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Liposomal entrapment of the neutrophil-derived peptide indolicidin endows it with in vivo antifungal activity

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Abstract

Indolicidin, a cationic tridecapeptide amide isolated from the granules of bovine neutrophils, has been found to possess potent antimicrobial activity in vitro but its nonselective toxicity could restrict its therapeutic utility. We found that the concentration at which indolicidin disrupts washed human red blood cell membranes coincided with the concentration at which indolicidin self associates. Because of a preponderance of hydrophobic residues, we believed that indolicidin would partition into liposomes which would restrict its exchange with biological tissues and consequently reduce its toxicity. Fluorescence spectroscopy of indolicidin added to 100 nm liposomes comprised of POPC, POPC/cholesterol (60:40 mol%), DPPC, or DPPC/cholesterol (60:40) revealed a large blue-shift and an increase in intensity of the emission profile indicating insertion into the bilayer. Of the lipids tested, POPC exhibited the highest degree of indolicidin binding as determined by fluorescence and encapsulation efficiency. By sequestering indolicidin within the lipid bilayer of 100 nm POPC liposomes we significantly reduced its toxicity to CHO/K1 cells. Likewise, the systemic toxicity of liposomal indolicidin in Balb/c mice was decreased dramatically relative to aqueous solutions; the maximum dose at which no deaths occurred was 0.4 mg/kg for free indolicidin versus 40 mg/kg for indolicidin-POPC. Because of this decrease in toxicity, we were able to administer liposomally encapsulated material at significantly higher concentrations than unencapsulated aqueous material and achieve efficacy in treating animals systemically infected with *Aspergillus fumigatus*. Liposomal but not free indolicidin was found to be effective in obtaining cures. This report is the first description of the in vivo therapeutic activity of a neutrophil-derived antimicrobial peptide and suggests that liposomal treatment modalities will provide effective strategies for endowing this class of compounds with pharmacological utility.

Keywords: Antimicrobial agent; Antifungal agent; Liposome; Peptide; Indolicidin

1. Introduction

H-Ile-Leu-Pro-Trp-Lys-Trp-Pro-Trp-Trp-Pro-Trp-Arg-Arg-NH_2

Indolicidin is an antimicrobial tridecapeptide amide isolated from the cytoplasmic granules of bovine neutrophils [1,2]. Structurally, indolicidin is unique among known host defense peptides due to its high (39%) tryptophan content [3].

The mechanism of action of indolicidin is not clearly understood but may involve the disruption of cell membranes. Thus, in solution, in addition to a broad spectrum of antifungal and antibacterial activities, it is also toxic to mammalian cells. This lack of selectivity could significantly restrict its clinical use.

In recent years, much interest has centered upon the therapeutic applications of liposomes and lipid complexes [4]. In many cases these systems have effectively reduced the toxicity of important drugs without significant compromise to efficacy [5,6]. In fact, as determined in recent clinical trials involving both water and lipid soluble compounds [7–9], this approach has permitted dosing protocols

Abbreviations: POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; DPPC, 1,2-dipalmitoylphosphatidylcholine; FATMLVs, freeze-thaw multilamellar vesicles; LUVETs, large unilamellar vesicles by extrusion technique; CHO, Chinese hamster ovary; LD_{10}, lethal dose 10%, dose at which 10% animals die; IC_{50}, inhibitory concentration 50%, dose at which 50% inhibition occurs; PBS, phosphate-buffered saline; RBC, red blood cell; FBS, fetal bovine serum.

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that would otherwise produce toxicity and consequently has opened new therapeutic opportunities. Now we describe a liposome formulation of indolicidin and compare its toxicity in vitro and in vivo to aqueous indolicidin. Furthermore, we compare the efficacy of a single maximally tolerated intravenous dose of liposomal indolicidin to aqueous indolicidin in a mouse model of systemic aspergillosis. Taken together, these data demonstrate that a striking reduction in overall toxicity and improvement in therapeutic efficacy can be achieved for this compound by providing it with a liposomal carrier system. Such liposomal systems may well provide heretofore not available clinical opportunities for antimicrobial peptides.

2. Materials and methods

2.1. Chemicals

Indolicidin (acetate salt), was synthesized [2] and purified as previously described [2,10]. Preparations were determined to be homogeneous by reversed phase HPLC, acid-urea PAGE, FAB-MS and by sequence analysis. Final stock preparations were quantified by amino acid analysis prior to lyophilization. 1-Palmitoyl-2-oleoyliphosphatidylcholine (POPC) and 1,2-dipalmitoylphosphatidylcholine (DPPC) were purchased from Avanti Polar Lipids (Birmingham, Alabama). Cholesterol, Hepes, and NaCl were purchased from Sigma Chemical (St. Louis, MO) and all other reagents were of the highest purity available.

2.2. Biochemical assays

Indolicidin concentrations were measured spectrophotometrically by dissolving samples in ethanol and comparing absorbance to A_{280} of an amino acid analyzed standard solution. In these studies we used a Shimadzu Model 160 spectrophotometer (Shimadzu, Columbia, MD, USA). Phospholipid concentration was determined by the procedure of Chen [11].

2.3. Preparation of liposomes

Freeze-thaw multilamellar vesicles (FATMLVs) were made as previously described [12]. Briefly, lipid in chloroform was dried to a thin film using a rotary evaporator and the thin dried film was hydrated with a buffer comprised of 10 mM Hepes and 150 mM NaCl at pH 7.4. For liposomes containing indolicidin the lipid and indolicidin were co-dissolved in organic solvent by first dissolving indolicidin in methanol and then adding the lipid in chloroform. After evaporation and rehydration, the liposome suspensions were frozen (liquid nitrogen) and thawed (40°C water bath) five times, thereby producing freeze-thaw multilamellar vesicles (FATMLVs) [12]. To make large unilamellar liposomes by extrusion (LUVETs), the FATMLVs were passed 10 times through 0.1 μm double stacked Nuclepore filters using an extruder device (Lipex Biomembranes Vancouver, BC, Canada). For samples containing DPPC the extrusion was done at 50°C. Sizes of liposomes were determined by light scattering using a Nicomp Model 270/370 Submicron Particle Sizer. For all studies, LUVETs had a mean diameter between 98 and 118 nm.

2.4. Spectroscopy

Right angle light scattering of indolicidin in buffer solution (no liposomes) was performed using a PTI Alfa Spectrofluorometer (PTI Instruments, Princeton, NJ). Excitation wavelength was set at 500 nm. Scattered light was quantitated by scanning between 460 and 540 nm (maximum at 500 nm), and the area under the curve was plotted versus indolicidin concentration. Spectrophotometric analysis demonstrated that indolicidin does not absorb light between 460 and 540 nm.

The fluorescence of indolicidin in buffer solution (10 mM Hepes, 150 mM NaCl at pH 7.4) was measured using the above mentioned spectrofluorometer and quartz cuvettes. The excitation maximum was found to be 285 nm. With the excitation wavelength fixed at 285 nm, emission spectra were taken between 320 and 450 nm (emission maximum at 353 nm). The area under the emission curve was plotted versus indolicidin concentration. Indolicidin-free liposomes (100 nm diameter) were prepared as described above. The emission spectra of buffer, indolicidin-free liposomes in buffer, and indolicidin in buffer with and without liposomes (0.5 μg/ml indolicidin and 0.1 mg/ml lipid) were measured between 300 nm and 500 nm (excited at 285 nm). Spectra of the buffer solution alone and empty liposomes were subtracted from the appropriate indolicidin spectra to correct for scattering.

2.5. Hemolytic activity

Hemolysis was measured as previously described [13]. Briefly, an equal volume of sample was mixed with washed 4% (v/v) human red blood cells in phosphate-buffered saline and incubated with constant agitation for 20 h at 37°C. Following low speed centrifugation ($\sim 1000 \times g$ for 10 min) an aliquot of the supernatant was taken and diluted with buffer solution and the absorbance at 550 nm measured. The controls for 0 and 100% hemolysis were established by mixing the 4% red blood suspension 1:1 with either buffer solution or distilled water, respectively.

2.6. Cytotoxicity

The specific inhibition of in vitro proliferation of CHO/K1 cells by free, liposomal indolicidin or empty (indolicidin-free) liposomes was monitored by a [^{3}H]thymidine incorporation assay [14]. In brief, $2 \cdot 10^4$ cells/well were plated onto 96 well flat-bottomed mi-

crotiter plates in RMP-1640 medium supplemented with 10% FBS, and kept at 37°C in a humidified atmosphere of 5% CO_2. Cells were exposed to various concentrations of either empty (indolicidin-free) liposomes, phosphate-buffered saline (PBS), free indolicidin or liposomal indolicidin and cultured for 4 h at 37°C. Cells treated with various formulations of indolicidin were incubated for another 8 h with an added 0.5 μCi/well of [^{3}H]thymidine (specific activity 50 Ci/mmol) (ICN Biomedicals, USA). Cells were harvested on 934AH filter paper with a Brandel M-96 harvester (Brandel, MD, USA). [^{3}H]Thymidine incorporation was determined by liquid scintillation counting.

2.7. Animal models

For in vivo toxicity, groups of male Balb/c mice (5/group, weight 20–22 g) were injected with various doses of free indolicidin (0.4–12 mg/kg) or liposomal indolicidin (20–160 mg/kg) in 0.2 ml of pyrogen-free saline via the tail vein. Normal saline or suspensions of indolicidin-free liposomes were also injected in separate groups of mice. The mice were observed for 14 days to determine long term consequences although death usually occurred within 15 min. The LD_{10} dose was estimated according to method of Litchfield and Wilcoxon [15].

For in vivo efficacy, an infected animal model was established using *Aspergillus fumigatus* strain 36607 which was obtained from ATCC and maintained in Malt extract agar medium (Blakeslee's Formula). In preliminary experiments three groups (10/group) of male Balb/c mice (body weight 18–22 g) were injected via the tail vein with $8 \cdot 10^7$, $4 \cdot 10^7$, or $2 \cdot 10^7$ spores of *A. fumigatus* suspended in 0.2 ml sterile phosphate-buffered saline (PBS). Animals treated with either $8 \cdot 10^7$ or $4 \cdot 10^7$ spores died within 2 days but animals treated with $2 \cdot 10^7$ fungal spores survived for 4–5 days. In all subsequent experiments, we used a dose of $2 \cdot 10^7$ *A. fumigatus* spores for infection.

Five groups of mice (10/group) were injected with $2 \cdot 10^7$ *A. fumigatus* spores administered intravenously via the tail vein. 6 h following infection the first and second groups received either 2 mg/kg free indolicidin or liposomal indolicidin via the tail vein. The third and fourth groups were given either 40 mg/kg liposomal indolicidin or the same amount of lipid dosed as empty liposomes. An additional control group was injected with 0.2 ml PBS. The therapeutic efficacy of free indolicidin and liposomal indolicidin was measured by quantifying percent surviving animals as a function of time after infection.

3. Results and discussion

Although indolicidin is highly soluble in water (≥ 20 mg/ml) we explored the possibility that because of its preponderance of hydrophobic residues, indolicidin might self associate at the higher concentrations. We found the 90° angle light scattering of this molecule increased dramatically when its concentration exceeded approx. 50 μg/ml (Fig. 1). In similar experiments we plotted the area under the curve of the fluorescence emission profile of indolicidin scanned over 325–450 nm (excited at 285 nm) as a function of concentration and again found a discontinuity near 50 μg/ml confirming a concentration dependent self association of this peptide (Fig. 2). The importance of this self association became apparent when we examined indolicidin toxicity. Interestingly, the concentration corresponding to self association coincided with the onset of indolicidin in vitro hemolytic activity (Fig. 3) mirroring activity not unexpected for a membrane active agent [16].

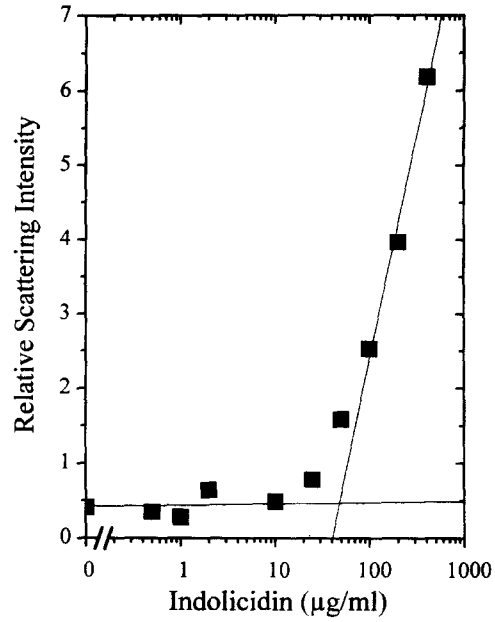

Fig. 1. Light scattering (90° angle) of aqueous indolicidin. Indolicidin was placed in buffer solution (10 mM Hepes, 150 mM NaCl at pH 7.4) and the amount of light scattered at 90° was measured using a spectrofluorometer with excitation and emission wavelengths set at 500 nm. The lines are linear regression fits of the first five data points and the last three data points. The intersection occurs at ~ 50 μg/ml indolicidin.

The molecular details of how indolicidin permeabilizes RBC membranes were unclear but its character, adsorption and disruption of membranes invited comparison to detergents and their permeabilization of membranes [16]. In particular, the self association of indolicidin suggested that it might partition into bilayer membranes to avoid aqueous exposure of its hydrophobic domains. Using this strategy, we believed we could entrap indolicidin into liposomes where its availability and therefore acute toxicity would be reduced making it a more appealing candidate for antimicrobial therapy. To gauge the extent to which the peptide would interact with various membranes, we mixed indolicidin with preformed single lamellar liposomes (100 nm)

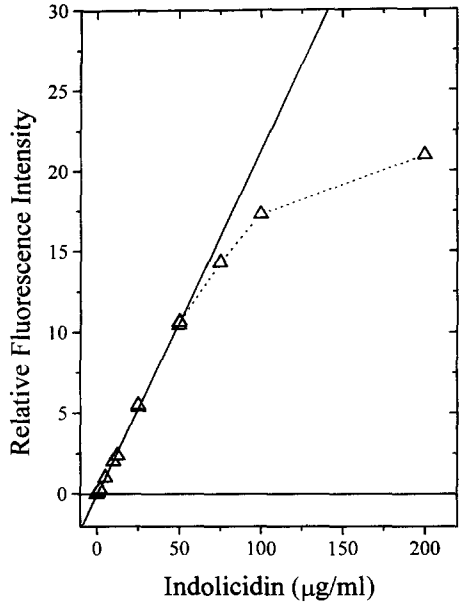

Fig. 2. Relative fluorescence of aqueous indolicidin. Indolicidin was added to buffer solution and the fluorescence emission spectrum scanned between 320 and 450 nm with the excitation wavelength set at 285 nm. The relative fluorescence spectra (areas under the curve for the emission spectra) are plotted as a function of indolicidin concentration. The solid line is a linear regression fit of the first 13 data points, $r = 0.999$ (the highest three indolicidin concentrations were excluded). The deviation from linearity occurred above 50 μg/ml indolicidin which could be the result of self quenching but is more likely due to an inner filter effect derived from the increased light scattering.

at room temperature comprised of either solid (DPPC) or fluid phase (POPC) lipid, and examined fluorescence emission profiles (Fig. 4). The blue-shift in emission maximum, as well as increase in fluorescent intensity suggested the partitioning of tryptophan residues from buffer solution into the comparatively less polar environment of the lipid bilayer.

Although qualitatively it appeared that POPC liposomes were afforded the largest degree of partitioning, no quantitative assessment could be made from the fluorescence data. However, when we examined the retention of indolicidin by POPC liposomes formed by the freeze thaw procedure, we found that essentially all of the added indolicidin was associated whereas only ~ 20% entrapment would be expected based on aqueous distributions. Similarly made dispersions of DPPC/cholesterol (60:40) liposomes, the lipid system showing the least indolicidin adsorption/penetration by fluorescence, retained only ~ 20% of the initial amount of indolicidin consistent with entrapment into the aqueous volume of these systems. In this study, the initial aqueous concentrations of indolicidin and lipid were held at 0.33 mg/ml and 20 mg/ml, respectively, and repeated centrifugation with supernatant replacement was used to remove unbound/unentrapped material and the indolicidin to lipid ratio determined as described in Materials and methods. This preference for partitioning into fluid phase bilayers is not unique to indolicidin but rather has been shown for other amphipathic molecules (i.e., anesthetics [17,18]). Inclusion of cholesterol in POPC and DPPC membranes reduced the fluorescence intensity of indolicidin. Because we do not know the depth of indolicidin penetration into the bilayer we can not fully assess the role of chain fluidity (as affected by cholesterol) upon indolicidin partitioning. However, consistent with our results, cholesterol has been shown to reduce the partitioning of anesthetic into both fluid and gel phase bilayers [18] suggesting that acyl chain mobility may have only marginal impact upon the ability of these classes of agents to associate with membranes.

We realized at this point that we could take advantage of indolicidin's high affinity for POPC bilayer membranes and entrap it with high efficiency (100%). To make the sample size more homogeneous we next turned to extrusion to make the liposomes reproducibly at 100 nm mean diameter. Using this 100 nm POPC formulation we assessed the cytotoxicity of free indolicidin and liposome-bound indolicidin against normal CHO/K1 cells using a [^{3}H]thymidine incorporation assay (Fig. 5). Comparison between free indolicidin and liposomal indolicidin clearly showed that POPC associated indolicidin was less toxic to normal mammalian cells. However, there was some toxicity observed when higher concentrations of liposomal indolicidin were used. The 50% growth inhibitory concentra-

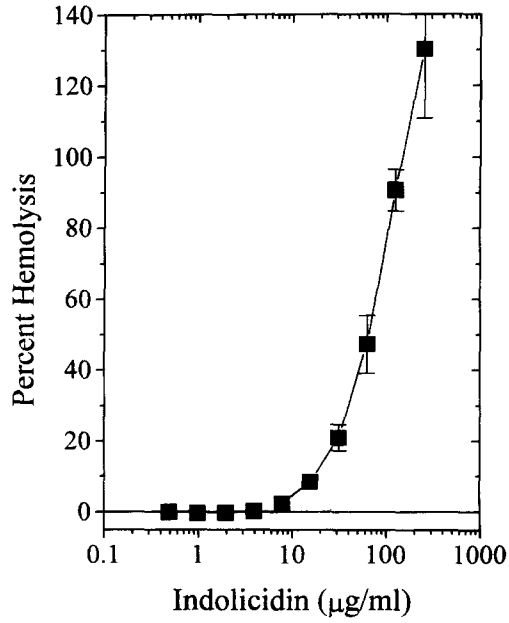

Fig. 3. Hemolytic activity of aqueous indolicidin. Indolicidin was incubated with washed human red blood cells for 20 h at 37°C (see Materials and methods for details). Error bars represent the standard deviation for three experiments (note: each experiment is the average of two to three replicates). Hemolytic activity increased dramatically between 10 and 100 μg/ml indolicidin. Percent hemolysis values greater than 100% were obtained because indolicidin was apparently more efficient than the distilled water control at lysing red blood cells; the '100%' control using distilled water does not yield complete hemolysis but is used for comparative purposes.

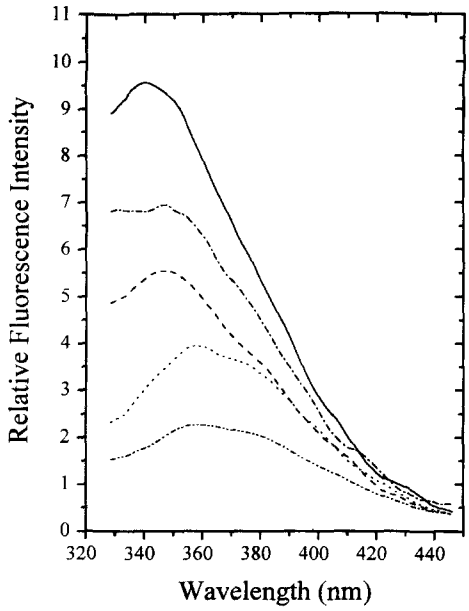

Fig. 4. Fluorescence emission profiles of indolicidin in 100 nm liposomes and free indolicidin in buffer solution. Spectra from highest to lowest intensity are of indolicidin in POPC liposomes (—), in POPC/cholesterol (60:40 mole ratio) liposomes (– · –), in DPPC liposomes (– – –), DPPC/cholesterol (60:40) liposomes (· · · · · ·), and in buffer solution (– · · –). Lipid and indolicidin concentrations were 0.1 mg/ml and 5 μg/ml, respectively. The emission maximum is blue shifted by 18 nm for indolicidin in POPC liposomes (λ_{max} = 340.5 nm) as compared to buffer solution (λ_{max} = 358.5 nm). Each spectrum is the average of five scans.

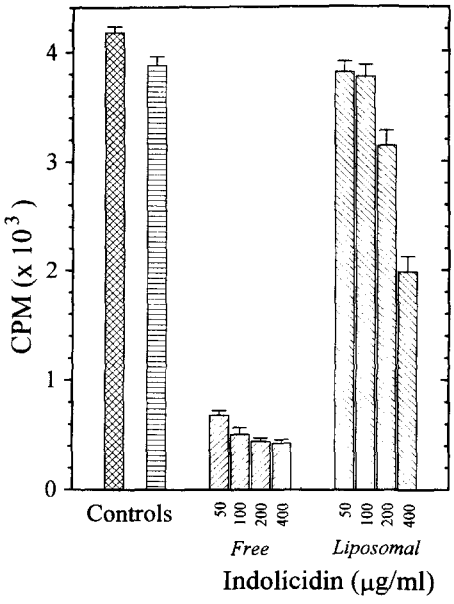

Fig. 5. Growth inhibition of CHO/K1 cells by free indolicidin and liposomal indolicidin after 12 h as a function of indolicidin concentration. Cells were first incubated for 4 h and then 0.5 μCi/well [^{3}H]thymidine was added with a further 8 h incubation in media. Indolicidin comprised 5 mol% of a mixture with POPC 100 nm LUVETs. Controls were run using PBS (crosshatch lines) and empty liposomes (horizontal lines). Results are expressed as the means ± S.D. of eight replicates.

Table 1
Indolicidin in vivo toxicity in Balb/c mice

Indolicidin (mg/kg)	Mortality
0.4	0/5
1.2	1/5
4.0	3/5
12.0	5/5

Four groups of mice (5/group) were injected with various doses of free indolicidin in 0.2 ml of pyrogen-free saline via the tail vein. Normal saline was also injected in a separate group of mice but no toxicity was observed.

tion (IC_{50}) of free indolicidin was much lower than that of liposomal indolicidin, 30 versus 479 μg/ml. The higher IC_{50} for liposomal indolicidin was probably a result of the sequestration of indolicidin within the liposomes which would restrict the amount of material available to interact with cells. Results similar to those reported for the thymidine incorporation assay were obtained from an assay in which CHO/K1 cell proliferation was measured directly by counting cell numbers (not shown).

We next evaluated the POPC liposomal indolicidin formulation in Balb/c mice (see Table 2). The data from Table 1 Table 2were used to estimate the LD_{10} of free indolicidin. Free indolicidin had an estimated LD_{10} of 0.9 mg/kg whereas liposomal indolicidin had a significantly reduced toxicity ($LD_{10} \geq 59$ mg/kg). Symptoms reflecting the acute toxicity of free indolicidin and liposomal indolicidin were similar and included gasping and convulsions most likely neurological in origin. These in vivo results were consistent with the in vitro CHO/K1 data. We postulate that the reduced toxicity of indolicidin entrapped in liposomes may be due to (i) altered interactions of indolicidin with mammalian cells or (ii) decreased availability because liposomes release entrapped indolicidin slowly, restricting its concentration below that required for deleterious effects.

Table 2
Toxicity of liposomal indolicidin in Balb/c mice

Indolicidin (mg/kg)	Mortality
20	0/5
40	0/5
80	1/5
150	4/5
160	5/5

Five groups of mice (5/group) were injected with various doses of liposomal indolicidin (5 mol% in POPC 100 nm LUVETs) in 0.2 ml of pyrogen-free saline via the tail vein. Normal saline and indolicidin-free 100 nm POPC liposomes were also injected in separate groups of mice but no toxicity was observed with these controls. To find the maximum mol%age of indolicidin that could be introduced safely into mice we escalated the mol% of indolicidin (up to 15 mol%) in POPC liposomes. However, these systems showed immediate lethality in mice (data not shown). This lethality was not observed at similar doses when indolicidin comprised up to 5 mol% of the mixture with POPC liposomes.

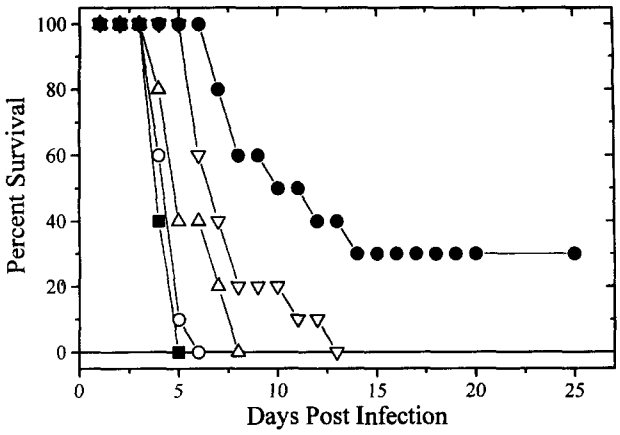

Fig. 6. Therapeutic efficacy of a single dose of free indolicidin and liposomal indolicidin against systemic aspergillosis in Balb/c mice. Liposomes were 100 nm POPC LUVETs containing indolicidin at 5 mol%. Mice (10/group) were infected intravenously with $2 \cdot 10^7$ *A. fumigatus* spores via tail vein. After 6 h of spore challenge, the first and second groups received 2 mg/kg of either free indolicidin (△) or liposomal indolicidin (▽) intravenously. The third and fourth groups were treated either with 40 mg/kg of liposomal indolicidin (●) or with a similar dose of indolicidin-free liposomes (○). The fifth group was treated with 0.2 ml of 10 mM Hepes-buffered saline (■).

The reduced toxicity of the liposomal formulation encouraged us to develop an animal model for systemic fungal infection to evaluate and compare the therapeutic efficacy of free indolicidin and liposomal indolicidin. Two mg/kg of free indolicidin had no significant effect on the survival of mice infected with *A. fumigatus* (nor did this dose appear to cause any toxic deaths in infected animals) whereas 20% of mice treated with a similar dose of liposomal indolicidin survived 10 days (Fig. 6). Although escalating the dose of free indolicidin was prohibited by its toxicity, Fig. 6 shows that survival was considerably increased by treating the animals with 40 mg/kg of liposomal indolicidin; a 30% long term survival was achieved. The surviving mice were killed after the 25th day with no sign of disease in their lungs upon gross pathological inspection. Lungs of the terminated mice were plated on Malt extract agar medium and no *A. fumigatus* was detected after 3 days of incubation at 30°C. It is well documented that liposomal association can improve the therapeutic index of systemically toxic drugs [4] but the exact mechanism for improved therapeutic efficacy with liposomal indolicidin is not clearly understood. Possible explanations for reduced toxicity and improved efficacy may include the rapid uptake of liposomes by macrophages giving indolicidin less opportunity to be systemically active. In disease, the migration of macrophages to the site of infection would provide a mechanism for accomplishing high local concentrations of indolicidin at target sites. Alternatively, uptake of liposomal indolicidin by macrophages might augment their activity.

The observations presented here demonstrate the usefulness of liposomes as carriers in improving the therapeutic index of indolicidin and perhaps other neutrophil-derived antimicrobial peptides in the treatment of fungal infections. The therapeutic utility of intravenously infused free indolicidin appears to be limited due to its toxicity. However, the in vivo study described here demonstrates for the first time that a non-toxic liposomal formulation of indolicidin is efficacious in treating systemic murine aspergillosis.

Acknowledgements

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