J. Pharm. Pharmacol. 1988, 40: 171-175
Received August 13, 1987

Association of Liposome-entrapped [^{3}H]Methotrexate with Thioglycollate-elicited Macrophages In-vitro

W. C. FOONG* AND K. L. GREEN

School of Pharmacy, Portsmouth Polytechnic, White Swan Road, Portsmouth PO1 2DT, UK

Abstract—The association of free or liposome-entrapped [^{3}H]methotrexate ([^{3}H]MTX) with thioglycollate-elicited macrophages was investigated in-vitro. [^{14}C]Cholesteryl oleate was incorporated into the liposomes as a lipid marker. [^{3}H]MTX association with the macrophages was 5 to 9-fold higher with liposome-entrapped [^{3}H]MTX than with the free drug. Macrophage-liposome association was biphasic, temperature-dependent and saturable at high liposomal lipid concentration. A high liposome cholesterol (CH) content or the presence of 2,4-dinitrophenol or colchicine also reduced macrophage-liposome association.

Dingle and colleagues (Dingle et al 1978; Shaw et al 1979) have shown that the anti-inflammatory effects of cortisol injected into arthritic joints are greatly enhanced when the drug is entrapped in liposomes. Similarly we have found that liposome-entrapped methotrexate (MTX) injected intra-articularly suppresses an experimental arthritis more effectively than free MTX (Foong & Green 1983a). Liposome-entrapped [^{3}H]MTX was shown to be retained in the injected joint significantly longer than when the free drug was injected, but the uptake of liposomal [^{3}H]MTX by the inflamed synovium was relatively low. Thus, of the ^{3}H recovered from the injected joint three days later, most remained in the synovial fluid with less than 10% associated with the synovium (Foong & Green 1983b). Despite this, the synovium is known to be actively phagocytic (Adam 1966) and in inflamed joints there is also a heavy infiltration of leucocytes and macrophages. Since intra-articular liposome therapy might be expected to be enhanced if lipsome uptake by the synovium is more complete, the objective of the present study was to investigate some of the factors affecting the association of liposome-entrapped [^{3}H]MTX with thioglycollate-elicited rabbit peritoneal macrophages.

Materials and Methods

Materials

[3′,5′,7-^{3}H]Methotrexate sodium (160 mCi mmol^{-1}) ([^{3}H]MTX) and cholesteryl [1-^{14}C]oleate (58 mCi mmol^{-1}) were obtained from Amersham International. Foetal calf serum (FSC) and minimum essential medium Eagles (MEM) containing Earle's salts with 20 mM Hepes buffer but deficient in L-glutamine and sodium bicarbonate was obtained from Flow Laboratories. Cholesterol (CH), dicetylphosphate (DCP), egg phosphatidylcholine type VE (PC), colchicine, 2,4-dinitrophenol, EDTA, penicillin G-benzyl, streptomycin sulphate and trypsin were from Sigma. Other materials were obtained as follows: Brewer's thioglycollate medium (Difco), propranidid (Bayer), pentobarbitone sodium (May & Baker), trypan blue (BDH), Triton X-100 (BDH) and heparin (Evans).

Rabbits were inbred of the Old English strain, 2·0–2·4 kg.

Liposome preparation

[^{3}H]MTX was entrapped in the aqueous phase of negatively charged multilamellar liposomes prepared by prolonged shaking (20 h) at 20°C with lipids in the molar ratio PC:CH:DCP, 5:0:1; 10:5:2 or 5:5:1 as previously described (White et al 1983). In addition [^{14}C]cholesteryl oleate was incorporated as a lipid marker contributing 0·2 mol % of the total lipid content. Free and liposome-entrapped [^{3}H]MTX were separated by repeated washing and centrifuging at 40 000 g (r_{av} 8·2 cm) at 4°C for 20 min. The liposomes had a mean diameter of 1·07 μm as measured by photon correlation spectroscopy.

Collection of rabbit peritoneal macrophages

Brewer's thioglycollate medium, heat sterilized, was stored in the dark for one week before use. Rabbits were injected with the thioglycollate medium (65 mL kg^{-1} i.p.) under brief anaesthesia induced with propranidid (50 mg kg^{-1} i.v.). Three days later the rabbits were anaesthetized with pentobarbitone sodium (40 mg kg^{-1} i.v.), a mid-line abdominal incision made and the peritoneal fluid collected by lavaging with 5 × 10 mL heparinized phosphate-buffered saline pH 7·4 (PBS) taking aseptic precautions. The peritoneal fluid was withdrawn by suction into a collecting vessel on ice, centrifuged at 225 g for 10 min at 4°C and the supernatant discarded. The cells were resuspended in MEM, centrifuged and washed three times with MEM supplemented with 10% heat-inactivated FCS. Total cell counts were performed using a haemocytometer chamber and differential cell counts obtained by staining smears with Leishman's or with haematoxylin and eosin and counting 2000 cells. Cell viability was assessed by the ability of cells to exclude trypan blue. Cells were counted in a haemocytometer chamber using PBS containing 0·17% trypan blue as the cell diluting fluid. $1{\cdot}06 \times 10^9 \pm 1{\cdot}86 \times 10^7$ peritoneal cells were recovered from each rabbit, of which 86·9 ± 3·1% were macrophages, 9·3 ± 2·8% lymphocytes and 3·8 ± 0·9% granulocytes (mean ± s.e. mean, n = 6). Cell viability was 93·4 ± 3·2% (n = 6).

* Present address: College of Pharmacy, Dalhousie University, Halifax, Nova Scotia, Canada, B3H 1NJ.
Correspondence to: K. L. Green, School of Pharmacy, Portsmouth Polytechnic, White Swan Road, Portsmouth PO1 2DT, UK.

Incubation of peritoneal cells

Aliquots containing 1×10^6 viable cells in 0·5 mL MEM with 10% FCS were transferred to polystyrene cell culture tubes and incubated at 37°C for 18 h. After incubation, non-adherent cells were decanted and the adherent cells washed three times with 3 mL MEM containing 10% FCS. 90·9 ± 2·3% (n = 24) of the peritoneal cells were adherent, the non-adherent cells being predominantly lymphocytes.

Incubation of macrophages with liposomes

MEM, 1 mL, with 10% FCS and liposomes containing [^{3}H]MTX and [^{14}C]cholesteryl oleate were added to the adherent cells. After incubation at 4°C or 37°C for specified times, the supernatant was decanted and the adherent cells gently washed three times with 3 mL PBS. The cells were harvested by adding 0·5 mL of a solution containing 0·25% trypsin and 0·02% EDTA in PBS and incubating at 37°C for 10 min. The cell suspension obtained was decanted and the culture tubes washed with the trypsinizing fluid containing 0·1% Triton X-100 with brief sonication (10 s) to remove adherent material. ^{3}H- and ^{14}C-content of the pooled suspension was measured by liquid scintillation counting.

Preliminary studies established that the optimum number of macrophages for incubation in the cell culture tubes was 1×10^6 cells or less. When larger numbers of cells were incubated in each tube with radiolabelled liposomes, reduced amounts of ^{3}H and ^{14}C were associated with each cell. 1×10^6 cells were routinely incubated in each tube.

Liposome stability

The stability of [^{3}H]MTX liposomes in the cell culture medium was assessed by measuring the release of [^{3}H]MTX from liposomes at a concentration of 1 mM lipid, incubated in 2 mL MEM with 10% FCS for 4 h at 37°C. The liposome suspension was then centrifuged at 105 000 *g* (r_{av} 8·2 cm) at 4°C for 20 min and the amount of [^{3}H]MTX in both the supernatant and in the liposome pellet measured by liquid scintillation counting.

Statistics

Results were expressed as mean ± s.e. mean, statistical significance being determined using an unpaired, two-tailed Student's *t*-test.

Results

Liposome stability

Liposomes with a high CH content (PC:CH:DCP, 5:5:1 molar ratio) showed the greatest stability when incubated in the culture medium used to maintain macrophages, retaining 91·7 ± 1·0% (n = 4) of entrapped [^{3}H]MTX after 4 h incubation at 37°C. Liposomes containing PC:CH:DCP, 10:5:2 molar ratio retained 88·1 ± 3·8% (n = 4), and CH-free liposomes (PC:DCP, 5:1 molar ratio) retained 54·9 ± 5·2% (n = 4) of entrapped [^{3}H]MTX after 4 h incubation in the cell culture medium.

Effect of increasing lipid concentration on cell-liposome association

Liposomes (PC:CH:DCP, 5:5:1 molar ratio) in a final concentration of 0·33, 1·0, 3·33, 10·0 or 33·3 mM lipid were incubated at 37°C with 1×10^6 adherent cells. Although the total amount of ^{3}H and ^{14}C associated with the macrophages increased on incubation with increasing concentration of liposomes, the efficiency of cell-liposome association decreased. Thus the highest percentage of liposomal ^{3}H and ^{14}C to become cell-associated was when the liposome concentration was 0·33 mM lipid (Fig. 1).

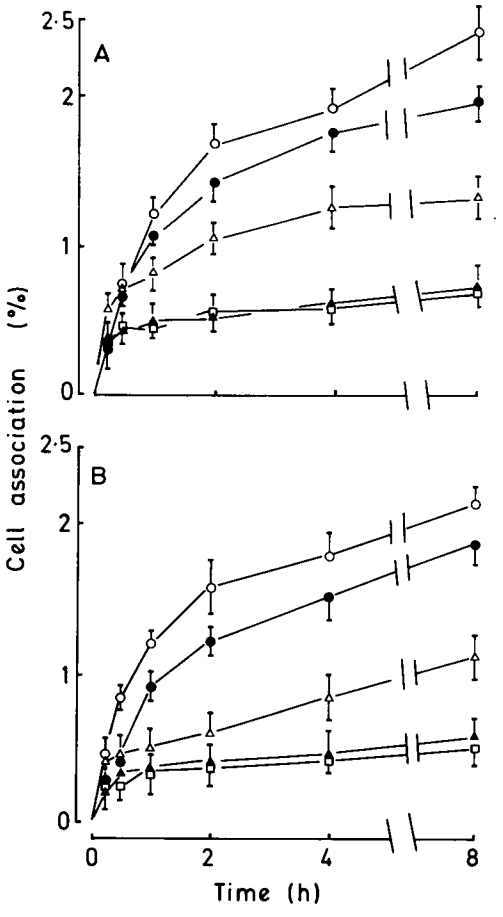

FIG 1. Effect of liposomal lipid concentration on cell-liposome association. 1×10^6 cells were incubated at 37°C with liposomes (PC:CH:DCP, 5:5:1 molar ratio), containing [^{3}H]MTX and [^{14}C]cholesteryl oleate as a lipid marker. Final lipid concentration was 0·33 (○), 1·0 (●), 3·33 (△), 10·0 (▲) or 33·33 (□) mM. Results expressed as percentage of liposomal ^{14}C (A) or ^{3}H (B) which became cell-associated. Mean ± s.e. mean, (n = 6).

If association of liposomes with macrophages was due to internalization of intact liposomes, the ratio of ^{14}C:^{3}H in the liposomes and in the macrophages should be identical. This was the case when macrophages were incubated with liposomes in a concentration of 0·33 mM lipid, but when the liposome concentration exceeded 1 mM lipid, a greater proportion of the [^{14}C]lipid marker (approx. 1·4-fold, $P < 0·01$) was associated with the macrophages.

Effect of CH content of liposomes on cell-liposome association

When macrophages were incubated at 37°C with liposomes of various lipid composition, the amount of liposomal ^{3}H and ^{14}C associated with the macrophages was found to be inversely related to the CH content of the liposomes (Fig. 2). Thus after 4 h incubation with CH-free liposomes (PC:DCP,

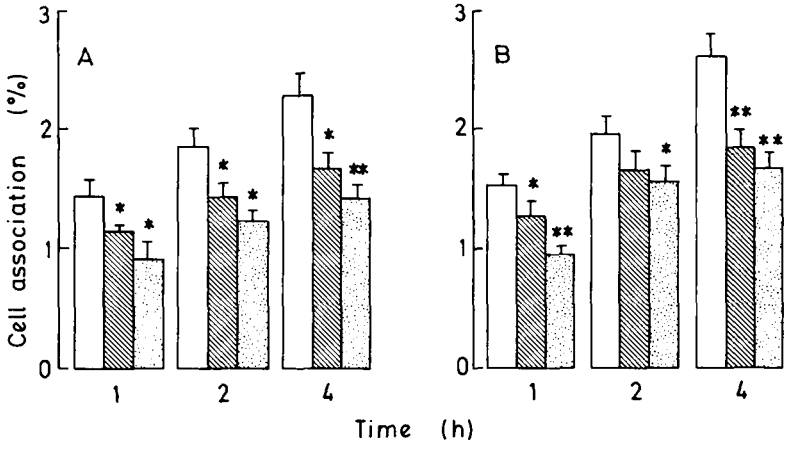

FIG 2. Effect of liposome CH content on cell-liposome association. 1×10^6 cells were incubated at 37°C with liposomes in a final lipid concentration of 1 mM. Liposomes, PC:DCP, 5:1 molar ratio (open columns), PC:CH:DCP, 10:5:2 molar ratio (hatched columns) and PC:CH:DCP, 5:5:1 molar ratio (spotted columns), contained [^{3}H]MTX with [^{14}C]cholesteryl oleate as a lipid marker. Results expressed as percentage of liposomal ^{3}H (A) or ^{14}C (B) which became cell-associated. Mean ±s.e. mean (n=6). $*P<0.05$, $**P<0.01$ significantly different from CH-free liposomes (Student's *t*-test).

5:1 molar ratio), cell association of ^{3}H was 61±12% greater and ^{14}C was 50±10% greater than when incubated with liposomes containing 45·4 mol % CH (PC:CH:DCP, 5:5:1 molar ratio).

Surprisingly, the ratio of ^{14}C:^{3}H cell associated was approximately one, regardless of the CH content of the liposomes (Fig. 2). Since CH-free liposomes were relatively unstable, leaking approximately 45% entrapped [^{3}H]MTX after 4 h incubation, it was anticipated that this would be reflected in the ratio of ^{14}C:^{3}H associated with the macrophages.

Effect of temperature on cell-association of free or liposome-entrapped [^{3}H]MTX

Macrophages were incubated at 4°C or 37°C for 30 min and then incubated at the same temperature for 4 h in the presence of [^{3}H]MTX (1 μCi mL^{-1}) in solution or entrapped in liposomes. When macrophages were incubated with [^{3}H]MTX in solution at 37°C, 0·25±0·09% became cell-associated (Fig. 3). This was 5·8-fold less than when macrophages were incubated with similar amounts of [^{3}H]MTX entrapped in CH-rich liposomes (PC:CH:DCP, 5:5:1 molar ratio), and 9-fold less than when incubated with CH-free liposomes (PC:DCP, 5:1 molar ratio). Incubation of macrophages at 4°C almost completely inhibited cell-association of free [^{3}H]MTX (0·02±0·01%). Cell-association of liposomal [^{3}H]MTX at 4°C was reduced to 0·81±0·17% with CH-free liposomes, and to 0·35±0·13% with CH-rich liposomes (Fig. 3). Low temperature inhibited cell-association of liposomal [^{3}H]MTX and [^{14}C]cholesteryl oleate to a similar extent ($P>0.05$), irrespective of the CH content of the liposomes.

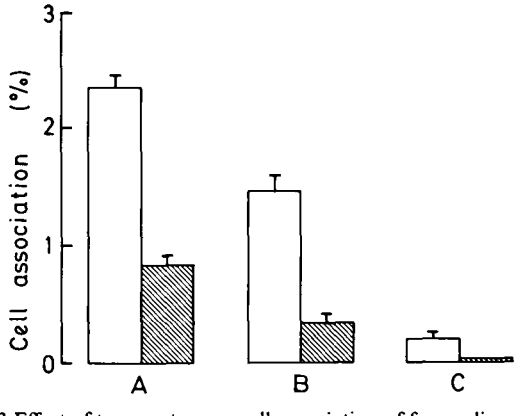

FIG 3. Effect of temperature on cell-association of free or liposome-entrapped [^{3}H]MTX. 1×10^6 cells were incubated for 4 h at 37°C (open columns) or 4°C (hatched columns) with (A) CH-free liposomes (PC:DCP, 5:1 molar ratio) or (B) CH-rich liposomes (PC:CH:DCP, 5:5:1 molar ratio) or (C) free ^{3}H-MTX in solution.

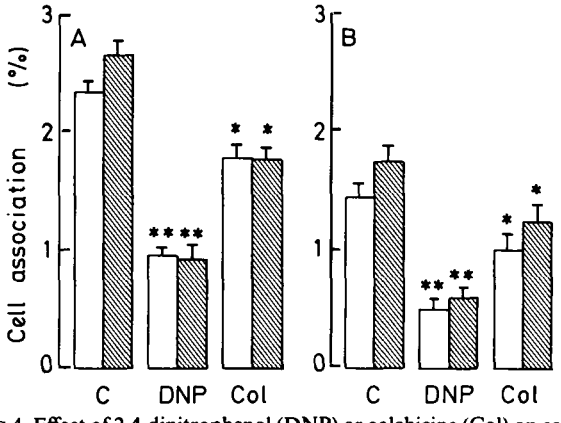

FIG 4. Effect of 2,4-dinitrophenol (DNP) or colchicine (Col) on cell-association of liposome-entrapped [^{3}H]MTX (open columns) or [^{14}C]cholesteryl oleate (hatched columns). 1×10^6 cells were incubated at 37°C for 30 min alone or with 2,4-dinitrophenol (100 μg mL^{-1}) or colchicine (100 μg mL^{-1}). (A) CH-free liposomes (PC:DCP, 5:1 molar ratio) or (B) CH-rich liposomes (PC:CH:DCP, 5:5:1 molar ratio) were added to a final lipid concentration of 1 mM, the concentration of inhibitor, if present, being kept constant, and the cells incubated at 37°C for 4 h. Mean ±s.e. mean, (n=6). $*P<0.05$, $**P<0.01$ significantly different from corresponding control (Student's *t*-test).

Effect of 2,4-dinitrophenol or colchicine on cell-liposome association

Adherent cells were incubated at 37°C for 30 min alone or with 2,4-dinitrophenol (100 μg mL^{-1}) or colchicine (100 μg mL^{-1}). [^{3}H]MTX liposomes were then added to obtain a final lipid concentration of 1 mM. The concentration of inhibitor, if present, was maintained at 100 μg mL^{-1}. Neither colchicine nor 2,4-dinitrophenol appeared to affect the adherance of the macrophages to the culture tubes. 2,4-Dinitrophenol reduced cell-association of both liposomal ^{3}H and ^{14}C from CH-free and CH-rich liposomes by 60–70% (Fig. 4). Colchicine caused a 20–30% reduction in cell-association of liposomal ^{3}H and ^{14}C.

Discussion

Entrapment [^{3}H]MTX in negatively charged liposomes was shown to increase [^{3}H]MTX associated with macrophages in culture 5- to 9-fold compared with [^{3}H]MTX in solution. The association of liposomal [^{3}H]MTX and [^{14}C]cholesteryl oleate with macrophages appeared to be biphasic, the initial rapid association being followed by a slower phase. Incubation of macrophages with a high concentration of liposomes progressively reduced the percentage of [^{3}H]MTX and [^{14}C]cholesteryl which became cell associated, presumably due to saturation of the mechanism(s) involved in liposome uptake. Similar saturable uptake processes have been reported with Kupffer cells in-vitro (Dijkstra et al 1984, 1985). This has been exploited to reduce accumulation of systemically administered liposomes in the liver by predosing with 'empty' liposomes (Abra et al 1980).

In the present study, when macrophages were incubated with liposomes at low liposomal lipid concentrations, cell-association of liposomal [^{3}H]MTX was similar to that of the [^{14}C]lipid marker. However, with liposomal lipid concentrations greater than 1 mM, cell-association of the [^{14}C]lipid marker exceeded that of [^{3}H]MTX approximately 1·4-fold. It is unclear why such differences occurred, but it is possible that leakage of liposome-entrapped [^{3}H]MTX occurred in the immediate vicinity of the macrophages. The initial event in cell-liposome interaction is thought to be adsorption and/or fusion, followed by endocytosis (Pagano & Weinstein 1978). Endocytosis is energy-dependent and at high liposome concentrations is likely to be saturable, liposomes adhering to the cell surface remaining there for some time before being internalized. Perturbation of the liposome bilayers due to cell adhesion would promote leakage of entrapped [^{3}H]MTX, leaving 'empty' [^{14}C]-labelled liposomes associated with the macrophages. Such a mechanism could account for the relatively selective association of [^{14}C]cholesteryl oleate with the macrophages at high liposome concentrations.

Previous studies have shown that liposomes with a high CH content are relatively stable in biological fluids (White et al 1983). This was confirmed in the current study, but liposome association with peritoneal macrophages was reduced by a high liposomal CH content ($P<0·01$). The influence of CH on cell-liposome association was even more marked if the poor stability of CH-free liposomes is taken into consideration. Thus, after 4 h incubation in the cell culture medium, approximately 45% entrapped [^{3}H]MTX had leaked from CH-free liposomes and this [^{3}H]MTX in solution would only be slowly taken up by macrophages. In-vivo studies have shown that liposome uptake by the spleen and liver is similarly depressed by a high liposome CH content (Patel et al 1983). Furthermore, the degradation of liposomes taken up by macrophages appears to be inhibited by a high CH content (Johnson 1975) and the cytotoxicity of liposome-entrapped MTX is reduced by liposomal CH (Todd et al 1982). Margolis et al (1978) suggested that the incorporation of CH into liposomes decreases the fluidity of the vesicle bilayers, so that they are less likely to fuse with cell membranes. It has also been suggested that the transfer of CH from CH-rich liposomes to the cell membrane will stabilize the latter and depress cell-liposome interactions (Robertson & Poznansky 1985). In view of this, liposomes with a high CH content, although having the advantage of being stable in biological fluids, may not necessarily be suitable as drug carriers in-vivo.

Several processes may be involved in cell-liposome interactions, including adsorption, fusion and endocytosis. Incubating peritoneal macrophages at 4°C instead of 37°C caused a 60–70% reduction in cell-liposome association, similar to results obtained by Stendahl & Tagesson (1977). This suggests that cell-liposome association involves an energy-dependent process. The small degree of cell-liposome association observed at 4°C could be due to external binding of liposomes to cells. However, cell-liposome association will also be influenced by the fluidity of the lipid bilayer and this will be reduced at low temperatures (Huang & Pagano 1975; Poste & Papahadjopoulos 1976). Several workers have shown that macrophages are capable of internalizing liposomes by endocytosis (Pratten et al 1981; Stevenson et al 1983) and that preincubation of cultured cells with metabolic inhibitors reduces cell-liposome association (Poste & Papahadjopoulos 1976; Dijkstra et al 1985). Pratten et al (1981) showed that 2,4-dinitrophenol completely inhibited the association of liposomes with rat macrophages and suggested that cell-liposome association involved an active energy-dependent process such as endocytosis, rather than mere external binding. However, in the present study the same concentration of 2,4-dinitrophenol only reduced the association of liposomes with macrophages by approximately 60%. Colchicine, an inhibitor of microtubule assembly, reduced macrophage-liposome association by 20–30%. Similarly, Wu et al (1981) reported that colchicine inhibited the uptake of aminomannose-targeted liposomes by approximately 20%. Dijkstra et al (1985) showed that colchicine reduced the uptake of liposomal insulin by Kupffer cells by 55%, and concluded that colchicine slows down the uptake of liposomes and their subsequent transport to the lysosomal compartment.

Conclusion

This study was instigated to ascertain whether it might be possible to enhance the uptake of liposome-entrapped MTX or similar drugs by phagocytic cells in inflamed joints. To prevent saturation of cell-liposome uptake mechanisms it would appear to be important to minimize the amount of lipid injected and therefore drugs used must be potent and entrapped with high efficiency. It would also appear that the uptake of liposome-entrapped drugs could be enhanced by reducing the CH content of the liposomes. With PC

liposomes it may not be possible to avoid the use of CH without compromising liposome stability, since with a low CH content they rapidly lose entrapped [^{3}H]MTX in the presence of rheumatoid synovial fluid (White et al 1983).

Acknowledgements

This work was supported by a grant from the Nuffield Foundation. We thank Dr H.W.R. Rattle for the photon correlation spectroscopy measurements.

References

Abra, R. M., Bosworth, M. E., Hunt, C. A. (1980) Liposome disposition in vivo. VI. Effects of pre-dosing with liposomes. Res. Commun. Chem. Pathol. Pharmacol. 29: 349–360

Adam, W. S. (1966) Fine structure of the synovial membrane: Phagocytosis of colloidal carbon from the joint cavity. Lab. Invest. 15: 680–691

Dijkstra, J., van Galen, W. J. M., Hulstaert, C. E., Kalicharan, D., Roerdink, F. H., Scherphof, G. L. (1984) Interaction of liposomes with Kupffer cells in vitro. Exp. Cell Res. 150: 161–176

Dijkstra, J., van Galen, W. J. M., Scherphof, G. L. (1985) Effects of (dihydro) cytochalasin B, colchicine, monensin and trifluoperazine on uptake and processing of liposomes by Kupffer cells in culture. Biochim. Biophys. Acta 845: 34–42

Dingle, J. T., Gordon, J. L., Hazelman, B. L., Knight, C. G., Page Thomas, D. P., Phillips, N. C., Shaw, I. H., Fildes, F. J. T., Oliver, J. E., Jones, G., Turner, E. H., Lowe, J. S. (1978) Novel treatment for joint inflammation. Nature, Lond. 271: 372–373

Foong, W. C., Green, K. L. (1983a) Treatment of an experimental arthritis with liposome-entrapped methotrexate. Br. J. Pharmacol. 80: 692P

Foong, W. C., Green, K. L. (1983b) Pharmacokinetics of liposome-entrapped methotrexate injected into arthritic joints. J. Pharm. Pharmacol. 83 (Suppl): 84P

Huang, L., Pagano, R. E. (1975) Interaction of phospholipid vesicles with cultures mammalian cells. J. Cell Biol. 67: 38–48

Johnson, S. M. (1975) The inability of macrophages to digest liposomes containing a high proportion of cholesterol. Biochem. Soc. Trans. 3: 160–161

Margolis, L. B., Dyatovitskaya, E. V., Bergelson, L. D. (1978) Cell-lipid interactions. Cell attachment to lipid substrates. Exp. Cell Res. 111: 454–474

Pagano, R. E., Weinstein, J. N. (1978) Interactions of liposomes with mammalian cells. Ann. Rev. Biophys. Bioeng. 7: 435–468

Patel, H. M., Tuzel, N. S., Ryman, B. E. (1983) Inhibitory effect of cholesterol on the uptake of liposomes by liver and spleen. Biochim. Biophys, Acta 761: 142–151

Poste, G., Papahadjopoulos, D. (1976) Lipid vesicles as carriers for introducing materials into cultured cells: Influence of vesicle lipid composition on mechanism(s) of vesicle incorporation into cells. Proc. Natl. Acad. Sci. 73: 1603–1607

Pratten, M. K., Millard, P. C., Lloyd, J. B. (1981) Uptake by rat peritoneal macrophages of ^{125}I-labelled polyvinylpyrrolidone entrapped within liposomes. Biosci. Rep. 1: 125–133

Robertson, D. L., Poznansky, M. J. (1985) The effect of non-receptor mediated uptake of cholesterol on intracellular cholesterol metabolism in human skin fibroblasts. Biochem. J. 232: 553–557

Shaw, I. H., Knight, G. C., Page Thomas, D. P., Phillips, N. C., Dingle, J. T. (1979) Liposome-incorporated corticosteroids. I. Interaction of liposomal cortisol palmitate with inflammatory synovial-membrane. Br. J. Exp. Pathol. 60: 142–150

Stendahl, O., Tagesson, C. H. R. (1977) Interaction of liposomes with polymorphonuclear leucocytes. I. Studies on the mode of interaction. Exp. Cell Res. 108: 167–274

Stevenson, M., Baillie, A. J., Richards, R. M. (1983) Enhanced activity of streptomycin and chloramphenicol against intracellular Escherichia coli in the J774 macrophage cell line mediated by liposome delivery. Antimicrob. Agents Chemother. 24: 742–749

Todd, J. A., Modest, E. J., Rossow, P. W., Tokes, Z. A. (1982) Liposome encapsulation enhancement of methotrexate sensitivity in a transport resistant human leukemic cell line. Biochem. Pharmacol. 31: 541–546

White, G. L., Foong, W. C., Green, K. L., (1983) The influence of cholesterol on the stability of liposomes containing methotrexate. Biochem. Soc. Trans. 11: 305–306

Wu, P. S., Tin, G. W., Baldeschwieler, J. D. (1981) Phagocytosis of carbohydrate-modified phospholipid vesicles by macrophages. Proc. Natl. Acad. Sci. 78: 2033–2037