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Amikacin liposomes: characterization, aerosolization, and in vitro activity against *Mycobacterium avium-intracellulare* in alveolar macrophages

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Summary

A feasible way to treat pulmonary *Mycobacterium avium-intracellulare* (MAI) infections is inhalation of liposome-encapsulated antimycobacterial agents aimed at infected alveolar macrophages (AM). To this end, amikacin-containing liposomes consisting of soy phosphatidylcholine (SPC), hydrogenated SPC (HSPC) and phosphatidylglycerol (PG) or cholesterol (CH) were prepared by extrusion and characterized with respect to drug content, encapsulation efficiency, drug retention in lung lavage fluid, stability during aerosolization, and in vitro efficacy against MAI in murine AM. Unencapsulated amikacin was separated by dialysis and ion-exchange (Amberlite IRC50) adsorption which was found to be fast, complete and less cumbersome than dialysis. Drug content increased linearly with drug concentration for a fixed lipid concentration from 1 to 12% for SPC, and from 5 to 21% for SPC/PG (7:3 molar ratio) liposomes, while the amikacin content remained constant at 0.5–1% (SPC) and 1.5–2% (SPC/PG) over a lipid concentration range of 20–160 mg/ml for a fixed amikacin concentration. With increasing lipid concentration (10–160 mg/ml), the encapsulation efficiency increased linearly for SPC liposomes (2–20%), and in a saturable fashion for SPC/PG liposomes (1–35%). Aerosolization (Collison nebulizer) over 80 min resulted in loss of content of approx. 20% (SPC and HSPC) and 30–40% (SPC/PG and SPC/CH), respectively. Incubation with fresh lung lavage fluid at 37°C resulted in poor retention of amikacin in SPC/PG liposomes, whereas SPC and SPC/CH liposomes essentially retained the drug over 24 h. A dose of 20 μg/ml amikacin when encapsulated within SPC/PG liposomes was approx. 100-times more efficacious against intracellular MAI in the infected alveolar macrophage model in vitro, than an equivalent concentration of free amikacin, indicating that uptake of drug-carrying liposomes by infected macrophages is operative.

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Introduction

Atypical mycobacteria, including *Mycobacterium avium-intracellulare* (MAI), are facultative

intracellular bacilli which frequently cause pulmonary disease in humans. MAI resists killing once ingested by pulmonary alveolar macrophages. The organism is able to reside and multiply intracellularly, eventually lysing and killing the host macrophage and promoting re-infection and occasionally dissemination (Frehel et al., 1986). MAI has emerged recently as major pathogen in individuals with compromised cell-mediated immunity and other chronic debilitating medical conditions (Iseman et al., 1985; Woods and Washington, 1987). MAI is relatively resistant in vitro to many antituberculous agents at concentrations effective against *Mycobacterium tuberculosis* (Kuze et al., 1981). Consequently, no predictably efficacious therapy is available, although empirical therapy with combinations of four to six antituberculous drugs occasionally produces clinical improvement and even cure in some patients (Rosenzweig, 1979; Davidson et al., 1981; Horsburgh et al., 1987). However, therapy is commonly complicated by the toxicity and side effects encountered with oral administration of such combinations (Frehel et al., 1986; Horsburgh et al., 1987).

While aminoglycoside antibiotics are active against MAI in vitro, they are currently not feasible for prolonged therapy in pulmonary MAI infection for three reasons: (i) the need for parenteral administration; (ii) the insignificant partitioning of parenterally administered aminoglycoside into the lung fluid (Mombell et al., 1981); and (iii) their nephrotoxic side effects (Lietman and Smith, 1983). Amikacin levels in bronchial fluid have been reported to be only about 18% of the corresponding plasma levels (Mombell et al., 1981).

Recent evidence from several laboratories suggests liposomal antibiotics to be more efficacious in the treatment of intracellular microbial infections compared to the corresponding drug solution (for review, see Swenson et al., 1988). Accordingly, liposomal amikacin has been shown in vitro to be more efficacious than an amikacin solution in MAI-infected macrophage culture (Bermudez et al., 1987), and parenterally when administered to mice with disseminated MAI infection (Düzgünes et al., 1988; Cynamon et al., 1989; Bermudez et al., 1990). The same effect has been shown for liposomal gentamicin (Bermudez et al., 1990; Klemens et al., 1990). Interestingly, all authors who examined organ-specific effects of intravenously administered liposomal drugs (Düzgünes et al., 1988; Cynamon et al., 1989; Klemens et al., 1990) reported reduction of the number of viable organisms in liver and spleen, whereas the number of viable organisms persisting in the lung was not significantly reduced.

Since MAI resides and multiplies in alveolar macrophages, we have proposed aerosolized delivery of liposome-encapsulated amikacin as a feasible way to deliver chronically amikacin to pulmonary tissues, specifically to MAI-infected alveolar macrophages. To this end, we have investigated the physicochemical characteristics of liposome aerosols (Niven and Schreier, 1990; Niven et al., 1991, 1992), the interaction of liposomes in vitro with macrophage function in the alveolar macrophage model (Gonzalez-Rothi et al., 1991), the effects of chronic liposome aerosol delivery to mice (Myers et al., 1989), and liposome aerosol safety in human volunteers (Thomas et al., 1991). Here, we describe the development and characterization of liposomal amikacin formulations suitable for aerosol delivery, their stability in lung lavage fluid and in vitro efficacy in MAI-infected alveolar macrophages.

Materials and Methods

Chemicals and reagents

Tobramycin, Triton X-100, CM Sephadex (C25), mercaptoethanol, and Amberlite IRC 50 were obtained from Sigma Chemical Co., St. Louis, MO. Sodium pentanesulfonate, sodium sulfate, boric acid, potassium hydroxide, acetic acid and *o*-phthaldialdehyde were from Fisher Scientific, Springfield, NJ. Amikacin was a gift from Bristol-Myers Squibb, Princeton, NJ. Liposome components were soy phosphatidylcholine (SPC) and hydrogenated soy phosphatidylcholine (HSPC) (Phospholipon 90 and 90H, American Lecithin Co., Atlanta, GA), egg phosphatidylglycerol (PG) (Avanti Polar Lipids, Alabaster, AL), and cholesterol (CH) (99% GC grade, Sigma).

Quantitative determination of amikacin

Amikacin was determined by an HPLC reversed-phase ion pair chromatography with online post-column derivatization and fluorescence detection as described in detail elsewhere (Wichert et al., 1991). For drug content determination, 50 μl liposome dispersion was diluted with 950 μl Triton X-100 (6% in water). A sample of this solution was mixed with internal standard (40 μg tobramycin/ml). Ion pair concentrate was used to adjust the ionic strength of the sample to the mobile phase. The injection volume was 10 μl.

As the presence of Triton X-100 and phospholipids had no effect on calibration, calibration curves were prepared in water without addition of Triton X-100 and lipids. Calibration curves were linear over the investigated concentration range with a good correlation coefficient (r = 0.999). Assay precision (inter-day) ranged from 3.4% (20 μg/ml) to 13.3% (0.4 μg/ml), with an accuracy of 94.9–101.4% in the concentration range of 1.0–20.0 μg/ml.

For the in vitro antibiotic efficacy test (see below) amikacin was analyzed in the cell culture supernatant by a fluorescence polarization immunoassay (TDX, Abbott). In this system, amikacin molecules in the solution (but not encapsulated amikacin) compete with fluorescein-labeled amikacin for binding sites on anti-amikacin antibodies. The amikacin concentration is inversely proportional to the degree of fluorescence polarization measured. The sensitivity of the assay is 0.6 μg/ml.

Preparation of liposomes

Multilamellar liposomes (MLV) were prepared by extrusion according to Olson et al. (1979). Lipid formulations employed were: (i) SPC; (ii) HSPC; (iii) SPC/PG 7:3 (molar ratio); (iv) SPC/CH 7:3 (molar ratio). Lipids were combined in $CHCl_3$ and the solvent was stripped off under vacuum on a Büchi rotoevaporator. The thin lipid film was hydrated with an aqueous solution of amikacin by mechanical agitation on a wrist-action shaker for 2 h at 65°C. The lipid dispersion was extruded three times through polycarbonate membrane filters of 0.4 μm pore size (Nuclepore Inc., Pleasanton, CA). The volume-average diameter was determined with a laser particle sizer (NICOMP model 370, Particle Sizing Systems Inc., St. Barbara, CA). Prior to extrusion, the diameter of the liposomes was $\gg 1$ μm, except for SPC/PG 7:3 liposomes. Extrusion through 0.4 μm polycarbonate membranes effectively reduced the mean diameter of all preparations to the range from 290 to 490 nm.

For the antimicrobial efficacy tests, liposomes were prepared under aseptic conditions in a laminar flow hood. All glassware was heat sterilized. Extrusion equipment and filter membranes were autoclaved. Samples were tested for sterility by plating on sheep blood agar and incubation at 37 °C overnight prior to experimentation.

Separation of unencapsulated amikacin

Unencapsulated amikacin was removed either by dialysis or by adsorption to the ion-exchange resin Amberlite IRC 50. Samples were dialyzed exhaustively in Spectra/Por 2 dialysis tubes against phosphate-buffered saline (PBS) for 48 h at 4 °C, with three buffer changes. Alternatively, Amberlite IRC 50 ion exchanger was loaded with NH_4^+ as counterion by washing the resin three times each with 0.2 N NH_4OH and 1 M $(NH_4)_2SO_4$, and finally with water until the eluate was neutral. The 20-fold amount of Amberlite (NH_4^+ form) relative to amikacin was added to the liposome dispersion ($\approx$ 100 μg amikacin/ml). The mixture was agitated for 30 min, the Amberlite was allowed to settle and the liposome dispersion was removed and analyzed for amikacin by HPLC.

Drug content and encapsulation efficiency

Drug content (D.C.) was expressed as the fraction of drug mass encapsulated relative to the total mass (drug plus lipid):

$$\text{D.C.} = [\text{amikacin}]/([\text{amikacin}] + [\text{lipid}])$$

Encapsulation efficiency (E.E.) is defined as the fraction of encapsulated drug relative to the initial total amount of drug in the solution:

$$\text{E.E.}(\%) = [\text{amikacin}]_{\text{enc}}/[\text{amikacin}]_{\text{tot}} \times 100$$

Amikacin adsorption onto empty liposomes

Samples of 500 μl liposomes (100 mg/ml) were mixed with 500 μl amikacin solution (0.06–133.9 μg/ml) and incubated under agitation for 2 h. Liposomes were removed by centrifugation (Eppendorf centrifuge, 16000 $\times g$, 15 min), and the amikacin concentration in the supernatant was determined.

Liposome aerosolization

Amikacin-containing liposomes were aerosolized using a Collison nebulizer and samples were collected over an 80 min nebulization period, centrifuged and the unencapsulated drug determined in the supernatant by HPLC. The methodology employed is described in detail elsewhere (Niven and Schreier, 1990; Niven et al., 1991).

Liposome stability in lung lavage fluid

Lung lavage was performed and alveolar macrophages harvested from Balb/c mice as described elsewhere (Gonzalez-Rothi et al., 1991). Liposomes were incubated in pooled, cell-free whole lung lavage fluid at 37°C. The total drug concentration in the incubation medium was between 15 and 20 μg/ml. At each time point, the dispersion was mixed, a sample removed, diluted with PBS, centrifuged and the amikacin concentration in the supernatant determined by HPLC. The total drug concentration was determined after disrupting the liposomes with 6% Triton X-100.

In vitro antibiotic efficacy test

An in vitro macrophage infection model for MAI as described by Crowle et al. (1984) was adapted to a microtiter version for mouse alveolar macrophages (Straub et al., 1990). The MAI strain used (1T66) was originally isolated from a patient with pulmonary disease at the VA Medical Center, Gainesville, FL. It was cultured in Middlebrook 7H9 broth (Difco), harvested in the exponential growth phase and adjusted to a density of approx. 1.2×10^9 bacilli/ml using a McFarland standard. MAI were stored at −70°C for use in the macrophage infection studies. Tris buffer, pH 7.4, and 100 μg/ml of penicillin were used to resuspend the cell pellet. The cells were pipetted in duplicate into 96-well, flat bottom tissue culture plates (Linbro, Flow Labs., McLean, VA) at a density of 10^5 cells/well. Nonadherent cells were rinsed off and the adherent macrophages incubated for 24 h in Dulbecco's Modified Eagle Medium (DMEM) with 15% heat-inactivated fetal bovine serum (FBS). After 24 h inoculation (day 0) with approx. 2×10^6 bacilli, MAI not taken up by the macrophages were rinsed off vigorously and fresh culture medium was added.

The amikacin treatment was started 24 h after initial infection of macrophages (day 1) by adding amikacin solution or liposomal amikacin in concentrations of 20 μg/ml in culture medium. Culture medium alone was used in the control wells. On days 1, 2, and 3, plates were removed from the incubator. The supernatant was aspirated and saved for amikacin analysis. Supernatant samples were analyzed directly for amikacin using a fluorescence polarization immunoassay as described above. Since only amikacin in solution competes for antibody binding, no separation of liposomes was required. Macrophage monolayers were washed twice with warm Hanks Balanced Salt Solution (HBSS) (37°C) and lysed with sodium dodecyl sulfate (SDS) to release intracellular MAI. After neutralizing the SDS with 20% bovine serum (Sigma), serial dilutions of macrophage lysates were plated on Middlebrook 7H10 agar plates. The agar plates were incubated and the numbers of viable MAI colony forming units (CFU) for each agar plate were enumerated. The number of CFU was indicative of viable intracellular organisms retained within macrophages. Thus, the lower the CFU, the more efficacious the respective regimen would be.

Results and Discussion

Drug separation by dialysis and Amberlite IRC 50 adsorption

Removal of unencapsulated amikacin by either dialysis or adsorption on Amberlite was equivalent as the results summarized in Table 1 indicate. Thus, it can be concluded that ion exchange

TABLE 1

Final concentrations of encapsulated amikacin in various liposome formulations following separation of unencapsulated drug by either dialysis or adsorption to the ion exchange resin Amberlite IRC 50

Lipid composition [a] (molar ratio)	Encapsulated amikacin concentration [b] (mg/ml)	
	Unencapsulated drug separated by:	
	Dialysis	Ion-exchange resin
SPC	2.07 (0.09) [c]	2.01 (0.14)
SPC:PG (7:3)	5.76 (0.47)	5.96 (0.48)
SPC:CH (7:3)	1.49 (0.06)	1.49 (0.44)

[a] Total lipid concentration was 50 mg/ml.
[b] Initial amikacin concentration was 20 mg/ml.
[c] Numbers in parentheses = SD ($n = 6$).

is both a suitable, and, with respect to time and ease of manipulation, the preferred method to remove unencapsulated amikacin.

Drug content and encapsulation efficiency

At a constant lipid concentration, the drug content increased linearly with increasing amikacin concentrations (Fig. 1A). The maximum drug content, under the given experimental conditions, was about 15 and 21% for SPC and SPC/PG liposomes, respectively. For a constant amikacin concentration, drug content was independent of the lipid concentration over a range of 20–160 mg/ml (SPC, 0.5–1%; SPC/PG, 1.5–2%). Lipid concentrations higher than 160 mg/ml yielded very viscous preparations which were difficult to filter.

The encapsulation efficiency of amikacin increased linearly with increasing lipid concentrations (Fig. 1B), although it appeared to plateau at the highest concentrations of SPC/PG (140–160 mg/ml).

The results of the adsorption of amikacin to preformed liposomes over a wide amikacin concentration range (0.06–133.9 mg/ml) are summarized in Fig. 2. The resulting final amikacin concentration in the supernatant was found to be not significantly different from the original drug concentration prior to incubation with liposomes, even for the lowest concentrations used, suggesting negligible adsorption to the liposome surface.

The results reported here are in general agreement with relevant findings reported in the literature. Similar values for drug content have been reported for liposomal gentamicin and streptomycin (Barza et al., 1984; Tadakuma et al., 1985). We found negatively charged liposomes (SPC/PG) to have a higher amikacin content than neutral liposomes (SPC), similar to observations made for streptomycin, gentamicin and salbutamol sulfate (Dees et al., 1985; Farr et al., 1989; Bermudez et al., 1990). In order to assess whether this

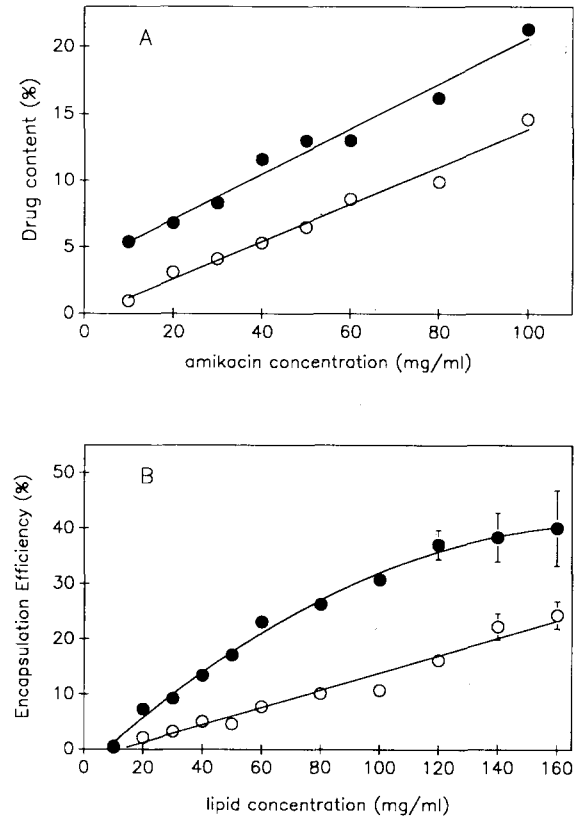

Fig. 1. (A) Drug content of liposomes as a function of increasing amikacin concentrations. Lipid concentration was kept constant at 50 mg/ml. (B) Amikacin encapsulation efficiency of liposomes as a function of increasing lipid concentration. Amikacin concentration was kept constant at 5 mg/ml. Average values ± SD of three experiments are shown for the three highest concentrations. SPC (○); SPC/PG 7:3 (●).

phenomenon was caused by ionic drug-lipid interactions, or by the larger entrapped volume resulting from charge repulsion between the individual liposome bilayers, adsorption of amikacin onto preformed liposomes was studied. Since adsorption to the liposome surface appeared to be negligible, the larger entrapped volume of charged liposomes (Szoka and Papahadjopoulos, 1980) is most likely responsible for their relatively higher drug content.

Liposome aerosolization

The retention of amikacin within liposomes while being aerosolized with a Collison nebulizer over 80 min was quite acceptable for all formulations (Fig. 3). SPC and HSPC liposomes released approx. 20% of their original content over the nebulization period, whereas loss of amikacin from SPC/PG and SPC/CH liposomes was 30–40%. However, it should be borne in mind that, under clinical conditions, a desirable aerosolization time is more in the order of 15–20 min, rather than 80 min, at which time none of the formulations would be expected to release more than 20% of its original content. The increased loss of amikacin from both SPC/PG and SPC/CH liposomes is in contrast to findings with the model substance carboxyfluorescein which was greatly retained when encapsulated in liposomes

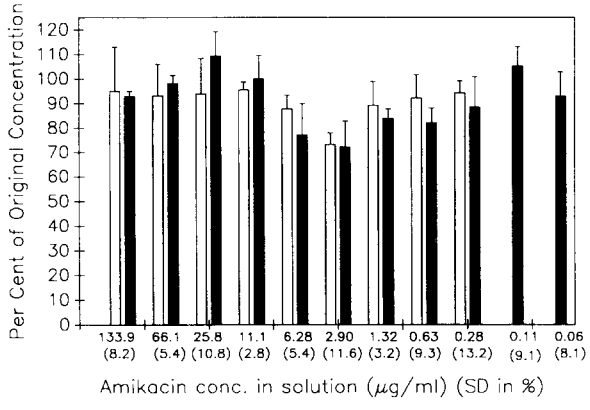

Fig. 2. Adsorption of amikacin to the outer surface of liposomes. The change in amikacin concentration in solution (in percent of original concentration) following incubation with liposomes is shown for drug concentrations ranging from 0.06 to 133.9 μg/ml. Bars indicate average values ± SD ($n = 3$). SPC (○); SPC/PG 7:3 (●).

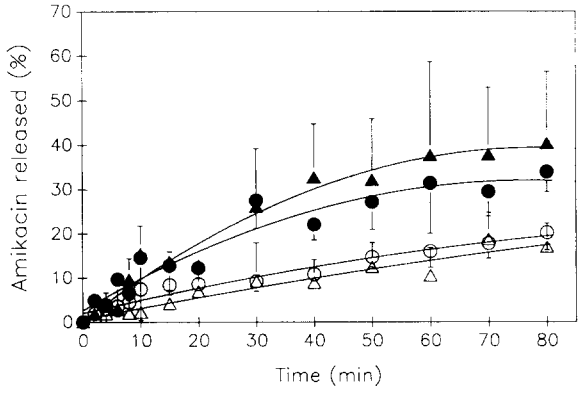

Fig. 3. Loss of liposome-encapsulated amikacin during aerosolization with a Collison nebulizer over an 80 min period. Bars indicate average values ± SD. Each experiment was performed on 3 different days. SPC (○); HSPC (△); SPC/PG 7:3 (●); SPC/CH 9:1 (▲).

of such compositions (Niven and Schreier, 1990). While Niven and Schreier (1990) reported a similar unexpectedly poor retention of marker in the presence of 10 mol% cholesterol (which was speculated to be due to phase separation), the reasons for the poor retention of amikacin in SPC/PG liposomes are not clear at this point. However, the system employed here may not be entirely comparable to that used by Niven and Schreier (1990), since carboxyfluorescein is a partially negatively charged molecule, while amikacin is a polycation at physiological pH.

While unexpected with respect to their relative behavior, the overall release pattern of the liposome formulations investigated is in agreement with studies by Niven et al. (1991) who showed that loss of encapsulated marker was directly related to the liposome diameter, relative to the aerosol mass median aerodynamic diameter which was determined for the Collison nebulizer to be approx. 1.7 μm (Niven and Schreier, 1990). Liposomes with average diameters of 400 nm or less retained encapsulated marker more effectively than larger liposomes.

Liposome stability in lung lavage

The stability of liposomes in the pulmonary physiological milieu was simulated in vitro using lung lavage fluid from mice. When incubated with

fresh lung lavage fluid at 37°C in a humidified atmosphere, SPC, HSPC and SPC/CH liposomes retained the drug practically quantitatively, whereas drug loss was significant from SPC/PG liposomes (Fig. 4).

It is known that liposomes interact with components of biological fluids including serum albumin and immunoglobulins (Scherphof et al., 1984), fibronectin (Rossi and Wallace, 1983; Schreier et al., 1987) and apolipoproteins (Guo et al., 1980) as a function of their composition and net surface charge, and that, as a consequence of this interaction, encapsulated material is released to a varying degree (Hunt, 1982). While plasma protein interaction determines the extent of disruption of the liposome membrane, thus loss of encapsulated material in the systemic circulation, lung lavage fluid is characterized by a low protein content, low enzymatic activity, but high concentration of lung surfactant which consists essentially of dipalmitoylphosphatidylcholine with minor fractions of other phospholipids, cholesterol and hydrophobic proteins (Jobe and Ikegami, 1987). The fate of exogenous phospholipids administered as dry powder or as liposomes to the lungs of experimental animals has been documented in the literature (for review, see Mihalko et al., 1988). However, further studies are needed to investigate the nature and extent of interaction of drug-carrying liposomes with lung lavage fluid,

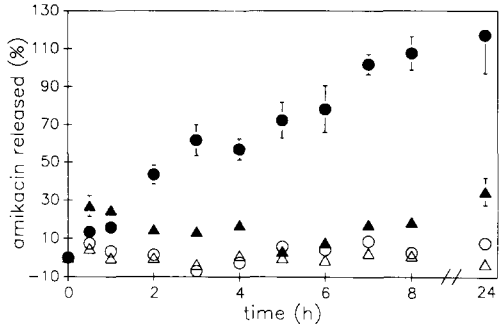

Fig. 4. Loss of liposome-encapsulated amikacin during incubation in diluted lung lavage fluid over 24 h. Average measurements ± SD are shown ($n = 3$). Error bars are not shown on all data points for clarity of plot. SPC (○); HSPC (△); SPC/PG 7:3 (●); SPC/CH 9:1 (▲).

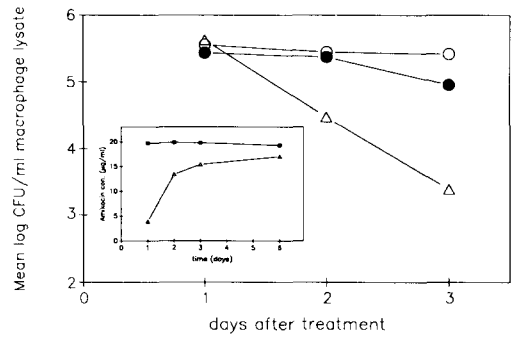

Fig. 5. Killing of *Mycobacterium avium-intracellulare* (MAI) (strain 1T66) in infected mouse pulmonary alveolar macrophages. Macrophages were infected with MAI for 24 h. On day 1, cells were exposed to 20 μg/ml amikacin in solution or encapsulated within SPC/PG liposomes. Macrophages were lysed at daily intervals following infection and treatment, and viable MAI were plated on mycobacterial growth agar and incubated for 14 days prior to colony-forming units (CFU) counting. Control (untreated) (○); 20 μg/ml amikacin solution (●); 20 μg/ml amikacin in SPC/PG liposomes (▲).

and the resulting release of encapsulated material.

In vitro efficacy tests

MAI-infected alveolar macrophages in culture were exposed to a 20 μg/ml dose of amikacin, both in solution and encapsulated within SPC/PG liposomes. As shown in Fig. 5, killing efficacy of the amikacin solution was modest compared to control, whereas the liposomal preparation caused a log-linear kill of intracellular MAI over 3 days. Although samples were initially monitored up to 6 days, the validity of these data was questionable due to potentially compromised cell viability over this extended period of time. The 6-day data set was therefore omitted from the results.

Amikacin was monitored in the incubation medium of the macrophage plate. Amikacin solution was found to remain approximately at the initial 20 μg/ml level, while originally encapsulated amikacin was released relatively rapidly (50–75%) from the liposomes over 24–48 h (inset, Fig. 5), which was not unexpected in light of prior findings in aerosolization and lung lavage exposure studies as described above. Notably, attempts to detect intracellular amikacin failed.

Yet, since significant killing was found with the liposomal preparation despite extracellular release (i.e. loss) of encapsulated drug, rapid phagocytosis of liposomes must have occurred which is not an unreasonable assumption. It is substantiated by the fact that incubation of MAI-infected alveolar macrophages with a mixture of drug solution and empty liposomes showed the same killing rates as amikacin solution alone, and that empty liposomes had no effect on the mycobacterial growth (data not shown).

The phospholipid composition SPC/PG does not appear to be the lipid composition of choice based on the relatively poor stability that this combination exhibited during incubation with lung lavage fluid (see above). However, in a preceding study (Gonzalez-Rothi et al., 1991), liposomes made from unsaturated phospholipids were found to be phagocytized more avidly than liposomes made from saturated phospholipids. Furthermore, we have evidence that partially negatively charged liposomes are more avidly taken up by pulmonary alveolar macrophages than neutral or strongly negatively charged liposomes (C. Cacace, R.J. Gonzalez-Rothi, and H. Schreier, unpublished observation). Similarly, Dees et al. (1985) reported that negatively charged gentamicin liposomes were more effective against intracellular *Brucella* infections than neutral liposomes. Hence, the selection of SPC/PG liposomes is justified based on their expected biological interaction, rather than their physical stability.

Employing an encapsulated fluorescent marker and dark-field fluorescence microscopy, Gonzalez-Rothi et al. (1991) showed that liposomes were taken up by alveolar macrophages and released their contents inside lysosomal vacuoles. Here we demonstrate that this process can profoundly enhance the efficacy of an antibiotic drug against the facultative intracellular bacillus MAI.

In summary, evidence is provided that amikacin liposomes may be a promising and potent compound to treat pulmonary MAI infections via the inhalatory route of administration. Although a speculation at this time, it is interesting to note that stable lipid compositions which retain drug effectively and, therefore, are most attractive from a pharmaceutical point of view, may not necessarily provide the best therapeutic outcome. It may therefore be imperative to develop auxiliary technologies for the in vitro stabilization of liposomes, lyophilization being one rather elegant solution to the problem. Experiments toward that end, as well as experiments to determine the type of lipid mixture with which optimal killing conditions against MAI can be achieved are now underway in our laboratory.

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