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Liposomes Promote Pulmonary Glucocorticoid Delivery

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The beneficial effects of glucocorticoids in treating pulmonary inflammatory disorders are complicated by systemic adverse effects. Thus, a possible reduction in dosage and dosing frequency would be advantageous, particularly for patients requiring high doses of the drug. We believe that this can be achieved by developing formulations that increase the retention of glucocorticoids in the lung and a liposome-based drug delivery system may be useful. In the present study, we examined the pulmonary delivery of a liposomal glucocorticoid formulation. Male adult rats were intratracheally instilled with free [^{3}H]dexamethasone (DEX) or [^{14}C]liposome-entrapped [^{3}H]dexamethasone (L-DEX) (800 μg DEX/kg body weight) and animals were killed at different times within a 72-h treatment period. Pulmonary retention of [^{3}H]DEX in animals instilled with free DEX was found to be approximately 1.5% of the administered dose 4 h post-instillation, with no radioactivity detectable 24 h post-instillation. Liposome encapsulation of the drug altered the pulmonary retention of DEX with about 34% and 8% of radioactivity remaining in the lung at 4 and 24 h post-instillation, respectively. The intratracheal instillation of free DEX or L-DEX reduced the number of leukocytes in peripheral blood to a similar extent (50% of control values) at 4 h. However, unlike free-DEX-treated animals whose leukocyte counts returned to control levels by 24 h, the circulating leukocyte counts of L-DEX-treated animals remained depressed in the same period. Furthermore, DEX-induced changes in ACTH levels were less evident in animals treated with the liposomal formulation than those treated with free DEX. Our data suggest that the administration of liposome-entrapped DEX has the distinct advantage of enhancing the anti-inflammatory activity of the drug and therefore, possibly reducing its need for frequent administration.

Keywords: Liposomes, Glucocorticoid, Dexamethasone, Lung, Drug delivery

TRODUCTION

ammation is a predominant feature of pulmon- diseases. Glucocorticoids are steroidal anti- inflammatory drugs which are, in most cases, successfully used to treat a wide spectrum of severe experimental and clinical inflammatory lung conditions (Nicholson, 1982; Jerome *et al.*, 1990;

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Williams and Yarwood, 1990; Barnes, 1995). The beneficial effects of glucocorticoids, however, are counterbalanced by several deleterious effects (Barnes and Pedersen, 1993; Check and Kaliner, 1990). For example, treatment with glucocorticoids such as dexamethasone is known to cause hypothalamic–pituitary–adrenal (HPA) axis suppression by reducing corticotrophin (ACTH) production, which then leads to reduced cortisol secretion by the adrenal gland (Barnes and Pedersen, 1993; Check and Kaliner, 1990).

The systemic adverse effects of an inhaled glucocorticoid depend on the amount of drug that is present in the circulation. This is of great concern, particularly for patients who require high doses of the anti-inflammatory agent. A reduction in the dosage and frequency of dosing in these patients would be convenient, not only as a measure to avoid the adverse drug effects, but also to improve the compliance of these patients with the medication (Utiger, 1993). Thus, it is considered desirable to develop glucocorticoid preparations efficacious at the target site, but with weak systemic activity. In this study, we attempted to deliver a glucocorticoid incorporated in liposomes directly to the lung, with the aim of attaining high pulmonary levels of the drug with minimal systemic effects. Furthermore, the potential of the lung to serve as a depot for sustained drug release was also assessed.

Liposomes are phospholipid vesicles composed of lipid bilayers enclosing an aqueous compartment. Hydrophilic molecules can be encapsulated in the aqueous spaces and lipophilic molecules can be incorporated into the lipid bilayers. Liposomes provide an efficient delivery system because they are biocompatible, biodegradable and relatively non-toxic (Shek and Barber, 1986; Shek *et al.*, 1990). As a drug delivery system, liposomes can significantly change the pharmacokinetic and pharmacodynamic fate of a compound by enhancing drug uptake, delaying loss of rapidly cleared drugs and reducing drug toxicity (Gregoriadis, 1991; Fielding, 1991; Gregoriadis and Florence, 1993).

The present study was carried out to determine whether the encapsulation of dexamethasone in liposomes would enhance the retention of the drug in the lung and reduce its systemic adverse effects following the intratracheal instillation of the liposomal preparation.

MATERIALS AND METHODS

Chemicals

Dexamethasone was purchased from Sigma Chemical Co. (St. Louis, MO). Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids (Alabaster, AL). [^{3}H]Dexamethasone and [^{14}C]DPPC were obtained from Dupont Canada, Inc. (Mississauga, Ont.). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Liposomal Dexamethasone (L-DEX)

Liposome-entrapped dexamethasone was prepared from a mixture of DPPC and dexamethasone in a 9 : 1 molar ratio. The lipids were dissolved in chloroform–methanol (2 : 1 v/v) and ^{14}C-DPPC (specific activity 2.5 mCi/mmole) and ^{3}H-DEX (specific activity 5 mCi/mmole) were added as tracers. The lipid mixture was dried in a water bath at 40°C under a stream of helium to a thin film coating the interior surface of the glass vessel. Any traces of solvent were removed by placing the vessel under vacuum for at least 1 h. The dried lipid was hydrated with 1.0 mL of 5 mM potassium phosphate buffer, pH 6.5, containing 3 mM EDTA, and then vortexed to form multilamellar vesicles. The multilamellar vesicles were extruded (10 times) with an extruder (Lipex Biomolecules, Vancouver, BC) through two stacked polycarbonate filters of 400 nm pore size using a helium pressure of 100–200 lb/in^2. Non-entrapped DEX was removed by washing the liposomes twice in 5 mM potassium phosphate buffer, pH 6.5, and pelleting at 110,000*g* for 1 h at 5°C in a Beckman L8-70 ultracentrifuge. Aliquots of supernatants and pellets were counted for ^{3}H and ^{14}C radioactivity by employing a

Beckman LS-5801 liquid scintillation counter. The liposomes were diluted with 5 mM potassium phosphate buffer, pH 6.5, to give a final DEX concentration of 200 μg DEX/150 μL suspension.

Animals

Male Sprague-Dawley rats (220–250 g body weight) were purchased from Charles River Canada, Inc. (St. Constant, Quebec). All animals were housed in stainless-steel cages with free access to pelleted purina laboratory chow and tap water. The animals were exposed to alternate cycles of 12 h light and darkness. Animals used in this study were treated and cared for in accordance with guidelines recommended by the Canadian Council on Animal Care, and the experimental protocol was approved by the institutional animal care committee.

Administration of Free DEX or L-DEX to Animals

The endotracheal intubation technique of Brain *et al.* (1976) for drug administration in rats was adopted. All animals received 200 μg of DEX either as the free or liposomal drug in a total volume of 150 μL. Instillations were administered between 0800 and 0900 hours. Control animals received an equivalent volume of the buffered solution.

Experimental Design

To examine whether the encapsulation of DEX in liposomes could enhance drug retention in the lung, animals ($n = 5$ per group) were treated intratracheally with a single dose of free DEX or L-DEX and killed at different times within a 72-h treatment period. The distribution of DEX in the lungs and other tissues of treated animals was assessed by measuring the [^{3}H]DEX label in tissue homogenates.

Tissue Preparation

Rats were anaesthetized with an intraperitoneal injection of sodium phenobarbital (50 mg/kg). Blood was collected in EDTA-containing tubes by cardiac puncture. Lungs were removed immediately after decapitation and were rinsed with ice-cold saline to remove excess blood. All subsequent steps were carried out at 0–4°C. Following rinsing, tissues were weighed and finely minced. Approximately 1 g of tissue sample was homogenized with a Brinkman Polytron in a sufficient volume of ice-cold 0.25 M sucrose in 5 mM Tris-HCl buffer, pH 6.5, to produce a 20% homogenate.

Tissue Analysis

Tissue homogenates were digested with 0.5 mL solvable (Dupont Canada, Inc.) for 24 h and then bleached with 0.2 mL of 30% H_2O_2. This was subsequently incubated at 25°C for 1 h. After this, 10 mL of Formula-989 scintillation cocktail was added; the samples were then vortexed and counted for radioactivity in a Beckman LS 5801 scintillation counter.

Measurement of White Blood Cells in Peripheral Blood

The EDTA-treated blood samples were used to measure white blood cell counts using an automated Coulter JT hematology analyzer (Coulter Electronics Inc., FL).

Measurement of ACTH Concentrations in Plasma

Determination of plasma ACTH concentrations was carried out by using a specific RIA kit (Diagnostic Products Corporation, CA) according to the manufacturer's direction.

Statistical Analysis

Results were analyzed by one-way analysis of variance (ANOVA) (Gad and Weil, 1994). The level of significance was accepted at $p < 0.05$.

RESULTS

Liposomal Dexamethasone Preparation

The relatively insoluble dexamethasone was found to incorporate well in the liposome preparation at an entrapment efficiency of $34.4 \pm 1.9\%$ and the loading capacity was found to be $50.3 \pm 3.6\,\mu g$ DEX/mg DPPC. The size of the entruded vesicles was $231 \pm 32\,nm$. At a molar ratio of 9:1 DPPC:DEX, the extruded liposome preparation did not form aggregates and remained stable for at least a week when kept at 4°C.

Pulmonary Retention of [^{3}H]DEX

The retention of radioactive DEX in the lungs of rats treated intratracheally with free DEX or L-DEX is shown in Fig. 1 (upper panel). Recovery of [^{3}H]DEX from lung homogenate was approximately 50% of the initial dose 0.5 h after the administration of L-DEX, reaching a maximum level (57% of initial dose) 1 h later; thereafter, [^{3}H]DEX levels decreased in a time-dependent manner. On the other hand, the radioactivity retained in the lungs of rats 0.5 h after intratracheal instillation of free DEX was approximately 26% of initial dose and declined to a non-detectable level 4 h later, in sharp contrast to the 36% drug retention in L-DEX-treated animals.

Systemic Concentrations of [^{3}H]DEX

The concentrations of radioactive DEX in the blood of rats treated intratracheally with free DEX or L-DEX is shown in Fig. 1 (lower panel). Recovery of [^{3}H]DEX from blood was approximately 1% of the initial dose 0.5 h after the administration of L-DEX, reaching a maximum level of 1.8% of initial dose 4 h later; thereafter, [^{3}H]DEX levels decreased in a time-dependent manner. On the other hand, the radioactivity detected in the blood of rats 0.5 h after intratracheal instillation of free DEX was approximately 5% of initial dose and declined to a non-detectable level 4 h later.

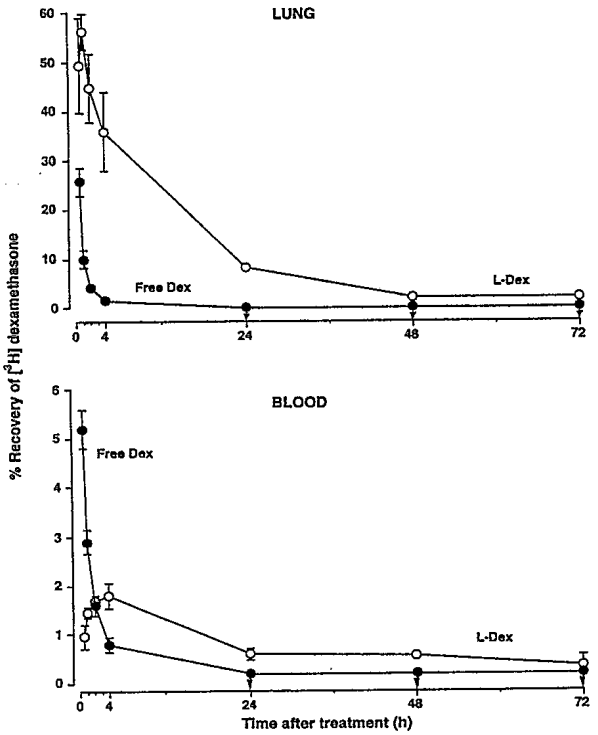

FIGURE 1 Recovery of ^{3}H-label from lung homogenates (upper panel) and blood (lower panel) following the intratracheal instillation of free DEX or L-DEX. Either drug formulation was labelled and prepared as described in Materials and Methods. Lungs of treated animals were removed for the determination of radioactive counts at various time periods after intratracheal instillation of each drug. Each point represents the mean percentage of recovered dose ± SE of five animals. The inverted arrows denote undetectable levels of radioactivity.

[^{3}H]DEX and [^{14}C]DPPC Distribution in Lungs of Rats Treated with L-DEX

To investigate whether the retention of [^{3}H]DEX in the lungs of L-DEX-treated rats was directly related to that of [^{14}C]DPPC-labelled liposomes, the distribution of ^{3}H- and ^{14}C-radioactivity was examined. As shown in Fig. 2, the retention of the two isotopes displayed similar profiles with a constant $^3H/^{14}C$ ratio of approximately 1.0 up to 4 h post-instillation; thereafter, the ratio declined due to a higher pulmonary retention of the liposomal label.

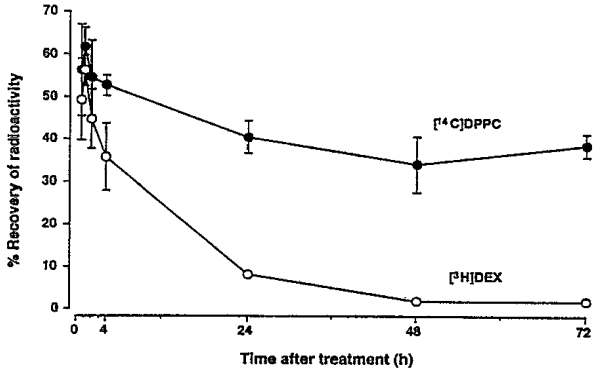

FIGURE 2 Recovery of ^{3}H and ^{14}C labels from lung homogenates following the intratracheal instillation of [^{3}H]DEX entrapped in ^{14}C-labelled liposomes. The liposomal DEX was prepared as described in Materials and Methods. Lungs of treated animals were removed at various time periods for the determination of ^{3}H and ^{14}C counts after the intratracheal instillation of L-DEX. Each point represents the mean ± SE of five animals.

Effect of DEX Treatments on White Blood Cell Counts in Peripheral Blood

The administration of dexamethasone is known to cause a redistribution of WBC from the peripheral blood to other tissues. In this study, the WBC concentration in the peripheral blood of animals treated with free DEX was significantly reduced by almost 27% during the first hour, with a suppression to about 50% by 4 h post-instillation (Fig. 3). The depressed WBC concentration, however, returned to control levels by 24 h of treatment and remained unchanged for the remaining experimental period. In contrast, WBC concentrations in the peripheral blood of L-DEX-treated animals were reduced to more or less the same extent as that observed in animals treated with free DEX, but remained suppressed throughout the experimental period.

Effect of DEX Treatments on Plasma ACTH Concentrations

The systemic adverse effects of inhaled glucocorticoids are most readily determined by assessing the pituitary–adrenal function by measuring plasma ACTH concentrations. In the present study,

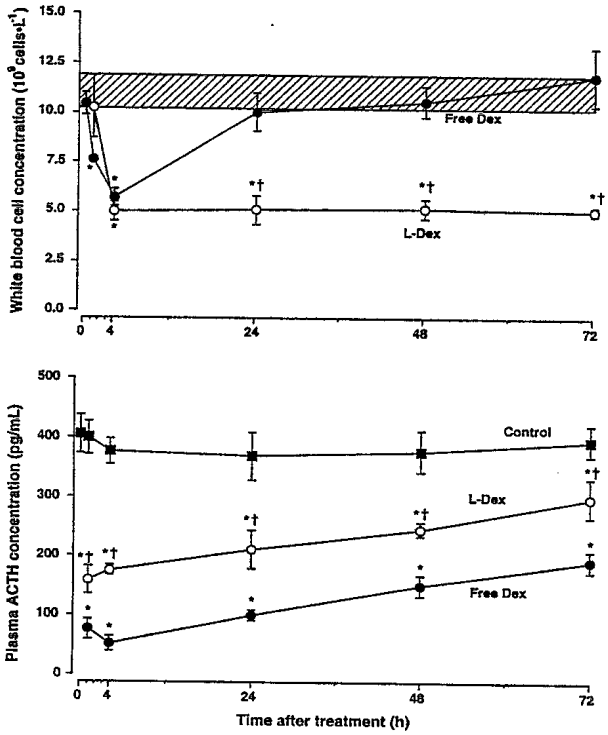

FIGURE 3 Effect of free DEX or L-DEX treatment on circulating WBC concentrations (upper panel) and plasma ACTH (lower panel). Each drug formulation was prepared as described in Materials and Methods. Blood was collected from control and drug-treated animals at various time periods after intratracheal instillation of free DEX or L-DEX. Each point represents the mean ± SE of five animals. (*) denotes a significant difference ($p < 0.05$) compared with the mean value of the control group instilled with the buffer solution. (†) denotes a significant difference ($p < 0.05$) compared with the mean value of the group of animals instilled with free DEX. The shaded area in the upper panel represents the normal WBC concentration of control animals.

intratracheal instillation of free DEX or L-DEX resulted in a significant reduction in plasma ACTH concentrations which were less marked in animals treated with L-DEX. Furthermore, animals treated with L-DEX displayed a faster recovery in plasma ACTH concentrations when compared to those of free DEX-treated animals.

DISCUSSION

Orally and systemically administered glucocorticoids have been used for the treatment of pulmonary

inflammatory disorders, such as asthma and the acute respiratory distress syndrome. The beneficial effects of these drugs, however, are counterbalanced by their systemic adverse effects, including suppression of the hypothalamic–pituitary–adrenal axis. In an attempt to avoid systemic adverse effects, glucocorticoids are now delivered directly to the lung in an aerosol form. However, a therapeutically undesirable aspect of pulmonary drug delivery is the rapid translocation of most drugs from the lung to the systemic circulation. Although inhalation therapy generally promotes high topical efficacy and low systemic adverse effects, the development of a sustained-release glucocorticoid formulation should be of benefit to patients, who require large and more frequent drug doses.

Intratracheally administered DEX was shown to be cleared very rapidly from the lung, and only traces of radiolabelled DEX remained in the lung by 4 h post-instillation. Other investigators have shown that in animals given DEX, via the tracheal route, 50% of the steroid was absorbed from the lung at 1.7 min (Burton and Schanker, 1974). In the present study, although a detailed pharmacokinetic profile of DEX was not examined, the elimination of free DEX following intratracheal instillation was also rapid, with only about 10% remaining in the lung by the first hour. In contrast, the pulmonary retention of DEX instilled directly into the lung as a liposomal formulation was prolonged, with 56% of the drug remaining in the lung at the 1-h time-point. Our data corroborate with the findings by other investigators (Taylor *et al.*, 1989; Fielding and Abra, 1992) who reported that the encapsulation of sodium cromoglycate or terbutaline prolonged the retention of the encapsulated drug in the lung, thus minimizing the drug from distributing into the circulation.

In our study, DEX was incorporated in liposomes prepared from DPPC, which is known to be the major lipid component of alveolar surfactant and is relatively non-toxic. DPPC preparations have also been used in the prevention or treatment of certain lung conditions, such as the respiratory distress syndrome and in the selective delivery of drugs to the lung and other tissues (Van Golde *et al.*, 1988; Jobe, 1993; Gregoriadis and Florence, 1993; Shek *et al.*, 1994). DPPC was chosen primarily because of its compatibility with intrinsic lung surfactants. The sustained drug release effect observed was found sufficient to mediate a demonstrable change in anti-inflammatory parameters. It is obvious that the entrapment efficiency, release kinetics, and therapeutic efficacy of other drugs may well be different when other types of liposomes are used. For example, the entrapment of salbutamol is higher in liposomal vesicles with a negative charge (Farr *et al.*, 1989); pulmonary absorption and solute release in the lung is dependent upon liposomal lipid composition (Kellaway *et al.*, 1985); and drug retention upon nebulization for intratracheal delivery is dependent on the type of phospholipid used (Taylor and Farr, 1993).

The relatively higher pulmonary retention of DEX in L-DEX-treated animals may be attributable to the distribution characteristics of ^{14}C-labelled liposomes or liposomal fragments. This interpretation is consistent with our finding that the $^{3}H/^{14}C$ ratio observed in the lung homogenates was relatively constant within the initial 4 h of treatment. The ability of liposomes to increase the retention of drugs following their direct delivery to the lung is well documented and it is because of this property that liposomes are used as a drug delivery system. The reduction in the ratio at later time-points may be explained by leakage of the drug from the liposomes and elimination of liposomes from the lung by pulmonary clearance mechanisms (Schreier *et al.*, 1993; Taylor and Newton, 1992).

The leukopenic effect of L-DEX suggests that the liposomal formulation may be more beneficial as an anti-inflammatory agent. The prolonged suppressive effect of the liposomal formulation perhaps can be attributed to a slower but continuous release of the drug into the systemic circulation. In light of these observations, it may be concluded that the lung can serve as a depot for sustained drug release, provided that an appropriate drug formulation is used. In the case of dexamethasone delivery, the liposomal formulation appears effective.

Another benefit of the liposomal formulation is that liposomes are normally taken up by macrophages and other inflammatory cells, known to play important roles in the pathogenesis of lung injury (Schreier *et al.*, 1993; Taylor and Newton, 1992). The intracellular uptake of dexamethasone is known to impair the chemotactic ability of neutrophils and suppress the release of toxic metabolites (proteases and reactive oxygen species) from phagocytes. A successful reduction by liposomal dexamethasone in toxic metabolite release from phagocytes may contribute to reduce tissue damage associated with inflammation. For the liposome-entrapped dexamethasone to be effective, it should be reasonably resistant to intralysosomal degradation. In this regard, it has been reported that dexamethasone stabilizes lysosomal membranes and inhibits phagocytosis (Ackermann and Beebe, 1975; Wallace and Whittle, 1988). Dexamethasone and other glucocorticoids have been used extensively in the treatment of shock because of their ability to neutralize the effects of lysosomal enzymes, which also contribute to tissue injury (Trachte and Lefer, 1978; Bradley, 1979). In this study, the ability of liposomal dexamethasone to suppress the white blood cell count strongly suggests that most, if not all of the liposomal dexamethasone may well have escaped lysosomal degradation.

Glucocorticoid drugs suppress the function of the hypothalamic–pituitary–adrenal axis by reducing the secretion of corticotropin (ACTH) and this effect has been attributed to the concentration of the drug in the circulation (Check and Kaliner, 1990; Barnes and Pedersen, 1993; Barnes, 1995). In this study, the plasma DEX concentrations in the first two hours in free DEX-treated animals were exceedingly high compared to that in L-DEX-treated animals. The initial surge of exogenous glucocorticoid appears to trigger a powerful suppression of the hypothalamic–pituitary–adrenal axis, as evidenced by a fairly long-lasting inhibition of ACTH production. Therefore, the greater reduction in plasma ACTH concentration in the free DEX group perhaps represents the residual effect of the initially high blood concentrations of the drug.

In conclusion, the results of the present study suggest that the advantages of liposome-entrapped DEX over the free drug are: (i) prolonged retention of the drug in the lung; (ii) reduced systemic side effects of the drug; and (iii) prolonged anti-inflammatory activity. Furthermore, the sustained drug-release property of liposomes in the lung may reduce the need for frequent drug dosing to keep an excessive pulmonary inflammatory response under control.

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