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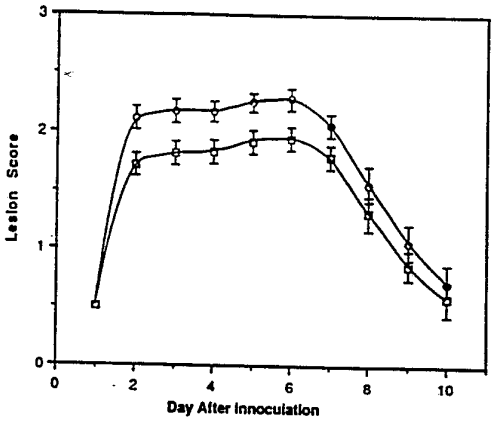
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**(54) Title:** TOPICAL DELIVERY OF PEPTIDES/PROTEINS ENTRAPPED IN DEHYDRATION/REHYDRATION LIPOSOMES

**(57) Abstract**

This invention concerns a topical delivery of small peptides/proteins into the deep tissue of the skin via intradermal permeation. Small peptides/proteins entrapped in liposomes prepared by dehydration/rehydration method and delivered topically via an intradermal route are useful in treatment of various diseases by depositing the entrapped peptide into the epithelial cells of the deeper tissue of the skin. In particular, this invention is useful in suppression of the development of skin lesions induced by virus, bacteria, inflammation or other causes by treatment of the infected epithelial cells with liposome entrapped small peptide able to reduce the damage to the epithelial cells or inhibit viral replication and expression.




o Virus Control

□ Skin Lipids DRV + Entrapped IFN

TOPICAL ACTIVITY OF SKIN LIPOSOMAL DRVs WITH ENTRAPPED INTERFERON-ALPHA (5.4 x $10^6$ IU/ML) AGAINST HSV-I IN THE CUTANEOUS GUINEA PIG MODEL.

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# TOPICAL DELIVERY OF PEPTIDES/PROTEINS ENTRAPPED IN DEHYDRATION/REHYDRATION LIPOSOMES

## BACKGROUND OF THE INVENTION

### Field of the Invention

This invention concerns a topical delivery of small peptides/proteins, in particular interferons, into the deep tissue of the skin via intradermal permeation. Small peptides/proteins entrapped in liposomes prepared by dehydration/rehydration method and delivered topically via a intradermal route are useful in treatment of various diseases by depositing the entrapped peptide into the epithelial cells of the deeper tissue of the skin. In particular, this invention is useful in suppression of the development of skin lesions induced by virus, bacteria, inflammation or other causes by treatment of the infected epithelial cells with liposome entrapped small peptide able to reduce the damage to the epithelial cells or inhibit viral replication and expression.

### Related Disclosures

There are many diseases which affect the epithelial skin cells which could be succesfully treated without submitting the patients to a systemic treatments with large doses of drugs which ususally cause unwanted or undesirable side effects.

Among these diseases belong the diseases such as viral and bacterial infections, skin allergies, inflammations, hormonal disturbances, cancerous or

proliferative growths, sarcomas such as Kaposi sarcoma, warts such as genital warts, psoriasis or alopecia.

Many of these diseases may be advantageously treated by peptide drugs such as antiviral and antibacterial peptides, hormones, antiallergens and others small proteins or peptides. However, as will immediately become apparent, some of these protein or peptides, such as for example epidermal growth hormone, are very potent and may be quite harmful when given in large quantities systemically parenterally or, in the alternative, they may be quickly inactivated by various proteases in the gastrointestinal tract when given orally.

Thus, while it is possible to treat some of these diseases systemically by administering large dosages of therapeutically effective peptide drugs specific to each of these diseases parenterally, more often than not the dosage required for such systemic parenteral treatment is excessively large and has unwanted and harmful side effects. When administered topically, the drugs often lose their effectivity because of their inability to penetrate into the stratum corneum.

It would be therefore advantageous to have available a topical treatment for these diseases which would eliminate a need for systemic administration of large amounts of drugs or avoid ineffective topical treatments.

One of the primary targets for the topical treatment with peptide drugs are viral infections, in particular herpes virus. Infections caused by herpes viruses are among the oldest known to man. Recurrent herpes simplex labialis has been reported to affect almost one-half of the population of the United States, and 25% of those affected have frequent and/or severe recurrences. Genital herpes is currently an epidemic venereal disease affecting more than five million Americans.

Although many forms of therapy have been tested,

none has proven profoundly beneficial in decreasing the severity and frequency of the clinical manifestations of these herpetic conditions. Can. Med. Assoc. J., 125:23 (1981).

Once established, herpes virus particles retreat into the nerve trunk and remain latent in the asymptomatic period. During that time they are located in ganglia and are thus inaccessible to therapy. Therefore, the succesful control of herpes labialis rests on the ability to suppress the virus when it begins to re-express itself. For that purpose a delivery of an effective antiviral agent into the living epidermal tissues of the skin during the prodromal stage of lesion development would seem to be the most effective. It is believed that virus replication and lateral spread in the basal layer can be arrested during this stage by either chemotherapeutic or immunotherapeutic drugs such as interferon. Can. Med. Assoc. J., 125:23(1981).

Antiviral agents interferons are peptide macromolecules of molecular weight in the range of 20,000. They are produced in cell cultures or host tissues in response to infection with active or inactivated virus and are capable of inducing a state of resistance to superinfection with related or unrelated virus. Interferons are small proteins which interfere with viruses other than the one which provoked their formation, but are much more effective in the cells of the species in which they were evoked than in others.

Interferon seems to have especially high potential for the treatment of herpes, condylomata acuminata and other similarly manifested disease states. However, systemic regimens of interferon adequate to suppress skin symptomology often results in adverse system effects and still may not overcome the inaccessibility of the target tissue to the drug. Interferon drug delivery thus

remains the singularly most limiting factor to the effective treatment of herpes and other like conditions.

Thus, it would be desirable to have available the interferon delivery system which would avoid and prevent adverse systemic effect but still deliver the antiviral drug to the target cell.

Similarly to herpes, many other cellular diseases such as bacterial infections, inflammations, allergic reactions, cellular metabolic or hormonal disturbances face the same problem. Unless there is available convenient and effective method for delivering the drug intradermally directly to the cell located in deep skin tissues, these diseases can only be treated systemically.

Thus, it would be desirable to have available the peptide/protein delivery system which would avoid and prevent adverse systemic reactions but still deliver the peptide/protein drug to the target cell.

Intereferon's antiviral function seems to be of preventive character. Its antiviral activity rests in transmitting messages to other cells to protect them from virus invasion. Interferons also seem to cause induction of several enzymes that impair viral replication at different stages. Thus, although interferon seems to have especially high potential for the treatment of herpes, and other similarly manifested viral disease states, it cannot protect an already infected cell.

Additionally to its preventive function, interferons may enhance host immunity by increasing the lymphocytotoxic activity of natural killer lymphocytes. Data from a number of recent clinical studies described in Chemotherapy, 32:537 (1986), demonstrate that interferon is most effective when used prophylactically rather than clinically for both genital warts and herpes infections and show that recombinant interferon-alpha (IFN-alpha) is highly effective in patients with human

papillomavirus-related genital warts and that the low-dose regimen ($1.5 \times 10^6$ Units) is at least equivalent to the high dose treatment ($18 \times 10^6$ U) and that recipients of the higher dose of IFN-alpha ($3 \times 10^6$ U) had few outbreaks of genital herpes lesions, a shorter period of viral shedding, less itching, and a faster healing time, while the lower dose of IFN-alpha ($1 \times 10^6$ U) was not effective. J.Infect. Dis., 154:437 (1986).

The combined evidence of these and other studies indicate that interferon's ability to activate macrophages plays a much more important role in its therapeutic effectiveness, particularly as a prophylactic, than was previously recognized and that interferon acts more as an immunomodulator than an antiviral agent and the way in which the initial episode is treated with interferon can affect the subsequent course of the disease, namely recurrences. Genitourin. Med., 62:97 (1986).

The limited usefulness of cytotoxic antiviral acyclovir for the treatment of herpes and related diseases may be attributed to its inability to act as an immunosuppressive agent.

The tissue level of interferon needed to arrest virus replication is not known. Considerable evidence demonstrates that low levels of endogenous interferon exist in the normal tissues of animals and man. This interferon is presumed to constitute an important part of the natural barrier to viral infection. For example, the J. Infect. Dis. 133: A6 (1976) describes the pharmacokinetics of human leukocyte interferon administered intravenously to rabbits which detected the presence of low levels (35-350 units/ml) of baseline activity against vesicular stomatitis virus in rabbit sera and also in the sera of other laboratory animals and

of humans. Although interferon was not isolated and characterized, these findings strongly point to the existence of low endogenous levels of interferon in normal tissues. Importantly, the antiviral activity of interferon was attributed to a specific alpha-interferon or an acid-labile alpha-interferon.

When systemic routes of administration are used to deliver antiviral drugs to extravascular sites, sufficient amount of the drug has to be administered to account for the drug's distribution through all tissues of the body. Therefore, systemic regimens adequate to suppress skin symptomology often result in adverse systemic effects and still may not overcome the inaccessibility of the target tissue to the drug. In these regards, drug delivery remains the singularly most limiting factor to the effective treatment of herpes. It is well recognized that an optimal prophylactic and therapeutic regimen must include (i) convenient route of administration, (ii) lack of side effects, and (iii) good therapeutic benefit. Since large doses of interferon must be given parenterally to achieve a reasonable therapeutic benefit, criteria (i) and (ii) are not met.

It would therefore be advantageous to have a convenient route of drug administration which would achieve a maximal therapeutic effect at a target organ with minimal amount of drug thus avoiding undesirable adverse systemic effect and achieving the optimal prophylactic and therapeutic regimen.

Over the last five years, a number of clinical studies have reported various degrees of success when herpes infections and genital warts were treated with topical applications of interferon. For example Antibiotiki, 28:848(1983) reported that an ointment containing pig leukocytic interferon had a pronounced therapeutic effect in herpetic affections of the face

skin and genitalia. Int. J. Clin. Pharmacol. Ther. Toxicol., 19:498 (1981) reported that treatment of labial and genital herpes with ointments containing human leukocyte interferon resulted in less frequent relapses and smaller lesion sizes.

J. Am. Acad. Dermatol., 5:989 (1986) reported the end of new lesion formation, and scabbing and healing of lesions were improved in patients with recurrent genital herpes who were treated with alpha-interferon combined with a surfactant fungicide nonoxynol-9 in a cream base. Lancet, 23:150 (1988) reported that topical application of interferon-beta in a carboxymethyl cellulose gel base during herpes eruptions reduced the mean number of recurrences and the duration of eruptions in patients with herpes involving either the lips or the genitals. When this gel was applied at the time of eruptions, there were no recurrences for at least a year in 10 of 12 cases of genital herpes treated.

Despite the above, there have been some clinically disappointing outcomes of topical interferon therapy. Antimicrob. Agents Chemother. 31:1137 (1987) reported in a study of 94 patients with recurrent genital herpes, that when aqueous solutions of alpha-2a interferon were applied to unroofed vesicles, the placebo was as effective as the drug-containing formulation with respect to the duration of viral shedding, the time of crusting, and the time to healing of herpetic lesions. The lack of efficacy of the interferon formulation described above may well have been caused by the failure of the delivery vehicle (a simple aqueous solution) to facilitate transport of interferon through the skin. This hypothesis is supported by the report in Antimicrob. Agents Chemother., 25:10 (1989) showing that efficacy of topically administered acyclovir is dependent on the ability of acyclovir to penetrate the skin and thus on

the delivery vehicle used and by a recent study described in J. Interfer. Res., 7:213 (1987) showing that therapeutic efficacy of recombinant interferon-alpha evaluated against experimental dorsal cutaneous HSV-1 infection of guinea pigs, was tied to the delivery system and the timing of its use.

It would be, therefore, beneficial to have available an interferon-containing formulation which would allow the maximal efficacy of transfer of interferon by permeation through the skin.

Liposomes recently have received much attention in the search of a more effective means of delivering intrinsically active drugs to their tissue targets. Liposomes are microscopic vesicles consisting of one or more concentric lipid bilayers enclosing an equal number of aqueous compartments. Introduced as a model membrane system, they have increased our understanding of biological membrane structure and function. More recently, liposomes are being viewed as potential carriers for site-directed delivery of drugs such as insulin, enzymes, antimicrobials, anti-tumor agents and biological response modifiers. Am. N.Y. Acad. Sci., 308:281 (1988). The attractiveness of liposomes as drug carriers lies in their ability to encapsulate and physically protect drugs, and their potential to selectively concentrate or deliver drugs at or to various body sites, even to the point of facilitating the transport of some drugs across biological membranes. Liposomes are generally nontoxic and readily metabolized, which adds measurably to their attractiveness.

The effectiveness of liposomes as drug carriers have been proven in many instances. For example, the lipid constituents of the liposomes have been shown to greatly affect drug entrapment, shelf life stability, the location of a drug in the liposome, the stability of drug

and liposome in physiological environment, the pharmacokinetics and tissue specificity of both the liposome and entrapped drug, the ability of the liposome and/or entrapped drug to penetrate cell membranes, and, most importantly of all, the pharmacological activity of the encapsulated drug. J. Viro., 41:575 (1982); J. Interferon Res., 1:495 (1981) and 2:117 (1982). Since the lipid constituents of the liposomes are easily manipulated, the new more efficatious delivery systems may be designed for specific types of drug delivery via liposomes.

Both fibroblast and leukocyte interferons have been incorporated successfully into liposomes. Studies reported in J. Interferon Res., 3:161 (1983) show that the physical location and the extent of incorporation of interferon in liposomes as well as interferon's stability and antiviral activity are dependent on lipid composition of the liposome.

The first suggestion of liposomes' usefulness as drug carriers by the topical route was described in Life Sci., 26: 1473 (1986) which reported that the percutaneous absorption of a liposomally encapsulated drug, triamcinolone, through rabbit skin was reduced relative to when the drug was applied in an ointment but that the concentration of liposomally delivered drug was greatly increased locally into epidermis and dermis, suggesting that the liposomes penetrate and cross biological membranes in order to reach selected tissue locations.

Subsequently, it was shown in Int. J. Pharm., 20:139 (1989) and J. Cont. Release, 2:61 (1988) that intact liposomes themselves do not penetrate intact skin but that they facilitate deposition of drugs associated with liposomal bilayers into the skin.

A successful treatment of cutaneous virus infections with interferon relies on an ability to effectively deliver interferon to the infected cells. Thus far it has been impossible, even with liposome encapsulated interferon, to obtain adequate tissue levels to control herpes and other skin viruses by conventional topical routes of drug administration.

Thus, a pharmaceutical composition suitable for topical administration of small peptides/proteins and antiviral interferons which would overcome the disadvantages of systemic administration and provide adequate and effective delivery of peptides/proteins and interferons into infected cells and assure tissue levels of these drugs able to control herpes and other skin diseases would be extremely valuable.

It has been now discovered that the method of preparation of liposomes may be very important with respect to the physicochemical behavior and ultimate therapeutic efficacy of these liposomal systems. Of particular importance was the discovery that when the small peptide/protein or interferon is encapsulated in traditionally prepared liposomes, it lacks therapeutic efficacy. When, however the polypeptide liposomes are prepares by a technique which facilitates its association with bilayers, the polypeptide penetrates intact skin and is extraordinarily therapeutically active. Such technique has been found to include dehydration and rehydration of liposomes. Although the mechanism of physical entrapment and the exact location and distribution of polypetide in dehydration/rehydration liposome vesicles (DRVs) are not quite clear, the large amounts of polypetide are taken up by DRVs, a significant amount of the polypetide is internalized within DRVs and liposomally entrapped polypetide is stable for at least

one year while maintaining its antiviral activity under these conditions.

Thus, it is a primary object of this invention to provide a suitable system for efficatious topical delivery of small peptides, polypetides and interferons by way of a liposomal delivery system.

SUMMARY

One aspect of this invention is a pharmaceutical composition for a topical liposomal intradermal delivery through stratum cormeun of peptides/proteins normally nonpenetrating skin.

Another aspect of this invention is the topical liposomal formulation with encapsulated small peptide which provides enhanced skin penetration and increased bioavailability of the peptide underlining target tissue in cells.

Another aspect of this invention is the method of intradermal delivery of liposomally entrapped small peptide into diseased cells.

Yet another aspect of this invention is the method of treatment of diseased cells and tissues by administering the composition containing peptide drug of this invention intradermally to the human or animal skin surface.

Still another aspect of this invention is the process of preparing the topical pharmaceutical liposome composition with entrapped peptide.

Yet another aspect of this invention is the method of intradermal delivery of liposomally entrapped interferon into virus-infected cells.

Still another aspect of this invention is the pharmaceutical composition for the topical intradermal delivery of liposome encapsulated antiviral interferon.

Another aspect of this invention is the topical liposomal formulation with encapsulated interferon which

provides enhanced skin penetration and increased bioavailability of the interferon in cells.

Yet another aspect of this invention is the method of treatment of viral infected cells and tissues by administering the composition of this invention containing interferon intradermally to the human or animal skin surface.

Still another aspect of this invention is the process of preparing the topical pharmaceutical liposome composition with entrapped interferons.

## PREFERRED EMBODIMENTS

Preferred embodiments of this invention are liposome formulations comprising egg lecithin, cholesterol and phosphatidylserine with about 5-30% of encapsulated small peptide prepared by dehydration/rehydration method.

More preferred embodiments of this invention are liposome formulations comprising dimyristoyl-phosphatidylcholine, cholesterol and phosphatidylserine in ratio 2:1:0.33 with about 15-30% of entrapped small peptide.

The most preferred embodiments of this invention are liposome formulations comprising ceramide, cholesterol, palmitic acid and cholesteryl sulfate in molar ratio 4:2.5:2.5:1 with encapsulated peptide.

## BRIEF DESCRIPTION FO FIGURES

Figure 1 illustrates the topical antiviral activity of peptide interferon-alpha in aqueous solution on HSV-I lesions, compared to virus control in the cutaneous guinea pig model.

Figure 2 illustrates the topical antiviral activity of peptide interferon-alpha entrapped in water-in-oil emulsion on HSV-I lesions, compared to virus control in the cutaneous guinea pig model.

Figure 3 illustrates the topical antiviral activity against HSV-I of interferon-alpha entrapped in negatively

charged EL:CH:PS-MLVs compared to virus control in the cutaneous guinea pig model.

Figure 4 illustrates the topical antiviral activity against HSV-I of interferon-alpha entrapped in negatively charged EL:CH:PS-LUVs compared to virus control in the cutaneous guinea pig model.

Figure 5 illustrates topical activity of interferon-alpha entrapped in negative EL:CH:PS-DRVs prepared by dehydration/rehydration method compared to virus control in the cutaneous guinea pig model.

Figure 6 illustrates the topical activity of interferon-alpha entrapped in negatively charged DMPC:CH:PS-DRVs prepared by dehydration/rehydration method, compared with virus control in the cutaneous guinea pig model, expressed in lesion score.

Figure 7 illustrates the topical activity, expressed in lesion score, of interferon-alpha entrapped in skin lipids CM:CH:PA:CHS-DRVs compared to virus controls in the cutaneus guinea pig model.

Figure 8 illustrates the topical activity of skin lipid CM:CH:PA:CHS-DRVs containing free interferon-alpha compared to virus control in a cutaneous guinea pig model and expressed as lesion.

## DETAILED DESCRIPTION OF THE INVENTION

### Definitions

The term "phospholipid" means and includes lipids such as dimyristoylphophatidylcholine (DMPC), cholesterol (CH), distearoylphosphatidylcholine (DSPC), egg lecithin (EL), phosphatidylserine (PS), stearylamine (SA), cholesterol (CH), cholesterol sulfate (CHS), phosphatidic acid (PPA), phosphatidylglycerol (PG), phosphatidylcholine (PC), phoshatidylinositol (PI), cardiolipin (CL), plasmalogens (PM), sphingomyelin (SM), bovine brain ceramides (CM) and palmitic acid (PA). These phospholipids may be fully saturated or partially

hydrogenated. They may be naturally occurring or synthetic.

The term "liposome" means and includes liposome vesicles such as multilamellar vesicles (MLVs), large unilamellar vesicles (LUVs) usually larger than 100 nm, small unilamellar vesicles (SUVs) closed bilayer vesicles of about 25-50 nm, and dehydration/rehydration vesicles (DRVs) which are large unilamellar or oligolamellar liposomes formed during the dehydration by fusion of small vesicles into multilammelar film which effectively encapsulate large amounts of the drug between successive layers and upon rehydration results in relatively large vesicles.

The term "peptide" include all small proteins and peptides/proteinsincluding polypetides with molecular weight between 900 and 50,000, whether naturally occuring in animals or humans or arteficially prepared and/or sythesized or purified.

Preparation Procedures

I. Liposomes

Preparation of Liposomes

Liposome type, size, lipid composition and charge affect the degrees of drug entrapment or encapsulation, the drug's location in the liposome, stability, pharmacokinetics, tissue specificity and ability of the liposome and/or entrapped drug to penetrate cell membranes and exert their pharmacological effect.

Lipid Composition and Charge

Liposomes of the current invention can be neutral, such as formed from egg lecithin (EL) and cholesterol (CH), positively charged such as those containing stearylamine in combination with egg lecithin and cholesterol, or negatively charged such as those containing phosphatidylserine (PS) phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylcholine

(PC), phosphatidic acid (PPA), dimyristoylphosphatidylcholine (DMPC) or distearoylphosphatidylcholine (DSPC). Positive liposomes containing EL:CH:SA (2:1:0.33) were found to be slightly irritating to the skin. Neutral liposomes of this invention tended to floculate to large aggregates within one week of storage. Although both positive and negative liposomes are contemplated to be within the scope of this invention, the most preferred liposomes suitable for practicing this invention are negatively charged liposomes, both MLVs and LUVs, comprising EL:CH:PS or DMPC:CH:PS in molar ratio from 1:0.5:0.01 to 3:3:1, preferably in molar ratios of 2:1:0.33 prepared by dehydration/rehydration technique.

Additionally, so called "skin lipid" liposomes were prepared from lipids with compositions similar to those found in stratum corneum. These skin liposomes were preferably made of the following lipids: bovine brain ceramides (CM) cholesterol (CH), palmitic acid (PA) and cholesteryl sulfate (CHS).

Unlike all other biologic membranes, stratum corneum does not contain phospholipids, but consists primarily of ceramides (40%), cholesterol (25%), fatty acids such as palmitic acid (25%) and cholesteryl sulfate (10%). Liposomes prepared using lipid compositions using the above lipids formed stable skin liposomes. Variation of the mole ratios of the components of skin lipids were shown to affect phase transitions of their bilayers and stability of the resulting structures. The skin lipid liposomes as a intradermal drug delivery system and/or as a model membrane system were tested side by side with negative liposomes, both prepared by dehydration/rehydration.

Liposomes best suitable to practice this invention are those liposomes prepared by dehydration/rehydration

technique (DRVs), described infra, which are superior to MLVs and LUVs prepared by other techniques with respect to interferon entrapment and antiviral activity as determined by the cutaneous herpes guinea pig model. MLVs and LUVs prepared by standard techniques were used as control liposomes for comparison of bioeffectivity of new compositions, particularly the skin lipid liposomes.

Multilamellar Liposomes (MLV)

MLVs were prepared by standard procedures known in the art. The various lipid mixtures were dissolved in chloroform and rotary-evaporated using any suitable method such as drying under nitrogen. The flask containing the thin lipid film was stored under vacuum from 5-48 hours, preferably overnight, to facilitate removal of residual solvent. The lipid film was then resuspended at a temperature above the phase transition temperature of the used phospholipid in a suitable buffer such as calcium-magnesium free phosphate buffered saline (pH 7.0) containing various concentrations of a peptide such as interferon, hormone and such others, in the presence of albumin. The mixture was vortexed for from 5-60 minutes preferably 10-30 minutes and all free, nonentrapped peptide was preferably removed by passage through a suitable Sephadex G-75 column or by repeated centrifugation at 100,000 g. However, even if free drug was not removed, leakage from the aqueous compartment was minimized since its external thermodynamic activity approximates its thermodynamic activity in the aqueous compartments of the liposomes.

Control empty liposomes were prepared as above, but in the absence of interferon.

Large Unilamellar Vesicles (LUV)

LUVs can be prepared by any suitable method such as by the reverse-phase evaporation (REV) process disclosed in U.S. Patent 4, 529, 501, incorporated by reference,

thin-film hydration, sonication, high shear fragmentation, freeze-drying, and preferably by the extrusion method, all above methods well known in the art.

Large unilamellar and oligolamellar vesicles with high entrapment efficiencies have been formed by a method reported in BBA, 816:1 (1985). During extrusion MLVs are repeatedly extruded through very small pore diameter polycarbonate membranes (0.1 um) under high pressure (up to 250 psi) so that their average diameter becomes progressively smaller reaching a minimum of 100 nm after about 5-10 passes. As the MLVs are forced through the small pores, successive layers are peeled off until only one remains.

To prepare extrusion type liposomes for these studies, the extrusion apparatus (The Extrudor obtained from the Lipex Biomembranes Inc., Vancouver, B.C., Canada) is fitted with 100 nm pore size polycarbonate membrane filters (Nucleopore Corporation, Pleasanton, CA).

Control or empty liposomes are prepared as above, but in the absence of the peptide.

Dehydration/Rehydration Liposomes (DRV)

The DRV were prepared by the method described in Liposome Technology, 1:19-28 (1984), CRC Press, Ed. G. Gregoriadis.

In this method, empty sonicated vesicles are mixed in an aqueous solution containing the solute of peptide desired to be encapsulated and the mixture is dried under a stream of nitrogen. During the dehydration, the small vesicles fuse to form a multilamellar film that effectively sandwiches the solute molecules between successive layers. Upon rehydration, large vesicles are produced which have encapsulated a significant proportion

of the solute. Control empty liposomes were prepared as above, but in the absence of peptide.

The above described method may be advantageously modified and used for large scale production. The method depends mainly on controlled drying and rehydration processes and does not require extensive use of organic solvents, detergents, or dialysis systems. The peptide is thus never in contact with organic solvents or detergents.

Physical Characterization of Liposomes

Size and Morphology

The size distribution of the liposomes was determined by a combination of light microscopy and electron microscopy. A Nikon Diaphot inverted microscope was used to visualize liposomes having diameters > 0.5 micrometers. Examination of uncharged neutral liposomes with the light microscope revealed flocculation problems long before they were apparent with the naked eye.

Electron microscopy was used to determine size distribution of the smaller vesicles (0.5 microns in diameter). Three hundred-mesh copper or stainless steel grids were cleaned ultrasonically in glacial acetic acid and coated with formvar. A Denton 502 evaporator was used to coat the grids with carbon. Negative staining of liposomes was carried out by placing a small drop of the vesicle sample on a freshly prepared grid surface and drawing off the excess with filter paper. A drop of 2% sodium phosphotungstate or 2% ammonium molybdenate at pH 7.4 was placed on the grid and allowed to stain for 30 seconds. Excess stain was removed, the grids dried, and viewed with a JEM 100 CX electron microscope operated at 75 KV.

For determination of morphological changes of the liposomes on exposure to interferon, the technique of freeze-fracture electron microscopy was used. Liposome

samples were centrifuged at 100,000 g and suspended in 30% glycerol in buffer. Droplets of the sample (approximately 10 ml) were mounted on gold cops and quickly frozen in lipid Freon 22. The samples were stored in liquid nitrogen until used, at which time they were placed in a Balzers BA 360M freeze etching device at -150$^0$C and shadowed with platinum within two seconds after the last fracture. After replication with carbon, the samples were removed from the chamber and cleaned in 1% hypochlorite solution. After rinsing with double distilled water, the replicas were mounted on copper grids and studied in a JOEL Model JEM 100-CS electron microscope.

After the liposomes have been characterized by electron microscopy, quasi-elastic light scattering (QELS) was used as a quality control check for studied liposomal preparations. Presently, a Langley-Ford LSA-2 spectrometer containing a 5 mW helium-neon laser light source (wavelength=632.8nm) and a fixed scattering angle of 90 is used. Liposomes are examined using a sample time of 2.9 x $10^{-5}$ seconds. Calculations or particle size diameter were performed by a Model 1096 CM64 autocorrelator using 64 channels.

Surface Charge of the Liposomes

Proteins and polypeptides interact with liposome bilayers by way of the interaction of proteins with bilayers. Such interaction depends on hydrophobic associations of the protein with the phospholipid which are facilitated by initial electrostatic binding. In general, peptides/proteinssuch as interferon do not penetrate phospholipid monolayers and bilayers, but it seems that the polypeptide, when incorporated in liposomes, adsorbs to polar head groups of the bilayer. The effect of interferon on the on the surface charge of liposomes was studied in order to determine the extent of

its bilayer association. A model 501 Lazer Zee Meter was used to determine the electrophoretic mobility (zeta potential) of the liposomes. This instrument is extremely sensitive since it does not tract individual particles but rather adjusts the image to produce a stationary cloud of particles using a rotating prism technique. A number of studies have shown that the phospholipid content of the liposomes affects the extent of entrapment of peptides/proteinssuch as interferon within bilayers. Determination of electrophoretic mobility of the various liposomes in the presence and absence of peptide was used in demonstrating the extent of these interactions. For a given liposome composition, zeta potential was determined for: (i) "blank" liposomes; (ii) "blank liposomes incubated with peptide; (iii) liposomes containing entrapped peptide; and (iv) liposomes containing entrapped peptide after trypsin treatment.

Determination of Degree of Peptide Entrapment

To calculate the theoretical amount of peptide that can be trapped nonspecifically in a given liposomal system, it is necessary to know internal volume of the liposomes (volume of the aqueous compartments). Internal volume was measured by the method described in Hoppe-Seyler's Physiol. Chem., 362:1051 (1981). Liposomes were prepared in buffer containing $K_3(CN)_6$ (250 mOsM) and a sample passed over a previously washed and swollen Sephadex G-25 column to separate free and trapped solute. The amount of $K_3(CN)_6$ present inside the liposome (the aqueous phase entrapment) was determined from the absorbance at 420 nm after lysis of the liposome by Triton X-100.

The amount of captured peptide was determined by a number of methods. First, liposomes containing peptide,

in this instance $^{14}$C-Recombinant Leukocyte A Interferon, were used for screening procedure for capture efficiency. Samples of the liposomal dispersions were incubated with Triton X-100 (0.5%) for one hour to completely disrupt the liposomes and free the entrapped interferon. The amount of interferon captured was determined by scintillation counting and the amount of lipid was determined by the method described in J. Biol. Chem., 66:375 (1925). In addition and in order to remove all free interferon or other peptide, these systems were exposed to trypsin treatment to destroy the interferon not internalized within the liposomes. These procedures allowed for quick elimination of systems which inefficiently internalize interferon. The liposomal formulations which pass the screening procedure were tested using the biological peptide assay previously described.

The degree of peptide interferon entrapment for the various liposomes was determined as follows. Two aliquots of 100 ul were removed and one aliquot frozen for future assay to determine total peptide interferon (Total IFN). The second aliquot was placed in a Beckman centrifuge tube and centrifuged at 148,000 x g in a Beckman Airfuge for 30 minutes. The supernatant was withdrawn and the pellet washed 3 times with 100 ul HEPES buffer. The supernatant with the washings was frozen for future assay for determination of free peptide interferon (Free INF). The pellet was dissolved in 500 ul of 0.4% sodium deoxycholate in HEPES (previously shown not to interfer with the interferon assay) and samples were frozen for future assay to determine the amount of entrapped peptide interferon (Entrapped IFN).

In order to determine the percent of pellet-associated interferon that is free or bound to the

outside surface of the bilayer (trypsin sensitive), the procedure described in Infect. Immun., 31:1099 (1981), was used. Interferon, either free or contained within the liposomes was incubated with trypsin (50 mg/ml) for 30 minutes at 37°C, at which point antitrypsin (150mg/ml) was added for an additional 30 minutes. Controls were run by incubating samples at 37°C in buffer for at least one hour. Samples were then assayed for interferon activity, either directly in the case of free interferon or after detergent lysis in the case of liposomally entrapped interferon. Trypsin destroyed 99.9% of the activity of free interferon.

The same assays as those described above are used for all other peptides/proteinswhich are covered by the scope of this invention.

Stability of Liposomally Entrapped Peptide

The stability of liposomal systems is a complex issue. The overall stability includes a number of parameters: (i) morphology of the liposomes; (ii) chemical stability of the liposomal lipids; (iii) chemical stability of the entrapped drug; and (iv) integrity of the liposomes with respect to drug, i.e. their leakiness. Liposomally entrapped peptide interferon dispersions were stored at 4, 25, and 37°C. At weekly intervals for one month and at monthly intervals thereafter, samples were analyzed to monitor the following:

1. Physical changes: Light and electron microscopy was used to observe the morphological changes, e.g., liposomal size and size distribution, evidence of fusion and evidence of flocculation.

2. Chemical stability: Lipid peroxidation was monitored by the appearance of conjugated dienes, as determined by increased UV absorption in the 230-260 nm

range and lipid hydrolysis will be monitored by appearance of lyso-PC, as determined by phospholipid extraction followed by separation of PC and lyso-PC by TLC.

3. Antiviral activity: Samples were withdrawn at appropriate time intervals and processed as described above for determination of degree of entrapment. The interferon activity was determined and compared with the value at time zero. Analysis of supernatants and pellets determination of the percent of interferon that leaked from the liposomes but remained active in the free state. The data obtained for the 25 and 37°C samples constituted our screening procedure and allowed the choice of the more stable systems for longer term stability testing and evaluation of transport properties into the skin and the intradermal activity in the cutaneous herpes guinea pig model.

Active Compounds

Active compounds of this invention are either peptides or proteins which are under normal circumstances penetrating very little or nonpenetrating through the skin and therefore, their topical therapeutic efficacy is limited or nonexistent. The primary purpose of this invention is to provide a means for these peptides/proteins to reach the target tissue and/or tissue cells underlining the stratum corneum by allowing or enhancing the penetration through the stratum corneum, such penetration having been achieved via encapsulation in liposomes.

Compounds encapsulated in liposomes prepared by the above described procedure include but are not limited to peptides, as defined in Definitions, with molecular weight from 900 to 50,000. All peptides/proteinsand other small molecules which would be active and useful as antivirals, antiinflammatories, antiproliferatives,

antibacterial, antiallergenic, antitumorous, or for hormone treatments, for treatment of Kaposi Sarcoma, Psoriasis, Alopecia, genital warts and for other therapeutical uses may be advantageously formulated into liposomes of the current invention and administered intradermally.

The examples of the active compounds normally nonpenetrating through the skin and or stratum corneum suitable to be encapsulated into the topical liposomal formulation for intradermal penetration into and through the stratum corneum to the underlining target tissue are peptides, such as TCMP-80-F-cell modulatory peptide, bradykinin antagonist, Anaritide, Auriculin atrial peptide, pentagetide; tumor necrosis factors; vaccines such as hepatitis B vaccine, Escherichia coli vaccine, HIVAC-1e vaccine, Vaxsyn HIV-1, conjugate vaccine for haemophilus influenzae, cancer vaccine, malaria vaccines, Factor VIII:C, endogenous human insulin or of recombinant DNA origin, endogenrous somatotropin, or of recombinant DNA origin, human growth hormone, tissue plasminogen activator, MAb; anticoagulants or thrombolytic agents such as prourokinase, colony stimulating factors such as granulocyte/colony stimulating factor, granulocyte macrophage/colony stimulating factor; dismutases; such as superoxide dismutase, PEG-SOD superoxide dismutase erythropoietin such as Epogen, Marogen, Eprex; interferons such as interferon-alpha, interferon-alpha 2a, interferon-alpha 2b, human leukocyte interferon-alpha, recombinant human interferon-beta, interferon gamma, interferon-concensus; interleukins such as interleukin-2, recombinant human interleukin-2, recombinant human interleukin-2/LAK cell therapy, recombinant human interleukin-2/Roferon-A combination; monoclonal antibodies such as Anti-Leu-2 MAb, MAb-L6, Centoxin, Panorex, ovarian RT, Centorel

antiplatelet MAb, Onco-Rad MAb, ADDC agent MAb, Onco Scint CR 103 MAb, Melanoma I-131 MAb, Orthoclone OKT3 MAb, Xomen-E5 MAb, XomaZyme-Mel MAb, XomaZyme -H65, other peptides such as thymrosin or Factor VIII C, antibodies such as endotoxin antibodies, toxins such as diphteria toxin, immunotoxins, and others. Also suitable to be formulated in the liposomal composition claimed therein are ANF atrial naturetic factor, TPA, prourokinase, erythropoietin, hGH, EGF epidermal growth factor, angiogenesis factor, lipocortin, cyclosporin, glucoproteins, calcitonin gene-relaxed peptide, 1L-1, IL-2, 1L-3 multi-CSF, IL-4 B-cell GF, GM-CSF, M-CSF CSF-1, G-CSF, TNF-alpha, TNF-beta, Mullerian inhibitory substance, Muromonab-$CD_3$, MAb/immunotoxin, hepatitis B surface Ag, herpes II surface Ag, malaria Ag, HIV Ag, bGH, pGH, BoIFN-alpha, BoIL-2, HuIFN-alpha, Eg, Bo, Po fertility hormones FSH and, LH, pseudo-rabies Ag, recombinant factor VIII, fibronectin, insulin-like growth factor I, recombinant alpha-1-antitrypsin, asparaginase, adenosine deaminase, recombinant soluble human CD4, glucocerebrosidase, thymiopeptin TP5, and other peptides or protein whether of endogenons or recombinant origin or whether naturally occuring in man or animal or synthetic.

The current invention uses primarily the peptide interferon in particular interferon-alpha as illustration fo its utility, however, the use of all other peptides/proteinsand other molecules falling within the scope of the Definitions is contemplated under the scope of this invention.

Interferons Used

Recombinant Leukocyte A Interferon was obtained from Hoffmann-La Roche, Inc., Nutley, NJ, as a lyophilized powder for injection. It contains approximately 2 X $10^9$ units/ml when reconstituted (specific activity is

approximately equal to 2 x $10^8$ units/mg). $^{14}C$-Recombinant Leukocyte A Interferon (approximately 10 microcuries/300 micrograms) was also to be supplied by Hoffmann-La Roche, Inc.

Interferon Assay

A biological assay described in Can. J. Microbiol., 21:1247 (1975) was selected for measurements of peptide interferon for the following two reasons: (i) the level of sensitivity enabling detection of picograms of interferon protein exceeds that of conventional radio-immunoassay systems, and (ii) a bioassay able to distinguish between biologically active interferon molecules or inactivated interferon protein.

The above cited procedure was used with slight modifications. Briefly, growth medium was removed from 96-well microtiter plate cultures of human embryonic lung cells (HEL) that have reached confluency. To dilute samples containing interfon, Eagle's minimum essential medium with Earle's salts (MEM(E)) supplemented with 2% fetal bovine serum and antibiotics was placed in a sterile container and 110 ul was added to each well with a multitipped pipetter. Fifty ul of each peptide interferon sample or an appropriate peptide interferon standard containing 1000 international reference units were pipetted into the first well ($10^{-0.5}$ dilution) of an 8-well row with a micropipette. Each sample was titrated in duplicate or triplicate. With a multitipped pipetter, the samples were diluted directly over the cells in half log 10 increments by transferring 50 ul serially through the eight wells. Appropriate cell and virus controls were included. After incubation for 5 hours or more at $37^0C$, the peptide interferon samples were removed. Cultures were then rinsed with HEPES-buffered saline (HBS). Twenty-five ul of a suspension containing

approximately 50 plague-forming units of vesicular stomatitis virus in MEM(E) supplemented with 2% fetal bovine serum and antibiotics were added to each well (supplemented MEM(E) alone was added to cell control wells) and incubated for 1 hour at $37^0$C in a humidified 4% $CO_2$-enriched atmosphere. Unabsorbed virus was carefully removed and a methyl cellulose overlay medium supplemented with 2% fetal bovine serum and (50 ul) antibiotics pipetted into each well. After 16-24 hours of additional incubation at $37^0$C and 4% $CO_2$, plaques were counted microscopically or they were developed for visualization by staining. Staining was performed by removing the overlay medium and adding 50 ul of crystal violet solution per well. After 3 minutes, excess stain was removed by rinsing with HBS. The plaques were counted, and the end point was calculated as described infra.

In the plaque reduction assay, one unit of interferon was contained in the highest dilution of a sample that inhibits 50% of the challenge virus plaques. Dose-response relationships were constructed by linearly regressing prohibit values of the percent inhibition of plaque formation against log interferon concentrations. The 50% inhibitory ($I_{50}$) concentrations and the 95% confidence intervals was calculated from the regression lines when necessary, additional calculations were made to express the results in international reference units.

This assay would be applicable for all antiviral peptides/proteinsuseful in practicing this invention.

Methods Related to Interactions of Peptide with Phospholipid and Skin Lipid Monolayers and Bilayers

Liposomally encapsulated peptide is transferred from the phospholipid liposome into the skin. Peptide such as interferon encapsulated in liposomes, in particular in

liposomal DRVs, does not penetrate liposomal bilayers but associates with the bilayers' polar head groups. In this manner, the liposomes act as a donor and stratum corneum of the skin as a recipient.

Monolayer Studies

Polypeptide-lipid interactions method was used to determine interaction of peptide with monolayer. In this technique a lipid was spread on the surface of a buffer, the peptide was injected into the subphase, and the extent of the resulting interaction was determined by measuring the change in surface pressure. This techniques provided insight into protein interactions with artificial and natural lipid bilayers. The monolayer penetration studies were performed by a modification of the constant-area monolayer technique procedure in J. Pharm. Sci., 2:244 (1983).

The used lipid mixtures correspond to the various liposomal lipid compositions tested. Individual lipid components of these mixtures were tested. The experiments were performed at $25^0$C and $35^0$ using a Rosano Surface Tensiometer (Laboratory Products Inc., Boston, MA) equipped with a sandblasted platinum Wilhelmy plate to measure surface tension. The subphase consisted of 90.0 ml of 0.05M HEPES at pH 7.0 containing sodium chloride to adjust the ionic strength to 0.2. The pure lipid or lipid mixture was spread from a suitable solvent in amounts sufficient to produce the initial surface pressure. A stationary needle with a removable glass syringe was used to deliver varying amounts of IFN-alpha in 0.2 ml increments beneath the surface into the subphase. Surface tension reading were taken every 5 minutes until no further changes were observed (<0.05 dyne/cm) for 30 minutes. Surface pressure, was calculated as the difference in surface tension in the absence of the lipid film and that of the film-covered

surface. The change in surface pressure was calculated as the difference in surface pressure of the lipid film upon injection of protein into the subphase and that of the film in the absence of protein, i.e., at its initial surface pressure. Of particular interest was a comparing peptide's interactions with monolayers prepared from phospholipids and skin lipids.

Lipid Transfer Studies

In order to study the transfer of interferon from phospholipid liposomes (the drug delivery system) to liposomes prepared from stratum corneum lipids (the model membrane system), the terbium-dipicolinic acid assay described in Nature, 281:690 (1979) to study nonspecific transfer of aqueous compartment markers was used. The following four populations of liposomes were prepared: (i) phospholipid liposomes containing 2.5 mM $TbCl_3$, 50 mM DPA and 50 mM sodium citrate; (ii) skin lipid liposomes containing 50 mM DPA; (iii) phospholipid liposomes containing 2.5 mM $TbCl_3$, 50 mM DPA and 50 mM sodium citrate; and (iv) skin lipid liposomes containing 2.5 mM $TbCl_3$, 50 mM DPA and 50 mM sodium citrate. Vesicles were separated from nonencapsulated material by passage of the dispersion through a 1 x 45 cm column of Sephadex G-75. Mixtures (1:1 v/v) of liposome preparations (i) and (ii) were incubated at $35^0C$ and fluorescence measurements carried out at various time intervals. Maximum Tb fluorescence (that obtained upon complete mixing of the two liposome populations) was determined by measuring the fluorescence intensity of a quantity of liposome preparation (iii) or (iv) equal to that of the mixture of preparation (i) and (ii).

The specific transfer of peptide interferon was studied by incubation of phospholipid liposomes containing $^{14}C$-interferon with a population of skin lipid

liposomes. The two liposome populations were separated using neomycin reductively coupled to sepharose 2B as the stationary phase in column chromatography. The method quantitatively separates liposomal populations based on the affinity of the various negatively charged lipids for neomycin. For example, liposomes of EL containing as little as 10 mole% $PIP_2$ were quantitatively retained in 0.2 M NaCl while liposomes containing PS are recovered to 70-97%. The results of this study were compared with the terbium-dipicolinic acid study.

Effect of Phospholipid Liposomes on Stratum Corneum Lipids

Liposomally entrapped peptide interferon penetrates the full thickness of the skin and may alter the structural characteristics of the lipids associated with the stratum corneum. Association of the skin lipids with phospholipid liposomes results in slight alterations of stratum corneum lipid composition leading to the partial destruction of its bilayer orientation and its permeability barrier. Thermograms of skin lipid liposomes before and after incubation with phospholipid liposomes were compared. Additionally, stratum corneum obtained from guinea pigs and cadavers by the procedure described in Pharm. Res., 5:140 (1988) were tested by comparing their thermograms before and after incubation with phospholipid liposomes for various periods of time. Deviations from their characteristic thermograms, particularly peaks associated with lipid domains, are excellent indicators of changes in degree of lipid mixing, phase separation and phase transitions, e.g. bilayer to hexagonal phase.

A Perkin Elmer DSC.2C scanning calorimeter, upgraded with a data station and necessary software for data analysis, was used for these studies. The liposomal dispersions or rinsed stratum corneum was centrifuged and

wet pellets were placed in hermetically sealed sample pans. The reference pan contained an equal amount of buffer. All scans obtained from a heating rate of $5^0$C/min. and a range setting of 1 mcal/sec. Indium standard was used to calibrate the calorimeter. The thermograms obtained were analyzed for changes in phase transition temperature, phase separation behavior and enthalpies of the transition peaks.

Methods Related to Determination of Liposomally Entrapped Peptide's Diffusion Through Intact Skin Preparation of Membranes

Excised guinea pig skin in Franz diffusion cells according to method described in J. Infect. Dis., 153:64 (1986) was used to determine the skin penetration for the efficacy of topical antiviral peptides/proteinstogether with membranes prepared with hairless mouse skin, hairless guinea pig skin, and human cadaver skin. The skin membrane used contained the full epidermis, the dermis, and a thin membranous covering at the base of the dermis. Membranes such as these were trimmed to size and clamped between the diffusion cell chambers. Stratum corneum free membranes of all used skin membranes were prepared by tape stripping the skin and isolating the whole epidermis and the dermis for separate study by the gentle use of heat ($60^0$C for about 60 seconds). Such membrane preparations were useful in showing interferons' diffusive mobility in deeper skin strata.

Diffusion Cell Description

A diffusion cell system which allows application of material to the skin surface in facsimile to the way drugs are topically used was required for this phase of study. To avoid inherent deficiencies of this type of cells such as inefficient temperature control, large hydrodynamic diffusion layers and tendency for bubble

formation under the dermis, a flow-through finite dose diffusion cell manufactured by Crown Glass, Sommerville, NJ was used. The flow-through cell consists of a 1.0 ml receiver compartment having an inlet and an outlet to allow flow of solvent. In the operation of the flow-through cells, the receptor fluid was pumped from a temperature-controlled reservoir into and through the cell by a peristaltic pump (Rainin Rabbit, Rainin, Woburn, MA). After exiting the cell, the fluid enters a length of Teflon tubing and the drops which emerge from the end of each of the tubings are collected in test tubes situated in an automatic fraction collector (Isco, Lincoln, NE). The collector allows for simultaneous collection from a number of cells and replacement of test tubes with a fresh set at predetermined intervals. The distance traversed by the fluid in the outlet tubing is minimized so that the time of fraction collection correlates well with the time of skin absorption. The effective area for diffusion for the flow-through cells is about 0.8 $cm^2$. The flow-through cells are made of glass and are jacketed for temperature control and studies can be performed at various flow rates to ascertain the influence of flow rate on permeation.

The membrane was placed in its housing and the receiver compartment was filled with calcium-magnesium free phosphate buffered saline of pH 7.0, containing about 1.25 mg/ml albumin. Care was taken to ensure fluid contact over the entire skin undersurface so that no bubbles appeared. Temperature was maintained at $37^0C$. Liposomal systems that showed reasonable degrees of entrapment and stability as well as their respective controls (free interferon with and without empty liposomes) were intently spread as evenly as possible with a small Teflon spatula made for the purpose in the

donor compartment. Liposomal dispersions containing radiolabelled interferon were used to show the presence of radiolabel into and through the layers of the skin. Different concentrations of liposomes and different concentrations of the peptide interferon within the liposomes were tested. Each permeation profile was used to show the diffusional lag time (time to establish a measure flux) and also to indicate the rate of permeation and the amount permeated as a function of time. Side-by-side comparison of profiles thus readily revealed which of several applications delivered drug in a preferred fashion, more promptly and in greater amount in the early stages of permeation. With peptide interferon it is desirable that a burst of material passes into the living epidermal mass so it can immediately shore itself against viral replication. Those systems which show reasonable indication of uptake were assayed by the plaque reduction assay to determine if biologically active interferon molecules and not inactivated interferon protein were transported.

Measurement of Tissue Strata Concentration

The therapeutic efficacy of topical antiviral drugs depends upon how soon the drug reaches the basal cell layers and attains a concentration sufficient to inhibit virus replication. Thus, the viral replication is a sensitive measure of the drug efficacy. The stratum corneum, in addition to serving as a rate limiting barrier, also functions as a reservoir for drugs.

The skin stripping method and radiolabeled $^{125}I$-interferon was used for rapid range-finding. Skin was exposed to free and liposomally entrapped peptide interferon as described in the diffusion cell studies. At various times the skin was removed from the cell and wiped free of surface retained material with alcoholic swabs. Scotch tape was applied over the conditioned skin

area, pressed tightly to the skin, and then pulled away. The tape and adhering cells were digested with the aid of a tissue oxidizer and assayed by liquid scintillation counting. The amount of tissue harvested from a single tape stripping was determined by taring the tape and weighing again post stripping. Polyester tape was found to be more suitable and was used for this purpose since cellophane tape was found to be too hygroscopic to allow accurate weighing. Surface adsorbed peptide was accounted for by comparing the first tape stripping with subsequent strippings. 10-15 strippings are generally required to completely remove the stratum corneum. These were pooled in sets of three so that the drug levels at 5 successive depths were roughly estimated. Once the horny layer was removed, the underlying tissue was excised and further sectioned, using 30 seconds of $60^0$ heat to effect separation of the skin at the epidermal-dermal junction. Since the heating was of such short duration, the activity of peptide was not affected.

Systems showing reasonable strata concentrations were tested using the biological assay method. The tissue was homogenized, quick frozen on dry ice and thawed once, minced with scissors, and homogenized using a Tissumizer (Tekmar Co., Cincinnati, OH) in ice-cold HEPES-buffered saline (pH 7.4) containing 1.25 mg/ml albumin. The effects of tissue extraction (heating at $60^0$C for 30 seconds and homogenization) on the biological activity of interferon was determined by spiking duplicate samples of tissue with known quantities of interferon immediately after removal of the horny layer.

Test of Drug Delivery on Human Cadaver Skin

Liposomal vehicles promotion of drug delivery of peptides/proteinsinto human skin was determined as an intermediate step to clinical assessment of peptide

activity. Human cadaver skin was substituted for guinea pig skin in the above described experiment using the liposomal systems of delivery which appear most effective in the guinea pig skin work. One-inch wide strips of abdominal skin taken fresh from autopsy was treated with $60^0$C water for two minutes. This procedure frees the epidermis from underlying tissue. Epidermal membranes suitable for the finite dose cell were cut from these and studied as previously described.

Methods Related emonstration of the Intradermal Delivery of Liposomally Entrapped Interferon Into Virus-Infected Cells

Measurements of tissue strata concentration in live guinea pigs were done using the following method. Dorsal skins of living guinea pigs using Hill Top Chamber adhesive chambers were used for determination of interferon or other antiviral peptide levels. These chambers can be retained by unrestrained animals for a number of days. Sorptive pad inserts soaked with the vehicles of interest was kept in contact with the skin in this way for a period of similar duration to the in vitro permeation experiments. Upon removal of the chamber, interferon concentrations in the various strata of the skin were biologically assayed as previously described.

Determination of Antiviral Activity in a Herpes Guinea Pig Model

The cutaneous herpes guinea pig model was selected for use as the disease state most closely resembling that seen in human beings in clinical appearance and duration. The model represents a marked improvement over herpes virus infection models seen in other animals.

The severity of the infection expressed as a lesion score was used to determine the topical activity of liposomally encapsulated interferon as illustrated in Example 6. In addition, dermal toxicity measured by

erythema and indurations was measured and determination made whether it is induced by either free or entrapped interferon or by the liposomes themselves.

To quantitate the antiviral effect of interferon preparations, the scoring system of Alenius and Oberg Arch. Virol., 58:277 (1978) was used with photographs serving as standards for scores of 1,2,3, III, II, and I. After inoculation of the guinea pigs with HSV-1 as described above, the inoculated areas were scored daily for 9-11 days. All scoring was done blind. Time to healing was noted also for each test formulation. Statistical significance was determined using techniques of profile analysis, paired t-tests and analysis of variance.

In separate experiments virus titers in infected skin were measured to determine if the interferon preparations were inhibiting viral replication. Guinea pigs were sacrificed and the individual areas of inoculation sites excised. Skin samples were frozen and thawed once, minced with scissors, and homogenized (Tissumizer, Tekmar Co., Cincinnati, OH) in ice-cold HEPES-buffered saline at pH 7.4 (102) containing 100 Units of penicillin and 100 ug of streptomycin per ml. The suspension was centrifuged at 900 x g and the centrifugate stored at -76$^0$C for subsequent assay in BHK-21/4 cells according to the procedure Antimicrob. Agents Chemother., 9:120 (1976).

The topical activity of small peptide molecules and interferons was also tested on white human skin obtained from human cadavers. Skin was from subjects between 20 and 70 years of age was used without regard to gender. For health reasons, restrictions were placed on the cause of death. No diseased skin was accepted. Time of death, the age, the gender and the site from where the skin was removed was noted.

Utility

The compositions of this invention prepared by dehydration/rehydration method are extremely suitable and efficacious in intradermal delivery of peptide. Moreover, these compositions have high entrapment of the peptides/proteins and are very stable.

The invention concept that direct transfer of peptide/protein drug from liposomes to skin occurs only when the drug is associated with the bilayer has been tested. Several liposomal formulation consisting of negative MLVs or LUVs prepared by standard methods, negative DRVs prepared by dehydration/rehydration method and skin lipid liposomes DRVs prepared by dehydration/rehydration method all having encapsulated peptide interferon, were tested against the virus control using the cutaneous herpes guinea pig model described in Example 7. The formulations used are described in Examples 2 and 3.

The topical activity of aqueous formulation and water-in-oil formulations of interferon also tested, is shown in Figures 1 and 2. Neither aqueous or water-in-oil formulation have shown a difference in their topical activity when compared to the untreated virus control.

Similarly, the topical activity of the negative MLVs or LUVs interferon formulations prepared by standard method did not differ from that of the virus control as seen in Figures 3 and 4.

On the other hand, the interferon was transported through guinea pig skin when incorporated in negative DRVs and skin lipid DRVs prepared by dehydration/rehydration method and these formulations were able to reduce lesion scores, as illustrated in Figures 5,6 and 7. This indicates that the method of liposmal preparation is the most important factor in reducing lesions in the cutaneous guinea pig model. It

appears that the dehydration and subsequent rehydration of the liposomes facilitates partitioning of the peptide into the liposomal bilayer at a point where it is positioned for transfer into the lipid compartment of the stratum corneum. This finding is supported by the results shown in Figure 7, where the skin lipids (CE:CH:PA:CHS) DRV liposomes appear to be even more efficacious than the phosphalipid based on negative DRVs shown in Figures 5 and 6.

Figure 8 further support this finding because there the empty skin liposomes are used containing nonentrapped free peptide/protien interferon. As can be seen, there is no difference between topical activity of the empty skin liposomes and free interferon and the virus control.

DRV derived liposomes provide further advantages in that the entrapment of the peptide in the DRV liposomes prepared by standard methods as illustrated in Example 4 and Table 1. In all DRVs the entrapment is almost twice as high or more.

The DRV derived liposomes show also much better stability against MLVs. For up to 10 months, the total entrapment of the peptide in DRV liposomes is around 95%, representing essentially no change in entrapment from the 92% at one month.

These and other aspects of this invention become apparent from the following examples which intend to illustrate but not limit the current invention.

## Materials and Methods

Egg lecithin (EL), cholesterol (CH), cholesteryl sulfate (CHS), bovine brain ceramides (CM), palmitic acid (PA), and dimyristoylphosphatidylcholine (DMPC) were obtained from Sigma Chemical CO. (St. Louis, MO.). Phosphatidylserine (PS) was obtained from Avanti Polar Lipids (Birmingham, Ala.). Alpha-tocopherol was obtained

from Eastman Kodak Co. (Rochester, N.Y.). Lyophilized recombinant leukocyte A IFN in vials, each containing 18 x $10^6$ IU of IFN, 9 mg of sodium chloride, and 5 mg of human serum albumin, was supplied by Hoffmann-LaRoche Inc. (Nutley, J.J.). CH was recrystallized twice from ethanol. All other compounds were used as received. The S-148 strain of HSV type 1 (HSV-1) was provided by T.W. Schafer of Schering Corp., Bloomfield, N.J. Titration was performed by plaque reduction in BSC-1 cells as described previously.

Example 1

Nonliposome Interferon Formulations

An aqueous interferon solution containing 5.4 x $10^6$ IU of interferon alpha per ml, and interferon containing water-in-oil emulsion containing mineral oil buffered solution of arlacel 80, in ratio 6:3:1 with the same amount (5.4 x $10^6$ IU) of interferon were prepared and tested in the cutaneous guinea pig model and compared to an untreated virus control.

The results are summarized in Figures 1 and 2.

Example 2

Interferon Formulations

Three types of liposomes were prepared,tested and compared to the virus control.

In all cases, the of the formulation final volume was adjusted so that the concentration of total lipid was 100 umol/ml. The ratio of interferon (IFN) to human serum albumin was maintained at 4 x $10^6$ IU/mg and the final IFN-alpha concentration was 5.4 x $10^6$ IU/ml of suspension.

The effect of lipid composition was tested by preparing negative liposomes of EL-CH-PS and DMPC-CH-PS at molar ratios of 2:1:0.33. An antioxidant alpha-tocopherol [1%] was added to all liposomes containing

EL. Liposomes were also prepared from lipids with compositions similar to those found in the stratum corneum using lipids CM:CH:PA:CHS in molar ratio (4:2.5:2.5:1).

MLV. Multilamellar liposomes (MLV) were prepared by standard procedures such as herein described thin-film.

The lipid mixture containing EL:CH:PS in molar ratio 2:1:0.33 alpha-tocopherol in 1% amount was added, and the mixture was dissolved in chloroform and rotary evaporated under nitrogen. The flask containing the thin lipid film was then stored overnight under vacuum to facilitate removal of residual solvent. The lipid film was resuspended at a temperature above the phase transition temperature of the phospholipid in calcium- and magnesium-free phosphate-buffered saline (pH 7.0), containing IFN and human serum albumin, and the mixture was vortexed for 10 to 30 min. The free drug was not removed, since leakage from the aqueous liposomal compartment was minimized when the external thermodynamic activity of the drug approximated its thermodynamic activity in the aqueous compartments of the liposomes.

LUV. Large unilamellar vesicles (LUV) were prepared with an extrusion apparatus fitted with 100-nm-pore-size Nuclepore polycarbonate membrane filters. The method utilizes observations when MLVs are repeatedly extruded through 0.1 um pore diameter polycarbonate membranes under 250 lb of pressure per inch (2), their average diameter becomes progressively smaller, reaching a minimum of 100 nm after about 5 to 10 extrusions. As the MLV are forced through the pores, successive layers are peeled, until one intact bilayer remains. The MLVs prepared as above were submitted to repeated extrusion until LUVs were prepared.

Both MLVs and LUVs containing INF prepared by standard methods were tested against the virus controls. The results are shown in Figures 3 and 4.

Example 3

Dehydration/Rehydration liposomes

Dehydration/rehydration liposomes were prepared by the method of Kirby and Gregoriadis Liposome Technology, Vol.1, p.19-28 (1980) CRC Press.

Briefly, empty sonicated vesicles were mixed with an aliquot of IFN stock solution containing $5.4 \times 10^6$ IU/mml of INT-alpha. The mixture was dried under a stream of nitrogen. During dehydration, the small vesicles fused to form a multilamellar film that effectively sandwiched the INF solute molecules between successive bilayers. Upon rehydration, large vesicles which had encapsulated a significant proportion of the solute were produced. All three DRV liposome INF formulation and one formulation of empty DRV liposomes wee tested against virus control in cutaneous guinea pig model. The results are show in Figures 5-8.

Example 4

Interferon Entrapment

This example illustrates the degree of interferon entrapment in various liposomes.

Various types of liposomes were prepared according to Table 1, and the volume adjusted so that the final concentration of total lipid was 100 umole/ml, and the final IFN-alpha concentration was $1.8 \times 10^7$ I.U. per ml of suspension.

Two aliquots of 100 ul were removed and one aliquot was frozen for future assay and marked Total IFN after its volume was brought up to 1 ml with 1.25% HSA in HEPES. The second aliquot was placed in a Beckman centrifuge tube and centrifuged at 148,000 x g in a

Beckman Airfuge for 30 minutes. The supernantant was withdrawn and the pellet was washed 3 times with 100 ul HEPES buffer. The weight of the pellet was approximately 200 mg. 200 ul of 1.25% HSA in HEPES were then added and the volume was brought up to 1 ml with HEPES and the samples were frozen for future assay (Entrapped IFN). The pellet was dissolved in 500 ul of 0.4% sodium deoxycholate in HEPES (previously shown not to interfere with interferon assay), and the volume was brought to 1 ml with HSA solution. The samples were then frozen for future assay and designated Free IFN. Mass balance (Total IFN=Entrapped IFN + Free IFN) was always obtained within the sensitivity of the interferon assay. The percent of interferon entrapped for the various types of liposomes tested is shown in the entrapment of interferon in positive, negative and neutral vesicles are compared to entrapment in DRVs. The results are show in Table 1.

Table 1

| | Percent of IFN Entrapment | |
|---|---|---|
| Lipid Composition | MLV | DRV |
| EL:CH:PS | 10 | 19 |
| EL:CH:SA | 6 | 11 |
| EL:CH | 3 | 19 |
| DMPC:CH:PS | 12 | 26 |
| DMPC | 2 | 3 |

## Example 5

## Liposomal Interferon

This example illustrates the stability of liposome interferon formulations over the 12 month storage period.

The particle size distribution of the MLVs and DRVs (Table 2) showed no apparent changes over the 12 month storage period as determined using light microscopy.

Electron micrographs of supernatant samples at 0-12 month storage periods gave no evidence of significant amounts of smaller vesicles (<5% on a volume basis).

In order to evaluate the stability of both free and entrapped interferon when incorporated into liposomal formulations and to determine the leakage of entrapped interferon from the liposomes, two ml of each of the liposomal suspensions tested were stored in a refrigerator at $2^0$ -$4^0$C. At various times, two aliquots of 100 ul were removed and processed and assayed as described in the previous sections. The percent of free and entrapped interferon remaining (IFN%=100 at time zero) for the various types of liposomes tested is at various time periods is shown in the following table.

Table 2

| Time (Months) | DMPC:CH (MLV) %Entr. | % Free | EL:CH:SA(DRV) %Total | EL:CH:PS(DRV) %Entr. | %Free |
|---|---|---|---|---|---|
| 1 | 90 | 75 | | 92 | 90 |
| 2 | 105 | 102 | 90 | | |
| 3 | 88 | 75 | | 102 | 99 |
| 4 | 95 | 77 | 102 | | |
| 5 | | | | 102 | 98 |
| 6 | | | 72 | | |
| 7 | | | | 92 | 91 |
| 8 | | | 83 | | |
| 9 | | | | 95 | 115 |
| 10 | | | 83 | | |

## Example 6

### Biological Activity of Liposome Interferon Formulations

This example illustrates the biological activity of liposome interferon formulations.

The Franz diffusion cell was used for these experiments. The cell had an effective area for

diffusion of 0.785 $cm^2$, and the receiver compartment volumes ranged from 4.6 to 5.0 ml. The receiver compartment was filled with solvent, and the membrane of the hairless guinea pig intact skin was placed over the upper opening of the receiver, in contract with the liquid. A rubber o-ring was placed around the outer edge of the membrane and the upper cell cap was clamped into place. Small magnetic stirrers at the bottom of the receiver compartment stirred the contents. 0.3 ml of each formulation according to Table 3 was placed in one of 9 donor compartments.

Skin from the same hairless guinea pig was used in all 9 diffusion cells of a given experiment. The receiver compartment contained 1.25% HSA in HEPES buffer. For sampling, 1 ml was withdrawn from receiver compartment and replaced with 1 ml 1.25% HSA in HEPES buffer. For each of the formulations tested, cells 1-3 contained the liposome formulation (IFN= $1.8 \times 10^6$ U/ml); cells 4-6 contained the aqueous IFN control ($1.8 \times 10^6$ IU/ml).

The following chart summarizes the total amount of biologically active interferon transported at 48 hours. In all cases no interferon could be detected in the receiver compartment with any of the control formulations tested.

Table 3

| Formulation | Percent Recovered |
|---|---|
| EL:CH:PS MLVs | 0 |
| 5% Azone Dispersion | 0 |
| 25% DMSO Dispersion | 0 |
| EL:CH:PS:LUVs | 0 |
| EL:CH:PS DRVs | 1.7 (S.D. = 1.06) |

Example 7

Topical Activity of Liposomally Encapsulated Interferon

This example illustrates topical activity of liposome encapsulated interferon against HSV-I in the cutaneous guinea pig model.

The cutaneous herpes guinea pig model was used to test whether intradermally delivered liposomally entrapped interferon will enter virus-infected cells. The severity of the infection as measured by cumulative lesion scores during infection and the time to healing was used to determine the antiviral effects of free and liposomally encapsulated interferon.

Adult female hairless guinea pigs (Crl:IAF(HA)BR), supplied by Charles River Laboratories, Inc., Wilmington, MS, weighing 300-400 g were used. The backs of the animals were divided into six squares with a marking pen. In the center of each area 25 ul of strain 148 herpes simplex virus type 1 (HSV-1) at a titer of $3.2 \times 10^6$ PFU/ml was applied. The virus was inoculated under anesthesia with a spring-loaded vaccination instrument (Sterneedle Gun, Panray Division, Ormont Drug Co., Englewood, NJ) which was released 10 times producing inoculations 0.75 mm deep on each skin area. The procedure was essentially according to Am. N.Y. Acad. Sci., 284:624 (1977).

The three areas on the left or right sides of each animal was treated topically one to three times per day for five days beginning 24 hours after inoculation with varying concentrations and amounts of free and encapsulated interferon. The results of the studies on the effects of the amount of interferon within the liposome and the effects of the total number of liposomes in vitro transport of interferon was used to determine how these parameters influence desired levels of drug in the receiver compartment, and more importantly, in the

skin. The contralateral sites received either no treatment or vehicle alone and served as control site(s). Any dermal toxicity due to the test preparations were noted and scored as none (0), very slight (±), slight (+), moderate (++), or severe (+++).

To quantitate the antiviral effect of interferon preparations, the scoring system of Alenius and Oberg was used. All of the cutaneous guinea pig model experiments were performed using hairless guinea pigs. A wide variety of formulations depicted in Figures 1-8 were evaluated for therapeutic efficacy against experimental dorsal cutaneous HSV-I infection. Lesion score vs. time plots are shown for all the formulations tested.

The effects of liposomal charge, composition and method of preparation were tested by using negatively charged DRVs, LUVs or MLVs EL:CH:PS in molar ratio 2:1:0.33, or DMPC:CH:PS in molar ratio 2:1:0.33, and dehydration/rehydration skin liposomes CE:CH:PA:CHS in molar ratio 4:2.5:2.5:1.

In all cases, the final volume was adjusted so that the concentration of total lipid 100 umole/ml. The effect of liposomal type was tested by using MLVs, DRVs and LUVs. With the exception of the set of experiments performed in the absence of human serum albumin (HSA), the ratio of interferon to HSA was maintained as $1 \times 10^6$ IU:0.25 mg. An aqueous interferon solution, an interferon-containing water-in-oil emulsion (mineral oil: buffered solution:Arlacel 80: 6:3:1) and a commercial topical antiviral product (Acyclovir) were tested using the same model. The results are shown in Figures 1-8.

In order to gain a greater degree of sensitivity and reduce intersubject variability, the "side-by-side" technique, where each contralateral site served as a control site was used for the remainder of the experiments in the phase of the study. Plots of lesion

score differences wherein a value of zero indicates no difference from that of the control vs. time more clearly show the efficacy, or lack thereof, of various interferon-containing formulations.

## Example 8

## Liposome Interferon Dermal Irritation

This example illustrates that the dermal irritation of liposome encapsulated interferon.

Representative liposomal formulations were tested for dermal toxicity. Only the liposomal formulations containing stearylamine (positively charged liposomes) caused a slight redness upon twice daily application for three days. The neutral and negatively charged liposomes whether MlVs, LUVs or DRVs demonstrated no reddening or produced a very slight redness. The degree of irritation was independent of the liposomal type (MLV, LUV or DRV) and was related to lipid composition. Stearylamine and other positively charged substances, i.e., quaternary ammonium compounds, have been previously reported to be skin irritants, and therefore the findings of their irritability was not surprising.

WHAT IS CLAIMED:

1. A topical composition comprising a liposomally encapsulated peptide or protein normally very little penetrating or nonpenetrating through a skin.

2. The topical composition of Claim 1 wherein the liposomes are prepared by dehydration/rehydration method.

3. The topical composition of Claim 2 wherein the liposomes allow the normally very little penetrating or nonpentrating peptide or protein to penetrate intradermally through the stratum corneum to the underlining target tissue.

4. The composition of Claim 3 wherein the liposomes are negatively charged.

5. The composition of Claim 4 wherein the peptide has molecular weight between 900 and 50,000.

6. The composition of Claim 5 wherein the peptide is selected from the group consisting of interferons, hormones, enzymes and immunostimulators.

7. The composition of Claim 6 wherein the peptide is interferon.

8. The composition of Claim 6 wherein the peptide is hormone.

9. The composition of Claim 6, wherein the peptide is immunostimulator.

10. The composition of Claim 6 wherein the liposome lipids are selected from the group consisting of egg lecitin, cholesterol, phosphatidylserine, dimyristoyl, phosphatidycholine, ceramide, palmitic acid and cholesteryl sulfate.

11. The composition of Claim 10 wherein the liposomes comprised of egg lecitin, cholesterol and phosphatidylserine and optionally alpha-tocopherol.

12. The composition of Claim 11 wherein the molar ratio of egg lecitin to cholesterol to phosphatidylserine is 2:1:0.33.

13. The composition of Claim 10 wherein the liposomes comprise dimyristoylphosphatidylcholine, cholesterol and phosphatidylserine.

14. The composition of Claim 13 wherein the molar ratio of dimyristoylphosphatidylcholine to cholesterol to phosphatidyserine is 2:1:0.33.

15. The composition of Claim 10 wherein the liposomes comprise ceramide, cholesterol, palmitic acid and cholesteryl sulfate.

16. The composition of Claim 15 wherein the molar ratio of ceramide to cholesterol to palmitic acid to cholesteryl sulfate is 4:2.5:2.5:1.

17. The composition of Claim 12 wherein the peptide is interferon.

18. The composition of Claim 14 wherein the peptide is interferon.

19. The composition of Claim 16 where the peptide is interferon.

20. A method of treatment of viral diseases comprising a therapeutically effective amount of a antiviral peptide or protein encapsulated in liposomes prepared by dehydration/rehydration method administered topically to a subject in need of such treatment.

21. The method of Claim 18 wherein the antiviral compound is interferon.

22. A process for preparing a topical composition of peptide encapsulated in liposomes comprising steps

(a) preparing empty sonicated liposomes;

(b) mixing said liposomes with a peptide or protein solution;

(c) encapsulating said peptide or protein into the liposomes;

(d) dehydrating said liposomes; and

(e) rehydrating said liposomes.

FIG. 1

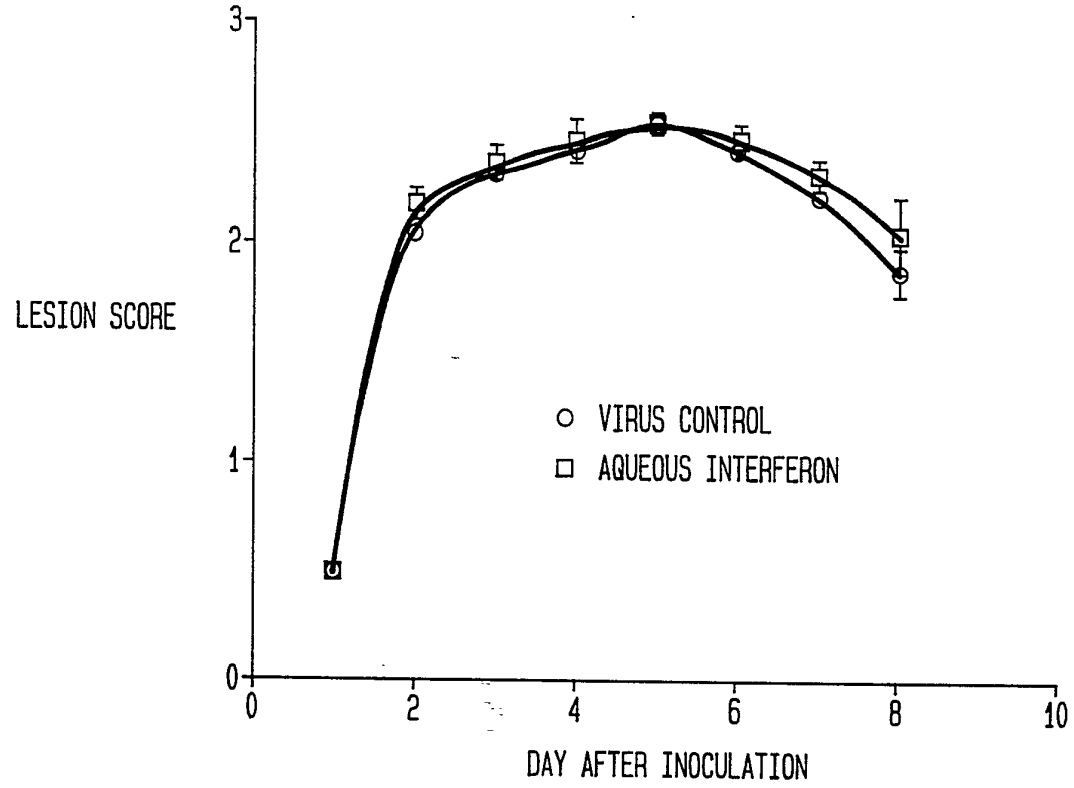

LESION SCORE
3
2
1
0
0
2
4
6
8
10
VIRUS CONTROL
AQUEOUS INTERFERON
DAY AFTER INOCULATION

FIG. 2

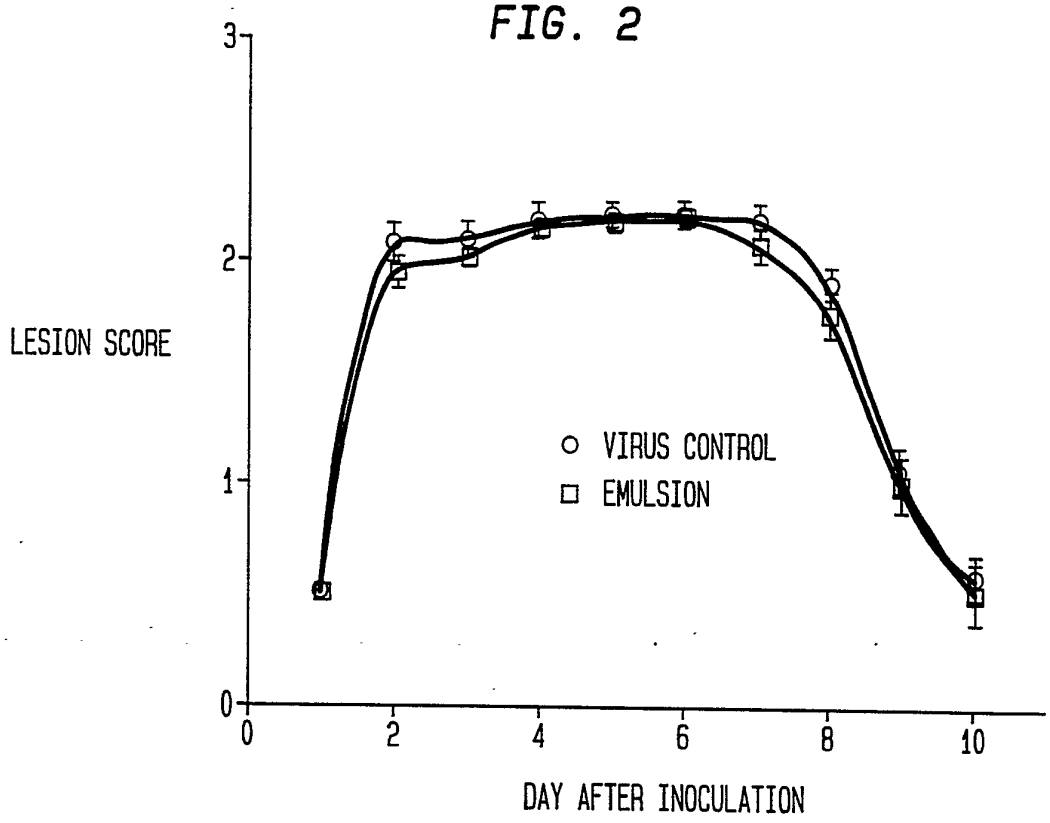

LESION SCORE
3
2
1
0
0
2
4
6
8
10
VIRUS CONTROL
EMULSION
DAY AFTER INOCULATION

FIG. 3

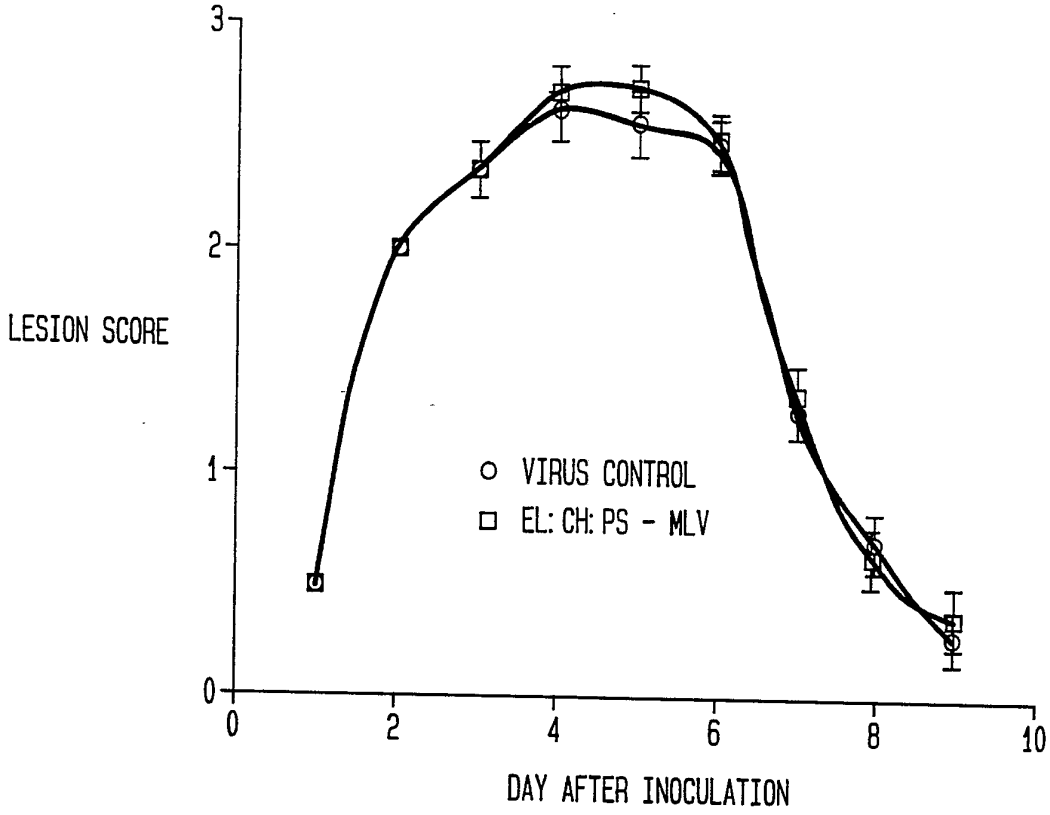

LESION SCORE
3
2
1
0
0
2
4
6
8
10
VIRUS CONTROL
EL: CH: PS - MLV
DAY AFTER INOCULATION

FIG. 4

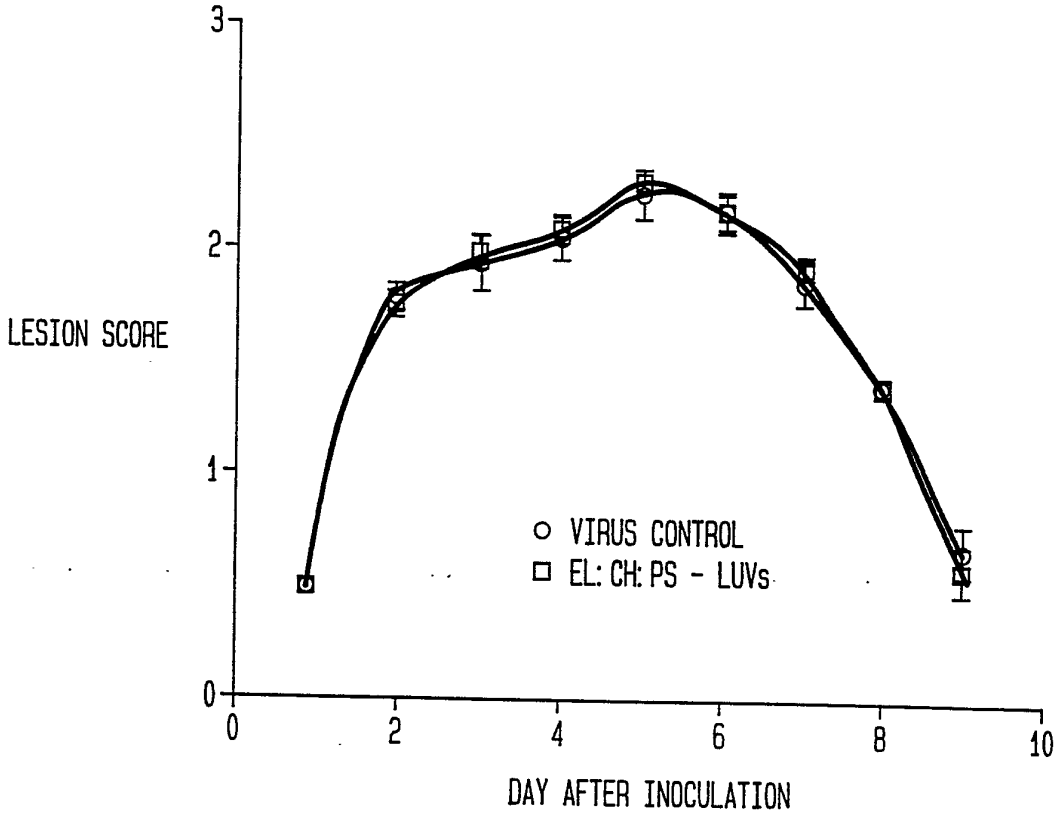

LESION SCORE
3
2
1
0
0
2
4
6
8
10
VIRUS CONTROL
EL: CH: PS - LUVs
DAY AFTER INOCULATION

FIG. 5

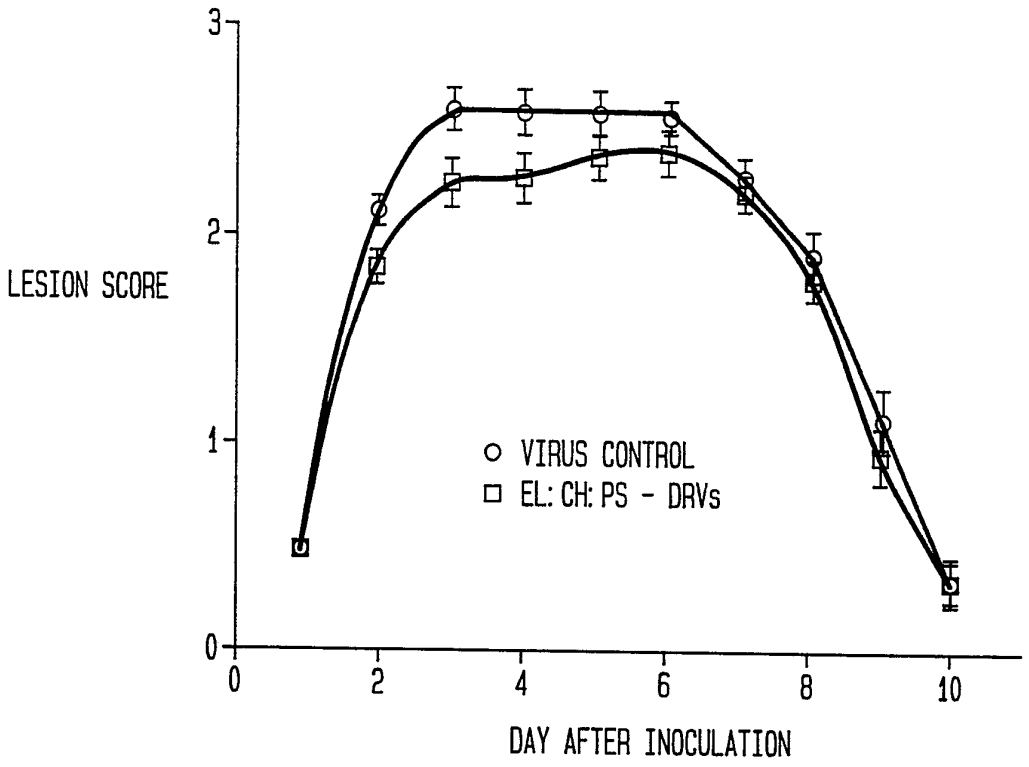

LESION SCORE
3
2
1
0
0
2
4
6
8
10
VIRUS CONTROL
EL: CH: PS - DRVs
DAY AFTER INOCULATION

FIG. 6

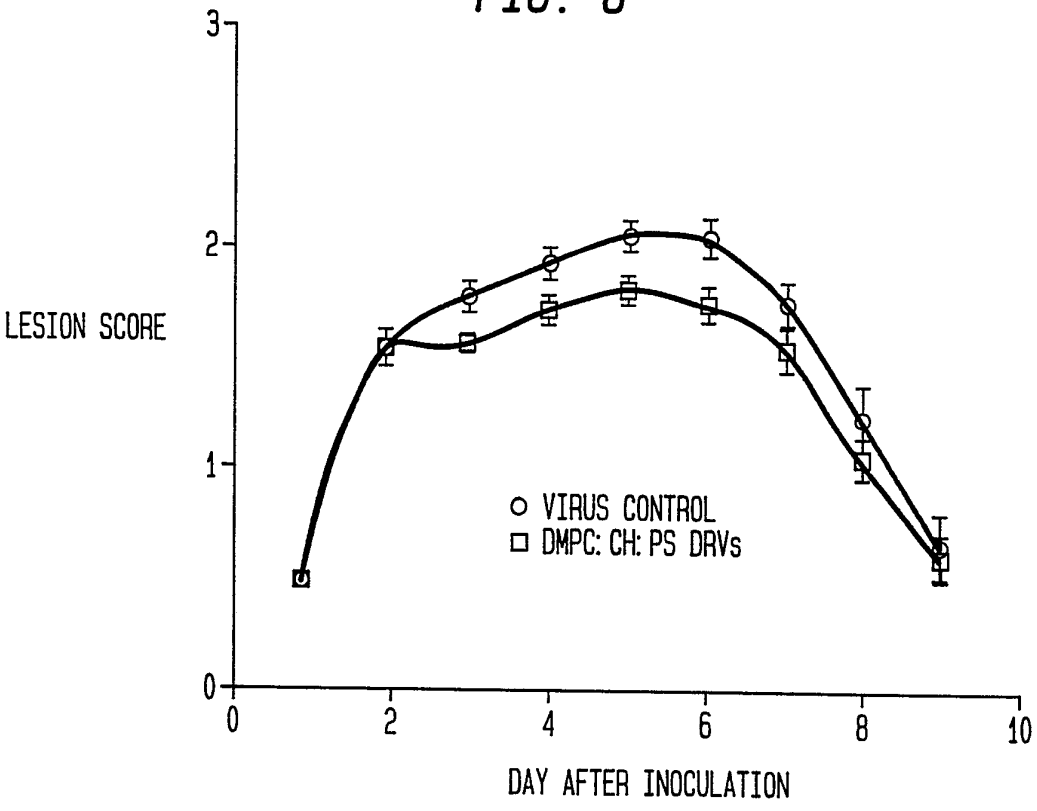

LESION SCORE
3
2
1
0
0
2
4
6
8
10
VIRUS CONTROL
DMPC: CH: PS DRVs
DAY AFTER INOCULATION

FIG. 7

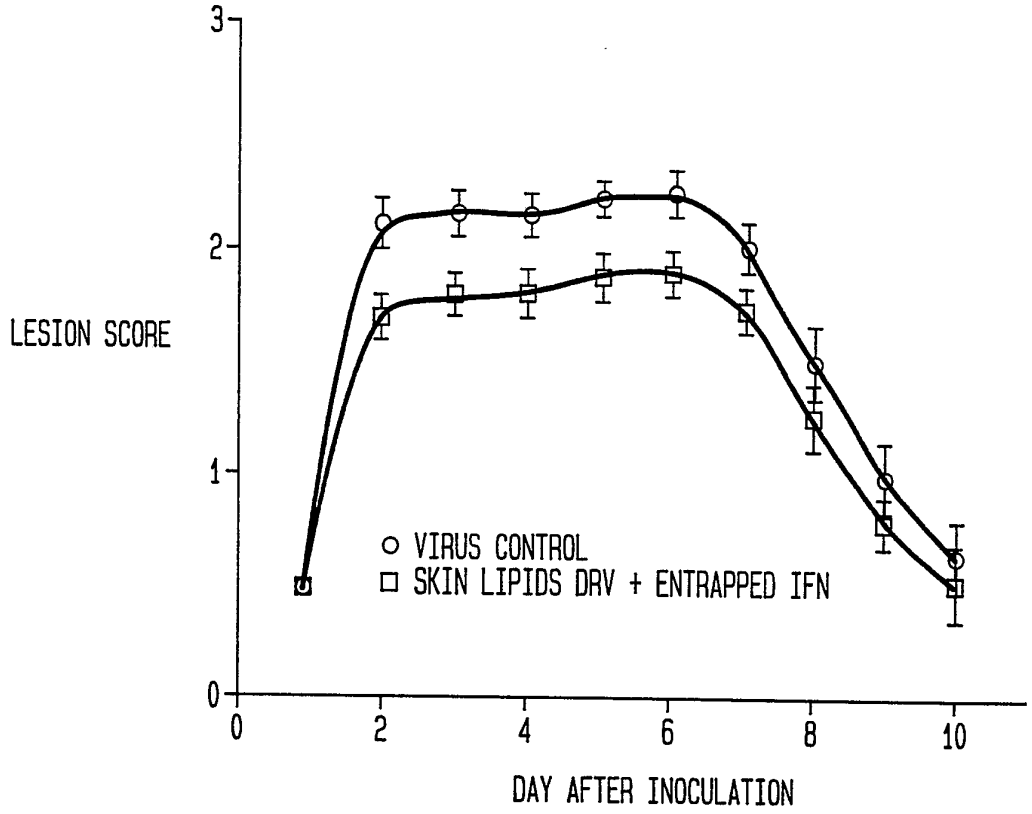
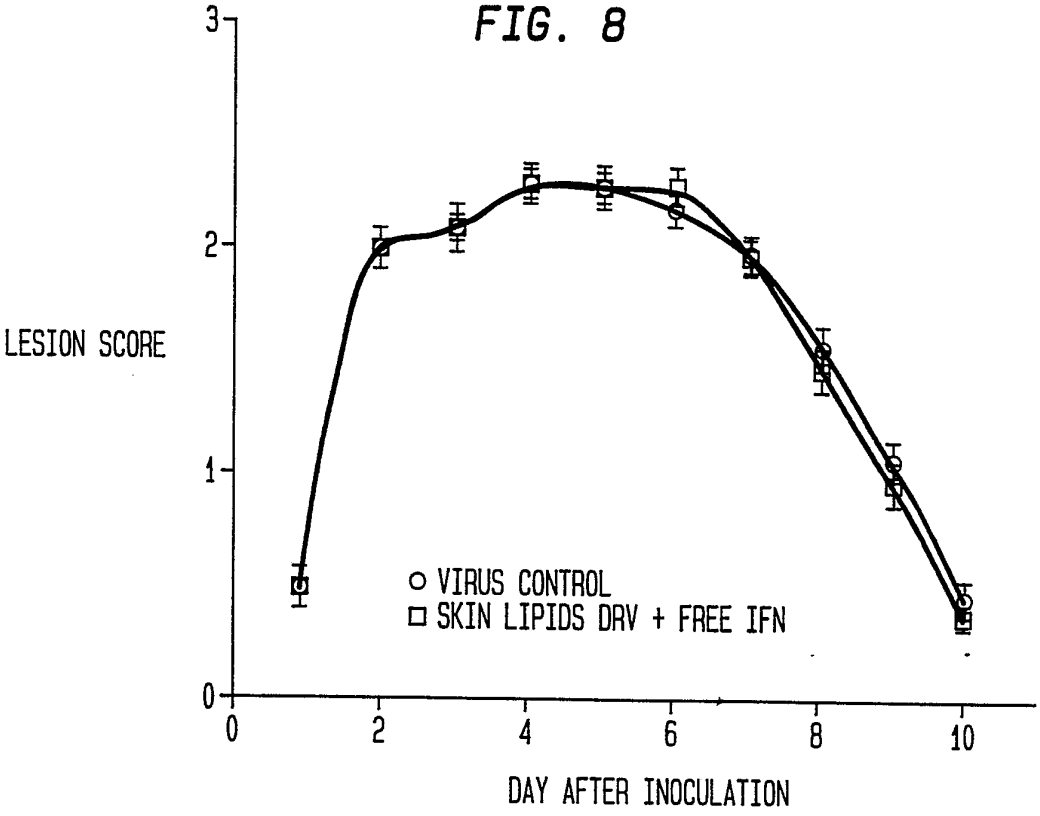

LESION SCORE
3
2
1
0
0
2
4
6
8
10
DAY AFTER INOCULATION
VIRUS CONTROL
SKIN LIPIDS DRV + ENTRAPPED IFN

FIG. 8


LESION SCORE
3
2
1
0
0
2
4
6
8
10
DAY AFTER INOCULATION
VIRUS CONTROL
SKIN LIPIDS DRV + FREE IFN

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/04316

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: A 61 K 9/127, 37/66

**II. FIELDS SEARCHED**

Minimum Documentation Searched [7]

| Classification System | Classification Symbols |
|---|---|
| IPC[5] | A 61 K |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

**III. DOCUMENTS CONSIDERED TO BE RELEVANT** [9]

| Category * | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No. [13] |
|---|---|---|
| P,X | Chemical Abstracts, volume 111, no. 12, 18 September 1989, (Columbus, Ohio, US), N. Weiner et al.: "Topical delivery of liposomally encapsulated interferon evaluated in a cutaneous herpes guinea pig model", see page 349, abstract 102643q & Antimicrob. Agents Chemother. 1989, 33(8), 1217-21 (Eng). -- | 1-22 |
| P,X | Chemical Abstracts, volume 112, no. 12, 19 March 1990, (Columbus, Ohio, US), K. Egbaria et al.: "Topical delivery of liposomally encapsulated interferon evaluated by in vitro diffusion studies", see page 382, abstract 104683x & Antimicrob. Agents Chemother. 1990, 34(1),107-10 (Eng). -- | 1-22 |

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* Special categories of cited documents: [10]

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**IV. CERTIFICATION**

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 16th October 1990 | 30. 10. 90 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | R.J. Eernisse |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
|---|---|---|
| Y | EP, A, 0172007 (SYNTEX (U.S.A.)) 19 February 1986 see page 5, line 23 - page 6, line 5; page 43, lines 11-22; claims | 1-7,10,11,13, 20-22 |
| Y | Chem. Pharm. Bull., volume 32, no. 6, June 1984, T. Ohsawa et al.: "A novel method for preparing liposome with a high capacity to encapsulate proteinous drugs : freeze-drying method", pages 2442-2445 see the whole article | 1-3,5-7,20-22 |
| Y | US, A, 4515736 (DEAMER) 7 May 1985 see the whole document, in particular column 4, lines 34-42 | 4,10,11,13 |
| A | WO, A, 89/05151 (THE LIPOSOME COMP.) 15th June 1989 see page 2, line 19 - page 3, line 36; page 11, line 31 - page 12, line 9; claims 1-8 | 8,9 |
| X | DE, A, 3301951 (A. NATTERMANN & CIE) 26 July 1984 see claims 1-8,11-14; page 11, lines 8-24; page 12, lines 5-14; page 13, example 4 | 1-5,10,11,22 |

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9004316 SA 39242

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 26/10/90
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| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| EP-A- 0172007 | 19-02-86 | CA-A- 1264669 | 23-01-90 |
| US-A- 4515736 | 07-05-85 | None | |
| WO-A- 8905151 | 15-06-89 | EP-A- 0389570 | 03-10-90 |
| DE-A- 3301951 | 26-07-84 | None | |