Liposomes as Possible Carriers for Lactoferrin in the Local Treatment of Inflammatory Diseases

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Liposomes prepared from naturally occurring biodegradable and nontoxic lipids are good candidates for local delivery of therapeutic agents. Treatment of arthritis by intra-articular administration of anti-inflammatory drugs encapsulated in liposomes prolongs the residence time of the drug in the joint. We have previously shown that intra-articular injection of human lactoferrin (hLf), a glycoprotein that possesses anti-inflammatory and antimicrobial activities, into mice with collagen-induced arthritis reduces inflammation. We have now investigated the possibility of using liposome-entrapped hLf as a delivery system to prolong hLf retention at sites of local inflammation such as the rheumatoid joint. Entrapment of hLf in negatively charged liposomes enhanced its accumulation in cultured human synovial fibroblasts from rheumatoid arthritis (RA) patients, compared with positively charged formulations or free protein. However, in the presence of synovial fluid, positively charged liposomes with entrapped hLf were more stable than the negatively charged formulations. *In vivo* experiments in mice with collagen-induced arthritis showed that the positive liposomes were more efficient in prolonging the residence time of hLf in the inflamed joint as compared with other liposomes. Thus, the amount of hLf retained in the joint after 2 hr was 60% of the injected dose in the case of positive liposomes and only 16% for negative pH-sensitive liposomes. The results suggest that entrapment of hLf in positively charged liposomes may modify its pharmacodynamic profile and be of therapeutic benefit in the treatment of RA and other local inflammatory conditions. [Exp Biol Med Vol. 226(6):559–564, 2001]

Key words: liposomes; human lactoferrin; rheumatoid arthritis; human synovial fibroblasts; human synovial fluid

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Liposomes have been proposed as efficient carriers for controlled drug delivery. They are able to entrap hydrophilic drugs in the large aqueous interior and lipophilic drugs inserted in the lipid bilayer. Derived from naturally occurring, biodegradable, and nontoxic lipids, they are good candidates for local targetting of therapeutic agents to the site of interest, while reducing systemic toxicity (1–3).

In the case of arthritic diseases, it has been reported that the intra-articular administration of anti-inflammatory drugs encapsulated in liposomes shows prolonged residence in the joint and reduction of inflammation (4–6). For example, liposome-entrapped methotrexate injected intra-articularly was 10-fold more potent than the free drug in suppressing the development of arthritis (7, 8). Moreover, the greater retention of the drug at the injection site should reduce potential adverse systemic effects (4, 9).

Lactoferrin (Lf) is an iron-binding glycoprotein of the transferrin family that can modulate the inflammatory response by binding potentially toxic-free iron (10). This mechanism could be important locally at the site of inflammation, such as in the rheumatoid joint. In rheumatoid arthritis (RA), iron-binding proteins are often not able to bind all the iron that accumulates in synovial tissue and fluid, resulting in the presence of potentially harmful "free" iron. Previous work has shown that Lf can bind "free" iron in synovial fluid and can reduce inflammation when injected intra-articularly into mice with collagen-induced arthritis (11). It is, therefore, possible that intra-articular administration of human Lf (hLf) entrapped in liposomes may prolong the residence time in joint and thus have therapeutic potential in RA and other local inflammatory conditions.

The aim of this study was to examine the properties of Lf prepared in various liposome formulations (positive and negative charge). We have examined the ability of each liposome formulation to release Lf into the cytoplasm of human synovial fibroblasts, and the stability of Lf-containing liposomes in the presence of synovial fluid from RA patients. We have also studied the effect of liposomal formulation on Lf retention in the joint and tissue distribu-

tion following intra-articular injection into mice with collagen-induced arthritis.

Materials and Methods

Materials. Liposomal lipids, phosphatidylcholine (PC), phosphatidylserine (PS), stearylamine (SA), dioleoylphosphatidylethanolamine (DOPE), dipalmitoylphosphatidylethanolamine (DPPE), cholesterol (Chol), cholesteryl hemisuccinate (CHEMS), hLf, and bovine collagen (type II) were purchased from Sigma Chemicals (St. Louis, MO). ^{125}I was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). All other chemicals were of analytical grade as used as purchased.

Liposome Preparation. Negatively charged pH-insensitive liposomes were prepared with PC, Chol, and PS in a molar ratio of 5:5:1, while pH-sensitive liposomes were composed of DOPE:CHEMS in a 6:4 molar ratio. The positively charged liposomes contained DPPE:Chol:SA in a 5:5:1 molar ratio. It has been shown that Chol and its derivatives have the capacity to improve liposomal membrane stability in the presence of serum or plasma (8, 12–14), hence, all types of liposomes were formulated with a high content of Chol or CHEMS. Inclusion of SA in liposomal membranes reduced the release of the encapsulated drug in comparison with the neutral formulation (3).

The lipids were dissolved in chloroform: methanol solution (95:5) and a thin, dry film of these lipids was made on the surface of a round bottom flask by evaporating the organic solvent in a rotary evaporator. The film was suspended in PBS containing ^{125}I-hLf and was incubated for 5 hr at room temperature to facilitate the annealing process (15). Five freeze/thaw cycles were performed to obtain a suitable size and a maximum efficiency of hLf incorporation (16). Free hLf was removed by centrifugation (1 hr, 40,000 rpm, 4°C) and the liposomal pellet was washed twice with PBS. The amount of protein entrapped in liposomes was measured by BCA protein assay after the solubilization of liposomal samples in 0.1% Triton X-100. This method resulted in approximately 40% of the initial amount of hLf being entrapped.

Protein Labelling. hLf was labelled with ^{125}I by a modification of the chloramine-T method as described previously (17).

Cell Culture. Synovial tissue was obtained from patients undergoing joint replacement. All subjects gave their informed consent. Human synovial fibroblasts were obtained by collagenase digestion of freshly collected synovial tissue. The tissue was washed four times in Hanks' balanced salt solution (Gibco, Paisley, UK), cut into very small pieces, and then digested in a 0.2% (w/v) solution of collagenase type IV prepared in RPMI-1640 medium containing 10% (v/v) fetal calf serum and penicillin-streptomycin. The mixture was incubated for 3 hr at 37°C with occasional gentle shaking. After incubation, the suspension was filtered through a sterile 100-μm filter (Fred Baker, Runcorn, UK), centrifuged, and the cells were resuspended in PBS supplemented with 0.5% bovine serum albumin. The fibroblasts were selected from macrophages using MACS CD14 microbeads (Miltenyi, Woking, UK), according to the manufacturer's instructions. The negatively selected flow-through from the column was collected to obtain fibroblasts. Purity of cells was determined by staining an aliquot with antibody to human fibroblasts (Serotec, Oxford, UK). Cells were routinely grown in RPMI-1640 medium supplemented with 10% fetal calf serum, passaged every 3 days, and seeded at 2×10^6 cells/75 cm^2 flask.

Liposome/Cell Interaction. Human synovial fibroblasts were seeded at 10^6 cells per well in 6-well plates (Costar) and were cultured for 24 hr. They were then incubated in 2 ml of RPMI-1640 (10% fetal calf serum) with different liposomal formulations (100 μM lipid final concentration in the medium) containing ^{125}I-hLf (20 μg/ml concentration in the medium) at 37°C for various times. It has been previously shown that this concentration of liposomes is nontoxic (18). A similar amount of free hLf was incubated with additional cell cultures as control. At the end of each experiment the supernatant was removed and the fibroblasts were washed twice. They were detached from the plates by incubating with trypsin/EDTA (Gibco) at 37°C for 5 min, centrifuged (1500 rpm, 10 min), and the radioactivity in the pellet measured was in a gamma counter (LKB, Croydon, UK). Trypsin treatment did not affect association of free or liposome-entrapped Lf with the cells (data not shown).

Liposome Stability in Synovial Fluid. Stability of different liposomal formulations was investigated by measuring the release of entrapped ^{125}I-hLf in the presence of synovial fluid obtained from RA patients during routine diagnostic or therapeutic aspiration, as described previously (11).

Synovial fluid (90 μl) was incubated at 37°C with 10 μl of liposome-entrapped ^{125}I-hLf. Final lipid and Lf concentrations were 100 μM and 20 μg/ml, respectively. After different incubation times, the samples were diluted to 1 ml with PBS, and were then centrifuged at 30,000 rpm for 30 min to sediment the liposomal pellet. The amount of ^{125}I-hLf released into supernatant was measured.

To determine whether Lf in the supernatant was intact, it was precipitated by adding 20% (final concentration) of trichloroacetic acid, as described (17, 19).

Collagen-Induced Arthritis. The collagen-induced murine model of rheumatoid arthritis as described by Wooley *et al.* (20) was used in these studies. Male dba/1 mice, 6 to 8 weeks old, weighing approximately 20 g, were obtained from Harlan-Olac (Bicester, UK) and were allowed to acclimatise for 1 week. They were then injected intradermally with 0.2 mg of bovine collagen (Type II, Sigma, Poole, UK) emulsified in Freund's complete adjuvant, in a total volume of 100 μl, into two sites at the base of the tail. Three weeks later each mouse received an additional injection of 0.2 mg of collagen in 100 μl of phosphate-buffered saline (PBS), intraperitoneally. Mice were

then monitored daily for arthritis according to the degree of swelling, erythaema, and ankylosis (20).

Biodistribution of ^{125}I-hLf in Arthritic Mice. Liposomes containing ^{125}I-hLf were injected intra-articularly into mouse joints that had shown severe arthritis (extensive swelling and erythaema) for at least 3 days. Rear paws were injected with 50 µl of liposomes (1 µmol lipid per mouse) containing 1 mg of ^{125}I-hLf, while front paws, due to their smaller size, were injected with 25 µl. Similar amounts of free ^{125}I-hLf were injected into the joints as a control. Mice were sacrificed at intervals up to 24 hr and radioactivity in the joint, blood, and tissues was determined. Total activity in the blood was calculated by assuming a total blood volume of 2 ml. The ^{125}I-hLf activity recovered in each tissue was calculated as a percent of injected dose.

Results

Human Lf Delivery into Fibroblasts using Liposomes as Carriers. To determine the ability of liposomes to deliver Lf to fibroblasts, liposomes with different surface charges containing entrapped ^{125}I-lactoferrin were used in cell interaction experiments with human synovial fibroblasts from RA patients. Free labelled Lf was used as a control. As shown in Figure 1, all liposomal formulations showed a greater capacity to deliver Lf to fibroblasts than free Lf. pH-sensitive negatively charged liposomes were most effective, delivering about twice as much as pH-insensitive or positive liposomes, which in turn were about twice as effective as free Lf. Similar experiments showed that the net uptake at 37°C was approximately 60% of liposome-entrapped Lf and 50% of free Lf after subtracting the values obtained at 4°C (binding) (data not shown).

Stability of Liposomes with Entrapped Lf in the Presence of Human Synovial Fluid. Negatively charged liposomes (pH-insensitive and pH-sensitive) and positive liposomes, all with entrapped ^{125}I-Lf, were incubated with synovial fluid from an RA patient, and the amount of labelled protein released into the supernatant was measured after different incubation times. The results (Fig. 2) revealed that in all cases, most of the labelled Lf had been released from the liposomes after 24 hr of incubation. The positive liposomes were marginally more stable with 70% of the radioactive protein released compared with 80% of the ^{125}I-Lf from pH-sensitive negative liposomes and 88% from the pH-insensitive liposomes. Similar results were obtained using synovial fluids from four other RA patients (data not shown).

Tissue Distribution of Free and Liposome-Entrapped Lf following Intra-Articular Injection into Mice with Collagen-Induced Arthritis. We have previously shown that human Lf injected intra-articularly into mice with collagen-induced arthritis can reduce the degree of joint inflammation (11). It was therefore of interest to establish whether any of the liposomal formulations could prolong the residence time of hLf in the joints of the mice with collagen-induced RA. Since positive liposomes were the most stable in synovial fluid (Fig. 2) and negative pH-

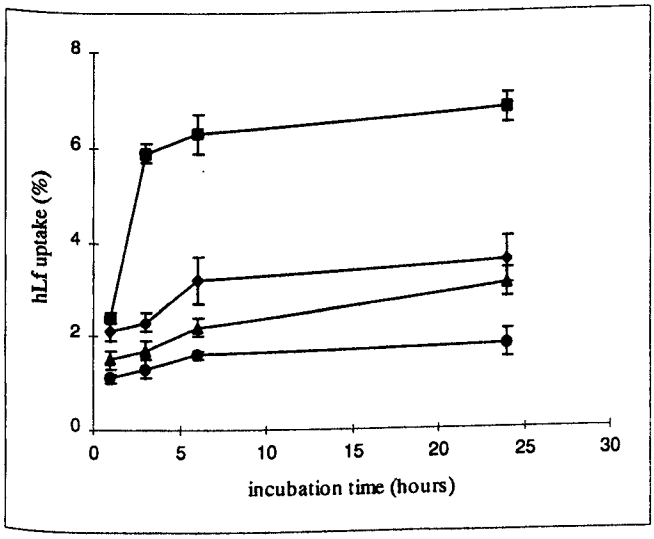

Figure 1. Kinetics of uptake of free and liposome-entrapped ^{125}I-hLf by human synovial fibroblasts from RA patients. ♦ - hLf entrapped in negative pH-insensitive liposomes (PC:Chol:PS; 5:5:1). ■ - hLf entrapped in negative pH-sensitive liposomes (DOPE:CHEMS; 6:4). ▲ - hLf entrapped in positive liposomes (DPPE:Chol:SA; 5:5:1). ● - free hLf. Free and liposome-entrapped ^{125}I-hLf formulations were incubated in RPMI/10% fetal calf serum at 37°C for 24 hr, with human synovial fibroblasts from RA patients. Liposomal lipid concentration was 100 µM and hLf was 20 µg/ml. The amount of ^{125}I-hLf associated with the fibroblasts was calculated as the percentage of the initial amount of free or liposome-entrapped radiolabeled protein added to the medium. Each point is represented as the means ± SD, n = 6.

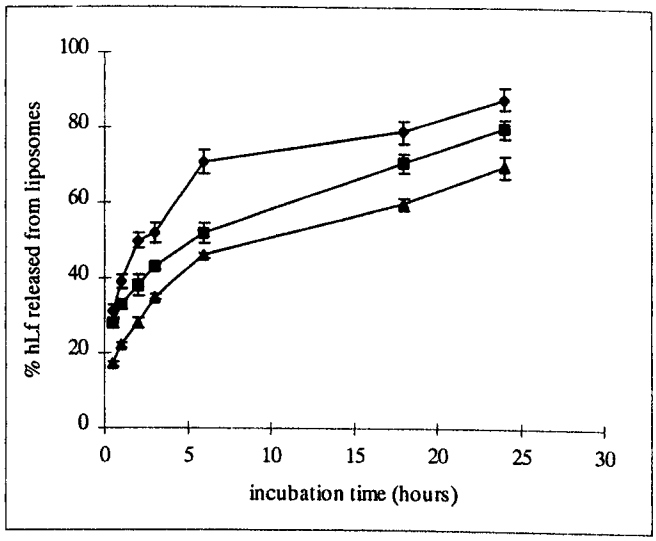

Figure 2. Kinetics of release of ^{125}I-hLf entrapped in different liposomal formulations in the presence of synovial fluid from RA patients. ♦ - hLf entrapped in negative pH-insensitive liposomes (PC:Chol:PS; 5:5:1). ■ - hLf entrapped in negative pH-sensitive liposomes (DOPE:CHEMS; 6:4). ▲ - hLf entrapped in positive liposomes (DPPE:Chol:SA; 5:5:1). 10 µl of liposomes (100 µM final lipid concentration in medium) containing ^{125}I-hLf (20 µg/ml final concentration in medium) were incubated with 90 µl of synovial fluid at 37°C for 24 hr. The amount of ^{125}I-hLf released from liposomes was measured in the supernatant and calculated as the percentage of the initially-entrapped protein released. Each point is represented as the means ± SD, n = 5.

sensitive liposomes were the most effective at delivering Lf to fibroblasts (Fig. 1), these two formulations containing ^{125}I-Lf were compared with free Lf.

Lf entrapped in positive liposomes was retained longer in the injected joint compared with Lf entrapped in negative liposomes, which was retained less well than free hLf (Fig. 3a). After 2 hr, 60% of the injected dose in positive liposomes remained at the site of injection compared with 16% for negative pH-sensitive liposomes and 28% of free Lf. Even after 24 hr, 15% of the injected dose entrapped in positive liposomes was still present in the joint, whereas Lf in pH-sensitive liposomes had almost completely disappeared (only 0.1% remaining) and 2% of free Lf remained.

Significant but lesser amounts (up to about 7% of the injected dose of labelled Lf) were also found in the blood (Fig. 3B) and liver (Fig. 3C) of injected animals. Relative concentrations in the blood generally paralleled those in the injected joint, but liver concentrations showed less variation between the different formulations. Other tissues examined (spleen, kidney, or lungs) never contained more than 1% of the total injected dose at any time (data not shown). Similarly, Lf was not detected in noninjected joints from the same animal, even when these were also inflamed.

Discussion

Lf possesses various biological activities, some of which have potential prophylactic or therapeutic potential for treatment of infectious and inflammatory diseases (21). In particular, we have previously found that injection of Lf into the joints of mice with collagen-induced or septic arthritis reduces joint inflammation (22). However, a major problem with this and other potential therapeutic uses of Lf is that once it enters the circulation, it is rapidly cleared by the liver (22). Furthermore, it is uncertain whether free Lf is readily taken up by cells, except by hepatocytes involved in its catabolism (23). Thus, the therapeutic potential of Lf could be improved if more effective ways could be found to introduce it into cells and ensure its retention at target tissues *in vivo*.

To address these questions, we have examined the ability of liposomes to enhance cellular uptake and tissue retention of Lf, as this approach has been successful in optimising efficient delivery of drugs to target tissues (24). Various liposomal formulations have been used, with differing charge and stability characteristics. In the present work we have used positively-charged liposomes and two different types of negatively-charged liposomes varying in their sensitivity to low pH, which will affect stability within different endosomal compartments of the cell (25).

To assess the capacity of liposomes to enhance uptake of Lf into cells, we studied their effect on uptake of Lf by human fibroblasts obtained from patients with RA. This is a particularly appropriate model in relation to possible therapeutic uses of Lf in arthritic diseases, and moreover, fibroblasts normally take up Lf very poorly (M. Trif, J.M. Telfer, A. Roseanu, J.H. Brock, unpublished data). All formulations enhanced Lf uptake, with the pH-sensitive negatively charged liposomes being most effective. Uptake was biphasic, there being rapid uptake up to 6 hr followed by a slower accumulation thereafter. The reason for this is unknown, but

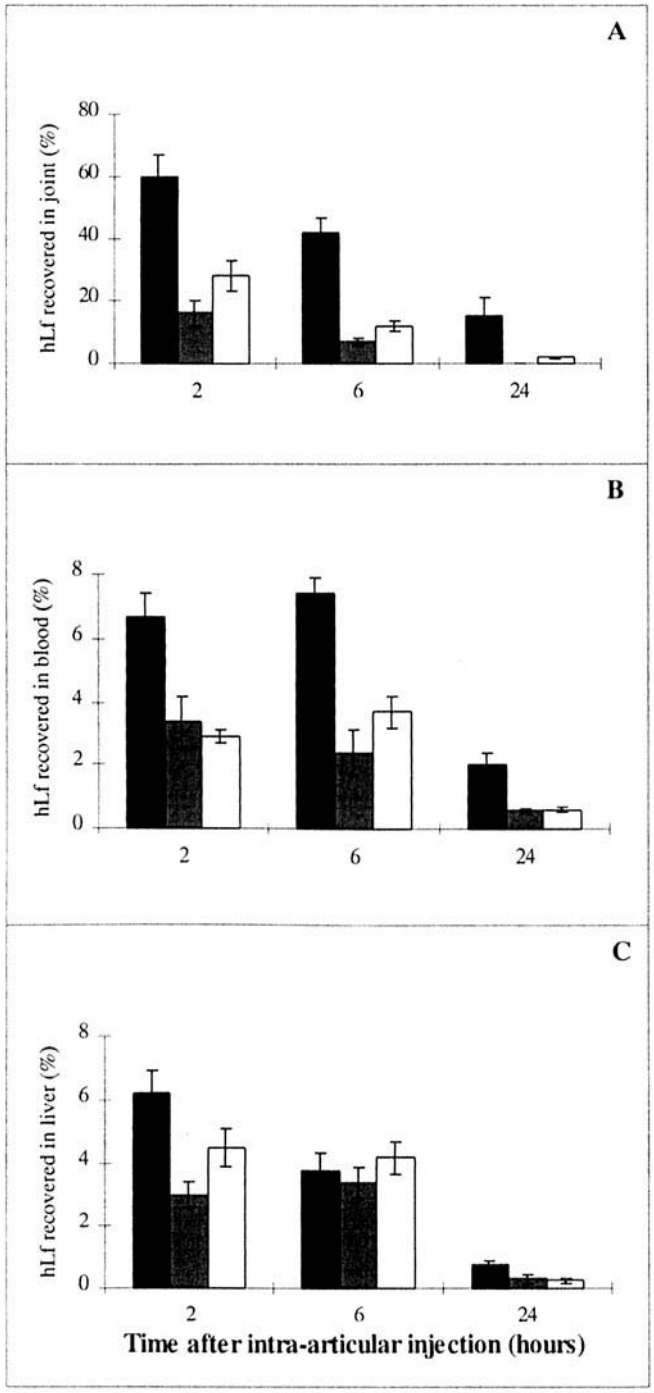

Figure 3. The effect of liposomal formulation on ^{125}I-hLf retention in the inflamed joint (3A), blood (3B), and liver (3C) after intra-articular injection into mice with collagen-induced arthritis. ■ - hLf entrapped in positive liposomes (DPPE:Chol:SA; 5:5:1). ▨ - hLf entrapped in negative pH-sensitive liposomes (DOPE:CHEMS; 6:4). □ - free hLf. Each liposomal formulation was tested on 3 to 4 mice using a dose of 1 μmol lipid and 1 mg of ^{125}I-hLf per mouse in rear paws, and one-half of this amount in front paws. Controls received the same amount of free hLf per joint. Mice were sacrificed 2, 6, and 24 hr after injection. The recovered ^{125}I-hLf was calculated as the percentage of the injected dose. The horizontal bar represents the SD.

may be due to establishment of an equilibrium between uptake and release. Kinetic experiments could help to resolve this question.

Our results confirm previous work showing that negatively charged liposomes enhanced the intracellular accumulation of entrapped material compared with positive or neutral ones (26–28). It has been shown that the presence of the positively charged compound stearylamine (SA) in the liposomal membrane delays the interaction with cells and slows their uptake (3, 29).

Since any attempt to use liposomes in the treatment of human arthritic disease is likely to initially involve interaction between liposomes and synovial fluid, it was important to determine whether this fluid would affect liposome stability. All three formulations tended to disintegrate in the presence of synovial fluid, though positively charged liposomes were somewhat more stable, with over one-half the formulation remaining intact for at least 5 hr (Fig. 2). These results are in agreement with previous reports indicating that positive liposomes containing SA showed higher stability in serum (29, 30). The stability of negative liposomes was found to be dependent on the lipid composition of the liposomal bilayer (28, 31, 32). Our experiments indicate that pH-sensitive liposomes were more stable than pH-insensitive ones (Fig. 2). These data may also be relevant for assessing the stability of liposome-entrapped Lf in other local inflammatory conditions, especially when oedema is present.

The mechanism(s) by which Lf exerts anti-inflammatory and anti-microbial activity remain unclear, although the ability of Lf to bind iron with high affinity suggests that it may act by rendering "free" iron unavailable to act as a catalyst for the production of damaging free radicals (33). Such a mechanism, if active in an arthritic joint, would require Lf to remain there for some time. We have, therefore, investigated whether liposome-entrapment could increase the residence time of Lf in the joint following injection into the inflamed joints of mice with collagen-induced arthritis. For this we elected to test positively charged liposomes, which showed the best stability in synovial fluid, and pH-sensitive negatively charged liposomes, which were most effective in promoting Lf uptake by fibroblasts.

Free Lf disappeared rapidly from the injected joint, with two-thirds lost by 2 hr and only 2% remaining at 24 hr (Fig. 3). However, entrapment in positively charged liposomes markedly enhanced retention time, with close to 50% still present after 6 hr and 15% at 24 hr. Surprisingly, Lf entrapped in pH-sensitive negatively charged liposomes was lost from the joint even more rapidly than the free protein, it being virtually undetectable by 24 hr. Why this should be so is unclear, but it is possible that retention of Lf in the joint is assisted by its native positive charge (pI ~ 9.0) (34), and that entrapment in a negatively charged liposome neutralizes this charge. In contrast, entrapment in positively charged liposomes could enhance binding to cell membranes and thus increase still further retention in the joint.

It was of interest that Lf, either free or liposome-entrapped, when lost from the injected joint, was found only in the blood and liver. The liver is the major, if not the only, site of Lf catabolism, and these results suggest that Lf leaving the joint is first released from the liposomes, whereupon in enters the bloodstream and is transported to the liver. However, the loss of some Lf from the joint while still entrapped in liposomes cannot be entirely ruled out, as systemic delivery of large liposomes also results in their uptake by the liver (29, 35). The lack of significant amounts of Lf in other tissues, or in other joints even when these were also inflamed, suggests that liposome-entrapped Lf is unlikely to localize elsewhere in the body and perhaps cause unwanted side effects, e.g., alterations to iron metabolism. Furthermore, very little radioactivity was found in the kidney, suggesting that degradation of Lf to low-molecular weight products did not occur to any extent.

In conclusion, these results suggest that positively charged liposomes are more appropriate than other liposome formulations in modifying the pharmacodynamic profile of Lf, and may provide an efficient therapeutic agent for the treatment of arthritic and other inflammatory diseases.

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