Technetium-99m labelled liposomes to image experimental arthritis

Otto C Boerman, Wim J G Oyen, Gert Storm, M Luisa Corvo, Louis van Bloois, Jos W M van der Meer, Frans H M Corstens

Abstract

Objectives—Liposomes sterically stabilised with polyethylene glycol (PEG) labelled with technetium-99m were tested for their ability to image adjuvant arthritis in a rat model.

Methods—Adjuvant arthritis was induced in the ankle joint of the left hind foot by injection of *Mycobacterium butyricum* in Freund's incomplete adjuvant in the foot pad. Seven days later animals received the following radiopharmaceuticals labelled with ^{99m}Tc (a) non-PEG-liposomes, (b) PEG-liposomes or (c) non-specific human polyclonal IgG. For each of the radiopharmaceuticals the in vivo distribution of the radiolabel was monitored both scintigraphically as well as by counting the dissected tissues at two, eight, and 24 hours after injection.

Results—The pharmacokinetics of the radiopharmaceuticals differed considerably (half life in the blood: PEG-liposomes (18 hours) > ^{99m}Tc -IgG (3 hours) > non-PEG liposomes (1 hour)). The inflamed focus was visualised with each of the agents. The uptake of each of the radiopharmaceuticals in the inflamed ankle region correlated with their residence time in the blood (inflamed joint uptake: PEG liposomes (1.15% injected dose (ID)/g) > ^{99m}Tc -IgG (0.35% ID/g) > non-PEG-liposomes (0.05% ID/g)). Quantitative analysis of the images showed that the inflamed ankle to background ratio was highest with the PEG-liposomes (7.5 at 24 hours after injection), while with the other two agents this ratio did not exceed 4.

Conclusion—This study shows that ^{99m}Tc -labelled PEG-liposomes may be an excellent agent to visualise arthritis. Increased label uptake in the inflamed joint and increased target to background ratios can be obtained with PEG-liposomes because of their long circulating properties. In addition to their use as vehicles for scintigraphic imaging of arthritis PEG-liposomes might also be used for the site specific delivery of antirheumatic drugs.

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Departments of
Nuclear Medicine
O C Boerman
W J G Oyen
F H M Corstens

and Internal Medicine
J W M van der Meer

University Hospital
Nijmegen, the
Netherlands

Department of
Pharmaceutics,
Utrecht Institute for
Pharmaceutical
Sciences, University
Utrecht, the
Netherlands
G Storm
M L Corvo
L van Bloois

Departamento de
Biotecnologia,
IBQTA-INETI,
Portugal
M L Corvo

Correspondence to:
Dr O C Boerman,
Department of Nuclear
Medicine, University
Hospital Nijmegen, PO Box
9101, 6500 HB Nijmegen,
the Netherlands.

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inflammatory activity in the involved joints is important, not only for accurate early diagnosis, but also to monitor the state of the disease and to evaluate the effect of the therapeutic regimen. However, because considerable interobserver and intraobserver variability exists, there is a continued interest in the development of an objective, quantitative method to assess disease activity in the involved joints.

The use of radiopharmaceuticals in the detection of arthritis activity has the advantages of permitting direct imaging by means of whole body scintigraphy, while joints that are difficult to assess clinically or radiographically can be studied as well. A wide range of technetium-99m labelled radiopharmaceuticals has been proposed to visualise rheumatoid arthritis: nanocolloid, methylene diphosphonate (MDP), ^{99m}Tc -IgG (^{99m}Tc -labelled human immunoglobulin G). The conventional bone scan with ^{99m}Tc -MDP is a sensitive test to study bone diseases. It has been shown, however, that in patients presenting with rheumatoid arthritis bone scans frequently show increased uptake in the affected joints unrelated to disease activity.^{1–3} In the late 1980s liposomes have been tested to image rheumatoid arthritis.⁴ However, the recognition of these liposomes by cells of the mononuclear phagocyte system (MPS) caused relative high uptake in liver and spleen, concomitant with rapid blood clearance and low uptake in the target. In recent years, it has been shown that inclusion of polyethyleneglycol (PEG), conjugated to phosphatidylethanolamine, in the lipid bilayer, considerably decreased the recognition of the liposomes by MPS cells.⁵ It is postulated that the flexible and hydrophilic PEG-tails cause the formation of a water mantle around the liposomes. As a result the interaction of liposomes with macromolecules from the surrounding solution is reduced and thus the liposomes are protected from destruction or opsonisation and recognition by MPS cells.⁶ As a result PEG-liposomes have an increased residence time in the blood.⁷ In previous studies we have shown that radiolabelled PEG-liposomes accumulate in infectious and inflammatory foci^{8–10} because of the locally enhanced capillary permeability. The prolonged residence time in the blood enables the PEG-liposomes to extravasate to a relatively high degree at sites of enhanced vascular permeability. Therefore, radiolabelled PEG-liposomes may also have potential to visualise joints affected by rheumatoid

The clinical course of rheumatoid arthritis is highly variable, with most patients suffering from persistent but fluctuating disease activity and variable episodes of more active and more quiescent inflammation. The assessment of the

arthritis. Moreover, PEG-liposomes can also be 'loaded' with drugs and thus could potentially be used for site directed delivery of antirheumatic drugs.

In this study we compared the imaging potential of ^{99m}Tc -PEG-liposomes with that of non-PEG-liposomes and the commercially available imaging agent ^{99m}Tc -IgG, in rats with adjuvant arthritis.

Methods

ANIMAL MODEL

Adjuvant arthritis was induced in the left foot of randomly bred male Wistar rats (3 month old, body weight 300 g) as described by Weichman.¹¹ After ether anaesthesia, 0.1 ml of a suspension of *Mycobacterium butyricum* (Difco Labs, Detroit, MI) in Freund's incomplete adjuvant (Sigma Chemical Co, St Louis, MO) (10 mg/ml) was injected in the foot pad of the left foot. Six days after induction, accumulation of mononuclear cells in the loose connective tissues of the foot can be appreciated at histological examination. Seven days after the inoculation, the respective radiopharmaceuticals were injected via the tail vein.

PREPARATION OF LIPOSOMES

Partially hydrogenated egg-phosphatidylcholine with an iodine value of 40 (PHEPC) prepared as described previously¹² was obtained from Asahi Chemical Industry Co (Ibarakiken, Japan). Cholesterol and glutathione were obtained from Sigma (St Louis, MO). For the preparation of PEG-liposomes the polyethyleneglycol (PEG) 1900 derivative of DSPE (a kind gift of Sequus Inc, Menlo Park, CA), while for the preparation of the non-PEG-liposomes underivatised DSPE (Avanti Polar lipids, Montreal,) was used as an ingredient.

A chloroform/methanol mixture (10/1, v/v) containing DSPE, PHEPC, and cholesterol was prepared at a molar ratio of 0.15:1.85:1. A lipid film was formed by rotary evaporation followed by high vacuum to remove residual organic solvent. The lipid film was dispersed at room temperature in 50 mM glutathione in HEPES buffer (10 mM HEPES, 135 mM NaCl, pH 7.5) at a phospholipid concentration of 120 mM. The liposomes were sequentially extruded through polycarbonate filters of 200, 100, 80, and 50 nm pore size (Poretics, Livermore, CA). Untrapped glutathione was removed by gel filtration on a 10DG column (Bio-Rad, Richmond, CA). The particle size distribution was determined by dynamic light scattering (Malvern 4700 system, Malvern, UK). The liposomes had a mean size of 120 nm.

LABELLING PROCEDURES

Preformed glutathione containing non-PEG-liposomes or PEG-liposomes were labelled with ^{99m}Tc essentially as described previously.¹³ ^{99m}Tc was transported by *d,l*-hexamethylpropylene amine oxime (HMPAO) through the bilayer and trapped irreversibly in the internal aqueous phase caused by reduction by the encapsulated glutathione. Briefly, 2.0 ml of

liposomes (75 mmol phospholipid/ml) were incubated for 15 minutes at room temperature with 20 mCi ^{99m}Tc -HMPAO. Removal of unencapsulated ^{99m}Tc -HMPAO was achieved by gel filtration on a 10DG column (Bio-Rad) eluted with 5% glucose.

A Technescan-HIG vial (Mallinckrodt Medical, Petten, the Netherlands) was reconstituted with 20 mCi ^{99m}Tc -pertechnetate according to the manufacturer's instructions. The resulting ^{99m}Tc -IgG was used without any further purification.

BIODISTRIBUTION STUDIES

Seven days after the inoculation of *M butyricum* in the foot pad, 45 rats were divided randomly into three groups of 15 rats. Each group was injected with 100 μCi of either ^{99m}Tc -labelled non-PEG-liposomes, PEG-liposomes or ^{99m}Tc -IgG via the tail vein.

At two, eight, and 24 hours after injection, five rats of each group were killed with 30 mg intraperitoneally injected phenobarbital. Blood was obtained by cardiac puncture. After cervical dislocation, several tissues (inflamed left ankle, right ankle, muscle, liver, spleen, kidney, intestine, right femur, and bone marrow from the right femur) were dissected, weighed, and their activity was measured in a shielded well type gammacounter. To correct for physical decay and to permit calculation of the uptake of the radiopharmaceuticals in each organ as a fraction of the injected dose, aliquots of the injected dose were counted simultaneously. Inflamed ankle to contralateral ankle uptake ratios (IA/CA) were calculated.

IMAGING PROTOCOL

Adjuvant arthritis was induced in another nine rats as described above. Seven days later, groups of three rats received 300 μCi of ^{99m}Tc -labelled non-PEG-liposomes, PEG-liposomes or ^{99m}Tc -IgG via the tail vein. Rats were anaesthetised (halothane/nitrous oxide/oxygen) and were placed prone on a single head gammacamera equipped with a parallel hole low energy collimator (Orbiter, Siemens Inc, Hoffmann Estates, IL). The three groups of rats were imaged synchronously at five minutes, one, two, four, six, and 24 hours after injection. Symmetric 15% windows were used for the 140 keV energy peak. Images (300 000 counts per image) were obtained and stored in a 256×256 matrix.

The scintigraphic results were analysed by drawing regions of interest over the heart region, the inflamed foot, over the normal contralateral foot—used as a background region—and over the whole animal. Inflamed foot to background ratios and percentage residual activity in the inflamed foot (inflamed foot to whole body ratio) were calculated.

STATISTICAL ANALYSIS

Values are given as mean (SD). Statistical analysis was performed using the one way analysis of variance test.

Results

LABELLING AND QUALITY CONTROL OF THE RADIOPHARMACEUTICALS

The labelling efficiency of Technescan HIG 10 minutes after reconstitution with 20 mCi $^{99m}\text{TcO}_4$ was 97% as determined by instant thin layer chromatography (Gelman Labs, Ann Arbor, MI). The non-PEG and the PEG-liposomes were labelled with ^{99m}Tc -HMPAO with a labelling efficiency of 73% and 76%, respectively. Analysis of a sample of each of the purified labelled liposome preparations on a BioRad 10DG column indicated that more than 96% of the radioactivity was associated with the liposomes.

BIODISTRIBUTION STUDIES

Table 1 shows the biodistributions of the ^{99m}Tc -labelled non-PEG-liposomes, PEG-liposomes, and ^{99m}Tc -IgG in rats with adjuvant arthritis induced in the left foot. The non-PEG-liposomal preparation cleared very rapidly from the blood: two hours after injection blood concentrations had fallen to values as low as 0.17 (0.03)% ID/g. These non-PEG-liposomes showed relatively high uptake in liver and spleen early after injection (2.65 (0.20) and 5.10 (1.09)% ID/g, respectively at two hours after injection). In contrast, blood clearance of the PEG-liposomes was much slower (fig 1A). Blood concentrations of the ^{99m}Tc -IgG preparation were intermediate between those of the two liposomal preparations at all time points (fig 1A).

The three radiopharmaceuticals also showed considerable differences in uptake in the inflamed ankle (table 1). With the PEG-liposomes this uptake increased over time up to a value of 1.15 (0.18)% ID/g at 24 hours after injection, while the inflamed ankle uptake with the non-PEG-liposomes and the ^{99m}Tc -IgG remained almost at the same level throughout the study period (0.1% ID/g and 0.5% ID/g, respectively) as shown in figure 1B. At eight hours and 24 hours after injection the IA/CA ratio obtained with the PEG-liposomes was significantly higher than the ratio obtained with the other two radiopharmaceuticals ($p < 0.05$). The IA/CA ratios obtained with the PEG-liposomes increased in time up to a value of 7.51 (0.91) (fig 2).

Figure 1 Blood concentration (A) and uptake in the inflamed ankle (B) of the ^{99m}Tc -labelled non-PEG-liposomes, PEG-liposomes, and ^{99m}Tc -IgG in rats with adjuvant arthritis in the left ankle. Five rats per group were used. Error bars represent SD.

Both liposomal formulations showed relatively high uptake of the radiolabel in the spleen. At 24 hours after injection splenic uptake was 4.0 (0.8)% ID/g and 11.1 (1.5)% ID/g for non-PEG and PEG-liposomes, respectively. With the ^{99m}Tc -IgG preparation uptake in the spleen was much lower (0.34 (0.04) % ID/g, 24 hours after injection). With this radiopharmaceutical the kidney was the organ with the highest activity (7.62 (1.29)% ID/g, 24 hours after injection).

IMAGING STUDIES

The inflamed foot was visualised with each of the radiopharmaceuticals included in this study. Figure 3 shows the image 24 hours after injection of the rats that received ^{99m}Tc -labelled non-PEG-liposomes, PEG-liposomes or

Table 1 Biodistribution of ^{99m}Tc -non-PEG-liposomes, ^{99m}Tc -PEG-liposomes, and ^{99m}Tc -IgG in rats with adjuvant arthritis two, eight, and 24 hours after injection (%ID/g 5 rats/group)

	Non-PEG-liposomes			PEG-liposomes			^{99m}Tc -IgG		
	2 hours	8 hours	24 hours	2 hours	8 hours	24 hours	2 hours	8 hours	24 hours
Blood	0.17 (0.03)	0.07 (0.01)	0.03 (0.01)	4.27 (0.28)	3.49 (0.33)	2.42 (0.33)	2.79 (0.27)	1.19 (0.10)	0.50 (0.09)
Inflamed ankle	0.10 (0.01)	0.11 (0.04)	0.05 (0.01)	0.49 (0.08)	0.77 (0.04)	1.15 (0.18)	0.46 (0.05)	0.44 (0.05)	0.35 (0.03)
Unaffected ankle	0.03 (0.00)	0.03 (0.01)	0.02 (0.00)	0.14 (0.02)	0.14 (0.02)	0.16 (0.02)	0.16 (0.01)	0.11 (0.02)	0.08 (0.01)
Muscle	0.01 (0.00)	0.01 (0.00)	0.00 (0.00)	0.05 (0.01)	0.06 (0.02)	0.04 (0.01)	0.05 (0.01)	0.05 (0.02)	0.02 (0.01)
Bone marrow	0.29 (0.08)	0.25 (0.09)	0.24 (0.07)	0.72 (0.17)	0.83 (0.09)	1.03 (0.16)	0.76 (0.08)	0.56 (0.12)	0.32 (0.05)
Femur	0.08 (0.02)	0.03 (0.01)	0.03 (0.01)	0.16 (0.03)	0.11 (0.02)	0.17 (0.02)	0.20 (0.02)	0.09 (0.02)	0.07 (0.02)
Lung	0.16 (0.05)	0.09 (0.02)	0.07 (0.02)	0.95 (0.12)	1.08 (0.23)	0.70 (0.07)	0.94 (0.22)	0.57 (0.12)	0.27 (0.05)
Spleen	5.01 (1.09)	5.04 (1.21)	4.01 (0.80)	6.15 (0.47)	7.84 (0.95)	11.1 (1.46)	0.87 (0.11)	0.55 (0.09)	0.34 (0.04)
Kidney	3.68 (0.45)	3.56 (0.71)	2.97 (0.43)	1.41 (0.24)	1.73 (0.12)	2.60 (0.36)	7.84 (0.79)	7.80 (0.90)	7.62 (1.29)
Liver	2.65 (0.20)	1.81 (0.36)	1.28 (0.43)	1.16 (0.23)	0.98 (0.21)	0.78 (0.31)	1.24 (0.10)	0.74 (0.07)	0.33 (0.08)
Intestine	0.12 (0.02)	0.05 (0.01)	0.02 (0.00)	0.62 (0.28)	0.71 (0.20)	0.60 (0.04)	0.50 (0.05)	0.25 (0.04)	0.13 (0.02)
IA/UA ratio	2.97 (0.35)	4.97 (2.48)	2.96 (0.54)	3.53 (0.62)	5.58 (1.01)	7.51 (0.91)	2.92 (0.49)	3.9 (0.67)	4.27 (0.65)

Data shown as mean (SD).

Figure 2 Inflamed ankle to unaffected ankle ratio obtained with non-PEG-liposomes, PEG-liposomes, and ^{99m}Tc-IgG two hours, eight hours, and 24 hours after injection. The biodistribution data of five rats per group were used. Error bars represent SD.

Figure 3 Scintigrams of rats with adjuvant arthritis imaged 24 hours after injection of ^{99m}Tc-labelled PEG-liposomes, non-PEG-liposomes, and ^{99m}Tc-IgG.

Figure 4 Quantitative analysis of the scintigraphic images of rats (three rats per group) injected with ^{99m}Tc-labelled non-PEG-liposomes, PEG-liposomes, and ^{99m}Tc-IgG. The activity in the inflamed foot was assessed. The whole body activity at five minutes after injection was set at 100%ID. Error bars represent SD.

^{99m}Tc-IgG. Quantitative analysis of the images clearly showed the main differences in circulatory half life of the three agents studied. The initial half life of the activity from the heart region of the PEG-liposomes, the non-PEG-

liposomes, and the ^{99m}Tc-IgG was 18 hours, one hour, and three hours, respectively (data not shown). Furthermore, quantitative analysis of the images confirmed the superiority of the PEG-liposomes: from four hours onwards the uptake in the inflamed foot was significantly higher for the PEG-liposomes as compared with the other preparations ($p < 0.03$) (fig 4). Activity in the contralateral foot was low for each of the radiopharmaceuticals. Consequently inflamed foot to background ratios were highest for the PEG-liposomes as well ($7.4 (1.1) v \leq 3.6 (1.5)$, 24 hours after injection).

Discussion

This study showed that in a rat model PEG-liposomes preferentially localise to arthritic joints, and thus PEG-liposomes potentially could be used (a) to image rheumatoid arthritis or (b) for the site specific delivery of antirheumatic drugs. The diagnosis of rheumatoid arthritis in patients with typically established disease is relatively easily made, but may be more difficult early in the course of the disease. An accurate clinical and diagnostic evaluation in patients is important because early treatment to suppress the inflammatory process may prevent progressive damage to articular structures. Furthermore, accurate assessment of the status of the disease allows a more objective way to evaluate the efficacy of the therapeutic regimen.

Liposomes, sterically stabilised with PEG, have been shown to preferentially localise to infectious foci.^{14,15} In previous studies we have shown that sterically stabilised liposomes labelled with gamma-emitters can be used to image infectious and inflammatory foci in soft tissue.⁸⁻¹⁰ This study in rats with adjuvant arthritis showed that ^{99m}Tc-PEG-liposomes are superior to both ^{99m}Tc-non-PEG-liposomes as well as ^{99m}Tc-IgG to image experimental arthritis. The PEG-liposomes were superior in terms of both absolute uptake in the target tissue as well as target to background ratios. Several studies in patients with rheumatoid arthritis have shown that ^{99m}Tc-IgG is a useful imaging agent for the clinical evaluation of rheumatoid arthritis,^{2,16,17} further supporting the clinical potential of ^{99m}Tc-labelled PEG-liposomes. In addition, the finding that PEG-liposomes displayed the highest absolute uptake in the inflammatory foci suggests that PEG-liposomes could also be exploited for site specific delivery of antirheumatic drugs. It has been shown in a rat model that liposomal methotrexate is more effective than the free drug in adjuvant arthritis.¹⁸ We have studied the therapeutic potential of using liposomes with encapsulated oxygen derived free radical scavengers in the model used in this study (Corvo *et al*, unpublished data).

The performance of the non-PEG-liposomes in this study was comparable with the results obtained in previous studies: an optimised formulation of non-PEG-liposomes evaluated in a rat model of adjuvant arthritis revealed an initial half life of one to two hours, with an IA/CA ratio of 4.¹⁹ In clinical studies

these non-PEG-liposomes labelled with ^{99m}Tc localised to inflamed joints and not to non-affected joints.^{1, 4, 20}

Previous studies have shown that the interaction of PEG-liposomes with mononuclear cells in the circulation is minimal. Therefore, most probably the uptake of the PEG-liposomes in the inflammatory foci is mainly a result of extravasation caused by the locally increased vascular permeability. This would explain why uptake of the long circulating liposomes in the inflamed area is much higher than the uptake of the non-PEG-liposomes: the persistently high blood concentrations of the PEG-liposomes facilitate the continued extravasation of the PEG-liposomes at the inflammatory focus. The mechanism of the subsequent retention of the liposomes in the inflammatory focus remains to be elucidated. The increased blood volume in the inflamed foot can only partly explain the increased uptake of PEG liposomes in the inflamed foot, because the IA/CA ratio increased with time. The IA/CA ratio obtained immediately after injection is a result of the increased vascularity in the inflamed foot and is relatively low.

Non-specific human polyclonal IgG has been shown to localise to foci of rheumatoid arthritis.^{2, 17, 21} Initially it was proposed that the IgG was trapped specifically because of the interaction with Fc receptor positive inflammatory cells.²² However, further studies have shown that the role of Fc receptor interaction in the accumulation of IgG in inflammatory foci is minimal.²³ Therefore, scintigraphic detection of rheumatoid arthritis with either IgG or liposomes both represent non-specific targeting methods, exploiting the increased capillary permeability in inflammatory foci. Consequently, it is expected that imaging with a liposome based radiopharmaceutical cannot discriminate rheumatoid arthritis from other inflammatory processes in joints. The role of radiolabelled liposomes in the diagnostic investigation of patients with rheumatoid arthritis has to be determined in clinical studies.

In conclusion, this study shows that PEG-liposomes may be powerful vehicles for scintigraphic imaging of the joints affected by rheumatoid arthritis as well as for the targeted delivery of antirheumatic drugs.

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