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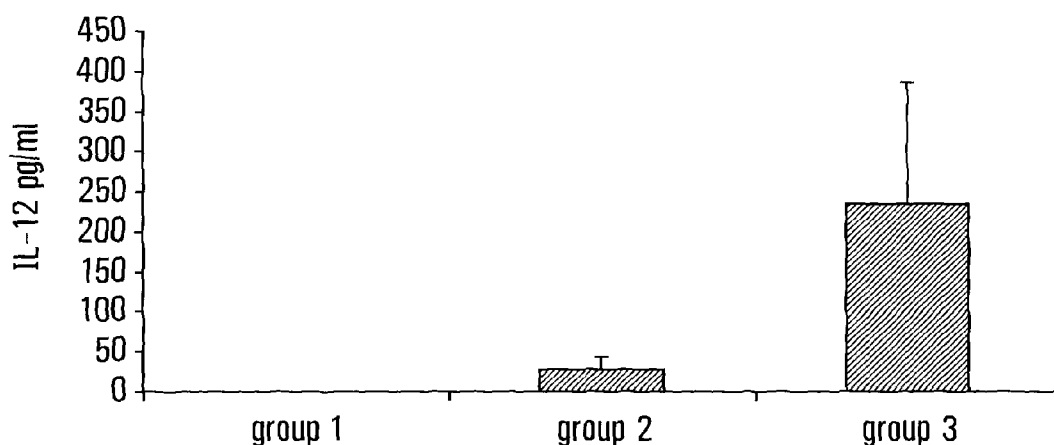
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(54) Title: USE OF LIPOSOMES IN A CARRIER COMPRISING A CONTINUOUS HYDROPHOBIC PHASE FOR DELIVERY OF POLYNUCLEOTIDES IN VIVO

**FIG. 1**

(57) Abstract: Compositions comprising liposomes are frequently used to deliver nucleic acids to a mammal for exogenous expression of a therapeutic protein, suppression of the expression of a disease-causing protein, or to induce an immune response against a disease-causing pathogenic cell or a cancer cell. The liposome-nucleic acid complex of these compositions are normally suspended as a dispersed phase in a pharmaceutically-acceptable aqueous carrier that constitutes a continuous phase to form an oil-in-water emulsion. Using a continuous phase of a hydrophobic carrier, instead of an aqueous carrier, to carry the dispersed phase of liposomes encapsulating nucleic acids, a composition is produced that is more effective in delivering nucleic acids to a mammalian subject in addition to being encapsulated in the liposomes, the nucleic acids may also be present in the hydrophobic carrier as a water-in-oil emulsion.

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Use of liposomes in a carrier comprising a continuous hydrophobic phase for delivery of polynucleotides *in vivo*

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of and priority to United States Provisional Patent Application No. 60/975,602 filed on September 27, 2007 and United States Provisional Patent Application No. 61/061,303 filed June 13, 2008, both of which are hereby incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present application relates to the use of a composition comprising liposomes and a continuous hydrophobic phase as a vehicle for delivering polynucleotides *in vivo*.

BACKGROUND OF THE INVENTION

There has been much research into effective introduction of nucleic acids into target cells and tissues for use in e.g. gene therapy. Such nucleic acids may be e.g. sequences encoding a gene product or instead short sequences of nucleotides that correspond to the sense or antisense sequence of specific genes or their products and hence have a direct effect on the expression of these genes and/or their products.

There continue to exist problems in delivering the nucleic acids to the correct target site and to a sufficient number of target cells. Nucleic acids are subject to nuclease attack and are often unable to cross cell membranes. A wide variety of delivery methods have been proposed, including microinjection, scrape loading, and

receptor-mediated endocytosis. Lipid-based carrier systems, including those involving use of liposomes, are frequently used to package the therapeutic nucleic acids. However, the use of liposomes may pose problems such as poor encapsulation efficacy and rapid clearance from circulation. There may also be problems in packaging enough nucleic without increasing the size of the liposome to the point where delivery to the target tissues is impaired. Accordingly, there exists a need to develop liposome-based delivery systems for targeting nucleic acids to the correct target tissue.

SUMMARY OF THE INVENTION

In one aspect, the invention provides a composition comprising: a carrier comprising a continuous phase of a hydrophobic substance; liposomes; and a polynucleotide.

In another aspect, the invention provides a method for delivering a polynucleotide to a subject, comprising administering the composition as described above to a subject.

Other aspects and features of the present invention will become apparent to those of ordinary skill in the art upon review of the following description of specific embodiments of the invention in conjunction with the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

In the figures, which illustrate embodiments of the invention by way of example only:

Figure 1 illustrates IL-12 expression potential in cells isolated from lymph nodes 8 days post-injection.

Figure 2 illustrates IL-12 expression potential in cells isolated from lymph nodes 8 days post-injection. IL-12 protein levels detected from each group of mice were averaged.

Figure 3 illustrates green fluorescent protein (GFP) expression potential in cells isolated from lymph nodes 8 days post-injection. Lymph node cells from animals of Group 1 (GFP in PBS), Group 2 (GFP/liposome/hydrophobic carrier), Group 3 (GFP/hydrophobic carrier) and Group 4 (GFP/liposome) contained detectable GFP expressing cells above background fluorescence represented by a horizontal line. Background fluorescence was estimated using lymph node cell fluorescence counts from control animals of Group 5 (liposome/hydrophobic carrier, no GFP), and Group 6 (untreated mice). P values were calculated using the student T test.

Figure 4 illustrates CD11b/CD11c positive lymph node cells showing GFP expression at 8 days post-vaccination. Data presented in Figure 1 was re-analyzed to compare the number of CD11b/CD11c and GFP positive cells in lymph nodes from animals of Groups 1-6 (described in Figure 1). The number of CD11b/CD11c positive, GFP positive cells was calculated as a percentage of total lymph node cells.

Figure 5 illustrates inhibition of plasmid IL-12 induced IL-12 protein expression in cells isolated from lymph nodes, following injection of IL-12 siRNA. IL-12 protein levels detected from each group of mice were averaged. Lymph node cells are from Group 1 (pORF-mIL12

plasmid alone, no siRNA), Group 2 (pORF-mIL12 plasmid, IL12-siRNA in PBS), Group 3 (pORF-mIL12 plasmid, IL12-siRNA in liposome/ hydrophobic carrier) and Group 4 (untreated, naïve mice). p-values were calculated using Students t test.

Figure 6 illustrates inhibition of ovalbumin-induced IL-12 protein expression in cells isolated from lymph nodes, following injection of IL-12 siRNA. IL-12 protein levels detected from each group of mice were averaged. Lymph node cells are from Group 1 (ovalbumin in CFA on day-0, no siRNA), Group 2 (ovalbumin in CFA, IL12-siRNA in PBS, day minus 1), Group 3 (ovalbumin in CFA, IL12-siRNA in liposome/hydrophobic carrier, day minus 1), Group 4 (ovalbumin in CFA, IL12-siRNA in PBS, day plus 1), Group 5 (ovalbumin in CFA, IL12-siRNA in liposome/hydrophobic carrier, day plus 1) and Group 6 (untreated naïve mice). p-values were calculated using Students t test.

DETAILED DESCRIPTION

The invention provides compositions useful for delivering a polynucleotide to a subject.

Polynucleotides

The use of polynucleotides as described herein refers specifically to polynucleotides that contain sequences that correspond largely to the sense or antisense sequence of specific genes or their products, and hence have a direct effect on the expression of these genes and/or their products. For example, the use of polynucleotides that contain gene coding sequences affects the transcription and/or translation of the genes of interest in cells that uptake such polynucleotides. Similarly, the use of RNA interference polynucleotides affects the expression of

specific genes of interest by directly affecting the levels of mRNA in cells that uptake such nucleotides. This differs significantly from other polynucleotide-based molecules such as CpG and polyIC adjuvants, which do not act through the presence of gene specific sequences. Furthermore, polynucleotide-based adjuvants are believed to modulate an immune response in a non-specific manner, and their actions start at the site of vaccination where they interact with extracellular receptors to enhance the activity of immune cells in a non-specific manner. In some cases, polynucleotide-based adjuvants are internalized whereby they exert their effects by interacting with intracellular receptors, similarly leading to the activation of downstream pathways, and resulting collectively in the enhancement of immune cell activity to aid in the generation of an immune response. Such adjuvants do not directly affect the expression of specific genes that are being targeted by polynucleotide constructs as contemplated herein. Such adjuvants do not directly interact with the expression products of targeted genes, nor do they contain sequences that correspond to the sense or antisense sequence of targeted genes.

In one embodiment, the composition is useful for enhancing the expression of a polypeptide-encoding polynucleotide *in vivo*. In other embodiments, the polynucleotide may not encode a polypeptide, but may instead be e.g. a polynucleotide comprising or encoding an antisense RNA or other molecule that is not a polypeptide. In some embodiments, the compositions comprise a polynucleotide of interest, optionally operably linked to regulatory sequences suitable for directing protein expression from the polynucleotide (e.g. a promoter), liposomes, and a carrier comprising a continuous phase of a hydrophobic substance.

Compositions of the invention were demonstrated to increase polypeptide expression from plasmid DNA, as measured by ELISA, in a murine model, relative to plasmid DNA suspended in phosphate buffered saline. Compositions of the invention were also demonstrated to increase polypeptide expression from plasmid DNA, as measured by immunofluorescence, in a murine model, relative to plasmid DNA suspended in phosphate buffered saline, Incomplete Freund's Adjuvant (IFA) or in liposomes with no oil.

As used herein the term "polynucleotide" encompasses a chain of nucleotides of any length (e.g. 9, 12, 18, 24, 30, 60, 150, 300, 600, 1500 or more nucleotides) or number of strands (e.g. single-stranded or double-stranded). Polynucleotides may be DNA (e.g. genomic DNA or cDNA) or RNA (e.g. mRNA) or combinations thereof. They may be naturally occurring or synthetic (e.g. chemically synthesized). It is contemplated that the polynucleotide may contain modifications of one or more nitrogenous bases, pentose sugars or phosphate groups in the nucleotide chain. Such modifications are well-known in the art and may be for the purpose of e.g. improving stability of the polynucleotide.

As used herein, the term "polypeptide" or "protein" means any chain of amino acids, regardless of length (e.g. 4, 6, 8, 10, 20, 50, 100, 200, 500 or more amino acids) or post-translational modification (e.g., glycosylation or phosphorylation). Both terms are used interchangeably.

The compositions of the invention are useful for delivering polynucleotides of all kinds to a subject *in vivo*. In some embodiments, the polynucleotide is not

expressed as a protein in the subject, but rather encodes e.g. an antisense RNA, an interfering RNA, a catalytic RNA, or a ribozyme. In some embodiments, the polynucleotide encodes a polypeptide to be expressed *in vivo* in a subject. The invention is not limited to the expression of any particular type of polypeptide. The polypeptide may be, merely by way of illustrative examples, an antigen, an antibody or antibody fragment, an enzyme, a cytokine, a therapeutic protein, a chemokine, a regulatory protein, a structural protein, a chimeric protein, a nuclear protein, a transcription factor, a viral protein, a TLR protein, an interferon regulatory factor, an angiostatic or angiogenic protein, an apoptotic protein, an Fc gamma receptor, a hematopoietic protein, a tumor suppressor, a cytokine receptor, or a chemokine receptor.

Representative antigens include, without limitation: those derived from Cholera toxoid, tetanus toxoid, diphtheria toxoid, hepatitis B surface antigen, hemagglutinin, neuraminidase, influenza M protein, PfHRP2, pLDH, aldolase, MSP1, MSP2, AMA1, Der-p-1, Der-f-1, Adipophilin, AFP, AIM-2, ART-4, BAGE, alpha-fetoprotein, BCL-2, Bcr-Abl, BING-4, CEA, CPSF, CT, cyclin D1Ep-CAM, EphA2, EphA3, ELF-2, FGF-5, G250, Gonadotropin Releasing Hormone, HER-2, intestinal carboxyl esterase (iCE), IL13Ralpha2, MAGE-1, MAGE-2, MAGE-3, MART-1, MART-2, M-CSF, MDM-2, MMP-2, MUC-1, NY-EOS-1, MUM-1, MUM-2, MUM-3, p53, PBF, PRAME, PSA, PSMA, RAGE-1, RNF43, RU1, RU2AS, SART-1, SART-2, SART-3, SAGE-1, SCRN 1, SOX2, SOX10, STEAP1, surviving, Telomerase, TGFbetaRII, TRAG-3, TRP-1, TRP-2, TERT, or WT1; those derived from a virus, such as Cowpoxvirus, Vaccinia virus, Pseudocowpox virus, Human herpesvirus 1, Human herpesvirus 2, Cytomegalovirus, Human adenovirus A-F, Polyomavirus, Human papillomavirus,

Parvovirus, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Human immunodeficiency virus, Orthoreovirus, Rotavirus, Ebolavirus, parainfluenza virus, influenza A virus, influenza B virus, influenza C virus, Measles virus, Mumps virus, Rubella virus, Pneumovirus, Human respiratory syncytial virus, Rabies virus, California encephalitis virus, Japanese encephalitis virus, Hantaan virus, Lymphocytic choriomeningitis virus, Coronavirus, Enterovirus, Rhinovirus, Poliovirus, Norovirus, Flavivirus, Dengue virus, West Nile virus, Yellow fever virus and varicella; those derived from a bacterium, such as Anthrax, Brucella, Candida, Chlamydia pneumoniae, Chlamydia psittaci, Cholera, Clostridium botulinum, Coccidioides immitis, Cryptococcus, Diphtheria, Escherichia coli O157: H7, Enterohemorrhagic Escherichia coli, Enterotoxigenic Escherichia coli, Haemophilus influenzae, Helicobacter pylori, Legionella, Leptospira, Listeria, Meningococcus, Mycoplasma pneumoniae, Mycobacterium, Pertussis, Pneumonia, Salmonella, Shigella, Staphylococcus, Streptococcus pneumoniae and Yersinia enterocolitica; or those derived from a protozoa, e.g. Plasmodium falciparum.

RNA interference (RNAi) is a sequence specific post-transcriptional gene silencing mechanism, which is triggered by double-stranded RNA such as small (or short) interference RNA (siRNA) and single stranded intracellular RNA such as microRNA (miRNA), both of which can cause degradation of mRNAs homologous in sequence to siRNA or miRNA (Fire et al, 1998, Nature, 391:806-811; Montgomery et al, 1998, PNAS, 95:15502-15507; Elbashir et al, 2001, Nature, 411:494-498). RNAi is a conserved pathway common to plants and mammals that suppress expression of genes with complementary sequences (Hannon and Rossi, 2004, Nature, 431:371-378; Meister and Tuschl, 2004, Nature, 431,

343-349). RNAi was first observed in lower organisms, such as plants or nematodes. In these systems, long dsRNAs serve as effective triggers of RNAi. Long dsRNAs are not the actual triggers but are degraded by the endoribonuclease Dicer into small effector molecules called siRNAs. In mammals, Dicer processing occurs as a complex with the RNA-binding protein TRBP. The nascent siRNA associates with Dicer, TRBP, and Ago2 to form the RNA-Induced Silencing Complex (RISC) that mediates gene silencing (Chendrimada et al, 2005, Nature, 436:740-744). Once in RISC, one strand of the siRNA (the passenger strand) is degraded or discarded while the other strand (the guide strand) remains to direct sequence specificity of the silencing complex. The Ago2 component of RISC is a ribonuclease that cleaves a target RNA under direction of the guide strand.

Although long dsRNAs (several hundred bp) are commonly employed to trigger RNAi in *C. elegans* or *D. melanogaster*, these molecules will activate the innate immune system and trigger interferon (IFN) responses in higher organisms. RNAi can be performed in mammalian cells using short RNAs, which generally do not induce IFN responses. Many researchers today employ synthetic 21-mer RNA duplexes as their RNAi reagents, which mimic the natural siRNAs that result from Dicer processing of long substrate RNAs. An alternative approach is to use synthetic RNA duplexes that are greater than 21-mer in length, which are substrates for Dicer (Tuschl, T. 2002, Nature Biotechnology, 20:446).

Recently developed Dicer-substrate RNAs (DsiRNAs) are chemically synthesized RNA duplexes that have increased potency in RNA interference (Kim et al, 2005, Nat Biotechnol,

23:222-226). DsiRNAs are processed by Dicer into 21-mer siRNAs and designed so that cleavage results in a single, desired product. This is achieved through use of a novel asymmetric design where the RNA duplex has a single 2-base 3'-overhang on the AS strand and is blunt on the other end; the blunt end is modified with DNA bases. This design provides Dicer with a single favorable PAZ binding site that helps direct the cleavage reaction. Functional polarity is introduced by this processing event, which favors AS strand loading into RISC, and the increased potency of these reagents is thought to relate to linkage between Dicer processing and RISC loading (Rose et al, 2005, Nucleic Acids Res, 33:4140-4156). The Dicer-substrate approach can result in reagents having as much as 10-fold higher potency than traditional 21-mer siRNAs at the same site.

miRNA, first described in 1993 (Lee et al, 1993, Cell 75:843-854), are single-stranded RNA molecules of about 21-23 nucleotides in length, which regulate gene expression. miRNAs are encoded by genes that are transcribed from DNA but not translated into protein (non-coding RNA); instead they are processed from primary transcripts known as *pri-miRNA* to short stem-loop structures called *pre-miRNA* and finally to functional miRNA. Mature miRNA molecules are partially complementary to one or more messenger RNA (mRNA) molecules, and their main function is to downregulate gene expression. Although miRNA is generated within the cell and is highly conserved, it rarely has perfect complementarity with mRNA sequences. However, miRNA can affect protein translation and mRNA decay by binding to its imperfectly matched target sites on 3' UTR region of mRNA, which also requires Ago protein (not necessarily Ago2 as in seen in siRNA). Comparing and contrasting siRNA with miRNA shows that if siRNA hits an imperfect complementary target on

3' UTR, it behaves similar to microRNA, and if a miRNA hits a perfectly matched target on a mRNA, it can behave like an siRNA. Hence, although structurally different, both siRNA and miRNA might possess similar biological functions in the cells of host animal, with some differences in the mechanism of action.

Thus, RNAi molecules, siRNA and/or miRNA, provide a powerful tool for inhibiting endogenous gene expression and thereby could provide a means to effectively modulate biological responses. Studies have shown that RNAi can be induced in antigen presenting dendritic cells (DC) to polarize immune responses. By transfecting DC with synthetic siRNA specific for cytokine IL-12 p35 sub-unit, it was possible to inhibit bioactive IL-12 which subsequently led to Th2 polarization (Hill et al, J Immunology, 2003, 171:691). Similarly, modification of professional antigen presenting cells with siRNA *in vivo* has been used to enhance cancer vaccine potency (Kim et al, Cancer Research, 2005, 65:309-316). In this study, co-administration of DNA vaccine encoding human papilloma virus type 16 E7 with siRNA targeting key pro-apoptotic proteins Bax and Bcl-2 was shown to prolong the life span of antigen expressing DCs in the lymph nodes, enhancing antigen-specific CD8 T cell responses that had potent anti-tumor effects against an E7-expressing tumor model in vaccinated mice. Thus, there is a good prospect for the use of siRNA for silencing specific undesirable responses during effective vaccination against infectious/autoimmune diseases, cancer and during transplantation. Efficient delivery of siRNA to the intracellular compartment of cells of interest is critical for the success of such strategies, requiring the use of enhanced delivery formulations.

siRNA may be a naturally occurring or synthetic double stranded nucleotide (RNA) chain of varying length. siRNA can be duplexes, usually but not always limited to, 20 to 25-nt long that have 19 base pair central double stranded domain with terminal 2-base 3' overhangs. siRNA can be further modified chemically to enhance its *in vivo* efficacy, induce nuclease-resistance to prevent degradation and enhance stability. In this regard, the anti-sense strand may have either a free 5'-OH or 5'-phosphate terminus, the latter results in natural Dicer processing and represents the active form of the molecule. siRNA may have phosphorothioate or boranophosphate modification of the internucleoside linkage to improve nuclease stability and prolong life of the duplex when exposed to serum or other nuclease sources. siRNA may have modifications at 2' position, for example, 2'-O-methyl RNA residue incorporation to retain full potency compared with unmodified RNA, retaining stability in serum and significantly reducing the risk of potential IFN responses in the cell. siRNA may also have 2'-fluoro modification, which is usually incorporated selectively at pyrimidine bases, to improve stability and potency.

siRNA and miRNA used as mediators of RNAi may be used as targets in, but not limited to, various infectious diseases, autoimmune/allergic diseases, heart diseases, metabolic disorders, solid tumors/cancers, hematological disorders/cancers.

In embodiments of the present invention, the polynucleotide in the composition may be a polynucleotide for use in RNAi, including, without limitation, an siRNA, an miRNA, a long dsRNA for cleavage by Dicer, or a DsiRNA, all as described above.

It has been demonstrated in the present invention that injection of IL-12 siRNA nucleotide sequences *in vivo* when formulated in a liposome/continuous hydrophobic carrier resulted in better inhibition of IL-12 protein expression induced by IL-12 plasmid than did IL-12 siRNA formulated in a PBS carrier. Similar results were achieved in experiments concerning inhibition of ovalbumin-induced IL-12 protein expression in cells isolated from lymph nodes, following injection of IL-12 siRNA. Again, IL-12 siRNA formulated in a liposome/continuous hydrophobic carrier gave superior results to IL-12 siRNA formulated in a PBS carrier.

The subject may be any subject to which it is desired to deliver a polynucleotide. The subject is preferably a vertebrate, such as a bird, fish or mammal, preferably a human.

The polynucleotide may delivered in various forms. In some embodiments, a naked polynucleotide may be used, either in linear form, or inserted into a plasmid, such as an expression plasmid. In other embodiments, a live vector such as a viral or bacterial vector may be used.

Depending on the nature of the polynucleotide and the intended use, one or more regulatory sequences that aid in transcription of DNA into RNA and/or translation of RNA into a polypeptide may be present. For example, if it is intended or not required that the polynucleotide be transcribed or translated, such regulatory sequences may be absent. In some instances, such as in the case of a polynucleotide that is a messenger RNA (mRNA) molecule, regulatory sequences relating to the transcription process (e.g. a promoter) are not required, and protein expression may be effected in the absence of a promoter. The skilled

artisan can include suitable regulatory sequences as the circumstances require.

In some embodiments, the polynucleotide is present in an expression cassette, in which it is operably linked to regulatory sequences that will permit the polynucleotide to be expressed in the subject to which the composition of the invention is administered. The choice of expression cassette depends on the subject to which the composition is administered as well as the features desired for the expressed polypeptide.

Typically, an expression cassette includes a promoter that is functional in the subject and can be constitutive or inducible; a ribosome binding site; a start codon (ATG) if necessary; the polynucleotide encoding the polypeptide of interest; a stop codon; and optionally a 3' terminal region (translation and/or transcription terminator). Additional sequences such as a region encoding a signal peptide may be included. The polynucleotide encoding the polypeptide of interest may be homologous or heterologous to any of the other regulatory sequences in the expression cassette. Sequences to be expressed together with the polypeptide of interest, such as a signal peptide encoding region, are typically located adjacent to the polynucleotide encoding the protein to be expressed and placed in proper reading frame. The open reading frame constituted by the polynucleotide encoding the protein to be expressed solely or together with any other sequence to be expressed (e.g. the signal peptide), is placed under the control of the promoter so that transcription and translation occur in the subject to which the composition is administered.

Promoters suitable for expression of polynucleotides in a wide range of host systems are well-known in the art. Promoters suitable for expression of polynucleotides in mammals include those that function constitutively, ubiquitously or tissue-specifically. Examples of non-tissue specific promoters include promoters of viral origin. Examples of viral promoters include Mouse Mammary Tumor Virus (MMTV) promoter, Human Immunodeficiency Virus Long Terminal Repeat (HIV LTR) promoter, Moloney virus, avian leukosis virus (ALV), Cytomegalovirus (CMV) immediate early promoter/enhancer, Rous Sarcoma Virus (RSV), adeno-associated virus (AAV) promoters; adenoviral promoters, and Epstein Barr Virus (EBV) promoters. Compatibility of viral promoters with certain polypeptides is a consideration since their combination may affect expression levels. It is possible to use synthetic promoter/enhancers to optimize expression (see e.g. US patent publication 2004/0171573).

An example of a tissue-specific promoter is the desmin promoter which drives expression in muscle cells (Li et al. 1989, Gene 78:243; Li & Paulin 1991, J. Biol. Chem. 266:6562 and Li & Paulin 1993, J. Biol. Chem. 268:10403). Other examples include artificial promoters such as a synthetic muscle specific promoter and a chimeric muscle-specific/CMV promoter (Li et al. 1999, Nat. Biotechnol. 17:241-245; Hagstrom et al. 2000, Blood 95:2536-2542).

Useful vectors are described in numerous publications, specifically WO 94/21797 and Hartikka et al. 1996, Human Gene Therapy 7:1205.

As noted above, the polynucleotide of interest, together with any necessary regulatory sequences, may be delivered naked, e.g. either alone or as part of a plasmid, or may be delivered in a viral or bacterial or bacterial vector.

Whether a plasmid-type vector, or a bacterial or viral vector is used, it may be desirable that the vector be unable to replicate or integrate substantially in the subject. Such vectors include those whose sequences are free of regions of substantial identity to the genome of the subject, as to minimize the risk of host-vector recombination. One way to do this is to use promoters not derived from the recipient genome to drive expression of the polypeptide of interest. For example, if the recipient is a mammal, the promoter is preferably non-mammalian derived though it should be able to function in mammalian cells, e.g. a viral promoter.

Viral vectors that may be used to deliver the polynucleotide include e.g. adenoviruses and poxviruses. Useful bacterial vectors include e.g. *Shigella*, *Salmonella*, *Vibrio cholerae*, *Lactobacillus*, Bacille bilié de Calmette-Guérin (BCG), and *Streptococcus*.

An example of an adenovirus vector, as well as a method for constructing an adenovirus vector capable of expressing a polynucleotide, is described in U.S. Patent No. 4,920,209. Poxvirus vectors include vaccinia and canary pox virus, described in U.S. Patent No. 4,722,848 and U.S. Patent No. 5,364,773, respectively. Also see, e.g., Tartaglia et al. 1992, Virology 188:217 for a description of a vaccinia virus vector and Taylor et al. 1995, Vaccine 13:539 for a reference of a canary pox.

Poxvirus vectors capable of expressing a polynucleotide of interest may be obtained by homologous recombination as described in Kieny et al. 1984, Nature 312:163, so that the polynucleotide is inserted in the viral genome under appropriate conditions for expression in mammalian cells.

With respect to bacterial vectors, non-toxicogenic *Vibrio cholerae* mutant strains that are useful for expressing a foreign polynucleotide in a host are known. Mekalanos et al. 1983, Nature 306:551 and U.S. Patent No. 4,882,278 describe strains which have a substantial amount of the coding sequence of each of the two *ctxA* alleles deleted so that no functional *cholerae* toxin is produced. WO 92/11354 describes a strain in which the *irgA* locus is inactivated by mutation; this mutation can be combined in a single strain with *ctxA* mutations. WO 94/01533 describes a deletion mutant lacking functional *ctxA* and *attRS1* DNA sequences. These mutant strains are genetically engineered to express heterologous proteins, as described in WO 94/19482.

Attenuated *Salmonella typhimurium* strains, genetically engineered for recombinant expression of heterologous proteins are described in Nakayama et al. 1988, Bio/Technology 6:693 and WO 92/11361.

Other bacterial strains which may be used as vectors to express a foreign protein in a subject are described for *Shigella flexneri* in High et al. 1992, EMBO 11:1991 and Sizemore et al. 1995, Science 270:299; for *Streptococcus gordonii* in Medaglini et al. 1995, Proc. Natl. Acad. Sci. USA. 92:6868; and for Bacille Calmette Guerin in Flynn 1994, Cell. Mol. Biol. 40 (suppl. I):31, WO 88/06626, WO 90/00594, WO 91/13157, WO 92/01796, and WO 92/21376.

In bacterial vectors, the polynucleotide of interest may be inserted into the bacterial genome or remain in a free state as part of a plasmid.

Liposomes

Liposomes are completely closed lipid bilayer membranes containing an entrapped aqueous volume. Liposomes may be unilamellar vesicles (possessing a single bilayer membrane) or multilamellar vesicles characterized by multimembrane bilayers, each bilayer may or may not be separated from the next by an aqueous layer. A general discussion of liposomes can be found in Gregoriadis G. *Immunol. Today*, 11:89-97, 1990; and Frezard, F., *Braz. J. Med. Bio. Res.*, 32:181-189, 1999. As used herein and in the claims, the term "liposomes" is intended to encompass all such vesicular structures as described above, including, without limitation, those described in the art as "niosomes", "transfersomes" and "viroosomes".

Any liposomes may be used in this invention, including liposomes made from archaebacterial lipids. Any amphipathic lipid with at least one fatty acid chain containing at least 4 carbons, typically about 4 to 28 carbons in length may be used. The fatty acid chain may contain any number of saturated and/or unsaturated bonds. Amphipathic lipids contemplated may be phospholipids, sphingolipids, sphingomyelin, cerobrocides, gangliosides. Particularly useful liposomes use phospholipids and unesterified cholesterol in the liposome formulation. The cholesterol is used to stabilize the liposomes and any other compound that stabilizes liposomes may replace the cholesterol. Other liposome stabilizing compounds are known to those skilled in the art. For example, saturated

phospholipids produce liposomes with higher transition temperatures indicating increased stability.

Phospholipids that are preferably used in the preparation of liposomes are those with at least one head group selected from the group consisting of phosphoglycerol, phosphoethanolamine, phosphoserine, phosphocholine and phosphoinositol. More preferred are liposomes that comprise lipids which are about 94-100% phosphatidylcholine. Such lipids are available commercially in the lecithin Phospholipon® 90 G (Phospholipid GmbH, Germany) or lecithin S100 (Lipoid GmbH, Germany). Other preferred phospholipids include cationic lipids such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride (DOTIM).

When unesterified cholesterol is also used in liposome formulation, the cholesterol is usually used in an amount equivalent to about 10% of the amount of phospholipid. If a compound other than cholesterol is used to stabilize the liposomes, one skilled in the art can readily determine the amount needed in the composition.

Liposome compositions may be obtained, for example, by using natural lipids, synthetic lipids, sphingolipids, ether lipids, sterols, cardiolipin, cationic lipids and lipids modified with poly (ethylene glycol) and other polymers. Synthetic lipids may include the following fatty acid constituents: lauroyl, myristoyl, palmitoyl, stearoyl, arachidoyl, oleoyl, linoleoyl, erucoyl, or combinations of these fatty acids.

Carriers

The carrier of the composition comprises a continuous phase of a hydrophobic substance, preferably a liquid hydrophobic substance. The continuous phase may be an essentially pure hydrophobic substance or a mixture of hydrophobic substances. In addition, the carrier may be an emulsion of water in a hydrophobic substance or an emulsion of water in a mixture of hydrophobic substances, provided the hydrophobic substance constitutes the continuous phase. Further, in another embodiment, the carrier may function as an adjuvant.

Hydrophobic substances that are useful in the compositions as described herein are those that are pharmaceutically and/or immunologically acceptable. The carrier is preferably a liquid but certain hydrophobic substances that are not liquids at atmospheric temperature may be liquefied, for example by warming, and are also useful in this invention. In one embodiment, the hydrophobic carrier may be a PBS/FIA emulsion.

Oil or water-in-oil emulsions are particularly suitable carriers for use in the present invention. Oils should be pharmaceutically and/or immunologically acceptable. Preferred examples of oils are mineral oil such (especially light or low viscosity mineral oil), vegetable oil (e.g., soybean oil), nut oil (e.g., peanut oil). A low viscosity mineral oil such as Drakeol® 6VR is preferred in some embodiments. In a further embodiment, the oil is a mannide oleate in mineral oil solution, commercially available as Montanide® ISA 51. Animal fats and artificial hydrophobic polymeric materials, particularly those that are liquid at atmospheric temperature or that can be liquefied

relatively easily, may also be used. Mixtures of different hydrophobic substances, such as mixtures that include one or more different oils, animal fats or artificial hydrophobic polymeric materials, may be used.

Additional Components

The composition may further comprise one or more additional components that may complement or enhance the function of the polypeptide to be expressed in the subject. For example, if the encoded polypeptide is a vaccine antigen, an additional component, such as an adjuvant, may be present. The term "adjuvant" refers to a compound or mixture that enhances the immune response to an antigen. An adjuvant can serve as a tissue depot that slowly releases the antigen and also as a lymphoid system activator that non-specifically enhances the immune response (Hood et al, Immunology, 2d ed., Benjamin/Cummings: Menlo Park, C.A., 1984; see Wood and Williams, In: Nicholson, Webster and May (eds.), Textbook of Influenza, Chapter 23, pp. 317-323). Often, a primary challenge with an antigen alone, in the absence of an adjuvant, will fail to elicit a humoral immune response. It should be noted that the polynucleotide of interest to be delivered to the subject may itself function as an adjuvant, or may encode a polypeptide that constitutes an adjuvant (e.g. IL-12, IFN-gamma, or Granulocyte-Macrophage Colony Stimulating Factor ("GM-CSF")).

In some embodiments, suitable adjuvants include, but are not limited to, alum, other compounds of aluminum, Bacillus of Calmette and Guérin (BCG), TiterMax®, Ribi®, incomplete Freund's adjuvant (IFA), saponin, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, Corynebacterium parvum, QS-21, and

Freund's Complete Adjuvant (FCA), adjuvants of the TLR agonist family such as CpG, polyIC, falgellin, lipopeptides, peptidoglycans, imidazoquinolines, single stranded RNA, lipopolysaccharides (LPS), heat shock proteins (HSP), and ceramides and derivatives such as alpha Gal-cer. Suitable adjuvants also include cytokines or chemokines in their polypeptide or DNA coding forms such as, but not limited to, GM-CSF, TNF-alpha, IFN-gamma, IL-2, IL-12, IL-15, IL-21.

The amount of adjuvant used depends on the amount of antigen and on the type of adjuvant. One skilled in the art can readily determine the amount of adjuvant needed in a particular application.

A wide range of pharmaceutically acceptable adjuvants, excipients, etc. are known in the art and may be used in the compositions of the invention: See, for example, Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985) and The United States Pharmacopoeia: The National Formulary (USP 24 NF19) published in 1999.

If an additional component in the composition is a polypeptide, a polynucleotide encoding the additional polypeptide may instead be provided, in the same manner as for the polynucleotide encoding the polypeptide of primary interest. Such polypeptides could be expressed from the same or separate expression vectors, or could be expressed in the form of a fusion protein.

Formulation of Compositions

Methods for making liposomes are well known in the art: see, for example, Gregoriadis (1990) and Frezard (1999), both cited previously. Any suitable method for

making liposomes may be used in the practice of the invention. Liposomes are typically prepared by hydrating the liposome components that will form the lipid bilayer (e.g. phospholipids and cholesterol) with an aqueous solution, which may be pure water or any other physiologically compatible solution such as saline, e.g. phosphate-buffered saline (PBS).

In an embodiment, a liposome component or mixture of liposome components, such as a phospholipid (e.g. Phospholipon® 90G) and cholesterol, may be solubilized in an organic solvent, such as a mixture of chloroform and methanol, followed by filtering (e.g. a PTFE 0.2 µm filter) and drying, e.g. by rotary evaporation, to remove the solvents.

Hydration of the resulting lipid mixture may be effected by e.g. injecting the lipid mixture into an aqueous solution or sonicating the lipid mixture and an aqueous solution. During formation of liposomes, the liposome components form single bilayers (unilamellar) or multiple bilayers (multilamellar) surrounding a volume of the aqueous solution with which the liposome components are hydrated.

In some embodiments, the liposomes are then dehydrated, such as by freeze-drying or lyophilization, and subsequently reconstituted with an aqueous solution.

The liposomes are combined with the carrier comprising a continuous hydrophobic phase. This can be done in a variety of ways.

If the carrier is essentially water-free, and is composed solely of a hydrophobic substance or a mixture of hydrophobic substances (e.g. use of a 100% mineral oil

carrier), the liposomes may simply be mixed with the hydrophobic substance, or if there are multiple hydrophobic substances, mixed with any one or a combination of them.

If instead the carrier comprising a continuous phase of a hydrophobic substance contains a discontinuous aqueous phase, the carrier will typically take the form of an emulsion of the aqueous phase in the hydrophobic phase, such as a water-in-oil emulsion. Such compositions may contain an emulsifier to stabilize the emulsion and to promote an even distribution of the liposomes. In this regard, emulsifiers may be useful even if water-free carrier is used, for the purpose of promoting an even distribution of the liposomes in the carrier. Typical emulsifiers include mannide oleate (Arlacel™ A), lecithin, Tween™ 80, and Spans™ 20, 80, 83 and 85. Typically, the weight to volume ratio (w/v) of hydrophobic substance to emulsifier is in the range of about 5:1 to about 15:1 with a ratio of about 10:1 being preferred.

The liposomes may be added to the finished emulsion, or they may be present in either the aqueous phase or the hydrophobic phase prior to emulsification.

The polynucleotide to be expressed may be introduced at various different stages of the formulation process. In this section, the term "polynucleotide" includes the polynucleotide in naked form including in a plasmid such as an expression plasmid, or in a live vector such as a bacteria or virus.

More than one polynucleotide may be incorporated into the composition. For example, two or more polynucleotides encoding different proteins may be incorporated into the composition, or a polynucleotide

encoding a protein may be present as well as a polynucleotide encoding an antisense RNA or interfering RNA. Proteins may be expressed as the fusion product of two different polynucleotides. More than one polynucleotide may be under the control of the same regulatory elements, e.g. two or more polynucleotides under transcriptional control of a single promoter.

In some embodiments, the polynucleotide is present in the aqueous solution used to hydrate the components that are used to form the lipid bilayers of the liposomes (e.g. phospholipid(s) and cholesterol). In this case, the polynucleotide will be encapsulated in the liposome, present in its aqueous interior. If the resulting liposomes are not washed or dried, such that there is residual aqueous solution present that is ultimately mixed with the carrier comprising a continuous phase of a hydrophobic substance, it is possible that additional polynucleotide may be present outside the liposomes in the final product. In a related technique, the polynucleotide may be mixed with the components used to form the lipid bilayers of the liposomes, prior to hydration with the aqueous solution.

In an alternative approach, the polynucleotide may instead be mixed with the carrier comprising a continuous phase of a hydrophobic substance, before, during, or after the carrier is combined with the liposomes. If the carrier is an emulsion, the polynucleotide may be mixed with either or both of the aqueous phase or hydrophobic phase prior to emulsification. Alternatively, the polynucleotide may be mixed with the carrier after emulsification.

The technique of combining the polynucleotide with the carrier may be used together with encapsulation of the

polynucleotide in the liposomes as described above, such that polynucleotide is present both within the liposomes and in the carrier comprising a continuous phase of a hydrophobic substance.

Generally, the composition may comprise about 0.1 to 5 mg polynucleotide per ml of the composition and about 1 mg to 300 mg liposomes per ml of the composition.

If the composition contains one or more additional components (e.g. an adjuvant), the additional component(s) can be incorporated in the composition together with the polynucleotide at the same processing step, or separately, at a different processing step. For instance, the polynucleotide and the additional component may both be present in the aqueous solution used to hydrate the lipid bilayer-forming liposome components, such that both the polynucleotide and additional component become encapsulated in the liposomes. Alternatively, the polynucleotide may be encapsulated in the liposomes, and the additional component mixed with the carrier comprising a continuous phase of a hydrophobic substance. It will be appreciated that many such combinations are possible.

In some embodiments, the polynucleotide and the additional component may be in the form of a complex, in which they are in intimate contact at least prior to incorporation into the composition. Complexing may but need not necessarily involve a chemical linkage, such as covalent bonding.

The compositions as described herein may be formulated in a form that is suitable for oral, nasal, rectal or parenteral administration. Parenteral administration includes intravenous, intraperitoneal,

intradermal, subcutaneous, intramuscular, transepithelial, intrapulmonary, intrathecal, and topical modes of administration. The preferred routes are intramuscular, subcutaneous and intradermal to achieve a depot effect. In practice, a depot effect is achieved when the therapeutic agent remains at the site of injection for more than about one hour.

The injection site may be anywhere close to, or directly into a lymph node, for example. Alternatively, the injection site may be directly into a spleen, a tumour or other diseased tissue. The volume that may be injected is within the professional judgment of the clinician. The volume depends on the injecting device used and the site of injection. When the injection is intramuscularly or subcutaneous, the injection volume may be about 2 mL. When needleless injection is used, the volume may be as low as 0.05 mL. The volume may be increased by injecting multiple sites.

Kits and Reagents

The present invention is optionally provided to a user as a kit. For example, a kit of the invention contains one or more of the compositions of the invention. The kit can further comprise one or more additional reagents, packaging material, containers for holding the components of the kit, and an instruction set or user manual detailing preferred methods of using the kit components for a desired purpose.

Uses

The invention finds application in any instance in which it is desired deliver a polynucleotide to a subject.

Many such applications will be in the treatment or prevention of disease. Representative applications of the invention include cancer treatment and prevention, gene therapy, adjuvant therapy, infectious disease treatment and prevention, allergy treatment and prevention, autoimmune disease treatment and prevention, neuron-degenerative disease treatment, and arteriosclerosis treatment.

Prevention or treatment of disease includes obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent of disease, stabilisation of the state of disease, prevention of development of disease, prevention of spread of disease, delay or slowing of disease progression, delay or slowing of disease onset, conferring protective immunity against a disease-causing agent and amelioration or palliation of the disease state. Prevention or treatment can also mean prolonging survival of a patient beyond that expected in the absence of treatment and can also mean inhibiting the progression of disease temporarily, although more preferably, it involves preventing the occurrence of disease such as by preventing infection in a subject.

The skilled artisan can determine suitable treatment regimes, routes of administration, dosages, etc., for any particular application in order to achieve the desired result. Factors that may be taken into account include, e.g.: the nature of a polypeptide to be expressed; the disease state to be prevented or treated; the age, physical condition, body weight, sex and diet of the subject; and other clinical factors.

The invention is further illustrated by the following non-limiting examples.

EXAMPLES

Example 1

In order to demonstrate the ability to enhance the expression of a protein-coding nucleotide sequence *in vivo* using a formulation comprising of liposomes and a continuous hydrophobic carrier, a model expression plasmid engineered with IL-12 complete coding sequence was selected. The IL-12 plasmid was formulated in liposome first, and the resulting IL-12 plasmid/liposome preparation was emulsified in a model water-in-oil emulsion with the continuous hydrophobic carrier consisting of mineral oil. *In vivo* expression was determined by examining the IL-12 expression potential in cells isolated from lymph node in the proximity of the injection site.

Pathogen free, female C57BL/6 mice, 6-8 weeks of age, were obtained from Charles River Laboratories (St Constant, Quebec, Canada) and were housed according to institutional guideline with water and food *ad libitum*, under filter controlled air circulation.

Murine IL-12 plasmid, pORF-mIL-12 was purchased from InvivoGen, San Diego, California, USA. The plasmid, supplied as lyophilized in GT100 *E. coli* bacteria transformed by pORF-mIL-12, was reconstituted in LB medium and streaked on an ampicillin-LB agar plate and incubated overnight at 37°C. Bacteria were grown from single colony in TB medium supplemented with ampicillin using. Plasmid DNA was purified from large scale bacterial cultures using

endo-free Maxi- or Mega-prep kits (Qiagen, Mississauga, Ontario, Canada) to ensure complete removal of LPS.

Multilamellar liposomes were prepared by hydrating a 10:1 (W/W) mixture of dioleoyl phosphatidylcholine (DOPC) and cholesterol using phosphate buffered saline (PBS) containing IL-12 plasmid at a final concentration of 1 milligram/milliliter. Liposomes were then extruded through a 200 nm polycarbonate membrane using a manual extruder (Avanti lipids, Alabaster, AL, USA). The final liposome preparation was subsequently emulsified in Incomplete Freund's adjuvant (Sigma, Oakville, Ontario, Canada), a mineral oil based oil carrier, by mixing an equal volume of liposomes containing IL-12 plasmid and incomplete Freund's adjuvant.

Three groups of mice containing three mice each (n=3) were injected subcutaneously in the right flank above the base of the tail as follows: Group 1 was injected with phosphate buffered saline, Group 2 with 50 micrograms of IL-12 plasmid in PBS, and Group 3 with 50 micrograms of IL-12 plasmid formulated as described above in a liposome/continuous hydrophobic carrier. All injections were 100 microliters in volume. Draining lymph nodes from all mice were collected 8 days post-injection. Lymph nodes were dissected and single cell suspensions was cultured in vitro at a concentration of 2×10^6 cell/ml in RPMI medium supplemented with 10% FBS, penicillin/streptomycin, 2- β -mercaptoethanol and L-glutamine in 6 well plates for 48h. Cell culture supernatants were collected and stored frozen in aliquots until used for IL-12 protein quantification.

Efficacy of IL-12 gene expression in the lymph nodes was determined by quantifying levels of IL-12 protein secreted into the cell culture supernatants. IL-12 protein quantification was performed by enzyme linked immunosorbent assay (ELISA), using a commercial IL-12 specific ELISA kit (Peptrotech, Rocky Hill, NJ, USA). Briefly, anti-IL-12 capture antibody was coated overnight on ELISA plates and samples and standard IL-12 were added. After thorough washes, biotinylated anti-IL-12 detection antibody was added and incubated for 2h at room temperature and washed. Following incubation with Avidin-HRP conjugates and washes, ABTS liquid substrate (Sigma, St Louis, MO) was used for color development. Absorbances were read at 405 nm using a microtiter plate reader and IL-12 concentrations were extrapolated using a standard curve that was generated using IL-12 standards supplied in the kit.

IL-12 levels secreted by lymph node cells isolated from Groups 1-3 are shown in Figure 1. Lymph node cells isolated from mice injected with PBS (Group 1) did not secrete detectable IL-12 protein into the culture supernatants. Low levels of IL-12 protein were secreted by lymph node cells isolated from mice injected with IL-12 plasmid in PBS. However, lymph nodes cells isolated from mice injected with IL-12 plasmid in a formulation comprising liposomes in a continuous hydrophobic carrier secreted a significantly higher level of IL-12, suggesting improved delivery of protein-coding nucleotide sequences *in vivo* and resulting in enhanced protein expression.

Example 2

Pathogen free, female C57BL/6 mice, 6-8 weeks of age, were obtained from Charles River Laboratories

(St Constant, Quebec, Canada) and were housed according to institutional guideline with water and food ad libitum, under filter controlled air circulation.

Murine IL-12 plasmid, pORF-mIL-12 was purchased from InvivoGen, San Diego, California, USA. The plasmid, supplied as lyophilized in GT100 E. coli bacteria transformed by pORF-mIL-12, was reconstituted in LB medium and streaked on an ampicillin-LB agar plate and incubated overnight at 37°C. Bacteria were grown from single colony in TB medium supplemented with ampicillin using. Plasmid DNA was purified from large scale bacterial cultures using endo-free Maxi- or Mega-prep kits (Qiagen, Mississauga, Ontario, Canada) to ensure complete removal of LPS.

Multilamellar liposomes were prepared by hydrating a 10:1 (W/W) mixture of purified soy-derived mixture of phospholipids (phospholipid S100, provided by Lipoid GmbH) and cholesterol using phosphate buffered saline (PBS) containing IL-12 plasmid at a final concentration of 0.8 milligram/milliliter. Liposomes were then extruded through a 200 nm polycarbonate membrane using a manual extruder (Avanti lipids, Alabaster, AL, USA). The final liposome preparation was subsequently emulsified in Incomplete Freund's adjuvant (Sigma, Oakville, Ontario, Canada), a mineral oil based oil carrier, by mixing an equal volume of liposomes containing IL-12 plasmid and incomplete Freund's adjuvant. The final injected volume for each mouse was 100 microliters.

Six groups of mice containing five mice each (n=5) were injected subcutaneously in the right flank above the base of the tail as follows: Group 1 mice were injected with 40 micrograms of IL-12 plasmid in PBS, Group 2 mice with

40 micrograms of IL-12 plasmid formulated as described above in a liposome/continuous hydrophobic carrier, Group 3 mice with 40 micrograms of IL-12 plasmid formulated in a continuous hydrophobic carrier (incomplete Freund's adjuvant water-in-oil emulsion) without liposomes, Group 4 mice with 40 micrograms of IL-12 plasmid formulated in liposomes as described above but without a continuous hydrophobic carrier, Group 5 mice with a control formulation consisting of liposome/continuous hydrophobic carrier without IL-12 plasmid, and Group 6 mice remained untreated. All injections were 100 microliters in volume. Draining lymph nodes from all mice were collected 8 days post-injection. Lymph nodes were dissected and single cell suspensions was cultured in vitro at a concentration of 2×10^6 cell/ml in RPMI medium supplemented with 10% FBS, penicillin/streptomycin, 2- β -mercaptoethanol and L-glutamine in 6 well plates for 48h. Cell culture supernatants were collected and stored frozen in aliquots until used for IL-12 protein quantification.

Efficacy of IL-12 gene expression in the lymph nodes was determined by quantifying levels of IL-12 protein secreted into the cell culture supernatants. IL-12 protein quantification was performed by enzyme linked immunosorbent assay (ELISA), using a commercial IL-12 specific ELISA kit (Peprotech, Rocky Hill, NJ, USA). Briefly, anti-IL-12 capture antibody was coated overnight on ELISA plates and samples and standard IL-12 were added. After thorough washes, biotinylated anti-IL-12 detection antibody was added and incubated for 2h at room temperature and washed. Following incubation with Avidin-HRP conjugates and washes, ABTS liquid substrate (Sigma, St Louis, MO) was used for color development. Absorbances were read at 405 nm using a microtiter plate reader and IL-12 concentrations were

extrapolated using a standard curve that was generated using IL-12 standards supplied in the kit.

IL-12 levels secreted by lymph node cells isolated from Groups 1-6 are shown in Figure 2. Lymph node cells isolated from mice injected with IL-12 plasmid in PBS (Group 1) did not secrete detectable IL-12 protein into the culture supernatants. However, lymph nodes cells isolated from mice injected with IL-12 plasmid in a formulation comprising liposomes in a continuous hydrophobic carrier (Group 2) secreted a considerably higher level of IL-12. Lymph nodes cells isolated from mice from Group 3, Group 5 and Group 6 secreted significantly lower levels of IL-12 protein and lymph nodes cells isolated from mice from Group 4 did not secrete detectable IL-12 protein into the culture supernatants. In this experiment, superior IL-12 protein expression was achieved using a liposome/hydrophobic carrier formulation relative to formulations lacking either or both of liposomes and the continuous hydrophobic carrier.

Example 3

In order to demonstrate the ability to enhance the expression of a protein-coding nucleotide sequence *in vivo* using a formulation comprising of liposomes and a continuous hydrophobic carrier, a model expression plasmid engineered with green fluorescent protein (GFP) complete coding sequence was selected. The GFP plasmid was formulated in liposome first, and the resulting GFP plasmid/liposome preparation was emulsified in a model water-in-oil emulsion with the continuous hydrophobic carrier consisting of mineral oil. *In vivo* uptake and expression was determined by examining the GFP expression potential in cells isolated from lymph node in the proximity of the injection site.

Pathogen free, female C57BL/6 mice, 6-8 weeks of age, were obtained from Charles River Laboratories (St Constant, Quebec, Canada) and were housed according to institutional guideline with water and food ad libitum, under filter controlled air circulation.

Synthetic GFP plasmid, pMOD-GFPSh (catalog name, pmod-zgfpsh) was purchased from InvivoGen, San Diego, California, USA. The plasmid was transformed into XL1 strain of *E.coli* bacteria, grown in LB medium and streaked on an ampicillin-LB agar plate and incubated overnight at 37°C. Bacteria were grown from single colony in TB medium supplemented with ampicillin. Plasmid DNA was purified from large scale bacterial cultures using endo-free Maxi- or Mega-prep kits (Qiagen, Mississauga, Ontario, Canada) to ensure complete removal of LPS.

Multilamellar liposomes were prepared by hydrating a 10:1 (W/W) mixture of purified soy-derived mixture of phospholipids (phospholipid S100, provided by Lipoid GmbH) and cholesterol using phosphate buffered saline (PBS) containing GFP plasmid at a final concentration of 0.8 milligram/milliliter. Liposomes were then extruded through a 200 nm polycarbonate membrane using a manual extruder (Avanti lipids, Alabaster, AL, USA). The final liposome preparation was subsequently emulsified in Incomplete Freund's adjuvant (Sigma, Oakville, Ontario, Canada), a mineral oil based oil carrier, by mixing an equal volume of liposomes containing GFP plasmid and incomplete Freund's adjuvant. The final injected volume for each mouse was 100 microliters which delivered 40 micrograms plasmid per dose per mouse.

Six groups of mice containing four mice each (n=4) were injected subcutaneously in the right flank above the base of the tail as follows: Group 1 mice were injected with 40 micrograms of GFP plasmid in PBS, Group 2 mice with 40 micrograms of GFP plasmid formulated as described above in a liposome/continuous hydrophobic carrier, Group 3 mice with 40 micrograms of GFP plasmid formulated in a continuous hydrophobic carrier (incomplete Freund's adjuvant water-in-oil emulsion) without liposomes, Group 4 mice with 40 micrograms of GFP plasmid formulated in liposomes as described above but without a continuous hydrophobic carrier, Group 5 mice with a control formulation consisting of liposome/continuous hydrophobic carrier without GFP plasmid, and Group 6 mice remained untreated. All injections were 100 microliters in volume. Draining lymph node from all mice were collected 8 days post-injection. Lymph nodes were dissected and single cell suspensions was prepared. Efficacy of GFP gene expression in the lymph nodes was determined by two color immunofluorescence staining for detecting GFP-positive cells. Cells were stained with phycoerythrin-conjugated CD11b and CD11c antibodies for identifying antigen presenting cells in channel FL2 along with GFP detection in channel FL1. Samples were run through a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA). At least 3×10^5 events were collected for each sample to enhance the accuracy of detection of GFP positive cells.

Number of lymph node cells isolated from Group 1 to Group 6, expressing GFP (and thus are positively identified in flow cytometric analysis), are shown in Figure 3. Lymph nodes isolated from mice injected with GFP plasmid in PBS (Group 1) showed low level of GFP protein expressing cells (<10). However, lymph nodes isolated from mice injected with GFP plasmid in a formulation comprising

liposomes in a continuous hydrophobic carrier (Group 2) showed almost four-fold increase in GFP positive cells. Further, lymph nodes cells isolated from mice from Group 3 and Group 4 showed significantly lower number of GFP protein expressing cells compared to mice in Group 2. Among mice in Group 5 and Group 6, minimal fluorescence was detected which was attributed to background auto-fluorescence events as these mice did not receive GFP plasmid injections. Moreover, the majority of GFP positive cells were detected within the population of CD11b/CD11c positive lymph node cells. The results of a re-analysis of lymph node cells targeting specifically cells that are CD11b/CD11c and GFP double positive correlate with the finding presented in Figure 3, with at least a 4 fold increase in GFP expression in CD11b/CD11c positive cells from Group 2 (Figure 4). This re-analysis also confirmed that no specific fluorescence can be detected in lymph node cells from control Groups 5 and 6. This observation confirmed the specificity of GFP positive cell detection in this experiment. Thus, in the present experiment, superior GFP plasmid uptake and protein expression was achieved using a liposome/hydrophobic carrier formulation relative to formulations lacking either or both of liposomes and the continuous hydrophobic carrier.

Example 4

In order to demonstrate the ability to inhibit the expression of a protein-coding nucleotide sequence *in vivo* by siRNA against given protein-coding nucleotide sequence, using a formulation comprising of liposomes and a continuous hydrophobic carrier, a model expression plasmid engineered with IL-12 complete coding sequence and siRNA sequence for IL-12 was selected. The IL-12 plasmid was formulated in liposome first, and the resulting IL-12 plasmid/liposome

preparation was emulsified in a model water-in-oil emulsion with the continuous hydrophobic carrier consisting of mineral oil. One day before IL-12 plasmid injection, siRNA was injected in either PBS or in liposome/water-in oil emulsion with the continuous hydrophobic carrier consisting of mineral oil. *In vivo* functional activity of siRNA was determined by examining the IL-12 expression potential in cells isolated from lymph node in the proximity of the injection site.

Pathogen free, female C57BL/6 mice, 6-8 weeks of age, were obtained from Charles River Laboratories (St Constant, Quebec, Canada) and were housed according to institutional guideline with water and food ad libitum, under filter controlled air circulation.

Murine IL-12 plasmid, pORF-mIL-12 was purchased from InvivoGen, San Diego, California, USA. The plasmid, supplied as lyophilized in GT100 *E. coli* bacteria transformed by pORF-mIL-12, was reconstituted in LB medium and streaked on an ampicillin-LB agar plate and incubated overnight at 37°C. Bacteria were grown from single colony in TB medium supplemented with ampicillin. Plasmid DNA was purified from large scale bacterial cultures using endo-free Maxi- or Mega-prep kits (Qiagen, Mississauga, Ontario, Canada) to ensure complete removal of LPS.

siRNA against murine IL-12 was purchased from Ambion Applied Biosystems, Austin, TX, USA. This lyophilized product was >95% pure by analytical HPLC and contained less than 10EU of endotoxin by LAL assay. siRNA was dissolved in sterile phosphate buffered saline (PBS) before formulating for injection.

Multilamellar liposomes were prepared by hydrating a 10:1 (W/W) mixture of dioleoyl phosphatidylcholine (DOPC) and cholesterol using phosphate buffered saline (PBS) containing IL-12 plasmid at a final concentration of 1.6 milligram/milliliter. Liposomes were then extruded through a 200 nm polycarbonate membrane using a manual extruder (Avanti lipids, Alabaster, AL, USA). For every 500 microliters of a liposome/IL12 suspension, an equal volume of a mineral oil carrier (MontanideTM ISA 51, Seppic, France) was added to form a water-in-oil emulsion with the liposome suspension contained in the water phase of the emulsion and the oil forming the continuous phase, acting as a hydrophobic carrier.

In order to induce effective levels of IL-12 expression, three groups of mice containing three mice each (n=3) were all injected subcutaneously in the right flank above the base of the tail on day-0 with 40 micrograms of IL-12 plasmid formulated as described above in a liposome/continuous hydrophobic carrier in 50 microliters volume. Additionally, on day minus 1, Group 1 mice were injected with vehicle only, Group 2 mice were injected with 40 micrograms IL-12 siRNA in PBS and Group 3 mice were given 40 micrograms IL-12 siRNA in a liposome/continuous hydrophobic carrier, similar to the formulation used to deliver plasmid IL-12. All injections were given subcutaneously in the right flank above the base of the tail in 50 microlitre volume.

Draining lymph nodes from all injected mice and corresponding lymph nodes from three naïve mice (Group 4) were collected 8 days post-injection. Lymph nodes were dissected and single cell suspensions was cultured in vitro at a concentration of 2×10^6 cell/ml in RPMI medium

supplemented with 10% FBS, penicillin/streptomycin, 2- β -mercaptoethanol and L-glutamine in 24 well plates for 48h. Cell culture supernatants were collected and stored frozen in aliquots until used for IL-12 protein quantification.

Efficacy of siRNA injected in various formulations was determined by measuring the extent of inhibition in plasmid IL-12 induced IL-12 protein expression by lymph node cells. IL-12 protein quantification in cell culture supernatant was performed by enzyme linked immunosorbent assay (ELISA), using a commercial IL-12 specific ELISA kit (Peptrotech, Rocky Hill, NJ, USA). Briefly, anti-IL-12 capture antibody was coated overnight on ELISA plates and samples and standard IL-12 were added. After thorough washes, biotinylated anti-IL-12 detection antibody was added and incubated for 2h at room temperature and washed. Following incubation with Avidin-HRP conjugates and washes, ABTS liquid substrate (Sigma, St Louis, MO) was used for color development. Absorbances were read at 405 nm using a microtiter plate reader and IL-12 concentrations were extrapolated using a standard curve that was generated using IL-12 standards supplied in the kit.

IL-12 levels secreted by lymph node cells isolated from Groups 1-4 are shown in Figure 5. Lymph node cells isolated from mice injected with IL-12 plasmid alone, but not siRNA against IL-12 (Group 1), secreted 270.4 picograms per millilitre of IL-12 into the culture supernatants. In Group 2 mice, injected with IL-12 siRNA in PBS, no significant inhibition in IL-12 protein secretion was observed. In contrast, when siRNA was delivered in liposome/continuous hydrophobic barrier, marked decrease in secreted IL-12 was observed, which was as low as that

observed with naïve mice. Moreover, lymph node cells from mice in Group 3 secreted significantly lower IL-12 compared to mice in Group 2 that received siRNA in PBS. This demonstrated an improved delivery of siRNA nucleotide sequences *in vivo* when injected in a liposome/continuous hydrophobic carrier that resulted in better inhibition of IL-12 protein expression induced by IL-12 plasmid.

Example 5

As a further example, to test the ability of IL-12 siRNA delivered in a liposome/continuous hydrophobic carrier to inhibit IL-12 secretion, ovalbumin antigen in complete Freund's adjuvant (CFA) was used to induce IL-12 secretion by lymph node cells. Protein antigen ovalbumin has been previously shown to induce IL-12 cytokine secretion in mice (Yotsumoto et al, 2007, Vaccine, 25:5256-5262), which could be inhibited using siRNA specific for IL-12 p35 (Hill et al, Journal of Immunology, 2003, 171:691; Ichim et al, Journal of Translational Medicine, 2006, 4:2, 1-11). In this example, mice injected with Ovalbumin in CFA subcutaneously were injected with IL-12 siRNA subcutaneously (either in PBS or in liposome/continuous hydrophobic carrier) one day before or one day after ovalbumin injection.

Pathogen free, female C57BL/6 mice, 6-8 weeks of age, were obtained from Charles River Laboratories (St Constant, Quebec, Canada) and were housed according to institutional guideline with water and food *ad libitum*, under filter controlled air circulation.

Mice were immunized subcutaneously in the right flank above the base of the tail with 5 micrograms of ovalbumin emulsified in CFA (Difco Laboratories, Detroit, MI) in 50 microlitre volume.

Multilamellar liposomes were prepared by hydrating a 10:1 (W/W) mixture of purified soy-derived mixture of phospholipids (phospholipid S100, provided by Lipoid GmbH) and cholesterol using phosphate buffered saline (PBS) containing IL-12 siRNA at a final concentration of 1.6 milligram/milliliter. Liposomes were then extruded through a 200 nm polycarbonate membrane using a manual extruder (Avanti lipids, Alabaster, AL, USA). For every 500 microliters of a liposome/IL12-siRNA suspension, an equal volume of a mineral oil carrier (Montanide™ ISA 51, Seppic, France) was added to form a water-in-oil emulsion with the liposome suspension contained in the water phase of the emulsion and the oil forming the continuous phase, acting as a hydrophobic carrier.

Five groups of mice (n=4) were all immunized subcutaneously on day-0 with ovalbumin formulated in complete Freund's adjuvant (CFA), and each group treated subcutaneously in the right flank above the base of the tail as follows: Group 1 mice were untreated, Group 2 mice with 40 micrograms of IL-12 siRNA in PBS on day minus one, Group 3 with siRNA formulated as described above in a liposome/continuous hydrophobic carrier on day minus one, Group 4 with siRNA in PBS on day plus one and Group 5 with siRNA in liposome/continuous hydrophobic carrier on day plus one, while Group 6 consisted of non-vaccinated and untreated naïve mice. All injections were 50 microliters in volume. Draining lymph nodes from all mice were collected 8 days post-ovalbumin injection. Lymph nodes were dissected and single cell suspensions was cultured in vitro at a concentration of 2×10^6 cell/ml in RPMI medium supplemented with 10% FBS, penicillin/streptomycin, 2- β -mercaptoethanol and L-glutamine in 24 well plates for 48h. Cell culture

supernatants were collected and stored frozen in aliquots until used for IL-12 protein quantification.

Efficacy of siRNA injected in two different formulations was determined by measuring the extent of inhibition in ovalbumin-induced IL-12 protein expression by lymph node cells. IL-12 protein quantification was performed by enzyme linked immunosorbent assay (ELISA), using a commercial IL-12 specific ELISA kit (Peprotech, Rocky Hill, NJ, USA). Briefly, anti-IL-12 capture antibody was coated overnight on ELISA plates and samples and standard IL-12 were added. After thorough washes, biotinylated anti-IL-12 detection antibody was added and incubated for 2h at room temperature and washed. Following incubation with Avidin-HRP conjugates and washes, ABTS liquid substrate (Sigma, St Louis, MO) was used for color development. Absorbances were read at 405 nm using a microtiter plate reader and IL-12 concentrations were extrapolated using a standard curve that was generated using IL-12 standards supplied in the kit.

IL-12 levels secreted by lymph node cells isolated from Groups 1-6 are shown in Figure 6. Lymph node cells isolated from mice in Group 1, which did not receive siRNA injection against IL-12, secreted more than 300 picograms per millilitre of IL-12 into the culture supernatants. In Group 2 mice, injected with IL-12 siRNA in PBS on day minus one or in Group 4 mice injected with IL-12 siRNA in PBS on day plus one relative to ovalbumin immunization, no significant inhibition in IL-12 protein secretion was observed. In contrast, when siRNA was delivered in liposome/continuous hydrophobic barrier either on day minus one (Group 3) or on plus one (Group 5), marked decrease in secreted IL-12 was observed ($p=0.003$ and $p=0.004$).

respectively), which was as low as that observed with lymph node cells from naïve mice. Moreover, lymph node cells from mice in Group 3 and Group 5 secreted significantly lower IL-12 compared to mice in Group 2 and Group 4 that received siRNA in PBS. This shows an effective and improved delivery of siRNA nucleotide sequences *in vivo* when injected in a liposome/continuous hydrophobic carrier that resulted in complete inhibition of IL-12 protein expression induced by ovalbumin antigen.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Unless defined otherwise all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of

the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

CLAIMS:

1. A composition comprising:

a carrier comprising a continuous phase of a hydrophobic substance;

liposomes; and

a polynucleotide.
2. The composition according to claim 1, wherein the carrier is an oil or a water-in-oil emulsion.
3. The composition according to claim 2, wherein the oil is a natural oil or a synthetic oil.
4. The composition according to claim 3, wherein the oil is mineral oil, a vegetable oil or a nut oil.
5. The composition according to any one of claims 1 to 4, wherein the carrier comprises a mixture of oils.
6. The composition according to any one of claims 1 to 5, wherein the liposomes comprise a phospholipid.
7. The composition according to any one of claims 1 to 6, wherein the liposomes comprise cholesterol.
8. The composition of any one of claims 1 to 7, wherein the liposomes comprise dioleoyl phosphatidylcholine (DOPC) and cholesterol.
9. The composition according to any one of claims 1 to 8, wherein the polynucleotide encodes an antisense RNA, an interfering RNA, a catalytic RNA, a ribozyme, or a polypeptide.

10. The composition according to any one of claims 1 to 9, further comprising an adjuvant.

11. The composition according to any one of claims 1 to 10, wherein the polynucleotide is operably linked to a promoter functional in mammalian cells.

12. The composition according to any one of claims 1 to 11, wherein said polynucleotide is inserted in an expression plasmid.

13. The composition according to any one of claims 1 to 12, comprising a bacterial or viral vector containing said polynucleotide.

14. The composition according to any one of claims 1 to 13, wherein said polynucleotide is present:

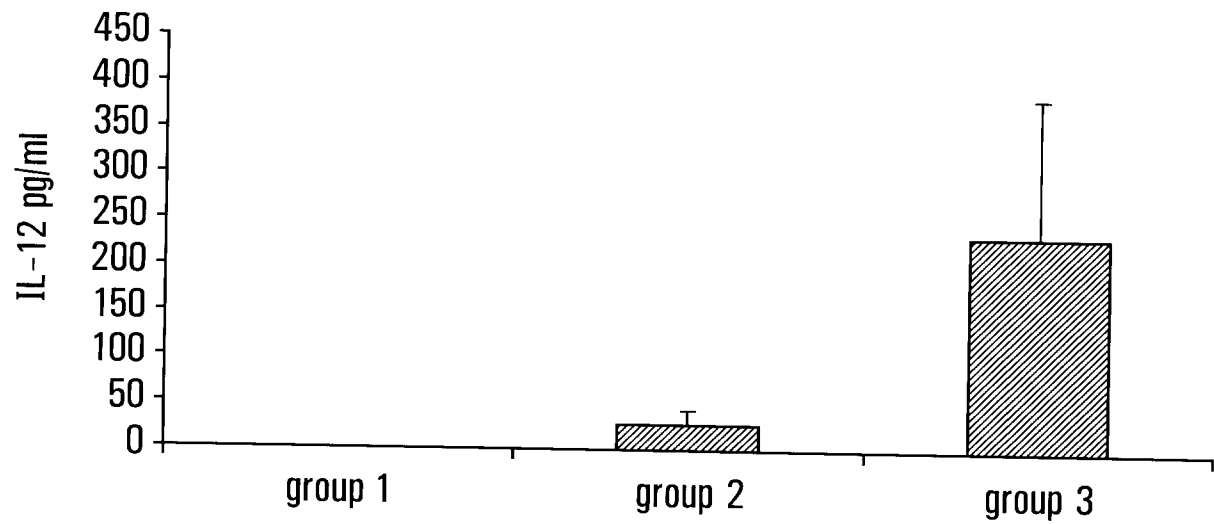
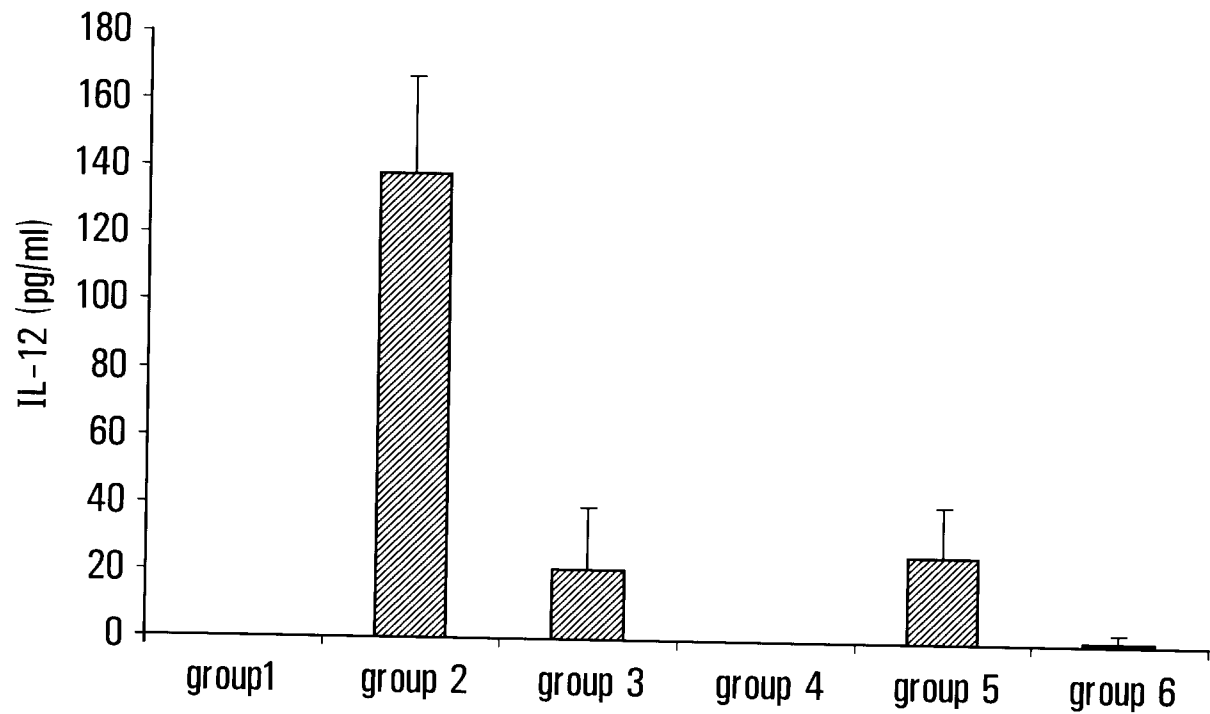
- (a) encapsulated within said liposomes;
- (b) exterior to said liposomes; or
- (c) both encapsulated within said liposomes and exterior to said liposomes.

15. The composition according to claim 10, wherein said adjuvant is present:

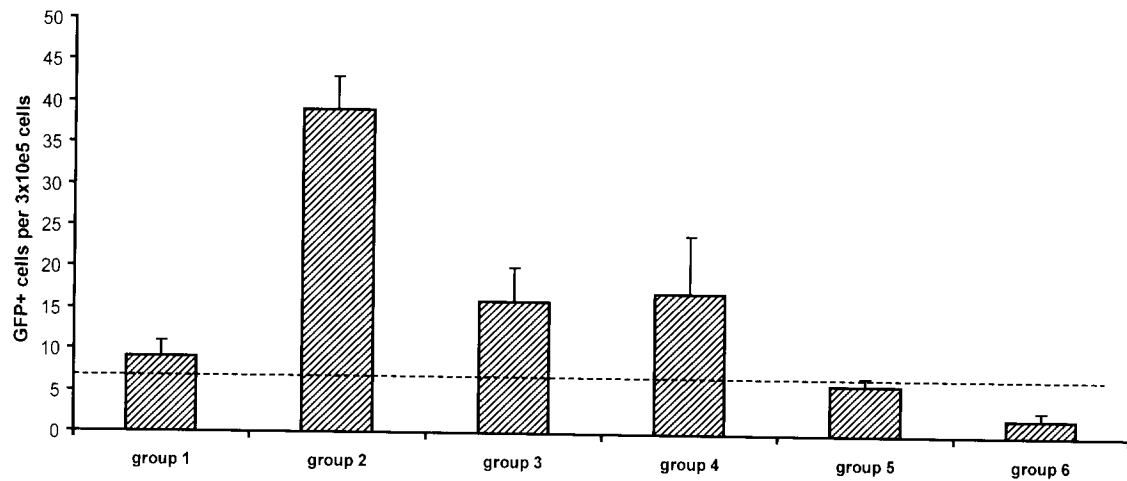
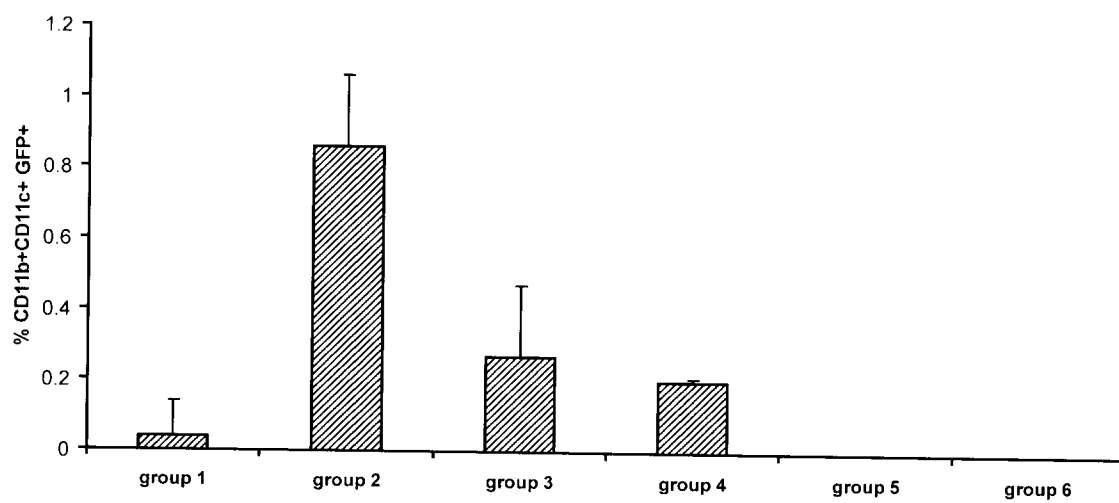
- (a) within said liposomes;
- (b) exterior to said liposomes; or
- (c) both within said liposomes and exterior to said liposomes.

16. A kit, comprising: a composition of any one of claims 1 to 15; and instructions for using said composition to deliver a polynucleotide to a subject.

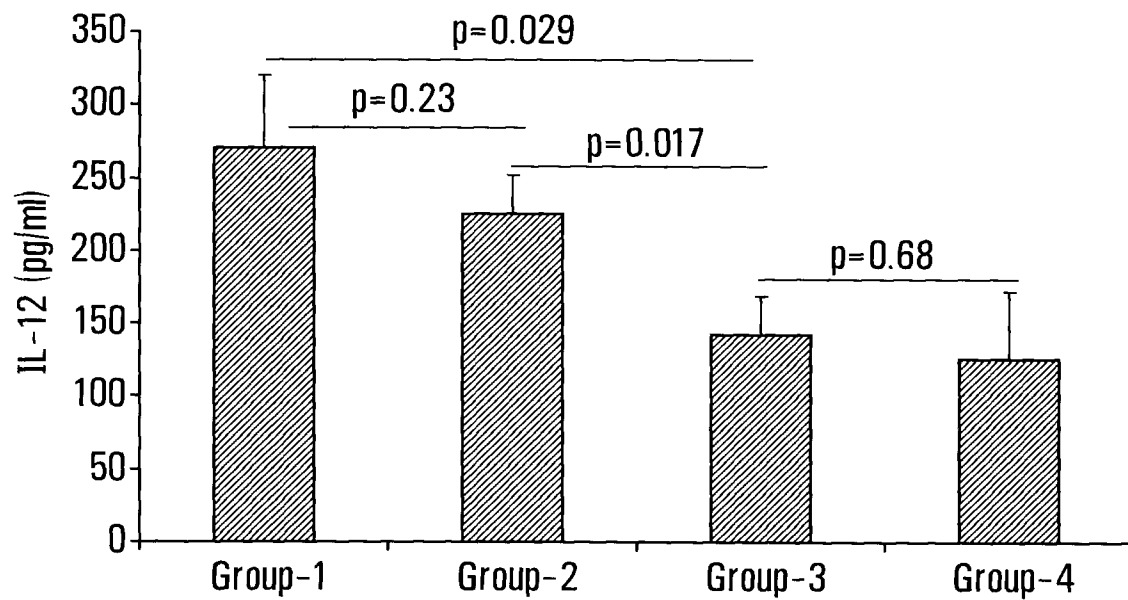
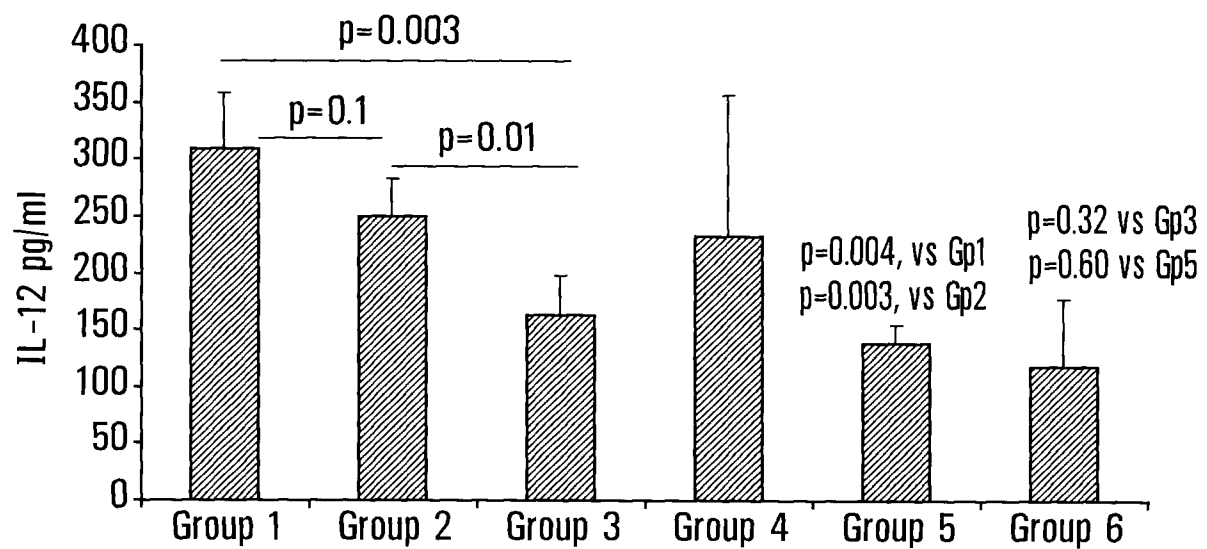
17. A method for delivering a polynucleotide to a subject, comprising administering the composition of any one of claims 1 to 15 to said subject.
18. The method according to claim 17, wherein said subject is a mammal.
19. The method according to claim 17, wherein said mammal is a human.
20. A method for making a composition comprising combining liposomes, a polynucleotide, and a carrier comprising a continuous phase of a hydrophobic substance.
21. The method according to claim 20, wherein said polynucleotide is encapsulated in said liposomes.
22. The method according to claim 20 or 21, wherein the liposomes are dehydrated before they are combined with said carrier.
23. The method according to any one of claims 20 to 22, wherein said polynucleotide is a polynucleotide as defined in any of the preceding claims.
24. The method according to any one of claims 20 to 22, wherein said liposomes are liposomes as defined in any of the preceding claims.
25. The method according to any one of claims 20 to 22, wherein the carrier is a carrier as defined in any of the preceding claims.
26. A composition produced by the method according to any one of claims 20 to 25.

1/3**FIG. 1****FIG. 2**

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**FIG. 3****FIG. 4**

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**FIG. 5****FIG. 6**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2008/001678

A. CLASSIFICATION OF SUBJECT MATTER

IPC: **A61K 48/00** (2006.01), **A61K 31/7088** (2006.01), **A61K 47/44** (2006.01), **A61K 9/127** (2006.01), **C12N 15/10** (2006.01), **C12N 15/88** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: **A61K 48/00** (2006.01), **A61K 31/7088** (2006.01), **A61K 47/44** (2006.01), **A61K 9/127** (2006.01), **C12N 15/10** (2006.01), **C12N 15/88** (2006.01)

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

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Databases: Canadian Patent Database, Delphion, QWEB, CAPIus, Pubmed and Scopus. **Keywords:** liposome, lipid, hydrophobic carrier, continuous phase, oil, mineral oil, water-in-oil, oil-in-oil, emulsion, adjuvant, gene therapy, transfection, VacciMax® and Immunovaccine.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/041832 A1 (DAFTARIAN P. M. et al.) 19 April 2007 Whole document	1 - 7, 10 and 14 - 26
X	MANSOUR, M. et al. Therapy Of Established B16-F10 Melanoma Tumors By A Single Vaccination Of CTL/T Helper Peptides In VacciMax®. J TRANSL MED (Electronic) 23 April 2007 Vol. 5:20, pages 1 - 8 [retrieved on 2007-11-05] Retrieved from the Internet: <URL: http://www.translational-medicine.com/content/5/1/20 > ISSN 1479-5876 Whole document	26 1 - 7, 14 and 16 -

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

5 November 2008 (05-11-2008)

Date of mailing of the international search report

30 December 2008 (30-12-2008)

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Authorized officer

Jacinth Abraham 819- 934-7598

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2008/001678

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DAFTARIAN P. M. et al. Rejection Of Large HPV-16 Expressing Tumors In Aged Mice By A Single Immunization Of VacciMax® Encapsulated CTL/T Helper Peptides. J TRANSL MED (Electronic) 7 June 2007 Vol. 5:26, pages 1 - 9 [retrieved on 2007-11-05] Retrieved from the Internet: <URL: http://www.translational-medicine.com/content/5/1/26> ISSN 1479-5876 Whole document</p>	1 - 7, 14 and 16 - 26
X	<p>WO 2006/113679 A2 (SOOD, A. K. et al.) 26 October 2006 Whole document</p>	1 - 26

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/CA2008/001678**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. ☒ Claim Nos. : **17 - 19**
because they relate to subject matter not required to be searched by this Authority, namely :

Claims **17 - 19** are directed to a method for treatment of the human or animal body by surgery or therapy which the International Search Authority is not required to search. However, this Authority has carried out a search based on the alleged effects or purposes/uses of the composition defined in claims **17 - 19**.
2. ☐ Claim Nos. :
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :
3. ☐ Claim Nos. :
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2008/001678

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO 2007/041832 A1	19-04-2007	AU 2006301891A1	19-04-2007
		CA 2523032A1	07-04-2007
		CA 2533705A1	13-07-2007
		CA 2542212A1	07-04-2007
		CA 2622464A1	19-04-2007
		EP 1948225A1	30-07-2008
<hr/>			
WO 2006/113679 A2	26-10-2006	AU 2006236453A1	26-10-2006
		CA 2605068A1	26-10-2006
		EP 1874793A2	09-01-2008
		JP 2008536874T	11-09-2008
		WO 2006113679A3	26-07-2007