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Experimental pulmonary delivery of cyclosporin A by liposome aerosol

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Abstract

The utilization of CsA–liposome for aerosol delivery by jet nebulizers has potential advantages for clinical development including: aqueous compatibility, sustained pulmonary release to maintain therapeutic drug levels and facilitated delivery to alveolar macrophages and pulmonary lymphocytes. Inhalation of cyclosporin A (CsA)–dilauroylphosphatidylcholine (DLPC) liposome aerosols will theoretically result in localized and sustained delivery of therapeutic CsA concentrations within the lung as an alternative to local immunotherapy for pulmonary diseases. In the lung, targeted delivery of therapeutic CsA concentrations would require lower dosages than via conventional intravenous or oral routes of administration. Potential benefits from targeted lung delivery could include reduced systemic toxicity and prolonged immunosuppressive activity. Aerosol delivery systems have been developed to deposit drugs directly onto pulmonary surfaces at the sites of disease within the lung. A novel HPLC method for tissue analysis of CsA–liposomes is developed and utilized with a solid-phase extraction method to measure CsA recovered from Balb/c mouse lung tissues. A concentrated formulation containing 5 mg CsA–37.5 mg DLPC/ml was nebulized with an Aerotech II nebulizer generating an aerosol particle size distribution (mass median aerodynamic diameter (MMAD)) of 1.7 μm and geometric standard deviation (GSD) of 2.0. After a 15-min aerosol exposure, little of no CsA was detected in the blood, liver, kidney or spleen. The lung contained the highest organ CsA levels with high immunosuppressive activity demonstrating effective pulmonary targeting of the CsA–DLPC liposome aerosol. The results of this system will be utilized as the experimental basis for future pharmacokinetic, toxicological, immunosuppression and other biological studies. © 1998 Elsevier Science B.V.

Keywords: Liposome; Aerosol; Cyclosporin A; HPLC; Nebulizer; Lung

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1. Introduction

Immunologically mediated lung diseases, such as allergy, hypersensitivity, chronic severe asthma, obliterative bronchiolitis, and pulmonary sarcoidosis have proven difficult to treat by conventional oral or intravenous drug therapies or may be complicated by the development of serious toxic side effects resulting from long-term systemic treatments. For example, potent immunosuppressive drugs like the cyclic peptide cyclosporin A (CsA) have demonstrated differential clinical effectiveness against pulmonary T-lymphocyte subsets, possibly due to poor penetration of CsA into the lung from the systemic circulation (Atkinson et al., 1983; Martinet et al., 1988; Reid et al., 1988). In contrast, oral systemic CsA has proven to be effective for the treatment of chronic, severe asthma by an unknown mechanism (Alexander et al., 1992; Lock et al., 1996). Thus, the site of drug action, its mode and route of delivery may determine therapeutic effectiveness.

Many different lung diseases have been successfully treated through the clinical utilization of aerosol delivery systems to deposit drugs directly onto pulmonary surfaces at the sites of disease within the lung. Recent studies have demonstrated that it is possible to deliver CsA directly to the lung in an aerosol form (Dowling et al., 1990; Muggenburg et al., 1990). Data of canine and rat models indicates that the aerosolized CsA is effective in preventing allograft rejection with reduced dose compared to intravenous or oral dosage forms (Dowling et al., 1990; Keenan et al., 1992; Blot et al., 1995). A study of aerosolized CsA in lung recipients with refractory chronic rejection has demonstrated that the drug stabilizes pulmonary function and can be inhaled without systemic toxicity (Iacono et al., 1996).

The development of liposomal formulations compatible with aerosol delivery with jet nebulizers has expanded the possibilities for more effective utilization of aerosol-based therapies (Farr et al., 1985; Gilbert et al., 1988; Niven and Schreier, 1990; Schreier et al., 1993; Taylor and Farr, 1993). The utilization of liposomes for aerosol delivery has many potential advantages for clinical development, including: aqueous compatibility, sustained pulmonary release to maintain therapeutic drug levels (Hung et al., 1995), and facilitated intra-cellular delivery particularly to alveolar macrophages and lymphocytes (Schreier et al., 1993; Taylor and Farr, 1993). We have developed a variety of liposomal drug formulations, including CsA–liposomes, for aerosol lung delivery in preclinical (Gilbert et al., 1993; Waldrep et al., 1993, 1994b, 1997a, 1994a; O'Riordan et al., 1997) and clinical studies (Vidgren et al., 1994; Gilbert et al., 1997b; Waldrep et al., 1997b). Using gamma scintigraphy, nebulizer systems were evaluated and selected for aerosol targeting of the peripheral lung regions (Vidgren et al., 1994).

The purpose of this study was to develop an optimized, targeted pulmonary delivery system in Balb/c mice utilizing CsA–DLPC liposome aerosols as the experimental basis for future pharmacokinetic, toxicological, immunosuppression, and other biological studies.

2. Materials and methods

2.1. Preparation of CsA–liposomes

CsA–liposomes were produced by lyophilization from *t*-butanol as previously described (Waldrep et al., 1993, 1997a; Gilbert et al., 1997a). For nebulization and pulmonary aerosol delivery, formulations were produced containing 25 mg CsA (Chemwerth, Woodbridge, CT, USA) with 187.5 mg DLPC (1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC; Avanti Polar Lipids, Alabaster, AL, USA) at an optimal CsA:DLPC ratio of 1:7.5 by weight. Just prior to nebulization, multi-lamellar vesicular (MLV) liposomes were produced by adding 5 ml of ultra-pure water above the DLPC phase transition temperature to deliver the desired final standard drug concentration of CsA 5 mg:DLPC 37.5 mg/ml. The mixture is incubated for 30 min at room temperature with intermittent mixing to produce multilamellar vesicular (MLV) liposomes. Aliquots are taken for determination of drug concentration by HPLC. After swelling, the CsA–DLPC liposome formulations were

characterized by microscopy under polarized light and by quasi-elastic light scattering with a Nicomp Model 370 Submicron Particle Sizer as previously described (Waldrep et al., 1997a).

2.2. CsA–DLPC liposome aerosol particle size distribution

CsA–DLPC liposome aerosols were generated using an efficient, high-output continuous-flow Aerotech II nebulizer (ATII; CIS-US, Bedford, MA, USA) flowing at 10 l/min (Waldrep et al., 1997a). Aerodynamic particle sizing of the drug–liposome aerosols was measured as previously described using an Andersen 1 ACFM non-viable ambient particle sizing sampler operated at 28.3 l/min (Graseby Andersen Division, Smyrna, GA, USA) and equipped with an artificial throat as a simulator of the human respiratory system (Waldrep et al., 1993, 1994a,b, 1997a,b; Vidgren et al., 1994). After determination of the CsA and DLPC concentrations for each stage by HPLC, the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of the drug–liposomes calculated using a computer by plotting on a log probability plot. The MMAD and GSD are determined by the liposomal drug content distributed within the array of droplets comprising the aerosol (Waldrep et al., 1997a).

2.3. Nebulized CsA–liposome aerosol output rates

Aerosol samples containing CsA–DLPC liposomes were collected in water using an All Glass Impinger (AGI-4, Ace Glass, Vineland, NJ, USA) as previously described (Waldrep et al., 1993, 1997a). The AGI-4 impinger device was utilized with the collecting flask containing 10 ml of water to which the aerosol was introduced through a calibrated glass tube and critical orifice delivering the jet of aerosol 4 mm above the bottom of the flask. The system was operated by a vacuum pump (through connector tubes with negative pressure differential compensation as described) using a sampling period of 1 min.

2.4. CsA and DLPC analysis by HPLC

The CsA in DLPC liposomal formulations (to determine CsA drug content and liposome association) in aerosol samples was determined by HPLC as previously described (Waldrep et al., 1997a). Aerosol samples for parallel CsA and DLPC analysis were dissolved directly in methanol. CsA was measured using a Supelcosil LC-1 (5.0 cm × 4.6 mm, 5 μm) column (Supelco, Bellefonte, PA, USA) at 75°C with acetonitrile:methanol:water (50:20:30, v/v/v) mobile phase (Charles et al., 1988). Peaks were detected at 214 nm using the multi-wavelength UV detector and quantified with the Millennium 2010 Chromatography Manager (Waters, Millford, MA, USA) The limits of detection were 10 ng CsA. Radio-labeled CsA ([mebmt-β-^{3}H]CsA; Amersham Life Sciences Division, Arlington Heights, IL, USA) was used to confirm the identity of the CsA peak. A modification of the HPLC protocol of Grit and Crommelin (1992) has been utilized to measure DLPC in aerosol samples. A Waters Nova-Pak silica column (15 cm × 3.9 mm, 4 μm) was used at ambient, room temperature with acetonitrile, methanol, and water (64:28:8, v/v/v) mobile phase. Peaks were detected with a mass evaporative detector (Sedex 55, Sedere, France) and quantified with the Water's Millennium 2010 Chromatography Manager.

2.5. Pulmonary delivery of CsA–DLPC aerosol

Balb/c mice (Harlan-Sprague Dawley; Houston, TX, USA) were utilized in these experiments. CsA–DLPC liposome aerosols were generated with an ATII nebulizer connected via a T-tube (with a one-way valve distal to the outlet port) inserted into one end of an enclosed 4-l closed, plexi-glass chamber where the flowing liposome aerosol saturates the internal chamber, exiting out through a distally situated outlet hole. A wire mesh grid is used to minimize contact with cage surfaces. The mice were treated in the chambers for a single 15-min exposure to a continuously flowing CsA–DLPC liposome aerosol ($n = 10$ per group). As an added worker safety precaution, all of the aerosols will be generated within a biologi-

cal safety cabinet with an HEPA filter and exhausted to the outside.

2.6. Solid-phase extraction and drug analysis of lung tissues

After inhalation, the mice were sacrificed by methoxyflurane (Pitman-Moore, Mundelein, IL, USA) anesthesia followed by cervical dislocation. 10 μg of cyclosporin D (CsD; Sandoz Research Institute, East Hanover, NJ, USA) (10 μl of a 1-mg/ml stock solution) was added to the weighed tissue samples as an internal standard and the tissues were homogenized for 1 min in 1 ml of sterile water with a Wig-L-Bug (Crescent Dental Manufacturing, Lyons, IL, USA) in polypropylene tubes containing five glass beads (4 mm borosilicate) per tube. The homogenized slurry was then mixed with 2 ml of 98% acetonitrile:2% methanol solution, and this volume was centrifuged ($1000 \times g$, 20 min) to obtain a clear supernatant. Five ml of sterile water was added to dilute the tissue extraction. Meanwhile Sep-Pak® Plus C_{18} cartridges for solid-phase extraction (Waters, Milford, MA, USA) were prepared for use. First these columns were activated with 5 ml of 95% ethanol and then washed with 5 ml of sterile water before layering the extracted supernatant onto the column. The sample was applied slowly to the column and all fluid was allowed to drain from the resin bed. The column was washed again with 5 ml of sterile water and then with 5 ml of 50% acetonitrile. The sample was finally eluted with 1.5 ml of methanol and 0.5 ml of sterile water. The eluted material was evaporated and then reconstituted in CsA–HPLC mobile phase, described above. These samples were then analyzed by HPLC to determine the concentration and extraction efficiency of the CsA. Data was expressed as μg CsA/g tissue analyzed.

2.7. Inhibition of lymphocyte blastogenesis in vitro by CsA extracted from mouse lung tissues after aerosol delivery of CsA–DLPC liposomes

For testing the biological activity of CsA delivered to the lung by liposome aerosol, an in vitro immune response system was utilized. A primary immune response was generated within the lung-associated mediastinum (MLN) lymph nodes after intranasal immunization. Under methoxyflurane anesthesia Balb/c mice (Harlan-Sprague Dawley, Houston, TX, USA; $n = 5$) were immunized intranasally with 35 μl of alum-precipitated ovalbumin (AP-OVA, 87.5 μg) supplemented (1:1, v/v) with *Bordetella pertussis*-adsorbed vaccine (Michigan Dept. Public Health, Lansing, MI, USA). This protocol was demonstrated to deliver at least 75% or more of the inoculum to the lungs (Vidgren et al., 1994). The mice were sacrificed 7 days post-immunization and the MLN removed by micro-dissection and the lymphocytes isolated for in vitro analysis. The proliferation assay consisted of alterations in the stimulation of lymphocytes after activation with the sensitizing antigen ovalbumin or with the non-specific T-cell mitogen, Concanavalin A (Con A; Sigma, St. Louis, MO, USA). To test the biological activity of aerosol-delivered CsA–DLPC, lung CsA was isolated from mouse pulmonary tissues using solid-phase extraction and quantification by HPLC as described above. Immunosuppressive activity was determined by co-culture of lung-extracted CsA with antigen- or mitogen-stimulated lymphocytes. The uptake of [^{3}H]TdR (Amersham Life Sciences Division, Arlington Heights, IL, USA) into DNA was determined at 48–72 h. Inhibition of specific or non-specific lymphocyte activation was demonstrated by reduction in the antigen-specific stimulation or in the inhibition of mitogen responsiveness.

2.8. Statistical analysis

Aerosol samples analyzed in parallel for CsA and DLPC content were subjected to statistical evaluation using InStat 2.00 (Macintosh; GraphPad, San Diego, CA, USA). The Spearman correlation coefficient was utilized as an indication of statistical significance of paired aerosol distribution of CsA and DLPC (O'Riordan et al., 1992; Smaldone et al., 1992).

3. Results

3.1. Aerosol studies

For these aerosol experiments, a liposomal formulation containing 5 mg CsA–37.5 mg DLPC/ml (w/w ratio) was utilized in conjunction with an efficient, high output nebulizer (ATII) operated at a 10-l/min flow rate. The CsA–DLPC liposomal formulations were standardized in this system by aerosol performance criteria of MMAD/GSD and output. Parallel analyses of both CsA and DLPC in aerosol samples demonstrated a similar and statistically significant distribution profile for both CsA and DLPC liposome carrier (Fig. 1). A Spearman correlation coefficient of 0.9878 and $p < 0.0001$ suggests that the CsA and DLPC remain associated upon nebulization and aerosol generation. The aerosol particle size distribution of these nebulized CsA–DLPC liposomes was measured to be MMAD = 1.7 μm and GSD = 2.0 by CsA distribution in the Andersen Cascade Impactor, and MMAD = 1.8 μm and GSD = 1.8 for DLPC as determined from the mean 10 aerosol analyses. A mean DLPC/CsA ratio of 7.2 was calculated for aerosol samples and virtually unchanged from the initial starting ratio of 7.5. The stability of the CsA–DLPC aerosol formulation was also demonstrated by MMAD and GSD values which remained unchanged over 15 min of nebulization. Non-encapsulated CsA in the reservoir after nebulization was minimal and detected at a level of only 181–233 ng/ml.

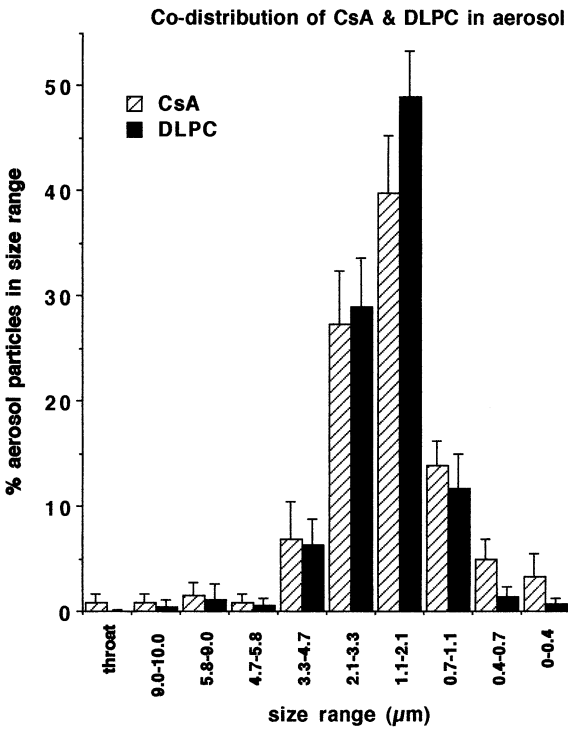

Fig. 1. Aerosol distribution profile of CsA and DLPC components of CsA–DLPC liposomes nebulized with an Aerotech II at a flow rate of 10 l/min as determined by the Andersen Cascade Impactor sampled at 7–8 min. The liposome formulations contained 5 mg CsA–37.5 mg DLPC per ml; with a total 25 mg CsA–187.5 mg DLPC. The data (mean ± standard deviation; $n = 10$ analyses) represent the fractional percentage of total CsA and DLPC recovered on each stage of the impactor with associated size cut-off in μm. The mass median aerodynamic diameters (MMAD) and geometric standard deviations (GSD) were calculated on a log-probability plot.

The total CsA–DLPC aerosol output of the system was determined with the AGI-4 impinger and is demonstrated in Fig. 2. From a standard input of CsA–DLPC liposomes containing 5 mg CsA/ml (25 mg total) there was an approximate aerosol output of 1 mg of CsA nebulized per minute with a slight time-dependent increase due to concentration effects in the reservoir. The mean nebulization efficiency of this system was determined (CsA reservoir input minus vial residual/CsA aerosol output calculated by integration of AGI data) as 54% or 13.37 mg CsA aerosolized. This CsA–DLPC liposome aerosol system was employed in subsequent animal experiments.

3.2. HPLC tissue analysis of CsA–liposomes

There have been numerous published studies reporting analysis of CsA in peripheral whole blood, serum, and/or plasma using immunoassays or HPLC (Tjandra-Maga et al., 1990). These different methods measure either the whole CsA molecule or its metabolites. The HPLC method is both highly effective, sensitive, and the most accurate. Analysis of CsA by HPLC requires the implementation of reversed-phase columns (typically C_{18}) heated to elevated temperatures (70°C), high concentrations of polar organic solvents and water, and prolonged run times (Charles et al.,

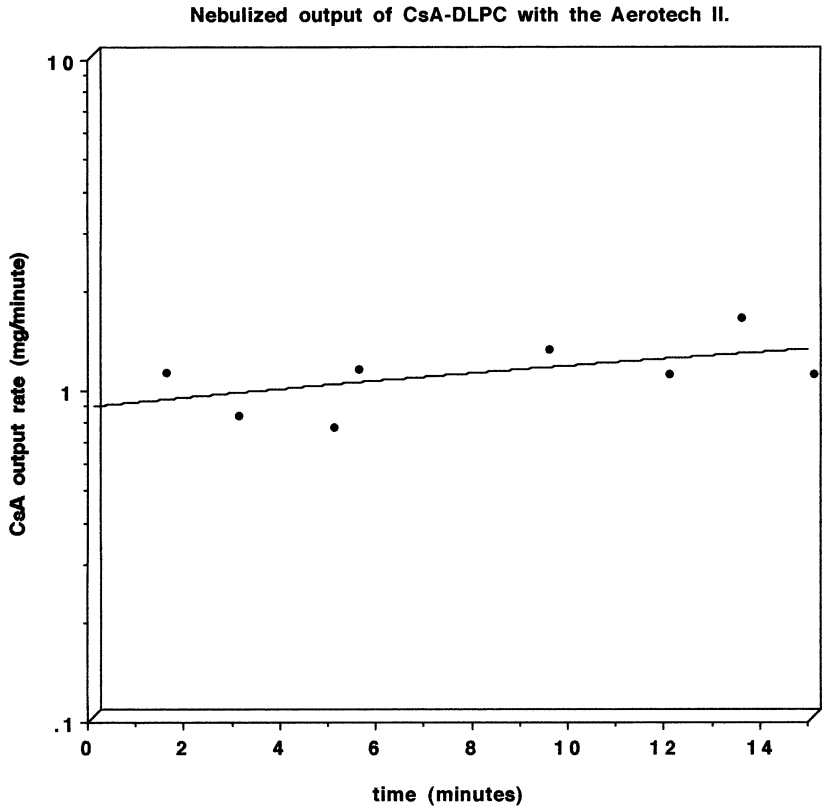

Fig. 2. Total aerosol output (mg/min) of 5 mg CsA–DLPC 37.5 mg/ml DLPC liposomes nebulized with an Aerotech II at a flow rate of 10 l/min and analyzed by the AGI-4 impinger. One-minute samples were taken from the aerosol at the designated intervals.

1988; Gilbert et al., 1993). In this system, utilization of a 5-cm C_3 column at 75°C produces a shorter run time of under 3 min as demonstrated in Fig. 3. The identity of the CsA peak was confirmed using ^{3}H-labeled CsA. Identical chromatograms were obtained for CsA extracted from CsA-DLPC liposomal formulations. The identity of CsA metabolites was not measured by this system.

A solid-phase extraction system was utilized to measure CsA recovered from Balb/c mouse lung tissues. Fig. 4 demonstrates the chromatographic profile of ^{3}H-spiked CsA–DLPC after extraction from lung. Similar results were obtained from other spiked tissues (blood, liver, kidney, and spleen). There were no peaks from normal lung tissue that co-eluted under the CsA peak. As with many tissue drug analysis systems, non-CsA contaminants or metabolites are evident in the chromatogram due the required implementation of a 214-nm wavelength detection system. The early elution profile of these contaminants did not interfere with detection of CsA or CsD. These results demonstrated that this novel CsA analysis method would be suitable for monitoring aerosol CsA–DLPC and deposition into the lungs and other organs of experimental animals.

3.3. Organ distribution of inhaled CsA–DLPC liposome aerosol in Balb/c mice

In order to test whether it was possible to selectively target lung tissues with this small-particle CsA–DLPC liposome aerosol, Balb/c mice were exposed for a 15-min inhalation interval. Tissue CsA levels were determined after solid-phase extraction as demonstrated in Fig. 5. The distribution of CsA was expressed as a standard-

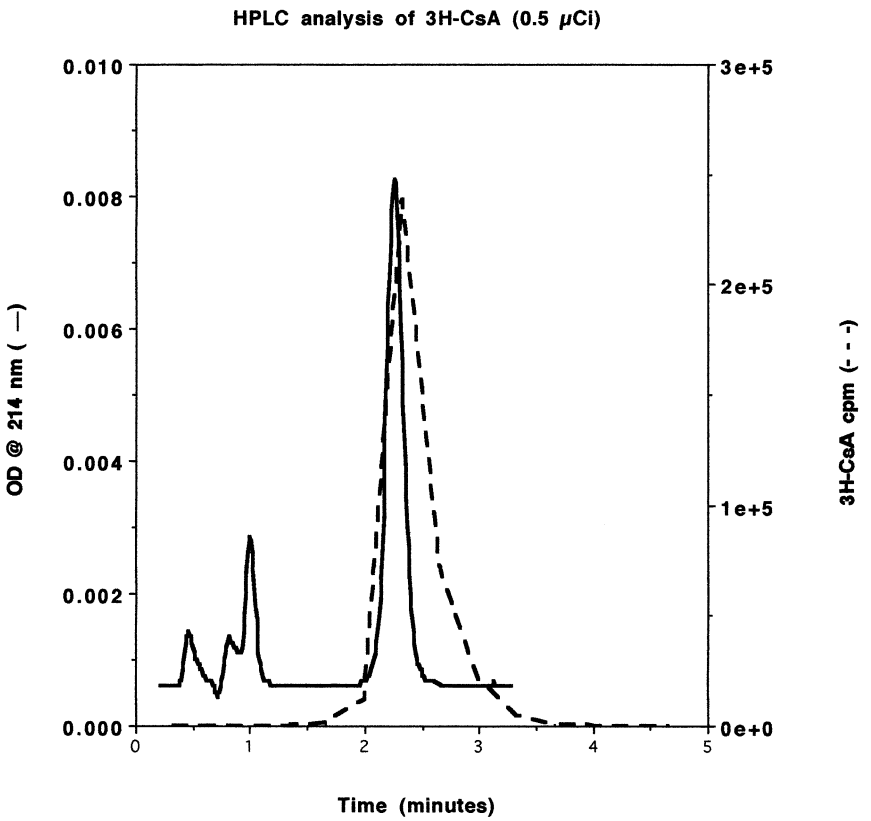

Fig. 3. HPLC profile of CsA on a Supelcosil LC-1 column (5 cm × 4.6 mm; C_3 column at 75°C). The CsA peak co-eluted with ^{3}H-labeled CsA. The mobile phase was acetonitrile:methanol:water (50:20:30, v/v/v). Peaks were detected at 214 nm.

ized μg/g of tissue analyzed. No CsA was detected in the kidney or spleen; 48 ± 48 ng/g were detected in blood, and 276 ± 391 ng/g were detected in liver. The lung contained the highest CsA levels with 5000 ± 1470 ng/g detected per gram of tissue. On a per gram tissue basis, the lung contained approximately 18-fold higher levels than the liver, and 104-fold higher levels than the peripheral blood, demonstrating effective pulmonary targeting of the CsA–DLPC liposome aerosol.

3.4. Inhibition of lymphocyte blastogenesis in vitro by CsA isolated from mouse lung tissues after aerosol delivery of CsA–DLPC liposomes

The results presented in Table 1 demonstrate that the CsA recovered from lung tissues following aerosol delivery retains its immunosuppressive activity. Inhibition of the uptake of [^{3}H]TdR by reduction in the antigen-specific stimulation (99% inhibition) or in the inhibition of mitogen responsiveness (95% inhibition) was highly significant. This level of inhibitory activity was equivalent to that previously demonstrated with the starting lot of CsA (data not presented). The inhibitory effects of the extracted lung CsA were most pronounced with OVA-stimulated MLN lymphocytes. These data indicate that, in this system, there is effective pulmonary targeting by the CsA–DLPC liposome aerosol.

4. Discussion

The fundamental therapeutic approach employing localized, topical delivery of pharmacological agents has been successfully utilized for treatment

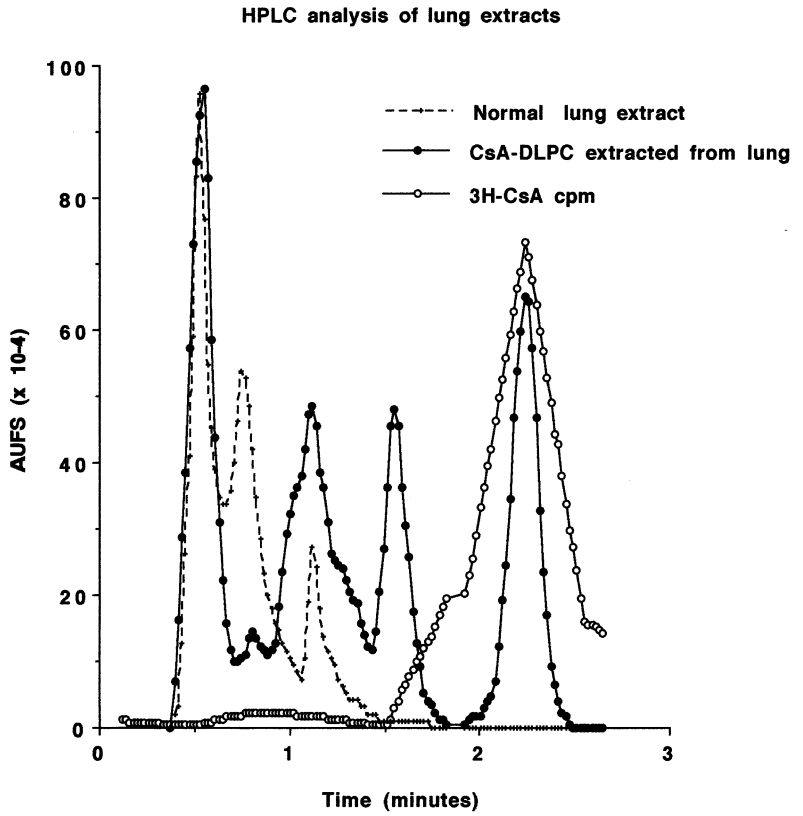

Fig. 4. HPLC chromatographic profile of ^{3}H-spiked CsA–DLPC liposomes (containing 10 μg CsA) after extraction from Balb/c lung. For comparison, a chromatogram of normal Balb/c lung extract is demonstrated. Injection volume was 10 μl (300 μl extract reconstitution volume).

of many different human organ systems. In the lung, many different infectious, inflammatory, and allergic diseases have been successfully treated employing aerosol delivery systems to deposit drugs directly onto the pulmonary surfaces (Kohler and Fleischer, 1991; Reed, 1991; Szefler, 1991; O'Doherty and Miller, 1993). The widespread utilization of aerosol technologies clearly demonstrates its effectiveness for therapeutic application for certain pulmonary diseases. While aerosol pulmonary delivery of water-insoluble, hydrophobic compounds has been limited. The recent development of liposomal formulations compatible with aerosol delivery has expanded the potential for more effective utilization with lipophilic drugs like CsA (Gilbert et al., 1993; Waldrep et al., 1993).

In this study, an efficient aerosol delivery system was employed through the implementation of concentrated liposomal formulations containing 5 mg CsA and 37.5 mg DLPC/ml nebulized via a high output, optimal nebulizer (ATII) (Smaldone et al., 1991; Keenan et al., 1997). The aerosol output of nebulized CsA–DLPC liposomes remained stable as demonstrated by parallel distribution of both CsA and DLPC in samples (Fig. 1), as described in other two-component aerosol systems (Smaldone et al., 1991; O'Riordan et al., 1992; Vidgren et al., 1994). A constant heterodisperse CsA–DLPC aerosol of MMAD = 1.7 μm and GSD = 2.0 over a 15-min operation was demonstrated in this system, and is within the optimal range for penetration into the lung periphery (Vidgren et al., 1994). A mean aerosol

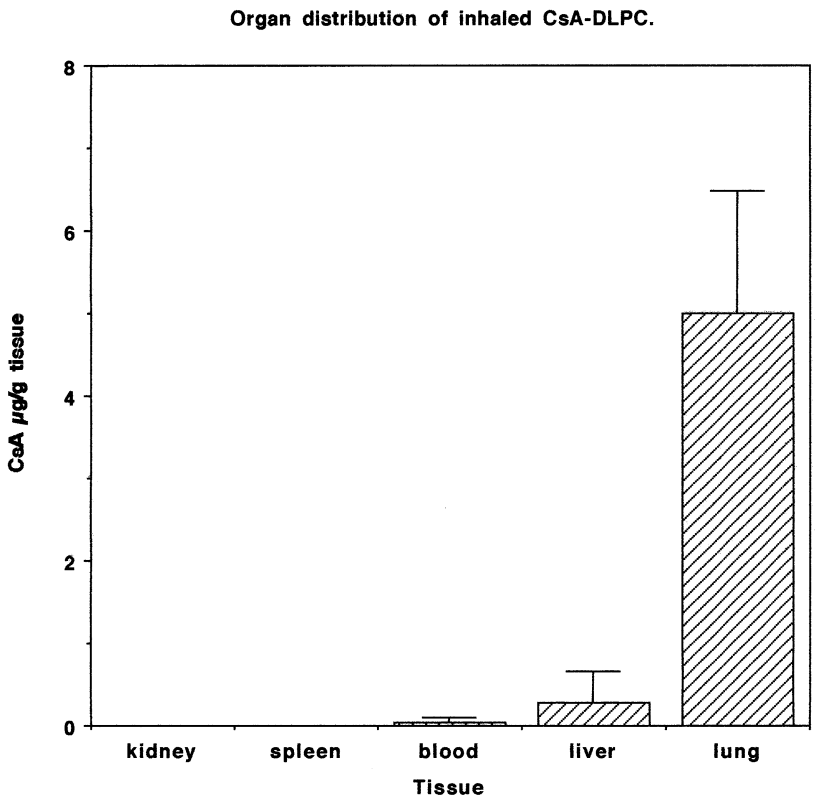

Fig. 5. Organ distribution of inhaled CsA–DLPC liposome aerosol in Balb/c mice after 15 min inhalation (5 mg CsA–37.5 mg DLPC/ml; 5 ml total nebulized volume with Aerotech II). Tissue CsA levels were determined after solid-phase extraction and expressed as a standardized $\mu g/g \pm$ standard deviation of tissue analyzed ($n = 10$).

output of 1 mg CsA/min was demonstrated (Fig. 2) with a nebulization efficiency of 54%. Effective lung targeting was with biologically active CsA was demonstrated in Figs. 3–5 and Table 1.

With this defined aerosol delivery system, it was thus possible to determine whether immunosuppressive CsA concentrations could be achieved within the lung tissues. Following a continuous 15-min inhalation, CsA tissue levels detected were approximately equivalent to 0.278 mg/kg body weight (excluding the tissues not analyzed). Lung levels were highest among the five tissues sampled (Fig. 5) and in the effective range for T-cell immunosuppression. Thus, the results of this study can serve as the experimental basis for future determination of the most effective therapeutic regimens to deliver pulmonary immunosuppression using CsA–DLPC liposome aerosols.

The potential clinical importance of CsA–liposome-based aerosol therapies as reported in this study is evident by the efficacy of CsA–ethanol/propylene glycol aerosols in pulmonary allograft rejection (Dowling et al., 1990; Keenan et al., 1992, 1997; Blot et al., 1995; Iacono et al., 1996). Stabilization of pulmonary function and improved histological rejection was noted in some patients after approximately 35 mg CsA deposition (O'Riordan et al., 1995; Iacono et al., 1996). The irritating properties of the ethanol vehicle (or propyleneglycol) used in these studies required lidocaine anesthesia of airways prior to aerosol (Iacono et al., 1996). The CsA–DLPC liposome aerosol system described here represents an non-irritating alternative (Schreier et al., 1993; Gonzalez-Rothi and Schreier, 1995) which is devoid of local and systemic toxicity (Schreier et al., 1993;

Gonzalez-Rothi and Schreier, 1995). This has been recently demonstrated by our group in preliminary animal and human studies (Gilbert et al., 1997a,b).

Utilization of systemically delivered CsA for treatment of most pulmonary diseases has been limited due to ineffectiveness and the high incidence of side effects (Lynch and McCune, 1997). The CsA–liposome aerosol system described in this study represents an experimental method to test the potential effectiveness of targeted pulmonary immunosuppression and increased depot effects and potency, as reported for many drug–liposomal formulations (Cullis et al., 1989). Furthermore, we have recently developed high-dose CsA–liposome aerosol formulations developed to deliver increased therapeutic dosages to the lung as indicated for different disease processes (Waldrep et al., 1997a). Implimentation of CsA–liposome aerosol therapies of varied range and/or frequency may be required for effective treatment of chronic bronchiolar asthma and a variety of other pulmonary diseases, such as pulmonary sarcoidosis and allergic hypersensitivities. The effective therapeutic regimens for CsA–DLPC liposome aerosol use for each pulmonary disease indication can only be determined experimentally and/or clinically. Studies are in progress to assess these issues.

Table 1
Inhibition of antigen/mitogen-induced lymphocyte blastogenesis in vitro by CsA isolated from mouse lung tissues after aerosol delivery of CsA–DLPC liposomes

Antigen/mitogen	Average (cpm)	% inhibition
Media	2171 ± 630	—
OVA	$13\,272 \pm 1487$	—
OVA + CsA	2173 ± 458	99.9 ($p = 0.004$)
Media	1033 ± 250	—
ConA	$24\,341 \pm 11\,762$	—
ConA + CsA	3041 ± 1374	95 ($p = 0.07$)

OVA, ovalbumin (250 μg/ml); ConA, concanavalin A (1 μg/ml); cpm, [^{3}H]TdR counts per minute average of $3 \pm$ standard deviation. % inhibition = 1 − (antigen or mitogen + CsA (cpm)) − media (cpm)/(antigen or mitogen (cpm)) − media (cpm).

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References

Alexander, A.G., Barnes, N.C., Kay, A.B., 1992. Trial of cyclsporine in corticosteroid-dependent chronic severe asthma. Lancet 339, 324–328.

Atkinson, K., Boland, J., Britton, K., Biggs, J., 1983. Blood and tissue distribution of cyclosporin in humans and mice. Transplant. Proc. 15, 2430–2433.

Blot, F., Tavakoli, R., Sellam, S., Epardeau, B., Faurisson, F., Bernard, N., Becquemin, M.H., Frachon, I., Stern, M., Pocidalo, J.J., Carbon, C., Bisson, A., Caubarrere, I., 1995. Nebulized cyclosporine for prevention of acute pulmonary allograft rejection in the rat: Pharmacokinetic and histologic study. J. Heart Lung Transplant. 14, 1162–1172.

Charles, B.G., Norris, R.L.G., Ravenscroft, P.J., 1988. A modified assay for cyclosporin A in blood using solid phase extraction with high performance liquid chromatography. Ther. Drug Monit. 10, 97–100.

Cullis, P.R., Mayer, L.D., Bally, M.B., Madden, T.D., Hope, M.J., 1989. Generating and loading of liposomal systems for drug delivery systems. Adv. Drug Del. Rev. 3, 267–282.

Dowling, R.D., Zenati, M., Burckart, G.J., Yousem, S.A., Schaper, M., Simmons, R.L., Hardesty, R.L., Griffith, B.P., 1990. Aerosolized cyclosporine as single-agent immunotherapy in canine lung allografts. Surgery (discussion on pp. 204–205) 108, 198–204.

Farr, S.J., Kellaway, I.W., Parry-Jones, D.R., Woolfrey, S.G., 1985. 99m-Technetium as a markers of liposomal deposition and clearance in the human lung. Int. J. Pharm. 26, 303–316.

Gilbert, B.E., Six, H.R., Wilson, S.Z., Wyde, P.R., Knight, V., 1988. Small particle aerosols of enviroxime-containing liposomes. Antiviral Res. 9, 355–365.

Gilbert, B.E., Wilson, S.Z., Garcon, N.M., Wyde, P.R., Knight, V., 1993. Characterization and administration of cyclosporine liposomes as a small-particle aerosol. Transplantation 56, 974–977.

Gilbert, B.E., Black, M.B., Waldrep, J.C., Bennick, J.B., Montgomery, C., Knight, V., 1997a. Cyclosporin A liposome aerosol: lack of acute toxicity in rats. Inhalation Toxicol. 9, 717–730.

Gilbert, B.E., Knight, C., Alvarez, F.G., Waldrep, J.C., Rodarte, J.R., Knight, V., Eschenbacher, W.L., 1997b. Tolerance of volunteers to cyclosporin A-dilaurylphospha-

tidylcholine liposome aerosol. Am. J. Respir. Crit. Care Med. in press.

Gonzalez-Rothi, R.J., Schreier, H., 1995. Pulmonary delivery of liposome-encapsulated drugs in asthma therapy. Clin. Immunother. 4, 331–337.

Grit, M., Crommelin, D.J., 1992. The effect of aging on the physical stability of liposome dispersions. Chem. Phys. Lipids 62, 113–122.

Hung, O.R., Whynot, S.C., Varnel, J.R., Shafer, S.L., Mezel, M., 1995. Pharmacokinetics of inhaled liposome encapasulated Fentanyl. Anesthesiology 83, 277–284.

Iacono, A.T., Keenan, R.J., Duncan, S.R., Smaldone, G.C., Dauber, J.H., Paradis, I.L., Ohori, N.P., Grgurich, W.F., Burckart, G.J., Zeevi, A., Delgado, E., O'Riordan, T.G., Zendarsky, M.M., Yousem, S.A., Griffith, B.P., 1996. Aerosolized cyclosporine in lung recipients with refractory chronic rejection. Am. J. Respir. Crit. Care Med. 153, 1451–1455.

Keenan, R.J., Duncan, A.J., Yousem, S.A., Zenati, M., Schaper, M., Dowling, R.D., Alarie, Y., Burckart, G.J., Griffith, B.P., 1992. Improved immunosuppression with aerosolized cyclosporine in experimental pulmonary transplantation. Transplantation 53, 20–25.

Keenan, R.J., Iocono, A., Dauber, J.H., Zeevi, A., Yousem, S.A., Ohori, N.P., Burckhart, G.J., Kawai, A., Smaldone, G.C., Griffith, B.P., 1997. Treatment of refractory acute allograft rejection with aerosolized cyclosporine in lung transplant recipients. J. Thorac. Cardiovasc. Surg. 113, 335–341.

Kohler, D. and Fleischer, W., 1991. Established Facts in Inhalation Therapy. Archis Verlag, Munich, p. 48.

Lock, S.H., Kay, A.B., Barnes, N.C., 1996. Double-blind, placebo-controlled study of cyclosporin A as a corticosteroid-sparing agent in corticosteroid-dependent asthma. Am. J. Respir. Crit. Care Med. 153, 509–514.

Lynch, J.P., McCune, W.J., 1997. Immunosuppressive and cytotoxic pharmacotherapy for pulmonary disorders. Am. J. Respir. Crit Care Med. 155, 395–420.

Martinet, Y., Pinkerton, P., Saltini, C., Spurzem, J., Muller-Quernheim, J., Crystal, R., 1988. Evaluation of the in vitro and in vivo effects of cyclosporine on the lung T-lymphocyte alveolitis of active pulmonary sarcoidosis. Am. Rev. Respir. Dis. 138, 1242–1248.

Muggenburg, B.A., Hoover, M.D., Griffith, B.P., Haley, P.J., Snipes, M.B., Wolf, R.K., Yeh, H.C., Burckart, G.J., Mauderly, J.L., 1990. Administration of cyclosporine by inhalation: a feasibility study in beagle dogs. J. Aerosol Med. 3, 1–13.

Niven, R.W., Schreier, H., 1990. Nebulization of liposomes. I. Effects of lipid composition. Pharm. Res 7, 1127–1133.

O'Doherty, M.J., Miller, R.F., 1993. Aerosols for therapy and diagnosis. Eur. J. Nucl. Med. 20, 1201–1213.

O'Riordan, T.G., Duncan, S.R., Burckhart, G.J., Griffith, B.P., Smaldone, G.C., 1992. Production of an aerosol of cyclosporine as a prelude to clinical studies. J. Aerosol Med. 5, 171–177.

O'Riordan, T.G., Iacono, A., Keenan, R.J., Duncan, S.R., Burckart, G.J., Griffith, B.P., Smaldone, G.C., 1995. Delivery and distribution of aerosolized cyclosporine in lung allograft recipients. Am. J. Respir. Crit. Care Med. 151, 516–521.

O'Riordan, T.G., Waldrep, J.C., Abraham, W.M., Mao, Y., Sabater, J.R., Sieiczak, M., Knight, V., 1997. Delivery of nebulized budesonide liposomes to the respiratory tract of allergic sheep. J. Aerosol Med. in press.

Reed, C.E., 1991. Aerosol steroids as primary treatment of mild asthma. New Engl. J. Med. 325, 425–426.

Reid, M., Gibbons, S., Kwok, D., Van Buren, C.T., Flechner, S., Kahan, B.D., 1988. Cyclosporin levels in human tissues of patients treated one week to one year. Transplant. Proc. 15, 2434–2437.

Schreier, H., Gonzalez-Rothi, R.J., Stecenko, A.A., 1993. Pulmonary delivery of liposomes. J. Control. Release 24, 209–223.

Smaldone, G.C., Fuhrer, J., Steigbigel, R.T., McPeck, M., 1991. Factors determining pulmonary deposition of aerosolized pentamidine in patients with human immunodeficiency virus infection. Am. Rev. Respir. Dis. 143, 727–737.

Smaldone, G.C., Dickinson, G., Marcial, E., Young, E., Seymour, J., 1992. Deposition of aerosolized pentamidine and failure of pneumocystis prophylaxis. Chest 101, 82–87.

Szefler, S.J., 1991. Glucocorticoid therapy for asthma: clinical pharmacology. J. Allergy Clin. Immunol. 88, 147–165.

Taylor, K.M.G., Farr, S.J., 1993. Liposomes for delivery to the respiratory tract. Drug Dev. Ind. Pharm. 19, 123–142.

Tjandra-Maga, B., Verbesselt, R., Scharpe, S., Verkerk, R., Lambert, W.E., Liedekerke, B.V., DeLeenheer, A., 1990. Comparison of cyclosporin A measurement in whole blood by six different methods. J. Clin. Chem. Clin. Biochem. 28, 53–57.

Vidgren, M., Waldrep, J.C., Arppe, J., Black, M., Rodarte, J.A., Cole, W., Knight, V., 1994. A study of 99m technetium-labeled beclomethasone dipropionate dilauroylphosphatidylcholine liposome aerosol in normal volunteers. Int. J. Pharm. 115, 209–216.

Waldrep, J.C., Scherer, P.W., Keyhani, K., Knight, V., 1993. Cyclosporin A liposome aerosol: particle size and calculated respiratory deposition. Int. J. Pharm. 97, 205–212.

Waldrep, J.C., Keyhani, K., Black, M., Knight, V., 1994a. Operating characteristics of 18 different continuous-flow jet nebulizers with beclomethasone dipropionate liposome aerosol. Chest 105, 106–110.

Waldrep, J.C., Scherer, P.W., Hess, G.D., Black, M., Knight, V., 1994b. Nebulized glucocorticoids in liposomes: aerosol characteristics and human dose estimates. J. Aerosol Med. 7, 1994.

Waldrep, J.C., Arppe, J., Jansa, K.A., Knight, V., 1997a. High dose cyclosporin A and budesonide-liposome aerosols. Int. J. Pharm. 152, 27–36.

Waldrep, J.C., Gilbert, B.E., Knight, C.M., Black, M.B., Scherer, P., Knight, V., Eschenbacher, W., 1997b. Pulmonary delivery of beclomethasone liposome aerosol in volunteers. Chest 111, 316–323.