J. Pharm. Pharmacol. 1997, 49: 960–971
Received April 2, 1997
Accepted July 11, 1997

Nebulized Interleukin 2 Liposomes: Aerosol Characteristics and Biodistribution

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Abstract

Although interleukin 2 (IL-2) has been associated with modest anti-tumour responses in man, treatment-related toxicity has limited its widespread use. The local delivery of liposomal formulations of interleukin 2 to the lung as aerosols has been demonstrated to be non-toxic, biologically active, and associated with regression of spontaneous pulmonary metastases in dogs. This study was undertaken to evaluate the physical and biological characteristics of nebulized interleukin 2 liposomes.

The aerosol droplet size distribution and the physical stability of interleukin 2 liposomes were examined in-vitro using an Andersen cascade impactor and studies of liposome entrapment of interleukin 2 before and after nebulization. The biological stability of interleukin 2 liposomes after nebulization was demonstrated using the CTLL-2 bioassay for interleukin 2. In-vivo studies of pulmonary biodistribution and clearance of inhaled technetium (^{99m}Tc)-labelled interleukin 2 liposomes were undertaken in a normal dog. Aerosols of free interleukin 2 and of interleukin 2 liposomes were compared in both in-vitro and in-vivo experiments. The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of interleukin 2 liposomes were 1·98 μm and 2·02, respectively. Independent analysis of aerosol particle-size distribution using the constitutive components of the interleukin 2 liposomes (interleukin 2 : lipid : HSA) demonstrated a close correlation of size distributions ($r = 0{\cdot}9445$; $P < 0{\cdot}001$). The entrapment of interleukin 2 in liposomes was $93 \pm 4{\cdot}3\%$ before nebulization and $90 \pm 8{\cdot}9\%$ after. After delivery to an anaesthetized dog, interleukin 2 liposome aerosols were deposited evenly throughout the lung (mean ± s.d. central lung-to-peripheral lung deposition was $1{\cdot}12 \pm 0{\cdot}03$). After approximately 24 h inhalation, interleukin 2 liposomes were retained within the lung and were taken up in part by the spleen.

The results of this study are indicative of the stability of this interleukin 2 liposome formulation to nebulization. Such nebulization might be an attractive immunotherapeutic strategy for treatment of pulmonary metastases and primary lung cancers.

Interleukin 2 (IL-2) immunotherapy has been associated with modest anti-tumour effects in both human and animal tumours. Widespread use of interleukin 2 has been limited by its narrow therapeutic index especially when administered in high-dose intravenous protocols (Anderson et al 1992a; Menzel et al 1993; Guida et al 1995). Toxicities associated with intravenous interleukin 2 have included fever, pulmonary vascular leakage, weight gain and anasarca, malaise, rigors, azotaemia, anaemia and thrombocytopaenia (Siegal & Puri 1991; Smith 1993). Adverse effects of interleukin 2 are dependent on the dose, route of delivery, and formulation (Anderson et al 1992b; Maas et al 1993).

Nebulization of a number of immunomodulators and chemotherapeutics has been described (Maasilta et al 1991; Waldrep et al 1993; Hodson 1995; Knight & Waldrep 1995). Aerosol therapy results in high pulmonary drug concentrations and relatively low systemic uptake of drug, thereby increasing the pulmonary therapeutic index of inhaled agents (Newman & Clarke 1983; Knight & Waldrep 1995). Inhalation of free human interleukin 2 has been shown to be effective against tumours and non-toxic in both animal models and in man (Huland et al 1992, 1994; Khanna et al 1994; Zhang & Liu 1994; Lorenz et al 1996).

Liposomal formulations of drugs have been used to reduce toxicity, to effect more favourable pharmacokinetics, and to maximize tissue targeting of several agents (Juliano & McCullough 1980; Ostro & Peiter 1989; Anderson et al 1994). Liposomal formulations of interleukin 2 have been shown to increase the circulating half-life of interleukin 2, to target tissues of the immune system (spleen, lymph nodes, bone marrow) and lungs, and to reduce toxicity, including pulmonary vascular leakage (Anderson et al 1990, 1992a; Bergers et al 1993; Kedar et al 1994). Nebulized liposomal formulations of cytarabine, beclomethasone and cyclosporin increase pulmonary drug deposition and extend pulmonary retention compared with aerosols of the free drug (Niven & Schreier 1990; Niven et al 1991; Schreier et al 1993; Knight & Waldrep 1995). Liposomal formulations of hydrophobic therapeutic agents such as interleukin 2 have been shown to be better suited to nebulization than non-liposomal formulations of the same agent (O'Riordan et al 1992).

It has been our interest to combine a liposomal formulation of interleukin 2 with aerosol delivery to provide an effective and non-toxic anti-cancer treatment for the lung (pulmonary metastases and primary lung cancers). We have demonstrated the safety and biological activity of inhaled liposomal for-

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mulations of interleukin 2 in normal dogs (Khanna et al 1996). Normal dogs receiving aerosols of interleukin 2 liposomes had significantly greater pulmonary immune activation (increased bronchoalveolar lavage cellular infiltration and increased tumour cytolysis by bronchoalveolar lavage effector cells) compared with dogs receiving aerosols of free interleukin 2, empty liposomes, or saline (Khanna et al 1996). On the basis of these results, dogs with spontaneously occurring pulmonary metastases and primary lung tumours were treated with inhaled interleukin 2 liposomes. In these tumour-bearing dogs, the lack of toxicity and anti-tumour activity of inhaled interleukin 2 liposomes was demonstrated (Khanna et al 1997).

In this report we characterize the physical and biological properties of inhaled interleukin 2 liposome aerosols. The specific objectives of this study included: documentation of the aerosol particle size of nebulized interleukin 2 liposomes; evaluation of the structural and biological stability of interleukin 2 liposomes to nebulization; and demonstration of the pulmonary deposition of nebulized interleukin 2 liposomes. The physical and biological characteristics of nebulized interleukin 2 liposomes were compared with those of aerosols of free interleukin 2 to determine if differences in these characteristics might explain the marked advantage in pulmonary anti-tumour immune activation associated with inhaled interleukin 2 liposomes over inhaled free interleukin 2 (Khanna et al 1996). The findings of these studies suggest that the nebulization of interleukin 2 liposomes results in physically and biologically stable aerosols that are highly respirable, deposit throughout the ventilated lung, and might then distribute to immune centres. Distribution of the inhaled interleukin 2 liposomes from the lung to immune centres was not demonstrated for aerosols of free interleukin 2 and might in part explain the previously demonstrated advantage of inhaled interleukin 2 liposomes over free interleukin 2.

Materials and Methods

Interleukin 2 formulations

Free interleukin 2 was an *Escherichia coli*-produced non-glycosylated human recombinant interleukin 2. Interleukin 2 liposomes were kindly provided by Biomira USA (formerly OncoTherapeutics; Cranbury, NJ) or prepared using identical techniques by the authors. This liposome preparation contains a synthetic lipid, dimyristoylphosphatidyl choline (DMPC; Avanti Polar Lipids, Alabaster, AL), human serum albumin (HSA; Baxter, Glenville, CA), and human recombinant interleukin 2 of the natural sequence (Hoffman LaRoche, Nutley, NJ; tecelleukin; specific activity $1{\cdot}4 \times 10^7$ international units mg^{-1}). Each millilitre of interleukin 2 liposomes contained 40 mg DMPC, $2{\cdot}0 \times 10^6$ international units interleukin 2 and 3·4 mg HSA. The method of preparation and size distribution of these multilamellar vesicles and their biological activity in the CTLL-2 interleukin 2 bioassay have been previously reported (Anderson et al 1990, 1992a, 1994).

Nebulization of interleukin 2 liposomes and free interleukin 2

Nebulization involved the use of a Bunn 400A compressor (John Bunn, Happauge, NY) operating at a flow rate of 6 L min^{-1} and a Puritan Bennet (Carlsbad, CA) twin-jet nebulizer. Interleukin 2 liposomes (1×10^6 international units interleukin 2 in 20 mg DMPC; 0·5 mL) were diluted in 3·5 mL sodium chloride (NaCl). Free interleukin 2 was diluted in 4·0 mL NaCl at $2{\cdot}5 \times 10^5$ international units mL^{-1}.

The efficiency of nebulization

The efficiency of nebulization of interleukin 2 liposomes and free interleukin 2 was determined by analysis of the mass of interleukin 2 liposomes or of free interleukin 2 solution delivered from the nebulizer, and of the amount of interleukin 2 (measured by ELISA; Quantikine, R&D Systems, Minneapolis, MN) delivered from the nebulizer.

Efficiency of delivery by mass. This was determined from the mass of diluted interleukin 2 liposomes or free interleukin 2 formulation in the nebulizer before nebulization relative to the mass retained in the nebulizer, nebulizer apparatus and tubing after nebulization.

Efficiency of interleukin 2 delivery. This was determined from the total amount of interleukin 2 (measured by ELISA) in the nebulizer bowl before nebulization relative to the amount of interleukin 2 remaining in the nebulizer, nebulizer apparatus and tubing after nebulization.

Characterization of aerosol size distribution

Characterization of the size distribution of aerosols of interleukin 2 liposomes and free interleukin 2 was undertaken with an Andersen 1 ACFM non-viable ambient particle sizing sampler (Andersen Instruments, Atlanta, GA) multistage cascade impactor. The Puritan Bennet twin-jet nebulizer, powered by the Bunn 400A air-compressor, was connected to one arm of a glass 'Y'-piece. The stem of the Y-piece was placed on top of the Andersen impactor. A vacuum pump (Graseby, Atlanta, GA) operated at 28·3 L min^{-1} was connected at the downstream end of the Andersen impactor. The remaining free arm of the Y-piece was open to room air, enabling air-pressure equilibration. Aerosol droplets from $<0{\cdot}4$ to 10·0 μm were collected (impacted) on eight impactor stages reflecting aerosol-droplet effective cut-off diameters (stage 0, 9·0-10·0 μm; stage 1, 5·8–9·0 μm; stage 2, 4·7–5·8 μm; stage 3, 3·3–4·7 μm; stage 4, 2·1–3·3 μm; stage 5, 1·1–2·1 μm; stage 6, 0·7–1·1 μm; stage 7, 0·4–0·7; filter stage $<0{\cdot}4$ μm). After delivery of a 2-mL volume of interleukin 2 liposomes or free interleukin 2 solution, the stainless steel Andersen impactor plates were lavaged with high-pressure NaCl (10 mL) and scraped using a sterile cell lifter (Costar, Cambridge, MA). Each sizing experiment was performed in triplicate. Material collected from plate washings were divided and stored at −20°C and −70°C for later analysis of interleukin 2 concentration, interleukin 2 bioactivity, HSA concentration and DMPC concentration. The efficacy of the plate-lavage technique was verified by placing the previously lavaged plates in a 10-mL bath of 90% ethanol for 10 min and measuring the residual protein concentration (DU-64 Spectrophotometer, Beckman, Schaumberg, MA) in the ethanol samples. The protein content of Andersen plates processed using the lavage technique was no greater than that of a clean impactor plate lavaged, scraped, and then placed in an ethanol bath, thereby demonstrating the efficacy of the technique.

Analysis of interleukin 2 liposome components and free interleukin 2

Interleukin 2 concentration. A commercially available ELISA for human interleukin 2 (Quantikine, R&D Systems, Minneapolis, MN) was used to determine interleukin 2 concentration from samples stored at −70°C.

Biological activity of interleukin 2. Interleukin 2 bioactivity was determined using the CTLL-2 bioassay (Anderson et al 1989) from samples stored at −20°C.

HSA concentration. The Micro BCA Protein Assay (Pierce, Rockford, IL) was used to determine HSA concentration. Liposomes were dissolved in Triton 100-X (Sigma, St Louis, MO) before HSA analysis. The contribution of interleukin 2 to the protein concentration calculated by this assay was in the pg mL^{-1} range and thought to be negligible relative to the HSA concentrations (which were in the $\mu g\ mL^{-1}$ range).

DMPC concentration. A modification of the HPLC protocol of Grit & Crommelin (1992), was utilized to measure DMPC concentration. Briefly, a Waters 717 WISP automatic sample injector and Waters Nova-Pak Silica column (15 cm × 3·9 mm, 4 μm; Waters, Milford, MA) were used with acetonitrile–methanol–water (64:28:8, v/v) as mobile phase. Peaks were detected with a evaporative detector (Sedex 55, Sedere, France) and quantified by means of Water's Millennium 2010 Chromatography Manager (Version 2·15). The DMPC standard was obtained from Avanti Polar Lipids (Alabaster, AL). Samples for analysis were dissolved in methanol (to dissolve the liposomes). The limit of HPLC detection was 100 ng DMPC.

Mass median aerodynamic diameter and geometric standard deviation

Mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were independently calculated using the percent interleukin 2, HSA and DMPC delivered to the Andersen impactor stages. The amount of interleukin 2 delivered to each stage relative to the total amount of interleukin 2 delivered from the nebulizer to the Andersen impactor enabled determination of the percent interleukin 2 on each impactor stage. The percentage of interleukin 2 impaction on each impactor plate (within an effective cut off diameter) was established. The distribution of interleukin 2 within aerosol droplets was determined for free interleukin 2 and interleukin 2 liposomes. Using the material collected from each Andersen stage after nebulization of interleukin 2 liposomes, the percent HSA and DMPC within an effective cut off diameter for particle size was also determined. The MMAD and GSD were determined using KaleidaGraph (Version 2·0, Synergy Software) as previously described (Waldrep et al 1993). The validity of the Andersen sampler methodology for calculating the MMAD and GSD has previously been verified using a laser particle-sizer (Waldrep et al 1993).

Interleukin 2 liposome physical stability

The physical stability of interleukin 2 liposomes after aerosol formation was evaluated by assessment of the percent liposomal entrapment of interleukin 2 before and after nebulization. Microcon 30 microconcentrators (Amicon, Beverly, MA) were used to separate liposome-entrapped interleukin 2 from the non-entrapped (free) interleukin 2 (MW 15 000) in the starting interleukin 2 liposome solution and in interleukin 2 liposomes collected after nebulization, delivered to the Andersen impactor plates (stage 5, 1·1–2·1 μm; stage 6, 0·7–1·1 μm; stage 7, 0·4–0·7 μm). Percent entrapment was calculated from:

$$\text{entrapment} = [\text{liposome-entrapped interleukin 2 (retentate)}/(\text{liposome-entrapped interleukin 2} + \text{free interleukin 2 (filtrate)})] \times 100$$

The physical stability of interleukin 2 liposomes after nebulization was supported by demonstrating high correlation of aerosol particle-size distribution determined by interleukin 2, HSA and DMPC impaction to the Andersen impactor. The amount of interleukin 2, HSA, or DMPC delivered to an impactor plate was expressed as a percentage of the total collected from all Andersen impactor plates. Correlation analysis was performed using percent interleukin 2 compared with percent DMPC, percent interleukin 2 compared with percent HSA, and percent DMPC compared with percent HSA delivered to each impactor plate.

Interleukin 2 aerosol biological stability

The biological activity (stability) of interleukin 2 liposomes and free interleukin 2 after nebulization was assessed by use of an all-glass impinger (ACE Glass, Vineland, NJ). Aerosols of interleukin 2 liposomes and free interleukin 2 were sampled by use of the impinger with an inflow rate of air of 12·5 L min^{-1}. The concentration of interleukin 2 in the nebulizer reservoir bowl and captured by the all-glass impinger from the nebulizer was determined by using the CTLL-2 bioassay.

Technetium (^{99m}Tc) radiolabelling

Radiolabelling of the interleukin 2 liposomes was undertaken using a technique described elsewhere (Vidgren et al 1995). Briefly, 1·0 mL of 0·68 mg mL^{-1} stannous chloride ($SnCl_2$; Merck, Darmstadt, Germany) solution in MilliQ (Millipore, Bedford, MA) water purged of oxygen (bubbled for 30 min with helium, then for 30 min with nitrogen, and finally for another 30 min with helium) was added to 0·5 mL DMPC interleukin 2 liposomes and vortex-mixed for 1 min. Technetium (^{99m}Tc; 50 mCi) was added to the stannous chloride-interleukin 2 liposome mixture and vortex-mixed for 1 min. The total volume was adjusted to 4·0 mL by addition of NaCl, vortex-mixed for 1 min, and then rocked for 30 min to establish equilibrium. Free interleukin 2 was labelled using the same methodology, except 1·0 mL of the stannous chloride reagent was added to $1{\cdot}0 \times 10^6$ international units of interleukin 2 (in 0·5 mL saline) and vortex-mixed for 1 min.

[^{99m}Tc]Interleukin 2 liposome label: efficiency and stability

The labelling efficiency and stability of the [^{99m}Tc]interleukin 2 liposome label was determined by comparing free ^{99m}Tc with entrapped (liposome associated) ^{99m}Tc by thin layer chromatography (TLC; ITLC-SG, Gelman Sciences, Ann Arbor, MI). [^{99m}Tc-interleukin liposomes (1 mL) were placed on a silica gel chromatography paper strip above a 0·9% NaCl water bath. The counts min^{-1} detected at the advancing NaCl front represent the free (non-labelled) ^{99m}Tc whereas the counts min^{-1} at the point of origin represent the liposome-

associated (labelled) ^{99m}Tc fraction. The labelling efficiency was determined from:

labelling efficiency = 100 × [liposome-labelled ^{99m}Tc/(liposome-labelled ^{99m}Tc + free ^{99m}Tc)]

The stability of labelling over time was assessed by determining the labelling efficiency 0 min, 20 min, 40 min, 1 h, 2 h, 8 h and 24 h after labelling. The labelling efficiency after nebulization of [^{99m}Tc]interleukin 2 liposomes was assessed by collecting nebulized labelled interleukin 2 liposomes in an all-glass impinger. The effect of ^{99m}Tc-labelling of interleukin 2 liposomes on the aerosol droplet size and size distribution (MMAD and GSD) was determined by use of the Andersen impactor. Andersen impactor plates were individually placed 10 cm above the centre of a gamma camera (Pho/Gamma LFOV, Searle, Des Plaines, IL) and the emitted counts min^{-1} measured from each plate. The MMAD and GSD of [^{99m}Tc]interleukin 2 liposomes were determined from the counts min^{-1} emitted from each impactor plate, as described above. The particle-size distribution, calculated from radioactivity counts, was then correlated with the particle-size distribution calculated from the interleukin 2 concentration, as described above. The labelling efficiency of ^{99m}Tc-labelled free interleukin 2 was undertaken as described for interleukin 2 liposomes.

In-vivo nebulization of [^{99m}Tc]interleukin 2 formulations

Radiolabelled interleukin 2 liposomes and free interleukin 2 were nebulized to a 20-kg female mixed-breed dog, anaesthetized with a butorphanol premedication (0·4 mg kg^{-1} i.m., Torbugesic, Fort Dodge, IA), thiopental (8 mg kg^{-1}, thiopental sodium, Gensia Labs, Irvine, CA) induction, and halothane (Halocarbon Labs, River Edge, NJ) maintenance as needed. Respiration was controlled by use of a mechanical ventilator (Ohio Veterinary Anesthesia Ventilator, Ohio Medical Products, Madison, WI) with a tidal volume of 650 mL, inspiratory flow pressure of 15 mm Hg, and a respiratory rate of 16 breaths min^{-1}. Radiolabelled interleukin 2 liposomes were delivered to the anaesthetized dog, by use of the nebulization equipment described above, through an endotracheal tube. Static images of the lung were taken at the start of nebulization and every 5 min until the completion of nebulization (16–20 min) in a ventro-dorsal projection, using the gamma camera. After delivery of the radiolabelled interleukin 2 liposomes, static images (in dorso-ventral and lateral projections) were taken 10, 20, 30, 60, 90 and 120 min after nebulization. General anaesthesia was discontinued 3 h after nebulization. Dorso-ventral projections, only, were then taken 3, 5, 7, 9, 13·5, 19·5 and 22·5 h after nebulization. Total lung counts min^{-1} and counts min^{-1} from defined regions of interest within the lung were documented at the indicated time-points and back-calculated for decay. Counts min^{-1} emitted from 0·1 mL whole blood were recorded at least as often as scintigraphic images were obtained. The whole-blood counts min^{-1} measured during and after nebulization were back-calculated for decay. Identical studies to those described for radiolabelled interleukin 2 liposomes were performed using inhaled ^{99m}Tc-labelled free interleukin 2. The inhalation studies with radiolabelled interleukin 2 liposomes and free interleukin 2 were repeated in triplicate. Inhalation studies using free [^{99m}Tc]pertechnate and [^{99m}Tc]diethylenetriaminepentaacetic acid (DTPA) were performed as controls. The same dog was used for all inhalation studies to eliminate the effects of anatomical differences among different dogs. The dog was isolated in a radioactive isolation area for animals within the University of Minnesota Veterinary Teaching Hospital (UM-VTH) for 72 h after each inhalation study.

All procedures involving animals were reviewed and approved by the Animal Care Committee and the Department of Radiation Protection at the University of Minnesota.

Statistical analysis

Descriptive statistics and comparisons of differences between means of data sets were calculated by use of InStat software (Macintosh version; Graph Pad Software, San Diego, CA). If standard deviations of compared data sets were equal an unpaired Students *t*-test was used. If standard deviations of data sets were unequal, an alternate Welch *t*-test was used. The correlation coefficient and its 95% confidence interval were determined using a Pearson line-correlation statistic. Statistically significant differences in data sets were defined by $P < 0.05$.

Results

Efficiency of delivery of nebulized interleukin 2 liposomes and free interleukin 2

Highly efficient delivery of interleukin 2 liposomes and free interleukin 2 was achieved by use of the nebulization conditions described. The mean efficiency of nebulization by mass was 91·4% for interleukin 2 liposomes and 92·9% for free interleukin 2 ($P = 0.20$). The mean efficiency of nebulization by interleukin 2 delivery was 89·3% for interleukin 2 liposomes and 82% for free interleukin 2 ($P = 0.39$). Thus at least 80% of the interleukin 2 within the nebulizer bowl was converted to aerosol.

Aerosol size distribution for interleukin 2 liposomes and free interleukin 2

Fig. 1 shows the aerosol particle-size distribution, established from the mean percent delivery of interleukin 2 to impactor stages, for nebulized interleukin 2 liposomes and free interleukin 2. The mean MMAD and GSD for aerosols of nebulized interleukin 2 liposomes were 1·98 μm and 2·02, respectively. The mean MMAD and GSD for aerosols of nebulized free interleukin 2 were 1·81 μm and 1·97, respectively. The MMAD for aerosols of free interleukin 2 was statistically smaller than that of interleukin 2 liposomes ($P = 0.02$); however, it is thought unlikely that this difference will be reflected in a significant difference between the biological effects of the aerosols. The mode aerosol droplet diameter for aerosols both of interleukin 2 liposomes and free interleukin 2 was 1·1 μm; over 90% of the aerosol droplets were less than 5·0 μm in diameter (Fig. 1). Given that the GSD for both nebulized interleukin 2 liposome and free interleukin 2 was greater than 1·0, they are described as heterodisperse aerosols. The difference in the GSD for interleukin 2 liposomes compared with free interleukin 2 was not statistically significant ($P = 0.86$).

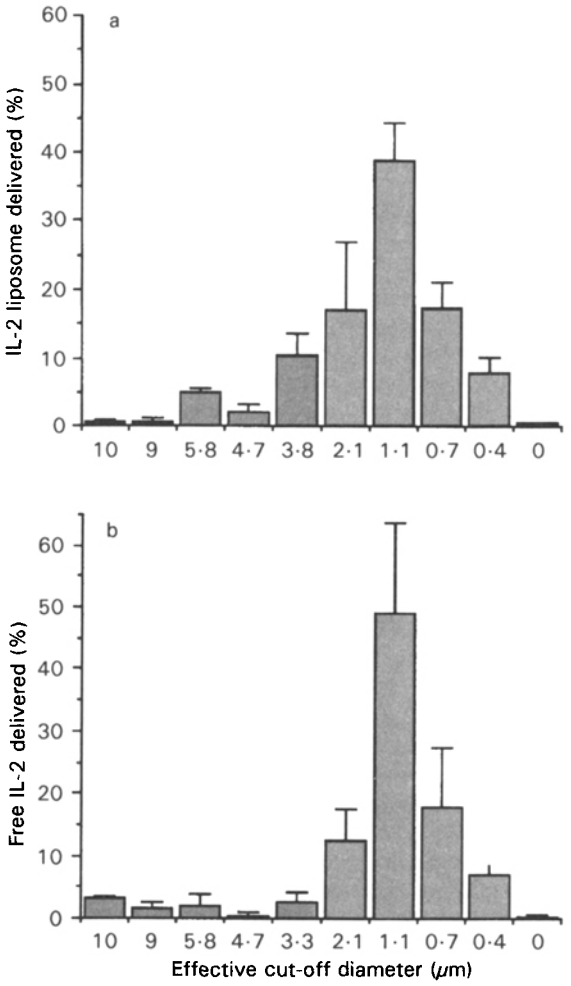


FIG. 1. Nebulization of interleukin 2 liposomes and free interleukin 2 results in highly respirable aerosol particles. The aerosol particle distribution of nebulized interleukin 2 liposomes (a) and nebulized free interleukin 2 (b) are similar. The aerosol size distribution is heterogeneous with a mode particle size of 1·1 μm. Aerosols of interleukin 2 liposomes and free interleukin 2 were delivered to an Andersen impactor and the impacted material was collected from the stainless steel impactor plates (each representing a specific aerosol size effective cut-off diameter) and analysed by ELISA for human interleukin 2.

The physical stability of interleukin 2 liposomes to aerosol formation

The liposome entrapment of interleukin 2 before nebulization was demonstrated to be 93 ± 4·3%. Nebulized interleukin 2 liposomes collected from the impactor plates, had a liposome entrapment of interleukin 2 of 90 ± 8·9%. The aerosol particle-size distribution, determined using the Andersen impactor, of interleukin 2 liposomes based on interleukin 2, HSA and DMPC impaction is presented in Fig. 2. MMAD/GSD for the interleukin 2 liposome aerosol, established from DMPC and HSA analysis were 1·93 μm/1·66 and 2·11 μm/2·57, respectively. The similarity in aerosol particle size of interleukin 2 liposomes, determined by independent analysis of the interleukin 2 liposome components (interleukin 2, DMPC, and HSA), was confirmed by linear regression analysis (Fig. 3). The correlation coefficient of the interleukin 2 liposome aerosol particle-size distribution as determined by interleukin 2 impaction compared with DMPC impaction was high ($r = 0.9621$; $P = 0.0001$). A similarly high correlation of the interleukin 2 liposome aerosol particle-size distribution was seen for interleukin 2 impaction compared with HSA impaction ($r = 0.9888$; $P = < 0.0001$), and HSA impaction compared with DMPC impaction ($r = 0.9216$; $P = 0.002$). Three-way linear correlation analysis for aerosol particle-size distribution established by interleukin 2, DMPC, and HSA impaction again supported the stability of interleukin 2 versus DMPC versus HSA association after nebulization ($r = 0.9445$; $P < 0.0001$). These results suggest that the components of the interleukin 2 liposome remain intact after nebulization.

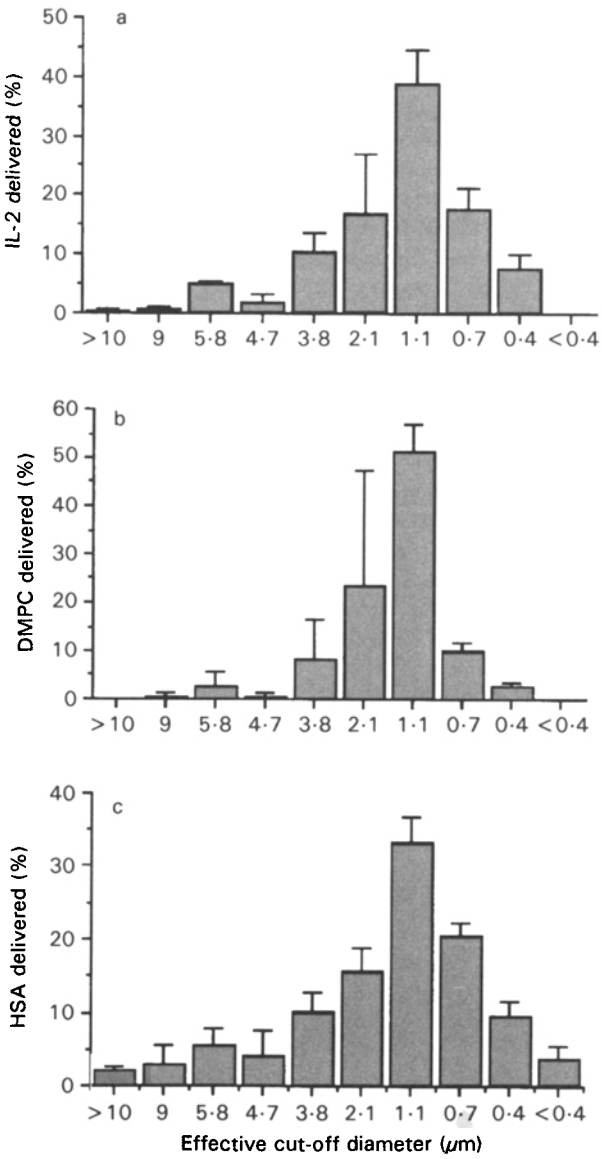


FIG. 2. The similarity in aerosol particle-size distributions determined by independent analysis of the interleukin 2 liposome components (a. interleukin 2, b. DMPC, c. HSA) after interleukin 2 liposome nebulization. Nebulized interleukin 2 liposomes were delivered to an Andersen impactor and the impacted material was collected from the stainless steel impaction plates was analysed independently for interleukin 2 concentration (using ELISA), DMPC concentration by HPLC, and HSA concentration by calorimetric protein analysis. Data are presented as the percent material impacted on each plate divided by the total material delivered to the impactor.

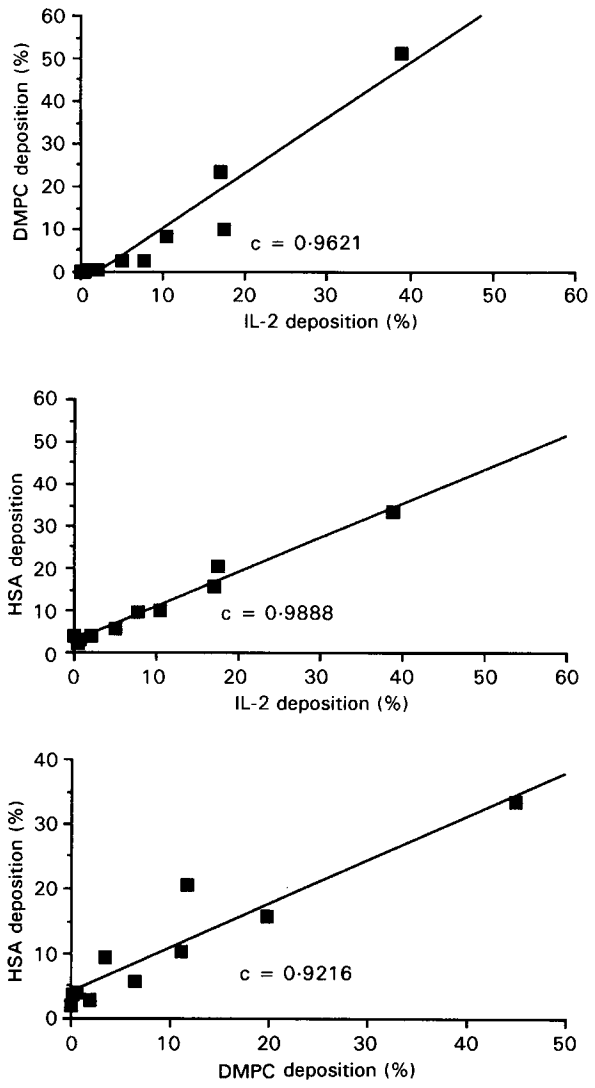


FIG. 3. The high correlation between aerosol particle-size distributions determined by a. interleukin 2 and DMPC, b. interleukin 2 and HSA, c. DMPC and HSA impaction after interleukin 2 liposome nebulization. Interleukin 2 liposomes were nebulized to an Andersen impactor. Each data point represents the material removed from a single stage of the Andersen impactor, analysed for interleukin 2 concentration (using ELISA), DMPC concentration by HPLC, and HSA concentration by calorimetric protein analysis. Data are presented as a scatter plot of the mean percent deposition on each impactor plate joined by a line of best fit.

The biological activity of nebulized interleukin 2 liposomes and free interleukin 2

The CTLL-2 bioassay was used to demonstrate the biological activity of interleukin 2 liposomes and free interleukin 2 after nebulization. Interleukin 2 biological activity was demonstrated in material captured by the all-glass impinger during nebulization, and in the nebulizer bowl after nebulization of interleukin 2 liposomes. Comparison of the interleukin 2 concentration, as determined by ELISA and by CTLL-2 bioassay, suggested that the biological activity of interleukin 2 liposomes did not significantly decline during nebulization. Identical analyses of samples of free interleukin 2 demonstrated the biological stability of free interleukin 2 during nebulization.

^{99m}Tc radiolabelling of interleukin 2 liposomes and free interleukin 2 protein

The mean labelling efficiency (^{99m}Tc association) was greater than 98% for interleukin 2 liposomes and greater than 99% for free interleukin 2. This high labelling-efficiency remained unchanged with time (0 min, 20 min, 40 min, 1 h, 2 h, 8 h and 24 h after labelling) and was not affected by nebulization (samples analysed after nebulization to the impinger). The MMAD/GSD values of radiolabelled interleukin 2 liposomes and radiolabelled free interleukin 2, as determined by quantification of the counts min^{-1} emitted from each Andersen impactor plate, were 1·68 μm/2·12 and 1·52 μm/2·14, respectively. There was high correlation between aerosol particle-size distribution for [^{99m}Tc]interleukin 2 liposomes and [^{99m}Tc] free interleukin 2, determined as counts min^{-1}, with the aerosol-particle distribution determined by measurement of interleukin 2 content (interleukin 2 liposomes: $r=0·9195$, $P=0·0002$; free interleukin 2: $r=0·9177$, $P=0·0002$). The aerosol particle size of [^{99m}Tc]interleukin 2 liposomes was similar to that of the unlabelled interleukin 2 liposome aerosols.

Nebulized interleukin 2 liposomes and free interleukin 2 distribute throughout the lung with prolonged pulmonary retention

Distribution of interleukin 2 liposomes and free interleukin 2 throughout the lung was seen immediately after nebulization. Retention within the lung approaching 24 h after nebulization was evident both for interleukin 2 liposomes and for free interleukin 2. Fig. 4 shows scintigraphic images obtained after nebulization of interleukin 2 liposomes and free interleukin 2 to a dog. At the completion of nebulization the images reveal aerosol deposition to the central and peripheral regions of the lung, with excellent retention in the lung periphery. The ratio of deposition in the central third of the ventilated lung region to deposition in the peripheral (i.e. non-central) ventilated lung regions was $1·12 \pm 0·03$ and $1·11 \pm 0·225$ (mean $\pm$ s.d.) for nebulized interleukin 2 liposomes and free interleukin 2, respectively. Clearance of radiolabelled interleukin 2 liposomes and free interleukin 2 from the lung was very slow (Fig. 5; extending over 22 h) compared with complete clearance seen after inhalation of free [^{99m}Tc]pertechnate (complete pulmonary clearance at 1 h) and after inhalation of DTPA (complete pulmonary clearance at 4 h). The elimination half-lives, $t_{\frac{1}{2}}$, of the final phase of pulmonary clearance of interleukin 2 liposomes and free interleukin 2 were 39 h and 46 h, respectively. Scintigraphic images taken 22 h after inhalation of [^{99m}Tc]interleukin 2 liposomes reveal persistence of the radiolabel within the lung and accumulation in part in the spleen (Fig. 4). Splenic accumulation was not seen after inhalation of ^{99m}Tc-labelled free interleukin 2 (Fig. 4).

Discussion

The findings of these studies suggest that aerosols of interleukin 2 liposomes are efficiently generated, physically and biologically stable, highly respirable, and seem to be retained in the lung after nebulization. More rapid pulmonary clearance and potential targeting of interleukin 2 liposomes to immune centres might in part explain the significant advantage of local (pulmonary) immune activation associated with inhaled inter-

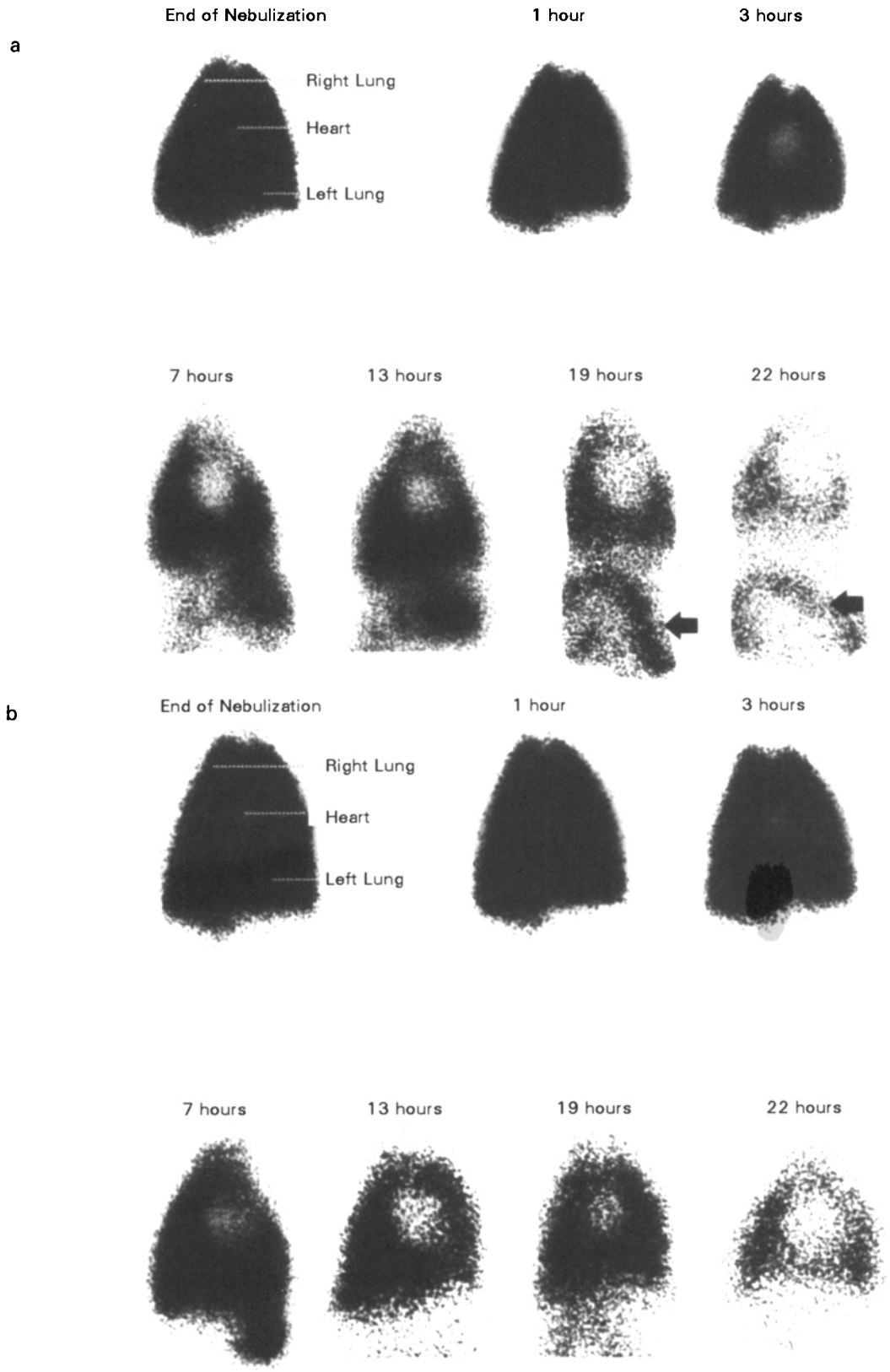
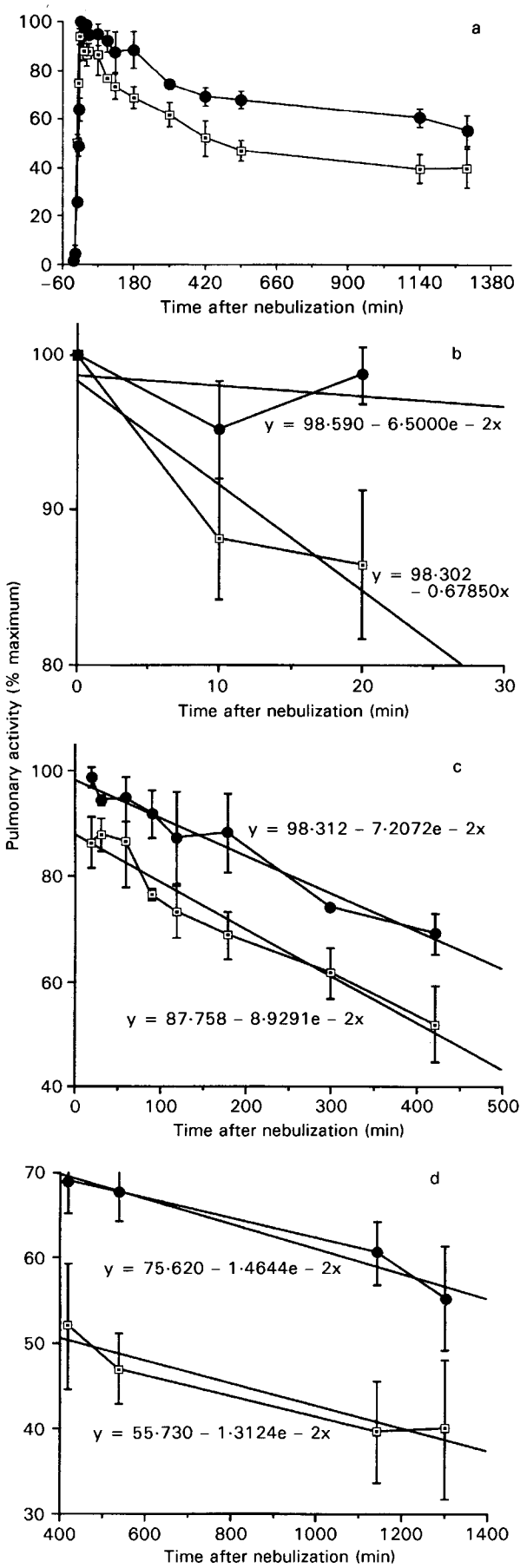


FIG. 4. Nebulized interleukin 2 liposomes and free interleukin 2 distribute throughout the ventilated lung after nebulization. Pulmonary scintigraphic images were generated after nebulization of [^{99m}Tc]interleukin 2 liposomes (a) and free [^{99m}Tc]interleukin 2 (b) to a normal anaesthetized dog. Static images were accrued over a 2-min acquisition time. The mean ($\pm$s.d.) central-to-peripheral deposition ratio of radiolabelled interleukin 2 liposomes and free interleukin 2 at the completion of nebulization was $1{\cdot}12 \pm 0{\cdot}03$ and $1{\cdot}11 \pm 0{\cdot}225$, respectively. Images acquired after nebulization of interleukin 2 liposomes for 22 h demonstrate accumulation in the lung and in the spleen. Images acquired after 22 h nebulization of free interleukin 2 demonstrate accumulation in the lung but not in the spleen.



leukin 2 liposomes compared with inhaled free interleukin 2 previously observed (Khanna et al 1996). There were no other differences between the physical or biological properties of the interleukin 2 liposome aerosol and those of the aerosol of free interleukin 2. Nebulized interleukin 2 liposomes might offer an attractive local immunotherapeutic strategy for the treatment of both pulmonary metastases and primary lung cancers.

The process of jet nebulization involves repeated cycles of aerosol formation and drug recapture in the nebulizer bowl before eventual delivery of the aerosol mist from the nebulizer. The drug within the nebulizer bowl is drawn towards a stationary bead in the high-speed nebulizer jet stream. Impaction with this bead then results in the generation of an aerosol. The created aerosol will either leave the nebulizer apparatus or, more likely, contact the housing of the nebulizer, coalesce as a droplet and then return to the nebulizer bowl (Martin 1990; Taylor et al 1990). This cycle is often repeated several times before the drug leaves the nebulizer. During the repeated cycles of aerosol formation considerable shearing forces are applied to the starting drug solution (Taylor et al 1990). A potential concern during nebulization of liposomal drug formulations, might be that shearing forces will result in the dissociation of the liposome from the drug (the DMPC liposome from the interleukin 2). Studies of the entrapment of interleukin 2 by the liposome before and after nebulization demonstrate the maintenance of high entrapment of the drug by the liposome, and support the physical stability of interleukin 2 liposomes during nebulization. The high correlation of aerosol particle size and aerosol particle-size distribution, established by independent analysis of the constitutive components of the interleukin 2 liposome (interleukin 2, DMPC, HSA), further support the physical stability of the interleukin 2 liposome to nebulization. Similar correlative studies of aerosol particle-size distribution have been undertaken to support the association of a radiolabel with a drug and the stability of a liposome drug formulation during nebulization (Smaldone et al 1991; O'Riordan et al 1992; Vidgren et al 1995).

FIG. 5. Nebulized interleukin 2 liposomes and free interleukin 2 are retained within the lung 24 h after inhalation (a). Pulmonary clearance of interleukin 2 liposomes (□) and free interleukin 2 (●) can be separated into three phases during the 24 h observation period. Radiolabelled interleukin 2 liposomes and free interleukin 2 were nebulized to an anaesthetized dog. The greatest value of counts min^{-1}, recorded within the ventilated lung, was defined as the maximum counts min^{-1} (100% counts min^{-1}). All other counts min^{-1} recorded were expressed as a percentage of this defined maximum counts min^{-1}. Figs 5b, 5c, and 5d are counts min^{-1} measured in the ventilated lung during the initial, intermediate and final phases, respectively, of pulmonary clearance of inhaled interleukin 2 liposomes and inhaled free interleukin 2. During the first phase of pulmonary clearance the slope of clearance for interleukin 2 liposomes from the lung was −0·679, the slope of clearance for free interleukin 2 was −0·065. The clearance slopes during the intermediate and final phases of pulmonary clearance were similar for interleukin 2 liposomes and free interleukin 2 (intermediate phase of clearance: interleukin 2 liposome clearance slope = −0·070; free interleukin 2 clearance slope = −0·089; final phase of clearance: interleukin 2 liposome clearance slope = −0·013; free interleukin 2 clearance slope = −0·015). Analysis of radiolabel uptake into the blood after inhalation of interleukin 2 liposomes and free interleukin 2 demonstrated less uptake of radiolabel in whole blood after inhalation of interleukin 2 liposomes compared with free interleukin 2 during the entire observation period. From 420 min after nebulization to the end of the observation period (approximately 24 h) the counts min^{-1} in whole blood remained constant for interleukin 2 liposomes but gradually increased after delivery of free interleukin 2 (data not shown).

The stability of the interleukin 2 liposome to the physical stresses of nebulization might be associated with the lack of liposome rigidity and high liposomal drug entrapment of the starting interleukin 2 liposome formulation. This formulation of interleukin 2 liposomes is prepared using a freeze-thaw-sonicate technique with DMPC lipid, HSA and interleukin 2, without the addition of cholesterol (Anderson et al 1994). The resulting liposomes are multilamellar and heterogeneous in size and the interleukin 2 is in close association with the multilamellar phospholipid structure of the liposome rather than encapsulated within the liposome. The addition of HSA to the formulation increases interleukin 2 entrapment and the biological shelf-life of the starting interleukin 2 liposome formulation. The absence of cholesterol in the formulation results in a liposome that is more flexible and less rigid than other parenteral liposomal formulations. The compliance and deformability of these liposomes might enable them to fracture easily, re-capture the hydrophobic interleukin 2 protein from the aqueous environment of the aerosol droplet, and spontaneously re-form during the process of formation of the aerosol. Large liposomes might readily fracture and re-form into smaller particles during nebulization and then enter smaller more respirable aerosol droplets (Taylor et al 1990). It is important to note that the aerosol droplet (characterized by the MMAD) consists of one or more smaller liposomes. Liposome formulations able to undergo such fracture and re-formation cycles, during nebulization, without disruption of the lipid-drug relationship, offer advantages in liposome physical stability, drug retention, and aerosol droplet size (respirability).

Further improvements in the formulation of the interleukin 2 liposome, for nebulization might include the use of dilauryl-phosphatidylcholine (DLPC) in place of DMPC. DLPC has a phase-transition temperature of $-2°C$, whereas the phase transition temperature of DMPC is 23°C. A lipid will behave more as a liquid than as a solid at temperatures above its phase transition temperature. The reduction in the air temperature within the nebulizer bowl during nebulization (16°C), will result in greater rigidity of DMPC liposomes compared with DLPC liposomes. The greater fluidity of DLPC liposomes might improve the success of liposome re-formation after nebulization. Previous workers have reported the potential advantage of DLPC lipids for the formulation of aerosols from liposomal drugs (Waldrep et al 1994b). The advantage of the higher phase-transition temperature of DMPC might be longer pre-nebulization shelf-life and greater interleukin 2 entrapment over time.

The biological stability of the interleukin 2 liposomes after nebulization was supported by the CTLL-2 bioassay. Interleukin 2 has been demonstrated to be remarkably resistant to heat, cold, and changes in pH (Anderson & Sorenson 1994). Recent studies have demonstrated that the biological activity of free interleukin 2 is adversely affected by sonication; however, liposomal formulations of interleukin 2 are not adversely affected by similar exposure to sonication (Mark Sorenson, personal communication). The biological activity of interleukin 2 liposomes and of free interleukin 2 was maintained by nebulization. Quantification of interleukin 2 activity is not as precise in interleukin 2 CTLL-2 bioassay as by interleukin 2 ELISA; however, our data do not show significant differences between the bioactivity of interleukin 2 in aerosols of interleukin 2 liposomes and that of aerosols of free interleukin 2. Thus the interleukin 2 protein remained biologically active after nebulization, whether the protein is liposome-formulated or not.

The nebulization conditions used in these studies enabled efficient generation of highly respirable aerosols. The aerosol particle size is the most important factor in predicting pulmonary deposition and distribution (Schreier et al 1993). The size of an aerosol is dependent on the nebulization equipment, the conditions used to operate the equipment, and the starting drug formulation (Niven & Schreier 1990; Niven et al 1991, 1992; Smaldone et al 1991). Of the variables that might effect aerosol particle size, the equipment used to generate and deliver the aerosol is thought to be most important (Smaldone et al 1991; Schreier et al 1993). The Puritan Bennet Twin-Jet nebulizer used in our studies has been favourably evaluated against several commercially available jet nebulizers for factors including the particle size of the generated aerosol (Waldrep et al 1994a). Respirable particles have been defined as having a maximum diameter of 5 μm (Morrow 1974; Brain & Valberg 1979). Aerosol particles larger than 5 μm impact or deposit in the upper respiratory tract or large conducting airways whereas inhaled particles that are much smaller than 1·0 μm can be exhaled without pulmonary impaction (Schreier et al 1993).

The high efficiency of delivery seen with interleukin 2 liposomes and free interleukin 2 might also be associated with the Puritan Bennet Twin-Jet nebulizer bowl and the low concentration of lipid in the starting interleukin 2 liposomes. The nebulizer bowl has a conical shape which reduces the volume of residue in the nebulizer bowl after nebulization compared with flat-bottomed nebulizer bowls. The concentration of lipid in the liposomal formulation used in these studies was reduced to 5 mg mL^{-1} by dilution with saline. This relatively low lipid concentration might also have contributed to the efficiency of delivery of the interleukin 2 liposomes. The high viscosity of some liposomal drug formulations can effect the efficiency of delivery and can be associated with considerable retention in the nebulizer bowl (Taylor et al 1990). The nebulization equipment and operating conditions used in these studies enabled the efficient generation of respirable aerosols. Future studies might consider optimization of interleukin 2 liposome nebulization by modification of nebulization equipment and interleukin 2 liposome formulations.

One might predict very good pulmonary delivery and significant peripheral airway deposition for inhaled interleukin 2 liposomes, given the MMAD of approximately 2·0 μm and the mode particle size of 1·1 μm (Knight & Waldrep 1995). Scintigraphic analysis of inhaled radiolabelled interleukin 2 liposomes and free interleukin 2 confirmed these expectations. The radiolabelling techniques utilized were effective, resulting in high-efficiency radiolabelling that was stable during the process of nebulization and that did not significantly affect the particle size of the interleukin 2 liposome or free interleukin 2 aerosols. Images obtained during and after nebulization of radiolabelled interleukin 2 liposomes showed deposition of the inhaled drug in all ventilated regions of the lung with a central-to-peripheral lung deposition ratio of 1·12 for interleukin 2 liposomes and 1·11 for free interleukin 2 aerosols. The use of the central-to-peripheral lung deposition ratio has been previously described as an index of regional deposition of inhaled particles. A central-to-peripheral lung deposition ratio of

approximately 1·0 suggests a uniform aerosol particle deposition in the peripheral airways and alveoli (Smaldone et al 1991). The uniform deposition of interleukin 2 liposomes in the entire ventilated lung might be important in the treatment of pulmonary metastases and primary lung tumours, which might have central or peripheral pulmonary distribution. Scintigraphic images in the intubated anaesthetized dog might enable prediction of the pulmonary deposition of nebulized interleukin 2 liposomes in patients presently undergoing this therapy at the University of Minnesota. It should be noted that the deposition of inhaled aerosols can be significantly influenced by pulmonary pathology (Schreier et al 1993), and that all scintigraphic studies were undertaken with a single healthy dog. Future pulmonary scintigraphic studies in dogs with spontaneous pulmonary metastases or primary lung tumours will be valuable in assessing the effects of pulmonary pathology on interleukin 2 liposome deposition patterns and predicting the inhaled dose of interleukin 2 liposomes.

The clearance of the radiolabelled interleukin 2 liposomes and free interleukin 2 from the lung (after nebulization) suggest a relatively long (approximately 24 h) retention of radiolabel within the lung after nebulization. Similar findings have been reported for several nebulized liposomal formulations (Barker et al 1994; Knight & Waldrep 1995; Vidgren et al 1995). The limited uptake of radiolabel in the trachea, oesophagus and stomach suggest that inhaled interleukin 2 liposomes and free interleukin 2 were not significantly cleared by muco-ciliary escalation and swallowing. The more rapid clearance of interleukin 2 liposomes compared with free interleukin 2 in the first 30 min after nebulization suggests the possibility of a different clearance rate or clearance pathway from the lung for the interleukin 2 liposomes. The initial phase of pulmonary radiolabel clearance for inhaled interleukin 2 liposomes was faster than for inhaled free interleukin 2; however, the uptake of radiolabel in whole blood after nebulization for radiolabelled interleukin 2 liposomes was not appreciably greater than free interleukin 2 uptake in whole blood. This might suggest that after clearance from the lung, interleukin 2 liposomes enter the systemic circulation for a short period of time only and then become distributed to other organs, or that alternative pathways (lymphatics) contribute to interleukin 2 liposome pulmonary clearance.

Parenteral administration of this interleukin 2 liposome formulation has previously been shown to be associated with biodistribution to immune centres (lymph nodes, spleen, bone marrow; Anderson et al 1992b). Uptake of the liposomes from distal pulmonary airways and lung parenchyma with rapid delivery to immune centres via the blood or lymphatics, or both, might explain the more rapid pulmonary clearance of inhaled interleukin 2 liposomes compared with free interleukin 2. Findings in a dog with pulmonary metastases and peripheral lymph node metastases from an appendicular osteosarcoma also support the hypothesis of lymphatic clearance or lymphoid targeting, or both, of inhaled interleukin 2 liposomes (Khanna et al 1997). In this dog the inhalation of interleukin 2 liposomes for 30 days resulted in complete regression of not only the pulmonary metastasis, but also the peripheral lymph node metastasis. Interestingly, this complete response was noted in association with significant pulmonary immune effector-cell activation, and without significant peripheral blood effector-cell activation (Khanna et al 1997).

Although the initial phases of pulmonary clearance of radiolabelled interleukin 2 liposomes and free interleukin 2 were different, the remaining phases (intermediate and final) were similar. The most interesting finding noted during pulmonary clearance of the inhaled interleukin 2 liposomes was the retention of the radiolabel within not only the lung but also the spleen approximately 24 h after nebulization of interleukin 2 liposomes. The accumulation of the radiolabel within the reticuloendothelial system might support the hypothesis that inhaled interleukin 2 liposomes are cleared from the lung and are then, in part, biodistributed to immune centres (including the spleen). We were unable to demonstrate concentration of radiolabel, after nebulization of interleukin 2 liposomes, within bronchial-associated lymphoid tissue, presumably because of the high radioactive background produced by the retained pulmonary radiolabel.

Although we have demonstrated the stability of the radiolabel to nebulization in-vitro, there might be some transfer of the inhaled radiolabel, in-vivo, from the liposome or the interleukin 2 to other pulmonary proteins (for example albumin; Vidgren et al 1995). The significance of this potential radiolabel transfer in not known. Conclusions from such pulmonary clearance studies must be made cautiously. Nevertheless, the differences between the clearance patterns of radiolabelled interleukin 2 liposomes compared with free interleukin 2 suggest the value of these scintigraphic studies in evaluating pulmonary clearance of inhaled interleukin 2 liposomes and free interleukin 2.

One objective of this study was to determine if advantages in the physical and biological characteristics of inhaled interleukin 2 liposomes could explain the significantly enhanced pulmonary immune activation associated with inhaled interleukin 2 liposomes compared with inhaled free interleukin 2 (Khanna et al 1996). Although there were no significant differences between the physical characteristics of the nebulized liposomal and free formulations of interleukin 2, the biodistribution of inhaled interleukin 2 liposomes to the spleen was not seen after nebulization of free interleukin 2. The combination of both pulmonary and lymphoid localization of inhaled interleukin 2 liposomes might in part explain the advantage in pulmonary immune activation of interleukin 2 liposomes compared with free interleukin 2. At this time we cannot discount the possibility that the enhanced pulmonary immune activation of inhaled interleukin 2 liposomes compared with free interleukin 2 inhalation is because of differences in cytokine profiles induced by these formulations, or possibly differences in effector cell interactions. Nonetheless, the ability to target the lung and the lymphoid centres with immunomodulators might be of considerable value in the treatment of pulmonary metastases and primary lung cancers, and of potentially infectious diseases involving the lung.

The results of this study suggest the stability of this interleukin 2 liposome formulation to nebulization. Interleukin 2 liposomes were efficiently nebulized, physically and biologically stable, highly respirable, and appeared to have extended pulmonary retention after nebulization. The stability of interleukin 2 liposomes to nebulization and the potential ability of inhaled interleukin 2 liposomes to target both the lung and immune centres are particularly attractive in the treatment of both pulmonary metastases and primary lung cancers. Neb-

ulized interleukin 2 liposomes might offer a valuable immunotherapeutic strategy for stimulation of the immune system and the management of both pulmonary metastases and primary lung cancers.

Acknowledgements

This research was supported in part by the Perlman Oncology Residency and the University of Minnesota Veterinary College Companion Animal Research Fund.

The authors acknowledge the technical assistance of Connie Callfas and Maria Crowley. We would also like to thank Drs Timothy Wiedman, Gary Johnson, Keith Skubitz, Mircea Popescu, Mary Neville, Mark Sorenson and Daniel Saltzman for their encouragement and helpful discussions during these studies.

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