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The incorporation of lipid-soluble antineoplastic agents into microemulsions—protein-free analogues of Low Density Lipoprotein

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Summary

Low Density Lipoprotein is a potential drug carrier system in cancer chemotherapy; however, the isolation and handling of the native particle is difficult. In this paper we discuss the incorporation of lipid-soluble antineoplastic agents into microemulsions which act as readily obtainable synthetic protein-free analogues of Low Density Lipoprotein. The addition of the cytotoxic drug to the dried lipids before treatment to form the microemulsion results in the partial incorporation of the drug into the microemulsion particles. Drug incorporation is related to the properties of the cytotoxic used as etoposide would not incorporate whereas approximately 60% of added methotrexate-α-benzyl-γ-cholesteryl diester was incorporated into the microemulsions. The particles formed appear to be stable with only a slight increase in size over the time period studied, and with no leakage of the entrapped drug. The activity of the microemulsion against L1210 murine leukaemia cells has been tested in vitro. In normal media or in media containing lipoprotein-deficient serum the particles were inactive at the concentrations used. However, in serum-free media the microemulsion exhibited growth inhibitory effects that were approximately 500 times less active than free methotrexate; this difference in activity would appear to be due to a differential interaction of the cell with the particles which occurs in serum-free media.

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Introduction

It is well known that one of the major limitations of systemic cancer chemotherapy are the side-effects associated with drug toxicity on normal replicating cells. Currently much attention is being focussed on reducing these side-effects by attaching antineoplastic agents to a carrier moiety (Goldberg, 1983) and thereby modifying the pharmacokinetics of drug substances. Several carriers have been utilized (see Goldberg, 1983) each with varying degrees of success, but many problems remain to be overcome if this technique is to be of clinical value. Recently Low Density Lipoproteins (LDL) have aroused interest as a novel carrier system (Counsell and Pohland, 1982) for antineoplastic drugs, with targeting potential due to the fact that dividing cells require large quantities of cholesterol for cell membrane synthesis.

Low Density Lipoprotein is a component of normal plasma whose physiological function is the transport of cholesterol; it exists as spherical particles (22 nm diameter) consisting of an apolar lipid core containing approximately 1300 molecules of cholesteryl ester surrounded by a polar coat composed of phospholipid, protein (Apoprotein B) and cholesterol (Deckelbaum et al., 1977). LDL is taken up by cells through a receptor-dependent pathway, whereupon the particle is internalized and delivered to the lysosomes where its components undergo hydrolysis. Gal et al. (1981) have demonstrated in tissue culture that replicating neoplastic cells (e.g. epidermoid cervical carcinoma (EC 50))metabolize LDL at a rate 20 times greater than cervical fibroblasts. Welsh et al. (1982) have shown in vivo, that a solid MAC 13 tumour in NMRI mice showed a greater receptor-dependent uptake of LDL over that of normal tissue.

However, attempts to utilize this pathway for the delivery of antineoplastic agents to cancer cells have been limited. The inclusion of 'Dioleyl methotrexate' into a reconstituted LDL particle has been reported (Krieger et al., 1979), but no data on the properties or final use of these loaded LDL particles has been seen. Rudling and colleagues (1983) have demonstrated the delivery of aclarubicin to human glioma cells in vitro, using an aclarubicin–LDL complex, showing that aclarubicin accumulation was dependant on LDL receptor activity, and that uptake of the complex inhibited growth of the glioma cells.

In this work we have studied the possibilities of including lipid-soluble antineoplastic agents into microemulsions of phospholipid and cholesteryl esters, which we follow Ginsburg et al. (1982) in using as synthetic protein free models of LDL. The manufactured particles were assessed for physical and chemical stability and L1210 murine leukaemia was challenged with the preparation in vitro under a variety of conditions.

Materials and Methods

Materials

All buffer salts and other reagents were of 'Analar' grade (B.D.H.), cholesteryl oleate (B.D.H.) was purified by column chromatography on silicic acid, using

heptane (6 column vols.) and toluene (2 column vols.) as eluent; purity was assessed by TLC, egg yolk phosphatidyl choline (Type III-E, 1 g/10 ml hexane solution, Sigma Chemicals, Dorset) was used without further purification. Methotrexate was the gift of Lederle Laboratories and etoposide of Bristol Laboratories. Methotrexate-α-benzyl-γ-cholesteryl diester ('methotrexate diester') was synthesized by a method analogous to that used by Rosowsky et al. (1981) and will be reported fully elsewhere (Halbert, Ph.D. Thesis, University of Strathclyde). Phosphate-buffered saline consisted of NaCl 137 mM, KCl 3 mM, Na_2HPO_4 8 mM, KH_2PO_4 1.5 mM, pH 7.4.

Photon correlation spectroscopy

A photon correlation spectrometer (Malvern Instruments, Model 4300) with 48 channels and later a Type 7027 (Malvern Instruments) with 60 channels were used in conjunction with a He/Cd laser (Linconix) operating at 441.6 nm with a power output of approximately 10 mW. All samples were measured after temperature equilibration at 25 ± 0.1°C and at an angle of 90° to the incident beam.

Analysis of PCS data

For a monodisperse system of small particles, the experimental data obtained is the second-order autocorrelation function $g^{(2)}(t)$ and it is related to the diffusion coefficient (D) by the relationship:

$$g^{(2)}(t) = 1 + e^{-2DK^2 \cdot t}$$

where $K = (4\pi n/\lambda)\ \sin(\theta/2)$, n is the refractive index of the solvent, λ the wavelength of the laser, θ the measurement angle and t is the delay time. Thus $[g^{(2)}(t) - 1]$ is an exponential curve and $\ln[g^{(2)}(t) - 1]$ is a straight line with a slope of $(-2DK^2)$ from which D is readily obtained. In polydisperse systems a family of exponentials will be obtained and plots of $\ln[g^{(2)} - 1]$ vs t are fitted to quadratic functions which provide the coefficient of t which is equal to the Z average diffusion coefficient (Koppel, 1972). The Z average diffusion coefficient is defined as $D_z = \Sigma n_i M_i^2 D_i / \Sigma n_i M_i^2$, n_i being the number of particles of molecular mass M_i with diffusion coefficient D_i, and from this was calculated the equivalent spherical hydrodynamic radius, assuming the applicability of the Stokes-Einstein equation. The normalized coefficient of t^2 gives the width of distribution expressed as the normalized variance of distribution, which is defined as $2K_2/K_1^2$, where K_1 is the coefficient of t and K_2 is the coefficient of t^2; values of NVD > 0.1 indicate an almost monodisperse system (Pusey, 1974), although this is an arbitrary demarcation.

Preparation of microemulsions

Microemulsions were prepared using a molar ratio of egg phosphatidyl choline–cholesteryl oleate of 2 : 1, by a method similar to that of Ginsberg et al. (1982). The appropriate quantities of phospholipid and cholesteryl oleate were shaken until the latter dissolved in the hexane. The solvent was removed under a

stream of N_2 and the residue vacuum-desiccated at 0–4°C overnight. The dried lipids were resuspended in buffer (0.1 M KCl, 0.01 M Tris, pH 8.0) to 1% w/v and the cloudy suspension sonicated under a N_2 atmosphere at a temperature of 53°C for 5 h in an MSE Soniprep Ultrasonic Disintegrator fitted with 9.5 mm probe. After sonication the material was filtered (1 μm filter) then centrifuged at 195,000 *g* for 30 min in an MSE Superspeed 75 centrifuge using an 8 × 14 ml titanium angle rotor at 4°C. All centrifugations were performed without braking. The upper 10% of the solution, containing particles which float at the background density of the buffer, was removed (Fraction S1). The remaining infranatant was adjusted to a density of 1.22 g/ml with solid KBr, and recentrifuged at 195,000 *g* for 2 h at 4°C. The top 20% of the tube volume including the gelatious lipid layer (Fraction S2) was removed and resuspended; slight sonication was occasionally required. This was then passed down a gel filtration column (Biogel P100, 2.2 × 12 cm) eluted with PBS. The microemulsion was obtained in (5 ml) fractions 3, 4 and 5 and concentrated by ultrafiltration (Diaflow Type XM 100A, Amicon) to the desired volume, filtered (0.22 μm) and packed aseptically under N_2. The material was stored at 4°C.

Microemulsions containing etoposide

The molar ratio of phosphatidylcholine to cholesteryl oleate was maintained at 2 : 1. A quantity of etoposide (approximately 2% with reference to the total weight of lipids) was added before the removal of the solvent. The dried lipids were then treated as stated in the method for the production of 'empty' microemulsions.

Microemulsions containing methotrexate diester

These were formed from a 2 : 1 molar lipid ratio; the methotrexate diester was added and an equal weight of cholesteryl oleate omitted; extra *n*-hexane was added to aid the dissolution of the methotrexate diester before removal of the solvent by N_2. After the first centrifugation no S1 fraction was removed but the remaining manipulations were as before.

Chemical analysis of microemulsions

The etoposide content of the microemulsion was determined by HPLC using as internal standard, *p*-dimethylaminobenzaldehyde, using a Bondepak C18 (0.45 × 25 cm) column; mobile phase = MeOH–H_2O (60 : 40) containing 1% v/v glacial acetic acid; flow rate = 1 ml/min and detector at 254 nm.

The methotrexate diester content of microemulsions was determined by UV spectroscopy. A 100 μl sample of microemulsions was diluted to 5 ml with acetonitrile, the mixture shaken and the precipitated residue removed by centrifugation (4000 rpm, 15 min). The absorbance of the supernatant was measured at 303 nm. Methotrexate diester standards were prepared in acetonitrile.

Phosphatidyl choline content

A 0.5 ml sample of microemulsion was dialyzed (Visking Tubing 18/32) against distilled water (2 × 4 litres) at 4°C. The bag contents were quantitatively removed and made to 10 ml with distilled water; 1 ml of this was digested with 1 ml of 60%

w/v perchloric acid for 1 h, heating until just boiling. Extra perchloric acid was added if necessary to prevent the material from drying out. The digested sample was made to a volume of 10 ml with distilled water, 2 ml transferred to a 10 ml volumetric flask, 0.5 ml 60% w/v perchloric acid, 1 ml 0.1% w/v ascorbic acid and 1 ml 5% w/v ammonium molybdate were added, the mixture made up to 10 ml with distilled water and after 10 min the absorbance at 700 nm measured.

Cholesteryl oleate content

A 100 μl sample of microemulsion was added to 5 ml of 0.05% w/v $FeCl_3 \cdot 6H_2O$ in purified glacial acetic acid, and 3 ml concentrated H_2SO_4 added. Absorbance measured at 560 nm after 30 min (Henly, 1957).

Physical analysis of microemulsions

Assessment of physical stability of microemulsions

The microemulsion was diluted with PBS to a level suitable for PCS measurement, filtered (0.22 μm) then incubated in a water bath at the desired temperature. In each experiment 10 measurements were taken daily of the size and polydispersity of the microemulsion particles and the result expressed as the mean.

Release of MTX from microemulsions containing methotrexate diester

An aliquot of the microemulsion was placed in a dialysis bag (Visking Tubing 18/32), 2 ml of PBS added, and dialyzed against PBS (50 ml) at 37 °C in a shaking water bath. Samples were removed daily from the exterior phase and analyzed for methotrexate content by HPLC. At the end of the experiment the dialysis bag contents were collected and analyzed for the diester by the method stated above. The samples were analyzed for MTX directly by HPLC using a Spherisorb ODS 5 μm (0.5 × 25 cm). Buffer = 0.1 M Tris : 0.1 M NaH_2PO_4 (50 : 50); mobile phase = buffer : MeOH (80 : 20); flow rate = 1 ml/min; injection loop = 100 μl; detector = 303 nm; range = 0.005 AUFS.

Tissue culture

L1210 cells were purchased from Flow Laboratories, Irvine, Scotland and maintained in medium (RPMI 1640) supplemented with 10% v/v foetal calf serum, and containing penicillin 100 IU/ml, streptomycin 100 μg/ml and 20 mM Hepes (Normal Media). Lipoprotein deficient medium contained 10% v/v lipoprotein-deficient serum instead of normal foetal calf serum, foetal calf serum-free medium consisted of normal medium without the addition of serum. Cells were grown normally in Nunclon flasks and experiments performed in Linbro (24 well) multiwell plates, 5 wells being used at each concentration. Experiments were carried out by harvesting cells in the exponential growth phase from normal media, pre-incubating at 37 °C in the respective experimental media for 24 h, collecting the cells then adjusting to the starting cell concentration (2.5×10^5 cells/ml) adding the material under test (in 0.1–0.2 ml PBS) and plating out into the Multiwell dish. In the case of the foetal calf serum-free experiments after pre-incubation for 24 h the cells were incubated at 37 °C with the material under test for 1 h; foetal calf serum was then added to 10% v/v and then the culture plated out. The incubation cell concentration

was arranged so that in addition of the foetal calf serum a cell concentration of 2.5×10^5/ml was obtained. For counting, two wells were sampled from each experiment every 24 h and the cells counted after dilution with PBS in a Coulter Counter (model Z_B, 100 μm counting tube), 4 counts were taken on each well and the result expressed as the average of the 8 counts.

Lipoprotein-deficient serum

Freshly thawed foetal calf serum was adjusted to a density of 1.2 $g \cdot ml^{-1}$ with solid KBr, and the lipoproteins floated by centrifugation at 40,000 rpm in a Beckman Ultracentrifuge for 7 h. The upper 10% of each tube was removed and the remainder dialyzed against buffer (17.3 mM Tris, 130 mM NaCl, 3.6 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, pH 7.6) at 4°C, then filtered (0.45 μm), assayed for protein content, sterilized by filtration (0.22 μm) and packed aseptically and stored at −20°C until use (Remsen and Shireman, 1981). The serum had approximately 30 $mg \cdot ml^{-1}$ protein content.

Results

Empty microemulsions

Data pertaining to the microemulsions is presented in Table 1. Less than half of the total lipid is isolated in Fraction S2; the cholesteryl ester/phosphatidyl choline molar ratio is approximately half that obtained by Ginsburg et al. (1982). The

TABLE 1

SIZE AND COMPOSITION OF EGG YOLK PHOSPHATIDYL CHOLINE, CHOLESTERYL OLEATE MICROEMULSION PARTICLES (FRACTION S2)

		Data from Ginsberg et al. (1982) after 120 min sonication
Egg yolk phosphatidyl choline percentage recovery	50 [a]	90
Cholesteryl oleate percentage recovery	36 [a]	90
Cholesteryl oleate/egg yolk phosphatidyl choline molar ratio	0.38 [a]	0.77
Surface area/volume ratio [b]	0.038	0.031
Estimated radius (nm) from (c)	8.0	9.6
Measured radius (nm)	21.8 ± 1.8 NVD 0.11 [d]	11.2 ± 1.0 [e]

[a] Mean, n = 2.

[b] Surface area of phospholipid head group (Ginsberg) (Σ $\bar{v}$ phospholipid + $\Sigma\bar{v}$ cholesteryl ester). Volumes of $\bar{v}$ from Rund and Luzzeti (1968).

[c] Surface area/volume for a sphere = $4\pi r^2/(4\pi r^3)/3 = 3/r$.

[d] PCS measurement, mean ± S.D., n = 3.

[e] Electron mircoscopy size (Ginsberg), mean ± S.D.

TABLE 2

INCORPORATION OF LIPID-SOLUBLE CYTOTOXIC AGENTS INTO EGG YOLK PHOSPHOLIPID CHOLINE, CHOLESTERYL MICROEMULSIONS

Cytotoxic	Quantity added [a]	Percentage recovery in S2 [b]	Measured PCS radius (nm), NVD
VP 16 (etoposide)	2	0	27.3, 0.2
VP 16 (etoposide)	2	0.12	30.8, 0.28
Methotrexate diester [c]	2	12 [d]	20.2, 0.14
Methotrexate diester [c]	4	60	18.4, 0.12
Methotrexate diester [c]	4	61	17.6, 0.17
Methotrexate diester [c]	10 [e]	38	30.1, 0.22

[a] Expressed as a percentage weight of cytotoxic compound to total weight dried lipid before sonication.
[b] Remaining material isolated in S1, infranatant S2, and column eluate.
[c] Methotrexate-α-benzyl-γ-cholesteryl diester.
[d] Remaining material isolated in infranatant S2, and as precipitated (pelleted) material after first centrifugation.
[e] Fraction S1 formed during first centrifugation.

particle radius derived from the surface area-to-volume ratio is similar to Ginsburg's, but the radius measured by PCS is about double the size of the previously reported microemulsion particles.

The physical stability of the microemulsion is presented in Fig. 1A in which the radius (PCS) is plotted with respect to time. At 4°C there is no increase in size after 14 days. At higher temperatures a gradual size increase occurs, amounting to approximately a 20% increase in radius over the whole time period. A concomitant increase in the measured NVD of the samples indicated an increased degree of polydispersity.

When tested against L1210 cells the microemulsions produce a minimal effect on cell growth in media containing normal or lipoprotein-deficient serum. At high concentrations an inhibitory effect on cells preincubated in media without foetal calf

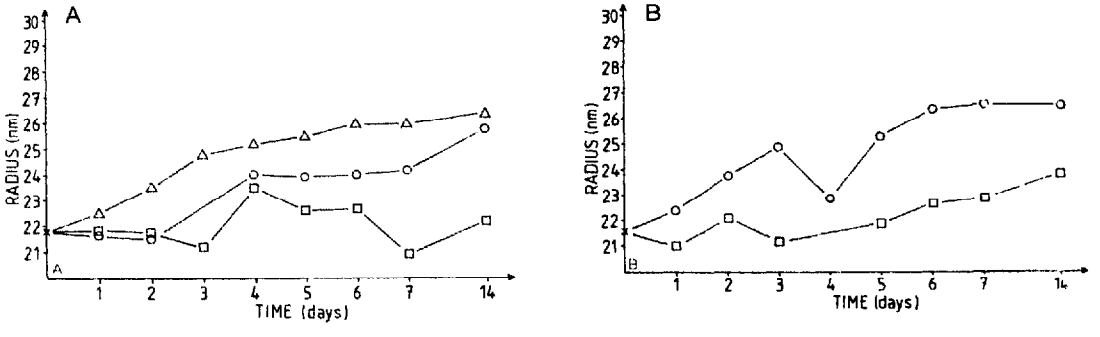

Fig. 1. A: Physical stability of microemulsions. □, 4°C, mean n = 3; △, 25°C, n = 1; ○, 37°C, n = 2. B: Physical stability of microemulsions containing methotrexate diester. □, 4°C, mean n = 4; ○, 37°C, mean n = 4.

serum is exhibited, and at lower concentrations the system appears to enhance cell growth under these conditions.

Attempts to incorporate useful amounts of etoposide into the microemulsions (Table 2) failed, but the observed particle size increased and the systems exhibited a greater degree of polydispersity.

Incorporation of methotrexate diester into emulsions

The addition of methotrexate diester to the dried lipids results in the partial inclusion of the material into the S2 fraction (Table 2) of the sonicated lipid. The drug also alters the sonication process so that the S1 fraction was only isolated in one experiment (see (e) in Table 2), and its inclusion to a certain concentration must therefore increase the density of the particles to a value greater than that of the buffer ($\rho = 1.006$). The measured size of the particles is smaller (in all but one case) than that of the native microemulsions, but the NVD values are higher. The quantity of diester incorporated into S2 varies according to the amount added, and there appears to be a maximum quantity that can be incorporated as addition of large amounts of methotrexate diester upsets the measured size and also causes an S1 fraction to separate out.

The physical stability of the microemulsions containing the methotrexate diester is presented in Fig. 1B. The microemulsions grow in size at 4°C over 14 days by about 10%. The increase in radius after 14 days at 37°C is approximately 23%, and the system apparently has reached some form of equilibrium at this stage.

No methotrexate is released from the microemulsions after 7 days at 37°C; 97% ($\pm$ 3.8, n = 3) of the added methotrexate diester can be recovered intact, confirming the minimal methotrexate release.

Inhibition of L1210 growth

Note that the concentrations referred to in the experiments with microemulsions containing methotrexate diester refer to the equivalent concentration of methotre-

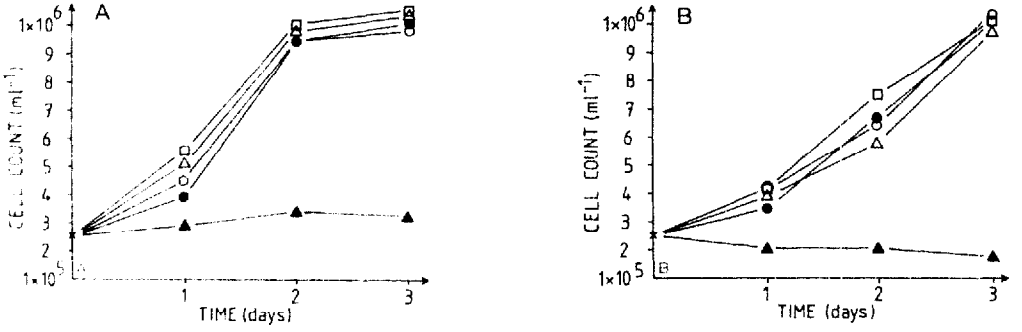

Fig. 2. A: inhibition of L1210 growth in normal media. □, control, mean n = 5; ▲, MTX 10^{-7} M, n = 5; △, MTX 10^{-8} M, n = 5; ●, microemulsion containing methotrexate diester 10^{-5} M, n = 3; ○, empty microemulsion at equivalent lipid concentration, n = 2. B: inhibition of L1210 growth in lipoprotein-deficient media. □, Control, mean n = 2; ▲, MTX 10^{-7} M, n = 2; △, MTX 10^{-8} M, n = 2; ●, microemulsion containing methotrexate diester 10^{-5} M, n = 1; ○, empty microemulsion at equivalent lipid concentration, n = 1.

xate present, and in the empty microemulsions refer to the use of the microemulsion at an equivalent concentration of egg phosphatidyl choline to that present in the methotrexate diester microemulsions at the relevant concentrations. The effect of microemulsions on the in vitro growth of L1210 murine leukaemia was studied in 3 different types of media.

In normal media 10^{-7} M methotrexate completely inhibits L1210 cell growth while at a concentration of 10^{-8} M methotrexate is ineffective (Fig. 2A). The addition of the methotrexate diester as a microemulsion at a concentration of 10^{-5} M shows only a slight effect on cell growth; the empty microemulsion at 10^{-5} M also exhibits a very slight effect on cell growth. Lower concentrations of the methotrexate diester in microemulsions have no discernable effect on cell growth (data not shown).

Lipoprotein-deficient media (Fig. 2B)

With the cells grown in medium containing 10% v/v lipoprotein-deficient serum, methotrexate at 10^{-7} M again completely inhibits cell growth, while at 10^{-8} M it now exhibits some degree of growth inhibition. Methotrexate diester in the microemulsion at a concentration of 10^{-5} M, also shows activity but less than that of methotrexate at 10^{-8} M. Similarly the empty microemulsions exhibit a slight growth inhibition at equivalent concentrations.

Foetal calf serum-free media

The results of these experiments are presented in Fig. 3A and B, which demonstrates that methotrexate diester microemulsions under these conditions inhibit cell growth at concentrations of 10^{-5} M, 10^{-6} M and 10^{-7} M. Empty microemulsions at a concentration of 10^{-5} M inhibit cell growth, but at lower concentrations (10^{-6} M and 10^{-7} M) enhance growth above that of control levels. This is represented in Fig.

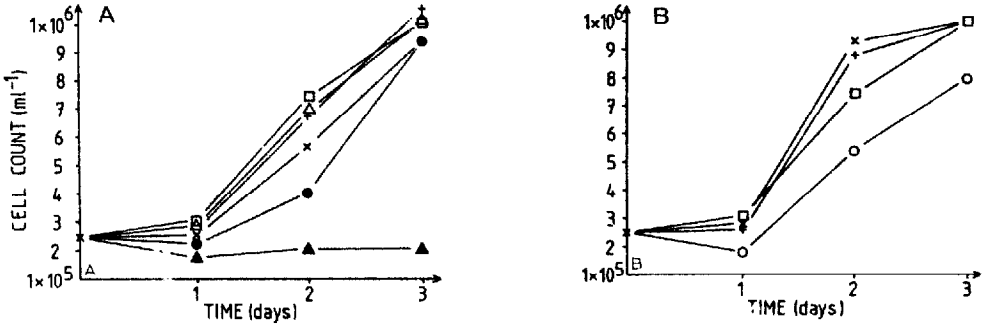

Fig. 3. A: inhibition of L1210 growth. Foetal calf serum-free media. □, control, mean n = 5; ▲, MTX 10^{-7} M, n = 5; △, MTX 10^{-8} M, n = 5; ●, microemulsion containing methotrexate diester 10^{-5} M, n = 3; ×, microemulsion 10^{-6} M, n = 3; + Microemulsion 10^{-7} M, n = 3. B: inhibition of L1210 growth. Foetal calf serum-free media. □, control, mean n = 5; ○, empty microemulsion at equivalent lipid concentration 10^{-5} M, n = 2; ×, empty microemulsion 10^{-6} M, n = 2; + empty microemulsion, n = 2.

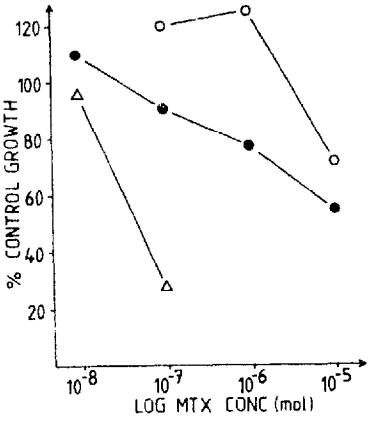

Fig. 4. Percentage inhibition of cell growth by microemulsions, in foetal calf serum-free media. (Control growth at 48 h = 100%.) △, free MTX; ●, microemulsion containing methotrexate diester; ○, empty microemulsion.

4 which illustrates that inclusion of MTX diester renders the microemulsion particles toxic to the L1210 cells under these conditions.

Discussion

The microemulsions formed in this study appear on initial examination to be different from those produced by Ginsberg et al. (1982), when the values for the S2 fractions are compared (Table 1). However, the original paper presents data obtained on this fraction at various times throughout the sonication period up to 300 min, and after a period of 80 min a microemulsion is produced with a cholesteryl ester/phospholipid molar ratio of approximately 0.4. The column chromatography elution profile of this microemulsion shows that it consists of a wide variety of particle sizes of differing cholesteryl ester/phospholipid ratios, especially when compared against the fraction obtained after 300 min sonication. This latter fraction elutes as a symmetrical peak which exhibits very little change in its cholesteryl ester/phospholipid molar ratio. It also contains over 90% of the lipid whereas fractions produced with a shorter sonication time contain less lipid. The measured radius of the particles produced in this study is approximately twice that found by Ginsberg et al., a discrepancy which may in part be contributed to by factors inherent in PCS measurement. The radius is calculated from the $\bar{Z}$ average diffusion coefficient which is weighted to larger particles, a fact which must come into play since the measured NVD indicates that the microemulsions are polydisperse. Also cholesteryl ester rich microemulsions formed by an injection technique had a medium diameter of 47 nm but did possess particles with a diameter as large as 90–100 nm which made up about 3% of the total number of particles (Via et al., 1982). Also the measurement is of the equivalent hydrodynamic radius which takes into account water of hydration, a factor which will lead to an increased size measurement. It would appear that the microemulsions discussed in this paper are

intermediate with respect to those obtained by the previous investigators, and this probably reflects upon a difference in the mode of manufacture.

The incorporation of lipid soluble cytotoxic agent into the microemulsion particles during their formation has been demonstrated; the failure to incorporate etoposide was disappointing. Etoposide is a podophyllotoxin derivative that has suffered solubility problems throughout its development (Canetta et al., 1982). The results, however, suggest that although etoposide may be incorporated partially into the microemulsion its water solubility (0.1–0.25 $mg \cdot ml^{-1}$) allows it to escape from the particle during handling and isolation leaving only trace quantities. Methotrexate diester is incorporated into the microemulsion particles more readily and in a reproducible manner to produce particles of a smaller size than the native microemulsion. The quantity of methotrexate diester incorporated varies with the quantity added, but above a certain limit the diester has the effect of greatly increasing the size of the particles. This is important since it will set a value on the maximum number of molecules of diester that can be accommodated without increasing particle size; this factor may limit the ability of this system to deliver sufficient quantities of cytotoxic agent to neoplastic cells.

The microemulsions appear to be physically stable at low temperatures with only small increases over the time period studied; the inclusion of methotrexate diester causes the microemulsion to gradually increase in size at 4°C which appears to be due to aggregation or coalescence and not increases in the individual particle sizes (Tucker and Florence, 1983). The differences in stability of the systems in conjunction with the non-separation of Fraction S1 in some of the experiments leads us to deduce that the methotrexate diester may increase the density and alter the internal structure of the particles. Further experiments would be required to determine the extent and exact nature of this interaction.

During incubation at 37°C no leakage of methotrexate from the microemulsion particles was detected, although the assay method employed would not detect the presence of methotrexate diesters or either of the mono-esters. However, most of the methotrexate diester was recovered at the end of the experiment, and the combination of these two results would indicate that the methotrexate diester microemulsion is chemically stable under these conditions. When tested against L1210 the methotrexate diester microemulsions exhibit no activity in normal media or in media containing lipoprotein-deficient serum; they do, however, display activity in the experiments conducted in the absence of foetal calf serum. These results would appear to be related to the various factors which could control the activity of the loaded microemulsion particles, for example, the dihydrofolate reductase inhibitory capacity of the methotrexate diester, the interaction of the microemulsion particles with the L1210 cells, and coupled with this the effect of growing cells in media without foetal calf serum.

Other investigators have synthesized *n*-dialkyl esters of methotrexate and have found that the di-*n*-octyl derivative was 13 times less active against dihydrofolate reductase than free methotrexate (Johns et al., 1973). The inhibitory activity of the ester was decreased as the alkyl chain-length increased, so that it is reasonable to assume that the cholesterol ester (C_{27}) will itself have a very weak dihydrofolate

reductase inhibitory capacity. In order to express growth inhibition it is probable that the ester must be hydrolyzed. Normal ester hydrolysis can be ruled out in view of the stability data presented in this paper, and although enzymatic hydrolysis is possible especially in the presence of serum esterases (Rosowsky et al., 1978), it would not explain the activity occurring only when the cells are incubated with the microemulsion in the absence of foetal calf serum.

It would appear therefore that the differences observed in the 3 types of media can be ascribed to varying degrees of interaction between the cell and the microemulsion particles. Normal cellular uptake of LDL is known to proceed via a high affinity receptor-dependant pathway that involves Apoprotein B which is situated on the surface of the LDL particles (Mahley and Innerarity, 1983). The microemulsion particles produced in this study have no surface protein and therefore any interaction with the cell is liable to be of a non-specific nature. Increasing the lipoprotein receptor activity by incubating the cells in lipoprotein-deficient serum may not necessarily increase the degree of interaction between the cells and the microemulsion particles. This would account for the similarity between the results in normal media and those in media containing lipoprotein-deficient serum. Consequently the inhibition seen in the foetal calf serum-free experiment must result from an increased interaction between the L1210 cells and the microemulsion particles over that occurring in experiments in the presence of serum. The interaction of lipid vesicles with L1210 cells has been studied (Szoka et al., 1979) in RPMI 1640 medium without serum. After incubation for 1 h only 0.42% of the lipid had become associated with the cells. In the conditions of this study using a 10-fold higher concentration of lipid, and cells that have been pre-incubated for 24 h in serum-free media, it is likely that the lipid interaction will be greater than this. It is possible that the presence of serum protein in media reduces the extent of the non-specific interaction by binding with the particles and coating the cell surface.

Based on the measured radius of the particles, and the measured phosphatidyl choline content of the microemulsions, it is possible to calculate the number of particles present in the sample by assuming that the area occupied by a phospholipid molecule in the particle surface is 0.65 nm^2 (Ginsberg et al., 1982). The number of particles per L1210 cell in the tissue culture experiments can then be calculated and at 10^{-5} M MTX concentration is equal to $8.76 \pm 3.76 \times 10^7$ (mean $\pm$ S.D., n = 5) particles per cell. If the L1210 cell is treated as a sphere of 10 μm diameter it would have a surface area of 3.14×10^8 nm^2. Using the calculated number of particles per cell in each experiment (at 10^{-5} M MTX) and assuming that these can pack onto the cell surface (without deformation) as discs of an equivalent radius, the cell coverage can be calculated. In this study the average cell coverage amounts to 360 ± 65 (mean $\pm$ S.D., n = 5) times the cell surface area at 10^{-5} M MTX. When the degree of lipid interaction previously quoted is taken into account it would mean a cell coverage of 1.5 times the cell surface area, or the interaction of 3.68×10^5 particles per cell.

The calculations presented above demonstrate that there is a large number of particles available per cell at the highest concentration and even allowing for the small interaction occurring in the absence of serum, it is enough to completely cover

the cell. The results presented in Figs. 3B and 4 show that at the highest concentration the empty microemulsions are themselves cytotoxic, a factor which is likely to be due to the number of particles interacting per cell causing the cells to be gorged with the lipid. At lower concentrations the empty microemulsions enhance cell growth over that of the control, an effect due to the microemulsion acting as a source of lipid without overwhelming cell metabolism? The methotrexate diester microemulsions exhibit a greater cytotoxic effect than the empty microemulsions at all concentrations tested (Fig. 4), a result indicative of a cytotoxic effect due to the drug after interaction of the microemulsion particles with the cell. This cytotoxicity could be ascribed to inhibition of dihydrofolate reductase by the ester after uptake and intracellular hydrolysis or due to a cytotoxic effect of the ester itself either on dihydrofolate reductase (unlikely in view of previous explanation) or on some other biochemical pathway. The latter explanation is supported by the ability of the di-butyl ester of MTX to block thymidine incorporation into the DNA of L1210 cells in serum-free media (Curt et al., 1976). The results presented in this study, however, do not provide enough unambiguous evidence on this point.

We have shown that it is possible to include antineoplastic agents into protein-free models of LDL to produce particles that are stable both physically and chemically. The loaded microemulsions also possess a degree of cytotoxicity when tested in vitro under certain conditions. The results indicate that the microemulsion system would be useful in assessing the suitability of lipid soluble cytotoxic agents for incorporation into LDL without resorting to the native material.

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