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The targeting of immunoliposomes to tumour cells (A431) and the effects of encapsulated methotrexate

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Immunoliposomes have been prepared from lipid mixtures of dipalmitoylphosphatidylcholine, wheat germ phosphatidylinositol and a reactive lipid (the *m*-maleimidobenzoyl-*N*-hydroxysuccinimide derivative of dipalmitoylphosphatidylethanolamine) which was conjugated to the *N*-succinimidyl-*S*-acetylthioacetate (SATA) derivative of a monoclonal antibody (H17E2) raised to human placental alkaline phosphatase (PLAP). The immunoliposomes were prepared by the extrusion technique (VETs) and by reverse phase evaporation (REVs) and were found to effectively target to immobilised PLAP and to PLAP or PLAP-like enzyme on the surface of a tumour cell line (A431) using an ELISA and autoradiography. The extent of binding to immobilised PLAP was a function of immunoliposomal lipid concentration, the weight-average number of antibody molecules per liposome ($\bar{P}_w$) and the liposome size. The effectiveness of methotrexate-loaded immunoliposomes in inhibiting the proliferation of A431 cells was investigated relative to equivalent levels of the free drug. The immunoliposomes prepared by the extrusion technique (VETs) inhibited growth of A431 cells but had no effect on the growth of a normal human fibroblastic cell line. Immunoliposomes prepared by reverse phase evaporation (REVs) were less effective in inhibiting A431 cell proliferation. The immunoliposomes probably enter the tumour cells largely by receptor-mediated endocytosis although other mechanisms of uptake cannot be excluded.

Introduction

The concept of targeting liposomes for drug delivery by attaching antibodies to their surfaces is now well established [1,2]. In principle for targeting to neoplastic human tissue it is desirable to use highly specific (monoclonal) antibodies however, this requirement must be balanced against the availability of antibody in sufficient quantity to carry out the necessary chemistry for covalent linking to the liposomal surface and to produce a sufficient quantity of immunoliposomes not only to study their value as drug delivery systems but also to characterise them by biochemical and physical methods. One antibody which can be obtained is relatively large quantities is the antibody (H17E2) raised against purified placental alkaline phosphatase (PLAP) [3]. There has been considerable interest in PLAP as a tumour marker arising from its presence in serum and tumour tissue extracts from a wide range of neoplasms. The work of Hamilton-Dutoit et al. [3], however, has confirmed that while PLAP and PLAP-like enzymes can be exploited as highly sensitive immunohistological markers for malignant germ cell tumours and carcinomas of the gastrointestinal tract the enzymes are present in several normal tissues including thymus, neonatal testis, endocervix and Fallopian tube. In this study we have investigated for the first time the use of the antibody H17E2 for targeting of liposomes and the delivery of the drug methotrexate to a tumour cell line. Although antibody H17E2 is a broad specificity monoclonal and in this respect falls short of a perfect antibody (if such exist) for targeting liposomes, the occurrence of PLAP and PLAP-like enzymes in tumour tissues together with the availability of H17E2 in sufficient quantity to carry out the necessary preparative and characterisation work makes it appropriate for a model study of targeting to tumour cells.

The target cell in this study is the human epidermoid carcinoma cell line A431 [4] which is believed to secrete and have PLAP bound to its plasma membrane surface [5–8]. Thus for A431 cells PLAP constitutes a potential target site for the study of the targeting of

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immunoliposomes with surface bound H17E2 antibody and the effects of antiproliferative drugs such as methotrexate carried by such immunoliposomes. Methotrexate is used in the treatment of both benign and malignant proliferative disorders [9] and immunoliposomes have received attention as potential carrier systems for methotrexate [10–12] and other cytoxic drugs such as daunomycin and adriamycin [13–15].

Here immunoliposomes have been prepared from phospholipid mixtures of phosphatidylcholines and phosphatidylinositol incorporating the *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) derivative of phosphatidylethanolamine which was used to conjugate the *N*-succinimidyl-*S*-acetylthioacetate (SATA) derivative of a monoclonal antibody (H17E2) raised to PLAP. The resulting immunoliposomes were characterised in terms of the numbers of surface-bound H17E2 antibody molecules. Permeability studies have shown that these immunoliposomes retain a permeability barrier in the presence of foetal calf serum [16]. The targeting of the immunoliposomes and their use as carriers of methotrexate to the tumour cell line A431 have been investigated. A preliminary abstract of some of this work has been published [17].

Materials and Methods

The materials used in the preparation of the immunoliposomes were very similar to those used in the preparation of lectin-bearing liposomes [19–21] and have been previously described [16]. The following materials were used for cell culture and autoradiography. Dulbecco's phosphate-buffered saline without calcium, magnesium and sodium carbonate (Cat. No. 041-04190), RPMI 1640 culture medium with L-glutamine (Cat. No. 041-01875H), penicillin-streptomycin solution (10000 units/ml penicillin, 10000 μg/ml streptomycin (Cat. No. 043-01360), trypsin (10 ×) 2.5% (Cat. No. 043-05090) and DMEM (Cat. No. 041-01966M) were obtained from Gibbs BRL, Life Technologies, UK. Foetal calf serum was from Advanced Protein Products, West Midlands, UK. Eosin (product No. 34197), Mayer's haemotoxylin (product No. 26110), colour index (ci) 75290, xam (product No. 36119) and xylene (product No. 36071) were obtained from BDH, Poole, Dorset, UK. Bioclone anti-A was obtained from Ortho Diagnostic Systems, New Jersey. K-5 gel emulsion was from Ilford Scientific Products, UK. Developer D19 was from Kodak, UK. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (product No. M2128), 3,3′,5,5′-tetramethylbenzidine (product No. T 2885), Thimerosal (product No. T512S) and Tween 20 (product No. P1379) were obtained from Sigma, Poole, Dorset, UK. ELISA plates (product No. 1 67008), cryotubes and 8 chamber incubation slides were from NUNC, Denmark. Sterile centrifuge tubes and tissue culture flasks (25 ml, product No. 3056 and 75 ml, product No. 3375) were from Costar, USA.

An initial culture of A431 cells and the antibody (H17E2) raised to PLAP were kindly donated by Mr. S. Beggs, Unilever Research, Colworth Laboratory, Bedford, UK. The antibody (an IgG with G1 heavy and κ light chains) was secreted from hybridoma cells cultured in hollow fibres and purified using a Protein A column. Human fibroblasts were obtained from a tissue biopsy on Dr. Karl Kadler of our department. Placental alkaline phosphatase (product No. 058A 0750) was from Calzyme Laboratories, University of Birmingham Research Park, Birmingham, UK. Horseradish peroxidase labelled mouse anti-IgG immunoconjugate (product No. P161) was from Dako, High Wycombe, Bucks, UK. All other reagents and solvents were of analytical grade and aqueous solutions were made up in double-distilled water.

Preparation of reactive lipid (DPPEMBS) and the SATA derivative of antibody H17E2

The MBS derivative of DPPE (DPPEMBS) was prepared as previously described [18,20].

The absence of contaminating residual DPPE was confirmed by thin layer chromatography. The SATA derivative of antibody H17E2 was prepared using the method of Duncan et al. [21]. SATA (2.5 μl of concentration 26 μmol in 200 μl dimethylformamide (DMF)) was added to the antibody (29 nmol in 1 ml of 40 mM phosphate buffer (pH 7.2) plus 0.01% w/v azide) and the resulting solution (SATA to antibody molar ratio 11.2) allowed to react at room temperature. The reaction was complete after 20 min, after which unreacted SATA was separated from derivatised antibody by gel filtration using a Sephadex G50 column (15 × 2 cm) pre-equilibrated with phosphate buffer (40 mM plus 0.01% w/v azide (pH 7.2)). Fractions (2.2 ml) were collected and scanned for protein content spectrophotometrically at 280 nm. The peak fractions were assayed for protein using a modification of the method of Lowry et al. [22] using the antibody (0–160 μg/100 μl) as the calibration standard.

The degree of derivatisation of the antibody was determined by deacetylation of bound SATA using 100 μl of hydroxylamine solution (40 mM NH_2OH plus 2 mM EDTA plus solid Na_2HPO_4 to pH 7.5) for each 1 ml of antibody solution for a period of 1 h. The sulphydryl content of the antibody was then assayed using DTNB by the Ellman method [23]. At the SATA to antibody molar ratio of 11.2 routinely used, 2.3 ± 0.4 SH groups per antibody were introduced.

Preparation of immunoliposomes

DPPC (18 mg), PI (2 mg), variable amounts of DPPEMBS (0–4 mg) and 200 μl [^{3}H]DPPC (2 μCi/ml) were dissolved in chloroform (10 ml) and methanol (2.5

ml) in a 500 ml round-bottomed flask. The organic solvent was removed by rotary evaporation at 60°C to yield a thin lipid film which was purged with nitrogen. The film was dispersed in 2 ml of nitrogen saturated phosphate-buffered saline (PBS, pH 7.2) at 60°C and shaken to form multilamellar vesicles (MLVs) which had a weight-average diameter of approx. 1000 nm. The MLVs were extruded through 0.1 μm pore size polycarbonate filters (Poretic) 10 × to produce VETs using a Lipex Biomembranes Extruder (Vancouver). The size of the MLVs and the resulting VETs was determined by photon correlation spectroscopy (PCS) using a Malvern autosizer model RR146.

The resulting VETs (2 ml) were conjugated with 1 ml of the deacetylated SATA derivative of the antibody (protein concentration 4.29 mg/ml) by incubation overnight at 4°C. The immunoliposomes were separated from the unreacted SATA derivative of the antibody by gel filtration using a Sepharose 4B column (25 cm × 2 cm) previously equilibrated with PBS. Immunoliposome peak fractions (2.2-ml) were assayed for protein content by the method of Wang and Smith [24], the lipid content by scintillation counting (100 μl-aliquots) and the size of the immunoliposomes was determined by PCS. These data were used to calculate the weight-average number of antibody molecules per immunoliposome ($\bar{P}_w$) and hence the area per antibody on the immunoliposome surface as previously described [20].

For the preparation of immunoliposomes by the technique of reverse phase evaporation identical lipid mixtures were taken as for VETs but they were dissolved in 3 ml of chloroform/methanol mixture (4:1, v/v) in a 50 ml round-bottomed flask. The organic solvent was removed by rotary evaporation at 60°C to form a thin lipid film. The film was re-dissolved in 6 ml chloroform/methanol (4:1, v/v) to which were added 3 ml of nitrogen-saturated PBS at 60°C. The mixture was gently shaken and sonicated for 3 min in a bath sonicator (Deacon FS 100) under nitrogen at 60°C. The resulting homogeneous emulsion was rotary evaporated at 60°C to remove the organic solvent and the aqueous suspension purged with nitrogen for 15 min. The suspension was incubated at 60°C to facilitate annealing of the vesicle bilayers. The size of the REVs was determined by PCS before conjugation with antibody as described above for VETs.

In the preparation of immunoliposomes encapsulating methotrexate, the drug was added at the hydration stage of the initial phospholipid film in sterile PBS. After preparation and conjugation of the immunoliposomes the extraliposomal methotrexate was removed together with the unreacted SATA derivative of the antibody by gel filtration on the Sepharose 4B column. The level of encapsulated methotrexate was determined by incubating an aliquot of immunoliposome suspension (0.3 ml) with 10% sodium n-dodecylsulphate (0.6 ml) at 60°C with periodic vortexing. The absorbance at 370 nm was used to determine the methotrexate concentration in the immunoliposomes with reference to a standard curve.

Cell culture

The growth medium for A431 cells consisted of sterile RPMI 1640 (500 ml) as supplied with added glutamine (300 mg/l), to which was added sterile solutions of foetal calf serum (50 ml), L-glutamine (5 ml, concentration 200 mM), sodium pyruvate (5 ml, concentration 100 mM) and gentamicin (2.8 ml, concentration 10 mg/ml).

The growth medium for normal fibroblasts consisted of sterile DMEM (500 ml) to which was added sterile solutions of glucose (4.5 g/l), foetal calf serum (56 ml), L-glutamine (5.6 ml, concentration 200 mM), sodium pyruvate (5.6 ml, concentration 100 mM), and 5.6 ml penicillin/streptomycin (10000 units/ml; 10000 μg/ml).

The A431 cells were subcultured under sterile conditions as follows: to a 75 ml tissue culture flask containing a 90% confluent monolayer of cells prewashed with 10 ml sterile PBS were added sterile PBS (9 ml), 10 × trypsin solution (1 ml). The flask was incubated (37°C) with gentle agitation until the cells detached from the flask. The cell suspension was transferred to a sterile centrifuge tube containing fresh growth medium (35 ml) and centrifuged for 3 min on a bench centrifuge (1200 rpm). After removal of the supernatant the bottom of the tube was gently tapped to disperse the cells which were then resuspended in 30 ml of fresh growth medium. Cell suspensions (10 ml) were then transferred to three 75 ml culture flasks containing 20 ml of fresh growth medium. The cells were cultured at 37°C in a 5% carbon dioxide atmosphere until confluent.

Determination of cell numbers

Assay of viable cells was carried out by measurement of their ability to reduce the yellow substrate 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (DTDT) to a blue product as described by Mossman [25]. Standard curves were prepared relating cell numbers to the absorbance of the blue reduction product as follows: a confluent monolayer of cells was suspended in 5 ml of growth medium and the cell numbers determined by haemocytometry. The cell suspension was serially diluted 1:1 in sterile Eppendorf tubes with fresh growth medium. Aliquots (100 μl) of the serially diluted cells and 10 μl of substrate (DTDT) were added to sterile microtitre plate wells and incubated at 37°C/5% CO_2 for 4 h. After incubation, acidified isopropanol (100 μl of 0.04 M HCl in isopropanol at 60°C) was added to each well to dissolve

the blue crystals formed. The absorbance was measured at 570 nm (reference 630 nm) using a Dynatech MR 610 plate reader coupled to an Apple IIe microcomputer.

Targeting of immunoliposomes to immobilised PLAP (ELISA)

The targeting of the immunoliposomes to human placental alkaline phosphatase (PLAP) was assessed using an ELISA. PLAP was immobilised on microtitre plates as follows: each plate well was incubated with 100 μl of glutaraldehyde (0.25% w/v) for 5 min at room temperature, the plate was blot dried on absorbent tissue and to each well was added 50 μl PLAP solution (5 μg/ml in PBS at pH 7.3) followed by incubation at room temperature for 1 h. The wells were washed with PBS (200 μl) and blot dried. Finally 100 μl gelatin solution (20 mg gelatin and 20 mg sodium azide in 100 ml PBS (pH 7.3)) were added to each well and incubated at room temperature for 1 h. The plates were sealed and stored at 4°C and used within 14 days.

Microtitre plates with immobilised PLAP were washed four times with 200 μl PBS containing Thimerosal (PBSTM). Immunoliposome solutions (100 μl) of the required concentration range in PBS were added to the wells and incubated for 1 h. The wells were then washed four times with 200 μl PBSTM, followed by the addition of mouse anti-IgG-horse radish peroxidase conjugate (50 μl). The plates were incubated at room temperature for 1 h followed by a further four washes with PBSTM (200 μl) after which the substrate (200 μl) of 3,3′,5,5′-tetramethylbenzidine (TMB) was added. The TMB substrate was prepared from 200 μl of a stock TMB solution (0.1 g in 10 ml of dimethylsulphoxide) plus 20 ml of a citrate (0.04 M)-phosphate (0.16 M) buffer (pH 6.5) plus 10 μl of hydrogen peroxide. The plates were gently shaken at room temperature for 45 min, after which time the reaction was stopped by addition of 50 μl of 1 M HCl. The absorbances were measured at 450 nm with a plate reader (see above)

Determination of the presence of PLAP on the surface of A431 cells

A suspension of rapidly growing A431 cells was used to make 100 μl additions to sterile microtitre plates which were then incubated at 37°C in a 5% carbon dioxide atmosphere until the cells were 90% confluent. Non-specific binding sites present on the plate were blocked by incubation of the confluent cell monolayers with 100 μl of casein solution (20 mg/ml) for 60 min. The cells were washed once with sterile PBS (100 μl) and incubated for 110 min with antibody H17E2 or a non-specific antibody (Bioclone anti-A). The degree of binding of each antibody to the cell monolayer was determined by the ELISA method described above.

Determination of immunoliposome binding to A431 cells by autoradiography

Aliquots (1 ml) of A431 cell suspension were added to incubation chambers on tissue growth slides which were incubated at 37°C in a 5% carbon dioxide atmosphere until the cells were 70% confluent. Liposomes and immunoliposomes (0.4 ml) labelled with 5 μCi [^{14}C]DPPC were incubated with the cells for various times upto 90 min. After incubation the cells were washed four times with sterile PBS (1.3 ml), followed by a 30 min incubation with paraformaldehyde (4% w/v). The incubation chambers were emptied and left to dry at room temperature for 90 min, after which they were delicately washed with water, dried and coated with a thin film of K-5 photographic emulsion in a dark room. The slides were placed in a light-proof container and stored at 4°C for 28 days, after which they were immersed in D19 developer for 5 min, washed twice with water immersed in fixative solution, washed for 10 min in cold running water and stained with haemotoxylin for 30s. and rewashed with cold running water containing bicarbonate to intensify the stain. The slides were stained with eosin for 1 min followed by sequential dehydration by immersion in 95% methanol for 2 min, 100% ethanol for 2 min and xylene for 2 min. After removal of excess xylene a coverslip pre-immersed in Xam was carefully pushed onto the sample, care being taken to exclude air bubbles. The slides were subjected to photomicroscopy.

Incubation of A431 cells with methotrexate

Aliquots (100 μl) of sterile methotrexate (0.01–10 μg/100 μl) were added to two identical microtitre plates containing a known number of cells growing in 100 μl of growth medium for 2, 13, 25 and 45 min. After each incubation the wells were washed once with fresh growth medium, followed by a further addition of growth medium (100 μl). One plate was immediately assayed for cell numbers using DTDT as above. The second plate was incubated at 37°C/5% carbon dioxide for 48 h after the addition of methotrexate and then assayed for cell numbers.

The effects of free and immunoliposome encapsulated methotrexate on A431 and human fibroblasts

To two microtitre plates containing a known number of A431 or human fibroblasts growing in 100 μl of growth medium were added immunoliposomes (100 μl) encapsulating methotrexate or free methotrexate at a concentration equivalent to that encapsulated for periods of 1, 5, 10, 20 and 30 min. After each incubation the wells were washed once with fresh growth medium followed by a further addition of fresh growth medium

(100 μl). One plate was immediately assayed for cell numbers and the other was incubated at 37°C/5% carbon dioxide for 48 h and then assayed for cell numbers.

Results

The affinity of the H17E2 antibody for the immobilized PLAP enzyme was assessed using the ELISA method. The results of targeting the antibody over a wide range of concentration are shown in Fig. 1. A characteristic sigmoidal antibody dilution curve was found which confirmed that the enzyme retained affinity for the antibody after immobilisation. Figs. 2(a) and 2(b) show the ELISA signals on targeting immunoliposomes prepared by the vesicle extrusion technique (VETs) and reverse phase evaporation (REVs), respectively, to immobilized PLAP. The VETs were in the size (weight-average diameter, $\bar{d}_w$) range 86 to 104 nm and the weight-average number of surface bound antibody ($\bar{P}_w$) were in the range 5.4 to 13.4. The ELISA signal increased with liposomal lipid concentration and with increase in $\bar{P}_w$ (Fig. 2a). The REVs covered a $\bar{d}_w$ range from 315 to 456 nm and a $\bar{P}_w$ range from 103 to 490. As for the VETs the ELISA signal increased with liposomal concentration and $\bar{P}_w$. In these experiments it is the amount of liposomally bound antibody associated with the immobilised PLAP which determines the size of the ELISA signal. The extent of immunoliposome adsorption to the immobilised PLAP would be expected to increase with liposome concentration and hence lipid concentration. The signal level would also be expected to increase with $\bar{P}_w$ as a consequence of increasing affinity of the immunoliposomes for the PLAP and/or because of the larger number of antibody molecules associated with each immunoliposome. In order to investigate whether the increase in ELISA signal was associated with increase in numbers of immunoliposomes adsorbed the extent of adsorption was measured using radiolabelled VETs. Fig. 3 shows the results of such an experiment for immunoliposomes ($\bar{P}_w = 6.2$, $\bar{d}_w = 109$ nm). The increase in ELISA signal reflects an increase in the number of adsorbed immunoliposomes and the ELISA signal reaches a plateau before the sites are saturated with immunoliposomes.

The presence of PLAP on the surface of A431 cells was confirmed by adsorption of the cells on a microtitre plate followed by addition of antibody and measurement of the ELISA signal (Fig. 4). The ELISA signal increased steeply with addition of antibody in the concentration range 1–30 μg/100 μl confirming the presence of PLAP on the cell surface. In a control experiment using another antibody (Bioscot anti-B) raised to blood group B antigens at equivalent concentrations no ELISA signal was obtained indicating no binding to the A431 cells.

Autoradiography studies were carried out to visualize the binding of the immunoliposomes to the A431 cells. Immunoliposomes and control liposomes with no surface-bound antibody were incubated with A431 cells for 30, 60 and 90 min. Fig. 5 shows the autoradiographs after 90 min. The dark grains arising from ^{14}C-labelled liposomes associated with the cells were counted. Fig. 6 shows the increase in the numbers of grains (n) relative to background (n_0) as a function of time for the immunolipsomes and the control liposomes. The values for immunoliposomes reach a plateau after 60 min and are approximately six times larger than for 'naked' liposomes.

A range of experiments were carried out to deter-

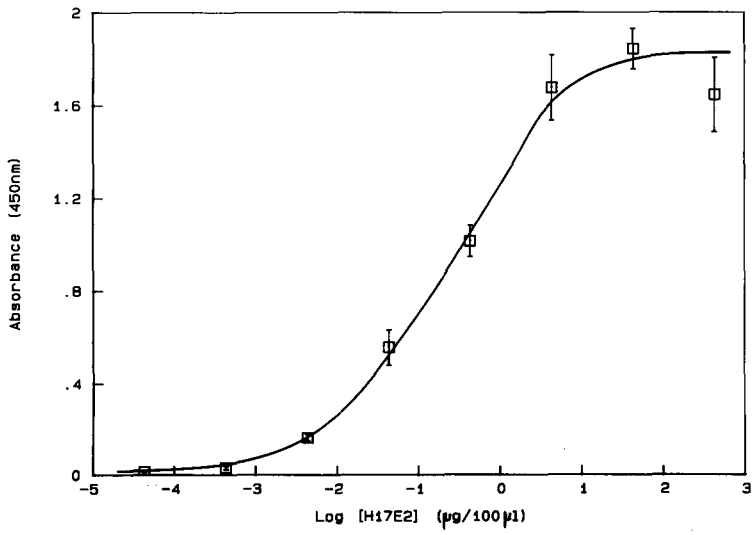

Fig. 1. Antibody (H17E2) dilution curve for an ELISA of immobilised placental alkaline phosphatase (PLAP). The error bars are based on six measurements.

mine the effectiveness of the immunoliposome-carried methotrexate on inhibiting the proliferation of A431 cells. Various control experiments were also done including the effect of 'free' methotrexate concentration on cell growth, the effect of drug-free immunoliposomes on cell growth, the effect of blocking of the PLAP sites on the cells, and the specificity of the immunoliposomes for A431 cells on comparison with their effects on fibroblast growth. The results are summarised in Table I and Figs. 7 and 8.

In Table I the % inhibition is calculated from the expression

$$\% \text{ Inhibition} = \left(\frac{n_f - n}{n_f - n_i}\right)100 \quad (1)$$

where n_i, n_f and n are the initial numbers of cells, the final number of cells after 48 h growth in the absence of test system (control) and the number of cells after 48 h growth in the presence of the test system, respec-

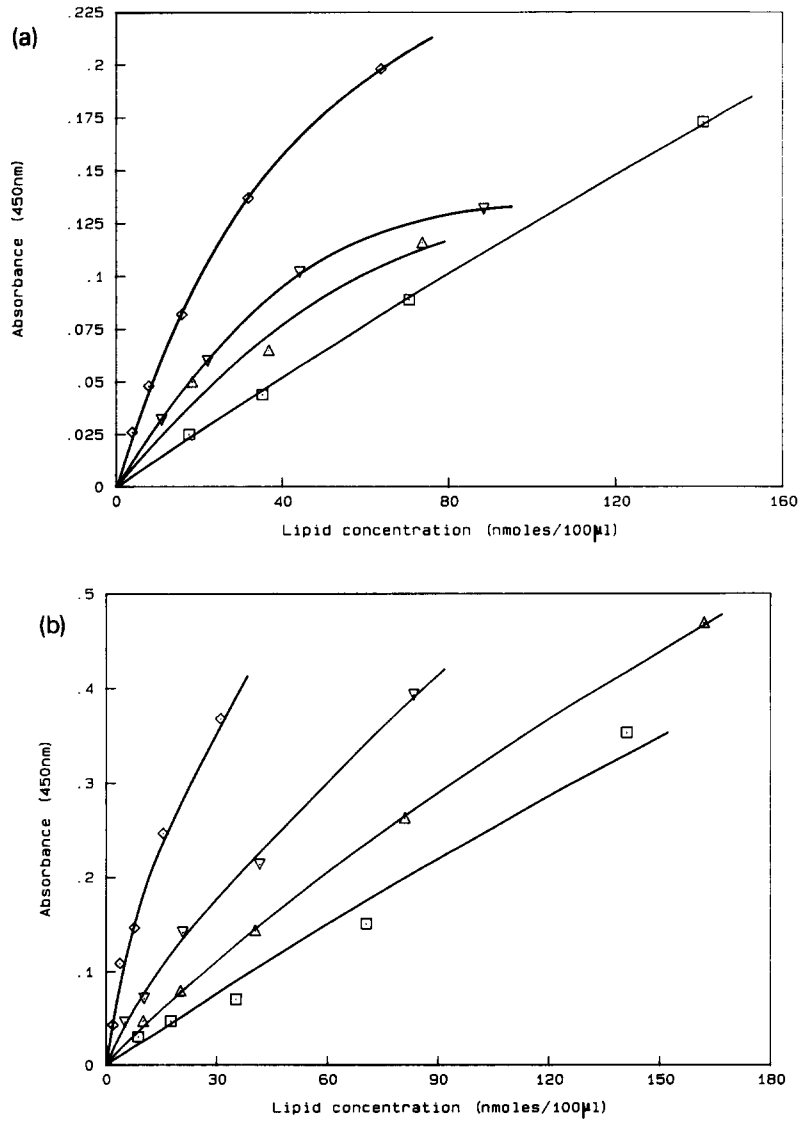

Fig. 2. (a) Targeting of immunoliposomes (DPPC/PI/DPPEMBS, weight ratio 1:0.11:0.25) prepared by extrusion (VETs) with surface-bound antibody (H17E2) to immobilised placental alkaline phosphatase (PLAP). The weight-average number of antibody molecules per liposome ($\bar{P}_w$), weight-average diameter ($\bar{d}_w$) and area per antibody on the liposome surface (A_s) are as follows. □, $\bar{P}_w = 5.4$, $\bar{d}_w = 104$ nm, $A_s = 6284$ nm^2/molecule; △, $\bar{P}_w = 6.8$, $\bar{d}_w = 102$ nm, $A_s = 4801$ nm^2/molecule; ▽, $\bar{P}_w = 9.0$, $\bar{d}_w = 94$ nm, $A_s = 3105$ nm^2/molecule; ◇, $\bar{P}_w = 13.4$, $\bar{d}_w = 86$ nm, $A_s = 1713$ nm^2/molecule. (b) Targeting of immunoliposomes (DPPC/PI/DPPEMBS, weight ratio 1:0.11:0.25) prepared by reverse phase evaporation (REVs) with surface-bound antibody (H17E2) to PLAP adsorbed from solution (5 μg/100 μl). ◇, $\bar{P}_w = 490$, $\bar{d}_w = 315$ nm, $A_s = 638$ nm^2/molecule; ▽, $\bar{P}_w = 208$, $\bar{d}_w = 300$ nm, $A_s = 1364$ nm^2/molecule; △, $\bar{P}_w = 107$, $\bar{d}_w = 348$ nm, $A_s = 3548$ nm^2/molecule; □, $\bar{P}_w = 103$, $\bar{d}_w = 456$ nm, $A_s = 6325$ nm^2/molecule.

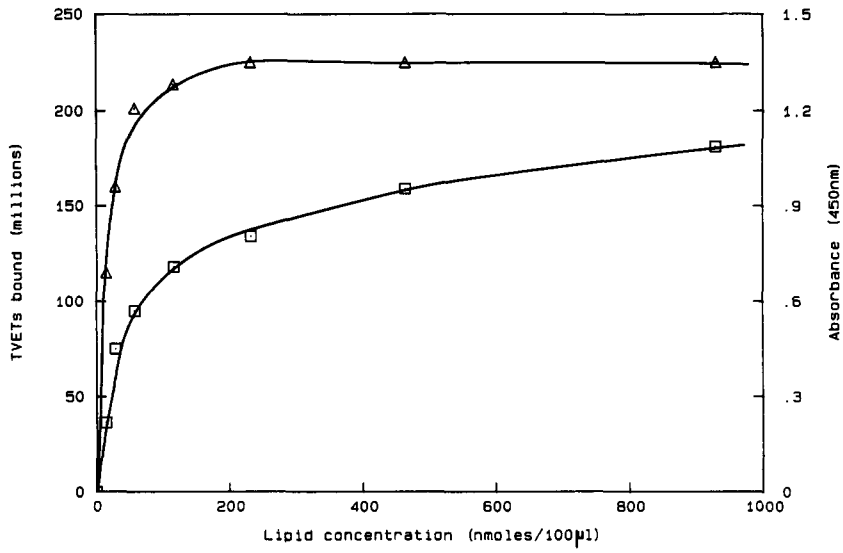

Fig. 3. The binding of immunoliposomes (targeted VETs, DPPC/PI/DPPEMBS, weight ratio 1:0.11:0.25, $\bar{P}_w = 6.2$, $\bar{d}_w = 109$ nm, $A_s = 5998$ nm^2/molecule) to immobilised PLAP adsorbed from solution (5 μg/100 μl) (□, left-hand axis) in relation to the ELISA signal (△, right-hand axis).

tively. The small negative and greater than 100% inhibition values arise from variations in cell numbers in multiple wells after 48 h growth and are not significantly different from 0 and 100%.

Methotrexate inhibited proliferation by 80% after 2–45 min exposure to the drug in the concentration range 0.01–10% (w/v). Fig. 7 shows a control experiment which demonstrates that methotrexate-free immunoliposomes incubated with A431 cells for up to 30 min have no effect on cell growth. The cell numbers after 48 h growth were the same as the control levels found in the absence of immunoliposomes. Also shown in Fig. 7 is the effect of incubation time of methotrexate-loaded immunoliposomes on cell growth after 48 h, relative to the equivalent concentration of free methotrexate. After 30 min incubation time the methotrexate-loaded immunoliposomes inhibit cell growth slightly more than an equivalent amount of free drug. In the experiments 81 000 cells per well grew to 134 000 cells per well in the absence of drug. After exposure of immunoliposome-carried drug for 30 min, followed by 48 h growth, there were 79 200 cells per well whereas cells treated with free drug equivalent to that in the immunoliposomes grew to 87 140 cells per well.

The cell surface binding sites (PLAP) can be partially blocked by initially incubating the cells with free antibody prior to delivery of methotrexate by immuno-

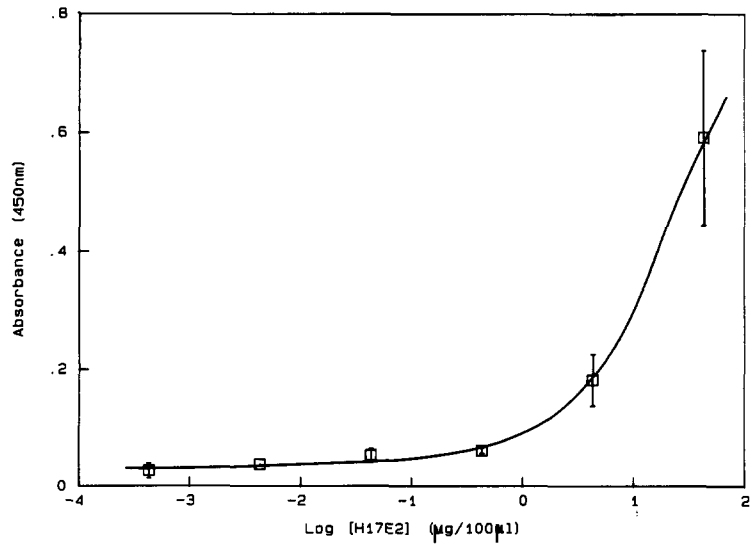

Fig. 4. Antibody (H17E2) dilution curve for an ELISA of placental alkaline phosphatase (PLAP) on the surface of immobilised A431 cells. The error bars are based on six experiments.

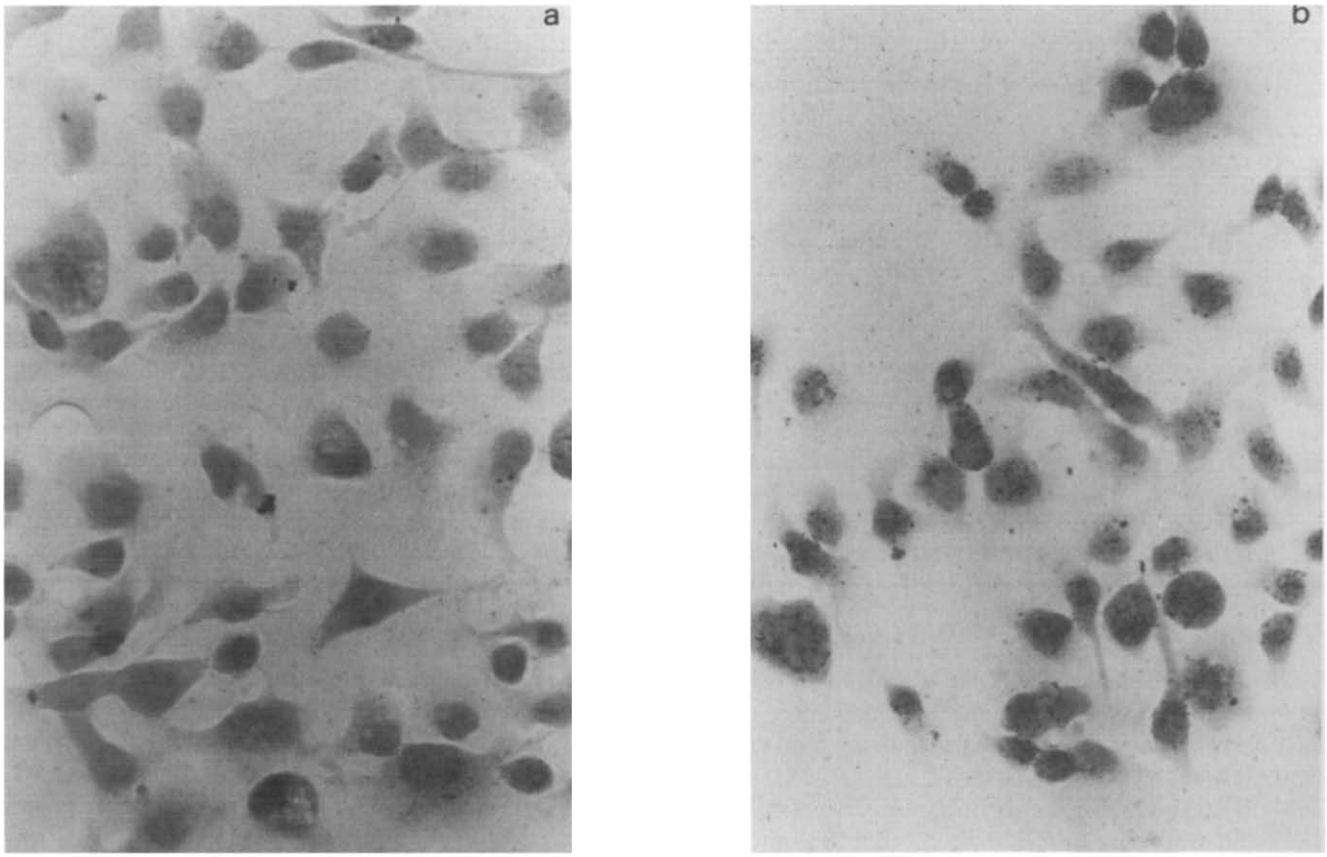

Fig. 5. Autoradiographs of (a) liposomes (DPPC/PI/DPPEMBS, weight ratio, 1:0.11:0.25, $\bar{P}_w = 0$, $\bar{d}_w = 104$ nm) and (b) immunoliposomes (DPPC/PI/DPPEMBS, weight ratio, 1:0.11:0.25, $\bar{P}_w = 6.0$, $\bar{d}_w = 104$ nm) targeted to A431 cells for 90 min.

liposomes (Table I). Growth after 48 h is greater after an initial exposure of the cells to free antibody although some inhibition of proliferation by immunoliposome-carried drug still occurs.

Similar experiments on human fibroblasts are shown in Fig. 8 which shows that immunoliposome carried

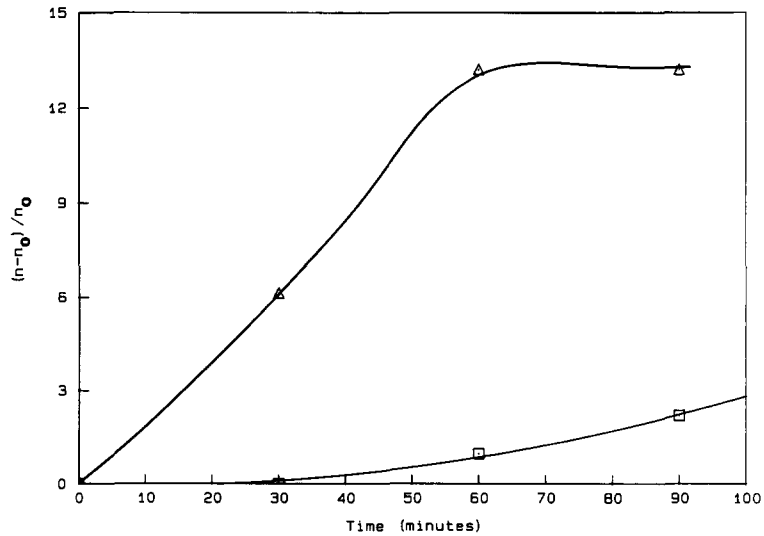

Fig. 6. Binding of liposomes and immunoliposomes as described in Fig. 5 to A431 cells as assessed by autoradiography. n and n_o are the number of grains associated per cell and the background number of grains, respectively.

TABLE I

Inhibition of A431 and fibroblast cell proliferation

System (Figure)	Cells/well n_i	Cells/well n_f [b] (control)	Cells/well n [b]	% Inhibition
A431 + methotrexate (MT) (0.01–10 μg/100 μl) 2 min (n [a] = 4)	5140 ± 750	9540 ± 1200	6080 ± 530	79
A431 + methotrexate (MT) (0.01–10 μg/100 μl) 45 min (n = 4)	5140 ± 750	9540 ± 1200	6000 ± 470	80
A431 + immunoliposomes Fig. 7 (n = 3)	81000 ± 12500	134000 ± 15000	134900 ± 1750	−1.7
A431 + [immunoliposomes-MT (0.53 μg/100 μl)] 30 min Fig. 7 (n = 3)	81000 ± 12500	134000 ± 15000	79160 ± 4300	103
A431 + [methotrexate (MT) (0.53 μg/100 μl)] Fig. 7 (n = 3)	81000 ± 12500	134000 ± 15000	87140 ± 580	88
A431 + [immunoliposomes[c]-MT (0.53 μg/100 μl)] antibody preincubation (5 μg/100 μl for 30 min) (n = 3)	81000 ± 12500	134000 ± 15000	105650 ± 4100	53
Fibroblasts + [immunoliposomes-MT (0.678 μg/100 μl)] Fig. 8 (n = 3)	6620 ± 380	9550 ± 460	9440 ± 80	3.8
Fibroblasts + MT (0.678 μg/100 μl) Fig. 8 (n = 3)	6620 ± 380	9550 ± 460	6500 ± 470	104
A431 + [immunoliposomes (REV)[d]-MT (0.34 μg/100 μl)] (n = 4)	6060 ± 320	9650 ± 840	8045 ± 140	45
A431 + methotrexate (MT 0.34 μg/100 μl) (n = 4)	6060 ± 320	9650 ± 840	5720 ± 180	109

[a] n = number of replicates.
[b] After 48 h growth.
[c] Composition DPPC/PI/DPPEMBS weight ratio 1:0.11:0.25, $\bar{P}_w = 3.8$, $\bar{d}_w = 105$ nm, $A_s = 9097$ nm^2/molecule.
[d] Composition DPPC/PI/DPPEMBS weight ratio 1:0.11:0.25, $\bar{P}_w = 156$, $\bar{d}_w = 282$ nm, $A_s = 1601$ nm^2/molecule.

methotrexate has no effect on fibroblast proliferation whereas the free drug progressively inhibits cell proliferation as the initial incubation time with the fibroblasts is increased.

Methotrexate carried by immunoliposomes prepared by the reverse phase evaporation method (REVs) having an approximately three times larger diameter than the VETs are not as effective in inhibiting cell proliferation (Table I).

Discussion

Immunoliposomes prepared by either the extrusion or reverse phase evaporation techniques were found to

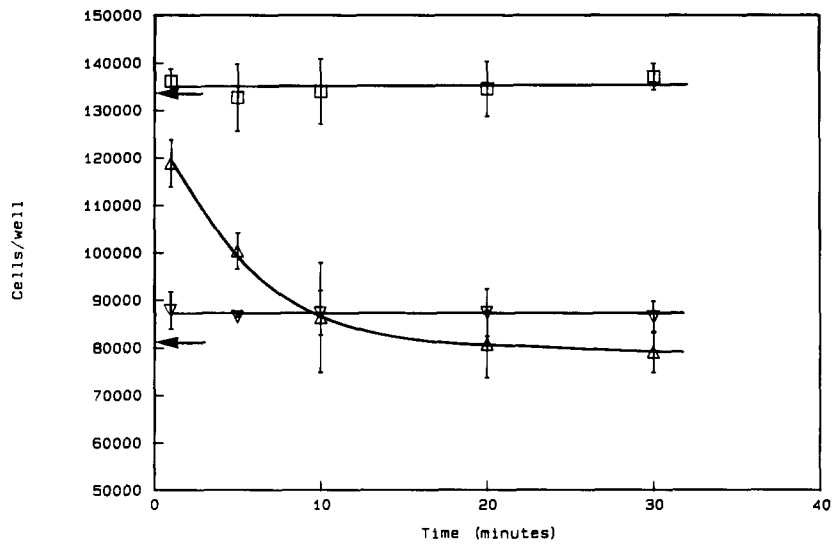

Fig. 7. (□) The effect of immunoliposomes (DPPC/PI/DPPEMBS, weight ratio 1:0.11:0.25, $\bar{P}_w = 5.8$, $\bar{d}_w = 99$ nm liposomal lipid concentration 3.84 mM) on the growth of A431 cells as a function of initial incubation time followed by 48 h growth. $n_i = 81000 \pm 12500$; $n_f = 134000 \pm 15000$. (△) The effect of methotrexate-loaded immunoliposomes (DPPC/PI/DPPEMBS, weight ratio 1:0.11:0.25, $\bar{P}_w = 3.8$, $\bar{d}_w = 105$ nm, $A_s = 9097$ nm^2/molecule, [methotrexate] = 0.53 μg/100 μl, liposomal lipid concentration 3.70 mM, molar ratio of lipid/drug = 317) on the growth of A431 cells as a function of time of initial incubation time followed by 48 h growth. $n_i = 81000 \pm 12500$; $n_f = 134000 \pm 15000$. (▽) The effect of free methotrexate at a concentration of 0.53 μg/100 μl on the growth of A431 cells (Control for above curve (△)). The lower and upper arrows denote the initial number of cells and the number of cells after 48 h growth respectively in control wells.

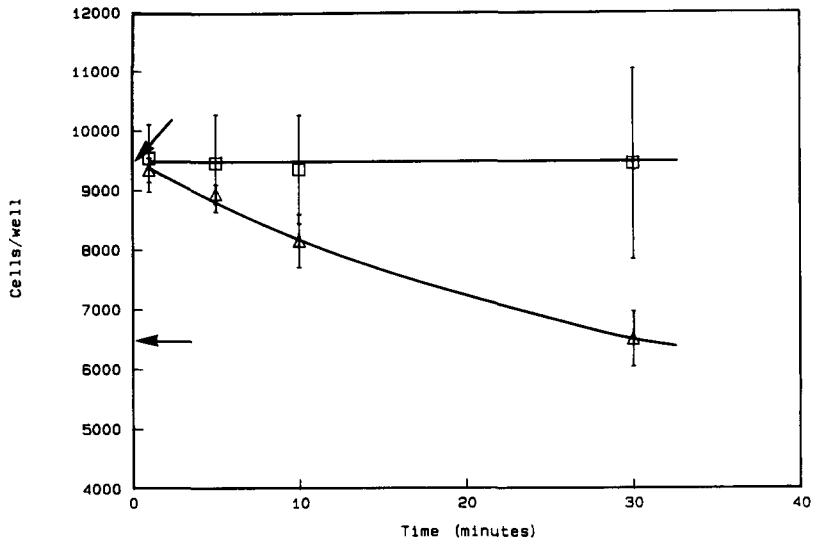

Fig. 8. (□) The effect of methotrexate-loaded immunoliposomes (DPPC/PI/DPPEMBS, weight ratio 1:0.11:0.25, $\bar{P}_w = 7.0$, $\bar{d}_w = 99.8$ nm, $A_s = 4445$ nm^2/molecule, [methotrexate] = 0.678 μg/100 μl, liposomal lipid concentration 3.59 mM, molar ratio lipid/drug = 241) on the growth of human fibroblasts as a function of initial incubation time followed by 48 h growth. $n_i = 6620 \pm 380$, $n_f = 9550 \pm 460$ (△). The effect of free methotrexate at a concentration of 0.678 μg/100 μl on the growth of human fibroblasts (Control for above (□)). The lower and upper arrows denote the initial number of cells and the number of cells after 48 h growth, respectively, in control wells.

be effective in targeting to immobilized PLAP (Fig. 2). The ELISA signal increases with liposomal lipid concentration and the number of antibody molecules per liposome ($\bar{P}_w$). The values of $\bar{P}_w$ for the VETs range from 5.4 to 13.4 while those for the REVs are much larger (103 to 490). The ELISA signal (absorbance) at a given immunoliposome lipid concentration would be expected to depend directly on $\bar{P}_w$ and the number of adsorbed immunoliposomes. At a given lipid concentration the number of immunoliposomes per unit volume of dispersion will vary inversely with the amount of lipid per immunoliposome. Since the amount of lipid per immunoliposome will depend on the surface area ($4\pi(\bar{d}_w/2)^2$) it might be expected that the ELISA signal will increase with the factor $\bar{P}_w/(\bar{d}_w)^2$. Fig. 9 shows a plot based on this argument for both types of immunoliposomes (VETs and REVs) for a given lipid concentration (30 nmol/100 μl). Despite the considerable differences in size and $\bar{P}_w$ between the VETs and REVs both give very similar linear plots consistent with

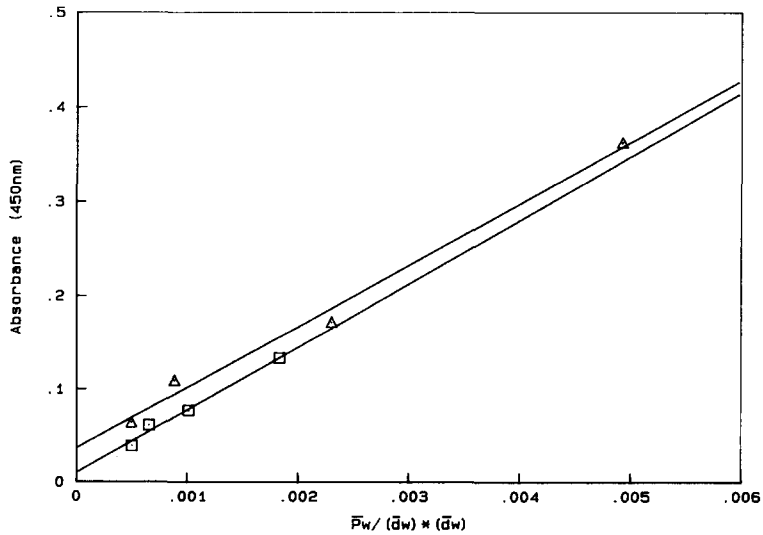

Fig. 9. Dependence of the ELISA signal for immunoliposomes targeted to immobilised placental alkaline phosphatase (PLAP) on the parameter $\bar{P}_w/(\bar{d}_w)^2$. □, Immunoliposomes prepared by the extrusion technique (VETs); △, immunoliposome prepared by reverse phase evaporation (REVs). The immunoliposome lipid concentration was constant at 30 nmol/100 μl.

the above assumptions and which obey the following equations.

$$\text{Abs}(450\text{ nm}) = 0.01 + 67.5\bar{P}_w/(\bar{d}_w)^2\text{VETs} \quad (2)$$

$$\text{Abs}(450\text{ nm}) = 0.04 + 65.3\bar{P}_w/(\bar{d}_w)^2\text{REVs} \quad (3)$$

For the REVs the proportions of antibody molecules per vesicle involved in binding the vesicle to the immobilised PLAP will most probably be smaller than for VETs, thus allowing more antibodies to bind the antibody-enzyme conjugate leading to slightly greater ELISA signals for the REVs.

The presence of PLAP or a PLAP-like enzyme on the surface of A431 cells was demonstrated using the ELISA (Fig. 4) and the targeting of the immunoliposomes to the A431 cells was shown by the autoradiography studies (Figs. 5 and 6). The autoradiography experiments show that binding of the immunoliposomes is not instantaneous, under the conditions used and saturation took approx. 60 min.

The effect of 'free' methotrexate on A431 cell growth (Table I) showed that the drug has an immediate effect on viable cell numbers which is independent of methotrexate concentration in the range 0.01–10 μg/100 μl and incubation time in the range 2–45 min. Reducing the methotrexate concentration by two orders of magnitude (to 0.1 ng/100 μl) was also found to inhibit proliferation. The rapid effect of methotrexate shows that the cells metabolism, specifically dihydrofolate reductase [9], is inhibited. The entry of methotrexate into the cell is probably via a reduced folate carrier system [26,27] which saturates at very low drug concentrations. The fact some cell growth still occurs after 48 h suggests that a small proportion of cells are either more resistant to methotrexate or are able to recover from the effects of the drug.

Immunoliposomes do not affect cell growth (Fig. 7) unless they are loaded with methotrexate. In contrast to free methotrexate the degree of growth inhibition depends on the time of incubation with methotrexate-loaded immunoliposomes, 100% inhibition occurs after 30 min incubation. If the A431 cells are pre-incubated with free antibody, growth inhibition by methotrexate-loaded immunoliposomes is reduced to approx. 50%, in contrast to 100% for cells not pre-incubated, suggesting that the PLAP binding sites could be partially blocked.

In contrast to the effects on A431 cells methotrexate-loaded immunoliposomes had no effect on the growth of normal human fibroblasts whereas free methotrexate inhibited growth in a time-dependent way (Fig. 8). The growth rate of the fibroblasts is much slower than for the A431 cells so that the uptake rate of methotrexate via the folate carrier may be lower, which would lead to the extent of growth being dependent on the time of incubation with free drug. These results demonstrate the specificity of the immunoliposomes for A431 tumour cells with surface PLAP or PLAP-like enzymes. Thus although inhibition of cell proliferation by the methotrexate-loaded immunoliposomes is not significantly greater than an equivalent amount of the free drug the immunoliposomes have a specificity for the tumour cell line. While this specificity should be advantageous for drug targeting the effectiveness of such a drug carrying system in vivo will depend on the distribution of PLAP and PLAP-like enzymes in normal and neoplastic tissue, which must await tests with animal models and include studies of the stability of the immunoliposomes in blood. The immunoliposomes would also be competing with PLAP or PLAP-like enzymes in the blood, however, should these arise from the metastasis of neoplastic tissue then the presence of circulating immunoliposomes encapsulation methotrexate might be beneficial as a means of preventing the proliferation of circulating malignant cells and hence of inhibiting metastasis.

Finally we consider the mechanism of uptake of methotrexate from the loaded immunoliposomes. The first step in the process is the binding of the immunoliposomes to the cell surface. The cell may then either take up the immunoliposomes by receptor-mediated endocytosis or the bound immunoliposomes may gradually release the drug which is taken up by the cells through the folate carrier system. Endocytic vesicles have a diameter of approx. 100 nm [28,29]. It is possible that at least the smaller immunoliposomes in a VET distribution with a weight-average diameter of approx. 100 nm could enter the cell by endocytosis. Increasing the average size of the immunoliposomes to approx. 280 nm diameter significantly reduces the extent of growth inhibition (Table I). There will be a much smaller proportion of small vesicles capable of being taken up by endocytosis in methotrexate-loaded REVs. While it is possible that other mechanisms may contribute to the uptake of methotrexate from immunoliposomes it seems likely that endocytosis plays a significant role.

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