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(57) Abstract

The present invention provides a method of treating a viral disease comprising administering to a mammal a polynucleotide construct comprising a polynucleotide encoding IFNω. The polynucleotide construct of the present invention can be administered free from associated with transfection facilitating agents or as a complex with at least one or more cationic lipids.

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Treatment of Viral Diseases Using an Interferon Omega Expressing Polynucleotide

Background of the Invention

Field of the Invention

The present invention relates to treatment of infectious diseases in mammals. Generally, the present invention provides methods of treating viral infections in a mammal by administering a polynucleotide construct comprising a polynucleotide encoding IFNω.

Related Art

Treatment of infectious diseases with an interferon (IFN) has traditionally involved repeat injections of large doses of recombinant protein. The interferons (IFNs) have been divided into two classes, type I IFN and type II IFN, on the basis of antigenicity, biochemical properties, and producer cell. IFNα, IFNβ, IFNω, and IFNτ are type I interferons and bind to the same α/β receptor, whereas IFNγ is a type II interferon and binds to the γ receptor (Petska, S., *Seminars in Oncology 24*:S19-S40 (1997)). The type I IFN's, IFNα and IFNβ, are naturally expressed in many cells upon viral infection, whereas the Type II IFN, IFNγ, is produced by activated T lymphocytes and natural killer (NK) cells.

IFNα is a naturally occurring protein which is produced by cells in the body to stimulate the immune system to respond to viral infections. IFNα systemic protein therapy has been used to treat certain types of cancer and infectious diseases. Recombinant IFNα polypeptide is approved for use in humans for hairy cell leukemia, AIDS-related Kaposi's sarcoma, malignant melanoma, chronic hepatitis B and C, chronic myleogenous leukemia, and condylomata acuminata (Baron, S. *et al.*, *JAMA 266*:1375 (1991)). However, effectiveness for such therapy is limited by dose-related complications such as flu-like symptoms of low-grade fever, nasal discharge and muscle pain, chronic fatigue, nausea,

vomiting, loss of appetite, low white blood count, low platelet count, anemia, and depression. The side effects are most severe for several hours after administration of the recombinant IFNα, but subsides as circulating levels of interferon diminish. Due to the short half-life of the protein in the serum (several hours) (Friedman, R., Interferons: A Primer, Academic Press, New York, p. 104-107 (1981); Galvani, D., and Cawley, J., Cytokine Therapy, Cambridge University Press, Cambridge, p. 114-115 (1992)), frequent repeat dosing (usually several times weekly) is required for effective treatment which then results in cycles of severe and subsiding side effects (Jones, G. and Itri, L., *Cancer 57*:1709-1715 (1986); Quesada, J., *et al., J. Clin. Oncol. 4*:234-243 (1986)). Clearly, there is a need for an improved delivery system for treating infectious diseases with IFNs.

IFNω has never been used for the treatment of infectious diseases, even in the form of a recombinant protein. IFNω was discovered independently by three different groups in 1985 (Capon, D.J., *et al., Mol. Cell. Biol. 5*: 768-779 (1985), Feinstein, S. *et al., Mol. Cell. Biol 5*:510 (1985); and Hauptmann and Swetly, *Nucl. Acids Res. 13*: 4739-4749 (1985)). Unlike IFNω, for which at least 14 different functional nonallelic genes have been identified in man, IFNω is encoded by a single functional gene. IFNω genes are believed to be present in most mammals, but have not been found in dogs or mice. The mature IFNω polypeptide is 172 amino acids and shares 60% sequence homology with the human IFNα's. Due to the sequence similarity with IFNα, IFNω was originally considered to be a member of a subfamily of IFNα, and was originally termed IFNα-$_{II}$. IFNω is a significant component (≈10%) of human leukocyte derived interferon, the natural mixture of IFN produced after viral infection (Adolf, G., *et al., Virology 175*:410 (1990)). IFNω has been demonstrated to bind to the same α/β receptor as IFNα (Flores, I., *et al., J. Biol. Chem. 266*: 19875-19877 (1991)), and to share similar biological activities with IFNα, including anti-proliferative activity against tumor cells *in vitro* (Kubes, M. *et al., J. Interferon Research 14*:57 (1994) and immunomodulatory activity (Nieroda, *et al., Mol. Cell. Differentiation 4*: 335-351 (1996)).

Summary of the Invention

The present invention is broadly directed to treatment of an infectious disease by administering into a tissue of a mammal a polynucleotide construct comprising a polynucleotide encoding a cytokine, or an active variant thereof. The polynucleotide construct is incorporated into the cells of the mammal, and a therapeutically effective amount of a cytokine is produced.

The present invention provides a method of treating a viral infection in a mammal, comprising administering to said mammal a polynucleotide construct comprising a polynucleotide selected from the group consisting of (a) a polynucleotide that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID NO:1 or the complement thereof, wherein said polynucleotide encodes a polypeptide having antiviral activity; (b) a polynucleotide that encodes a polypeptide comprising an amino acid sequence which, except for at least one but no more than 20 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 of SEQ ID NO:2, wherein said polypeptide has antiviral activity; (c) a polynucleotide that encodes a polypeptide comprising an amino acid sequence which, except for at least one but no more than 20 amino acid substitutions, deletions, or insertions, is identical to amino acids 1 to 172 of SEQ ID NO:2, wherein said polypeptide has antiviral activity; and (d) a polynucleotide encoding amino acids 21 to 172, 61 to 172, 41 to 172 or 86 to 172 of SEQ ID NO:2. The polynucleotide construct may be free from transfection facilitating compounds, or it may be complexed with one or more cationic compounds.

More specifically, the present invention provides a method for treating Hepatitis B and Hepatitis C.

The present invention provides the first description of treating infectious diseases using IFNω-encoding plasmid DNA. Compared to the recombinant protein delivery, the procedure described herein has several important advantages.

With *in vivo* administration of IFNω plasmid DNA, the IFNω is produced by cells in the body at lower levels for a sustained period. This delivery profile allows for less frequent administration, compared to the frequent high doses required for interferon protein, and thereby minimize the side effects while maintaining the therapeutic benefits of protein delivery. Furthermore, administration of plasmid vectors have not been found to induce significant toxicity or pathological immune responses as demonstrated in mice, pigs and monkeys (Parker, S. E., *et al.*, *Human Gene Ther. 6*:575-590 (1995); San, H., *et al.*, *Human Gene Ther. 4*:781-788 (1993)). Direct injection of plasmid DNA coding for a foreign major histocompatibility gene into human melanoma tumors resulted in no significant side effects or autoimmune responses (Nabel, G. J., *et al.*, *Proc. Natl. Acad. Sci. 90*:11307-11311 (1993)) in humans, and the direct intramuscular injection of a plasmid DNA encoding the malarial circumsporozoite protein (CSP from *Plasmodium falciparum)* was found to be safe and well tolerated in humans (Wang R. *et al.*, *Science* 282:476-480 (1988)).

Brief Description of the Figures

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same become better understood by reference to the following detailed description when considered in connection with the accompanying figures.

Figure 1 shows the plasmid map of VR4151 (SEQ ID NO:3). The cytomegalovirus immediate early gene promoter enhancer and 5′ untranslated sequences (5′ UTR + intron A) drive the expression of the hIFNω coding sequence. The transcriptional terminator region includes a polyadenylation and termination signal derived from the rabbit β-globin gene.

Figure 2 shows the pharmacokinetics of hIFω in nude mice following intramuscular (i.m.) injection of VR4151. Mice were injected i.m. with 100 μg of plasmid.

Figure 3 shows the dose response to a single i.m. administration of VR4151. Mice were injected with either 200 μg, 100 μg or 10 μg bilaterally or 100 μg unilaterally. Control mice received injections of 100 μg VR1055.

Figure 4 shows the pharmacokinetics of IFNω in nude mice. Mice were i.m. injected bilaterally on days 0 and 14 with 100 μg VR4151. Control mice were injected with 100 μg VR1055.

Figures 5A-B show the pharmacokinetics of IFNω in rats. In Figure 5A, rats received a single i.m. injection of either 0.1 mg or 1 mg of VR4151. In Figure 5B, rats received three consecutive i.m. injections of 1 mg VR4151.

Detailed Description of the Preferred Embodiments

The present invention is directed to treating a viral infection by administering *in vivo*, into a tissue of a mammal, a polynucleotide construct comprising a polynucleotide encoding a cytokine, or an active variant thereof. The polynucleotide construct is incorporated into the cells of the mammal *in vivo*, followed by production of a therapeutically effective amount of cytokine. Combinations of cytokine-encoding polynucleotides can also be administered.

More particularly, the present invention provides a method of treating a viral infection in a mammal, comprising administering to said mammal, a polynucleotide construct comprising a polynucleotide which encodes IFNω, or an active variant thereof, wherein said polynucleotide is expressed *in vivo* in an amount effective to treat the viral infection.

The term "mammal" is intended to encompass a singular "mammal" and plural "mammals," and includes, but is not limited to, primate mammals such as human, apes, monkeys, orangutans, and chimpanzees; canine mammals such as dogs and wolves; feline mammals such as cats, lions, and tigers; equine mammals such as horses, donkeys, deer, zebras, and giraffes; and bears. Preferably, the mammal is a human subject.

By "treatment" is meant reduction in symptoms, reduction in viral load, reduction in the rate of viral replication, and/or no worsening of symptoms, viral load, or viral replication over a specified period of time.

In further embodiments of the invention, the polynucleotide construct comprising a polynucleotide which encodes IFNω is delivered along with one or more polynucleotide constructs comprising a polynucleotide which encodes a different cytokine. The term "cytokine" refers to polypeptides, including, but not limited to, interleukins (e.g., IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, and IL-18), α interferons (e.g., IFNα), ω interferon (IFNω), β interferons (e.g., IFNβ), γ interferons (e.g., IFNγ), τ interferon (IFNτ), colony stimulating factors (CSFs, e.g., CSF-1, CSF-2, and CSF-3), granulocyte-macrophage colony stimulating factor (GMCSF), transforming growth factor (TGF, e.g.,.TGFα and TGFβ), and insulin-like growth factors (IGFs, e.g, IGF-I and IGF-II).

The polynucleotide sequences encoding IFNω polypeptides include the sequences encoding the complete IFNω and the mature IFNω proteins set forth in U.S. Patent No. 4,917,887; European Patent Publication No. 0 170 204 B1; and Capon, D.J., *et al., Molec. Cell. Biol. 5*: 768-779 (1985); Hauptmann, R. and P. Swetly, *Nucl. Acids Res.* 13: 4739-4749 (1985); Adolf, G.R., *et al., Biochim. Biophys. Acta 1089*: 167-174 (1991); Mege, D., *et al., J. Interf. Res. 11*: 341-350 (1991); Charlier, M., *et al., J. Interf. Res. 13* 313-322 (1993); Hughes, A.L., *J. Mol. Evol. 41*: 539-548 (1995); and Roberts, R.M., *et al., Prog. Nucl. Acid Res. Molec. Biol. 56*:287-325, edited by W.E. Cohn, Academic Press (1997), each of which is herein incorporated by reference. A polynucleotide sequence encoding IFNω is shown in SEQ ID NO:1. A full length IFNω amino acid sequence is shown as amino acids -23 to 172 of SEQ ID NO:2. A mature IFNω amino acid sequence is shown as amino acids 1 to 172 of SEQ ID NO:2. Useful fragments of IFNω are shown as amino acids 21 to 172, 41 to 172, 61 to 172 and 86 to 172 of SEQ ID NO:2.

The polynucleotide sequences encoding IFNα polypeptides include the sequences encoding the complete IFNα and the mature IFNα proteins set forth in U.S. Patent Nos. 4,530,901; 4,695,543; 4,695,623; 4,748,233; 4,892,743; 4,897,471; 4,973,479; 4,975,276; and 5,098,703; and in Pestka, S., *Methods Enzymol. 119*: 3-14 (1986); Hughes, A.L., *J. Mol. Evol. 41*: 539-548 (1995); and Roberts, R.M., *et al., Prog. Nucl. Acid Res. Molec. Biol. 56*:287-325, edited by W.E. Cohn, Academic Press (1997), each of which is herein incorporated by reference.

It will be recognized in the art that some amino acid sequences of the cytokine polypeptides described herein can be varied without significant effect on the functional activity of the polypeptides. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the polypeptide which determine activity. Such variations include deletions, insertions, inversions, repeats, and type substitutions. Guidance concerning which amino acid changes are likely to be phenotypically silent can be found in Bowie, J.U., *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science 247*:1306-1310 (1990). Variants of cytokines can be assayed according to the antiviral assay described herein. Amino acids that are critical for cytokine activity can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith *et al.*, *J. Mol. Biol. 224*:899-904 (1992) and de Vos *et al. Science 255*:306-312 (1992)).

The present invention further relates to using variants of the cytokine-encoding polynucleotide, which encode portions, analogs or derivatives of the cytokine. Variants may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. *Genes II*, Lewin, B., ed., John Wiley & Sons, New York (1985). Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially

preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the cytokine or portions thereof. Also especially preferred in this regard are conservative substitutions. For example, aromatic amino acids that can be conservatively substituted for one another include phenylalanine, tryptophan, and tyrosine. Hydrophobic amino acids that can be conservatively substituted for one another include leucine, isoleucine, and valine. Polar amino acids that can be conservatively substituted for one another include glutamine and asparagine. Basic amino acids that can be conservatively substituted for one another include arginine, lysine, and histidine. Acidic amino acids that can be conservatively substituted for one another include aspartic acid and glutamic acid. Small amino acids that can be conservatively substituted for one another include alanine, serine, threonine, methionine, and glycine.

Substitutions, deletions, or insertions can be made outside of the region encoding the shortest active fragment of the cytokine, without affecting the activity of the cytokine. Further, mutated proteins (or muteins) often retain a biological activity that is similar to that of the naturally occurring protein. For example, Gayle and coworkers (*J. Biol. Chem. 268* 22105-22111 (1993)) conducted an extensive mutational analysis of the human cytokine IL-1α. They used random mutagenesis to generate over 3,500 individual IL-1α mutants with an average of 2.5 amino acid changes per mutein over the entire length of the molecule. Multiple mutations were examined at every possible amino acid and, on average, each mutein's amino acid sequence was 98.4% identical to that of naturally occurring IL-1α. The investigators observed that most of the molecule could be mutated with little effect on either binding or biological activity, and that 75% of the molecule may not contribute significantly to the biological activity of the molecule.

Similarly, Gronenborn and colleagues (*FEBS Letters 231*: 135-138 (1988)) analyzed the receptor binding activity of six mutant IL-1α polypeptides. Each mutant contained a single amino acid alteration from the naturally occurring IL-1α polypeptide and was examined under four sets of experimental conditions. In this

study, the investigators found very little difference between the receptor binding activity of the mutants and naturally occurring IL-1α.

Further, Zurawski and colleagues (*EMBO J. 12*: 5113-5119 (1993)) studied residues 41-142 of mIL-2 by generating 1,090 muteins. The extent of the mutagenesis was such that there was an average of 11 different amino acid substitutions per naturally occurring amino acid residue, with the exception of the extreme N- and C-termini and residues 31-40. The mIL-2 muteins were assayed for specific activity and compared to that of naturally occurring mIL-2. The degree to which the specific activity was antagonized by a previously characterized mIL-2 mutant was also assessed. The investigators observed that, in the 149 residue mIL-2 protein, only 23 residues are important for interaction with IL-2R, 18 residues are presumed to be part of the structural core, and three additional residues are important for structure. 98 mIL-2 residues (or 65% of the protein) were assigned as relatively unimportant residues.

Additionally, a polynucleotide sequence used in the present invention can encode a polypeptide having one to twenty amino acid substitutions, deletions or insertions, either from natural mutations or human manipulation, relative to the full length or mature IFNα or IFNω. Preferably, no more than one to fifteen substitutions, deletions or insertions are present, relative to the full length or mature IFNα or IFNω (excluding the signal sequence). More preferably, no more than one to ten substitutions, deletions or insertions are present. Still more preferably, no more than one to five substitutions, deletions or insertions are present.

Polynucleotides used in the present invention also include those that hybridize under stringent conditions to polynucleotides encoding a cytokine, or the complement thereof, which encode a functional variant of the cytokine. By "stringent conditions" is intended a hybridization by overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (750 mM NaCl, 75mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA,

followed by repeatedly washing the filters (at least three times) in 0.1x SSC and 0.1% sodium dodecyl sulfate (w/v) for 20 minutes at about 65°C.

Thus, the present invention provides a method for treating a viral infection in a mammal, comprising administering to the mammal a construct comprising a polynucleotide selected from the group consisting of: (a) a polynucleotide that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID NO:1 or the complement thereof, wherein said polynucleotide encodes a polypeptide having antiviral activity; (b) a polynucleotide that encodes a polypeptide comprising an amino acid sequence which, except for at least one but no more than 20 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 of SEQ ID NO:2, wherein said polypeptide has antiviral activity; (c) a polynucleotide that encodes a polypeptide comprising an amino acid sequence which, except for at least one but no more than 20 amino acid substitutions, deletions, or insertions, is identical to amino acids 1 to 172 of SEQ ID NO:2, wherein said polypeptide has antiviral activity; and (d) a polynucleotide encoding amino acids 21 to 172, 41 to 172, 61 to 172 or 86 to 172 of SEQ ID NO:2.

A "polynucleotide construct" is a polynucleotide molecule that carries genetic information for encoding one or more cytokines. The polynucleotide material delivered to the cells *in vivo* can take any number of forms. It can contain the entire sequence or only a functionally active variant of a cytokine gene.

By "active variant" is intended a variant of a cytokine that displays the antiviral activity of the mature or full length cytokine. For example, a full length IFNω is set forth in amino acids -23 to 172 of SEQ ID NO:2. The corresponding mature IFNω is set forth in amino acids 1 to 172 of SEQ ID NO:2. Useful fragments of IFNω are shown as amino acids 21 to 172, 41 to 172, 61 to 172 and 86 to 172 of SEQ ID NO:2.

Assays of antiviral activity *in vitro* are well known to those of ordinary skill in the art. For example, one antiviral assay that can be used is outlined in Example 3, below. Briefly, plasmids expressing the IFNω variant are transfected

into cells, such as UM449 cells. The supernatant from the transfected cells is added onto cells which have been infected with Murine Encephalomyocarditis Virus (EMCV) at Multiplicity of Infection (MOI) of approximately 0.04. The viral cytopathic effect in infected cells treated with the supernatant is compared to untreated infected cells. A decrease in viral cytopathic effect in treated cells indicates that the IFNω or a variant thereof is active.

The polynucleotide construct comprises at least one polynucleotide (e.g., DNA, RNA, ribozyme, phosphorothioate, or other modified nucleic acid) encoding one or more cytokines. The polynucleotide can be provided in linear, circular (e.g. plasmid), or branched form; and double-stranded or single-stranded form. The polynucleotide can involve a conventional phosphodiester bond or a non-conventional bond (e.g., an amide bond as in peptide nucleic acid (PNA)). The choice of polynucleotide encoding a cytokine will depend on the desired kinetics and duration of expression. When long term delivery of the polynucleotide construct is desired, the preferred polynucleotide is DNA. Alternatively, when short term delivery is desired, the preferred polynucleotide is mRNA. RNA will be rapidly translated into polypeptide, but will be degraded by the target cell more quickly than DNA. In general, because of the greater resistance of circular DNA molecules to nucleases, circular DNA molecules will persist longer than single-stranded polynucleotides, and they will be less likely to cause insertional mutation by integrating into the target genome.

In one embodiment, the polynucleotide sequence encoding one or more cytokines is RNA. Most preferably, the RNA is messenger RNA (mRNA). Methods for introducing RNA sequences into mammalian cells is described in U.S. Patent No. 5,580,859, which is herein incorporated by reference. A viral alphavector, a non-infectious vector useful for administering RNA, may be used to introduce RNA into mammalian cells. Methods for the *in vivo* introduction of alphaviral vectors to mammalian tissues are described in Altman-Hamamdzic, S., *et al.*, *Gene Therapy 4*: 815-822 (1997), which is herein incorporated by reference.

Preferably, the polynucleotide sequence encoding one or more cytokines is DNA. In a DNA construct, a promoter is preferably operably linked to the polynucleotide encoding a cytokine. The promoter may be a cell-specific promoter that directs substantial transcription of the DNA only in predetermined cells. Other transcription control elements, besides a promoter, can be included in the polynucleotide construct to direct cell-specific transcription of the DNA.

An operable linkage is a linkage in which a polynucleotide sequence encoding a cytokine is connected to one or more regulatory sequence in such a way as to place expression of the cytokine sequence under the influence or control of the regulatory sequence(s). Two DNA sequences (such as a coding sequence and a promoter region sequence linked to the 5′ end of the coding sequence) are operably linked if induction of promoter function results in the transcription of mRNA encoding the desired polypeptide and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the expression regulatory sequences to direct the expression of the polypeptide, antisense RNA, or (3) interfere with the ability of the DNA template to be transcribed. Thus, a promoter region would be operably linked to a DNA sequence if the promoter was capable of effecting transcription of that DNA sequence.

Preferably, the polynucleotide construct is a circular or linearized plasmid containing non-infectious, nonintegrating nucleotide sequence. A linearized plasmid is a plasmid that was previously circular but has been linearized, for example, by digestion with a restriction endonuclease. The polynucleotide sequence encoding a cytokine may comprise a sequence which directs the secretion of the polypeptide.

"Noninfectious" means that the polynucleotide construct does not infect mammalian cells. Thus, the polynucleotide construct can contain functional sequences from non-mammalian (e.g., viral or bacterial) species, but the construct does not contain functional non-mammalian nucleotide sequences that facilitate infection of the construct into mammalian cells.

"Nonintegrating" means that the polynucleotide construct does not integrate into the genome of mammalian cells. The construct can be a non-replicating DNA sequence, or specific replicating sequences genetically engineered to lack the ability to integrate into the genome. The polynucleotide construct does not contain functional sequences that facilitate integration of the cytokine-encoding polynucleotide sequence into the genome of mammalian cells.

The polynucleotide construct is assembled out of components where different selectable genes, origins, promoters, introns, 5′ untranslated (UT) sequence, terminators, polyadenylation signals, 3′ UT sequence, and leader peptides, etc. are put together to make the desired vector. The precise nature of the regulatory regions needed for gene expression can vary between species or cell types, but shall in general include, as necessary, 5′ non-transcribing and 5′ non-translating (non-coding) sequences involved with initiation of transcription and translation respectively, such as the TATA box, capping sequence, CAAT sequence, and the like, with those elements necessary for the promoter sequence being provided by the promoters of the invention. Such transcriptional control sequences can also include enhancer sequences or upstream activator sequences, as desired.

The polynucleotide construct can be an expression vector. A typical mammalian expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the polypeptide coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRS) from retroviruses, e.g., RSV, HTLVI, HIVI, MPSV and the immediate early promoter of the cytomegalovirus (CMV IEP). However, cellular elements can also be used (*e.g.*, the human actin promoter, metallothionein promoter). In humans, CMV IEP is preferred. Suitable expression vectors for use in practicing the present invention include, for example, vectors such as PSVL and

PMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146), pBC12MI (ATCC 67109), VR1012, VR1055, and pcDNA3 (Invitrogen, San Diego, CA). All forms of DNA, whether replicating or non-replicating, which do not become integrated into the genome, and which are expressible, are within the methods contemplated by the invention.

The vector containing the DNA sequence (or the corresponding RNA sequence) which can be used in accordance with the invention can be a eukaryotic expression vector. Techniques for obtaining expression of exogenous DNA or RNA sequences in a host are known. See, for example, Korman *et al., Proc. Nat. Acad. Sci. (USA) 84*:2150-2154 (1987), which is herein incorporated by reference.

Secretion of a cytokine from a cell can be facilitated by a leader or secretory signal sequence. In a preferred embodiment, either the native leader sequence of a cytokine is used, or a functional derivative of that sequence that retains the ability to direct the secretion of the peptide that is operably linked to it. Alternatively, a heterologous mammalian leader sequence, or a functional derivative thereof, may be used. For example, the wild-type leader sequence may be substituted with the leader sequence of human tissue plasminogen activator or mouse β-glucuronidase.

For example, to facilitate secretion of a polypeptide comprising amino acids 86 to 172 of SEQ ID NO:2, the natural leader sequence of IFNω is used. The polynucleotide construct comprises a sequence encoding amino acids -23 to -1 of SEQ ID NO:2 directly 5′ to the sequence encoding amino acids 86 to 172 of SEQ ID NO:2. When the polynucleotide is expressed, the native leader sequence will direct secretion of the polypeptide comprising amino acids 86 to 172 of SEQ ID NO:2. The leader sequence is cleaved off, leaving only the polypeptide comprising amino acids 86 to 172 of SEQ ID NO:2.

For the methods of the present invention, a single polynucleotide construct containing more than one polynucleotide sequence encoding one or more molecules may be administered. For example, a single polynucleotide construct containing one polynucleotide encoding an IFNω and another polynucleotide

encoding an additional cytokine or an immunomodulatory molecule, *i.e.*, MHC class I antigen, viral antigen, and co-stimulatory molecule, can be administered.

Alternatively, more than one polynucleotide construct each containing polynucleotide sequences encoding one or more molecules may be co-administered or sequentially administered. For example, two polynucleotide constructs can be administered where one gene product enhances anti-viral efficacy of the other gene product. An IFNω-expressing polynucleotide construct can be co-injected with a polynucleotide construct encoding a different cytokine. Alternatively, one or more plasmids could be administered initially and other plasmid(s) could be administered subsequently at various time intervals.

When the single polynucleotide construct containing more than one polynucleotide encoding a polypeptide is DNA, preferably, each polynucleotide encoding a polypeptide will be operably linked to a separate promoter. Alternatively, the polynucleotides encoding polypeptides may be operably linked to the same promoter in order to form a polycistronic transcription unit wherein each sequence encoding a polypeptide is separated by translational stop and start signals. Transcription termination is also shared by these sequences. While both DNA coding sequences are controlled by the same transcriptional promoter, so that a fused message (mRNA) is formed, they are separated by a translational stop signal for the first and start signal for the second, so that two independent polypeptides result. Methods of making such constructs are disclosed in U.S. Patent Nos. 4,713,339, and 4,965,196, which are herein incorporated by reference.

When the single polynucleotide construct containing more than one polynucleotide encoding a polypeptide is RNA, preferably, there will be separate translational start and stop signals for each polypeptide-encoding sequence in order to produce two or more separate polypeptides.

In a preferred embodiment of the present invention, the polynucleotide construct is delivered as naked polynucleotide. By "naked" is meant that the polynucleotide construct is free from association with any delivery vehicle known in the art that can act to facilitate entry into cells, for example, from transfection-facilitating proteins, viral particles, liposomes, cationic lipids, and calcium phosphate precipitating agents.

In certain embodiments of the present invention, the polynucleotide construct is complexed with a cationic vehicle, which comprises a cationic compound, such as cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. A preferred cationic compound is a cationic lipid.

One or more cationic lipids can be complexed with the polynucleotide construct by ionic interaction. Generally, the complex then contacts the cell membrane and is transfected into the cell. This transfection mechanism is referred to as "lipofection," and is a highly efficient transfection procedure (Felgner *et al.*, *Proc. Natl. Acad. Sci. USA 84*:7413-7417, (Nov. 1987); and Felgner *et al.*, *Nature 337*:387-388 (1989) which are herein incorporated by reference).

For purposes of the present invention, lipid refers to a synthetic or naturally occurring compound that possesses both a lipophilic region and a polar region, commonly referred to as a head group. Preferred cationic compounds are cationic lipids. Cationic lipids are described in U.S. Pat. Nos. 4,897,355, 4,946,787, 5,049,386, 5,264,618, 5,279,833, 5,334,761, 5,429,127, 5,459,127, 5,589,466, 5,676,954, 5,693,622, 5,580,859, 5,703,055, and 5,578,475; and international publications WO 94/29469, WO 95/14381, 95/14651, 95/17373, 96/18372, 96/26179, 96/40962, 96/40963, 96/41873, and 97/00241, and documents cited therein, which are herein incorporated by reference. As illustrated in the above-cited patents and patent applications, cationic lipids comprise structural features that may be present in a variety of core molecular classes.

U.S. Patent No. 5,676,954 reports on the injection of genetic material, complexed with cationic lipid carriers, into mice. U.S. Patent Nos. 5,589,466,

5,693,622, 5,580,859, 5,703,055, and international patent application no. PCT/US94/06069 (publication no. WO 94/29469), which are herein incorporated by reference, provide methods for delivering DNA-cationic lipid complexes to mammals.

Examples of cationic lipids are 5-carboxyspermylglycine dioctadecylamide (DOGS) and dipalmitoyl-phophatidylethanolamine-5carboxyspermylamide (DPPES). Cationic cholesterol derivatives are also useful, including {3β-[N-N′,N′-dimethylamino)ethane]-carbomoyl}-cholesterol (DC-Chol). Dimethyldioctdecyl-ammonium bromide (DDAB), N-(3-aminopropyl)-N,N-(*bis*-(2-tetradecyloxyethyl))-N-methyl-ammonium bromide (PA-DEMO), N-(3-aminopropyl)-N,N-(*bis*-(2-dodecyloxyethyl))-N-methyl-ammonium bromide (PA-DELO), N,N,N-*tris*-(2-dodecyloxy)ethyl-N-(3-amino)propyl-ammonium bromide (PA-TELO), and N^1-(3-aminopropyl)((2-dodecyloxy)ethyl-N^2-(2-dodecyloxy)ethyl-1-piperazinaminium bromide (GA-LOE-BP) can also be employed in the present invention.

Non-diether cationic lipids, such as DL-1,2-dioleoyl-3-dimethylaminopropyl-β-hydroxyethylammonium (DORI diester), 1-O-oleyl-2-oleoyl-3-dimethylaminopropyl-β-hydroxyethylammonium (DORI ester/ether), and their salts promote *in vivo* gene delivery. Preferred cationic lipids comprise groups attached via a heteroatom attached to the quaternary ammonium moiety in the head group. A glycyl spacer can connect the linker to the hydroxyl group.

Preferred cationic lipids are 3,5-(N,N-dilysyl)-diaminobenzoyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl-β-hydroxyethylamine) (DLYS-DABA-DORI diester), 3,5-(N,N-di-lysyl)diamino-benzoylglycyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl-β-hydroxyethylamine) (DLYS-DABA-GLY-DORI diester), and (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide (DMRIE).

Also preferred are (±)-N,N-dimethyl-N-[2-(sperminecarboxamido)ethyl]-2,3-bis(dioleyloxy)-1-propaniminium pentahydrochloride (DOSPA), (±)-N-(2-aminoethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide (β-

aminoethyl-DMRIE or βAE-DMRIE) (Wheeler, *et al., Biochim. Biophys. Acta 1280*:1-11 (1996)), and (±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-l-propaniminium bromide (GAP-DLRIE) (Wheeler, *et al., Proc. Natl. Acad. Sci. USA 93*:11454-11459 (1996)), which have been developed from DMRIE.

Other examples of DMRIE-derived cationic lipids that are useful for the present invention are (±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-(*bis*-decyloxy)-1-propanaminium bromide (GAP-DDRIE), (±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-(*bis*-tetradecyloxy)-1-propanaminium bromide (GAP-DMRIE), (±)-N-((N"-methyl)-N'-ureyl)propyl-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide (GMU-DMRIE), (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide (DLRIE), and (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-*bis*-([Z]-9-octadecenyloxy)propyl-1-propaniminium bromide (HP-DORIE).

The lipids of the lipid-containing formulation can comprise a cationic lipid alone, or further comprise a neutral lipid such as cardiolipin, phosphatidylcholine, phosphatidylethanolamine, dioleoylphosphatidylcholine, dioleoylphosphatidyl-ethanolamine, 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), sphingomyelin, and mono-, di- or tri-acylglycerol. Other additives, such as cholesterol, fatty acid, ganglioside, glycolipid, neobee, niosome, prostaglandin, sphingolipid, and any other natural or synthetic amphiphiles, can also be used. A preferred molar ratio of cationic lipid to neutral lipid in these lipid-containing formulations is from about 9:1 to about 1:9; an equimolar ratio is particularly preferred. The lipid-containing formulation can further comprise a lyso lipid (*e.g.*, lyso-phosphatidylcholine, lysophosphatidyl-ethanolamine, or a lyso form of a cationic lipid).

Preferably, the cationic lipid is (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide (DMRIE) and the neutral lipid is 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) such that the mass ratio of polynucleotide construct to lipid is from about 10:1 and about 0.5:1. More

preferably, the mass ratio of polynucleotide construct to lipid is from about 5:1 and about 1:1. Still more preferably, the mass ratio of polynucleotide construct to lipid is about 5:1. DMRIE/DOPE has been shown to be effective for both *in vitro* (Felgner et al., *J. Biol. Chem.* 269:2550-2561, 1994) and *in vivo* transfection (Stopeck et al., *J. Clin. Oncol.* 15:341-349, 1997 and Rubin et al., *Gene Ther.* 4:419-425, 1997).

Lipid-containing pharmaceutical composition for use in a complex with the polynucleotide construct of the present invention can also comprise cationic lipid together with an effective amount of a lysophosphatide. The lysophosphatide can have a neutral or a negative head group. Lysophosphatidylcholine and lysophosphatidyl-ethanolamine are preferred, and 1-oleoyl lysophosphatidylcholine is particularly preferred. Lysophosphatide lipids are advantageously present in the lipid-containing formulation in a 1:2 ratio of lysolipid to cationic lipid. Lyso forms of a cationic lipid can also be used to promote polynucleotide delivery. These lyso forms are advantageously present in effective amounts up to about one-third of the total cationic lipid in the lipid-containing formulations.

In a formulation for preparing DNA/lipid complexes, the cationic lipid can be present at a concentration of between about 0.1 mole % and about 100 mole %, preferably about 5 mole % and 100 mole %, and most preferably between about 20 mole % and 100 mole %, relative to other compounds present in the formulation. The neutral lipid can be present in a concentration of between zero and about 99.9 mole %, preferably zero and about 95 mole %, and most preferably zero and about 80 mole %. In order to produce lipid vesicles having a net positive charge, the quantity of the positively charged component must exceed that of the negatively charged component. The negatively charged lipid can be present at between zero and about 49 mole %, and preferably between zero and about 40 mole %. Cholesterol or a similar sterol can be present at between zero to about 80 mole %, and preferably zero and about 50 mole %.

The polynucleotide to be delivered can be solubilized in a buffer prior to mixing with lipid vesicles. Suitable buffers include, for example, phosphate

buffered saline (PBS), normal saline, Tris buffer, and sodium phosphate vehicle (100-150 mM preferred). Insoluble polynucleotides can be solubilized in a weak acid or base, and then diluted to the desired volume with a neutral buffer such as PBS. The pH of the buffer is suitably adjusted, and moreover, a pharmaceutically acceptable additive can be used in the buffer to provide an appropriate osmolarity within the lipid vesicle.

A lipid solution comprising at least one amphipathic lipid can spontaneously assemble to form primary lipid vesicles, heterogeneous in size. Therefore, according to a preferred method, the lipids of the lipid-containing formulation, comprising at least one cationic lipid, are prepared by dissolution in a solvent such as chloroform and the mixture is evaporated to dryness as a film on the inner surface of a glass vessel. On suspension in an aqueous solvent, the amphipathic lipid molecules assemble themselves into primary lipid vesicles. These primary lipid vesicles are reduced to a selected mean diameter by means of a freeze-thaw procedure. Vesicles of uniform size can be formed prior to drug delivery according to methods for vesicle production known to those in the art; for example, the sonication of a lipid solution as described by Felgner *et al., Proc. Natl. Acad. Sci. USA 84*:7413-7417 (1987) and U.S. Pat. No. 5,264,618, which are herein incorporated by reference.

The constructs may be delivered to any tissue, including, but not limited to, 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, or connective tissue. Preferably, the construct is delivered to muscle. The muscle may be skeletal or cardiac. Most preferably, the construct is delivered to skeletal muscle.

Preferably, the polynucleotide construct is delivered to the interstitial space of tissues. "Interstitial space" 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.

The polynucleotide construct of the present invention, whether complexed with lipid or not, can be administered by any suitable route of administration, including intramuscularly, subcutaneously, intravenously, transdermally, intranasally, by inhalation, or transmucosally (i.e., across a mucous membrane). Similarly, the pharmaceutical composition of the present invention can by administered by any suitable route, including intramuscularly, into a cavity (e.g., intraperitoneally), subcutaneously, intravenously, transdermally, intranasally, by inhalation, or transmucosally (i.e., across a mucous membrane).

Any mode of administration can be used so long as the mode results in the expression of one or more cytokines in an amount sufficient to decrease the viral infection of a mammal. This includes needle injection, catheter infusion, biolistic injectors, particle accelerators (i.e., "gene guns", pneumatic "needleless" injectors, *e.g.*, Med-E-Jet (Vahlsing, H. *et al., J. Immunol. Methods* 171:11-22 (1994)), Pigjet (Schrijver, R. *et al., Vaccine* 15: 1908-1916 (1997)), Biojector (Davis, H. *et al., Vaccine* 12:1503-1509 (1994); Gramzinski, R. *et al., Mol. Med.* 4: 109-118 (1998))), gelfoam sponge depots, other commercially available depot materials, osmotic pumps (e.g., Alza minipumps), oral or suppositorial solid (tablet or pill) pharmaceutical formulations, and decanting or topical applications during surgery. The preferred mode is injection.

Determining an effective amount of substance to be delivered can depend upon a number of factors including, for example, the chemical structure and biological activity of the substance, the age and weight of the mammal, the precise condition requiring treatment and its severity, and the route of administration. The precise amount, number of doses, and timing of doses will be determined by the attending physician or veterinarian.

In humans, between 1 to 50 mg polynucleotide construct is delivered. Preferably, between 10 to 30 mg polynucleotide construct is delivered.

In certain embodiments, the polynucleotide construct is administered as a pharmaceutical composition. The pharmaceutical composition can be formulated according to known methods for preparing pharmaceutical compositions, whereby the substance to be delivered is combined with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their preparation are described, for example, in *Remington's Pharmaceutical Sciences*, 16th Edition, A. Osol, Ed., Mack Publishing Co., Easton, PA (1980), and Remington's Pharmaceutical Sciences, 19th Edition, A.R. Gennaro, Ed., Mack Publishing Co., Easton, PA (1995).

The pharmaceutical composition can be in the form of an emulsion, gel, solution, suspension, or other form known in the art. Optionally, it can contain one or more lipids as described above. In addition, the pharmaceutical composition can also contain pharmaceutically acceptable additives including, for example, diluents, binders, stabilizers, and preservatives. Administration of pharmaceutically acceptable salts of the polynucleotides described herein is preferred. Such salts can be prepared from pharmaceutically acceptable non-toxic bases including organic bases and inorganic bases. Salts derived from inorganic bases include sodium, potassium, lithium, ammonium, calcium, magnesium, and the like. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary, and tertiary amines, basic amino acids, and the like.

For aqueous pharmaceutical compositions used *in vivo*, sterile pyrogen-free water is preferred. Such formulations will contain an effective amount of the substance together with a suitable amount of vehicle in order to prepare pharmaceutically acceptable compositions suitable for administration to a human or animal.

A pharmaceutical composition can be in solution form, or alternatively, in lyophilized form for reconstitution with a suitable vehicle, such as sterile, pyrogen-free water. Both liquid and lyophilized forms will comprise one or more agents, preferably buffers, in amounts necessary to suitably adjust the pH of the injected solution.

The container in which the pharmaceutical formulation is packaged prior to use can comprise a hermetically sealed container enclosing an amount of the lyophilized formulation or a solution containing the formulation suitable for a pharmaceutically effective dose thereof, or multiples of an effective dose. The pharmaceutical formulation is packaged in a sterile container, and the hermetically sealed container is designed to preserve sterility of the pharmaceutical formulation until use. Optionally, the container can be associated with administration means and or instruction for use.

In certain embodiments of the invention, the polynucleotide constructs are delivered with additional, non-cytokine antiviral agents. Antiviral agents include, but are not limited to, protease inhibitors, nucleoside RT inhibitors, non-nucleoside RT inhibitors, fusion/binding inhibitors, and pyrophosphate analogue RT inhibitors.

Viral diseases which can be treated using the method of the present invention include chickenpox, shingles, rubella, influenza, rubeola, mumps, yellow fever, AIDS, mononucleosis, rabies, acute viral gastroenteritis, poliomyelitis, subacute sclerosing panencephalitis, encephalitis, Colorado tick fever, pharyngitis, croup, bronchiolitis, viral pneumonia, pleurodynia, aseptic meningitis, keratitis, conjunctivitis, viral leukemias, rabies, polio, myocarditis, hepatitis A, hepatitis B, hepatitis C, hepatitis D, hepatitis E; and any infections caused by adenoviruses, coxsackieviruses, parainfluenza viruses, respiratory syncytial virus, reovirus, cytomegalovirus, Epstein-Barr virus, herpes simplex viruses, herpes-zoster-varicella virus, rhinoviruses, rotaviruses, papolomaviruses, enteroviruses, paramyxoviruses, parvoviruses, apthoviruses, Ebola virus, Marburg virus, vesicular stomatitis virus, coronaviruses, Lassa virus, lymphocytic choriomeningitis virus, Machupo virus, Junin virus, and poxviruses.

In certain preferred embodiments, the method of the present invention is used to treat either Hepatitis B or Hepatitis C. In certain preferred embodiments, the polynucleotide construct encoding IFNω is administered with a polynucleotide encoding another cytokine to treat either Hepatitis B or Hepatitis C. The

polynucleotide encoding another cytokine may be part of the same construct encoding IFNω, or it may be part of a different construct. In certain embodiments, the other cytokine is IFNα, IL-2, or IL-12. In certain other preferred embodiments, other antiviral agents are administered with the polynucleotide construct encoding IFNω to treat either Hepatitis B or Hepatitis C. Other antiviral agents include levamisole, thymosin, thymus humoral factor-gamma 2, phosphonoformic acid trisodium, prednisone, interferon gamma, thymosin, levamisole, vidarabine, acyclovir, suramin, foscarnet, didanosine, and fialuridine; and nucleoside analogs such as penciclovir, ganciclovir, zidovudine, ribavirin, famciclovir, lamivudine (BioChem Pharma, Epivir/HBV), lobucavir, and adefovir dipivoxil.

Having now generally described the invention, the same will become more readily understood by reference to the following specific examples which are included herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

The present inventors have evaluated the therapeutic use of IFN-ω expressing plasmid DNA injected intramuscularly for the treatment of infectious disease. Described herein are 1) the *in vitro* characterization of the anti-viral biological activity of IFNs delivered by plasmid DNA; 2) *in vivo* expression (serum levels) of IFNω following intramuscular administration of IFNω plasmid DNA; and 3) the treatment regimen with IFNω plasmid DNA for patients with chronic Hepatitis B or C.

The IFNω plasmid DNA vectors used herein have been demonstrated to have potent anti-viral activity *in vitro*. The intramuscular injection of IFNω plasmid DNA result in systemic levels of the IFN protein. Moreover, therapeutic levels of IFN can be achieved following intramuscular administration of an IFN plasmid DNA in a very aggressive disease setting. Therefore, IFN plasmid DNA therapy is useful in the treatment of infectious disease.

Examples

Example 1: Construction of Expression Vectors

Two basic eukaryotic expression plasmid vectors, termed VR1012 and VR1055, can be used in the construction of interferon plasmids. These two blank plasmids differ only in transcriptional termination sequences. The backbone of both plasmids is derived from pUC19, with the beta-lactamase (ampicillin resistance) gene replaced by the aminoglycoside acetyltransferase (kanamycin resistance) gene from pET9a (Novagen, Madison, WI). Both plasmids direct eukaryotic gene expression from a cassette containing the human cytomegalovirus immediate early I gene promoter/enhancer, 5′ untranslated sequence, and intron A. Following these regulatory elements is a cloning polylinker for insertion of polypeptide coding sequences. Following the polylinker in VR1012 is the transcriptional terminator region from the bovine growth hormone gene. This region contains 3′ untranslated sequences and polyadenylation and termination signals. In VR1055, the transcriptional terminator region is derived from the rabbit beta-globin gene, and contains the polyadenylation and termination signals, but lacks any 3′ untranslated sequences.

Plasmid VR4101 (murine interferon a (mIFNα)) was constructed by cloning the murine interferon a cDNA into the vector VR1012 vector. The cDNA was obtained by amplifying the coding sequence from the plasmid RSV-a1 (Kelly, K.A. and P.M. Pitha, *Nucl. Acids Res. 13*: 805-823 (1985); Kelly, K.A. and P.M. Pitha, *Nucl. Acids Res. 13*: 825-839 (1985)). Plasmid VR4111 was constructed by transferring the coding sequences from VR4101 to the VR1055 cloning vector. The oligonucleotide primers used for polymerase chain reaction (PCR) were 5'-AACTGCAGATGGCTAGGCTCTGTGCT-3' (SEQ ID NO:4) and 5'-GAAGATCTTCATTTCTCTTCTCTCAG-3' (SEQ ID NO:5). Reaction conditions were 30 cycles of 94EC for 1 minute (denaturing), 58EC for 2 minutes (annealing), and 72EC for 1 minute (amplification).

Plasmid VR4102 (human interferon a (hIFNα)) was constructed by cloning the human interferon a gene into the VR1012 vector. The cDNA was obtained by amplifying the coding sequence from human genomic DNA prepared from a fresh blood sample. Plasmid VR4112 was constructed by transferring the coding sequence sequences from VR4102 to the VR1055 cloning vector. Genomic DNA was isolated using the QIAamp Blood Kit (Qiagen, Inc.). The oligonucleotide primers used for PCR were 5'-AACTGCAGATGGCCTC-GCCCTTTGCT-3' (SEQ ID NO:6) and 5'-CGGGATCCTTATTCCTTC-CTCCTTAATC-3' (SEQ ID NO:7). Reaction conditions were 30 cycles of 94EC for 1 minute (denaturing), 58EC for 2 minutes (annealing), and 72EC for 1 minute (amplification).

Plasmid VR4150 (human interferon T (hIFNω)) was constructed by cloning the human IFNω gene into the VR1012 cloning vector. The cDNA was obtained by amplifying the coding sequence from human genomic DNA prepared from a fresh blood sample. Plasmid VR4151 (SEQ ID NO:3) was constructed by transferring the coding sequence sequences from VR4150 to the VR1055 cloning vector. The oligonucleotide primers used for PCR were 5′-GCTCTAGATGGCCCTCCTGTTCCCT-3′ (SEQ ID NO:8) and 5′-GCGG-ATCCTCAAGATGAGCCCAGGTC-3′ (SEQ ID NO:9). Reaction conditions were 30 cycles of 94EC for 1 minute (denaturing), 58EC for 2 minutes (annealing), and 72EC for 1 minute (amplification).

Example 2: Purification of pDNA

pDNA was transformed into *Escherichia coli* DH10B-competent cells and grown in Terrific Broth (Sambrook, J. *et al.*, in: *Molecular Cloning: A laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, p. A.2 (1989)) complemented with 50 mg/ml kanamycin in a 1 Liter shaker flask. Cells were harvested by centrifugation at the end of the exponential growth phase (approximately 16 hr), typically yielding 10 grams of biomass net weight per liter. Covalently closed circular pDNA was isolated by a modified lysis procedure

(Horn, N.A. *et al.*, *Human Gene Therapy 6*: 565-573 (1995)) followed by standard double CsCl-ethidium bromide gradient ultracentrifugation with an average yield of approximately 5 mg per liter. Plasmids were ethanol precipitated and resolubilized in saline at 4°C and dialyzed against saline. Endotoxin content was determined by the *Limulus* Amebocyte Lysate assay (Associates of Cape Cod, Inc., Falmouth, MA). All plasmid preparations were free of detectable RNA. Endotoxin levels were less than 0.06 Endotoxin Units/μg of plasmid DNA. The spectrophotometric A260/A280 ratios were between 1.75 and 2.0.

Example 3: In Vitro Evaluation of Antiviral Activity of IFNw

To evaluate and quantitate the biological activity of IFNω and mIFNα, supernatants from UM449 cells transfected with the hIFNω plasmid DNA (VR4151), hIFNα plasmid DNA (VR4112), mIFNα plasmid DNA (VR4111), or the vector control (VR1055) were screened for anti-viral activity against murine encephalomyocarditis virus (EMCV) in both human (A549) and murine (L929) cells. All culture medium used in this and following examples was obtained from Life Technologies (Gaithersburg, MD), and all serum was obtained from HyClone (Logan, Utah).

In vitro transfection were performed as follows. UM449 cells (American Type Culture Collection, Rockville, MD) were plated at a concentration of 2×10^5 cells per well in a 6 well plate and incubated for 24 hours. Plasmid DNA and the lipid, DMRIE/DOPE (1:1) were each diluted to a concentration of 1 μg in 0.5 ml Optimem medium (Life Technologies, Gaithersburg, MD). The lipid DMRIE/DOPE consists of the cationic lipid (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide (DMRIE) and the neutral lipid dioleoylphosphatidylethanolamine (DOPE) at a 1:1 mol:mol ratio (Felgner et al., *J. Biol. Chem.* 269:2550-2561, 1994). The lipid mixture and the DNA mixture were then gently mixed. Medium was removed from the cells which

were rinsed gently with PBS, followed by addition of the DNA/lipid mixture (1 ml/well). After incubating the cells for 4-5 h at 37°C, one ml of Optimem with 30% fetal calf serum (FCS) was added to each well. Following an overnight incubation at 37°C, one ml of Optimem with 10% FCS was added to each well. Tissue culture supernatants were collected 48 h after the start of the *in vitro* transfection.

An antiviral assay was performed to evaluate the ability of the supernatants from the interferon plasmid DNA-transfected cells to protect murine L929 cells or human A549 cells from infection by murine encephalomyocarditis (EMC) virus (Assay performed at IIT Institute, Chicago, IL). *In vitro* transfections were performed as described above and supernatants were collected from cells transfected with either VR4151 (hIFNω), VR4112 (hIFNα), VR4111 (mIFNα) or VR1055 (control). Antiviral activity of the supernatants was performed by IIT Research Institute (Chicago, IL). Briefly, 2.5 x 10^4 L929 cells were plated into 96-well plates and incubated for 24 h. Tissue culture supernatants were serially diluted and added to the L929 cells which were incubated for another 24 h. Supernatants were then removed from the wells, the cells were washed and murine EMC virus was added to each well at a multiplicity of infection of 0.04. Assay plates were incubated further for 24 h followed by removal of supernatants, washing of wells, fixation with 5% formalin and staining with 1% crystal violet. Samples with interferon activity protected the cells from virus infection, resulting in darkly stained cell monolayers.

Supernatants from UM449 cells transfected with VR4151, VR4112, or VR4111 had antiviral activity of 30,000, 3,000 or 30 Units/ml, respectively, on human A549 cells. When evaluated for antiviral activity on the murine L929 cell line, supernatants from UM449 cells transfected with VR4151, VR4112, or VR4111 had antiviral activity of 300, 1000 and 30,000 Units/ml, respectively (Table 1) showing species specificity of the hIFNs for human cells and mIFNs for mouse cells.

Table 1. Antiviral Activity of interferon Plasmid DNA		
	Interferon (Units/ml)	
Plasmid	Human cell line	Murine cell line
VR4151 (hIFNω)	30,000	300
VR4112 (hIFNα)	3,000	1,000
VR4111 (mIFNα)	30	30,000

Example 4: Pharmacokinetics of IFNω in mice

Intramuscular injections and serum analysis. The muscle injections were performed using a 300 µl sterile tuberculin syringe fitted with a 28G 1/2 needle (Becton Dickenson) and a plastic collar cut from a 200 µl micropipette tip. The collar length was adjusted to limit the needle from penetrating further than 2 mm into the rectus femoris muscle. Serum samples were collected at the indicated time-points and analyzed using a hIFNω ELISA kit (Alexis, San Diego, CA) which was sensitive to 2 pg/ml.

Single dose pharmacokinetics. For single dose pharmacokinetic (PK) studies, nude mice received intramuscular (i.m.) injections on day 0 with 100 µg of VR4151 or VR1055 (50 µg/50 µl per leg, bilateral) in the rectus femoris. Serum samples were collected daily over a two week period and analyzed in the hIFNω ELISA kit. Serum samples were collected from 4-5 mice per day. Serum levels of IFNω were found as early as one day after injection (133 pg/ml). Peak serum levels were found on day 7 (648 pg/ml) and expression continued out to day 14 (134 pg/ml), the final time point of the study (Figure 2). Thus, interferon could be detected in the serum after a single i.m. injection of an interferon-encoding plasmid DNA.

Dose response pharmacokinetics. For the dose response PK studies, nude mice received i.m. injections on day 0 with 200, 100, or 10 µg of VR4151 or 100 µg of VR1055 (vector control) (bilaterally, 50 µg/50 µl per leg) in the rectus femoris. An additional group of mice received a single injection of 100 µg of

VR4151 (100 μg/100 μl) into one rectus femoris muscle. No significant differences in the serum levels of IFNω were observed following the intramuscular administration of 200 μg vs. 100 μg bilaterally vs. 100 μg administered to a single muscle. In all groups receiving VR4151, peak serum levels were found on Day 7 (approx. 300 - 425 pg/ml) and expression continued at approximately the same level in the 100 and 200 μg dose groups out to day 25 (100-125 pg/ml, Figure 3). A dose response was observed in the 10 μg dose group. For the latter group, the peak serum level was also found on Day 7, but it was approximately 8 fold lower than the higher dose groups. Serum levels persisted in the 10 μg dose group out to Day 21 (25 pg/ml), but at lower levels than the higher dose groups, being approximately 5X lower by Day 25 than the higher dose groups.

Repeat dose pharmacokinetics. Since the single dose PK studies with VR4151 pDNA in nude mice demonstrated that a single i.m. injection of VR4151 pDNA resulted in peak serum levels of IFNω at day 7 with a greater than 50% decline in serum levels by day 14, in follow up studies aimed at optimizing the injection regimen for sustained serum levels, repeat injection of IFNω pDNA was evaluated. Nude mice were injected intramuscularly with 100 μg of VR4151 (50 μg/50 μl per leg, bilateral) in the rectus femoris on days 0 and 14 (n=10). Control groups received 100 μg of VR1055 on corresponding times (n=3). Serum samples were collected every 3-4 days and assayed in a hIFNω ELISA. Cohorts of 5 mice per timepoint were bled from the VR4151-injected mice. All of the VR1055-injected mice were bled at each timepoint.

The i.m. injection of VR4151 pDNA on days 0 and 14 resulted in average peak serum values of 500 pg/ml hIFNω by day 7 after the first i.m. injection (Figure 4). The hIFNω serum levels declined to 260 pg/ml by day 10 after the first i.m. injection. After receiving a second i.m. injection of 100 μg of VR4151 on day 14, the hIFNω serum values rose to 460 pg/ml by 7 days later (day 21 after the first i.m. injection). Serum hIFNω values then declined to 225 pg/ml by day 27 after the first i.m. injection. Mice injected with the control pDNA VR1055 had 0 pg/ml hIFNω in the serum.

Therefore, the results show that the injections can be administered at 2 week intervals to maintain serum levels of IFNω (Figure 4), and suggest that a boost injection of VR4151 pDNA at day 14 can lead to increased serum levels up to day 21 and that serum levels decline at 14 days following the last intramuscular injection of the pDNA.

Example 5: Pharmacokinetics of IFNω in rats

Sprague Dawley rats (HSD, San Diego) were injected i.m. into the rectus femoris with VR4151. Rats received a single i.m. injection of either 0.1 mg (in 100 µl, 50 µl bilateral) or 1 mg (in 500 µl, 250 µl bilateral) of VR4151. An additional group of rats received 3 consecutive i.m. injections 1 mg of VR4151 (in 500 µl, 250 µl bilateral/day). VR4151 was diluted in 150 mM sodium phosphate buffer, pH7.2 for the i.m. injections. Serum samples were collected every 2-3 days and assayed in a hIFNω ELISA (Alexis, San Diego). Prebleeds of the rats (prior to i.m. injection) were used to determine background hIFNω levels and were subtracted from the final hIFNω values obtained at each timepoint after i.m. injection. Each group consisted of 4 rats.

Rats injected i.m. once with either 0.1 or 1 mg of VR4151 had an average of 9 and 11 pg/ml hIFNω, respectively, in the serum by day 6 after injection. By day 9, serum hIFNω levels were an average of 4 and 12 pg/ml for the groups injected once with either 0.1 or 1 mg VR4151, respectively. Rats injected i.m. with 1 mg VR4151 for 3 consecutive days had an average of 68 pg/ml hIFNω in the serum by day 5 after the first i.m. injection and 36 pg/ml hIFNω 4 days later.

Example 6: Treatment Regimen with IFNω Plasmid DNA for Patients with Chronic Hepatitis B or C

To decrease the chronic infection of Hepatitis B and C in human patients, 1-50 mg, preferably 10-30 mg of IFNω plasmid DNA in a pharmacologically acceptable carrier is injected to the patients biweekly or monthly. The therapy regimen is continued for a minimum of 24 weeks during which time the patients are monitored for levels of serum alanine aminotransferase, and serum HBsAg and HBV DNA for HBV patients, or HCV RNA in the case of HCV patients. In addition, liver biopsies are performed at the end of the treatment period. A successful outcome of the therapy is indicated by a normalization of serum alanine aminotransferase levels, a disappearance or decrease in detectable virus in the patient's serum, and histological improvement in the liver. In some cases, this therapy is used in conjunction with anti-virals such as lamivudine for HBV and ribavirin for HCV.

What Is Claimed Is:

1. A method for treating a viral infection in a mammal, comprising:

administering into a tissue of said mammal a non-infectious, non-integrating polynucleotide construct selected from the group consisting of:

(a) a polynucleotide that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID NO:1, wherein said polynucleotide encodes a polypeptide having antiviral activity;

(b) a polynucleotide that encodes a polypeptide having an amino acid sequence which, except for at least one but not more than 20 amino acid substitutions, deletions, or insertions, or any combination thereof, is identical to amino acids -23 to 172 of SEQ ID NO:2, wherein said polypeptide has antiviral activity;

(c) a polynucleotide that encodes a polypeptide having an amino acid sequence which, except for at least one but not more than 20 amino acid substitutions, deletions, or insertions, or any combination thereof, is identical to amino acids 1 to 172 of SEQ ID NO:2, wherein said polypeptide has antiviral activity; and

(d) a polynucleotide that encodes a polypeptide comprising amino acids 86 to 172 of SEQ ID NO:2;

wherein said polynucleotide is expressed *in vivo* in an amount effective to treat said viral infection.

2. The method of claim 1, wherein said polynucleotide is (a).

3. The method of claim 2, wherein said polynucleotide encodes amino acids -23 to 172 of SEQ ID NO:2.

4. The method of claim 2, wherein said polynucleotide encodes amino acids 1 to 172 of SEQ ID NO:2.

5. The method of claim 1, wherein said polynucleotide is (b).

6. The method of claim 1, wherein said polynucleotide is (c).

7. The method of claim 1, wherein said polynucleotide is (d).

8. The method of claim 7, wherein said polypeptide comprises amino acids 61 to 172 of SEQ ID NO:2.

9. The method of claim 8, wherein said polypeptide comprises amino acids 41 to 172 of SEQ ID NO:2.

10. The method of claim 9, wherein said polypeptide comprises amino acids 21 to 172 of SEQ ID NO:2.

11. The method of claim 1, wherein said polynucleotide construct is DNA comprising a promoter operably linked to said polynucleotide encoding said polypeptide.

12. The method of claim 11, wherein said polynucleotide construct is a plasmid.

13. The method of claim 1, wherein said polynucleotide construct is RNA.

14. The method of claim 1, wherein said polynucleotide construct is administered into a tissue selected from the group consisting of 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.

15. The method of claim 14, wherein said tissue is muscle.

16. The method of claim 15, wherein said muscle is skeletal.

17. The method of claim 15, wherein said muscle is cardiac.

18. The method of claim 1, wherein said construct is free from association with transfection-facilitating proteins, viral particles, liposomes, cationic lipids, and calcium phosphate precipitating agents.

19. The method of claim 1, wherein said construct is administered as a complex of said construct and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof.

20. The method of claim 19, wherein said cationic compound is a cationic lipid.

21. The method of claim 20, wherein said cationic lipid is selected from the group consisting of: 5-carboxyspermylglycine dioctadecylamide; dipalmitoyl-phophatidylethanolamine-5carboxyspermylamide; {3β-[N-N',N'-dimethylamino)ethane]-carbomoyl}-cholesterol; dimethyldioctdecyl-ammonium bromide; N-(3-aminopropyl)-N,N-(*bis*-(2-tetradecyloxyethyl))-N-methyl-ammonium bromide; N-(3-aminopropyl)-N,N-(*bis*-(2-dodecyloxyethyl))-N-methyl-ammonium bromide; N,N,N-*tris*-(2-dodecyloxy)ethyl-N-(3-amino)propyl-ammonium bromide; N^1-(3-aminopropyl)((2-dodecyloxy)ethyl)-N^2-(2-dodecyloxy)ethyl-1-piperazinaminium bromide; DL-1,2-dioleoyl-3-

dimethylaminopropyl-β-hydroxyethylammonium; 1-O-oleyl-2-oleoyl-3-dimethylaminopropyl-β-hydroxyethylammonium; 3,5-(N,N-dilysyl)-diaminobenzoyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl-β-hydroxyethylamine); 3,5-(N,N-di-lysyl)diamino-benzoylglycyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl-β-hydroxyethylamine); (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide; (±)-N,N-dimethyl-N-[2-(sperminecarboxamido)ethyl]-2,3-bis(dioleyloxy)-1-propaniminium pentahydrochloride; (±)-N-(2-aminoethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide; (±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-l-propaniminium bromide; (±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-(*bis*-decyloxy)-1-propanaminium bromide; (±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-(*bis*-tetradecyloxy)-1-propanaminium bromide; (±)-N-((N"-methyl)-N'-ureyl)propyl-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide; (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide; and (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-*bis*-([Z]-9-octadecenyloxy)propyl-1- propaniminium bromide.

22. The method of claim 20, wherein said complex further comprises one or more neutral lipids.

23. The method of claim 22, wherein said polynucleotide construct is complexed with DMRIE/DOPE.

24. The method of claim 1, wherein said polynucleotide construct is administered with a separate polynucleotide construct comprising a polynucleotide encoding a cytokine different than IFNω.

25. The method of claim 1, wherein said polynucleotide construct further encodes a cytokine different than IFNω.

26. The method of claim 1, wherein said viral infection is Hepatitis B.

27. The method of claim 26 wherein said polynucleotide construct is administered with an additional antiviral agent.

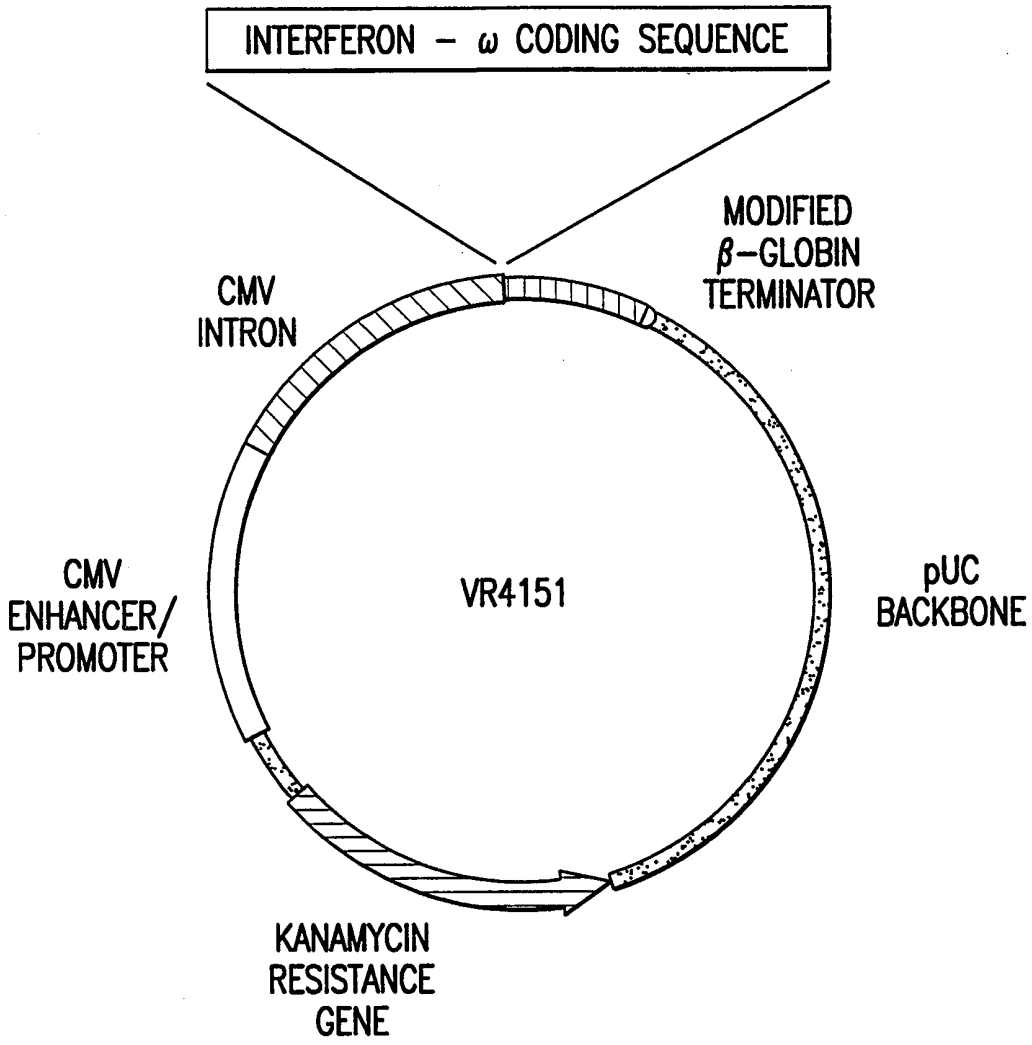
28. The method of claim 27, wherein said antiviral agent is selected from the group consisting of lamivudine, levamisole, thymus humoral factor-gamma-2, phosphonoformic acid trisodium, penciclovir, ganciclovir, zidovudine and ribavirine.

29. The method of claim 1, wherein said viral infection is Hepatitis C.

30. The method of claim 29, wherein said polynucleotide construct is administered with an additional antiviral agent.

31. The method of claim 30, wherein said antiviral agent is selected from the group consisting of levamisole, thymus humoral factor-gamma-2, phosphonoformic acid trisodium, penciclovir, ganciclovir, zidovudine and ribavirin.

32. The method of claim 1, wherein said mammal is human.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VR4151
pUC BACKBONE
KANAMYCIN RESISTANCE GENE

FIG.1

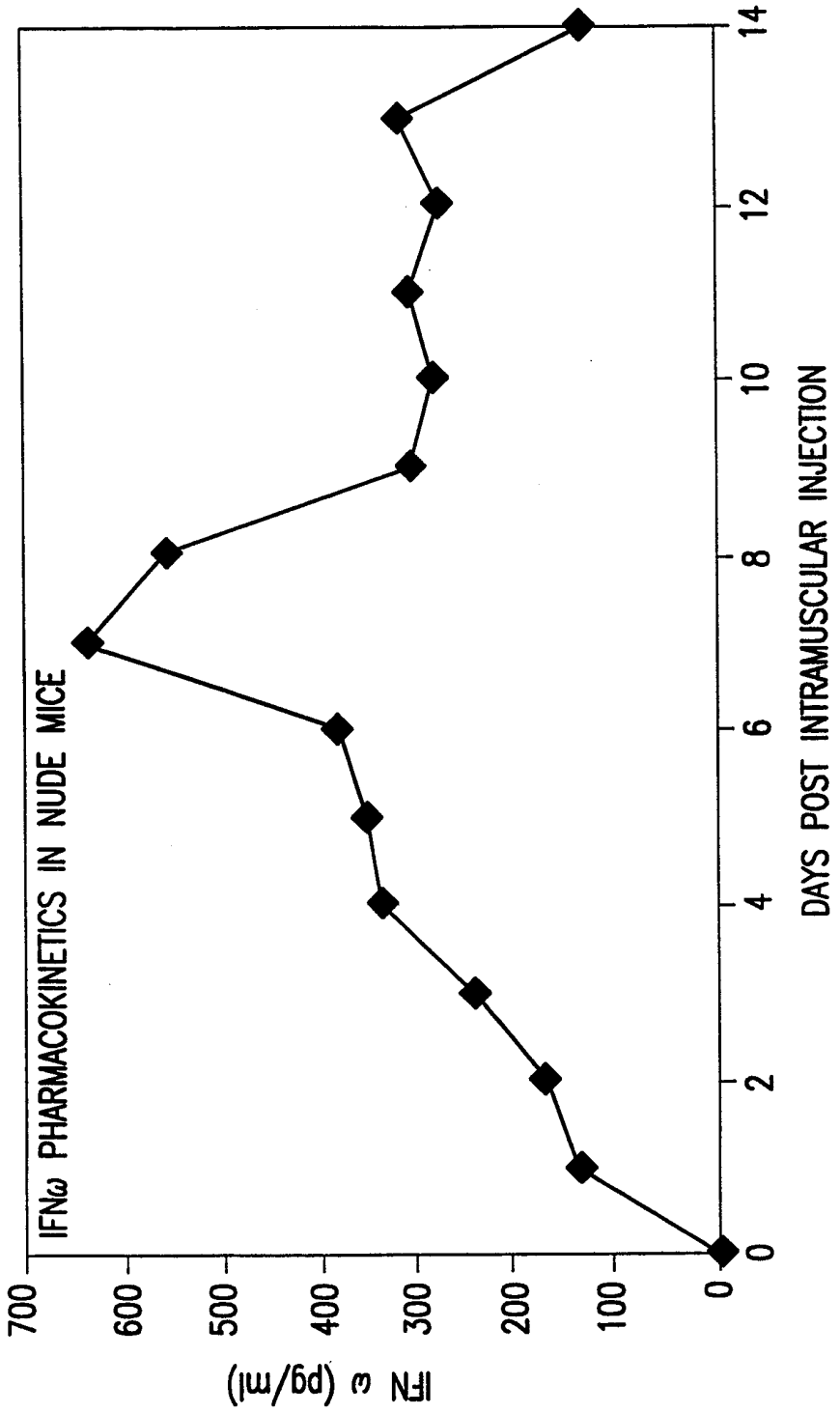

IFNω PHARMACOKINETICS IN NUDE MICE
IFN ω (pg/ml)
700
600
500
400
300
200
100
0
0
2
4
6
8
10
12
14
DAYS POST INTRAMUSCULAR INJECTION

FIG.2

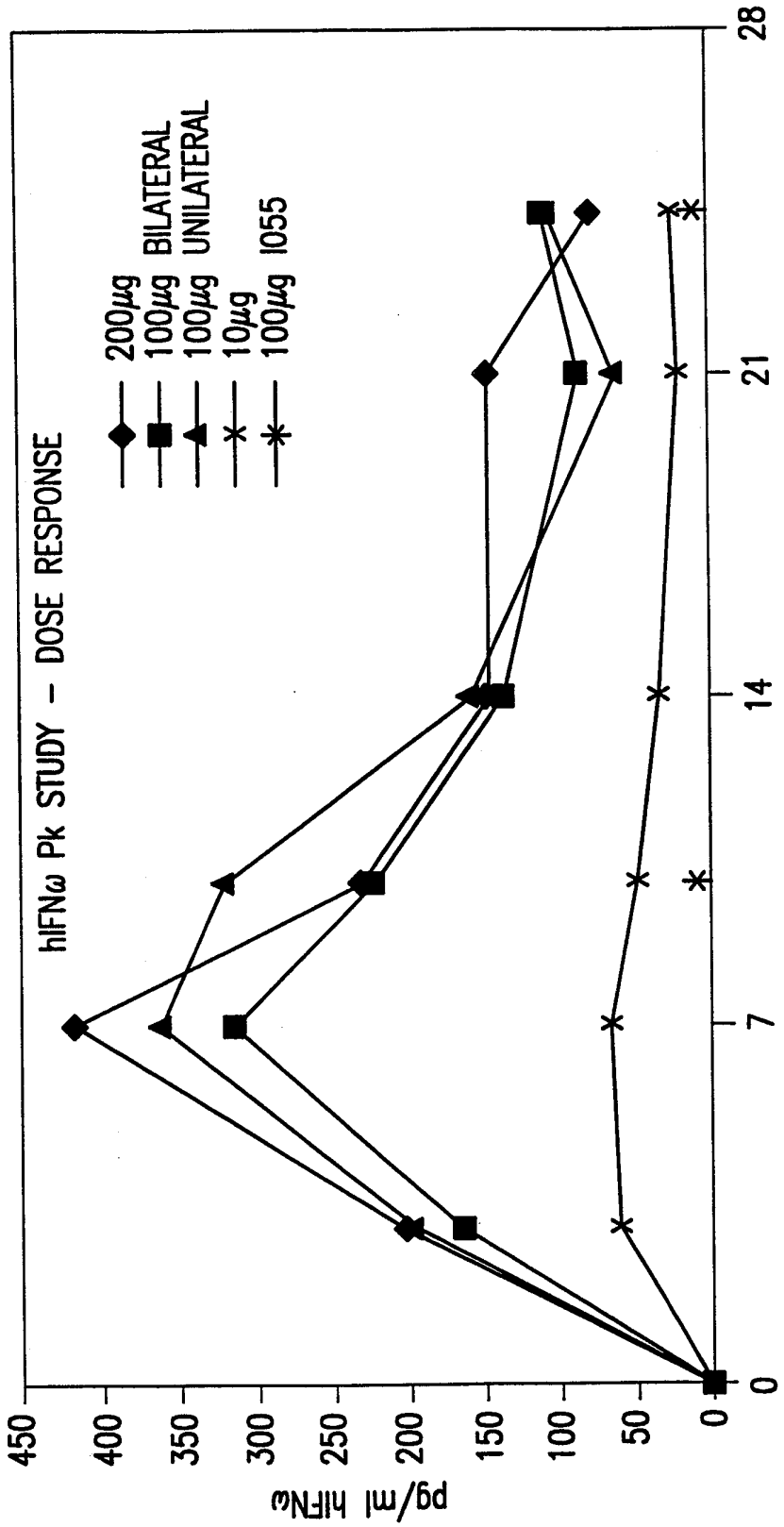
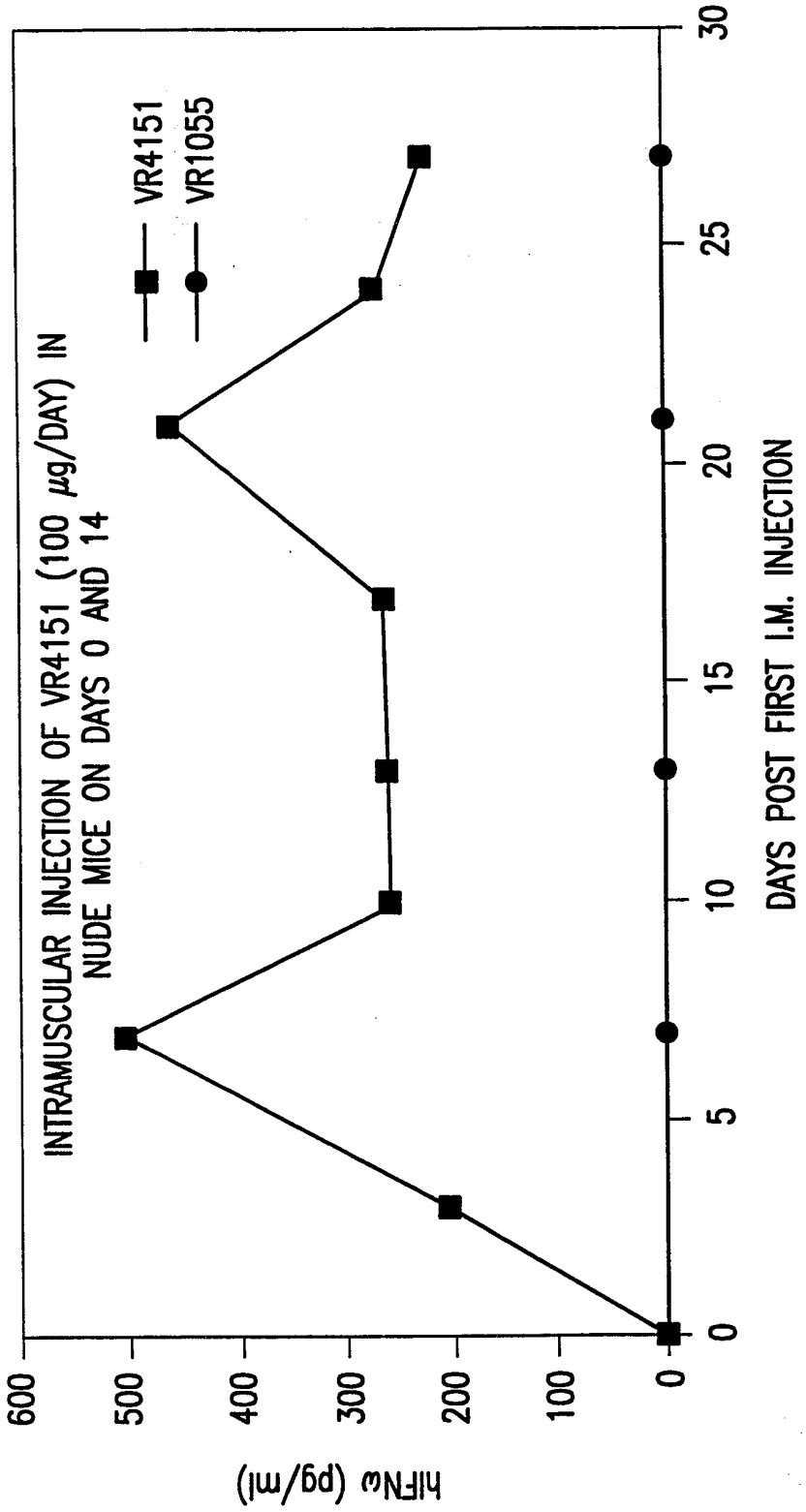

hIFNω Pk STUDY – DOSE RESPONSE
200μg BILATERAL
100μg UNILATERAL
100μg
10μg
100μg I055
pg/ml hIFNω
0
50
100
150
200
250
300
350
400
450
0
7
14
21
28
DAYS AFTER SINGLE I.M. INJECTION

FIG.3


INTRAMUSCULAR INJECTION OF VR4151 (100 μg/DAY) IN
NUDE MICE ON DAYS 0 AND 14
VR4151
VR1055
hIFNω (pg/ml)
0
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200
300
400
500
600
0
5
10
15
20
25
30
DAYS POST FIRST I.M. INJECTION

FIG.4

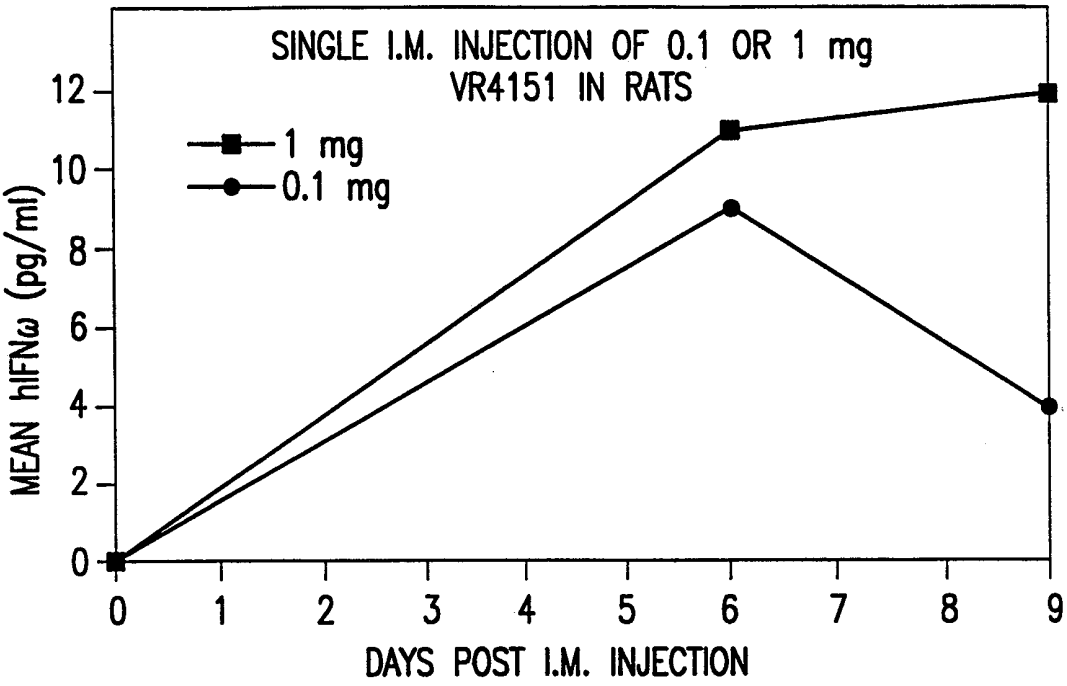
SINGLE I.M. INJECTION OF 0.1 OR 1 mg
VR4151 IN RATS
1 mg
0.1 mg
MEAN hIFNω (pg/ml)
0
2
4
6
8
10
12
0 1 2 3 4 5 6 7 8 9
DAYS POST I.M. INJECTION

FIG. 5A

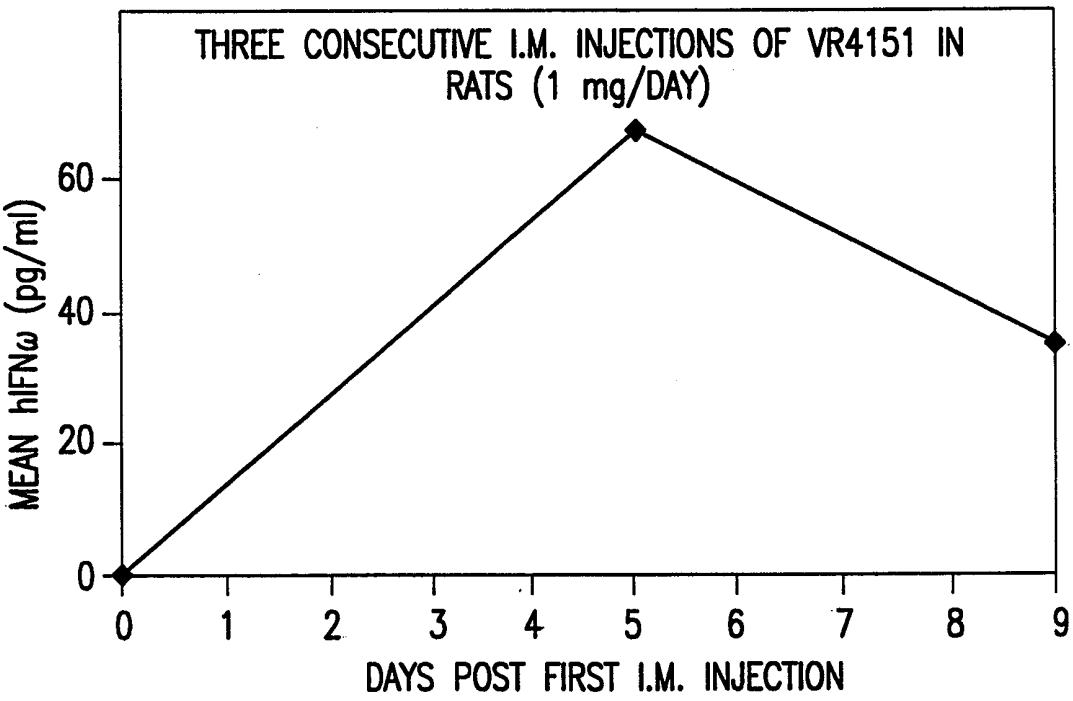
THREE CONSECUTIVE I.M. INJECTIONS OF VR4151 IN
RATS (1 mg/DAY)
MEAN hIFNω (pg/ml)
0
20
40
60
0 1 2 3 4 5 6 7 8 9
DAYS POST FIRST I.M. INJECTION

FIG. 5B

SEQUENCE LISTING

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Parker, Suezanne
Horton, Holly

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 10                  15                  20                  25

cct ttc ttg tgt ctc aag gac aga aga gac ttc agg ttc ccc cag gag   192
Pro Phe Leu Cys Leu Lys Asp Arg Arg Asp Phe Arg Phe Pro Gln Glu
                 30                  35                  40

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Met Val Lys Gly Ser Gln Leu Gln Lys Ala His Val Met Ser Val Leu
             45                  50                  55

cat gag atg ctg cag cag atc ttc agc ctc ttc cac aca gag cgc tcc   288
His Glu Met Leu Gln Gln Ile Phe Ser Leu Phe His Thr Glu Arg Ser
         60                  65                  70

tct gct gcc tgg aac atg acc ctc cta gac caa ctc cac act gga ctt   336
Ser Ala Ala Trp Asn Met Thr Leu Leu Asp Gln Leu His Thr Gly Leu
     75                  80                  85

cat cag caa ctg caa cac ctg gag acc tgc ttg ctg cag gta gtg gga   384
His Gln Gln Leu Gln His Leu Glu Thr Cys Leu Leu Gln Val Val Gly
 90                  95                 100                 105

gaa gga gaa tct gct ggg gca att agc agc cct gca ctg acc ttg agg   432
Glu Gly Glu Ser Ala Gly Ala Ile Ser Ser Pro Ala Leu Thr Leu Arg
                110                 115                 120

agg tac ttc cag gga atc cgt gtc tac ctg aaa gag aag aaa tac agc   480
Arg Tyr Phe Gln Gly Ile Arg Val Tyr Leu Lys Glu Lys Lys Tyr Ser
            125                 130                 135

gac tgt gcc tgg gaa gtt gtc aga atg gaa atc atg aaa tcc ttg ttc   528
Asp Cys Ala Trp Glu Val Val Arg Met Glu Ile Met Lys Ser Leu Phe
        140                 145                 150

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 10                  15                  20                  25

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                 30                  35                  40

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             45                  50                  55

His Glu Met Leu Gln Gln Ile Phe Ser Leu Phe His Thr Glu Arg Ser
         60                  65                  70

Ser Ala Ala Trp Asn Met Thr Leu Leu Asp Gln Leu His Thr Gly Leu
     75                  80                  85

His Gln Gln Leu Gln His Leu Glu Thr Cys Leu Leu Gln Val Val Gly
 90                  95                 100                 105

Glu Gly Glu Ser Ala Gly Ala Ile Ser Ser Pro Ala Leu Thr Leu Arg
                110                 115                 120

Arg Tyr Phe Gln Gly Ile Arg Val Tyr Leu Lys Glu Lys Lys Tyr Ser
            125                 130                 135

Asp Cys Ala Trp Glu Val Val Arg Met Glu Ile Met Lys Ser Leu Phe
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170
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cccgcctggc tgaccgccca acgacccccg cccattgacg tcaataatga cgtatgttcc 480

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ctccgcccca ttgacgcaaa tgggcggtag gcgtgtacgg tgggaggtct atataagcag 900

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