovir; or ganciclovir, Elvucitabine (ACH126, 433; beta-L-Fd4C, Achillion, Inc), clevudine (L-FMAU, 1-(2-fluoro-5methyl-β-L-arabinosyl uracil)), Entecavir (Britsol-Myers Squibb), telbivudine (β-L-thymidine (LdT, Idenix Pharmaceuticals)), β-L-2-deoxycytidine (L-dC), β-L-2-deoxyadenosine (L-dA), Efavirenz, Didanosine, Abacavir, Fialuridine, AM 365 (Amrad), XTL001 (XTL Biopharm), EHT899 (Enzo Biochem), MCC 478 (Eli Lilly), valLdC (valtorcitabine, Idenix Pharmaceuticals), ICN2001 (ICN Pharmaceuticals), Fluro L and D nucleoside (Pharmasset, Inc.), Racivir (Pharmasset, Inc.), robustaflavone (Advanced Life Sciences).

In addition, the composition can be enhanced by administering one or more other effective anti-HBV agent, for example, foscarnet (trisodium phosphonoformate), isoprinosine, levamizole, N-acetylcystine (NAC), PC1323 or polyadencyclic polyuridylic acid.

Compositions according to the present invention can be administered in combination and/or alternation with one or more other antiviral, anti-HIV, anti-HBV, anti-HCV or anti-herpetic agent or interferon, anti-cancer, antiproliferative or antibacterial agents, including other compounds of the present invention. Certain compounds according to the present invention may be effective for enhancing the biological activity of certain agents according to the present invention by reducing the

metabolism, catabolism or inactivation of other compounds and as such, are co-administered for this intended effect.

Immunomodulatory Agents

Immunomodulatory agents, such as immunostimulatory agents are used in combination with B-L-FTC.

The term "immunomodulatory" or "modulating an immune response" as used herein includes immunostimulatory as well as immunosuppressive effects. Immunomodulation is primarily a qualitative alteration in an overall immune response, although quantitative changes may also occur in conjunction with immunomodulation. Immunomodulation may involve an immune response that is shifted towards a "Th1-type" immune response, as opposed to a "Th2-type" immune response. Th1-type responses are typically considered cellular immune system (e.g., cytotoxic lymphocytes) responses, while Th2-type responses are generally "humoral", or antibody-based. Th1-type immune responses are normally characterized by "delayed-type hypersensitivity" reactions to an antigen, and can be detected at the biochemical level by increased levels of Th1-associated cytokines such as IFN-gamma, IL-2, IL-12, and TNF-beta, as well as IFN-alpha and IL-6, although IL-6 may also be associated with Th2-type responses as well. Th1-type immune responses are generally associated with the production of cytotoxic lymphocytes (CTLs) and low levels or transient production of antibody. Th2-type immune responses are generally associated with higher levels of antibody production, including IgE production, an absence of or minimal CTL production, as well as expression of Th2-associated cytokines such as IL-4. Accordingly, immunomodulation in one embodiment can be recognized by, for example, an increase in IFN-gamma and/or a decrease in IgE production in an individual treated in accordance with the methods of the invention as compared to the absence of treatment.

For example, immunostimulatory sequences may be used, such as those described herein above, as well as in: Krieg et al. (1989) *J Immunol.* 143:24482451; Tokunaga *et al.* (1992) *Microbiol. Immunol.* 36:55-66; Kataoka *et al.* (1992) *Jpn'. J Cancer Res.* 83:244-247; Yamamoto et al. (1 992) *J Immunol.* 148:4072-4076; Mojcik et al. (1993) *Clin. Immuno. and Immunopathol.* 67:13 0-13 6; Branda, et al. (1993) *Biochem. Pharmacol.* 45:2037-2043; Pisetsky *et al.* (1994) *Life Sci.* 54(2):101-107; Yamamoto *et al.* (1994a) *Antisense Research and Development.* 4:119-122; Yamamoto

et al. (1994b) *Jpn. J Cancer Res.* 85:775-779; Raz *et al.* (1994) *Proc. Nail. Acad. Sci. USA* 91:9519-9523; Kimura *et al.* (1994) *J Biochem.* (Tokyo) 116:991-994; Krieg *et al.* (1995) *Nature* 374:546549; Pisetsky et al. (1995) *Ann. N. Y Acad. Sci.* 772:152-163; Pisetsky (1996a) *J Immunol.* 156:421-423; Pisetsky (1996b) *Immunity* 5:303-310; Zhao
5 et al. (1996) *Biochem. Pharmacol.* 51:173-182; Yi *et al.* (1996) *J. Immunol.* 156:558-564; Krieg (1996) *Trends Microbiol.* 4(2):73-76; Krieg *et al.*(1996) *Antisense Nucleic AcidDrugDev.* 6:133-139; Klimnan et al. (1996) *Proc. Nad. Acad. Sci. USA.* 93:2879-2883; Raz *et al.* (1996); Sato *et al.* (1996) *Science* 273:3 52-3 54; Stacey *et al.* (1996) *J Immunol.* 157:2116-2122; Ballas *et al.* (1996) *J Immunol.* 157:1840-1845; Branda *et al.*
10 (1996) *J Lab. Clin. Med* 128:329338; Sonehara *et al.* (1996) *J Interferon and Cytokine Res.* 16:799-803; Klimnan *et al.* (1997) *J Immunol.* 158:3635-3639; Sparwasser *et al.* (1997) *Eur. J Immunol.* 27:16711679; Roman *et al.* (1997); Carson *et al.* (1997) *J Exp. Med* 186:1621-1622; Chace *et al.* (1997) *Clin. Immunol. and Immunopathol,* 84:185-193; Chu *et al.* (1997) *J Exp. Med* 186:1623-1631; Lipford *et al.* (1997a) *Eur. J*
15 *Immunol.* 27:2340-2344; Lipford *et al.* (1997b) *Eur. J Immunol.* 27:3420-3426; Weiner *et al.* (1997) *Proc. Natl.Acad Sci.* USA 94:10833-10837; Macfarlane *et al.* (1997) *Immunology* 91:586-593; Schwartz *et al.* (1997) *J Clin. Invest.* 100:68-73; Stein *et al.* (1997), *Antisense Technology*, Ch. 11 pp. 241-264, C. Lichtenstein and W. Nellen, Eds., IRL Press; Wooldridge et al. (1997) *Blood* 89:29942998; Leclerc et al. (1997) *Cell.*
20 *Immunol.* 179:97-106; Kline *et al.* (1997) *J Invest. Med* 45(3):282A; Yi et al. (1998a) *J Immunol.* 160:1240-1245; Yi *et al.* (1998b) *J Immunol.* 160:4755-4761; Yi *et al.* (1998c) *J Immunol.* 160:5898-5906; Yi *et al.* (1998d) *J Immunol.* 161:4493 -4497; Krieg (1998) *Applied Antisense Oligonucleotide Technology* Ch. 24, pp. 431-448, C.A. Stein and A.M. Krieg, Eds., Wiley-Liss, Inc.; Krieg *et al.* (1998a) *Trends Microbiol.* 6:23-27; Krieg et al.
25 (1998b) *J Immunol.* 161:2428-2434; Krieg *et al.* (1998c) *Proc. Nad. Acad. Sci. USA* 95:12631-12636; Spiegelberg *et al.* (1998) Allergy 53(45S):9397; Homer *et al.* (1998) *Cell Immunol.* 190:77-82; Jakob *et al.* (1998) *J Immunol.* 161:3042-3049; Redford *et al.* (1998) *J Immunol.* 161:3930-3935; Weeratna *et al.* (1998) *Antisense & Nucleic AcidDrug Development* 8:351-356; McCluskie *et al.* (1998) *J Immunol.* 161(9):4463-
30 4466; Gramzinski *et al.* (1998) *Mol. Med* 4:109-118; Liu *et al.* (1998) *Blood* 92:3730-3736; Moldoveanu *et al.* (1998) *Vaccine* 16: 1216-1224; Brazolot Milan *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95:15553-15558; Briode *et al.* (1998) *J Immunol.* 161:7054-7062; Briode *et al.* (1999) *Int. Arch. Allergy Immunol.* 118:453 -456; Kovarik *et al.*

(1999) *J Immunol.* 162:1611-1617; Spiegelberg et al. (1999) *Pediatr. Pulmonol. Suppl.* 18:118-121; Martin-Orozco *et al.* (1999) *Int. Immunol.* 11:1111- 1118; EP 468,520; WO 96/02555; WO 97/28259; WO 98/16247; WO 98/18810; WO 98/37919; WO 01/68116 PCT/US01/07839; WO99/33488; WO 99/51259 and WO 99/62923. See also Zimmermann et al. (1998) *J. Immunol.* 160:3627-3630; Krieg (1999) *Trends Microbiol* 7:64-65; U.S. Patent Nos. 5,663,153; 5,723,335; 5,849,719; and 6,174,872. See also WO 99/56755, WO 00/06588, WO 00/16804; WO 00/21556; WO 00/67023 and WO 01/12223.

Interferons that can be administered include: interferon alpha, pegylated interferon alpha, interferon alpha-2a, interferon alpha-2b, pegylated interferon alpha-2a, pegylated interferon alpha-2b ROFERON®-A (interferon alpha-2a, Roche), PEGASYS® (pegylated interferon alpha-2a, Roche), INTRON®A (Interferon alpha-2b, Schering Corporation), PEG-INTRON® (pegylated Interferon alpha-2b, Schering Corporation), interferon alpha, interferon beta, interferon gamma, interferon tau, interferon omega, INFERGEN (interferon alphacon-1) by InterMune, OMNIFERON (natural interferon) by Viragen, ALBUFERON by Human Genome Sciences, REBIF (interferon beta-1a) by Ares-Serono, Omega Interferon by BioMedicine, Oral Interferon Alpha by Amarillo Biosciences, interferon gamma- 1b by InterMune, SuperFeron (natural human multi-subtype IFN-alpha, Genetrol, Inc.), and HuFeron (human IFN-beta, Genetrol, Inc.).

Other immunomodulatory agents, such as cytokines, can also be used. For example, immunomodulatory agents selected from, but not limited to the following group including: Ribavirin (Ribapharm, Inc.), hepatitis B immune globulin (Nabi-HB intravenous, Nabi Pharmaceuticals), ZADAXIN™ (thymosin alpha 1, SCV-07 (SciClone Pharmaceuticals), Theradigm (Epimmune), anti-hepatitis B hyperimmune product (Cangene Corp), RC-529 (Corixa/Rhein Biochem), HYB2055 (Hybridon), ViroKine (human antiviral proteins, Genetrol, Inc.), Levovirin (Ribapharm, Inc.), interleukin-2 (IL-2), tumor necrosis factor-alpha, interleukin 1-beta, interleukin-12 (IL-12), Granulocyte-macrophage colony-stimulating factor (GM-CSF), polyadenylic-polyuridylic acid, thymosin alpha, Ampligen® (Hemispherx BioPharma), Polyadenur™ (Poly A:Poly U RNA, Hemispherx BioPharms), Oragen™ (Hemispherx BioPharms), Hepatitis B Virus (HBV)-specific Cytotoxic T Lymphocytes (CTL) (CellExSys, Inc.), therapeutic hepatitis b vaccine (Epimmune), PJ Hep B DNA prophylactic vaccine

(Powderject Pharmaceuticals), interleukin 4, interleukin 6, interleukin 7, and granulocyte colony stimulating factor.

Immunomodulatory agents include, but are not limited to, a molecule such as a chemokine or cytokine that affects either directly or indirectly an immune response. Non-limiting examples of immunomodulators include TH1 cytokines, and in particular, interferon, interferon-α, purified interferon-α, interferon-α2a, interferon-α2b, interferon-β, interferon-γ, consensus interferon, pegylated interferon, pegylated interferon-α, granulocyte macrophage colony-stimulating factor, interleukin, interleukin-2, and interleukin-12. In one embodiment, the immunomodulator is interferon, e.g., interferon-γ.

Immunostimulatory Sequences

The term "ISS" or "immunostimulatory sequence" as used herein refers to polynucleotide sequences, alone and/or complexed with MC, that effect a measurable immune response as measured in vitro, in vivo and/or ex vivo. Examples of measurable immune responses include, but are not limited to, antigen-specific antibody production, secretion of cytokines, activation or expansion of lymphocyte populations such as NK cells, CD4+T lymphocytes, CD8+T lymphocytes, B lymphocytes, and the like. Preferably, the ISS sequences preferentially activate a Th1-type response. A polynucleotide for use in the invention contains at least one ISS. As used herein, "ISS" is also a shorthand term for an ISS-containing polynucleotide.

A polynucleotide comprising an ISS (or a composition comprising such a polynucleotide) may be used in the methods and compositions disclosed herein. The immunomodulatory polynucleotide can contain at least one ISS or multiple ISSs. The ISSs can be adjacent within the polynucleotide, or they can be separated by additional nucleotide bases within the polynucleotide. Alternately, multiple ISSs may be delivered as individual polynucleotides.

ISS have been described in the art and may be readily identified using standard assays which indicate various aspects of the immune response, such as cytokine secretion, antibody production, NK cell activation and T cell proliferation. See, *e.g.*, WO 97/28259; WO 98/16247; WO 99/11275; Krieg *et al.* (1995); Yamamoto *et al.* (1992); Ballas *et al.* (1996); Klinman *et al.* (1997); Sato *et al.* (1996); Pisetsky (1996a);

Shimada *et al.* (1986) ; *Jpn. Cancer Res.* 77:808 816; Cowdery *et al.* (1996) *J Immunol.* 156:4570-4575; Roman *et al.* (1997); and Lipford *et al.* (1997a).

The ISS can be of any length greater than 6 bases or base pairs and generally comprises the sequence 5'-cytosine, guanine-3', preferably greater than 15 bases or base pairs, more preferably greater than 20 bases or base pairs in length. As is well-known in the art, the cytosine of the 5'-cytosine, guanine-3' sequence is unmethylated. An ISS may also comprise the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'. An ISS may also comprise the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, C3'. As indicated in polynucleotide sequences below, an ISS may comprise (ie., contain one or more of) the sequence 5'-T, C, G-3'. In some embodiments, an ISS may comprise the sequence 5'-C, G, pyrimidine, pyrimidine, C, G-3' (such as 5'-CGTTCG-3'). In some embodiments, an ISS may comprise the sequence 5'-C, G, pyrimidine, pyrimidine, C, G, purine, purine-3'. In some embodiments, an ISS comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine-3' (such as 5'-AACGTT-3').

In some embodiments, an ISS may comprise the sequence 5'-purine, T, C, G, pyrimidine, pyrimidine-3'.

In some embodiments, an ISS-containing polynucleotide is less than about any of the following lengths (in bases or base pairs): 10,000; 5,000; 2500; 2000; 1500; 1250; 1000; 750; 500; 300; 250; 200; 175; 150; 125; 100; 75; 50; 25; 10. In some embodiments, an ISS-containing polynucleotide is greater than about any of the following lengths (in bases or base pairs): 8; 10; 15; 20; 25; 30; 40; 50; 60; 75; 100; 125; 150; 175; 200; 250; 300; 350; 400; 500; 750; 1000; 2000; 5000; 7500; 10000; 20000; 50000. Alternately, the ISS can be any of a range of sizes having an upper limit of 10,000; 5,000; 2500; 2000; 1500; 1250; 1000; 750; 500; 300; 250; 200; 175; 150; 125; 100; 75; 50; 25; or 10 and an independently selected lower limit of 8; 10; 15; 20; 25; 30; 40; 50; 60; 75; 100; 125; 150; 175; 200; 250; 300; 350; 400; 500; 750; 1000; 2000; 5000; 7500, wherein the lower limit is less than the upper limit.

In some embodiments, the ISS includes any of the following sequences:

GACGCTCC; GACGTCCC; GACGTTCC; GACGCCCC; AGCGTTCC; AGCGCTCC; AGCGTCCC; AGCGCCCC; AACGTCCC; AACGCCCC; AACGTTCC; AACGCTCC; GGCGTTCC; GGCGCTCC; GGCGTCCC; GGCGCCCC; GACGCTCG;

GACGTCCG; GACGCCCG; GACGTTCG; AGCGCTCG; AGCGTTCG; AGCGTCCG; GCGCCCG; AACGTCCG; AACGCCCG; AACGTTCG; AACGCTCG; GGCGTTCG; GGCGCTCG; GGCGTCCG; GGCGCCCG. In some embodiments, the immunomodulatory polynucleotide includes the sequence 5'-TGACTGTGAACGTTCGAGATGA-Y (SEQ ID NO: 1).

In some embodiments, the ISS includes any of the following sequences:

GACGCU; GACGUC; GACGUU; GACGUT; GACGTU; AGCGUU; AGCGCU; AGCGUC; AGCGUT; AGCGTU; AACGUC; AACGUU; AACGCU; AACGUT; AACGTU; GGCGUU; GGCGCU; GGCGUC; GGCGUT; GGCGTU.

In some embodiments, the ISS includes any of the following sequences.

GABGCTCC; GABGTCCC; GABGTTCC; GABGCCCC; AGBGTTCC; AGBGCTCC; AGBGTCCC; AGBGCCCC; AABGTCCC; AABGCCCC; AABGTTCC; AABGCTCC; GGBGTTCC; GGBGCTCC; GGBGTCCC; GGBGCCCC; GABGCTCG; GABGTCCG; GABGCCCG; GABGTTCG; AGBGCTCG; AGBGTTCG; AGBGTCCG; GBGCCCG; AABGTCCG; AABGCCCG; AABGTTCG; AABGCTCG; GGBGTTCG; GBGCTCG; GGBGTCCG; GGBGCCCG; GABGCTBG; GABGTCBG; GABGCCBG; GABGTTBG; AGBGCTBG; AGBGTTBG; AGBGTCBG; AGBGCCBG; AABGTCBG; AABGCCBG; AABGTTBG; AABGCTBG; GGBGTTBG; GGBGCTBG; GGBGTCBG; GGBGCCBG, where B is 5-bromocytosine.

In some embodiments, the ISS includes any of the following sequences: GABGCUCC; GABGUCCC; GABGUTCC; GABGTUCC; GABGUUCC; GBGUUCC; AGBGTUCC; AGBGUTCC; AGBGCUCC; AGBGUCCC; AABGUCCC; ABGUUCC; AABGUTCC; AABGTUCC; AABGCUCC; GGBGUUCC; GGBGUTCC; GBGTUCC; GGBGCUCC; GGBGUCCC; GABGCUCG; GABGUCCG; GABGUUCG; ABGUTCG; GABGTUCG; AGBGCUCG; AGBGUUCG; AGBGUTCG; AGBGTUCG; AGBGUCCG; AABGUCCG; AABGUUCG; AABGUTCG; AABGTUCG; AABGCUCG; GGBGUUCG; GGBGUTCG; GGBGTUCG; GGBGCUCG; GGBGUCCG; GABGCUBG; GABGUCBG; GABGUUBG; GABGUTBG; GABGTUBG; AGBGCUBG; AGBGUUBG; AGBGUCBG; AGBGUTBG; AGBGTUBG; AABGUCBG; AABGUUBG; AABGUTBG; AABGTUBG;

AABGCUBG; GGBGUUBG; GGBGUTBG; GGBGTUBG; GGBGCUBG; GGBGUCBG, where B is 5-bromocytosine.

In other embodiments, the ISS comprises any of the sequences: 5'-TGACCGTGAACGTTCGAGATGA-3'; 5'-TCATCTCGAACGTTCCACAGTCA-3'; 5'-TGACTGTGAACGTTCCAGATGA-3'; 5'-TCCATAACGTTCGCCTAACGTTCGTC-3'; 5' -TGACTGTGAABGTTCCAGATGA-3', where B is 5-bromocytosine; 5'-TGACTGTGAABGTTCGAGATGA-3', where B is 5-bromocytosine, and 5'-TGACTGTGAABGTTBGAGATGA-3', where B is 5-bromocytosine.

An ISS and/or ISS-containing polynucleotide may contain modifications. Modifications of ISS include any known in the art, but are not limited to, modifications of the 3'-OH or 5'-OH group, modifications of the nucleotide base, modifications of the sugar component, and modifications of the phosphate group. Examples of such modifications are described below.

An ISS may be single stranded or double stranded DNA, as well as single or double-stranded RNA or other modified polynucleotides. An ISS may or may not include one or more palindromic regions, which may be present in the motifs described above or may extend beyond the motif. An ISS may comprise additional Ranking sequences, some of which are described herein. An ISS may contain naturally-occurring or modified, non-naturally occurring bases, and may contain modified sugar, phosphate, and/or termini. For example, phosphate modifications include, but are not limited to, methyl phosphonate, phosphorothioate, phosphoramidate (bridging or non-bridging), phosphotriester and phosphorodithioate and may be used in any combination. Other non-phosphate linkages may also be used. The oligonucleotides may comprise phosphorothioate backbones. Sugar modifications known in the field, such as 2'-alkoxy-RNA analogs, 2'-amino-RNA analogs and 2'-alkoxy- or amino-RNA/DNA chimeras and others described herein, may also be made and combined with any phosphate modification. Examples of base modifications include, but are not limited to, addition of an electron-withdrawing moiety to C-5 and/or C-6 of a cytosine of the ISS (*e.g.*, 5-bromocytosine, 5-chlorocytosine, 5-fluorocytosine, 5-iodocytosine).

The ISS can be synthesized using techniques and nucleic acid synthesis equipment which are well known in the art including, but not limited to, enzymatic methods, chemical methods, and the degradation of larger oligonucleotide sequences. See, for example, Ausubel *et al.* (1987); and Sambrook *et al.* (1989). When assembled 5 enzymatically, the individual units can be ligated, for example, with a ligase such as T4 DNA or RNA ligase. U.S. Patent No. 5,124,246. Oligonucleotide degradation can be accomplished through the exposure of an oligonucleotide to a nuclease, as exemplified in U.S. Patent No. 4,650,675.

The ISS can also be isolated using conventional polynucleotide isolation 10 procedures. Such procedures include, but are not limited to, hybridization of probes to genomic or cDNA libraries to detect shared nucleotide sequences, antibody screening of expression libraries to detect shared structural features and synthesis of particular native sequences by the polymerase chain reaction.

Circular ISS can be isolated, synthesized through recombinant methods, or 15 chemically synthesized. Where the circular ISS is obtained through isolation or through recombinant methods, the ISS will preferably be a plasmid. The chemical synthesis of smaller circular oligonucleotides can be performed using any method described in the literature. See, for instance, Gao *et al.* (1995) *NucleicAcids Res.* 23:2025-2029; and Wang et al. (1994) *Nucleic Acids Res.* 22:2326-2333.

20 The techniques for making oligonucleotides and modified oligonucleotides are known in the art. Naturally occurring DNA or RNA, containing phosphodiester linkages, is generally synthesized by sequentially coupling the appropriate nucleoside phosphoramidite to the 5'-hydroxy group of the growing oligonucleotide attached to a solid support at the 3'-end, followed by oxidation of the intermediate phosphite triester to 25 a phosphate triester. Once the desired oligonucleotide sequence has been synthesized, the oligonucleotide is removed from the support, the phosphate triester groups are deprotected to phosphate diesters and the nucleoside bases are deprotected using aqueous ammonia or other bases. See, for example, Beaucage (1993) "Oligodeoxyribonucleotide Synthesis" in *Protocols for Oligonucleotides and Analogs, Synthesis and Properties* 30 (Agrawal, ed.) Humana Press, Totowa, NJ; Warner et al. (1 984) *DNA* 3:401 and U.S. Patent No. 4,458,066.

The ISS can also contain phosphate-modified oligonucleotides. Synthesis of polynucleotides containing modified phosphate linkages or non-phosphate linkages is also know in the art. For a review, see Matteucci (I 997) "Oligonucleotide Analogs: an Overview" in *Oligonucleotides as TherapeuticAgents*, (D.J. Chadwick and G. Cardew,
5 ed.) John Wiley and Sons, New York, NY. The phosphorous derivative (or modified phosphate group) which can be attached to the sugar or sugar analog moiety in the oligonucleotides of the present invention can be a monophosphate, diphosphate, triphosphate, alkylphosphonate, phosphorothioate, phosphorodithioate or the like. The preparation of the above-noted phosphate analogs, and their incorporation into
10 nucleotides, modified nucleotides and oligonucleotides, *per se*, is also known and need not be described here in detail. Peyrottes *et al.* (1996) *NucleicAcids Res.* 24:1841-1848; Chaturvedi *et al.* (1996) *Nucleic Acids Res.* 24:2318-2323; and Schultz *et al.* (1996) *Nucleic Acids Res.* 24:2966-2973. For example, synthesis of phosphorothioate oligonucleotides is similar to that described above for naturally occurring
15 oligonucleotides except that the oxidation step is replaced by a sulfurization step (Zon (1993) "Oligonucleoside Phosphorothioates" in *Protocols for Oligonucleotides and Analogs, Synthesis and Properties* (Agrawal, ed.) Humana Press, pp. 165-190). Similarly the synthesis of other phosphate analogs, such as phosphotriester (Miller *et al.* (1971) *JACS* 93:6657-6665), non-bridging phosphoramidates (Jager et al. (1988) *Biochem.*
20 27:7247-7246), N3' to P5' phosphorarnidates (Nelson *et al.* (1997) *JOC* 62:7278-7287) and phosphorodithioates (U.S. Patent No. 5,453,496) has also been described. Other non-phosphorous based modified oligonucleotides can also be used (Stirchak *et al.* (1989) *Nucleic Acids Res.* 17:6129-6141). Oligonucleotides with phosphorothioate backbones can be more immunogenic than those with phosphodiester, backbones and appear to be
25 more resistant to degradation after injection into the host. Braun *et al.* (1988) *J Immunol.* 141:2084-2089; and Latimer *et al.* (1995) *Mol. Immunol.* 32:1057-1064.

ISS-containing polynucleotides used in the invention can comprise ribonucleotides (containing ribose as the only or principal sugar component), deoxyribonucleotides (containing deoxyribose as the principal sugar component), or, as
30 is known in the art, modified sugars or sugar analogs can be incorporated in the ISS. Thus, in addition to ribose and deoxyribose, the sugar moiety can be pentose, deoxypentose, hexose, deoxyhexose, glucose, arabinose, xylose, lyxose, and a sugar

"analog" cyclopentyl group. The sugar can be in pyranosyl or in a furanosyl form. In the ISS, the sugar moiety is preferably the furanoside of ribose, deoxyribose, arabinose or 2'-O-alkylribose, and the sugar can be attached to the respective heterocyclic bases either in α or β anomeric configuration. Sugar modifications include, but are not limited to, 2'-alkoxy-RNA analogs, 2'-amino-RNA analogs and 2'-alkoxy- or amino-RNA/DNA chimeras. The preparation of these sugars or sugar analogs and the respective "nucleosides" wherein such sugars or analogs are attached to a heterocyclic base (nucleic acid base) per se is known, and need not be described here, except to the extent such preparation can pertain to any specific example. Sugar modifications may also be made and combined with any phosphate modification in the preparation of an ISS.

The heterocyclic bases, or nucleic acid bases, which are incorporated in the ISS can be the naturally-occurring principal purine and pyrimidine bases, (namely uracil or thymine, cytosine, adenine and guanine, as mentioned above), as well as naturally occurring and synthetic modifications of said principal bases.

Those skilled in the art will recognize that a large number of "synthetic" non-natural nucleosides comprising various heterocyclic bases and various sugar moieties (and sugar analogs) are available in the art, and that as long as other criteria of the present invention are satisfied, the ISS can include one or several heterocyclic bases other than the principal five base components of naturally-occurring nucleic acids. Preferably, however, the heterocyclic base in the ISS includes, but is not limited to, uracil-5-yl, cytosine-5-yl, adenine-7-yl, adenine-8-yl, guanine-7-yl, guanine-8-yl, 4-aminopyrrolo [2.3-d] pyrimidin-5-yl, 2-amino-4-oxopyrolo [2,3-d] pyrimidin-5-yl, 2-amino-4-oxopyrrolo [2.3-d] pyrimidin-3-yl groups, where the purines are attached to the sugar moiety of the ISS via the 9-position, the pyrimidines via the 1-position, the pyrrolopyrimidines via the 7-position and the pyrazolopyrimidines via the 1-position.

The ISS and/or IMP may comprise at least one modified base as described, for example, in the commonly owned international application WO 99/62923. As used herein, the term "modified base" is synonymous with "base analog", for example, "modified cytosine" is synonymous with "cytosine analog." Similarly, "modified" nucleosides or nucleotides are herein defined as being synonymous with nucleoside or nucleotide "analogs." Examples of base modifications include, but are not limited to, addition of an electron-withdrawing moiety to C-5 and/or C-6 of a cytosine of the ISS.

Preferably, the electron-withdrawing moiety is a halogen. Modified cytosines can include, but are not limited to, azacytosine, 5-bromocytosine, 5-chlorocytosine, chlorinated cytosine, cyclocytosine, cytosine arabinoside, 5-fluorocytosine, fluoropyrimidine, 5,6-dihydrocytosine, 5-iodocytosine, 5-nitrocytosine, 5-hydroxycytosine, and any other pyrimidine analog or modified pyrimidine. Preferred modified uracils are modified at C-5 and/or C-6, preferably with a halogen, and include, but are not limited to, bromouracil such as 5-bromouracil, chlorouracil such as 5-chlorouracil, fluorouracil such as 5-fluorouracil, iodouracil such as 5-iodouracil and hydroxyuracil. Also see, Kandimalla *et al*., 2001, *Bioorg. Med. Chem.* 9:807-813. See, for example, International Patent Application No. WO 99/62923. Other examples of base modifications include the addition of one or more thiol groups to the base including, but not limited to, 6-thio-guanine, 4-thio-thymine and 4-thiouracil. Additionally, some IMPs may comprise modified bases such as 7-deazaguanosine in place of any guanosine residue, or a modified cytosine selected from N4-ethylcytosine or N4-methylcytosine in place of any cytosine residue, including the cytosine of the 5'-CG-3'.

The preparation of base-modified nucleosides, and the synthesis of modified oligonucleotides using said base-modified nucleosides as precursors, has been described, for example, in U.S. Pat. Nos. 4,910,300, 4,948,882, and 5,093,232. These base-modified nucleosides have been designed so that they can be incorporated by chemical synthesis into either terminal or internal positions of an oligonucleotide. Such base-modified nucleosides, present at either terminal or internal positions of an oligonucleotide, can serve as sites for attachment of a peptide or other antigen. Nucleosides modified in their sugar moiety have also been described (including, but not limited to, e.g., U.S. Pat. Nos. 4,849,513; 5,015,733; 5,118,800; 5,118,802) and can be used similarly.

The ISS used in the methods of the invention may be produced as ISS-microcarrier complexes. ISS-microcarrier complexes comprise an ISS-containing polynucleotide bound to a microcarrier (MC). ISS-MC complexes comprise an ISS bound to the surface of a microcarrier (*i.e.*, the ISS is not encapsulated in the MC), adsorbed within a microcarrier (e.g., adsorbed to PLGA beads), or encapsulated within a MC (*e.g.,* incorporated within liposomes).

ISS-containing oligonucleotides bound to microparticles (SEPHAROSE® beads) have previously been shown to have immunostimulatory activity *in vitro* (Liang *et al.*,

(1996), *J Clin. Invest.* 98:1119 1129). However, recent results show that ISS-containing oligonucleotides bound to gold, latex and magnetic particles are not active in stimulating proliferation of 7TD1 cells, which proliferate in response to ISS-containing oligonucleotides (Manzel *et al.*, (1999). *Antisense Nucl. Acid Drug Dev.* 9:459-464).

5 Microcarriers are not soluble in pure water, and are less than about 50-60 μm in size, preferably less than about 10 μm in size, more preferably from about 10 μm to about 10 μm, 25 nm to about 5 μm, 50 nm to about 4.5 μm or 1.0 μn to about 2.0 μm in size. Microcarriers may be any shape, such as spherical, ellipsoidal, rod-shaped, and the like, although spherical microcarriers are normally preferred. Preferred microcarriers 10 have sizes of or about 50 mn, 200 nm, 1 μm, 1.2 μm, 1.4 μm, 1.5 μm, 1.6 μm. 1.8 μm, 2.0 μm, 2.5 μm or 4.5 μm. The "size" of a microcarrier is generally the "design size" or intended size of the particles stated by the manufacturer. Size may be a directly measured dimension, such as average or maximum diameter, or may be determined by an indirect assay such as a filtration screening assay. Direct measurement of 15 microcarrier size is typically carried out by microscopy, generally light microscopy or scanning electron microscopy (SEM), in comparison with particles of known size or by reference to a micrometer. As minor variations in size arise during the manufacturing process, microcarriers are considered to be of a stated size if measurements show the microcarriers are ± about 5-10% of the stated measurement. Size characteristics may 20 also be determined by dynamic light scattering. Alternately, microcarrier size may be determined by filtration screening assays. A microcarrier is less than a stated size if at least 97% of the particles pass through a "screentype" filter (i. e., a filter in which retained particles are on the surface of the filter, such as polycarbonate or polyethersulfone filters, as opposed to a "depth filter" in which retained particles lodge 25 within the filter) of the stated size. A microcarrier is larger than a stated size if at least about 97% of the microcarrier particles are retained by a screen-type filter of the stated size. Thus, at least about 97% microcarriers of about I 0 tim to about I 0 nm in size pass through a 1 0 μn pore screen filter and are retained by a 1 0 nm screen filter.

As above discussion indicates, reference to a size or size range for a microcarrier 30 implicitly includes approximate variations and approximations of the stated size and/or size range. This is reflected by use of the term "about" when referring to a size and/or

size range, and reference to a size or size range without reference to "about" does not mean that I 0 the size and/or size range is exact.

Microcarriers may be solid phase (*e.g.*, polystyrene beads) or liquid phase (*e.g.*, liposomes, micelles, or oil droplets in an oil and water emulsion). Liquid phase microcarriers include-liposomes, micelles, oil droplets and other lipid or oil-based particles. One preferred liquid phase microcarrier is oil droplets within an oil-in-water emulsion. Preferably, oil-in-water emulsions used as microcarriers comprise biocompatible substituents such as squalene. Liquid phase microcarriers are normally considered nonbiodegradable, but may be biodegradable liquid phase microcarriers may be produced by incorporation of one or more biodegradable polymers in the liquid microcarrier formulation. In one preferred embodiment, the microcarrier is oil droplets in an oil-inwater emulsion prepared by emulsification of squalene, sorbitan trioleate, TWEEN 80® in an aqueous pH buffer.

Solid phase microcarriers for use in ISS-microcarrier complexes may be made from biodegradable materials or nonbiodegradable materials, and may include or exclude agarose or modified agarose microcarriers. Useful solid phase biodegradable microcarriers include, but are not limited to: biodegradable polyesters, such as poly(lactic acid), poly(glycolic acid), and copolymers (including block copolymers) thereof, as well as block copolymers of poly(lactic acid) and poly(ethylene glycol); polyorthoesters such as polymers based on 3,9-diethylidene-2,4,8,10-tetraoxaspiro[5.5]undecane (DETOSU); polyanhydrides such as poly(anhydride) polymers based on sebacic acid, *p*-(carboxyphenoxy)propane, or *p*-(carboxyphenoxy)hexane; polyanhydride imides, such as polyanhydride polymers based on sebacic acid-derived monomers incorporating amino acids (*i.e.*, linked to sebacic acid by imide bonds through the amino-terminal nitrogen) such as glycine or alanine; polyanhydride esters; polyphosphazenes, especially poly(phosphazenes) which contain hydrolysis-sensitive ester groups which can catalyze degradation of the polymer backbone through generation of carboxylic acid groups (Schacht et al. (1996) *BiotechnoL Bioeng.* 1996:102); and polyamides such as poly(lactic acid-co-lysine). A wide variety of nonbiodegradable materials suitable for manufacturing microcarriers are also known, including, but not limited to polystyrene, polyethylene, latex, gold, and ferromagnetic or paramagnetic materials. Solid phase microcarriers may be covalently

modified to incorporate one or more moieties for use in linking the ISS, for example by addition of amine groups for covalent linking using amine-reactive crosslinkers.

The ISS-microcarrier complexes may be covalently or noncovalently linked. Covalently linked ISS-MC complexes may be directly linked or be linked by a crosslinking moiety of one or more atoms (typically the residue of a crosslinking agent). The ISS may be modified to allow or augment binding to the MC (*e.g.*, by incorporation of a free sulfhydryl for covalent crosslinking or addition of a hydrophobic moieties such as lipids, steroids, sterols such as cholesterol, and terpenes, for hydrophobic bonding), although unmodified ISS may be used for formation of noncovalent ISS-MC complex formation by electrostatic interaction or by base pairing (e.g., by base pairing at least one portion of the ISS with a complementary oligonucleotide bound to the microcarrier). ISS-containing polynucleotides may be linked to solid phase microcarriers or other chemical moieties to facilitate ISS-MC complex formation using conventional technology known in the art such as use of available heterobifunctional crosslinkers (e.g., succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate or its sulfo-derivatives for covalently linking an amine-derivatized microcarrier and an ISS modified to contain a free sulfhydryl) or by addition of compounds such as cholesterol (*e.g.*, by the method of Godard *et al.* (1995) *Eur. J Biochem.* 232:404-410) to facilitate binding to hydrophobic microcarriers such as oil droplets in oil-in-water emulsions. Alternatively, modified nucleosides or nucleotides, such as are known in the art, can be incorporated at either terminus, or at internal positions in the ISS. These can contain blocked functional groups which, when deblocked, are reactive with a variety of functional groups which can be present on, or attached to, the microcarrier or a moiety which would facilitate binding to a microcarrier. Certain embodiments of noncovalently linked ISS-MC complexes utilize a binding pair (*e.g.*, an antibody and its cognate antigen or biotin and streptavidin or avidin), where one member of the binding pair is bound to the ISS and the microcarrier is derivatized with the other member of the binding pair (*e.g.*, a biotinylated ISS and a streptavidin-derivatized microcarrier may be combined to form a noncovalently linked ISS-MC complex).

Non-covalent ISS-MC complexes bound by electrostatic binding typically exploit the highly negative charge of the polynucleotide backbone. Accordingly, microcarriers for use in non-covalently bound ISS-MC complexes are generally positively charged at

physiological pH (*e.g.*, about pH 6.8-7.4). The microcarrier may intrinsically possess a positive charge, but microcarriers made from compounds not normally possessing a positive charge may be derivatized or otherwise modified to become positively charged. For example, the polymer used to make the microcarrier may be derivatized to add positively charged groups, such as primary amines. Alternately, positively charged compounds may be incorporated in the formulation of the microcarrier during manufacture (*e.g.*, positively charged surfactants may be used during the manufacture of poly(lactic acid)/poly(glycolic acid) copolymers to confer a positive charge on the resulting microcarrier particles.

Solid phase microspheres are prepared using techniques known in the art. For example, they can be prepared by emulsion-solvent extraction/evaporation technique. Generally, in this technique, biodegradable polymers such as polyanhydrates, poly(alkyl-α-cyanoacrylates) and poly(α-hydroxy esters), for example, poly(lactic acid), poly(glycolic -acid), poly(D,L-lactic-co-glycolic acid) and poly(caprolactone), are dissolved in a suitable organic solvent, such as methylene chloride, to constitute the dispersed phase (DP) of emulsion. DP is emulsified by high-speed homogenization into excess volume of aqueous continuous phase (CP) that contains a dissolved surfactant, for example, polyvinylalcohol (PVA) or polyvinylpirrolidone (PVP). Surfactant in CP is to ensure the formation of discrete and suitably-sized emulsion droplet. The organic solvent is then extracted into the CP and subsequently evaporated by raising the system temperature. The solid, microparticles are then separated by centrifugation or filtration, and dried, for example, by lyophilization or application of vacuum, before storing at 4 °C.

Generally, to prepare cationic microspheres, cationic lipids or polymers, for example, 1,2-dioleoyl-1,2,3-trimethylammonlopropane (DOTAP), cetyltrimethylammonium bromide (CTAB) or polylysine, are added either to DP or CP, as per their solubility in these phases.

Physico-chemical characteristics such as mean size, size distribution and surface charge of dried microspheres may be determined. Size characteristics are determine, for example, by dynamic light scattering technique and the surface charge was determined by measuring the zeta potential.

Generally, ISS-containing polynucleotides can be adsorbed onto the cationic microspheres by overnight aqueous incubation of ISS and the particles at 4 °C. Microspheres are characterized for size and surface charge before and after ISS association. Selected batches may then evaluated for activity as described herein.

5 An ISS-containing polynucleotide may be administered before, during and/or after exposure to a virus. An ISS polynucleotide may also be administered before, during and/or after infection by a virus. An ISS polynucleotide may also be administered before or after onset of symptoms of virus infection. Accordingly, administration of ISS-containing polynucleotide may be at various times with respect to exposure to, infection 10 by and/or onset of symptoms by infection by virus. Further, there may be one or more administrations. If the ISS-containing polynucleotide is administered on multiple occasions, the ISS may be administered on any schedule selected by the clinician, such as daily, every other day, every three days, every four days, every five days, every six days, weekly, biweekly, monthly or at ever longer intervals (which may or may not 15 remain the same during the course of treatment). Where multiple administrations are given, the IS S-containing polynucleotide may be given in 2, 3, 4, 5, 6, 7, 8, 9, 10 or more separate administrations. Generally, but not necessarily, an interval of at least about three days is necessary to allow effect of ISS-containing polynucleotides.

When ISS-containing polynucleotide is administered to an individual at risk of 20 exposure to virus (*i.e.*, before infection), ISS-containing polynucleotide is preferably administered less than about 14 days before exposure to virus, preferably less than about 10 days before exposure to virus, more preferably less than about 7 days before exposure to virus, even more preferably less than about 5 days before exposure to virus. In some embodiments, ISS-containing polynucleotide is administered about 3 days before 25 exposure to virus.

In a further embodiment, the ISS-containing polynucleotide is administered after exposure to a virus, but prior to appearance of symptoms. Preferably, the ISS containing polynucleotide is administered less than about three days after exposure, more preferably less than about one day, 12 hours, six hours or two hours after exposure, if the time of 30 exposure is known or suspected.

In another embodiment, the ISS containing polynucleotide is administered after appearance of at least one symptom of virus infection. For example, ISS containing polynucleotide is administered within about 28, 21, 14, 7, 5 or 3 days following appearance of a symptom of virus infection. However, some infected 10 individuals exhibiting symptoms will already have undertaken one or more courses of treatment with another therapy. In such individuals, or in individuals who failed to appreciate the import of their symptoms, the ISS-containing polynucleotide may be administered at any point following infection.

Additionally, treatments employing an ISS-containing polynucleotide may also be employed in conjunction with other treatments or as 'second line' treatments employed after failure of a 'first line' treatment.

ISS polynucleotides may be formulated in any form known in the art, such as dry powder, semi-solid or liquid formulations. For parenteral administration ISS polynucleotides preferably administered in a liquid formulation, although solid or semisolid formulations may also be acceptable, particularly where the ISS polynucleotide is formulated in a slow release depot form. ISS polynucleotides are generally formulated in liquid or dry powder form for topical administration, although semi-solid formulations may occasionally be useful.

ISS polynucleotide formulations may contain additional components such as salts, buffers, bulking agents, osmolytes, antioxidants, detergents, surfactants and other pharmaceutically-acceptable excipients as are known in the art. Generally, liquid ISS polynucleotide formulations made in USP water for injection and are sterile, isotonic and pH buffered to a physiologically-acceptable pH, such as about pH 6.8 to 7.5.

ISS-containing polynucleotides may be formulated in delivery vehicles such as liposomes, oil/water emulsion or slow release depot formulations. Methods of formulating polynucleotides in such forms are well known in the art.

ISS-containing polynucleotide formulations may also include or exclude

immunomodulatory agents such as adjuvants and immunostimulatory cytokines, which are well known in the art.

A suitable dosage range or effective amount is one that provides the desired reduction of symptoms and/or suppression of viral infection and depends on a number of factors, including the particular respiratory virus, ISS sequence of the polynucleotide, molecular weight of the polynucleotide and route of administration. Dosages are generally selected by the physician or other health care professional in accordance with a variety of parameters known in the art, such as severity of symptoms, history of the patient and the like. Generally, for an ISS-containing polynucleotide of about 20 bases, a dosage range may be selected from, for example, an independently selected lower limit such as about 0.1, 0.25, 0.5, 1, 2, 5, 10, 20, 30, 40, 50, 60, 80, 100, 200, 300, 400 or 500 µm/kg up to an independently selected upper limit, greater than the lower limit, of about 60, 80, 100, 200, 300, 400, 500, 750, 1000, 1500, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000 or 10,000 µm/kg. For example, a dose may be about any of the following: 0.1 to 100 µm/kg, 0.1 to 50 µm/kg, 0.1 to 25 µm/kg , 0.1 to 10 µm/kg, 1 to 500 µm/kg, 100 to 400 µm/kg, 200 to 300 µm/kg, 1 to 100 µm/kg, 100 to 200 µm/kg, 300 to 400 µm/kg, 400 to 500 µm/kg, 500 to 1000 µm/kg, 500 to 5000 µm/kg, or 500 to 10,000 µm/kg. Generally, parenteral. routes of administration may require higher doses of ISS compared to more direct application to infected tissue, as do ISS-containing polynucleotides of increasing length.

Polynucleotides comprising an ISS may be administered by systemic (e.g., parenteral) or local (e.g., topical) administration.

In one embodiment, the ISS-containing polynucleotide(s) is topically administered, such as respiratory mucosa (such as nasal passages or lung). Nasopharyngeal and pulmonary routes of administration include, but are not limited to, intranasal, inhalation, transbronchial and transalveolar routes. The ISS-containing polynucleotide may thus be administered by inhalation of aerosols, atomized liquids or powders. Devices suitable for administration by inhalation of ISS-containing compositions include, but are not limited to, nebulizers, atomizers, vaporizers, and metered-dose inhalers. Nebulizers, atomizers, vaporizers and metered-dose inhalers filled with or employing reservoirs containing formulations comprising the ISS-containing polynucleotide(s) are among a variety of devices suitable for use in inhalation delivery of the ISS-containing polynucleotide(s). Other methods of delivering to respiratory inucosa include delivery of liquid formulations, such as by nose drops.

In other embodiments, the ISS-containing polynucleotide is administered parenterally. Parenteral routes of administration include, but are not limited to, transdermal, transmucosal and direct injection. Parenteral administration by injection may be by any parenteral injection route, including, but not limited to, intravenous (IV), intraperitoneal (IP), intramuscular (IM), subcutaneous (SC) and intradermal (ID) routes.

Transdermal and transmucosal administration may be accomplished by, for example, inclusion of a carrier (*e.g.*, dimethylsulfoxide, DMSO), by application of electrical impulses (*e.g.*, iontophoresis) or a combination thereof. A variety of devices are available for transdermal administration which may be used in accordance with the invention, Because respiratory viruses infect cells of the respiratory tract, routes which deliver ISS polynucleotides to, the respiratory tract, such as inhalation and intranasal delivery (discussed above), are considered local routes of administration rather than systemic routes of administration, even though delivery through such routes are normally considered parenteral, systemic routes of administration.

IV, IP, IM and ID administration may be by bolus or infusion administration. For SC administration, administration may be by bolus, infusion or by implantable device, such as an implantable minipump (e.g., osmotic or mechanical minipump) or slow release implant. The ISS polynucleotide(s) may also be delivered in a slow release formulation adapted for IV, IP, IM, ID or SC administration. Administration by inhalation is preferably accomplished in discrete doses (e.g., via a metered dose inhaler), although delivery similar to an infusion may be accomplished through use of a nebulizer. Administration via the transdermal and transmucosal routes may be continuous or pulsatile.

Other Agents

Hepatitis B virus surface antigen vaccines also may be used in the methods and compositions disclosed herein. In one embodiment, B-L-FTC and a hepatitis B virus antigen vaccine, such as a surface antigen vaccine, is administered in combination or in alternation to a host in an effective amount for the treatment or prophylaxis of a hepatitis B virus infection, optionally incombination with another therapeutic agent, such as interferon.

Other vaccines that can be administered include Engerix-B® (GlaxoSmithKline), Recombivax HB® (Merck), Hepatitis B Vaccine (Recombinant), PJ Hep B DNA therapeutic vaccine (Powderject Pharmaceuticals), Hepavax-Gene® (DNA recombinant hepatitis B vaccine , Berna Biotech group), Gen H-B Vax™ (Chiron Corporation), Hepatavax-B® (Merck & Co.), Hevac B® (Pasteur), KGC® (Korea Green Cross), TGP 943™ (Takeda Chem, Japan), Gen Hevac B® (Pasteur, France), Bio-Hep-B™/Sci-B-Vac™ (Bio-Technology General, Israel), AG-3™, Hepagene™, and Hepacare™, (Medeva, UK, Evans UK).

In general, during alternation therapy, an effective dosage of each agent is administered serially, whereas in combination therapy, effective dosages of two or more agents are administered together. The dosages will depend on such factors as absorption, bio-distribution, metabolism and excretion rates for each drug as well as other factors known to those of skill in the art. It is to be noted that dosage values will also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens and schedules should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions. Examples of suitable dosage ranges can be found in the scientific literature and in the *Physicians Desk Reference*. Many examples of suitable dosage ranges for other compounds described herein are also found in public literature or can be identified using known procedures. These dosage ranges can be modified as desired to achieve a desired result.

IV. Gene Therapy

Another aspect of the present invention is using *in vivo* gene therapy methods to deliver immunomodulators in combination and/or alternation with B-L-FTC, and optionally in combination and/or alternation with at least one additional anti-HBV agent. Gene therapy methods relate to the introduction of nucleic acid (DNA, RNA, and antisense DNA or RNA) sequences into a host, such as an animal, and in particular a human, to increase the expression of the immunomodulator which may be operatively linked to a promoter and/or any other genetic elements necessary for its expression by

the target tissue. Such gene therapy and delivery techniques and methods are known in the art, see, for example, WO90/11092, WO98/11779; U.S. Pat. No. 5,693,622, 5,705,151, 5,580,859; Tabata H. et al. (1997) Cardiovasc. Res. 35(3):470-479, Chao J et al. (1997) Pharmacol. Res. 35(6):517-522, Wolff J. A. (1997) Neuromuscul. Disord. 7(5):314-318, Schwartz B. et al. (1996) Gene Ther. 3(5):405-411, Tsurumi Y. et al. (1996) Circulation 94(12):3281-3290.

The polypeptide vector constructs used in the gene therapy method are preferably constructs that will not integrate into the host genome nor will they contain sequences that allow for replication. Any strong promoter known to those skilled in the art can be used for driving the expression of DNA.

The polypeptide constructs may be delivered by any method that delivers injectable materials to the cells of an animal, such as, injection into the interstitial space of tissues (heart, muscle, skin, lung, liver, intestine and the like). The polypeptide constructs can be delivered in a pharmaceutically acceptable liquid or aqueous carrier.

The polypeptide construct can be delivered to the interstitial space of tissues within an animal, including the muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular fluid, mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels. They may be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression may be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts.

In a further embodiment of the invention, cells that are genetically engineered to express the immunomodulator are administered to a patient in vivo. Such cells may be obtained from the patient or an MHC compatible donor and can include, but are not

limited to fibroblasts, bone marrow cells, blood cells (e.g., lymphocytes), adipocytes, muscle cells, endothelial cells etc. The cells are genetically engineered in vitro using recombinant DNA techniques to introduce the coding sequence of polypeptides of the immunomodulator, or alternatively, by transduction (using viral vectors, and preferably vectors that integrate the transgene into the cell genome) or transfection procedures, including, but not limited to, the use of plasmids, cosmids, YACs, naked DNA, electroporation, liposomes, etc. The coding sequence of the immunomodulator can be placed under the control of a strong constitutive or inducible promoter or promoter/enhancer to achieve immunomodulator expression, and preferably secretion. The engineered cells which express and preferably secrete the immunomodulator can be introduced into the patient systemically, e.g., in the circulation, or intraperitoneally.

Alternatively, the cells can be incorporated into a matrix and implanted in the body, e.g., genetically engineered endothelial cells can be implanted as part of a lymphatic or vascular graft. (See, for example, Anderson et al. U.S. Pat. No. 5,399,349; and Mulligan & Wilson, U.S. Pat. No. 5,460,959).

When the cells to be administered are non-autologous or non-MHC compatible cells, they can be administered using well known techniques which prevent the development of a host immune response against the introduced cells. For example, the cells may be introduced in an encapsulated form which, while allowing for an exchange of components with the immediate extracellular environment, does not allow the introduced cells to be recognized by the host immune system.

Eukaryotic cells that may be transduced with the infectious viral particles containing a gene or gene fragment thereof for the expression of an immunomodulator include, but are not limited to, primary cells, such as primary nucleated blood cells, such as leukocytes, granulocytes, monocytes, macrophages, lymphocytes (including T-lymphocytes and B-lmphocytes), totipotent stem cells, and tumor infiltrating lymphocytes (TIL cells); bone marrow cells; endothelial cells; epithelial cells; keratinocytes; stem cells; hepatocytes, including hepatocyte precursor cells; hepatocytes, including hepatocyte precursor cells; fibroblasts; mesenchymal cells; mesothelial cells; and parenchymal cells.

In one embodiment, the cells may be targeted to a specific site, whereby the cells function as a therapeutic at such site. Alternatively, the cells may be cells that are not targeted to a specific site, and such cells function as a systemic therapeutic.

Transduced cells may be used, for example, in the treatment of HBV by introducing to host cells, such as blood cells that have been removed from a patient and expanded in culture, infectious viral particles in accordance with the present invention which contain genes that encode an immunomodulator. The cells can be expanded in number before or after transduction with the infectious viral particles containing the desired genes. Thus, the procedure is performed in such a manner that upon injection into the patient, the transformed cells will produce an immunomodulator in the patient's body.

The gene of the present invention carried by the transduced cells specifically comprises the sequence for an immunomodulator, but can be also comprise any sequence that directly or indirectly enhances the therapeutic effects of the cells. The gene carried by the transduced cells can also include sequences that allow the transduced cells to exert a therapeutic effect that it would not ordinarily have, such as a gene encoding a clotting factor useful in the treatment of hemophilia. The gene can encode one or more products having therapeutic effects. Examples of suitable genes include those that encode cytokines such as TNF, interleukins (interleukins 1-14), interferons (.alpha., .beta., .gamma.-interferons), T-cell receptor proteins and Fc receptors for antigen-binding domains of antibodies, such as immunoglobulins. Additional examples of suitable genes include genes that modify cells to "target" to a site in the body to which the cells would not ordinarily "target," thereby making possible the use of the cell's therapeutic properties at that site. For example, blood cells such as TIL cells can be modified, for example, by introducing a Fab portion of a monoclonal antibody into the cells, thereby enabling the cells to recognize a chosen antigen.

The most common types of vectors used in gene therapy are viruses. Scientists use viruses because they have a unique ability to enter a cell's DNA. Viruses used as vectors in gene therapy are genetically disabled; they are unable to reproduce themselves. Most gene therapy clinical trials rely on mouse retroviruses to deliver the desired gene. Other viruses used as vectors include adenoviruses, adeno-associated viruses, poxviruses and the herpes virus.

For example, cells from the patient are removed and grown in the laboratory. The cells are exposed to the virus that is carrying the desired gene. The virus enters the cells, and the desired gene becomes part of the cells' DNA. The cells grow in the laboratory and are then returned to the patient. This type of gene therapy is called *ex vivo*, which means "outside the body." The gene is transferred into the patient's cells while the cells are outside the patient's body. In other studies, vectors or liposomes (fatty particles) are used to deliver the desired gene to cells in the patient's body. This form of gene therapy is called *in vivo*, because the gene is transferred to cells inside the patient's body.

When viral vectors are used to carry genes into the body, they might alter more than the intended cells. Another danger is that the new gene might be inserted in the wrong location in the DNA, possibly causing cancer or other damage. In addition, when DNA is injected directly, or when a liposome delivery system is used, there is a chance that the DNA could be introduced into reproductive cells, producing inheritable changes.

Other concerns include the possibility that transferred genes could be "overexpressed," producing so much of the missing protein as to be harmful; that the viral vector could cause inflammation or an immune reaction; and that the virus could be transmitted from the patient to other individuals or into the environment.

There are many vectors known in the art. Any known vector can be used in the present invention. In a preferred embodiment of the present invention, the vector can target a specific cell type for specific gene delivery.

Adenoviral Vectors

Any of the adenoviral vectors can be used to transfect cells and/or cell lines to express and/or secrete an immunomodulator. Adenoviruses are non-enveloped viruses containing a linear double stranded DNA genome. While there are over 40 serotype strains of adenovirus, most of which cause benign respiratory tract infections in humans, subgroup C serotypes 2 or 5 are predominantly used as vectors. The life cycle does not normally involve integration into the host genome, rather they replicate as episomal elements in the nucleus of the host cell and consequently there is no risk of insertional

mutagenesis. The wild-type adenovirus genome is approximately 35 kb of which up to 30 kb can be replaced with foreign DNA (Smith, A. E. (1995). Viral vectors in gene therapy. Annual Review of Microbiology 49: 807-838, Verma, I. M. and Somia, N. (1997). Gene therapy - promises, problems and prospects. Nature 389: 239-242.). There are four early transcriptional units (E1, E2, E3 and E4) that have regulatory functions, and a late transcript, which codes for structural proteins. Progenitor vectors have either the E1 or E3 gene inactivated, with the missing gene being supplied in trans either by a helper virus, plasmid or integrated into a helper cell genome (human fetal kidney cells, line 293, Graham, F. L., Smiley, J., Russell, W. L. and Nairn, R. (1997). Characterization of a human cell line transformation by DNA from adenovirus 5. General Virology 36: 59-72.). Second generation vectors additionally use an E2a temperature sensitive mutant (Engelhardt, J. F., Litsky, L., and Wilson, J. M. (1994). Prolonged gene expression in cotton rat lung with recombinant adenoviruses defective in E2a. Human Gene Therapy 5: 1217-1229.) or an E4 deletion (Armentano, D., Zabner, J., Sacks, C., Sookdeo, C. C., Smith, M. P., St. George, J. A., Wadsworth, S. C., Smith, A. E. and Gregory, R. J. (1997). Effect of the E4 region on the presistence of transgene expression from adenovirus vectors. Journal of Virology 71: 2408-2416.). The most recent "gutless" vectors contain only the inverted terminal repeats (ITRs) and a packaging sequence around the transgene, all the necessary viral genes being provided in trans by a helper virus (Chen, H., Mack, L. M., Kelly, R., Ontell, M., Kochanek, S. and Clemens, P. R. (1997). Persistence in muscle of an adenoviral vector that lacks all viral genes. Proceedings of the National Academy of Sciences of the U.S.A. 94: 1645-1650.).

Adenoviral vectors are very efficient at transducing target cells in vitro and vivo, and can be produced at high titres (>10^{11}/mL). With the exception of Geddes, B. J., Harding, T. C., Lightman, S. L. and Uney, J. B. (1997). Long term gene therapy in the CNS: Reversal of hypothalamic diabetes insipidus in the Brattleboro rat by using an adenovirus expressing arginine vasopressin. Nature Medicine 3: 1402-1404.), who showed prolonged transgene expression in rat brains using an E1 deletion vector, transgene expression in vivo from progenitor vectors tends to be transient (Verma, I. M. and Somia, N. (1997). Gene therapy - promises, problems and prospects. Nature 389: 239-242.). Following intravenous injection, 90% of the administered vector is degraded in the liver by a non-immune mediated mechanism (Worgall, S., Wolff, G., Falck-

Pedersen, E. and Crystal R. G. (1997). Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration. Human Gene Therapy 8: 37-44.). Thereafter, an MHC class I restricted immune response occurs, using CD8+ CTLs to eliminate virus infected cells and CD4+ cells to secrete IFN-alpha which results in anti-adenoviral antibody (Yang, Y. and Wilson, J. M. (1995). Clearance of adenovirus-infected hepatocytes by MHC class I restricted CD4+ CTLs in vivo. Journal of Immmunology 155: 2564-2569.). Alteration of the adenoviral vector can remove some CTL epitopes, however the epitopes recognized differ with the host MHC haplotype (Sparer, T. E., Wynn, S. G., Clark, D. J., Kaplan, J. M., Cardoza, L. M., Wadsworth, S. C., Smith, A. E. and Gooding, L. R. (1997). Generation of cytotoxic T lymphocytes against immunorecessive epitopes after multiple immunizations with adenovirus vectors is dependent on haplotype. Journal of Virology 71: 2277-2284. Jooss, K., Ertl, H. C. J. and Wilson, J. M. (1998). Cytotoxic T-lymphocyte target proteins and their histocompatibility complex class I restriction in response to adenovirus vectors delivered to mouse liver. Journal of Virology 72: 2945-2954.). The remaining vectors, in those cells that are not destroyed, have their promoter inactivated (Armentano, D., Zabner, J., Sacks, C., Sookdeo, C. C., Smith, M. P., St. George, J. A., Wadsworth, S. C., Smith, A. E. and Gregory, R. J. (1997). Effect of the E4 region on the presistence of transgene expression from adenovirus vectors. Journal of Virology 71: 2408-2416.) and persisting antibody prevents subsequent administration of the vector.

Approaches to avoid the immune response involving transient immunosupressive therapies have been successful in prolonging transgene expression and achieving secondary gene transfer (Jooss, K., Yang, Y. and Wilson, J. M. (1996). Cyclophosphamide diminishes inflammation and prolongs expression following delivery of adenoviral vectors to mouse liver and lung. Human Gene Therapy 7: 1555-1566. Kay, M. A., Meuse, L., Gown, A. M., Linsley, P., Hollenbaugh, D., Aruffo, A., Ochs, H. D. and Wilson, C. B. (1997). Transient immunomodulation with anti-CD40 ligand and CTLA41g enhances presistence and secondary adenovirus-mediated gene transfer into mouse liver. Proceedings of the National Academy of Sciences of the U.S.A. 94: 4686-4691). A less interventionist method has been to induce oral tolerance by feeding the host UV inactivated vector (Kagami, H., Atkinson, J. C., Michalek, S. M., Handelman, B., Yu, S., Baum, B. J. and O'Connell, B. (1998). Repetitive adenovirus administration to

the parotid gland: role of immunolgical barriers and induction of oral tolerance. Human Gene Therapy 9: 305-313.). However, it is desirable to manipulate the vector rather than the host. Although only replication deficient vectors are used, viral proteins are expressed at a very low level which are presented to the immune system. The development of vectors containing fewer genes, culminating in the "gutless" vectors which contain no viral coding sequences, has resulted in prolonged in vivo transgene expression in liver tissue (Schiedner, G., Morral, N., Parks, R. J., Wu, Y., Koopmans, S. C., Langston, C., Graham, F. L., Beaudet, A. L. and Kochanek, S. (1998). Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. Nature Genetics 18: 180-183.). The initial delivery of large amounts of DNA packaged within adenovirus proteins, the majority of which will be degraded and presented to the immune system may still cause problems for clinical trials. Moreover the human population is heterogeneous with respect to MHC haplotype and a proportion of the population will have been already exposed to the adenoviral strain (Gahry-Sdard, H., Molinier-Frenkel, V., Le Boulaire, C., Saulnier, P., Opolon, P., Lengange, R., Gautier, E., Le Cesne, A., Zitvogel, L., Venet, A., Schatz, C., Courtney, M., Le Chevalier, T., Tursz, T., Guillet, J. and Farace, F. (1997). Phase I trial of recombinant adenovirus gene transfer in lung cancer. Journal of Clinical Investigation 100: 2218-2226.)

Until recently, the mechanism by which the adenovirus targeted the host cell was poorly understood. Tissue specific expression was therefore only possible by using cellular promoter/enhancers e.g. the myosin light chain 1 promoter (Shi, Q., Wang, Y. and Worton, R. (1997). Modulation of the specificity and activity of a cellular promotor in an adenoviral vector. Human Gene Therapy 8: 403-410.) and the smooth muscle cell SM22a promoter (Kim, S., Lin, H., Barr, E., Chu, L., Leiden, J. M. and Parmacek, M. S. (1997). Transcriptional targetting of replication-defective adenovirus transgene expression to smooth muscle cells in vivo. Journal of Clinical Investigation 100: 1006-1014.), or by direct delivery to a local area (Rome, J. J., Shayani, V., Newman, K. D., Farrell, S., Lee, S. W., Virmani, R. and Dicheck, D. A. (1994). Adenoviral vector mediated gene transfer into sheep arteries using a double-balloon catheter. Human Gene Therapy 5: 1249-1258.). Uptake of the adenovirus particle has been shown to be a two stage process involving an initial interaction of a fibre coat protein in the adenovirus with

a cellular receptor or receptors, which include the MHC class I molecule (Hong, S. S., Karayan, L., Tournier, J., Curiel, D. T., and Boulanger, P. A. (1997). Adenovirus type 5 fiber knob binds to MHC class I a2 domain at the surface of human epithelial and B lymphoblastiod cells. EMBO Journal 16: 2294-2306.) and the coxsackievirus-adenovirus receptor (Bergelson, J. M., Cunningham J. A., Droguett, G., Kurt-Jones, A. E., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L., and Finberg, R. W. (1997). Isolation of a common receptor for coxsackie virus B viruses and adenoviruses 2 and 5. Science 275: 1320-1323.). The penton base protein of the adenovirus particle then binds to the integrin family of cell surface heterodimers (Wickham, T. J., Mathias, P., Cheresh, D. A., and Nemerow, G. R. (1993). Integrins avb3 and avb5 promote adenovirus internalisation but not virus attachment. Cell 73: 309-319.) allowing internalisation via receptor mediated endocytosis. Most cells express primary receptors for the adenovirus fibre coat protein, however internalisation is more selective (Harris, J. D. and Lemoine, N. R. (1996). Strategies for targeted gene therapy. Trends in Genetics 12: 400-404.). Methods of increasing viral uptake include stimulating the target cells to express an appropriate integrin (Davison, E., Diaz, R. M., Hart, I. R., Santis, G. and Marshall, J. F. (1997). Integrin a5b1-mediated adenovirus infection is enhanced by the integrin-activating antibody TS2/16. Journal of Virology 71: 6204-6207.) and conjugating an antibody with specificity for the target cell type to the adenovirus (Wickham, T. J., Lee, G. M, Titus, J. A., Titus, J. A., Sconocchia, G., Bakacs, T., Kovesdi, I. and Segal, D. M. (1997b). Targeted adenovirus-mediated gene delivery to T cells via CD3. Journal of Virology 71: 7663-7669. Goldman, C. K., Rogers, B. E., Douglas, J. T., Sonsowski, B. A., Ying, W., Siegal, G. P., Baird, A., Campain, J. A. and Curiel, D. T (1997). Targeted gene delivery to karposis sarcoma cells via the fibroblast growth factor receptor. Cancer Research 57: 1447-1451.). The use of antibodies though increases the production difficulties of the vector and the potential risk of activating the complement system. By incorporating receptor binding motifs into the fibre coat protein, Wickham *et al.* (Wickham, T. J., Tzeng, E., Shears II, L. L., Roelvink, P. W., Li, Y., Lee, G. M., Brough, D. E., Lizonova, A. and Kovesdi, I. (1997a). Increased in vitro and in vivo gene transfer by adenovirus vectors containing chimeric fiber proteins. Journal of Virology 71: 8221-8229.) were able to redirect the virus to bind the integrin expressed by damaged endothelial or smooth muscle cells, or heparin sulphate receptors which is expressed by numerous cells types.

Adeno-Associated Viral Vectors

Any of the adeno-associated viral vectors can be used to transfect cells and/or cell lines to express and/or secrete an immunomodulator. Adeno-associated viruses (AAV) are non-pathogenic human parvoviruses, dependant on a helper virus, usually adenovirus, to proliferate. They are capable of infecting both dividing and non dividing cells, and in the absence of a helper virus integrate into a specific point of the host genome (19q 13-qter) at a high frequency (Kotin, R. M., Siniscalco, M., Samulski, R. J., Zhu, X. D., Hunter, L., Laughlin, C. A., McLaughlin, S., Muzyczka, N., Rocchi, M. and Berns, K. I. (1990). Site-specific integration by adeno-associated virus. Proceedings of the National Academy of Sciences of the U.S.A. 87: 2211-2215.). The wild type genome is a single stranded DNA molecule, consisting of two genes; rep, coding for proteins which control viral replication, structural gene expression and integration into the host genome, and cap, which codes for capsid structural proteins. At either end of the genome is a 145 bp terminal repeat (TR), containing a promoter.

When used as a vector, the rep and cap genes are replaced by the transgene and its associated regulatory sequences. The total length of the insert cannot greatly exceed 4.7 kb, the length of the wild type genome (Smith, A. E. (1995). Viral vectors in gene therapy. Annual Review of Microbiology 49: 807-838.). Production of the recombinant vector requires that rep and cap are provided in trans, along with helper virus gene products (E1a, E1b, E2a, E4 and VA RNA from the adenovirus genome). The conventional method is to cotransfect two plasmids, one for the vector and another for rep and cap, into 293 cells infected with adenovirus (Samulski, R. J., Chang, L., and Shenk, T. (1989). Helper free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. Journal of Virology 63: 3822-3828.). This method, however, is cumbersome, low yielding ($<10^4$ particles/ml) and prone to contamination with adenovirus and wild type AAV. One of the reasons for the low yield is the inhibitory effect of the rep gene product on adenovirus replication (Vincent, K. A, Piraino, S. T. and Wadsworth, S. C. (1997). Analysis of recombinant adeno-associated virus packaging and requirements for rep and cap gene products. Journal of Virology 71: 1897-1905.). More recent protocols remove all adenoviral structural genes and use rep

resistant plasmids (Xiao, X., Li, J. and Samulski, R. J. (1998). Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. Journal of Virology 72: 2224-2232.) or conjugate a rep expression plasmid to the mature virus prior to infection (Fisher, K. J., Kelley, W. M, Burda, J. F. and Wilson, J. M. (1996). A novel adenovirus-adeno-associated virus hybrid vector that displays efficient resue and delivery og the AAV genome. Human Gene Therapy 7: 2079-2087.).

In the absence of rep, the AAV vector will only integrate at random, as a single provirus or head to tail concatamers, once the terminal repeats have been slightly degraded (Rutledge, E. A. and Russell, D. W. (1997). Adeno-associated virus vector integration junctions. Journal of Virology 71: 8429-8436.). Interest in AAV vectors has been due to their integration into the host genome allowing prolonged transgene expression. Gene transfer into vascular epithelial cells (Maeda, Y., Ikeda, U., Ogasawara, Y., Urabe, M., Takizawa, T., Saito, T., Colosi, P., Kurtzman, G., Shimada, K. and Ozawa, K. (1997). Gene transfer into vascular cells using adeno-associated virus (AAV) vectors. Cardiovascular Research 35: 514-521.), striated muscle (Fisher, K. J., Jooss, K., Alston, J., Yang, Y., Haecker, S. E., High, K., Pathak, R., Raper, S. E. and Wilson, J. M. (1997). Recombinant adeno-associated virus for muscle directed gene delivery. Nature Medicine 3: 306-316. Herzog, R. W., Hagstrom, J. N., Kung, S., Tai, S. J., Wilson, J. M., Fisher, K. J. and High, K. A. (1997). Stable gene transfer and expression of human blood coagulation factor IX after intramuscular injection of recombinant adeno-associated virus. Proceedings of the National Academy of Sciences of the U.S.A. 94: 5804-5809.) and hepatic cells (Snyder, R. O., Miao, C. H., Patijn, G. A., Spratt, S. K., Danos, O., Nagy, D., Gown, A. M., Winter, B., Meuse, L., Cohen, L. K., Thompson, A. R. and Kay, M. A. (1997). Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. Nature Genetics 16: 270-275.) has been reported, with prolonged expression when the transgene is not derived from a different species. Neutralising antibody to the AAV capsid may be detectable, but does not prevent readministration of the vector or shut down promoter activity. It is possibly due to the simplicity of the viral capsid, that the immune response is so muted. As AAV antibodies will be present in the human population this will require further investigation. There has been no attempt to target particular cell types other than by localised vector delivery.

In particular, the adeno-associated vectors disclosed in U.S. Patent No. 5,693,531, which is hereby incorporated by reference, can be used, including AAVp5neo; pSV-β-Galactosidase; TRF169; LZ11; pSP72; pSP72nLacZ; pAdRSV4; pAdRSVnLacZ; AAVrnLac; SV40; pBluescriptSK; pSV40 ori AAV1; and pKMT11.

5

Retroviral Vectors

Any of the retroviral vectors can be used to transfect cells and/or cell lines to express and/or secrete an immunomodulator. Retroviruses are a class of enveloped viruses containing a single stranded RNA molecule as the genome. Following infection, 10 the viral genome is reverse transcribed into double stranded DNA, which integrates into the host genome and is expressed as proteins. The viral genome is approximately 10kb, containing at least three genes: gag (coding for core proteins), pol (coding for reverse transcriptase) and env (coding for the viral envelope protein). At each end of the genome are long terminal repeats (LTRs) which include promoter/enhancer regions and 15 sequences involved with integration. In addition there are sequences required for packaging the viral DNA (psi) and RNA splice sites in the env gene. Some retroviruses contain protooncogenes, which when mutated can cause cancers, however, in the production of vectors these are removed. Retroviruses can also transform cells by integrating near to a cellular protooncogene and driving inappropriate expression from 20 the LTR, or by disrupting a tumor suppresser gene. This event, termed insertional mutagenesis, though extremely rare could still occur when retroviruses are used as vectors.

Retroviral vectors are most frequently based upon the Moloney murine leukemia virus (Mo-MLV), which is an amphotrophic virus, capable of infecting both mouse cells, 25 enabling vector development in mouse models, and human cells, enabling human treatment. The viral genes (gag, pol and env) are replaced with the transgene of interest and expressed on plasmids in the packaging cell line. Because the non-essential genes lack the packaging sequence (psi) they are not included in the virion particle. To prevent recombination resulting in replication competent retroviruses, all regions of homology 30 with the vector backbone should be removed and the non-essential genes should be expressed by at least two transcriptional units (Markowitz, D., Goff, S. and Bank, A.

(1988). A safe packaging line for gene transfer: separating viral genes on two different plasmids. Journal of Virology 62: 1120-1124.). Even so, replication competent retroviruses do occur at a low frequency.

The essential regions include the 5' and 3' LTRs, and the packaging sequence lying downstream of the 5' LTR. Transgene expression can either be driven by the promoter/ enhancer region in the 5' LTR, or by alternative viral (e.g. cytomegalovirus, Rous sarcoma virus) or cellular (e.g. beta actin, tyrosine) promoters. Mutational analysis has shown that up to the entire gag coding sequence and the immediate upstream region can be removed without effecting viral packaging or transgene expression (Kim, S. H., Yu, S. S., Park, J. S, Robbins, P. D, An, C. S. and Kim, S. (1998). Construction of retroviral vectors with improved safety, gene expression, and versitility. Journal of Virology 72: 994-1004.). However the exact positioning of the transgene start codon and small alterations of the 5' LTR influence transgene expression (Rivire, I., Brose, K. and Mulligan, R. C. (1995). Effects of retroviral vector design on expression of human adenosine deaminase in murine bone marrow transplant recipients engrafted with genetically modified cells. Proceedings of the National Academy of Sciences of the U.S.A. 92: 6733-6737.). To aid identification of transformed cells selectable markers, such as neomycin and beta galactosidase, can be included and transgenes expression can be improved with the addition of internal ribosome sites (Saleh, M. (1997). A retroviral vector that allows co-expression of two genes and the versatility of alternate selection markers. Human Gene Therapy 8: 979-983.). The available carrying capacity for retroviral vectors is approximately 7.5 kb (Verma, I. M. and Somia, N. (1997). Gene therapy - promises, problems and prospects. Nature 389: 239-242.), which is too small for some genes even if the cDNA is used.

The retroviral envelope interacts with a specific cellular protein to determine the target cell range. Altering the env gene or its product has proved a successful means of manipulating the cell range. Approaches have included direct modifications of the binding site between the envelope protein and the cellular receptor, however these approaches tend to interfere with subsequent internalisation of the viral particle (Harris, J. D. and Lemoine, N. R. (1996). Strategies for targeted gene therapy. Trends in Genetics 12: 400-404.). By replacing a portion of the env gene with 150 codons from the erythropoietin protein (EPO), Kasahara et al. (Kasahara, N., Dozy, A. M. and Kan, Y. W.

(1994). Tissue-specific targetting of retroviral ligand-receptor interactions. Science 266: 1374-1376.) were able to target EPO receptor bearing cells with high affinity. Coupling an antibody to the viral particle with affinity for a second cell specific antibody via a streptovadin bridge, improves viral uptake, but internalisation tends to lead to viral degradation (Roux, P., Jeanteur, P., and Piechaczyk, M. (1989). A versatile and potentially general approach to the targeting of specific cell types by means of major histocompatibility complex class I and class II antigens by mouse ecotropic murine leukemia virus-derived viruses. Proceedings of the National Academy of Sciences USA 86: 9079-9083.). Neda et al (Neda, H., Wu, C. H., and Wu. G. Y. (1991). Chemical modification of an ecotropic murine leukemia virus results in redirection of its target cell specificity. The Journal of Biological Chemistry 266: 14143-14146.) treated viral particles with lactose which resulted in uptake by cells, principally hepatocytes, expressing asiaglycoprotein receptors. Subsequently there was efficient viral transgene expression, possibly due to acidification of the endosome allowing fusion of the viral envelope with the endosome membrane.

Viruses differ with respect to their tropisms, therefore by replacing the env gene with that of another virus, the host range can be extended, in a technique known as pseudotyping. Vesicular stomatitis virus G protein has been included in Mo-MLV derived vectors (Burns, J. C., Matsubara, T., Lozinski, G., Yee, J., Freidmann, T., Washabaugh, C. H. and Tsonis, P. A. (1994). Pantropic retroviral vector-mediated gene transfer, integration, and expression in cultured newt limb cells. Developmental Biology 165: 285-289.), which are also more stable when purified by ultracentrifugation. Recently, Qing (Qing, K., Bachelot, T., Mukherjee, P., Wang, X., Peng, L., Yoder, M. C., Leboulch, P. and Srivastava, A. (1997). Adeno-associated virus type 2 mediated transfer of ecotropic retrovirus receptor cDNA allows ecotropic retroviral transduction of established and primary human cells. Journal of Virology 71: 5663-5667.) improved transduction into numerous cell lines by first treating the recipient cells with an adeno-associated vector (discussed below) expressing the cellular receptor for retroviral envelope protein.

A requirement for retroviral integration and expression of viral genes is that the target cells should be dividing. This limits gene therapy to proliferating cells in vivo or ex vivo, whereby cells are removed from the body, treated to stimulate replication and

then transduced with the retroviral vector, before being returned to the patient. Ex vivo cells can be more efficiently transduced, due to exposure to higher virus titres and growth factors (Glimm, H., Kiem, H. P., Darovsky, B., Storb, R., Wolf, J., Diehl, V., Mertelsmann, R. and Kalle, C. V. (1997). Efficient gene transfer in primitive CD34+/CD38lo human bone marrow cells reselected after long term exposure to GALV-pseudotyped retroviral vector. Human Gene Therapy 8: 2079-2086.). Furthermore ex vivo treated tumor cells will associate with the tumor mass and can direct tumoricidal effects (Oldfield, E. H. and Ram, Z. (1995). Intrathecal gene therapy for the treatment of leptomeningeal carcinomatosis. Human Gene Therapy 6: 55-85.; Abdel-Wahab, Z., Weltz, C., Hester, D., Pickett, N., Vervaert, C., Barber, J. R., Jolly, D. and Seigler H. F. (1997). A phase I clinical trial of immunotherapy with interferon-gamma gene-modified autologous melanoma cells. Cancer 80: 401-412.).

Lentiviruses are a subclass of retroviruses that are able to infect both proliferating and non-proliferating cells. They are considerably more complicated than simple retroviruses, containing an additional six proteins, tat, rev, vpr, vpu, nef and vif. Current packaging cell lines have separate plasmids for a pseudotype env gene, a transgene construct, and a packaging construct supplying the structural and regulatory genes in trans (Naldini, L., Blmer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F. H., Verma I. M. and Trono, D. (1996). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272: 263-267.). Early results using marker genes have been promising, showing prolonged in vivo expression in muscle, liver and neuronal tissue (Blmer, U., Naldini, L., Kafri, T., Trono, D., Verma, I. M. and Gage, F. H. (1997). Highly efficient and sustained gene transfer in adult neurones with a lentivirus vector. Journal of Virology 71: 6641-6649. ; Miyoshi, H., Takahashi, M., Gage, F. H. and Verma, I. M. (1997). Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector. Proceedings of the National Academy of Sciences of the U.S.A. 94: 10319-10323. ; Kafri, T., Blmer, U., Peterson, D. A., Gage, F. H. and Verma, I. M. (1997). Sustained expression of genes delivered into liver and muscle by lentiviral vectors. Nature Genetics 17: 314-317). Interestingly the transgenes are driven by an internally engineered cytomegalovirus promoter, which unlike when in MoMLV vectors, is not inactivated. This may be due to the limited inflammatory response to the vector injection, which was equal in magnitude to the saline control (Blmer, U., Naldini, L.,

Kafri, T., Trono, D., Verma, I. M. and Gage, F. H. (1997). Highly efficient and sustained gene transfer in adult neurones with a lentivirus vector. Journal of Virology 71: 6641-6649.).

The lentiviral vectors used are derived from the human immunodeficiency virus (HIV) and are being evaluated for safety, with a view to removing some of the non-essential regulatory genes. Mutants of vpr and vif are able to infect neurones with reduced efficiency, but not muscle or liver cells (Blmer, U., Naldini, L., Kafri, T., Trono, D., Verma, I. M. and Gage, F. H. (1997). Highly efficient and sustained gene transfer in adult neurones with a lentivirus vector. Journal of Virology 71: 6641-6649; Kafri, T., Blmer, U., Peterson, D. A., Gage, F. H. and Verma, I. M. (1997). Sustained expression of genes delivered into liver and muscle by lentiviral vectors. Nature Genetics 17: 314-317.).

In a particular embodiment, the retroviral vectors pLXIN, pSIR, pLXSH, pLNCX, pLAPSN, pFB and pFB-Neo are used.

Herpes Simplex Viral Vectors

Any of the herpes simplex viral vectors can be used to transfect cells and/or cell lines to express and/or secrete an immunomodulator. Herpes simplex virus type 1 (HSV-1) is a human neurotropic virus, consequently interest has largely focused on using HSV-1 as a vector for gene transfer to the nervous system. The wild type HSV-1 virus is able to infect neurones and either proceed into a lytic life cycle or persist as an intranuclear episome in a latent state. Latently infected neurones function normally and are not rejected by the immune system. Though the latent virus is transcriptionally almost silent, it does possess neurone specific promoters that are capable of functioning during latency. Antibodies to HSV-1 are common in the human population, however complications due to herpes infection, such as encephalitis, are very rare.

The viral genome is a linear double stranded DNA molecule of 152 kb. There are two unique regions, long and short (termed UL and US) which are linked in either orientation by internal repeat sequences (IRL and IRS). At the non-linker end of the unique regions are terminal repeats (TRL and TRS). There are up to 81 genes (Marconi,

P., Krisky, D., Oligino, T., Poliani, P. L., Ramakrishnan, R., Goins, W. F., Fink, D. A. and Glorioso, J. C. (1996). Replication-defective herpes simplex virus vectors for gene transfer in vivo. Proceedings of the National Academy of Sciences USA 93: 11319-11320.), of which about half are not essential for growth in cell culture. Once these non essential genes have been deleted, 40-50 kb of foreign DNA can be accommodated within the virus (Glorioso, J. C., DeLuca, N. A. and Fink, D. J (1995). Development and application of herpes simplex virus vectors for human gene therapy. Annual Review of Microbiology 49: 675-710.). Three main classes of HSV-1 genes have been identified, namely the immediate-early (IE or alpha) genes, early (E or beta) genes and late (L or gamma) genes.

Following infection in susceptible cells, lytic replication is regulated by a temporally co-ordinated sequence of gene transcription. Vmw65 (a tegument structural protein) activates the immediate early genes (IP0, ICP4, ICP22, ICP27 and ICP477) that are transactivating factors allowing the production of early genes. The early genes encode genes for nucleotide metabolism and DNA replication. Late genes are activated by the early genes and code for structural proteins. The entire cycle takes less than 10h and invariably results in cell death.

The molecular events leading to the establishment of latency have not been fully determined. Gene expression during latency is driven by the latency associated transcripts (LATs) located in the IRL region of the genome. Two LATs (2.0 and 1.5kb) are transcribed in the opposite direction to the IE gene ICP0. LATs have a role in HSV-1 reactivation from latency (Steiner, I., Spivack, J. G., Lirette, R. P., Brown, S. M., MacLean, A. R., Subak-Sharpe, J. H. and Fraser, N. W. (1989). Herpes simplex virus type 1 latency-associated transcripts are evidently not evidently not essential for latent infection. EMBO Journal 8: 505-511.) and the establishment of latency (Sawtell, N. M. and Thompson, R. L. (1992). Herpes simplex virus type 1 latency-associated transcription unit promotes anatomical site-dependant establishment and reactivation from latency. Journal of Virology 66: 2157-2169.). Two latency active promoters that drive expression of the LATs have been identified (Marconi, P., Krisky, D., Oligino, T., Poliani, P. L., Ramakrishnan, R., Goins, W. F., Fink, D. A. and Glorioso, J. C. (1996). Replication-defective herpes simplex virus vectors for gene transfer in vivo. Proceedings

of the National Academy of Sciences USA 93: 11319-11320.) and may prove useful for vector transgene expression.

Two basic approaches have been used for production of HSV-1 vectors, namely amplicons and recombinant HSV-1 viruses. Amplicons are bacterially produced plasmids containing col E1 ori (an Escherishia coli origin of replication), OriS (the HSV-1 origin of replication), HSV-1 packaging sequence, the transgene under control of an immediate-early promoter and a selectable marker (Federoff, H. J., Geschwind, M. D., Geller, A. I. and Kessler, J. A. (1992). Expression of nerve factor in vivo from a defective herpes simplex virus 1 vector prevents effects of axotomy on sympathetic ganglia. Proceedings of the National Academy of Sciences USA 89: 1636-1640.). The amplicon is transfected into a cell line containing a helper virus (a temperature sensitive mutant) which provides all the missing structural and regulatory genes in trans. Both the helper and amplicon containing viral particles are delivered to the recipient. More recent amplicons include an Epstein-Barr virus derived sequence for plasmid episomal maintenance (Wang, S. and Vos, J. (1996). A hybrid herpesvirus infectious vector based on epstein-barr virus and herpes simplex virus type 1 for gene transfer into human cells in vitro and in vivo. Journal of Virology 70: 8422-8430.).

Recombinant viruses are made replication deficient by deletion of one the immediate-early genes e.g. ICP4, which is provided in trans. Though they are less pathogenic and can direct transgene expression in brain tissue, they are toxic to neurones in culture (Marconi, P., Krisky, D., Oligino, T., Poliani, P. L., Ramakrishnan, R., Goins, W. F., Fink, D. A. and Glorioso, J. C. (1996). Replication-defective herpes simplex virus vectors for gene transfer in vivo. Proceedings of the National Academy of Sciences USA 93: 11319-11320.). Deletion of a number of immediate-early genes substantially reduces cytotoxicity and also allows expression from promoters that would be silenced in the wild type latent virus. These promoters may be of use in directing long term gene expression.

Replication-conditional mutants are only able to replicate in certain cell lines. Permissive cell lines are all proliferating and supply a cellular enzyme to complement for a viral deficiency. Mutants include thymidine kinase (During, M. J., Naegele, J. R., OMalley, K. L. and Geller, A. I. (1994). Long-term behavioral recovery in Parkinsonian rats by an HSV vector expressing tyrosine hydroxylase. Science 266: 1399-1403.),

ribonuclease reductase (Kramm, C. M., Chase, M., Herrlinger, U., Jacobs, A., Pechan, P. A., Rainov, N. G., Sena-esteves, M., Aghi, M. Barnett, F. H., Chiocca, E. A. and Breakefield, X.. O. (1997). Therapeutic efficiency and safety of a second-generation replication-conditional HSV1 vector for brain tumor gene therapy. Human Gene Therapy 8: 2057-2068.), UTPase, or the neurovirulence factor g34.5 (Kesari, S., Randazzo, B. P., Valyi-Nagy, T., Huang, Q. S., Brown, S. M., MacLean, A. R., Lee, V. M., Trojanowski, J. Q. and Fraser, N. W. (1995). Therapy of experimental human brain tumors using a neuroattenuated herpes simplex virus mutant. laboratory Investigation 73: 636-648.).

Non-viral Vectors

Viral vectors all induce an immunological response to some degree and may have safety risks (such as insertional mutagenesis and toxicity problems). Further, their capacity is limited and large scale production may be difficult to achieve. Therefore, in one embodiment of the invention, non-viral methods of gene transfer are used, which may require only a small number of proteins, have a virtually infinite capacity, have no infectious or mutagenic capability and large scale production is possible using pharmaceutical techniques. There are three methods of non-viral DNA transfer, namely: naked DNA, liposomes and molecular conjugates.

Naked DNA (in the form of a plasmid) can be directly injected into muscle cells (Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A. and Felgner P. L. (1990). Direct gene transfer into mouse muscle in vivo. Science 247: 1465-1468.) or attached to gold particles that are bombarded into the tissue (Cheng, L., Ziegelhoffer, P. R. and Yang, N. S. (1993). In vivo promoter activity and transgene expression in mammalian somatic tissues evaluated by using particle bombardment. Proceedings of the National Academy of Sciences of the U.S.A. 90: 4455-4459.). The terms "naked" DNA or RNA refer to sequences that are free from any delivery vehicle that act to assist, promote, or facilitate entry into the cell, including viral sequences, viral particles, liposome formulations, lipofectin or precipitating agents and the like. Though not very efficient, this can result in prolonged low level expression in vivo. Unlike other gene therapies techniques, one major advantage of introducing naked nucleic acid sequences into target cells is the transitory nature of the immunomodulator synthesis in the cells.

Studies have shown that non-replicating DNA sequences can be introduced into cells to provide production of the desired immunomodulator for periods of up to six months. The simplicity of this method, and sustained expression has led to the development of DNA vaccines. Compared to conventional attenuated and protein based vaccines, they
5 are unaffected by pre-existing immunity e.g. due to maternal antibodies, relatively cheap, and can deliver a number of pathogen antigens on a single plasmid (Manickan, E., Karem, K. L., and Rouse, B. T. (1997). DNA vacines - a modern gimmick or a boon to vaccinology. Critical Reviews in Immunology 17: 139-154.). DNA vaccines are being developed for those pathogens where there is no existing vaccine e.g. HIV (Lekutis, C.,
10 Shiver, J. W., Liu, M. A., and Letvin, L. N. (1997). HIV1 env DNA vaccine administered to rhesus monkeys elicits MHC class II-restricted CD4+ T helper cells that secrete IFNg and TNFa. The Journal of Immunology 158: 4471-4477.) or the current vaccine not fully effective e.g. influenza (Macklin, M. D., McCabe, D., McGregor, M. W., Neumann, V., Meyer, T., Callan, R., Hinshaw, V. S. and Swain, W. S. (1998).
15 Immunization of pigs with a particle mediated vaccine to influenza A virus protects against challenge with homologous virus. Journal of Virology 72: 1491-1496.). By using a highly conserved gene Ulmer et al. (Ulmer, J. B., Donnelly, J. J., Parker, S. E., Rhodes, G. H., Felgner, P. L., Dwarki, J. J., Gromkowski, S. H., Deck, R., DeWitt, C. M., Friedman, A., Hawe, L. A., Laender, K. R., Martinz, D., Perry, H. C., Shiver, J.
20 Montgomery, D. L. and Liu, M. A. (1993). Heterologous protection against influenza by ingection of DNA encoding a viral protein. Science 254: 1745-1749.) were able to immunise mice against two serologically distinct influenza virus strains. In most cases however, DNA vaccines have not been shown to be better than the existing vaccines (Macklin, M. D., McCabe, D., McGregor, M. W., Neumann, V., Meyer, T., Callan, R.,
25 Hinshaw, V. S. and Swain, W. S. (1998). Immunization of pigs with a particle mediated vaccine to influenza A virus protects against challenge with homologous virus. Journal of Virology 72: 1491-1496.). The actual type of immune response can be controlled by cotransformation of a gene coding for the appropriate cytokine (Xiang, Z. and Ertl, H. C. (1995). Manipulation of the immune response to a plasmid-encoded viral antigen by
30 coinoculation with plasmids expressing cytokines. Immunity 2: 129-135.) and this method may prove useful in redirecting inappropriate immune responses (Manickan, E., Karem, K. L., and Rouse, B. T. (1997). DNA vacines - a modern gimmick or a boon to vaccinology. Critical Reviews in Immunology 17: 139-154.). Other uses for naked DNA

include cancer immunopotentiation (discussed below, Corr. M., Tighe, H., Lee. D., Dudler, J., Trieu, M., Brinson, D. C. and Carson, D. A. (1997). Costimulation provided by DNA immunisation enhances antitumor immunity. The Journal of Laboratory Investigation 159: 4999-5004.), repair of pancreatic insulin function (Goldfine. I. D., German, M. S., Tseng, H., Wang, J., Bolaffi, J. L., Chen, J. Olson, D. C. and Rothman, S. S. (1997). The endocrine secretion of human insulin and growth hormone by exocrine glands of the gastrointestinal tract. Nature Biotechnology 15: 1378-1382.), and stimulation of collateral blood vessel development (Takeshita, S., Tsurumi, Y., Couffinahl, T., Asahara, T., Bauters, C., Symes, J., Ferrara, N., and Isner, J. M. (1996). Gene transfer of naked DNA encoding for three isoforms of vascular endothelial growth factor endothelial growth factor stimulates collateral development in vivo. Laboratory Investigation 75: 487-501.). Expression of the gene product in muscle tissue can be improved by the coadministration of collagenase, papaverine and ischaemic conditions (Budker, V., Zhang, G., Danko, I., Williams, P. and Wolff, J. (1998). The efficient expression of intravascularly delivered DNA in rat muscle. Gene Therapy 5: 272-276.). The use of a muscle specific promoter (Skarli, M., Kiri, A., Vrbova, G., Lee, C. A. and Goldspink, G. (1998). Myosin regulatory elements as vectors for gene transfer by intramuscular injection. Gene Therapy 5: 514-520.) and other intragene regulatory sequences, such as the poly A and transcription termination sequence (Hartikka, J., Sawdey, M., Conefert-Jensen, F., Margalith, M., Barnhardt, K., Nolasco, M., Vahlsing, H. L., Meek, J., Marquet, M., Hobart, P., Norman, J. and Manthorpe, M. (1996). An improved plasmid DNA expression vector for direct injection into skeletal muscle. Human Gene Therapy 7: 1205-1217.) will also improve transgene expression.

For the naked polypeptide injection, an effective dosage amount of DNA or RNA will be in the range of from about 0.05 g/kg body weight to about 50 mg/kg body weight. Preferably the dosage will be from about 0.005 mg/kg to about 20 mg/kg and more preferably from about 0.05 mg/kg to about 5 mg/kg. Of course, as the artisan of ordinary skill will appreciate, this dosage will vary according to the tissue site of injection. The appropriate and effective dosage of nucleic acid sequence can readily be determined by those of ordinary skill in the art and may depend on the condition being treated and the route of administration. The route of administration is by the parenteral route of injection into the interstitial space of tissues, or other parenteral routes may also be used, such as,

inhalation of an aerosol formulation particularly for delivery to lungs or bronchial tissues, throat or mucous membranes of the nose. In addition, naked polypeptide constructs can be delivered to arteries during angioplasty by the catheter used in the procedure.

5 The immunomodulator may also be delivered in liposome formulations (such as those taught in Felgner P. L. et al. (1995) *Ann. NY Acad. Sci.* 772:126-139 and Abdallah B. et al. (1995) *Biol. Cell* 85(1):1-7) which can be prepared by methods well known to those skilled in the art. Liposomes are lipid bilayers entrapping a fraction of aqueous fluid. DNA will spontaneously associate to the external surface of cationic liposomes
10 (by virtue of its charge) and these liposomes will interact with the cell membrane (Felgner, J. H., Kumar, R., Sridhar, C. N., Wheeler, C. J., Tasi, Y. J., Border, R., Ramsey, P., Martin, M. and Felgner, P. L. (1994). Enhanced gene delivery system and mechanism studies with a novel series of cationic lipid formulations. Journal of Biological Chemistry 269: 2550-2561.). In vitro up to 90% of certain cell lines may be
15 transfected. By including a small amount of an anionic lipid in an otherwise cationic liposome the DNA can be incorporated into the internal surface of the liposome, thus protecting it from enzymatic degradation. (Crespo et al, 1996, cited in Alio, S. F. (1997). Long term expression of the human alpha1 antitrypsin gene in mice employing anionic and cationic liposome vector. Biochemical Pharmacology 54: 9-13.). To facilitate uptake
20 into the cell as endosomes, targeting proteins have been included in liposomes, e.g. anti-MHC antibody (Wang, C., and Huang, L. (1987). pH sensitive immunoliposomes mediate target-cell-specific delivery and controlled expression of a foreign gene in mouse. Proceedings of the National Academy of Sciences USA 84: 7851-7855.) transferrin (Stavridis, J. C., Deliconstantinos, G., Psallidopoulos, M. C., Armenakas, N.
25 S., Hadjiminas, D. J. and Hadjiminas J. (1986). Construction of transferrin-coated liposomes for in vivo transport of exogenous DNA to bone marrow erythroblasts in rabbits. Experimental Cell Research 164: 568-572.), and the Sendai virus or its F protein (Dzau, J. V., Mann, M. J, Morishita, R. and Kaneda, Y. (1996). Fusigenic viral liposome for gene therapy in cardiovascular disease. Proceedings of the National Academy of
30 Sciences USA 93: 11421-11425.). The Sendai virus additionally allows the plasmid DNA to escape from the endosome into the cytoplasm, thus avoiding degradation. The inclusion of a DNA binding protein (28 kDa high mobility group 1 protein) enhances

transcription by bringing the plasmid into the nucleus (Dzau et al, 1997 Am J Cardiol. 1997 Nov 6;80(9A):33I-39I).

Molecular conjugates consist of protein or synthetic ligands to which a DNA binding agent has been attached. Delivery to the cell can be improved by using similar
5 techniques to those for liposomes. Targeting proteins include asialoglycoprotein (Wagner, E., Cotten, M., Foisner, R. and Birnstiel, M. L. (1991). Transferrin-polycation-DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. Proceedings of the National Academy of Sciences USA 88: 4255-4259.), transferrin (Wu, C. H., Wilson, J. M. and Wu., G. Y. (1989). Targeting genes:
10 delivery and persistent expression of a foreign gene driven by mammalian regulatory elements in vivo. Journal of Biological Chemistry. 264: 16985-16987.), polymeric IgA (Ferkol, T., Kaetzel, C. S. and Davis, P. B. (1993). Gene transfer into respiratory epithelial cells by targeting the polymeric immunoglobin receptor. Journal of Clinical Investigation 92: 2394-2400.) and adenovirus (Madon, J. and Blum, H. E. (1996).
15 Receptor mediated delivery of hepatitis B virus DNA and antisense oligodeoxynucleotides to avian liver cells. Hepatology 24: 474-481.). Transgene expression tends to be transient and is limited by endosome/lysosomal degradation.

Illustrative and nonlimiting examples of the present invention are provided below. These examples are not intended to limit the scope of the invention.

20

EXAMPLES

The abbreviations used herein are meant to refer to the following:

Ad IFN	Adenovirus vector expressing woodchuck interferon gamma
Ad RFP	Adenovirus vector without woodchuck interferon gamma gene
CCC DNA	Covalently closed circular viral form of DNA
FTC	5-fluoro-1-(2*R*,5*S*)-1,3-oxathiolan-5-yl]cytosine
GFP	Green fluorescent protein

HBV	Hepatitis B Virus
IFN	Interferon
L-FMAU	1-(2-fluoro-5-methyl-β, L-arabinofuranosyl)thymine
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase chain reaction
Pfu	Plaque forming unit
RFP	Red fluorescent protein
RI	Replication intermediates
RT	Reverse Transcription
TUNEL	terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling assay
WHV	Woodchuck Hepatitis B Virus

Example 1

Administration of L-FMAU and B-L-FTC

In general, B-L-FTC was administrated following a protocol previously described by Cullen et al. (Cullen et al. *Antimicrob. Agents Chemother.* **1997**, *41*, 2076-2082). Specifically, B-L-FTC was injected intraperitoneally at 30mg/kg.

In experiments using L-FMAU, the compound was administered by intraperitoneal administration at a concentration described by Peek et al. (Peek et al. *Hepatology* **2001**, *33*, 254-66) and Zhu et al. (Zhu et al. *J. Virol.* **2001**, *75*, 311-22) (10 mg/kg).

Animals

The woodchuck hepatitis B virus infection model represents a useful model for the evaluation of antivirals since it mimics the history of viral infection observed in humans with chronic hepatitis B. Several studies have shown that this animal model is reliable for use in the study of new nucleoside analogs, particularly in terms of antiviral efficacy and toxicity.

Twelve captive-born woodchucks (*Marmotta monax*) of approximately one year of age and chronically WHV infected were used for *in vivo* experiments. The treatment protocols were performed from July to December 2000.

When adenovirus was injected, blood samples were collected daily during the ten days after the adenovirus injection and twice a week thereafter. These samples were further evaluated for viral markers as described below.

Interferon gene delivery system (Dr. Nassal, Freiburg, Germany)

The adenovirus vector was obtained according to procedures described by He et al. (He, T. C., et al. *Proc Natl Acad Sci U.S.A.* **1998**, *95*, 2509-14). The adenovirus vectors were replication defective due to deletions in the E1 and E3 region. A recombinant cassette with a coding cassette for the pre-IFN gamma gene (aa 1-143) under control of a CMV promoter associated with eCFP gene reporter under the control of SV40 promoter was inserted in the original E1 region (454-3500) (**Figure 1**). The active woodchuck IFN gamma production efficacy was assessed by protection from VSV infection of the woodchuck cell line WCH17.

The same adenovector containing only the pSV40-eCFP cassette was used as control. Adenoviral vectors were administered by intravenous injection to target the liver. The vectors were administered at concentrations of $3x10^7$ to $3x10^{11}$ pfu/animal, diluted in phosphate buffered saline (PBS).

Example 2

Evaluation of L-FMAU + B-L-FTC + Ad IFN

Eighteen woodchucks (*Marmota monax),* experimentally infected with WHV were purchased from Northeastern Wildlife (South Plymouth, N.Y.) The woodchucks
5 were chronically infected and were used for *in vivo* experiments.

Both B-L-FTC (30 mg/kg) and L-FMAU (10 mg/kg) were administrated intraperitoneally. Daily injections were performed during the first week, followed by injections every other day for the next 7 weeks.

A new recombinant cassette of the woodchuck interferon gamma (IFN) gene
10 under the control of a CMV promoter, which was associated with an eRFPnuc gene reporter under the control of a SV40 promoter was inserted in the original E1 region of the adenoviral vector (Ad IFN). The same adenovirus vector containing pSV40-controlled eGFPnuc alone was used as control (AdGFP) (provided by Dr. Michael Nassal, University of Freiburg, Germany).

15 For these experiments, intravenous injections of recombinant adenoviral vector expressing woodchuck interferon gamma were performed twice (at week 4 and week 8) with $3x10^{10}$ pfu per animal. The viral inoculation was validated in a previous study presented at the HBV meeting held in Amherst, MA, USA, August 2001 (see **Figure 2**).

During therapy, blood samples were collected every two days, and every day for
20 five days after each injection of Ad IFN. After drug withdrawal, blood samples were collected every two days until viral load began to increase, then once a week and, then twice a month.

Example 3

Liver biopsy

25 Surgical liver biopsy was performed after laparatomy under general anesthesia prior to therapy (TO), twice during treatment (M1, M2) and 4 months after drug withdrawal (M3). The on-treatment biopsies were made 4 days after each adenovirus

injection. A part of each sample was stored at -80°C for viral DNA analysis. Another part was fixed in formalin and embedded in paraffin for liver histology and immunostaining. The last part was fixed in 1% osmium tetroxide for electron microscopy study (TO and M2). A macroscopic liver examination was also performed during surgery.

Analysis of intrahepatic viral DNA synthesis

Intrahepatic viral DNA from infected woodchucks was extracted at the time of liver biopsy. After homogenization in 10mM Tris-HCl (pH 7.5), 10 mM EDTA, the liver samples were divided in two parts, one for isolation of total viral DNA (after proteinase K digestion, phenol-chloroform extraction followed by ethanol precipitation) and the other for isolation of non-protein bound covalently close circular (CCC) viral DNA (after SDS-KCl precipitation of protein bound DNA, phenol-chloroform extraction followed by ethanol precipitation). To normalize the amount of DAN in each sample used for Southern blot analysis, DNA concentration was determined by UV densitometric analysis and compared with previously quantified DNA after electrophoresis through agarose gel. Five micrograms of nucleic acids corresponding to total DNA or CCC DNA preparations were then subjected to electrophoresis through 1.2 % agarose gels, alkalin transfer by blotting to nylon membrane, and viral DNA was detected by a specific hybridization as described above.

Analysis of liver histology

Formalin-fixed liver biopsy tissue sections were stained with hematoxylin-eosin-safran (HES) stain and examined under a light microscope. The degree of hepatocyte necrosis (acidophilic bodies), the level of inflammation of the portal tract and the intralobular space, and the fibrosis stage were semi quantitatively assessed by using the Metavir score (*Hepatology*. **1994**;20(1 Pt 1):15-20). Liver biopsy sections were also assessed for steatosis, ductular proliferation, hepatocyte dysplasia, and hepatocellular carcinoma.

Liver histology examination revealed signs of acute exacerbation of hepatitis that were comparable in both groups of animals that received Ad IFN or Ad GFP vector while mild inflammatory activity was observed in the untreated animal.

Immunostaining studies performed on liver biopsy sections obtained prior to injection and D5 post injection with a polyclonal anti WHV antibody, showed a significant decrease of the number of infected hepatocytes in woodchucks A37 and A36.

Example 4

Analysis of viremia

Dot Blot Assay

Viremia was assessed by quantitative detection of woodchuck hepatitis B virus (WHV) DNA. WHV DNA was extracted from serum using HighPure PCR template DNA extraction kit (Roche daignostics), and samples corresponding to 50μL of serum were spotted on membranes (Biodyne B, 0.45 μM, Merck Eurolab) with serial dilutions of a WHV DNA standard. After fixation procedure, filters were hybridized with ^{32}P labeled full-length WHV DNA probe. A comparative analysis of phosphoImager scans (Amersham Pharmacia Biotech) was performed with the ImageQuant software. The detection limit of the assay was 6.10^7 copies/mL (200 pg/mL).

Endogenous Polymerase Assay (EPA)

The effect of Ad IFN on the synthesis of viral plus strand DNA was determined using WHV particles in an endogenous polymerase assay (EPA). For comparison, the DNA dependent DNA polymerase inhibitor, phosphonoformic acid (PFA) was used as control. The endogenous polymerase activity corresponds to the completion of viral plus strand DNA in mature particles containing relaxed circular DNA. WHV associated DNA polymerase activity was assayed by mixing 50μL of partially purified viral particles with 25μL of reaction buffer (Tris-HCl 50mM pH7.6, $MgCl_2$ 40mM, NH_4Cl 60mM, NP40 0.5%, β-mercaptoethanol 10mM) containing: (i) dATP, dCTP, dGTP (100μL each) and ^3H-dTTP (0.17μM). enzyme assays were performed as described by

Hantz, et al. (Hantz O, et al. 1992 *Virology*. Sep;190(1):193-200). Briefly, enzyme reactions were incubated at 37°C for 3 hours and spotted on GF/C fiber glass filters (Whatman), and extensively washed by trichloroacetic acid 5%. Incorporation of ^{3}H-dTTP was measured in a Beckman LS 6000SC scintillation counter. The limit of detection of this assay was 1,000 cpm/mL.

Real time PCR assay (Light Cycler)

In cases where viral titers were too low to be easily detected by Dot blot hybridization, real time PCR techniques were utilized (see for e.g. Dandri et al. *Hepatology* **2000**, *32*, 139-46). Real time PCR has been known to detect as few as 10 copies of human HBV DNA. Therefore, to complete the analysis of viremia, real time PCR was used to detect WHV DNA below $6x10^7$ copies/ml (200 pg/ml). The same WHV DNA standard was used for both the dot blot and real time PCR assays. PCR assays on Dot blot positive sample provided similar quantitative result. The lower limit of detection of this method was 25 WHV copies/μL.

Preliminary results of kinetics of the viral clearance and rebound in 3 woodchucks, analyzed by real time PCR, are presented in **Figure 10**. Viral titer was shown to reach a low value of 0.320 pg/ml, representing an average of $9x10^4$copies/ml. This technique could also be applied to further animal samples to determine the efficacy of B-L-FTC alone or in combination with immunomodulators, such as Ad IFN.

Example 5

Inoculum titer determination

To determine the antiviral efficacy and tolerance of the adenoviral vector inoculum, 4 animals received intravenous Ad-IFN injection at concentrations of 3.10^7 (A33), $3x10^9$ (A30) and $3x10^{11}$ pfu (A32 and A34).

The highest titered inoculum (3.10^{11} pfu) induced a marked drop of viral load, however all 2 animals died at day 2 and day 5 post injection (WHV DNA did drop between D0 and day of death: from 147 ng/ml to 70 ng/ml and from 1067 ng/ml to 265

ng/ml respectively). The $3x10^9$ pfu inoculum induced a transient decrease from 70 ng/ml to approximately 30 ng/ml for 6 days. The lowest inoculum did not induce a significant viremia decrease (**Figure 3**).

Example 6

5 *Efficacy study: evaluation of the antiviral effect of the Ad IFN vector*

Five woodchucks (A35, A36, A37, A38, A42) received $3x10^9$ pfu of the complete Ad IFN vector. A control group consisting of two woodchucks (A39, A40) received $3x10^9$ pfu of the Ad GFP vector, and one woodchuck (A41) was untreated (**Figures 4** and **5**).

10 In the Ad IFN treated group, a transient WHV DNA decrease was observed in 3 of 5 treated animals (A35 from 46 ng/ml to 20 ng/ml; A37 from 76 to 20 ng/ml and A42 from 20 to 5 ng/ml) after adenovirus inoculation. WHV DNA results were also assessed by EPA (**Figures 4** and **5**). Southern Blot Hybridization of intrahepatic WHV DNA showed a marked decrease of the viral DNA (from 16 to 76%, except A42) as well as
15 CCC DNA levels (from 2 to 76%) (**Figure 6**).

Example 7

Evaluation of L-FMAU and B-L-FTC.

Results showed a significant inhibition of viremia which was reduced approximately by 4 to 5 log units, down to the limit of detection of the Dot blot assay
20 (6.10^7 copies/ml) in all animals treated by B-L-FTC, L-FMAU, and Ad IFN (**Figure 7**). However, controls (untreated, Ad GFP) or woodchucks treated with Ad IFN alone showed no significant variation in viremia (**Figures 8** and **9**).

Treatment induced reduction of viral load was remarkably rapid. Viremia decreased to undetectable levels ($<6.10^7$/ml) in less than one to two weeks after the
25 beginning of the therapy.

Viremia in the animals treated with B-L-FTC, L-FMAU, and Ad IFN remained suppressed for various lengths of time following withdrawal of treatment. Furthermore, the viral load in treated animals did not return to pretreatment levels. Viremia level of two woodchucks (A52 and A49) treated with L-FMAU, B-L-FTC, and Ad IFN were still under detectable level at week 28 (6 months after drug withdrawal).

Results of combination therapy with an immunomodulator showed a rapid and significant decrease of viremia level (average of 5 $\log_{10}$ decrease within two weeks). This decrease is significant compared to results obtained with B-L-FTC and L-FMAU alone. In previous studies, viral titers in serum dropped about 56 and 1,000 fold respectively in the same time frame. This observation indicates that the antiviral activity of B-L-FTC and L-FMAU was improved when combined together with an immunomodulator. Moreover, this antiviral effect was prolonged over time with a delayed viral rebound. Viremia remained undetectable in dot blot assay ($<6.10^7$copies/ml) in serum samples of two animals (A49 and A52) 6 to 7 months after drug withdrawal. This prolonged effect is consistent with decreases in CCC DNA levels (-69% and -75% respectively). The delay in the rebound of viral replication following the cessation of therapy may reflect the time required to re-establish CCC DNA pools through reinfection of new hepatocytes and/or reamplification of existing intracellular pools of CCC DNA in infected cells.

Intrahepatic CCC DNA.

Southern blot analysis of intrahepatic viral DNA (**Figure 11**) demonstrated changes in the levels of intrahepatic WHV replication intermediates that were generally consistent with viremia patterns. A marked decrease in the level of viral DNA synthesis was observed in both B-L-FTC, L-FMAU treated groups, with 82 to 93% decrease in the level of intrahepatic viral DNA intermediates compared to the pretreatment of levels (**Figures 12** and **13**). While CCC DNA persisted in the liver at M1 and M2, other WHV DNA replicative intermediates decreased dramatically.

Moreover, CCC DNA was also reduced by 24 to 47% during L-FMAU B-L-FTC bitherapy. However, the inhibition of WHV replication by L-FMAU/B-L-FTC

administration was not followed by the clearance of intrahepatic viral CCC DNA, as demonstrated by southern blot analysis.

However in untreated woodchuck, total or CCC DNA increased over time by + 64% and + 77% respectively (**Figure 14**).

5 Compared to Cullen et al. (Cullen et al. *Antimicrob. Agents Chemother.* **1997**, *41*, 2076-2082), Zhu et al. (Zhu et al. *J. Virol.* **2001**, *75*, 311-22) and Peek et al. (Peek et al. *Hepatology* **2001**, *33*, 254-66) results, B-L-FTC combined with IFN and L-FMAU have a significant antiviral effect. Replicative intermediates decreased to undetectable levels in the liver one month after the beginning of the treatment. In contrast, the intrahepatic 10 CCC DNA levels declined at a much lower rate than replicative DNA. Combination of B-L-FTC and L-FMAU decreased viral CCC DNA 40% (versus a decrease of 98% for the DNA replication intermediates). This effect could be due to the antiviral effect of the L-FMAU alone as it was demonstrated by Peek, et al. (Peek et al. *Hepatology* **2001**, *33*, 254-66), who showed an average 10-fold decrease in CCC DNA). However, after 8 to 9 15 months of a treatment with a single drug, L-FMAU, drug resistant strains of WHV emerged (Zhou, T., et al. *J. Viron.* **2000**, *74*, 11754-63). L-FMAU in combination to B-L-FTC may avoid emergence of drug resistant strains and allow long-term treatment that could deplete the residual pool of CCC DNA.

Example 8

20 *Intrahepatic WHV DNA synthesis.*

Unlike the results with the combination therapy, Ad IFN alone did not affect total viral and CCC DNA levels in the liver (+44% and -6% respectively compared to + 43% for total DNA of Ad GFP group) **(Figure 15)**.

Example 9

Evaluation of hepatocytes transduction by the recombinant adenovirus vector

To determine the number of hepatocytes infected by adenovirus, immunostaining with commercial antibody directed against the Green Fluorescent Protein (GFP) was
5 performed. However, fluorescence observed was not specific when compared with controls, and may have been due to natural fluorescence of hepatocytes or perhaps biliary pigments.

Example 10

Histology examination

10 Examination of liver biopsy sections of animals treated with B-L-FTC combination therapy revealed inflammation with lobular lesions in the liver. All woodchucks of the group treated with Ad IFN showed increased inflammatory activity (**Figure 15**). Maximal inflammatory activity in Metavir score was observed in six biopsies in this group. In comparison, the group treated with L-FMAU and B-L-FTC did
15 not show similar profiles. Only three of the six woodchucks in this group showed an increase of the inflammatory activity. In control groups, Metavir score was stable over time, even in the group treated with Ad IFN alone.

Example 11

Analysis of viremia

20 Viremia could be analyzed using the dot blot assay for WHV DNA and quantified with a phosphoimager until viremia returns to pretreatment values.

If elimination of virus is to be confirmed, DNA extracted from serum under PCR conditions should be undetectable by the dot blot assay (viremia under 6.10^7 copies/ml). Furthermore, the DNA must be analyzed by a real time PCR (Light Cycler) assay. In

these examples, the real time PCR method was adapted for WHV detection DNA using specific primers

Example 12

Analysis of intrahepatic viral DNA synthesis

5 Liver sections of the a biopsy taken at 5 months post treatment were analyzed by southern blot hybridization to analyze intrahepatic viral DNA synthesis.

Example 13

WHV polymerase gene sequencing

Genome sequence from circulating virions could be analyzed after PCR 10 amplification of the PreS and the RT regions, to determine whether or not escape mutants had been selected by one of the antiviral protocols.

WHV DNA was amplified by PCR from serum collected either at the end of the treatment period or at the best point before the animal death. DNA from serum was amplified for 35 cycles (94°C for 1 min, 50°C for 1 min, and 72°C for 1 min) with Taq 15 polymerase and a specific primer pair (5'-AGATTGGTTGGTGCACTTCT-3 (nucleotides 385 to 403) and 5'-ATTGTCAGTGCCCAACA-3' (nucleotides 1468 to 1461), corresponding to the B and C domains of the reverse transcriptase gene, with reference to previously published sequences. To confirm that no viral DNA is present, another primer pair was used for nested PCR in samples found to be negative in the first 20 round of amplification, specifically 5'-GGATGTATCTGCGGCGTTT-3' (nucleotides 510 to 528) and 5'-CCCAAATCAAGAAAAACAGAACA-3' (nucleotides 953 to 931).

Example 14

Examination of liver mitochondria by electron microscopy

Liver biopsy sections were fixed in 1% osmium tetroxide in 150 mM sodium cacodylate HCI (pH 7.4), dehydrated in graded ethanol, and embedded with methanolic
5 uranyl acetate and lead citrate, and will be analyzed with a transmission electron microscope 1200EX to rule out mitochrondrial toxicity of the combination B-L-FTC therapy.

Example 15

Visualisation of the GFP or RFP

10 The number of cells infected by the adenovirus vector could be monitored by direct observation of a five-micrometers-thick deparaffinized liver tissue sections by confocal microscopy using appropriate filters.

Example 16

Evaluation of the number of WHV-infected hepatocytes

15 The number of VHW infected cells could be monitored by immunostaining for surface and capsid proteins. To do this, five-micrometers-thick deparaffinized liver tissue sections were incubated overnight with rabbit serum containing polyclonal antibody directed against the WHV core or pre-S proteins. The sections were thereafter incubation with a biotinylated goat anti-rabbit immunoglobulin G. The antigen-antibody
20 complex was revealed using streptadivin-horseradish peroxidase. Quantification of the number of infected hepatocytes expressing viral proteins was performed in representative areas of the liver sections.

Example 17

Analysis of hepatocytes turnover

The number of hepatocytes undergoing apoptosis could be determined using the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) method (Kerr, J.F.R., Wyllie, A.H. and Currie, A.R. 1972 *Br. J. Cancer* 26:239–257). The number of hepatocytes that progress in the cell cycle mitosis could be determined by immunostaining for PCNA.

This invention has been described with reference to its preferred embodiments. Variations and modifications of the invention, will be obvious to those skilled in the art from the foregoing detailed description of the invention. It is intended that all of these variations and modifications be included within the scope of this invention.

CLAIMS

What is claimed is:

5 1. A method for the treatment or prophylaxis of a host infected with hepatitis B virus comprising administering an effective amount of β-L-FTC or its pharmaceutically acceptable salt or prodrug in combination or alternation with an immunomodulator, optionally independently in pharmaceutically acceptable carriers.

10 2. The method of claim 1, wherein the immunomodulator is an immunostimulatory agent (ISS).

3. The method of claim 1, wherein the host is a human.

15 4. The method of claim 2, wherein the ISS comprises the sequence 5'-T, C, G-3'.

5. The method of claim 2, wherein the ISS comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3' or 5'-purine, purine, C, G, pyrimidine, 20 pyrimidine, C, C-3'.

6. The method of claim 2, wherein the ISS comprises a sequence selected from the group consisting of 5'-AACGTTCC-3', 5'-AACGTTCG-3', 5'-GACGTTCC-3' and 5'-GACGTTCG-3'.

25

7. The method of claim 2, wherein the ISS comprises the sequence 5'-TGACTGTGAACGTTCGAGATGA-3'.

8. The method of claim 2, wherein the ISS is a nucleic acid sequence 30 comprising an unmethylated CG sequence.

9. The method of claim 1, wherein the immunomodulator is a cytokine, adjuvant or antibody.

10. The method of claim 9, wherein the immunomodulator is a cytokine selected from the group consisting of interleukin 2, interleukin 4, interleukin 6, interleukin 7, interleukin 12, granulocyte-macrophage colony stimulating factor, granulocyte colony stimulating factor, interferon-gamma, and tumor necrosis factor-alpha.

11. The method of claim 1, wherein the immunomodulator is an interferon.

12. The method of claim 11, wherein the interferon is selected from the group consisting of pegylated interferon alpha 2a, interferon alphacon-1, natural interferon, albuferon, interferon beta-1a, omega interferon, interferon alpha, interferon gamma, interferon tau, interferon delta, consensus interferon and interferon gamma- 1b.

13. The method of claim 1, wherein the immunomodulator is a hepatitis B active antigen vaccine.

14. The method of claim 13, wherein the vaccine is a hepatitis B surface antigen vaccine.

15. The method of claim 13, wherein the vaccine is selected from Engerix-B® (GlaxoSmithKline), Recombivax HB® (Merck), Hepatitis B Vaccine (Recombinant), PJ Hep B DNA therapeutic vaccine (Powderject Pharmaceuticals), Hepavax-Gene® (DNA recombinant hepatitis B vaccine , Berna Biotech group), Gen H-B Vax™ (Chiron Corporation), Hepatavax-B® (Merck & Co.), Hevac B® (Pasteur), KGC® (Korea Green Cross), TGP 943™ (Takeda Chem, Japan), Gen Hevac B® (Pasteur, France), Bio-Hep-B™/Sci-B-Vac™ (Bio-Technology General, Israel), AG-3™, Hepagene™, and Hepacare™, (Medeva, UK, Evans UK).

16. The method of claim 1, further comprising administering bis pom PMEA (adefovir).

17. Use of an effective amount of β-L-FTC or its pharmaceutically acceptable salt or prodrug in combination or alternation with an immunomodulator, optionally independently in pharmaceutically acceptable carriers the manufacture of a medicament for the treatment of a host infected with hepatitis B virus.

5

18. The use of claim 17, wherein the immunomodulator is an immunostimulatory agent (ISS).

19. The use of claim 17, wherein the host is a human.

10

20. The use of claim 18, wherein the ISS comprises the sequence 5'-T, C, G-3'.

21. The use of claim 18, wherein the ISS comprises the sequence 5'-purine,

15 purine, C, G, pyrimidine, pyrimidine, C, G-3' or 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, C-3'.

22. The use of claim 18, wherein the ISS comprises a sequence selected from the group consisting of 5'-AACGTTCC-3', 5'-AACGTTCG-3', 5'-GACGTTCC-3' and 5'-

20 GACGTTCG-3'.

23. The use of claim 18, wherein the ISS comprises the sequence 5'-TGACTGTGAACGTTCGAGATGA-3'.

25 24. The use of claim 18, wherein the ISS is a nucleic acid sequence comprising an unmethylated CG sequence.

25. The use of claim 17, wherein the immunomodulator is a cytokine, adjuvant or antibody.

30

26. The use of claim 25, wherein the immunomodulator is a cytokine selected from the group consisting of interleukin 2, interleukin 4, interleukin 6, interleukin 7, interleukin 12, granulocyte-macrophage colony stimulating factor,

granulocyte colony stimulating factor, interferon-gamma, and tumor necrosis factor-alpha.

27. The use of claim 17, wherein the immunomodulator is an interferon.

5

28. The use of claim 27, wherein the interferon is selected from the group consisting of pegylated interferon alpha 2a, interferon alphacon-1, natural interferon, albuferon, interferon beta-1a, omega interferon, interferon alpha, interferon gamma, interferon tau, interferon delta, consensus interferon and interferon gamma- 1b.

10

29. The use of claim 17, wherein the immunomodulator is a hepatitis B active antigen vaccine.

30. The method of claim 29, wherein the vaccine is a hepatitis B surface

15 antigen vaccine.

31. The method of claim 30, wherein the vaccine is selected from Engerix-B® (GlaxoSmithKline), Recombivax HB® (Merck), Hepatitis B Vaccine (Recombinant), PJ Hep B DNA therapeutic vaccine (Powderject Pharmaceuticals),

20 Hepavax-Gene® (DNA recombinant hepatitis B vaccine , Berna Biotech group), Gen H-B Vax™ (Chiron Corporation), Hepatavax-B® (Merck & Co.), Hevac B® (Pasteur), KGC® (Korea Green Cross), TGP 943™ (Takeda Chem, Japan), Gen Hevac B® (Pasteur, France), Bio-Hep-B™/Sci-B-Vac™ (Bio-Technology General, Israel), AG-3™, Hepagene™, and Hepacare™, (Medeva, UK, Evans UK).

25

32. The use of claim 17, further comprising administering bis pom PMEA (adefovir).

FIGURE 1

ADENOVIRUS VECTOR FOR WODDCHUCK INTERFERON GAMMA DELIVERY

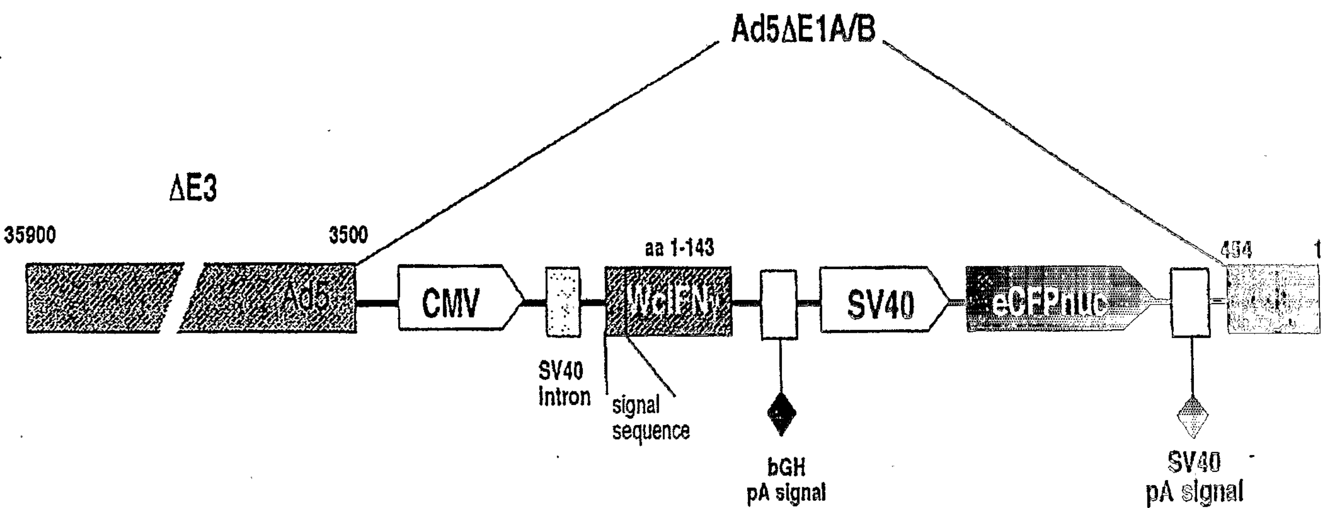


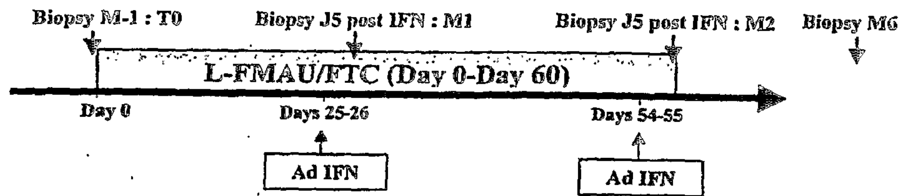
FIGURE 2

WOODCHUCK TREATMENT

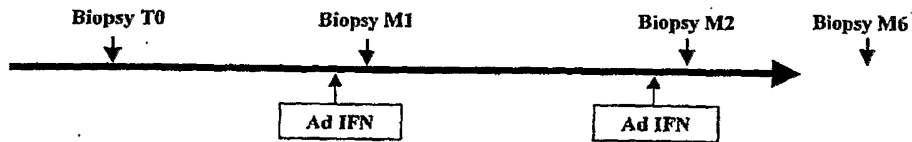
Treatment with L-FMAU 10 mg/kg FTC 30 mg/kg +/- injection of Ad IFN

➢ 8 Woodchucks treated with Ad IFN

- 6 woodchucks treated by L-FMAU + FTC with injection of Ad IFN : A43 A44 A45 A46 A47 A48

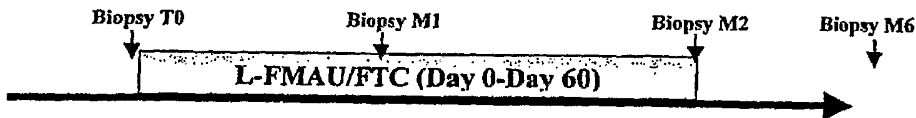


- 2 woodchucks without L-FMAU + FTC with injection of Ad IFN A38 A41



➢ 10 woodchucks controls

- 6 woodchucks treated by L-FMAU FTC without injection of Ad IFN A49 A50 A51 A52 A53 A54



- 2 woodchucks without antiviral treatment (L-FMAU FTC) but with injection of Ad GFP (W657 658)
- 1 without any treatment (W642)

FIGURE 3

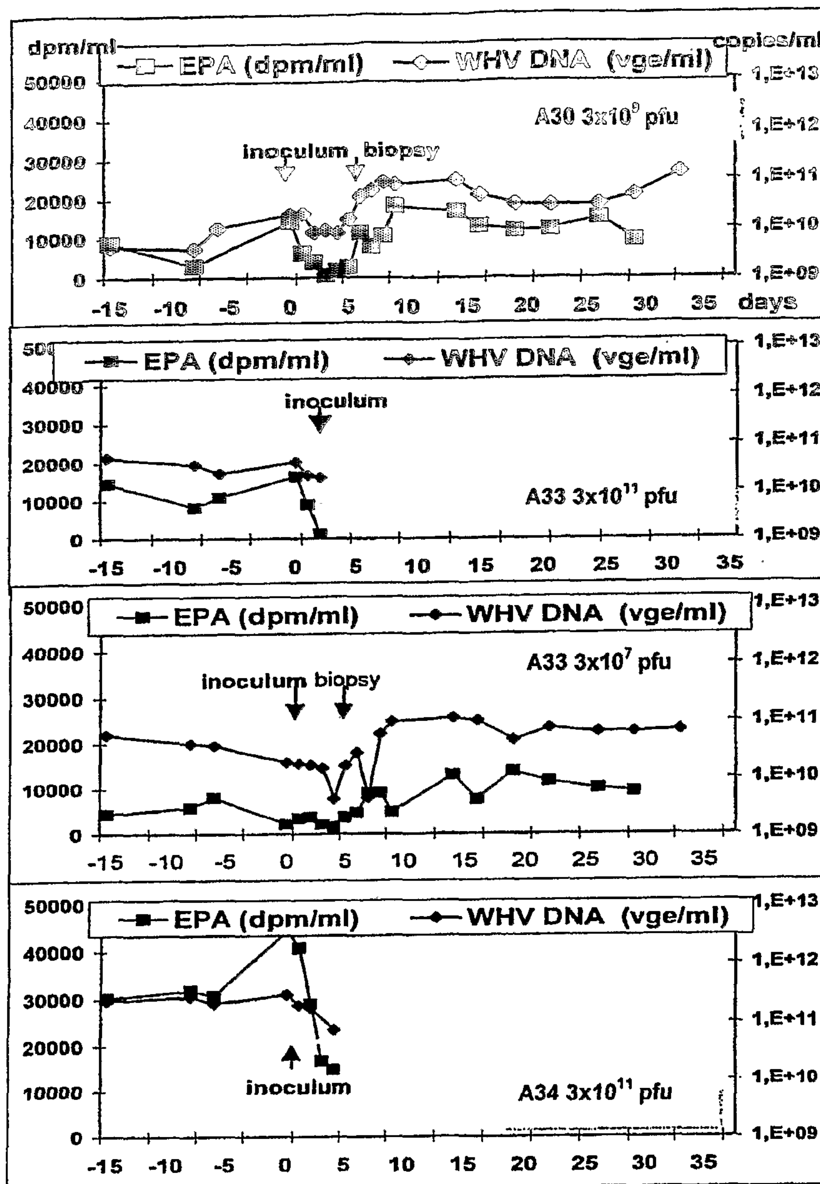


FIGURE 4

Ad-IFN-GFP GROUP VIREMIA FOLLOW UP

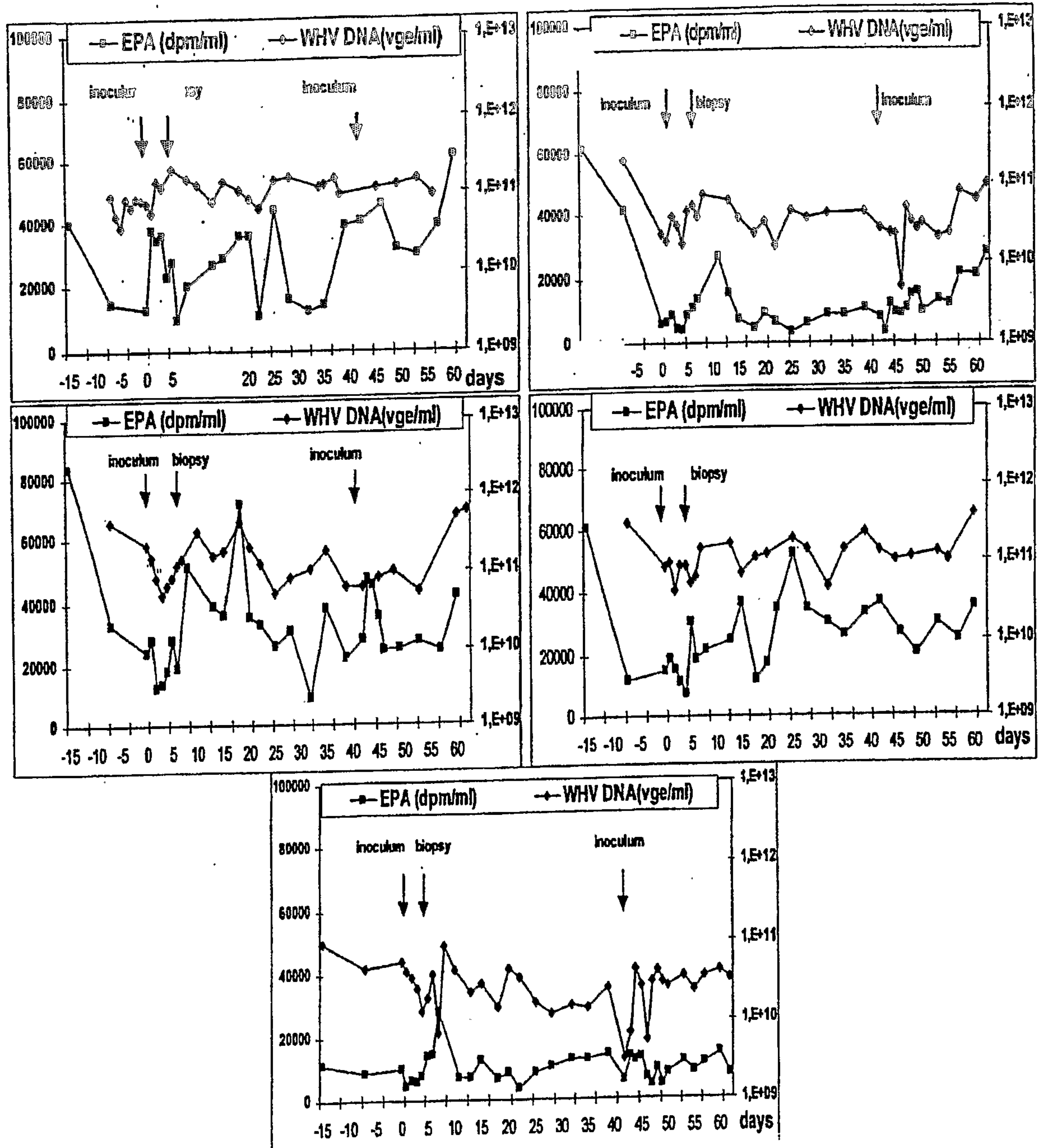
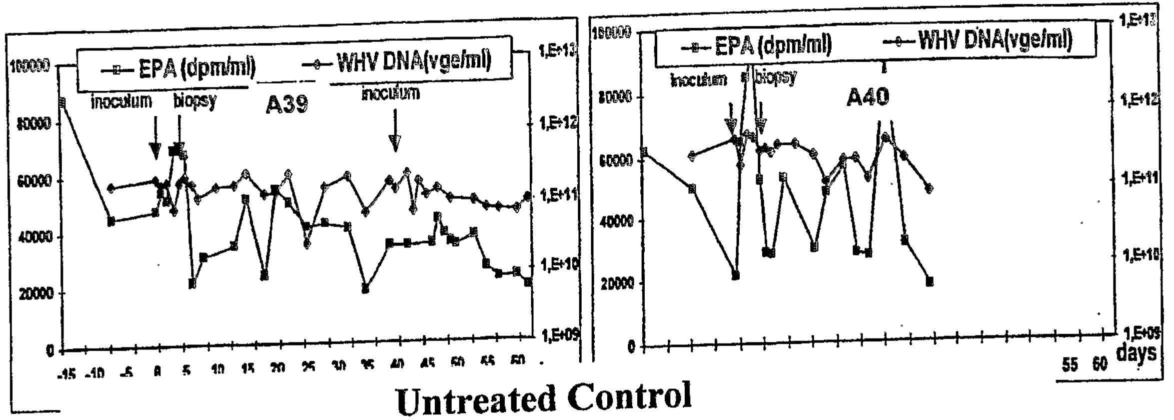


FIGURE 5

Ad-GFP CONTROL GROUP VIREMIA FOLLOW UP



UNTREATED WOODCHUCK VIREMIA FOLLOW UP

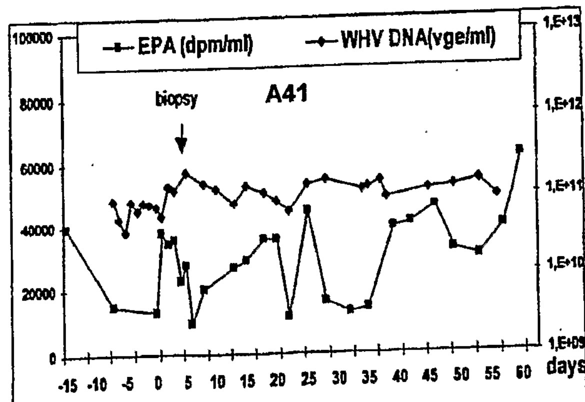


FIGURE 6

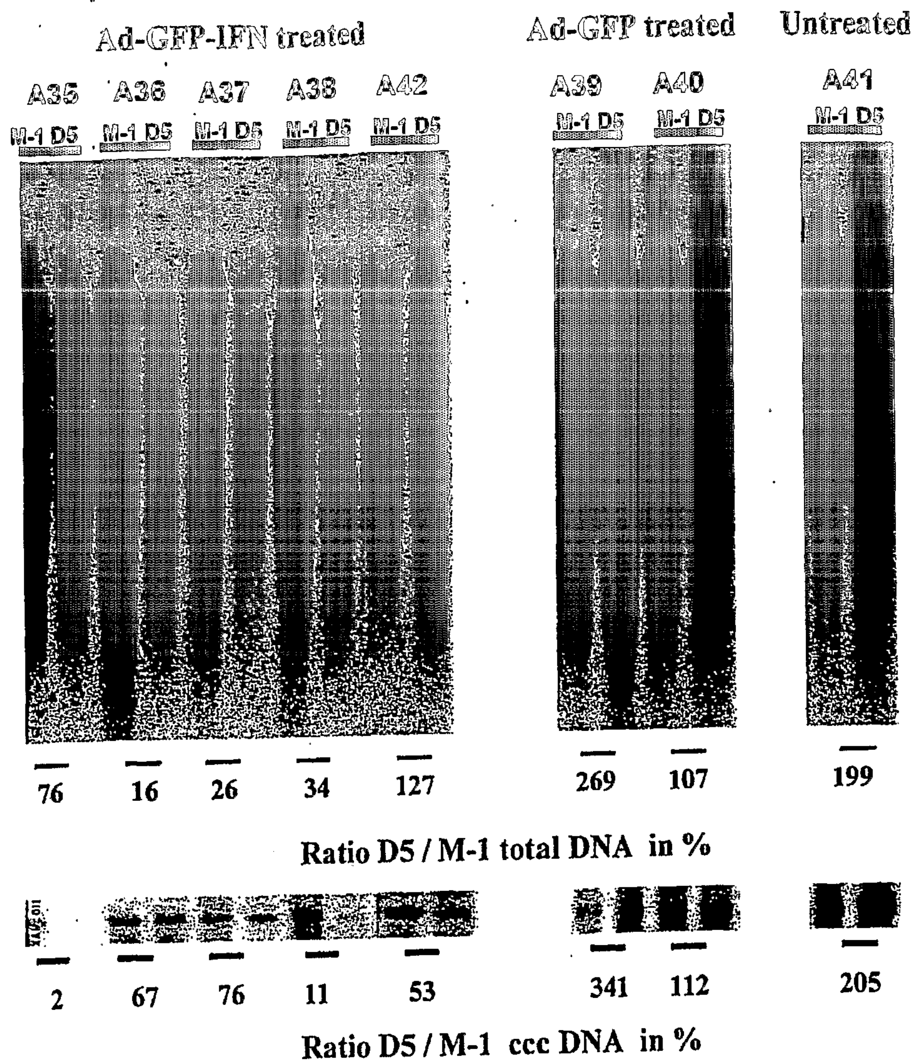


FIGURE 7

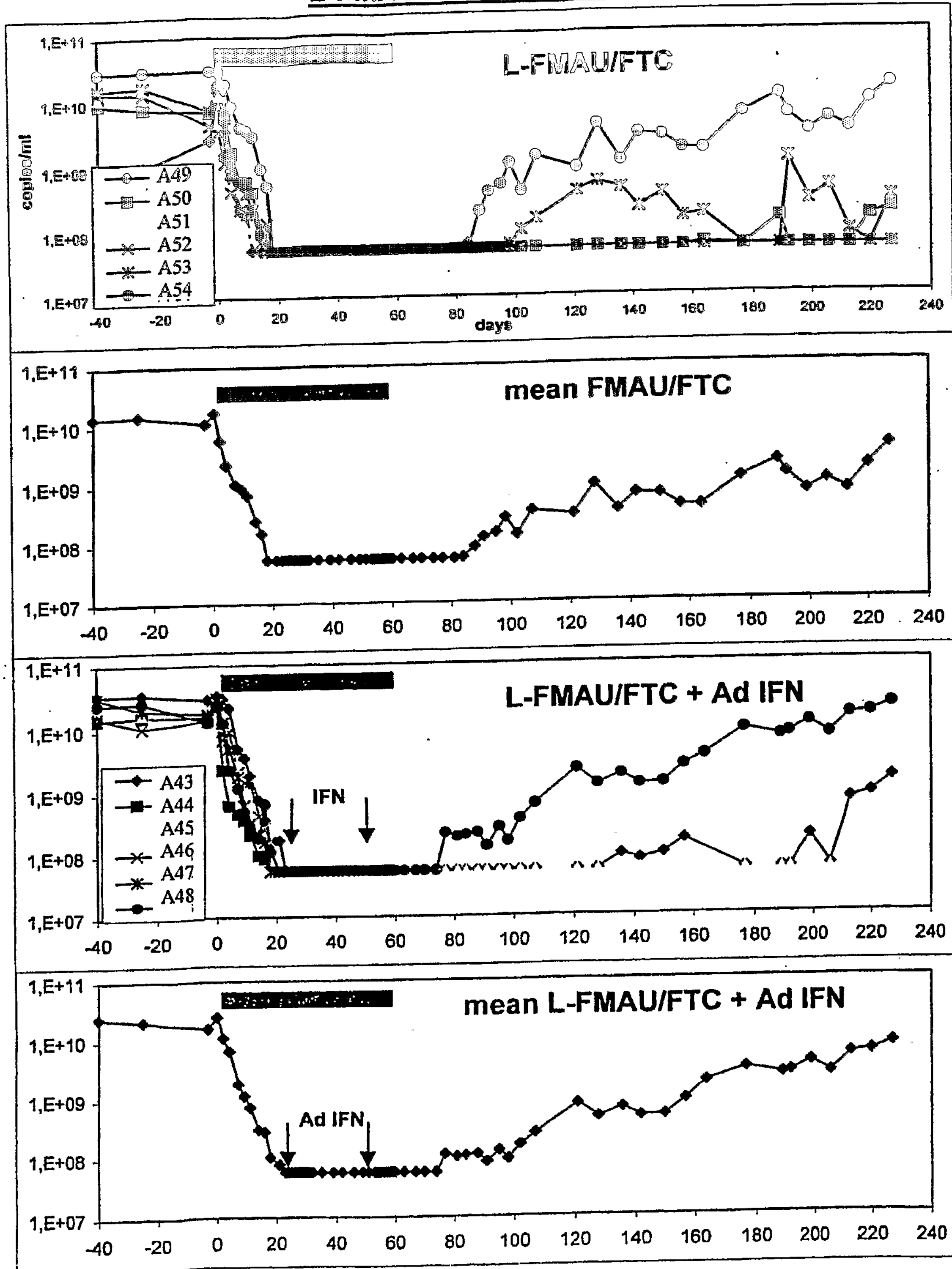


FIGURE 8

Viremia analysis by dot blot assay
Controls

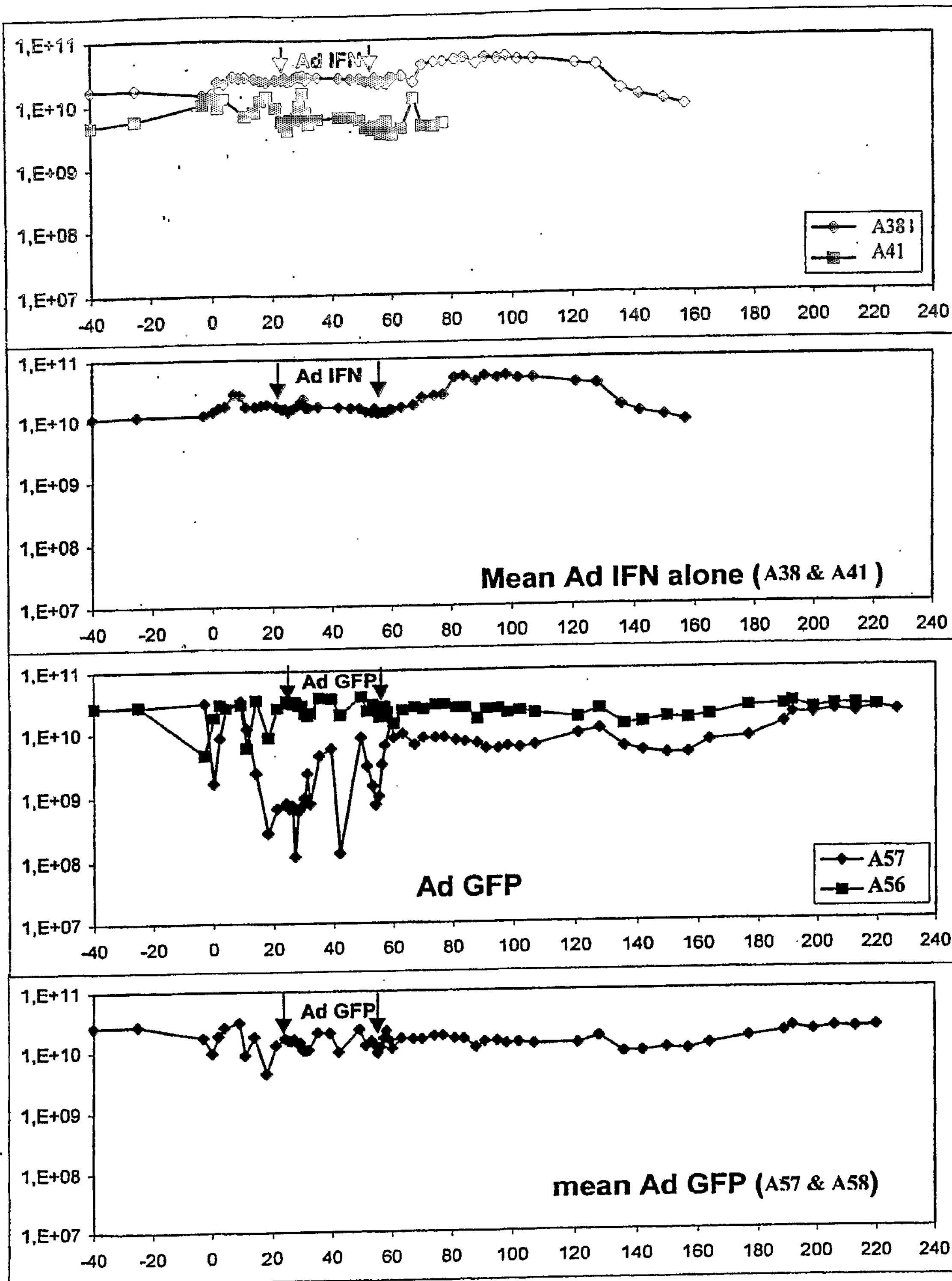


FIGURE 9

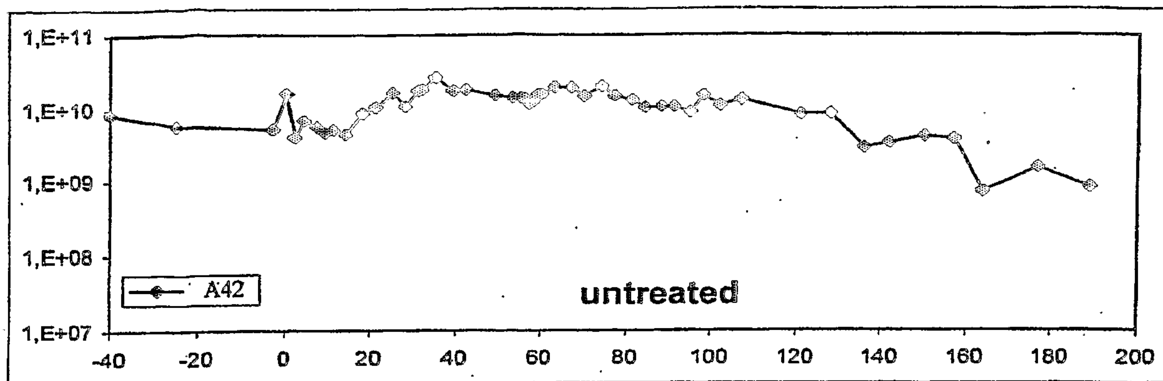


FIGURE 10

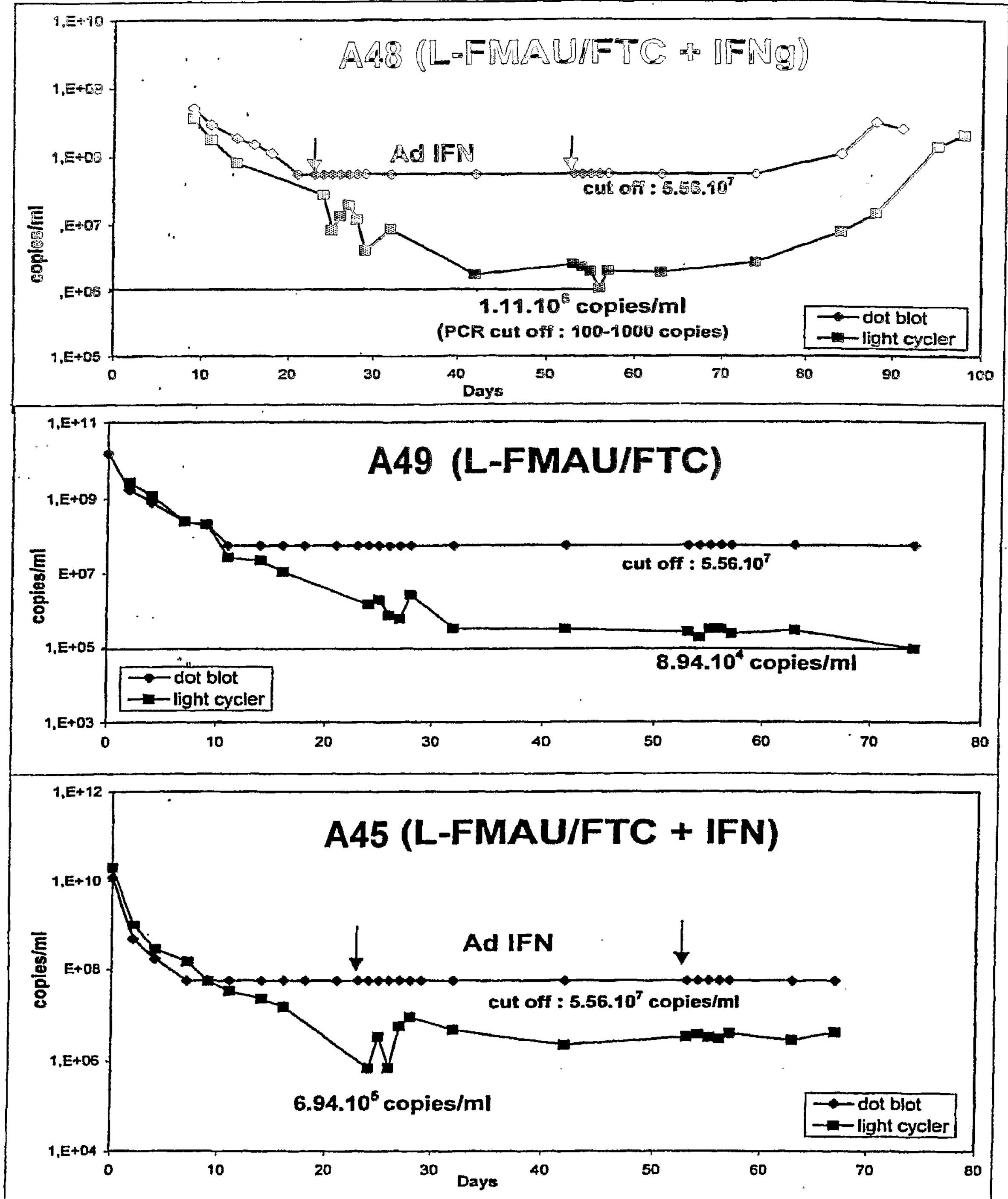


FIGURE 11

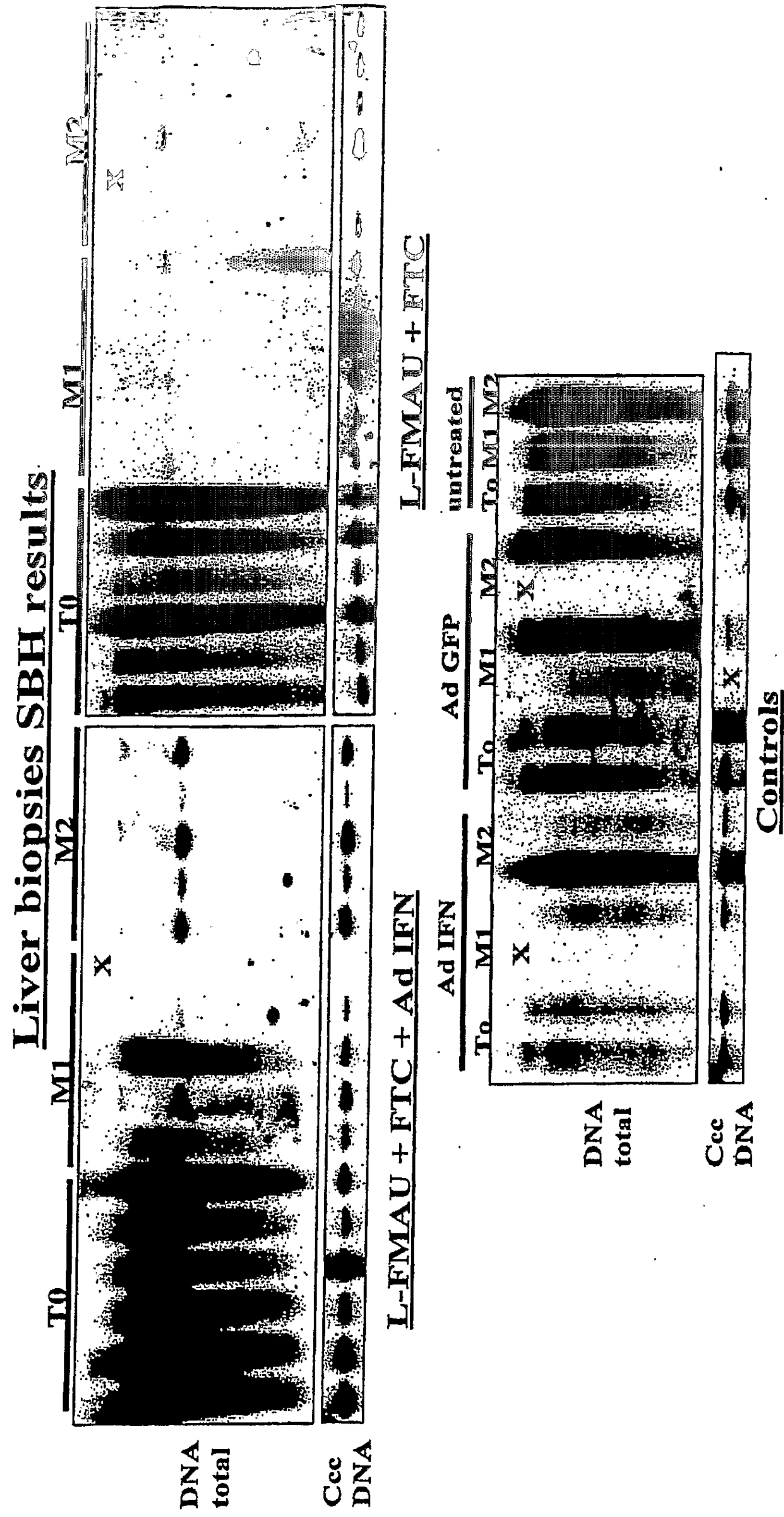


FIGURE 12

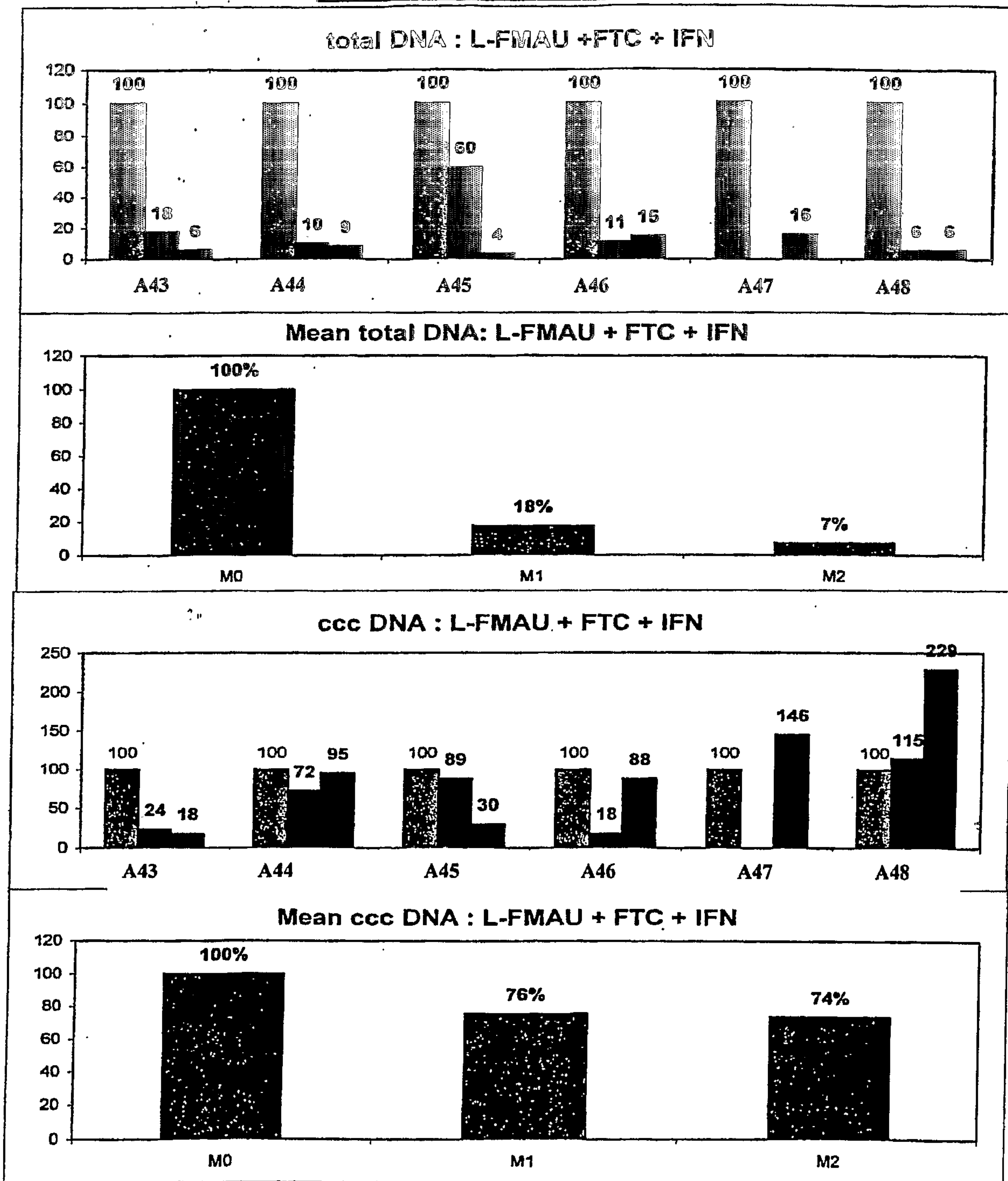


FIGURE 13

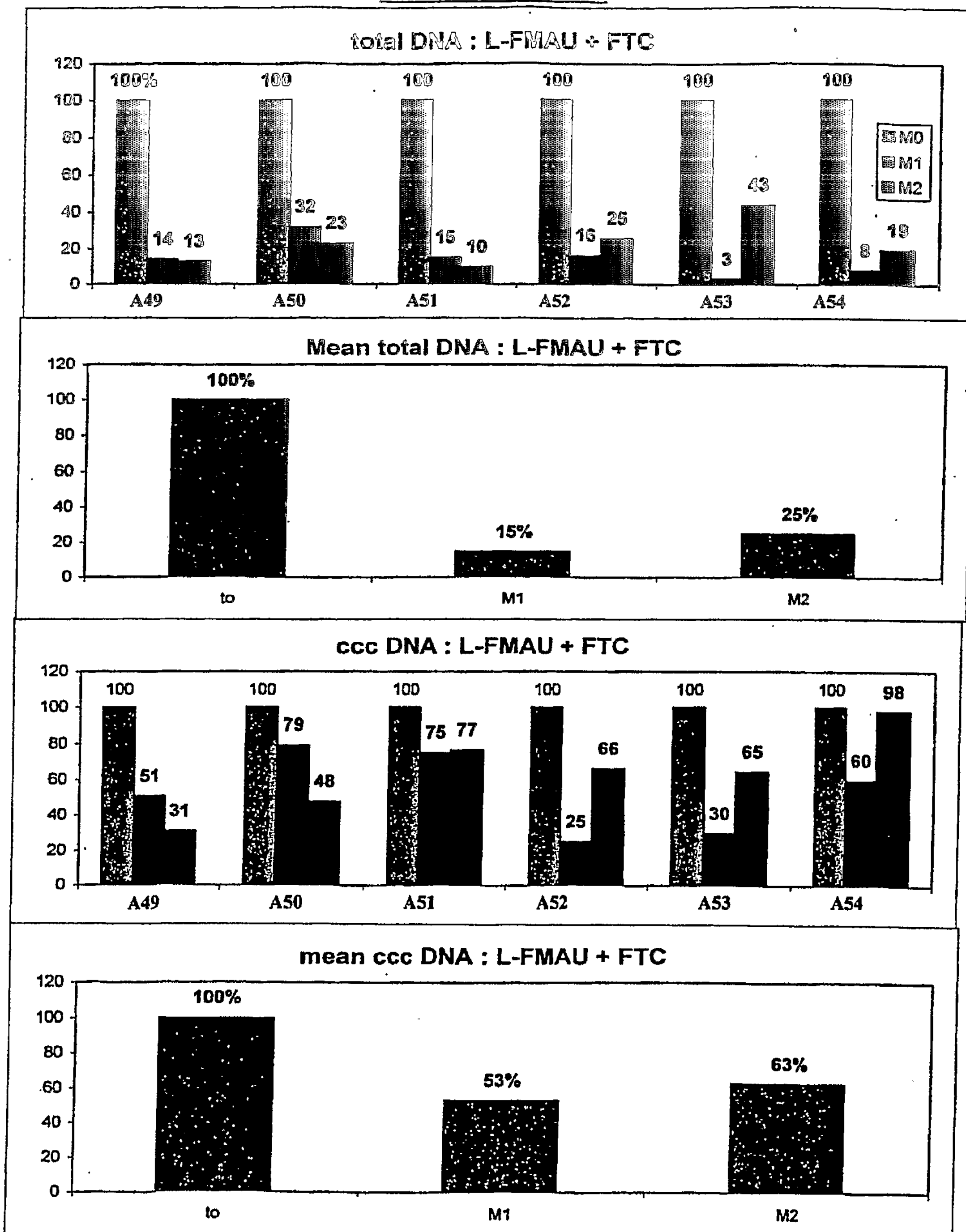


FIGURE 14

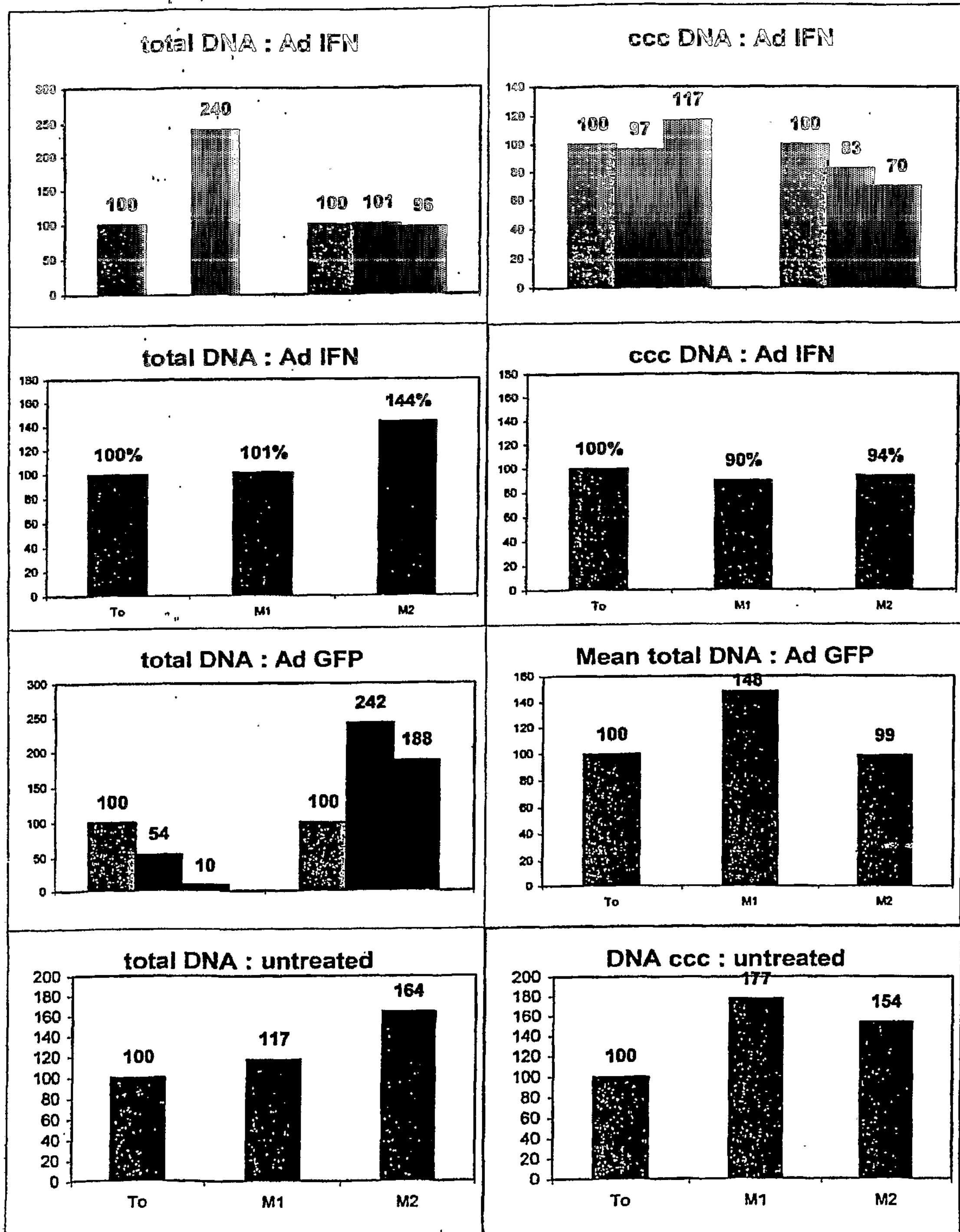


FIGURE 15