ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1983, p. 742–749
0066-4804/83/110742-08$02.00/0

Vol. 24, No. 5

Enhanced Activity of Streptomycin and Chloramphenicol Against Intracellular *Escherichia coli* in the J774 Macrophage Cell Line Mediated by Liposome Delivery

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

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

Received 19 May 1983/Accepted 25 August 1983

Streptomycin and chloramphenicol were entrapped within large neutral or anionic unilamellar vesicles of egg phosphatidylcholine prepared by an ether injection method. Both antibiotics in liposomal form were inactive against *Escherichia coli* in a simple tube dilution assay. A comparison was made of the activities of the free and liposomal forms of the antibiotics against *E. coli* located within the macrophages of the J774.2 murine cell line. The apparent intracellular antibacterial activity of both antibiotics was increased more than 10-fold by entrapment in neutral liposomes and in the case of chloramphenicol in anionic liposomes containing phosphatidylserine. Anionic liposomes containing phosphatidic acid were much less effective carriers than neutral liposomes for either antibiotic in this in vitro system. Incubation at 4°C of cells with liposomes containing antibiotic or carboxyfluorescein inhibited intracellular antibacterial activity and cell-associated fluorescence. The high intracellular activity of the liposomal antibiotics is consistent with their phagocytic uptake by the macrophages followed by intracellular liposomal degradation and antibiotic release. Liposomal modification of cellular uptake and intracellular distribution of antibiotics may be used to extend the activity of existing and new agents against intracellular infection of the reticuloendothelial system.

Although the selective targeting of drugs to tissues or cells in vivo by means of liposomes and other carrier systems does not seem feasible at present (15), the propensity of the reticuloendothelial system to remove particulates from the circulation (20) does offer a means of passive targeting. Thus, the experimental liposomal therapy of visceral leishmaniasis (1) is largely dependent on the avid hepatic uptake of drug-laden vesicles mediated by the fixed macrophages of the liver. Activation of the tumoricidal (7) and antimicrobial (8) activities of macrophages in vivo by using liposomal muramyl dipeptide and its analogs indicates that circulating phagocytes also serve as "targets" for agents administered in liposomal form.

It is logical, therefore, to consider liposomes as carriers of antibiotics to phagocytic cells, which in certain infections harbor microorganisms which can survive intracellularly. Antibiotics can only act against such intracellular infections if they can penetrate the phagocyte, although once within the cell, metabolism and localization of the antibiotic will also contribute to the overall antimicrobial activity. Such cellular pharmacokinetic parameters can be modified by the use of liposomes (14).

In simple in vitro culture, liposomal neomycin (10) and penicillins (5) were reported to be active against bacteria, although Stevenson et al. (21) showed that liposome entrapment markedly reduces the antimicrobial activity of chloramphenicol (CAP). In the rabbit, gentamicin distribution can be altered by the intravenous administration of a liposomal preparation of this antibiotic (13). The activity of dihydrostreptomycin against an intracellular infection in vitro is increased by liposome entrapment of the antibiotic, although the mechanism of action is not described (2).

In this paper we compare the activity of free and liposomal CAP and streptomycin (STREP), lipophilic and hydrophilic antibiotics, respectively, against *Escherichia coli* located within the cells of the J774 murine macrophage line.

MATERIALS AND METHODS

STREP sesquisulfate and crystalline CAP (both from Sigma Chemical Co.) were used as received, and STREP concentrations throughout this paper are in terms of this salt. 5,6-Carboxyfluorescein (CF) (Eastman Kodak) was purified (19) over activated charcoal and Sephadex LH20 (Pharmacia) before use. Ash-free cholesterol, L-α-phosphatidylcholine type III-E from frozen egg yolk (EPC); L-α-phosphatidyl-L-serine from

bovine brain (PS); and L-α-phosphatidic acid as the sodium salt from egg yolk (PA) were all purchased from Sigma. Phospholipids were checked for purity by thin-layer chromatography before use.

Escherichia coli NCTC 9001 were from the National Collection of Type Cultures, London, England. The J774.2 murine macrophage cell line was obtained from the Sir William Dunn School of Pathology, Oxford, England.

Phosphate-buffered saline (PBS) was 0.18% (wt/vol) NaCl buffered to pH 7.2 with 1.3×10^{-2} M Sorenson phosphate.

All reagents were of analytical reagent grade.

Liposome preparation. Liposomes were prepared by the ether injection method of Deamer and Bangham (6). Neutral liposomes were prepared from a mixture of EPC and cholesterol (7:2 molar ratio), and anionic liposomes were prepared from EPC, cholesterol, and PA or PS (7:2:1 molar ratio).

Forty micromoles of EPC plus other lipids were dissolved in 20 ml of diethyl ether, and this solution was slowly injected (0.25 ml min^{-1}) by means of an infusion pump through a no. 6 gauge needle into 4 ml of PBS aqueous phase maintained at 60°C with continuous N_2 purging. The aqueous phase additionally contained either 70 mg of STREP, 10 mg of CAP, or 300 mg of CF for the preparation of STREP, CAP, and CF liposomes.

Removal of nonentrapped solutes. Free STREP and CAP were removed from liposome suspensions by dialysis (Visking tubing) at 4°C against several changes of 1 liter of PBS. Liposomal CF suspensions were pelleted twice at 35,000 × *g* for 30 min at 5°C (MSE Superspeed 75 centrifuge). The supernatants were discarded, and the CF liposomes finally suspended in PBS by a 5-min bath sonication of the pellet.

Determination of liposomal solute entrapment. Small volumes (0.5 or 1 ml) of liposome suspension were diluted with an equal volume of propanol to disrupt the liposome bilayer structure. For the CAP and STREP liposome suspensions, the propanol was removed under reduced pressure at 50°C.

The remaining STREP solution was diluted in PBS, and the STREP concentration was determined by a tube dilution method against *E. coli* at 37°C in nutrient broth (Oxoid no. 2).

The CAP solution was either assayed microbiologically as for STREP or was extracted into two 25-ml volumes of chloroform:ethyl acetate (2:1 by volume) and the solvent extract evaporated to dryness under vacuum at 50°C and the residue was taken up in 80% (vol/vol) aqueous methanol for high-pressure liquid chromatography (HPLC).

A 1-ml propanol dilution of CF liposome suspension was diluted to 10 ml with water and fractionated by HPLC.

For CAP and CF, HPLC analysis of 2-μl samples was carried out at room temperature on a Constametric III (Laboratory Data Control) pump (flow rate, 2 ml min^{-1}) connected to a Spherisorb 10-μm ODS column (100 mm by 4.5 mm) fitted with a Copel ODS guard (20 by 4.5 mm). UV detection (Spectromonitor III; Laboratory Data Control) was at 254 nm at sensitivities (absorbance units per full scale deflection) of 0.05 for CAP and 0.20 for CF.

CAP samples were eluted in 80% (vol/vol) aqueous methanol as a single peak (R_f = 80 s). CF samples were eluted over a 20-min period with a linear gradient of 25 to 100% methanol in 0.5% (vol/vol) acetic acid as a double peak (R_f = 9.5 and 11.4 min, corresponding to the 6- and 5-isomers) (19).

The 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 aqueous phase used for liposome preparation.

Macrophage cultivation. The J774.2 murine macrophage cell line (18) maintained in complete tissue culture medium (TCM) consisting of RPMI 1640 supplemented with 5% (vol/vol) fetal calf serum and 2 mM L-glutamine and buffered to pH 7.2 with 20 mM (final concentration) HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-$NaHCO_3$.

For experiments, cells were grown to confluence (1 week at 37°C with daily changes of TCM) in 50-ml flat-bottomed culture flaskss (Nunclon) and then harvested by the addition of 20 ml of ice-cold TCM and shaking. Two-milliliter volumes of pooled cell suspension were added to the square wells (20 mm side) of tissue culture petri plates (Flow Laboratories), each well containing a 17-mm square cover slip, and after 60 min at 37°C the number of adherent cells per cover slip was determined by direct counting. A mean value of 10^6 macrophages per cover slip was obtained, and at this stage cell viability, as determined by trypan blue exclusion, was better than 98%.

Drug pretreatment of macrophages. Macrophage cover slip cultures were incubated at 37°C in 2 ml of TCM containing either free drug or liposomal drug suspension. The free CAP concentrations used were 80, 40, and 20 μg ml^{-1}, and the free STREP concentrations used were 500, 100, 50, and 10 μg ml^{-1}. Control cultures were incubated in 2 ml of antibiotic-free TCM. Free drug pretreatment periods were 1, 2, 3, 5, and 7 h.

The pretreatment time for liposomal STREP and CAP was 3 h, and in all liposome experiments 1 μmol of liposomal EPC was added per 10^6 cells in 2 ml of TCM. The quantity of liposomal drug added was thus dependent on entrapment efficiency (Table 1). As controls, macrophage cultures were incubated with empty liposomes plus equivalent free drug at 37°C or with liposomal drug at 4°C.

The pretreated cover slip cultures were dip washed

TABLE 1. Entrapment efficiency of CAP, STREP, and CF in unilamellar vesicles prepared from EPC

Vesicle type	Entrapment efficiency (liters mol^{-1}) of:[a]		
	CAP	STREP	CF
Neutral	4.16 (4)	4.53 (3)	1.41 (5)
Anionic (PS)	9.30 (1)		3.71 (2)
Anionic (PA)	8.18 (4)	10.23 (4)	4.93 (2)

[a] Entrapment efficiencies were calculated by assuming an intraliposomal solute concentration of 2.5 mg ml^{-1} for CAP, 17.5 mg ml^{-1} for STREP, and 75 mg ml^{-1} for CF, i.e., the concentrations used for phospholipid hydration. The values shown are lower than those obtained by Deamer and Bangham (6) for comparable ether vaporization liposomes. Numbers in parentheses are numbers of liposomal preparations assayed.

in saline to remove free drug or liposomes not taken up by the macrophages and then transferred to an *E. coli* cell suspension for phagocytosis of bacteria.

***E. coli* cultures.** *E. coli* cells from an 18-h static broth culture (Oxoid no. 2) at 37°C were suspended in TCM at a concentration of 2×10^6 to 2.5×10^6 viable cells ml^{-1} determined by surface plating. Bacterial cell suspensions in TCM were added to macrophage cover slip cultures within 15 min of preparation.

Phagocytosis of bacteria. The TCM of the macrophage cover slip cultures in the petri plate wells was replaced with 2 ml of TCM *E. coli* cell suspension such that the initial inoculum was between 4 and 5 bacteria per macrophage. Phagocytosis of bacteria was allowed to proceed at 37°C for 50 min with gentle agitation (ca. 0.5 Hz) on a platform shaker (New Brunswick Scientific), after which time the cover slips were removed to the wells of a second petri plate containing 2 ml of TCM and 500 μg of STREP sulfate per ml to inactivate uningested bacteria. After 20 min, each cover slip was dip washed through several changes of sterile normal saline at 37°C to remove the STREP.

The cover slip cultures were then transferred to TCM in the case of free antibiotic-pretreated macrophages or to TCM containing 30 μg of STREP per ml for liposome-treated macrophages and incubated at 37°C. Viable intracellular bacterial numbers were determined at 1½ and 4 h after infection, for free antibiotic-pretreated macrophages and at 1½-h intervals for up to 9 h after infection for liposome-treated cultures. Results (number of viable intracellular bacteria per macrophage) were calculated as a percentage of the 1½-h value, which was taken as the initial rate of infection (i.r.). Phagocytosis under the conditions described here gave reproducible values for i.r. (M. Stevenson, A. J. Baillie, and R. M. E. Richards, J. Pharm. Pharmacol., in press) so that this parameter was comparable for control and pretreated cells in the present experiments. Values for i.r. are detailed in the figure legends.

Determination of viable intracellular bacteria. The cover slips with adherent macrophages were vortexed for 5 min in 10 ml of sterile distilled water to effect hypotonic lysis, and then for each cover slip, two serial 10-fold dilutions of the hypotonic lysate were made in distilled water for surface counting.

In all experiments, cover slips were set up in triplicate, and phagocytosis was expressed as the mean number of CFUs recovered per macrophage. Throughout, significant differences were tested by the Student *t* test at the 95% probability level.

Macrophage uptake of CF liposomes. Macrophage cover slip cultures (10^6 cells) were incubated at 37 and 4°C in 2 ml of TCM liposomal CF suspension (1 μmol of EPC) with gentle agitation. At 1-, 2-, and 3-h intervals, cover slips were removed, rinsed with saline, and mounted on microscope slides in glycerol containing 5% (wt/vol) propyl gallate to minimize photobleaching (9). Photomicrographs (Fig. 6) were taken on a Fluoval microscope (Carl Zeiss, Jena, East Germany) under incident illumination, using a high-pressure mercury lamp (model HBO 202), an exciter filter (model B224g), and a barrier filter (model G247).

MIC determination. For determination of the minimal inhibitory concentration (MIC), a series of tubes representing a range of concentrations of free and liposomal CAP and STREP in nutrient broth (Oxoid no. 2) were inoculated with an 18-h broth culture of *E. coli* to a final cell density of 1×10^5 to 3×10^5 cells per ml. The tubes were incubated for 18 h at 37°C, and the number of viable bacteria in each tube was determined by surface plating.

RESULTS

The MICs of CAP and STREP were found to be 2.0 and 7.5 μg ml^{-1}, respectively, when tested against the strain of *E. coli* used here. For neutral liposomal CAP, concentrations of up to 38 μg ml^{-1} had no effect on the test organism and for neutral liposomal STREP, a concentration of 170 μg ml^{-1} was similarly ineffective. At the highest concentration studied, 360 μg of liposomal STREP per ml in neutral vesicles, although there was some 60% inhibition of growth over an 18-h period, an MIC value was not attained. Using anionic vesicles which had a higher entrapment efficiency (Table 1), we obtained an MIC of 800 μg ml^{-1} for liposomal STREP and an MIC of >113 μg ml^{-1} for liposomal CAP. However, after propanol disruption of the bilayer structure, CAP or STREP extracted from liposomes was found to have retained its expected antibacterial activity. In the case of liposomal CAP, the extracted antibacterial activity correlated with the CAP concentration determined by HPLC.

The very low antibacterial activity of the liposomal material would appear to be an index of the leakage of entrapped antibiotic across the bilayer. On the basis of the MIC value for liposomal STREP, it was calculated that ~10% of the anionic vesicle antibiotic content had escaped over an 18-h period. Bilayer disruption was thus a requirement for an antibacterial effect from the liposomal antibiotics. A similarly poor antibacterial effect has been described for liposomal CAP in small sonicated vesicles (21).

Inhibitory effects on intracellular bacterial growth quickly became apparent in experiments with free CAP, so that even after 1 h of pretreatment of macrophages with 40 and 80 μg of CAP per ml there was a marked reduction in the number of CFUs recovered (Fig. 1). The inhibitory effects became more obvious with increasing pretreatment time. Since, however, CAP is bacteriostatic for *E. coli*, there was no significant reduction in viable intracellular bacterial numbers to less than 100%, i.e., the initial level of infection, even after the longest pretreatment time of 7 h. At an extracellular free CAP concentration of 20 μg ml^{-1}, the bacteriostatic effect shown (Fig. 1) was not significant.

From the effects of free STREP on intracellular bacteria (Fig. 2), it appeared that this antibiotic penetrated much more slowly to the macrophage interior than CAP. Of the free STREP concentrations studied, 10, 50, 100, and 500 μg ml^{-1}, only the last was inhibitory, and the

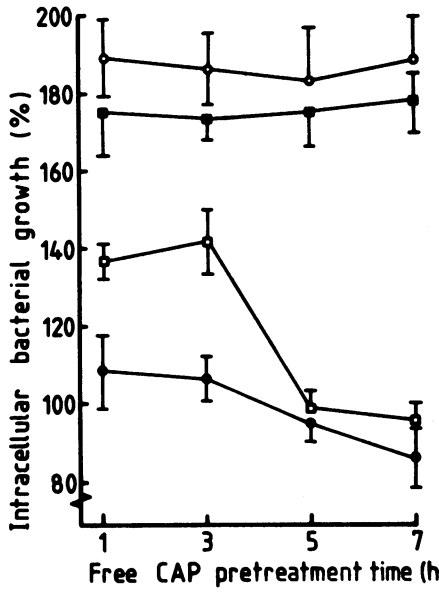

FIG. 1. Pretreatment time of J774.2 macrophages with free CAP and the growth of subsequently phagocytized *E. coli*. At each pretreatment time the i.r. (mean number of bacteria per macrophage) was determined at 1½ and 4 h after inoculation of the macrophage culture with *E. coli*. Bacterial growth is expressed as the percent change in bacterial numbers per macrophage between 1½ and 4 h postinfection. Symbols: ○, untreated control (n = 5, i.r. = 1.0); ■, 20 μg of CAP per ml (n = 5, i.r. = 0.5); □, 40 μg of CAP per ml (n = 4, i.r. = 1.0); ●, 80 μg of CAP per ml (n = 4, i.r. = 1.0). Vertical bars show standard error of the mean.

results for the other concentrations are not shown. At 500 μg of free STREP per ml, the inhibitory effect only became apparent after pretreatment times of greater than 2 h, and a minimum pretreatment time of about 6 h was required to actually decrease the viable intracellular bacterial numbers to less than the initial infection rate. For example a 7-h STREP pretreatment was bactericidal and reduced the intracellular bacterial population to some 53% of its initial value.

The apparent intracellular antibacterial activity of both antibiotics was markedly increased by entrapping them in neutral vesicles (Fig. 3 and 4) and, in the case of CAP, PS liposomes (Fig. 3). STREP was found to precipitate with PS during vesicle preparation, and hence no data was obtained for PS liposomal STREP. In spite of their high entrapment efficiency (Table 1), anionic PA vesicles containing either antibiotic were much less active than neutral or PS liposomes. In fact, for anionic PA liposomes containing CAP (Fig. 3) and STREP (Fig. 4), a significant inhibitory effect could only be established at 9-h postinfection. Cell-associated fluorescence elicited by exposure of macrophages to CF liposomes (Fig. 6) was not detected when PA vesicles were used.

The extracellular concentrations of liposomal CAP which caused intracellular bacteriostasis were 4.3 μg ml^{-1} (neutral vesicles) and 9.5 μg ml^{-1} (PS vesicles) (Fig. 3). For the 3-h pretreatment time of macrophages with liposomes, the concentration of free CAP required for bacteriostasis was 80 μg ml^{-1} (Fig. 1), so that liposome entrapment of CAP increased its activity in this system 10- to 20-fold.

Similarly, for STREP, a 40.5 μg ml^{-1} extracellular concentration in neutral liposomal form was sufficient to give an intracellular bactericidal effect (Fig. 4) comparable to that obtained with a free STREP concentration of 500 μg ml^{-1} (Fig. 2).

Pretreatment of macrophages with empty liposomes or empty liposomes plus free antibiotic at a concentration normally entrapped had no effect on subsequent intracellular bacterial numbers (Fig. 5). Similarly, pretreatment of macrophages with drug-loaded liposomes at 4°C (Fig. 5) had no effect on the viability of intracellular organisms. Incubation of macrophages with CF liposomes at 4°C also abolished the cell-associat-

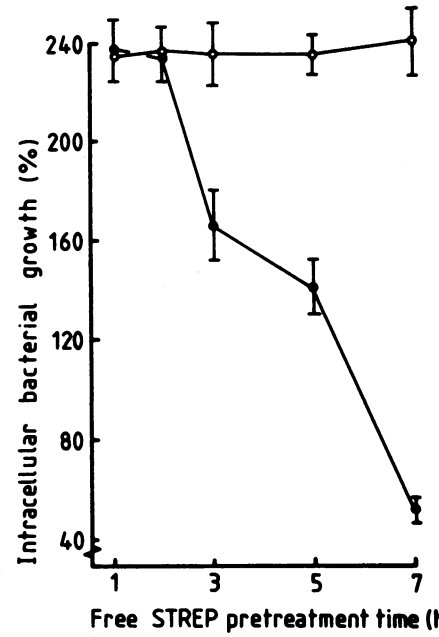

FIG. 2. Pretreatment time of J774.2 macrophages with free STREP and the growth of subsequently phagocytized *E. coli*. Conditions as described in the legend to Fig. 1. Symbols: ○, untreated control (n = 4, i.r. = 0.4); ●, 500 μg of STREP per ml (n = 4, i.r. = 0.4). Vertical bars show standard error of the mean.

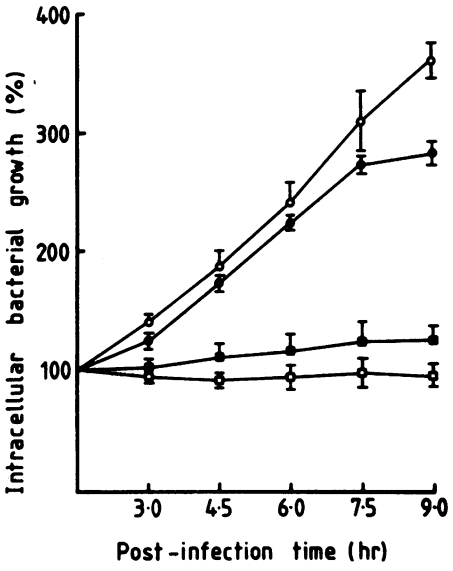

FIG. 3. Pretreatment (3 h) of J774.2 macrophages with liposomal CAP and the growth of subsequently phagocytized *E. coli*. After inoculation of pretreated macrophages with *E. coli*, the mean number of bacteria per macrophage was determined at 90-min intervals over a 9-h period. Bacterial growth (bacteria per macrophage) at each postinfection time is expressed as the percent change from the 1½-h i.r. Symbols: ○, untreated control (n = 12, i.r. = 0.8); ●, anionic (PA), liposomal CAP, 7.2 μg ml^{-1} (n = 9, i.r. = 0.8); ■, neutral liposomal CAP, 4.3 μg ml^{-1} (n = 9, i.r. = 0.9); □, anionic (PS), liposomal CAP, 9.5 μg ml^{-1} (n = 6, i.r. = 1.1). Vertical bars show standard error of the mean.

ed fluorescence observed (Fig. 6) after incubation at 37°C.

DISCUSSION

It would appear that the uptake of liposomes by the J774 macrophage was phagocytic since incubation of liposomes with cells at 4°C inhibited intracellular antibiotic activity (Fig. 5) and cell-associated CF fluorescence (Fig. 6). This uptake mechanism is in accord with the nonspecific phagocytic activity of these cells (17) and the phagocytic internalization of liposomes by other macrophage types (12).

The cell-associated fluorescence found (Fig. 6) after incubation of cells with CF liposomes at 37°C indicates dilution of the self-quenching vesicle aqueous contents in the cell cytoplasm (22), presumably after disruption of the vesicle bounding bilayer. The incubation time-dependent fluorescence shown in Fig. 6 thus lends support to the concept of vesicle degradation within the cell after phagocytosis, which in the case of STREP or CAP liposomes would liberate antibiotic close to any intracellular bacteria present. Such antibiotic release within the infected macrophage is a critical event since either antibiotic in liposomal form at the concentrations used here was inactive against *E. coli* per se.

The use of radiolabeled lipid or liposomal antibiotic, although allowing quantitation of cell-liposome association, does not quantify the all-important delivery to the cell interior (22) of free antibiotic. In fact, the intracellular antibacterial activity of liposomal antibiotic described in this paper may, like the observation of intracellular CF fluorescence, be regarded as a means of measuring the release of liposome contents within viable cells.

In the present system, therefore, the 3-h pretreatment of macrophages with liposomes must have allowed degradation of a sufficient number of internalized liposomes to establish an inhibitory antibiotic concentration within the cell. Although antibiotic is only required within an infected phagosome to influence the growth of ingested bacteria, it must be assumed that the entire intracellular volume is permeated by free antibiotic from degraded liposomes. A simple calculation shows that the contents of remarkably few vesicles of the type used here need be released within the cell to produce an inhibitory antibiotic concentration.

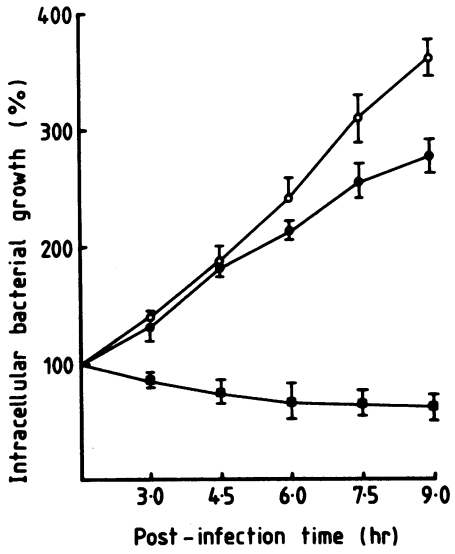

FIG. 4. Pretreatment (3 h) of J774.2 macrophages with liposomal STREP and the growth of subsequently phagocytized *E. coli*. Conditions as described in the legend to Fig. 3. Symbols: ○, untreated control (n = 12, i.r. = 0.8); ●, anionic (PA) liposomal STREP, 82.2 μg ml^{-1} (n = 9, i.r. = 0.8); ■, neutral liposomal STREP, 40.5 μg ml^{-1} (n = 9, i.r., = 1.1). Vertical bars show standard error of the mean.

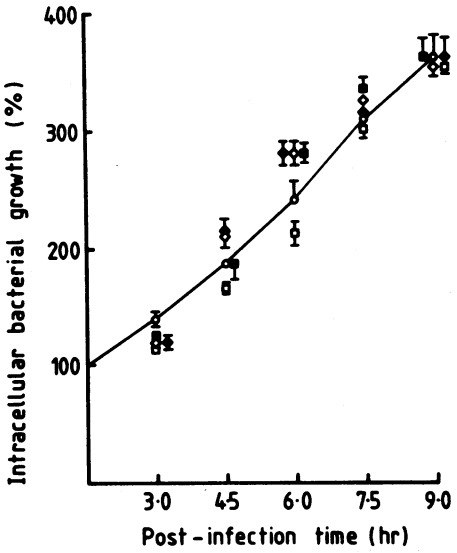

FIG. 5. Pretreatment (3 h) of J774.2 macrophages with empty liposomes plus free antibiotic (STREP or CAP) and drug-loaded liposomes at 4°C. Conditions as described in the legend to Fig. 3. Symbols: ○, untreated control (n = 12; i.r. = 0.8); □, empty neutral liposomes plus 5.0 μg of free CAP per ml (n = 3, i.r. = 0.8); ■, empty neutral liposomes plus 46.0 μg free STREP per ml (n = 3, i.r. = 0.8); ◇, neutral antibiotic-loaded liposomes at 4°C, 4.1 μg of CAP per ml (n = 4, i.r. = 0.9); ◆, neutral antibiotic-loaded liposomes at 4°C, 37.5 μg of STREP per ml (n = 4, i.r. = 0.9). Vertical bars show standard error of the mean.

If the vesicle bilayer thickness is taken as 5 nm and the area occupied by a phospholipid molecule as 0.65 nm^2, then a single 130-nm diameter neutral vesicle consisted of 1.5×10^5 EPC molecules, and 1 μmol of EPC represented some 4×10^{12} vesicles. For neutral vesicles, this weight of EPC represented a total entrapped volume of 4.16 μl (Table 1) with a concentration of 2.5 mg ml^{-1} for CAP liposomes. Each liposome thus contained 2.6×10^{-12} μg of CAP (8×10^{-21} mol).

The macrophage, if treated as a 16-μm diameter sphere, had a volume of 2.15×10^{-9} cm^3, so that for intracellular bacteriostasis of *E. coli* (MIC, 2 μg of CAP per ml), a minimum of 4.3×10^{-9} μg of CAP was required within the cell. To deliver this quantity of CAP it was calculated that the contents of approximately 1.6×10^3 liposomes were required. Similarly, the contents of about half this number of neutral STREP liposomes were required within each macrophage to attain an intracellular bactericidal concentration of STREP.

The comparable figures for anionic vesicles, which had higher entrapment efficiencies (Table 1), were of course lower.

The required number of liposomes calculated above is inversely proportional to liposome diameter, and for larger vesicles, which may well be present in the liposome preparations described here, this number is significantly reduced.

However, in the case of anionic PA liposomes

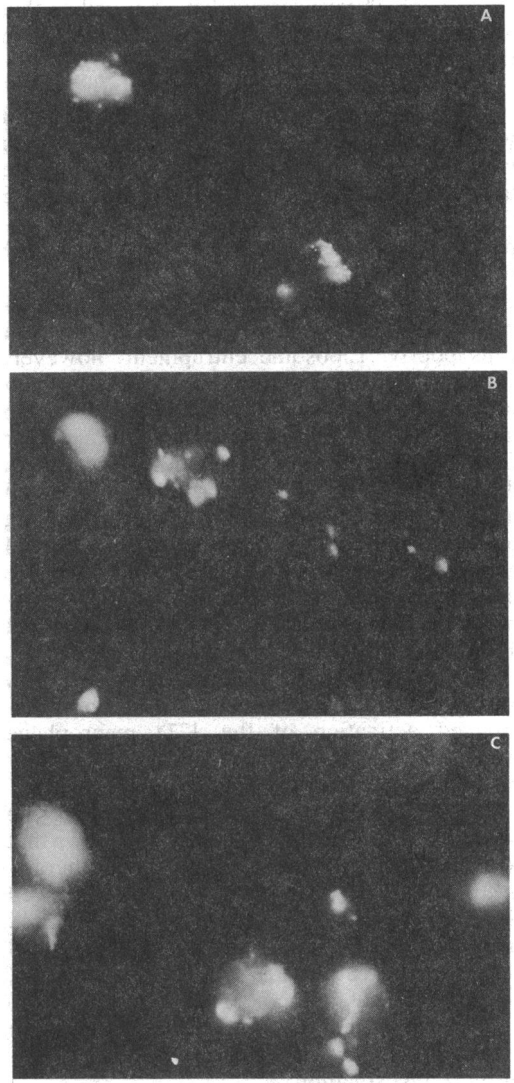

FIG. 6. UV photomicrographs of macrophage cover slip cultures at 1h (A), 2 h (B), and 3 h (C) after addition of liposomal CF. Each cover slip culture of 10^6 cells was given 1 μmol of phospholipid in anionic (PS) liposomal form, equivalent to 0.45 μmol of CF. A similar time-dependent accumulation of cell-associated fluorescence was observed by using neutral liposomes. Cells incubated with liposomal CF at 4°C, PA-containing liposomes at 37°C, or free CF (100 μmol) exhibited no fluorescence. A Zeiss-Jena Fluoval microscope was used with incident UV illumination.

it would appear that uptake by macrophages or degradation within macrophages was barely sufficient to allow attainment of an inhibitory intracellular antibiotic concentration, although the inhibition at 9 h postinfection seen with either antibiotic in PA liposomes (Fig. 3 and 4) indicated that some liposome uptake had occurred. Since each cover slip culture of 10^6 macrophages was given 1 μmol of EPC in liposomal form, there were 10^6 vesicles available per cell so that vesicle supply was an unlikely limiting factor in the present system.

The observed intracellular antibacterial activity of the free antibiotics (Fig. 1 and 2) correlated with their solubility characteristics, which would be expected to give a measure of passive uptake by cells. The comparatively lipid-soluble CAP is known to be concentrated within macrophages (11) and leucocytes (16), whereas highly water-soluble antibiotics such as penicillin G, cefazolin, cefamandole (11), dihydrostreptomycin (2), and STREP (3, 4) penetrate mammalian cells poorly. Liposome entrapment, however, still enhanced the apparent intracellular activity of CAP, which suggested that in addition to improved uptake by phagocytosis, intracellular CAP distribution was modified. Delivery of a discrete packet of antibiotic solution to the cell interior would, for example, delay partitioning of the drug into lipid-rich regions such as the cell membrane. Permeation of the cell from the interior rather than the exterior would also contribute to the enhanced activity of the liposomal antibiotic.

From the results with empty liposomes (Fig. 5), it would appear that there was no liposome-induced activation of the J774 macrophage which may have contributed to the high activity of the liposomal antibiotics.

The ability of circulating and fixed phagocytic cells to take up liposomes in vivo (15) shows that for some conditions liposome-mediated therapy is feasible. The present results, which showed that very few drug-loaded vesicles were required per phagocytic cell to achieve a pharmacological response, indicate that treatment of infections, bacterial, fungal, and protozoan, of the reticuloendothelial system in vivo is, as far as liposome carrier systems are concerned, an area worthy of more investigation.

ACKNOWLEDGMENTS

We thank S. Gordon, Sir William Dunn School of Pathology, Oxford, England, for the J774.2 cell line and the Science and Engineering Research Council, England, for financial assistance to M.S.

LITERATURE CITED

1. **Alving, C. R., E. A. Steck, W. L. Hanson, P. S. Loizeaux, W. L. Chapman, Jr., and V. B. Waits.** 1978. Improved therapy of experimental leishmaniasis by use of a liposome-encapsulated antimonial drug. Life Sci. **22**:1021–1026.
2. **Bonventre, P. F., and G. Gregoriadis.** 1978. Killing of intraphagocytic *Staphylococcus aureus* by dihydrostreptomycin entrapped within liposomes. Antimicrob. Agents Chemother. **13**:1049–1051.
3. **Brown, K. N., and A. Percival.** 1978. Penetration of antimicrobials into tissue culture cells and leucocytes. Scand. J. Infect. Dis. Suppl. **14**:251–260.
4. **Chang, Y. T.** 1969. Suppressive activity of streptomycin on the growth of *Mycobacterium lepraemurium* in macrophage cultures. Appl. Microbiol. **17**:750–754.
5. **Chowdhury, M. K. R., R. Goswami, and P. Chakbarti.** 1981. Liposome trapped penicillins in growth inhibition of some penicillin resistant bacteria, J. Appl. Microbiol. **51**:223–227.
6. **Deamer, D., and A. D. Bangham.** 1976. Large volume liposomes by an ether vaporization method. Biochim. Biophys. Acta **443**:629–634.
7. **Fidler, I. J., S. Sone, W. E. Fogler, D. Smith, D. G. Brown, L. Tarcsay, R. H. Gisler, and A. J. Schroit.** 1982. Efficacy of liposomes containing a lipophilic muramyl dipeptide derivative for activating the tumoricidal properties of alveolar macrophages in vivo. J. Biol. Resp. Modif. **1**:43–55.
8. **Fraser-Smith, E. B., D. A. Eppstein, M. A. Larsen, and T. M. Mathews.** 1983. Protective effect on a muramyl dipeptide analog encapsulated in or mixed with liposomes against *Candida albicans* infection. Infect. Immun. **39**:172–178.
9. **Giloh, H., and J. W. Sedat.** 1982. Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein conjugates by *n*-propyl gallate. Science **217**:1251–1255.
10. **Hodges, N. A., R. Mounajed, C. J. Olliff, and J. M. Padfield.** 1979. The enhancement of neomycin activity on *Escherichia coli* by entrapment in liposomes. J. Pharm. Pharmacol. **31**(Suppl.):85P.
11. **Johnson, J. D., W. Hand, J. B. Francis, N. King-Thompson, and R. W. Corwin.** 1980. Antibiotic uptake by alveolar macrophages. J. Lab. Clin. Med. **95**:429–439.
12. **Juliano, R.** 1982. Liposomes and the reticuloendothelial system: interactions of liposomes with macrophages and behaviour of liposomes *in vivo*. NATO Adv. Study Inst. Ser. Ser. A. Life Sci. **47**:285–300.
13. **Morgan, J. R., and K. E. Williams.** 1980. Preparation and properties of liposome-associated gentamicin. Antimicrob. Agents Chemother. **17**:544–548.
14. **Onaga, I. C., and A. J. Baillie.** 1980. The toxicity of liposomal chloramphenicol for *Tetrahymena pyriformis*. Int. J. Pharm. **7**:89–98.
15. **Poste, G., B. Corazon, A. Raz, P. Bugelski, R. Kirsch, and I. J. Fidler.** 1982. Analysis of the fate of systematically administered liposomes and implications for their use in drug delivery. Cancer Res. **42**:1412–1422.
16. **Prokesch, R. C., and W. L. Hand.** 1982. Antibiotic entry into human polymorphonuclear leucocytes. Antimicrob. Agents Chemother. **21**:373–380.
17. **Ralph, P., and I. Nakoinz.** 1975. Phagocytosis and cytolysis by a macrophage tumour and its cloned cell line. Nature (London) **257**:393–394.
18. **Ralph, P., J. Pritchard, and M. Cohn.** 1975. Reticulum cell sarcoma: an effector cell in antibody-dependent cell-mediated immunity. J. Immunol. **114**:898–905.
19. **Ralston, E., L. M. Hjelmeland, R. D. Klausner, J. N. Weinstein, and R. Blumenthal.** 1981. Carboxyfluorescein as a probe for liposome-cell interactions. Effect of impurities, and purification of the dye. Biochim. Biophys. Acta **649**:133–137.
20. **Scherphof, G., F. Roerdink, D. Hoekstra, J. Zbrowski, and E. Wisse.** 1980. Stability of liposomes in presence of blood constituents: consequences for uptake of liposomal lipid and entrapped compounds by rat liver cells, p. 179–209. *In* G. Gregoriadis and A. C. Allison (ed.), Liposomes in biological systems. John Wiley & Sons, Inc., New York.

21. **Stevenson, M., A. J. Baillie, and R. M. E. Richards.** 1981. Antibacterial activity of liposome entrapped chloramphenicol. J. Pharm. Pharmacol. **33**(Suppl):31P.
22. **Weinstein, J. N., R. Blumenthal, S. O. Sharrow, and P. A. Henkart.** 1978. Antibody-mediated targetting of liposomes. Binding to lymphocytes does not ensure incorporation of vesicle contents into the cells. Biochim. Biophys. Acta **509**:272–288.
23. **Weinstein, J. N., S. Yoshikami, P. Henkart, R. Blumenthal, and W. A. Hagins.** 1977. Liposome-cell interaction: transfer and intracellular release of a trapped fluorescent marker. Science **195**:489–492.