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Biodistribution of long-circulating PEG-liposomes in a murine model of established subcutaneous abscesses

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Abstract

The biodistribution of long-circulating PEG-liposomes in a subcutaneous mouse model of established mixed infection abscesses was investigated to assess their possible role as drug carriers in the treatment of small, undrainable intra-abdominal abscesses. There was a 10–30-fold greater localisation of ^{67}Ga-labelled PEG-liposomes in abscesses compared to uninfected normal skin samples. Over 3% of the injected dose (ID) of liposomes was present in the abscesses 24 h after liposome administration in contrast to 0.1% in normal skin sections. The percentage ID present in the liver, spleen and kidneys was 17%, 4% and 2% per organ respectively. Five days after liposome injection, 2% ID could still be recovered from the abscesses. Using colloidal gold-labelled PEG-liposomes, it was shown that there was a 4-fold greater density of liposome clusters in the subcutaneous tissue surrounding the capsule than in the core of the abscesses. The clusters within the abscesses were distributed evenly. We conclude that PEG-liposomes localise to a significant degree at the infection focus in our mouse model and may provide a new approach to the antimicrobial treatment of intra-abdominal abscesses.

Keywords: Polyethylene glycol-coated liposome; Abscess model; Vascular permeability; Tissue distribution; Colloidal gold liposome

1. Introduction

Despite improvements in the treatment of intra-abdominal abscesses, high rates of morbidity and mortality continue to be associated with these infections [1]. In our subcutaneous mouse model of established mixed infection abscesses, we have shown that treatment with broad-spectrum antimicrobials can result in a significant reduction in bacterial counts but only when very high doses were employed [2–4].

Treatment of established abscesses with antimicrobials encapsulated in long-circulating polyethylene glycol-coated (PEG)-liposomes may offer an alternative, more efficacious form of therapy. Previously, it has been demonstrated in other experimental animal models that these liposomes localise preferentially at the inflammatory sites of arthritis in rats [5], colitis in rabbits [6], local bacterial thigh muscle infection in rats [7], osteomyelitis in rabbits [8] and acute unilateral pneumonia of the left lung in rats [9]. Furthermore, compared to the free form of an antibiotic, therapeutic efficacy can be enhanced [10–12] and drug toxicity can be reduced [11,13] when antimicrobials are encapsulated in these liposomes.

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In this study, we have investigated the biodistribution of PEG-liposomes in the abscess model to assess their possible role as drug carriers in the treatment of small, undrainable intra-abdominal abscesses.

2. Materials and methods

2.1. Animals

Female specified pathogen free (SPF) BALB/c mice (IFFA Credo, l'Arbresle, France) 12–18 weeks and weighing 20–25 g were used throughout the study. The caecal contents of male SPF Swiss mice (Broekman Institute, The Netherlands) were used for the production of autoclaved caecal contents (ACC) [14]. All animals received water and food ad libitum. The study was approved by the Institutional Animal Care and Use Committee of the Erasmus University (Rotterdam, The Netherlands).

2.2. Bacterial strains

Bacteroides fragilis ATCC 23745 and *Escherichia coli* ATCC 25922 were used. Both strains were first passaged in BALB/c mice and standardised bacterial suspensions were made and stored at −80°C until required. Overnight cultures were obtained by inoculating 30 ml volumes of Wilkens Chalgren broth (Boom, Meppel, The Netherlands) with 0.1 ml of the standardised frozen bacterial suspensions and incubating aerobically (*E. coli*) or anaerobically (*B. fragilis)* at 37°C for 18 h.

2.3. Mouse model

The subcutaneous abscess model has been described previously [2]. Briefly, mice were inoculated (s.c.) on the right flank with 0.25 ml mixtures containing *B. fragilis*, 10^7 cfu, *E. coli*, 10^5 cfu, and ACC, 4 mg dry weight, in a ratio 1:1:2. The skin on the left flank was uninfected and used as a control. Vascular permeability and biodistribution studies were performed 3 days after inoculation on the established, well-encapsulated abscesses that developed within the subcutaneous tissue. Abscesses contained approx. 8 $\log_{10}$ cfu/abscess of both *B. fragilis* and *E. coli* strains.

2.4. Vascular permeability

A quantitative test described by Zhang et al. [15] was used to measure vascular permeability changes in the abscesses. Three mice were inoculated on the right flank with *B. fragilis*/*E. coli*/ACC and, after 3 days, were injected via the tail vein with a saline suspension of Evans blue dye (40 mg/kg) (Merck, Darmstadt, Germany). After 24 h, the mice were killed by CO_2 asphyxiation and the complete pelt was removed. Abscesses (including a section of surrounding skin) were removed using an 18 mm punch and an identical sized section of uninfected normal skin was similarly dissected from the left flank. The dye was extracted from the tissues by placing the abscess and skin sections in 2 ml formamide (Sigma, St. Louis, MO, USA) for 3 days. Absorbance was measured on an LKB Ultrospec Plus spectrophotometer (Pharmacia, Uppsala, Sweden) at a wavelength of 623 nm. Standards (0–20 μg Evans blue/ml) were also dissolved in formamide. Results were expressed as total Evans blue (μg) per abscess or skin section ± S.D.

2.5. Preparation and labelling of PEG-liposomes

PEG-liposomes were prepared as previously described [9]. Briefly, partially hydrogenated egg phosphatidylcholine (Asahi Chemical Industry, Ibaraki-ken, Japan), cholesterol (Sigma) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(PEG-2000) (Avanti Polar Lipids, Alabaster, AL, USA) were dissolved in chloroform:methanol in a round bottom flask in a molar ratio of 1.85:1:0.15, respectively. After evaporation, the lipid mixture was redissolved in 2-methyl-2-propanol (Sigma) and freeze dried overnight. The dried lipids were hydrated for 2 h in 10 mM HEPES/135 mM NaCl (Sigma) buffer pH 7.4, containing 5 mM desferal (Novartis, Basel, Switzerland). To obtain liposomes of approx. 100 nm, the hydrated lipid dispersion was sonicated for 8 min in a MSE Soniprep 150 (Sanyo Gallenkamp, Leicester, UK). The liposomes were labelled with ^{67}Ga-deferoxamine as outlined by Schiffelers et al. [16]. Previous studies have shown ^{67}Ga-deferoxamine to be an appropriate label for monitoring intact liposomes in the circulation because of the minimal translocation of radioactive labels to plasma proteins and the rapid renal clearance after release from the liposomes [17].

2.6. Biodistribution studies

The ^{67}Ga-labelled PEG-liposomes (100 μmole total lipid/kg) were administered i.v. via the tail vein to groups of six mice 3 days after bacterial inoculation of the right flank. At 24 h, 48 h, 72 h or 120 h after liposome injection, mice were killed by CO_2 asphyxiation and the abscesses and normal skin sections were removed as described in Section 2.4. The liver, spleen and kidneys were also removed. The radioactivity in the abscesses, skin and organs was counted in a Minaxi autogamma 5000 γ-counter (Packard, Meriden, CT, USA). The contribution of the radioactivity in the blood was subtracted from the total radioactivity measured in the abscesses, skin sections and organs as described previously [16]. Results were expressed as percentage injected dose (% ID) per abscess, skin section or organ ± S.D. The correlation between abscess weight and liposome localisation 24 h after injection was analysed by linear regression.

2.7. Preparation and tissue distribution of colloidal gold-labelled PEG-liposomes

Colloidal gold-labelled PEG-liposomes were prepared as described by Daemen et al. [18]. Briefly, the lipid film was prepared as outlined above and hydrated at 4°C with a filtered (0.2 μm) 1.1% (w/v) aqueous solution of $AuCl_2$ (Sigma) 4-fold diluted with sodium citrate (28 mM)/potassium carbonate (7 mM) buffer, pH 7.4. PEG-liposomes were sized by sonication as described above. During sonication the yellow suspension turned purple. Unencapsulated colloidal gold was removed by gel filtration of the liposome suspension over a Sephacryl SF S1000 column (Pharmacia) using HEPES/NaCl buffer as the eluent.

Two mice were injected i.v. with colloidal gold-labelled PEG-liposomes (approx. 10 μmol total lipid/kg) 3 days after bacterial inoculation. At 24 h after liposome injection, abscesses were removed as described previously, washed in three changes of 2.5% buffered formaldehyde (Merck), fixed in 10% buffered formaldehyde and embedded in paraffin. Sections of 5 μm were cut on a microtome and mounted on slides. Preparations were deparaffinised in two changes of xylene and hydrated. The colloidal gold-labelled PEG-liposomes were silver-enhanced using a silver enhancement kit (Sigma) according to the manufacturer's instructions. Eosin/haematoxylin was applied as a counterstain. Distribution of silver-enhanced colloidal gold clusters was evaluated by counting the number of clusters present in sectors measuring a total distance of 2.5 mm from the capsule. Measurements were made both within the abscess (capsule → abscess core) as well as outside the abscess (capsule → surrounding subcutaneous tissue). Each sector was divided into 10 sub-sectors each measuring 30 μm^2. In total, 40 different sectors were measured from two different abscesses and the results were expressed as mean number of clusters counted per 30 μm^2 ± S.D.

2.8. Statistical analysis

Differences in the vascular permeability and liposome biodistribution between uninfected normal skin compared to skin sections containing abscesses were analysed by the paired *t*-test.

3. Results

3.1. Vascular permeability

Differences in the vascular permeability of uninfected normal skin compared to skin sections containing subcutaneous abscesses were determined in mice by i.v. injection of Evans blue 3 days after bacterial inoculation. Unlike the normal skin, a distinct

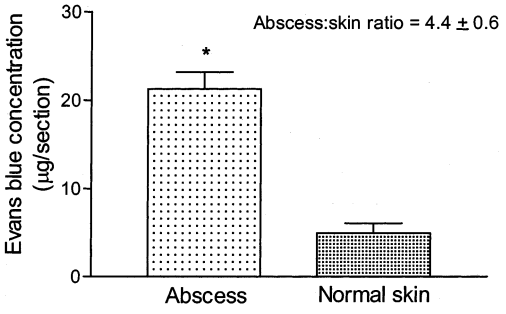

Fig. 1. The vascular permeability of infected (abscess) and uninfected normal skin sections in mice. Evans blue dye (40 mg/kg) was injected (i.v.) 3 days after inoculation (s.c.) with *B. fragilis–E. coli* and, after 24 h, abscesses and normal skin sections were removed. The mean μg Evans blue per abscess or normal skin section (± S.D.) is shown ($n = 3$, $^*P = 0.0008$ compared to uninfected normal skin).

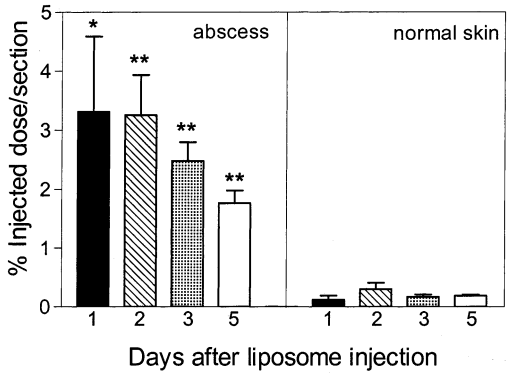

Fig. 2. Biodistribution of ^{67}Ga-labelled PEG-liposomes in infected (abscess) and uninfected normal skin sections in mice. Liposomes (100 μmole/kg) were injected (i.v.) 3 days after bacterial inoculation (s.c.) with *B. fragilis–E. coli* and, after 24 h, 48 h, 72 h or 120 h, abscesses and normal skin sections were removed. The mean % injected dose per abscess or normal skin section (±S.D.) is shown ($n=6$, $*P=0.0013$, $**P<0.0001$ compared to uninfected normal skin).

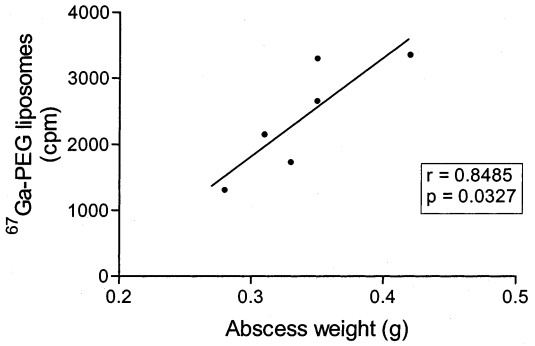

Fig. 3. Correlation between abscess weight and liposome localisation 24 h after injection (i.v.) of ^{67}Ga-labelled PEG-liposomes. Each point represents an individual abscess.

dark blue zone was evident around the abscesses. After formamide extraction of the dye, these differences were confirmed spectrophotometrically and indicated a 4-fold difference in the vascular permeability between abscess and normal skin sections, $P=0.0008$ (Fig. 1).

3.2. Biodistribution of ^{67}Ga-labelled PEG-liposomes

^{67}Ga-labelled PEG-liposomes, injected 3 days after bacterial inoculation, localised at the infectious focus with a 10–30-fold greater liposome concentration in the abscesses compared to normal skin sections (Fig. 2). Over 3% ID was present in the abscesses 24 h after liposome injection compared to 0.1% in normal skin ($P=0.0013$). The % ID present in the liver, spleen and kidneys at this time was 17±3.4, 4±0.6 and 2±0.3% per organ respectively (results not shown). Although abscess localisation gradually de-

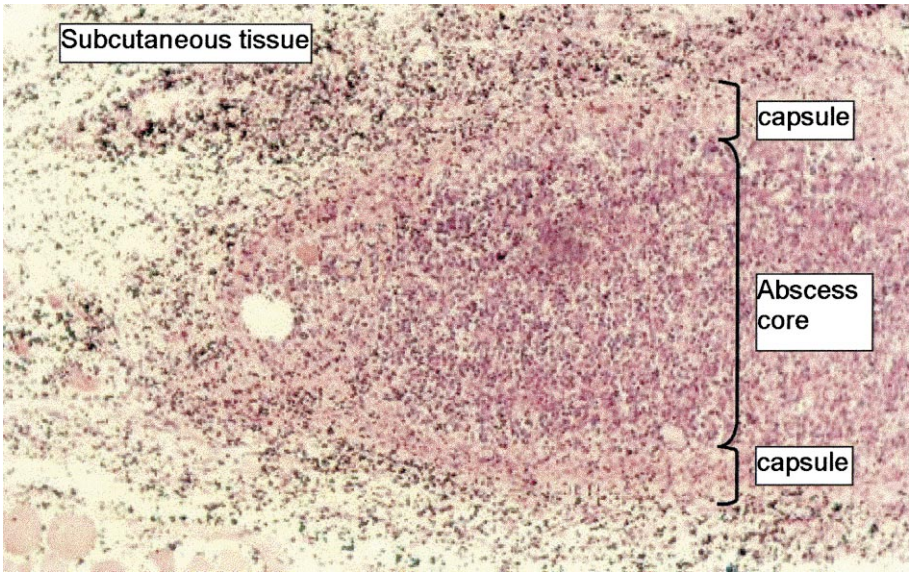

Fig. 4. Tissue distribution of colloidal gold-labelled PEG-liposomes in abscesses. Liposomes (10 μmole/kg) were injected (i.v.) 3 days after bacterial inoculation (s.c.) with *B. fragilis–E. coli* and, after 24 h, abscesses were removed. Liposomes were stained with silver enhancement kit (see text) and counterstained with eosin/haematoxylin. Liposome clusters are shown as black specks both within the abscess core and in the surrounding subcutaneous tissue (magnification ×400).

creased over the following days, approx. 2% ID could still be recovered from the site of infection after 5 days. In addition, an increase in abscess weight was also associated with a greater degree of liposome localisation 24 h after injection (Fig. 3).

3.3. Distribution of colloidal gold-labelled PEG-liposomes

Colloidal gold-labelled liposomes were used to evaluate the tissue distribution of i.v. injected liposomes at the cellular level. Micrographs showed an abundance of silver-enhanced colloidal gold clusters in the subcutaneous tissue surrounding the abscess capsule (Fig. 4). Clusters were also present inside the core of abscess, albeit at a lower density. Quantification of the clusters in 30 μm^2 areas showed that the capsule acted as a barrier to liposome penetration (Fig. 5). The density of clusters was approx. 4-fold higher in the surrounding subcutaneous tissue than inside the core of the abscess. Within the capsule, the clusters were distributed evenly.

4. Discussion

The aim of the present study was to investigate the biodistribution of PEG-liposomes in our mouse model to assess their possible role as drug carriers in the treatment of small, undrainable intra-abdominal abscesses. Previous studies have shown that treatment of these established abscesses is difficult [3,4]. Very high doses of trovafloxacin (well into the range of murine toxicity) were necessary to obtain more than a 2 $\log_{10}$ cfu/abscess reduction of both bacterial strains after 5 days of treatment [2]. These results emphasise the need for a more efficacious therapy for these mixed infections.

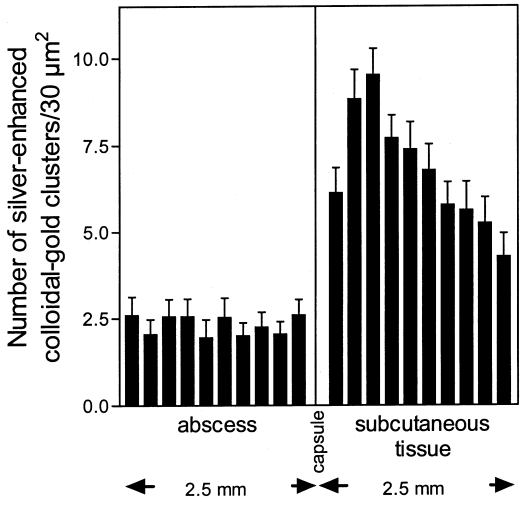

Fig. 5. Distribution of silver-enhanced colloidal gold clusters in sectors of 30 μm^2 measuring a total distance of 2.5 mm from the capsule both within the abscess core and outside in the surrounding subcutaneous (s.c.) tissue. Results are the mean ± S.D. of 40 measurements per 30 μm^2 sector.

One of the reasons for treatment failure of established abscesses may be sub-effective drug concentrations at the site of infection and/or limited penetration of the antimicrobial into the core of the abscesses [19]. In this respect, the encapsulation of antibiotics in liposomes may be beneficial in the treatment of these infections by altering the pharmacokinetics of the drug and enhancing therapeutic efficacy [10–12,20] as well as reducing any potential antibiotic toxicity [11,13]. PEG confers a steric stability that reduces liposome uptake by the mononuclear phagocytic system and results in a prolonged circulation time and an improved liposome localisation at inflammatory sites [16,21–24]. Indeed, the PEG-liposome blood concentration is the primary factor influencing the localisation of these liposomes at the site of infection in a rat model of acute unilateral pneumonia [24]. Furthermore, when an antibiotic is liposome-encapsulated, the doses and/or dosing frequency required for an effective treatment can be reduced compared to the free form of a drug [10–12].

In the present study, the 10–30-fold higher degree of liposome localisation at the site of the abscess (over 3% ID) compared to normal skin illustrates the targeting potential of PEG-liposomes in this model. Furthermore, the liposome recovery from the abscess, liver, spleen and kidneys was similar to that found at the site of infection and organs in other animal models [10,16]. The liposomes could be recovered from the abscesses for a considerable period of time (even at 5 days after injection). The preferential localisation is probably due to the increased vascular permeability facilitating local liposome extravasation. Increased capillary permeability has been reported recently to play a crucial role in the localisation of PEG-liposomes in infected rat lung tissue [25]. In addition, the positive linear relationship between abscess weight and liposome localisation sup-

ports an earlier report by Bakker-Woudenberg et al. [9] in which liposome localisation was linearly related to infected left lung weight. Presumably, the intensity of the inflammatory response, which is a prerequisite for liposome extravasation, is reflected by the weight (cell and fluid influx) at the site of infection.

Using colloidal gold-labelled liposomes, we have shown that PEG-liposomes penetrate the capsule of the abscess and are evenly distributed within the abscess core. However, liposome localisation was significantly greater in the subcutaneous tissue surrounding the abscess indicating the substantial barrier imposed on liposome penetration by the capsule. These results indicate that an antibiotic, if liposome-encapsulated, would be delivered to a lesser degree directly into the core of the abscess than in the surrounding tissues. Nevertheless, the liposomes remaining outside the capsule could be a source of released antibiotic in the free form which could more readily penetrate the abscess capsule.

The fluidity of the lipid bilayer is an important determinant in the release of encapsulated antibiotics from liposomes and, by varying liposome composition, a bilayer fluidity can be selected to ensure that drug retention in the bloodstream and drug release at the site of infection is optimised without compromising target localisation [16]. The potential of these liposomes, therefore, to preferentially localise and release effective concentrations of an antibiotic within the abscesses and surrounding tissues over a considerable period of time provides an interesting prospect for future studies and a possible new approach to the antimicrobial treatment of intra-abdominal abscesses.

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