J. Pharm. Pharmacol. 1984, 36: 824–830
Received March 12, 1984

Quantification of uptake of liposomal carboxyfluorescein by professional phagocytes in-vitro. A flow microfluorimetric study on the J774 murine macrophage cell line

M. STEVENSON, A. J. BAILLIE* AND R. M. E. RICHARDS

Department of Pharmacy, University of Strathclyde, Glasgow G1 1XW, UK

Unilamellar egg phosphatidylcholine/cholesterol liposomes containing carboxyfluorescein were prepared by an ether injection method. The ability of cells of the J774.2 murine macrophage cell line to incorporate the liposomal fluorophore during incubation at 37 °C was measured by flow microfluorimetry. Liposomes incorporating additional phosphatidylserine or phosphatidic acid were taken up much more avidly than those lacking these phospholipids and the greatest uptake of carboxyfluorescein was observed with the phosphatidylserine species. Calculation of the number of liposomes taken up, ≯0·2% of the number given, showed that this was an inefficient process. However these uptake data support previous findings based on the intracellular bactericidal activity of liposomal antibiotics determined in an identical in-vitro system.

The marked avidity of cells of the reticuloendothelial (RE) system for liposomes and other particulate material is well documented (Scherphof et al 1980) and is recognized (Poste et al 1982) as a severe limitation to the ability of such particulate systems to serve as drug carriers. For large liposomes, at least, it is now apparent that the major mechanism of uptake by RE cells is phagocytic both in-vitro (Hsu & Juliano 1982) and in-vivo (Poste et al 1982). Thus passive targeting of drugs to RE cells is eminently feasible and it would appear that the treatment of infection of this system may well benefit from the use of liposomes and other similar drug carriers.

In an in-vitro model of chronic infection of the RE system comprising cells of the J774 macrophage line (Ralph et al 1975) infected intracellularly with *Escherichia coli* (Stevenson et al 1984) we have shown (Stevenson et al 1983) that antibiotic inhibition of the growth of these intracellular bacteria is markedly enhanced by the use of a liposomal drug carrier system. The apparent intracellular activity of streptomycin is increased more than 10-fold and that of chloramphenicol more than 20-fold by the use of an appropriate liposomal system. Liposomal antibiotic is, however, inactive itself against *E. coli* and an inhibitory effect only becomes apparent after disruption of the bounding phospholipid bilayer of the liposome (Stevenson et al 1981). Similar bilayer disruption must presumably occur within the infected macrophage after phagocytosis of liposomes.

* Correspondence.

Inhibition of intracellular bacterial growth by liposomal antibiotic (Stevenson et al 1983) leads to the assumption that a minimal inhibitory antibiotic concentration is achieved within the infected macrophage. On this basis it was calculated that only a small proportion (≯0·05%) of the available liposomal drug need be released within the macrophage. Although representing the minimum value, such apparently inefficient uptake would have important implications for liposomal drug delivery to phagocytic cells in-vivo.

In an attempt to quantify vesicle uptake, and most importantly the delivery of their contents to the interior of the macrophage, we have substituted the self-quenching fluorophore carboxyfluorescein (CF) for antibiotic as the entrapped solute and measured cell-associated fluorescence by fluorescence-activated cell sorting (Weinstein et al 1977) and spectrofluorimetry. Although open to several criticisms (Szoka et al 1979) the use of liposomal CF does provide a measure of the release and transfer of solute from liposome to cell. Radiolabelled liposomal solute does not readily allow exclusive quantification of this transfer.

MATERIALS AND METHODS

5,6-Carboxyfluorescein (CF) (Eastman Kodak) was purified (Ralston et al 1981) over activated charcoal and Sephadex LH20 (Pharmacia) before use. Cholesterol, ash free (CHOL); L-α-phosphatidylcholine type III-E from frozen egg yolk (EPC); L-α-phosphatidyl-L-serine from bovine brain (PS);

L-α-phosphatidic acid, sodium salt from egg yolk (PA) (all Sigma). Phospholipids were checked for purity by thin layer chromatography before use. J774.2 murine macrophage cell line was obtained from the Sir William Dunn School of Pathology, Oxford.

Phosphate buffered saline (PBS) was pH 7·2, 1·3 × 10^{-2} M Sorensen's phosphate buffer containing 0·18% w/v NaCl.

All reagents were of analytical reagent grade.

Methods

Liposome preparation. Liposomes were prepared by the ether injection method of Deamer & Bangham (1976). Neutral liposomes were prepared from a mixture of EPC and CHOL (7:2 mol ratio) and 'anionic' liposomes from EPC:CHOL:PA or PS (7:2:1 mol ratio).

40 μmol EPC and the other lipids were dissolved in 20 ml diethyl ether and this solution slowly injected (0·25 ml min^{-1}) by means of an infusion pump through a No. 6 gauge needle into 4 ml PBS aqueous phase maintained at 60 °C with continuous N_2 purging, containing 300 mg CF (2 × 10^{-1} M). Liposome suspensions were stored (⩾24 h) in the hydrating solution before separation of the liposomes from the free CF by centrifugation.

Liposomal CF suspensions were twice pelleted at 3·5 × 10^4 g for 30 min at 5 °C (MSE Superspeed 75 centrifuge). The supernatants were discarded and the CF liposomes finally suspended in PBS by 5 min bath sonication of the pellet. Liposome suspensions were added to macrophage cultures immediately after centrifugation and washing.

Determination of liposomal CF. Small volumes (0·5 or 1 ml) of liposome suspension were diluted with an equal volume of propanol to disrupt the liposome bilayer structure, then further diluted with distilled water for determination of CF by spectrofluorimetry. The purity of liposomal CF which may influence leakage from liposomes and uptake by cells (Ralston et al 1981) was assured by hplc at room temperature (20 °C) of 2 μl samples of the diluted, disrupted liposome preparation on a Constametric III (Laboratory Data Control, LDC) pump, flow rate 2 ml min^{-1}, connected to a 100 × 4·5 mm 10 μm Spherisorb ODS column fitted with a 20 × 4·5 mm Copel ODS guard. Uv detection (Spectromonitor III, LDC) was at 254 nm at a sensitivity (absorbance units per full scale deflection) of 0·20.

CF samples were eluted over 20 min with a linear gradient of 25–100% methanol in 0·5% v/v acetic acid as a double peak, R_F = 9·5 and 11·4 min, corresponding to the 6- and 5-isomers (Ralston et al 1981).

Efficiency of solute entrapment (Table 1) in the liposome suspensions was calculated assuming an EPC concentration of 10 μmol ml^{-1} and an entrapped solute concentration equal to that in the hydrating aqueous solution used for liposome preparation (2 × 10^{-1} M).

Macrophage cultivation. Maintenance of the J744.2 murine macrophage cell line in complete tissue culture medium (TCM) has been described (Stevenson et al 1984).

For experiments, cells were grown to confluence (1 week at 37 °C with daily changes of TCM) in 50 ml flat-bottomed culture flasks (Nunclon) then harvested by addition of 20 ml ice cold TCM and shaking. 2 ml volumes of pooled cell suspension were added to the 20 mm square wells of tissue culture Petri plates (Flow Laboratories) and after 1 h at 37 °C the number of adherent macrophages per well was determined by direct counting. Cell viability by trypan blue exclusion was >98%.

Adherent macrophage cultures were incubated with gentle agitation at 37 °C in 2 ml TCM containing liposomal CF suspension so that 10^6 cells were dosed with 1 μmol EPC. The quantity of CF present in the incubation medium, determined retrospectively, was dependent on the entrapment efficiency of the liposomes and is shown in Table 2. After incubation for periods of up to 3 h, the TCM-liposome suspension was discarded and the adherent cultures washed with saline at 37 °C before addition of 2·5 ml ice-cold TCM. The cells were scraped off into suspension and kept on ice in small sealed tubes for flow microfluorimetry. As a control, cells were also incubated with empty liposomes (1 μmol EPC per 10^6 cells) in 2 ml TCM containing free CF (10^{-3} M). CF uptake was determined as described below.

Flow microfluorimetry. The fluorescence of macrophages exposed to CF liposomes was measured on a fluorescence activated cell sorter (FACS) (Becton Dickenson FACS II) using an argon laser tuned to 488 nm. The fluorescence of individual cells in sample populations of 0·5–1 × 10^4 cells was stored in a multi-channel analyser and used to construct a frequency distribution histogram of numbers of cells against channel number (fluorescence intensity). Channel number was converted to the equivalent quantity of CF required inside a cell to produce the fluorescence measured on the FACS. Throughout

Table 1. Uptake of liposomal CF by J774 macrophages. Macrophage monolayer cultures were incubated at 37 °C with 2 ml liposome suspension in TCM at a nominal concentration of 10^{-12} mol EPC per macrophage. Incubation mixtures with neutral (7EPC:2CHOL), PS(7EPC:2CHOL:1PS) and PA(7EPC:2CHOL:1PA) liposomes contained respectively: $8{\cdot}75 \times 10^5$ cells, $0{\cdot}9 \times 10^{-6}$ mol EPC, $2{\cdot}0 \times 10^{-6}$ mol CF; $5{\cdot}4 \times 10^5$ cells, $0{\cdot}54 \times 10^{-6}$ mol EPC, $1{\cdot}0 \times 10^{-6}$ mol CF; $5{\cdot}4 \times 10^5$ cells, $0{\cdot}54 \times 10^{-6}$ mol EPC, $0{\cdot}66 \times 10^{-6}$ mol CF. This corresponded to an effective dose (d) of liposomal CF (mol per cell) of neutral $2{\cdot}3 \times 10^{-12}$, PS $1{\cdot}8 \times 10^{-12}$ and PA $1{\cdot}2 \times 10^{-12}$. At 30 min intervals over a 3 h incubation, cell monolayers were rinsed with 0·9% w/v NaCl and the cells scraped off into suspension. Internalized CF (i) was determined by flow microfluorimetry (FACS) and the values shown are means for sample sizes of $0{\cdot}5$–$1{\cdot}0 \times 10^4$ cells. A macrophage cell volume of $2{\cdot}15 \times 10^{-9}$ ml was used to convert intracellular CF concentration (from FACS) to the weight of CF internalized shown. Total cell associated CF (t) was determined by spectrofluorimetry of the supernatants of cell supensions disrupted by repeated freezing and thawing.

	Incubation time (h)	t mol ($\times 10^{15}$)	t/d %	i mol ($\times 10^{15}$)	i/d %	i/t %
PS liposomes	0·5	4·00	0·22	0·83	0·045	20·75
	1·0	4·90	0·27	1·52	0·083	31·02
	1·5	5·45	0·30	1·78	0·097	32·66
	2·0	6·54	0·36	1·91	0·104	29·20
	2·5	7·27	0·40	2·06	0·112	28·34
	3·0	8·36	0·45	2·34	0·127	27·99
PA liposomes	0·5	3·16	0·26	0·75	0·063	23·73
	1·0	3·63	0·30	0·79	0·066	21·76
	1·5	4·73	0·39	0·99	0·083	20·93
	2·0	5·82	0·48	1·02	0·085	17·53
	2·5	5·45	0·45	1·06	0·088	19·45
	3·0	5·45	0·45	1·32	0·110	24·22
Neutral liposomes	0·5	4·18	0·18	0·73	0·032	17·46
	1·0	3·02	0·13	0·71	0·031	23·51
	1·5	6·17	0·29	0·81	0·035	12·07
	2·0	3·36	0·15	0·91	0·040	27·08
	2·5	6·22	0·27	0·95	0·041	15·27
	3·0	4·18	0·18	1·02	0·044	24·40

we have used the term internalized as implying phagocytosis of a liposome and the subsequent release of its contents.

Spectrofluorimetry. The concentration of aqueous CF solutions produced by disruption of liposomes or cells was measured spectrofluometrically (Perkin Elmer Model MPF3, excitation wavelength, 486 nm, emission, 514 nm) after dilution of the samples to between 0·1 and 1·0 μM CF with distilled water.

The free CF intracellular content of macrophages which had been incubated with liposomes was determined after hypotonic lysis of the cells in 3 ml distilled water and pelleting (3000*g* for 30 min) of the cell debris. Knowing the number of cells lysed, the mean intracellular content of free CF was obtained and this value, CF equivalent, was assigned to the mean channel number of the FACS histogram obtained for a macrophage suspension identical in terms of incubation time and liposome type used. The CF equivalent of the other channels was assigned by simple proportion.

Total cell associated CF, the sum of free intracellular CF, intracellular liposomal CF and liposomal CF adsorbed onto the cell surface, was determined after repeated freezing and thawing the macrophage suspension to disrupt cells and liposomes, and pelleting (3000*g* for 30 min) of the debris.

RESULTS

Fluorescence frequency histograms (Fig. 1) from the FACS experiments took the form of a single broad peak indicating that all cells in the populations had internalized liposomal CF. This was found at all incubation times studied and for each type of liposome used, neutral, PS or PA, so long as these preparations were undiluted (0·5 μmol EPC ml^{-1}). For PS liposomes at dilutions greater than ten fold, two histogram peaks were obtained, the less fluorescent of which represented a subpopulation which had not internalized a significant number of liposomes, having a mean channel number equivalent to control cells. These double-peaked histograms however probably represented limits imposed by liposome supply at the cell surface rather than two functionally dissimilar subsets within the macrophage population.

A common feature of all histograms obtained was their non-Gaussian distribution, with an obvious right skewing of the peaks; that is, in the direction of higher fluorescence. For cell populations incubated with each liposome type, the histogram peaks were

Table 2. Uptake of liposomal CF (vesicle equivalents) by J774 macrophages. Incubation mixtures detailed in legend to Table 1. Assuming monodisperse suspensions of unilamellar vesicles throughout, the dependence of entrapment efficiency on vesicle diameter can be determined. Based on an entrapped CF concentration of 2×10^{-1} M CF, the measured entrapment efficiencies (litre mol^{-1}) of 11·3 neutral, 9·4 PS and 6·2 PA liposomes were interpolated to liposome diameters of 350, 295 and 195 nm respectively. Treated as spheres with a bilayer thickness of 5 nm and a phospholipid molecular area of 0·65 nm^2 it was calculated that 10^{-6} mol EPC in liposomal form represented 5×10^{11} neutral, 8×10^{11} PS and $1{\cdot}8 \times 10^{12}$ PA liposomes. Thus the liposome dose was neutral, $5{\cdot}71 \times 10^5$, PS $1{\cdot}48 \times 10^6$ and PA $3{\cdot}33 \times 10^6$ vesicles per cell. These liposomes contained $4{\cdot}3 \times 10^{-18}$, $2{\cdot}6 \times 10^{-18}$ and $6{\cdot}0 \times 10^{-19}$ mol CF per liposome respectively and these values were used to derive vesicle equivalents from the data shown in Table 1.

	Incubation time (h)	Cell associated CF (vesicle equivalents per cell) Total	Internalized
PS liposomes	0·5	1499	311
	1·0	1854	575
	1·5	2058	672
	2·0	2476	723
	2·5	2749	779
	3·0	3162	885
PA liposomes	0·5	5099	1210
	1·0	5928	1290
	1·5	7692	1610
	2·0	9469	1660
	2·5	8877	1720
	3·0	8877	2150
Neutral liposomes	0·5	939	164
	1·0	681	160
	1·5	1516	183
	2·0	757	205
	2·5	1408	215
	3·0	943	230

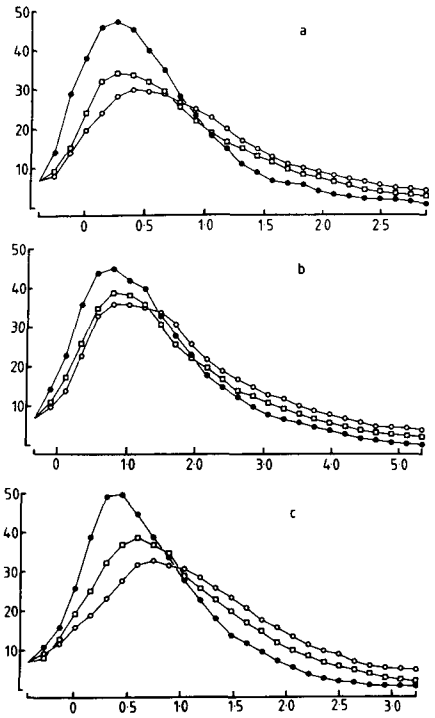

FIG. 1. Frequency distribution of macrophage fluorescence (CF equivalents) obtained by flow microfluorimetry after incubation of macrophages with CF liposomes (a, neutral; b, PS; c, PA liposomes) for 1, ●——●; 2, □——□ and 3, ○——○ h. [Ordinate shows cell number, abscissa shows cell fluorescence as CF equivalents (mol/cell × 10^{15}).] The histograms represent cell numbers of 10^4 (a) and 5×10^3 (b and c). Conditions as described in Table 1.

progressively right shifted with incubation time: that is, the cells became more fluorescent, and the extent of the skewing increased. After 3 h incubation, therefore, there were marked differences between mean and mode fluorescence values in any cell population. For example, in terms of CF equivalents internalized per cell the mean values (mol) were neutral $1{\cdot}02 \times 10^{-15}$, PS $2{\cdot}34 \times 10^{-15}$ and PA $1{\cdot}32 \times 10^{-15}$ (Table 1) and the modal values were neutral $5{\cdot}6 \times 10^{-16}$, PS $1{\cdot}07 \times 10^{-15}$ and PA $7{\cdot}3 \times 10^{-16}$ respectively (Fig. 1).

Another feature of the histograms was the wide distribution of fluorescence intensity such that there was a ten fold difference within a cell population. Size heterogeneity within the liposome preparations may contribute significantly to both the width and skewing of the distribution about the mean values observed.

Internalization of CF. After incubation of macrophages with liposomes (Table 1) the highest mean values for internalized CF were obtained with PS liposomes ($2{\cdot}34 \times 10^{-15}$ mol per cell after 3 h).

Hsu & Juliano (1982) showed that, based on the use of radio-labelled lipid, mouse peritoneal macrophages took up charged vesicles more readily than the neutral type and that uptake of anionic vesicles containing PS was almost four times that of the neutral vesicles.

For all liposome types there was an incubation time-dependent increase in internalized CF (Fig. 2) up to and including the longest incubation time of 3 h. However the major proportion of this CF was taken up within the initial 0·5 h incubation period. For each type of liposome there was no obvious relationship between the proportion of the total cell-associated CF which was internalized, and incubation time. The mean values (±s.e.m.) for the proportion (%) of the total cell-associated CF

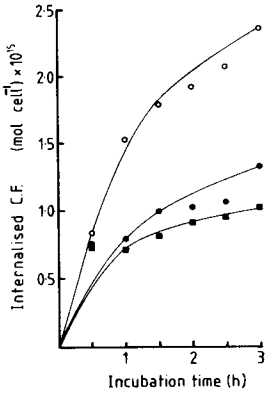

FIG. 2. Rate of internalization of liposomal CF by J774 macrophages at 37 °C. Mean values for cell fluorescence (CF equivalents) at each incubation time are plotted. Incubation conditions (described in Table 1) gave a liposomal CF dosage (mol × 10^{12} per macrophage) of neutral (■) 2·3, PS (○) 1·8 and PA (●) 1·2.

internalized were: neutral liposomes 20·0 ± 2·20, PS liposomes 28·3 ± 1·53 and PA liposomes 21·3 ± 0·95. These figures indicate that subsequent to association of liposomes with cells, neutral vesicles are internalized as efficiently as anionic vesicles. The greater transfer of liposomal CF to the cell interior obtained with anionic PA and PS vesicles, appears to reflect greater initial cell association rather than more efficient phagocytosis of the anionic vesicles. Similarly, there is evidence from a comparison of PA with PS liposome mediated CF delivery (Table 1), that although a somewhat greater proportion of the liposomal CF dose became cell-associated with PA liposomes, internalization of the contents of PS liposomes was greater.

The proportion of the liposomal CF available to each cell that was actually internalized was very small so that the 2·3 × 10^{-15} mol CF per cell measured in cells after 3 h incubation with PS liposomes represented 0·127% of the initial dose. However, converted to intracellular concentrations (cell volume 2·15 × 10^{-9} ml), the quantities of CF internalized, for example after 3 h incubation, gave values (×10^4 M) of 4·71 neutral, 10·90 PS and 6·11 PA liposomes. For the anionic PS and PA liposomes these concentrations were respectively 2·19 and 1·86 times greater than the corresponding extracellular CF concentrations. These results indicate the ability of a liposomal carrier system to load the cytoplasm of these phagocytic cells with entrapped solute against the apparent transmembranal concentration gradient. This loading effect was not observed in cells after incubation with neutral liposomes and the highest intracellular CF concentration achieved after 3 h was 47% of the extracellular concentration.

For cells incubated with empty liposomes plus free CF, internalization of the free fluorescent marker was at least an order of magnitude less than that of the liposomal form.

Internalization of vesicle equivalents. The results of the experiments described here may, instead of CF equivalents, be calculated in terms of vesicle equivalents (Blumenthal et al 1977). This is the number of liposomes whose total CF contents are required to be liberated within the cell to produce the fluorescence measured on the FACS. Three assumptions are involved in the calculation of the number of liposomes taken up by each macrophage and these are: the size of the vesicle and its configuration, that is uni- or multi-lamellar, and the concentration of entrapped solute within each vesicle. Inter- and intra-batch variation in all three parameters almost certainly occurs although the greatest variation is probably in vesicle size. We have already assumed that the intraliposomal CF concentration is 2 × 10^{-1} M (Methods) and if we also assume that all the vesicles in a population are unilamellar then entrapment efficiency becomes a measure of vesicle size.

For the three types of liposome, neutral, PS and PA, observed entrapment efficiencies (Table 2) translate into mean diameters of 350, 295 and 195 nm respectively. These values are greater than that quoted for a similar type of liposome by Deamer & Bangham (1976) who did, however, remove the larger vesicles from their preparation by filtration.

Sizing in this laboratory by photon correlation spectroscopy (PCS) (results not shown) of suspensions of empty liposomes prepared by the method used here, with the omission of CF from the hydrating solution, yielded a value for mean liposome diameter of 480 nm (neutral or anionic). Sizing by PCS, based on the Z-average hydrodynamic diffusion coefficient, is however heavily biased in favour of the larger vesicles in the sample under study. The normalized variance of the size distribution about the mean from the PCS data indicated a marked polydispersity in all suspensions studied. CF liposome suspensions were not sized by PCS.

The calculated CF content of the three types of liposome shown in Table 2 formed the basis of the calculation of CF uptake in terms of vesicle equivalents. It is apparent that this criterion increased the apparent avidity of the macrophages for anionic rather than neutral liposomes. The ranking of liposome type on the basis of number internalized,

PA > PS > neutral, was observed at all incubation times with about twice as many PA liposomes internalized than PS liposomes. However on the basis of the proportion of the dose internalized there was no significant difference between PA and PS liposomes although both were taken up by the cells more readily than neutral liposomes.

The inefficient internalization of liposomal CF described above was, of course, reflected in the efficiency of vesicle equivalents internalized, and the highest mean value of $2{\cdot}15 \times 10^3$ PA liposomes per cell after 3 h incubation, represents only 0·12% of the initial dose of $1{\cdot}8 \times 10^6$ vesicles per cell. Efficiency of internalization was found to be markedly dependent on the concentration of the liposome suspension given to the cells (Fig. 3) and it would appear that for PS liposomes at least, below a dosage level of about 10^5 liposomes per cell achieved by diluting the suspension, internalization was insignificant. Dilution experiments were not carried out with neutral or PA liposome suspensions.

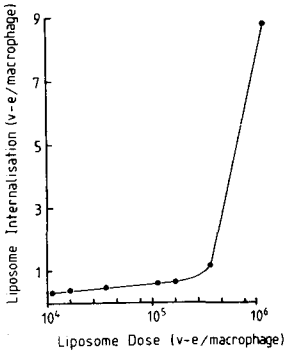

FIG. 3. The effect of liposome dose on internalization (vesicle equivalents, v-e) of anionic PS liposomes containing CF. J774 macrophages were incubated for 3 h at 37 °C with a series of dilutions of a PS liposomal CF suspension in TCM and the resultant mean macrophage fluorescence determined by flow microfluorimetry. Vesicle equivalents were calculated as described in the legend to Table 2.

DISCUSSION

The demonstration in a system essentially identical to that used here (Stevenson et al 1983) that streptomycin and chloramphenicol in liposomal form inhibit the growth of bacteria within the J774 macrophage shows that the minimal inhibitory concentration (MIC) of either antibiotic can be attained within the macrophage. The assumed diameter of 130 nm of the antibiotic-containing liposomes gave a calculated number of $8{\cdot}2 \times 10^2$ and $1{\cdot}7 \times 10^3$ neutral liposomes per cell to achieve intracellularly the MIC of streptomycin and chloramphenicol respectively. These numbers represent respectively 0·02 and 0·04% of the antibiotic liposome dose used.

The present results confirm that although a greater proportion of the liposome dose, 0·12%, is internalized, uptake of liposomes by the J774 line under these conditions is remarkably inefficient. However in spite of the apparently poor internalization figures, inhibitory quantities of antibiotic can be delivered to the interior of the phagocytic cells by small numbers of liposomes. Thus, the delivery of antibacterial quantities of liposomal solute to significant numbers of phagocytic cells, in-vivo, is feasible. Stimulated macrophages in-vitro or in-vivo could be expected to take up greater numbers of liposomes. It would also be interesting to know if the macrophage population displayed in-vivo the broad spectrum of liposome uptake ability observed here by FACS (Fig. 1), and to know its significance for in-vivo delivery of liposomal drug.

The present results do not explain the poor performance of PA liposomes as vehicles for antibiotic delivery to microorganisms located within these macrophages (Stevenson et al 1983). Here, although intracellular CF delivery was greatest with PS liposomes, PA liposomes delivered significantly greater quantities of CF to the cell interior than neutral liposomes, although the latter gave a much greater intracellular antibacterial effect. In both systems intracellular liposomal solute release is required, either to liberate antibiotic for bacterial inhibition or to liberate self quenched CF for macrophage fluorescence. The discrepancy between the results with PA liposomes obtained here and in the intracellular inhibition experiments may be explained either in terms of solute release rates from such vesicles or their intracellular destination. In either case the released antibiotic appears not to gain access in sufficient quantity to that cellular compartment containing the bacterial cells.

The marked inhibition of uptake achieved by dilution of the PS liposomes suspension is to be expected since phagocytosis of a vesicle by a cell must be preceeded by a collision between both, the chances of which will be reduced by dilution. Indeed it has been shown (Tomita & Kanegasaki 1982) that uptake of bacteria by macrophages may be increased by centrifugation which serves to promote collision between cell and bacterium.

At a constant dose of liposomal phospholipid, in this case 1 μmol EPC per 10^6 cells, a reduction in the number of liposomes available per cell may be achieved by increasing vesicle size instead of by dilution. Vesicle number, however, varies as the

square of vesicle linear dimension and the quantity of solute entrapped per vesicle as the cube of the linear dimension. There is thus an inverse relationship between vesicle size and that proportion of the liposomes presented to a cell which has to be internalized to deliver a particular quantity of solute to the cell interior. The influence of vesicle number and size on the arithmetic of solute delivery to the macrophage interior may be further complicated by the influence of the latter parameter on phagocytosis of the vesicles by the cells. Although a study of the phagocytosis of latex beads by Acanthamoeba (Weisman & Korn 1967) indicates that the kinetics of phagocytic uptake are independent of bead size, a recent report (Machy & Leserman 1983) indicating that the cell uptake of small unilamellar vesicles proceeded more rapidly than uptake of large reverse phase vesicles suggests that the dimensions of the coated pits on the cell surface is the limiting parameter. However the cells used here are professional phagocytes and liposome uptake by a classic phagocytic mechanism will tend to be limited by phagocytic vacuole size which will accommodate much larger particles.

The overall low uptake efficiency described here is probably a function of the in-vitro system used in which the macrophages are attached to a surface and the liposomes evenly distributed in the overlying TCM layer some 5 mm in depth. This is at least three orders of magnitude greater than the depth of the adherent macrophage monolayer (dimensions in μm) so that the effective liposome dose is proportionally reduced. In-vivo, vesicle supply, especially to the fixed phagocytes of the liver, would not be so limited.

Acknowledgements

The authors thank Dr Brian Young and Mrs E. McKenzie of the Beatson Research Institute, Glasgow for access to, and skilful assistance in the use of FACS, and to SERC for financial assistance to M. Stevenson.

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