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Topical Delivery of Liposomally Encapsulated Interferon Evaluated by In Vitro Diffusion Studies

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The topical delivery of several liposomal interferon formulations was evaluated by in vitro diffusion experiments in an effort to understand the effects of liposomal composition and method of preparation on the deposition of interferon into the stratum corneum and deeper strata of the skin. Application of liposomes prepared from lipids with a composition similar to that of the stratum corneum resulted in almost twice the amount of interferon being deposited in the deeper skin layers than did application of liposomes prepared from phospholipids. Topical application of "skin lipid" liposomes prepared by the dehydration-rehydration method was twice as effective as was topical application of liposomes prepared by the reverse-phase evaporation method with respect to their ability to deposit interferon into the skin strata where the basal cell layers reside. These results are consistent with the effects of liposomal composition and method of preparation on the ability of the formulation to reduce lesion scores in the cutaneous herpes simplex virus type 1 guinea pig model.

Genital herpes is currently an epidemic sexually transmitted disease. The successful treatment of cutaneous virus infections with interferon (IFN) depends on the ability to effectively deliver IFN to the infected cells. So far it has proven impossible to obtain adequate tissue levels to control herpes and other skin viruses by conventional avenues of drug administration. 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 most limiting factor to the effective treatment of herpes.

We recently reported that topical application of liposomally entrapped IFN caused a reduction of lesion scores in the cutaneous herpes simplex virus type 1 guinea pig model, whereas application of IFN formulated as a solution or as an emulsion was ineffective (9). For phospholipid-based liposomes, the method of preparation rather than the lipid composition of the bilayers appeared to be the most important factor for reducing lesion scores. We also showed that greater efficacy was observed when IFN was entrapped in liposomes prepared from lipids with a composition similar to that of the stratum corneum rather than from phospholipids. The present study describes the results of in vitro diffusion experiments in which several IFN formulations were tested in an effort to understand the effects of liposomal composition and of preparation method on the deposition of IFN into the various strata of the skin.

MATERIALS AND METHODS

Materials. Egg lecithin (EL), cholesterol (CH), cholesteryl sulfate (CS), bovine brain ceramides (CM), palmitic acid (PA), and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-free acid were obtained from Sigma Chemical Co. (St. Louis, Mo.). Phosphatidylserine (PS) was obtained from Avanti Polar Lipids (Birmingham, Ala.). α-Tocopherol was obtained from Eastman Kodak Co. (Rochester, N.Y.). Lyophilized recombinant leukocyte A IFN in vials, each containing 18×10^6 IU of IFN, 9 mg of sodium chloride, and 5 mg of human serum albumin, was supplied by Hoffmann-La Roche Inc. (Nutley, N.J.). ^{125}I-labeled lyophilized recombinant leukocyte A IFN, [^{14}C]EL, [^{14}C]PA, [^{3}H]CH, and [^{14}C]inulin were obtained from New England Nuclear Corp. (Boston, Mass.). CH was recrystallized twice from ethanol. All other compounds were used as received. All solvents used were of chromatographic (high-pressure liquid chromatography) grade, and the water used was double-distilled, deionized, and filtered with a Milli-Q system (Millipore Corp., Bedford, Mass.).

Formulations. Two types of liposomes were tested. In all cases, the final volume was adjusted so that the concentration of total lipid was ≈15 mg/ml. One percent α-tocopherol (an antioxidant) was added to the lipid phase of all liposomes. The ratio of IFN to human serum albumin was maintained at 4×10^6 IU/mg, and the final alpha IFN concentration was 5.4×10^6 IU/ml of suspension. The effect of lipid composition was tested by preparing liposomes containing EL-CH-PS at a molar ratio of 1:1:0.5, CM-CH-PA-CS at a molar ratio of 4:2.5:2.5:1, and CM-CH-CS at a molar ratio of 4.2:2.8:3. An aqueous IFN solution was also tested. For experiments testing the incorporation of liposomal bilayer lipids into the various skin strata, trace quantities of [^{14}C]EL and [^{3}H]CH were included in the EL-CH-PS formulation and trace quantities of [^{14}C]PA and [^{3}H]CH were included in the CM-CH-PA-CS formulation. For experiments testing the incorporation of an aqueous phase marker, [^{14}C]inulin was entrapped in the aqueous compartment of EL-CH-PS liposomes and free inulin was removed from the dispersion by centrifugation.

LUV. Large unilamellar vesicles (LUV) were prepared by a modification of the reverse-phase evaporation procedure of Szoka and Papahadjopoulos (8). Approximately 50 mg of total lipid, contained in a flask, was dissolved in 10 ml of a chloroform-methanol mixture (2:1 [vol/vol]). Five milliliters of 0.05 M HEPES buffer (pH 7.4) and enough additional methanol (up to 1.5 ml) were added to yield a clear solution after brief sonication. The organic solvents and a small amount of water were removed under vacuum at a temperature above the phase-transition temperature of the lipids by using a rotoevaporator. Solvent removal was continued until all foaming ceased. An appropriate sample of IFN stock solution containing trace amounts of radiolabeled IFN was

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added, and the mixture was hydrated at a temperature above the phase-transition temperature of the lipids for about 1 h. The resulting liposomal suspension was stored at 20°C overnight before use in the diffusion experiments.

DRV. Dehydration-rehydration liposomes (DRV) were prepared by a modification of the method of Kirby and Gregoriadis (5). Briefly, appropriate amounts of the various lipids, contained in flasks, were dissolved in chloroform-methanol (2:1 [vol/vol]). The solvents were removed by using a rotoevaporator under vacuum at a temperature above the phase-transition temperature of the lipids. The resulting film was dried overnight in a desiccator to remove any residual solvent. An appropriate sample of IFN stock solution in 0.05 M HEPES buffer (pH 7.4) containing trace amounts of radiolabeled IFN was added, and the mixture was hydrated at a temperature above the phase-transition temperature of the lipids for about 1 h. Intermittent vortexing was required for complete hydration. The resulting dispersion was dehydrated at 50°C under vacuum by using the rotoevaporator. When the liposomal suspension became very viscous, an amount of water equivalent to that removed was introduced into the viscous suspension. The liposomes were allowed to rehydrate for about 45 min at a temperature above the phase-transition temperature of the lipids. The dispersion was stored at 20°C overnight before use in the diffusion experiments.

Diffusion experiments. Previously frozen whole excised skin from hairless-guinea-pig abdomen was allowed to thaw gradually to room temperature. Any subcutaneous fat was removed carefully with a scalpel, and several pieces were cut from each specimen. Each piece of skin was mounted on a Franz diffusion cell (Crown Glass, Somerville, N.J.). The epidermal side of the skin was exposed to ambient conditions, while the dermal side was bathed with 0.05 M HEPES (pH 7.4) buffer solution containing 0.9% sodium chloride and 0.1% bovine serum albumin. The receiver solution was stirred continuously with a small Teflon-covered magnet. Care was exercised to remove any air bubbles between the underside of the skin and the solution in the receiver compartment. The temperature of the receiver compartment was maintained at 37°C.

Following mounting of the section of skin, 200 μl of formulation was added to the epidermal surface. A smaller amount of formulation was found to be insufficient to ensure uniform spreading across the entire exposed surface of the skin in the cell. A minimum of two cells were used for each IFN system, and duplicate experiments were carried out with sections of skin from different skin specimens for each formulation. All experiments were carried out with nonoccluded donor compartments. After 24 h, the experiments were stopped and the diffusion set-up was dismantled.

Assay of radiolabeled markers. After dismantling was completed, the donor compartment of the cell was rinsed carefully five times with 0.5 ml of buffer solution. The skin was removed and washed with 0.5 ml of buffer solution. Approximately five such washings were found to be sufficient to remove >99% of the formulation when determined at time zero. All washings were collected and assayed for radiolabel. Following the rinsing procedure, the skin patch was mounted on a board and a piece of adhesive tape (Scotch Magic Tape 810; 3M Commercial Office Supply Division, St. Paul, Minn.), 1.9 cm wide and about 6 cm long, was pressed firmly to the skin surface with a spatula. The tape was of sufficient size to cover the area of skin that was in contact with the formulation. Twelve such strippings were carried out, and each strip was analyzed separately for radiolabeled IFN, inulin, or lipid. Additionally, the remaining skin (after stripping of the stratum corneum) and the receiver compartment solution were also assayed for IFN, inulin, or lipid.

For the lipid or inulin analyses, each sample was placed in a Combustio-cone and burned in a tissue oxidizer (model 306 oxidizer; Packard Instrument Co., Inc., Downers Grove, Ill.). The separated radionucleotides were assayed with a scintillation counter. For the IFN analysis of the donor compartment washings, skin washings, and receiver compartment solution, enough trichloroacetic acid was added to samples so that the concentration of the protein precipitant was ≈5% (wt/vol). After overnight equilibration, the mixture was centrifuged in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.) for 15 min and the supernatants and precipitates were separately assayed with a 5000 series Gamma Minaxi γ-counter (Packard). For the IFN analysis of the skin samples (tape strippings and remaining skin), a 10% solution of trichloroacetic acid in HEPES buffer was added to the sample contained in a flask and the contents were thoroughly mixed on a Vortex mixer (The Vortex Manufacturing Co., Cleveland, Ohio) and further incubated for 24 h to allow intimate contact between the protein precipitant and the IFN residing in the skin tissue. The mixture was centrifuged, and the supernatant and precipitate were assayed. For each of the above-described procedures, the supernatant (representing the amount of free iodine label) accounted for approximately 15% of the radioactivity.

RESULTS

Table 1 shows the degree of deposition of bilayer lipids and entrapped aqueous marker (inulin) into the various strata of the skin 24 h after in vitro topical application of phospholipid-based and skin lipid liposomal formulations to full-thickness skin. The amount of lipids adhering to the stratum corneum surface was determined by analysis of the first two tape strippings. The amount of lipids penetrating the deeper stratum corneum was determined by analysis of the remaining tape strippings (3 through 12). The amount of lipids penetrating the deeper skin strata was determined by analysis of the remainder of the full-thickness skin. Note the excellent correlation of dual label for both liposomal systems tested. A mass balance of >96% was achieved after the donor compartment and skin rinses were accounted for. No lipid could be detected in the receiver compartment for any of the formulations tested. Table 1 also shows the ability of the liposomes to incorporate an entrapped aqueous-phase marker into the various skin strata. For both formulations, no inulin could be found in the receiver compartment.

Table 2 shows the degree of deposition of IFN into the various strata of the skin 24 h after in vitro topical application of the various formulations to full-thickness skin. The working definitions of stratum corneum surface, deeper stratum corneum, and deeper skin strata are the same as previously stated. A mass balance of >90% was achieved after the donor compartment and skin rinses were accounted for, and no IFN could be detected in the receiver compartment for any of the formulations tested.

DISCUSSION

Unlike virtually all other biologic membranes, the stratum corneum does not contain phospholipids. It is made primarily of CM (40%), CH (25%), fatty acids (primarily PA [25%]), and CS (10%). Lipid compositions similar to these have been

TABLE 1. Distribution of inulin and liposomal bilayer lipids in various strata of the skin 24 h after in vitro topical application of formulations to full-thickness skin

Formulation	Component	Distribution[a] on or in:			
		Stratum corneum surface	Deeper stratum corneum	Deeper skin strata	Combined donor cell and skin washings
EL-CH-PS liposomes	EL	55.0 ± 10.1	19.8 ± 9.2	2.3 ± 0.4	20.1 ± 4.2
	CH	50.1 ± 9.3	17.9 ± 5.1	2.0 ± 0.3	25.7 ± 5.1
Skin lipid liposomes	PA	48.2 ± 12.0	36.0 ± 10.1	8.9 ± 2.5	4.0 ± 2.2
	CH	44.1 ± 10.7	40.0 ± 13.5	7.4 ± 2.1	5.0 ± 1.7
Inulin solution	Inulin	1.2 ± 0.4	0	0	96.1 ± 8.0
Liposomally entrapped inulin	Inulin	32.6 ± 6.0	14.1 ± 4.0	2.9 ± 0.3	45.5 ± 10.2
	EL	36.0 ± 8.8	17.0 ± 4.9	2.9 ± 0.6	41.1 ± 14.9

[a] Expressed as a percentage (± standard deviation) of the amount of formulation applied (n = 8).

used to form stable liposomes (1, 2, 6, 10), and while such studies have been useful in elucidating the physicochemical characteristics of the stratum corneum, especially concerning its bilayer properties, we were the first to report that skin lipid liposomes are effective drug delivery systems (9). We demonstrated that topical application of IFN entrapped in liposomes prepared from lipids with a composition similar to that of the stratum corneum resulted in a greater reduction of lesion scores than did topical application of IFN entrapped in liposomes prepared from phospholipids when tested in a herpes simplex virus type 1 guinea pig model. The results in Table 1 clearly demonstrate that the bilayer lipids of skin lipid liposomes penetrate more efficiently than do the bilayer lipids of phospholipid-based liposomes into the deeper strata of the skin (tissue underlying the horny layer). Of equal importance, liposomes are able to carry their entrapped solutes into the skin, even to a depth beneath the stratum corneum. Inulin is a water-soluble marker and, when applied as an aqueous solution, is essentially incapable of permeating lipid membranes. The fact that the EL-CH and PA-CH lipid ratios are essentially maintained throughout the skin strata strongly suggests a molecular mixing of skin lipid liposomal bilayers and, to a lesser extent, phospholipid liposomal bilayers with those of the stratum corneum. The maintenance of the inulin-EL ratio, together with the observation that there is no breakthrough of inulin into the receiver compartment, even after 24 h, further suggests that a significant amount of water associated with the liposomal bilayers is carried into the deeper stratum corneum bilayers. The mechanism of this phenomenon is not clear, and we are presently pursuing kinetic and histological studies in an attempt to elucidate the mechanism.

TABLE 2. Distribution of IFN in various strata of the skin 24 h after in vitro topical application of IFN-containing formulations to full-thickness skin

Formulation	Distribution[a] on or in:			
	Stratum corneum surface	Deeper stratum corneum	Deeper skin strata	Combined donor cell and skin washings
Aqueous solution	8.5 ± 4.2	8.5 ± 7.1	0.6 ± 0.1	74.0 ± 0.2
EL-CH-PS LUV	46.1 ± 4.2	38.3 ± 2.1	2.6 ± 1.5	8.9 ± 2.8
Skin lipid LUV	5.5 ± 1.5	62.6 ± 5.5	4.5 ± 2.5	17.7 ± 5.1
Skin lipid DRV	6.6 ± 1.9	58.6 ± 7.9	9.6 ± 2.6	15.4 ± 3.0
CM-CH-CS DRV	7.4 ± 2.0	53.9 ± 8.5	12.8 ± 3.5	17.8 ± 1.5

[a] Expressed as a percentage (± standard deviation) of the amount of formulation applied (n = 3 to 5).

The results shown in Table 2 are consistent with the data shown in Table 1 and, more importantly, with the results reported for testing of various topically applied formulations in the cutaneous herpes simplex virus type 1 guinea pig model (9). Very little IFN penetrates the stratum corneum, and almost none can be found in strata underlying the horny layer when the peptide is applied as an aqueous solution. We (9) and others (3, 4) have shown that the topical application of IFN solutions fails to significantly reduce lesion scores.

For phospholipid-based liposomes, the method of preparation rather than the lipid composition of the bilayers appeared to be the most important factor for reducing lesion scores (9). For example, since EL-CH-PS liposomes prepared by the dehydration-rehydration method significantly reduced lesion scores whereas liposomes of the same composition prepared as MLV and LUV failed to elicit a pharmacological response, we speculated that the dehydration and subsequent rehydration of the liposomes facilitated partitioning of the IFN into liposomal bilayers, where the drug was positioned for transfer into the lipid compartment of the stratum corneum. Furthermore, greater efficacy was observed when IFN was entrapped in liposomes prepared from lipids with a composition similar to that of the stratum corneum rather than from phospholipids (9). We hypothesized that if the transfer of drug from liposomal bilayers to stratum corneum bilayers is an essential aspect of the mechanism by which liposomal entrapment facilitates drug transport into the skin, it would seem logical that the use of skin lipid liposomes would expedite this transfer. The theory we currently favor is fusion of the liposomal bilayers with the intercellular lipid, a unique cooperative drug-enhancer effect. In support of these observations, the data shown in Table 2 show that application of skin lipid LUV resulted in almost twice the amount of IFN being deposited in the deeper layers of the skin as did application of EL-CH-PS LUV. Of equal importance, skin lipid and CM-CH-CS liposomes prepared by the dehydration-rehydration method were more than twice as effective as were those prepared by the reverse-phase evaporation method with respect to their ability to deposit IFN into the skin strata where the basal cell layers reside.

The combined results reflect the relationships among liposomal bilayer lipid transfer into the skin, concomitant transfer of entrapped substances (inulin and IFN), and in vivo efficacy, as determined by the herpes simplex virus type

1 guinea pig model. Rougier et al. (7) reported that the absorption of a variety of drugs through the skin was proportional to the amount of drug recovered in the stratum corneum following a 30-min topical application. However, all of the drugs tested were capable of being transported from traditional formulations, e.g., solutions or emulsions. IFN, on the other hand, is clearly unable to permeate the stratum corneum when topically applied from such formulations, and the data from this study suggest that liposomal entrapment facilitates its penetration into the deeper skin strata where the basal cell layers reside.

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