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Selective transfer of a lipophilic prodrug of 5-fluorodeoxyuridine from immunoliposomes to colon cancer cells

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Abstract

A monoclonal antibody against the rat colon carcinoma CC531 was covalently coupled to liposomes containing a dipalmitoylated derivative of the anticancer drug FUdR as a prodrug in their bilayers. We investigated the in vitro interaction of these liposomes with CC531 target cells and the mechanism by which they deliver the active drug FUdR intracellularly to the cells by monitoring the fate of the liposomal bilayer markers cholesterol-[^{14}C]oleate and [^{3}H]cholesteryloleylether as well as the ^{3}H-labeled prodrug and colloidal gold as an encapsulated liposome marker. After binding of the immunoliposomes to the cell surface, only limited amounts were internalized as demonstrated by a low level of hydrolysis of liposomal cholesterol ester and by morphological studies employing colloidal gold-labeled immunoliposomes. By contrast, already within 24 h immunoliposome-incorporated FUdR-dP was hydrolyzed virtually completely to the parent drug FUdR intracellularly. This process was inhibited by a variety of endocytosis inhibitors, indicating that the prodrug enters and is processed by the cells by a mechanism involving an endocytic process, resulting in intracellular FUdR concentrations up to 3000-fold higher than those in the medium. Immunoliposomes containing poly(ethyleneglycol) (PEG) chains on their surface, with the antibody coupled either directly to the bilayer or at the distal end of the PEG chains were able to deliver the prodrug into the tumor cells at the same rate as immunoliposomes without PEG. Based on these observations, we tentatively conclude that during the interaction of the immunoliposomes with the tumor cells the lipophilic prodrug FUdR-dP is selectively transferred to the cell surface and subsequently internalized by constitutive endocytic or pinocytic invaginations of the plasma membrane, thus ultimately delivering the prodrug to a lysosomal compartment where

Abbreviations: FUdR, 5-fluoro-2′-deoxyuridine; FUdR-dP, 3′,5′-*O*-dipalmitoyl-FUdR; CC52, colon carcinoma CC531-specific antibody; FCS, fetal calf serum; PC, phosphatidylcholine; Chol, cholesterol; PEG, methoxy-poly(ethyleneglycol)$_{2000}$-distearoylphosphatidylethanolamine; MPB-PE, maleimido-4-(*p*-phenylbutyryl)phosphatidylethanolamine; Hz-PEG-DSPE, hydrazide-PEG-distearoylphosphatidylethanolamine

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hydrolysis and release of parent drug takes place. This concept allows for an efficient delivery of a liposome-associated drug without the need for the liposome as such to be internalized by the cells.

Keywords: Immunoliposome; 5-Fluorodeoxyuridine; Lipophilic prodrug; Selective transfer; Drug delivery; (Colon cancer cell)

1. Introduction

Liposomes have been extensively studied as delivery systems for cytostatic and cytotoxic drugs [1,2]. Hydrophilic drugs can be encapsulated in the inner aqueous phase and hydrophobic drugs can be incorporated into the bilayer of liposomes. The association of a drug with liposomes usually results in a drastic change in pharmacokinetics of the drug, including increased circulation half-life and marked changes in biodistribution, often resulting in reduced toxicities and/or improved therapeutic activities [1,3]. By modifying the liposome surface with poly(ethyleneglycol) (PEG), further improvement in drug circulation time can be achieved [4,5]. The extended circulation time of such liposomes has been shown to result in higher uptake into solid tumors by so-called passive targeting [5,6]. In order to achieve more specific tumor targeting, PEG liposomes have been developed with tumor cell-specific antibodies coupled to the lipid bilayer [7] or to the distal end of the PEG chains [7–9]. Two important barriers limiting successful drug targeting to tumors with liposomes still have to be overcome: (1) limited accessibility of target cells in solid tumors [10] and (2) a generally limited phagocytic capacity of tumor cells, preventing efficient delivery of the liposome-associated drug to its site of action, which for most anticancer drugs is intracellular. Although there are reports in literature describing internalization of immunoliposomes by tumor target cells [8,11], quantities are mostly low, while others report no uptake at all [9,12]. Even if any endocytic uptake of drug-containing liposomes occurs, the drug also needs to escape in an active form the endocytic/lysosomal compartment.

In a previous study we showed the efficacy of immunoliposomes containing a lipophilic prodrug of 5-fluorodeoxyuridine, FUdR-dipalmitate (FUdR-dP), towards rat CC531 colon cancer cells [13] The monoclonal antibody CC52 (murine IgG_1) that is coupled to the liposomes, recognizes a surface antigen on CC531 colon adenocarcinoma cells [14]. These immunoliposomes were ten times more efficient in delivering FUdR-dP to tumor cells than liposomes without antibody, despite a lack of uptake of the immunoliposomes themselves. In line with an earlier report, in which we postulated the transfer of the lipophilic prodrug FUdR-dP from liposomal bilayers to cell membranes [15], we proposed a similar mechanism for the transfer of FUdR-dP from immunoliposomes to target rat colon cancer cells.

In this study we investigated in detail the mechanism by which FUdR, associated by means of a lipid anchor with the bilayer of colon cancer cell-specific immunoliposomes, enters tumor target cells. For that purpose we followed the fate of the immunoliposomes labeled with the degradable cholesteryl-[^{14}C]oleate ester ([^{14}C]COA) and/or the non-degradable [^{3}H]cholesteryloleylether ([^{3}H]COE) in their bilayers and colloidal gold as an encapsulated marker. The fate of the liposomal prodrug was monitored with [6-^{3}H]uracil-labeled FUdR-dP. With the in vivo application of these liposomes in mind, we included in our studies the effect of attachment of PEG chains to the surface of the immunoliposomes as a means to increase circulation time. The antibody was coupled either directly to the liposomal surface by means of MPB-PE [16] or attached to the distal end of the PEG chains via hydrazide-PEG-DSPE [7,17].

2. Materials and methods

2.1. Materials

5-Fluoro-2′-deoxyuridine (FUdR), *N*-succinimidyl-*S*-acetylthioacetate (SATA) sodium periodate, *N*-acetylmethionine and cholesterol (Chol) were obtained from Sigma (St. Louis, MO, USA). Egg yolk phosphatidylcholine (eggPC), maleimido-4-(*p*-phenylbutyryl)phosphatidylethanolamine (MPB-PE) and methoxy-poly(ethyleneglycol)$_{2000}$-distearoylphosphatidylethanolamine (mPEG-DSPE) were pur-

chased from Avanti Polar Lipids (Birmingham, AL, USA). Hydrazide-PEG-DSPE (Hz-PEG-DSPE) was synthesized as described previously [18]. Cholesteryl-[^{14}C]oleate and [^{3}H]cholesteryloleylether were obtained from Amersham (Buckinghamshire, UK), 5-[6-^{3}H]fluoro-2′-deoxyuridine ([^{3}H]FUdR) was obtained from DuPont NEN (Wilmington, USA). Sephadex G-50 and Sepharose CL-4B were from Pharmacia (Uppsala, Sweden). All other chemicals were analytical grade or the best grade available.

2.2. Monoclonal antibody

The monoclonal antibody CC52 (IgG_1), recognizing a surface antigen on CC531 colon adenocarcinoma cells was developed in the department of Pathology, Leiden University Medical Center, the Netherlands [14] and was purified from culture supernatant by protein A-Sepharose (Pharmacia, Woerden, The Netherlands) chromatography, according to the manufacturers instructions.

2.3. Synthesis of radioactive 3′,5′-O-dipalmitoyl-FUdR (FUdR-dP)

Radioactively labeled FUdR-dP was synthesized as described earlier [19]. Briefly, 100 μCi of [^{3}H]FUdR was added to 4 μmol of FUdR dissolved in methanol. This mixture was dried under reduced nitrogen pressure, and subsequently 8 μmol of palmitoylchloride dissolved in dimethylacetamide was added. The mixture was incubated overnight under constant shaking at 40°C. Water was added to the mixture and the prodrug was extracted with chloroform/methanol (4:1). The purity of the FUdR-dP was checked by thin-layer chromatography on silica gel F254 plates (Merck, Darmstadt, Germany) with chloroform/methanol (95:5) as an eluent. The specific activity of the product was 25 μCi/μmol.

2.4. Liposome preparation

MPB liposomes were composed of eggPC, Chol and MPB-PE (23:16:1 molar ratio). MPB-PEG liposomes were composed of eggPC, Chol, MPB-PE and mPEG-DSPE (23:16:1:1.6 molar ratio). Hz-PEG liposomes were composed of eggPC, Chol, Hz-PEG-DSPE and mPEG-DSPE (23:16:1:0.6 molar ratio). 0.02 μmol [^{3}H]FUdR-dP was incorporated in the liposomes per μmol of lipid. When required, liposomes were labeled with trace amounts of [^{3}H]cholesteryloleylether ([^{3}H]COE) (1 μCi/μmol lipid) and/or cholesteryl-[^{14}C]oleate ([^{14}C]COA) (0.4 μCi/μmol lipid). Lipids dissolved in chloroform/methanol (9:1, v/v) were mixed and dried under nitrogen, dissolved in cyclohexane and lyophilized. The lipids were then either hydrated in HN-buffer (10 mM Hepes, 135 mM NaCl), pH 7.4 or, for coupling of antibodies to MPB-PE-containing liposomes, in HN-buffer, pH 6.7 or, for coupling of antibodies to Hz-PEG-DSPE-containing liposomes, in NaAc-buffer, pH 5.5 (100 mM sodium acetate, 70 mM NaCl). Gold containing liposomes were prepared by hydration of the dried lipid with cold gold chloride/citrate solution consisting of 3.18 mM $HAuCl_4$, 2.5 mM K_2CO_3, 10.2 mM trisodium citrate (pH 6.0–6.2) as described before [20]. Liposomes were sized by repeated extrusion through polycarbonate filters with a pore size of 50 nm (Costar Cambridge, MA, USA). using a high pressure extruder (Lipex Biomembranes, Vancouver, Canada). In the case of preparation of gold-containing liposomes, gold sols were formed by incubating the liposome suspension at 37°C for 30 min. Phospholipid phosphorus of each liposome preparation was determined by a phosphate assay after perchloric acid destruction [21]. Total liposomal lipid concentrations were calculated, taking into account the amount of cholesterol in the liposome preparations. Particle size and size distribution were determined by dynamic laser light scattering with a Nicomp model 370 submicron particle analyzer (Nicomp, Santa Barbara, CA, USA). The diameter of the liposomes was obtained from the volume distribution curves produced by the particle analyzer. Liposomes had an average diameter of 80 nm.

2.5. Coupling of antibodies to liposomes

The monoclonal antibody CC52 was coupled to MPB-PE-containing liposomes by a sulfhydryl–maleimide coupling method as described previously [16]. Briefly, free sulfhydryl groups were introduced in the CC52, using the heterobifunctional reagent SATA (*N*-succinimidyl-*S*-acetylthioacetate). Free SATA was separated from the derivatized CC52 by gel permeation chromatography using Sephadex G-50. Re-

active sulfhydryl groups were induced by deacetylating the acetylthioacetate-CC52 for 30 min by addition of 100 μl freshly prepared solution of 0.5 M Hepes, 0.5 M hydroxylamine–HCl, 0.025 M EDTA (pH 7.0) per ml of antibody solution. Deacetylated antibody and MPB-containing liposomes were incubated for 4 h at room temperature at a ratio of 0.3 mg antibody per μmol of liposomal lipid. 50 μl *N*-Ethylmaleimide (8 mM in HN-buffer, pH 7.4) was added per 300 μl antibody–liposome solution to cap unreacted sulfhydryl groups. Unconjugated antibody was separated from liposomes by flotation on a metrizamide gradient as described before [22]. Hereafter, CC52 liposomes were extensively dialyzed against HN-buffer, pH 7.4.

The monoclonal antibody CC52 was coupled to Hz-PEG-DSPE-containing liposomes via a hydrazone-linkage of the hydrazide moiety at the distal end of the PEG chains and oxidized carbohydrates in the Fc-region of the antibody as described previously [7,9,17]. Briefly, CC52 antibody was oxidized using 100 μl sodium periodate (10 mM) per ml of antibody solution for 1 h at room temperature. Unreacted periodate was quenched by adding 100 μl *N*-acetylmethionine (0.5 M) per ml of antibody solution. Oxidized antibody was incubated with Hz-PEG-DSPE-containing liposomes in a ratio of 0.3 mg antibody per μmol of total lipid, overnight at 4°C. Uncoupled antibody was separated from immunoliposomes by gel permeation chromatography using Sepharose CL-4B with HN-buffer (pH 7.4) as an eluent.

Immunoliposomes were characterized by determining protein content [23], phospholipid phosphorus content and particle size. The amounts of antibody coupled to liposomes were 70–160 μg/μmol lipid for CC52-MPB liposomes, 30–40 μg/μmol lipid for CC52-MPB-PEG, and 13 μg/μmol lipid for CC52-Hz-PEG liposomes, resulting in sizes of 140–180 nm, 90–120 nm and 100–140 nm, respectively. No aggregates were observed. Liposomes were stored at 4°C under nitrogen and used within two weeks after preparation.

2.6. Cell culture

CC531 colon adenocarcinoma is a 1,2-dimethylhydrazine-induced carcinoma of the colon of WAG/Rij-rats [24]. Cells were maintained in 75-cm^2 culture flasks (Costar, Cambridge, MA, USA) in RPMI 1640 medium with 25 mM Hepes (Gibco BRL, Breda, The Netherlands) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco), fresh L-glutamine (2 mM) and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively) (Gibco) at 37°C in a humidified atmosphere consisting of 5% CO_2 in air. Cells were subcultured at 80% confluence.

In all experiments cells were trypsinized (0.05% trypsin) and 1×10^5 cells were plated in 6-well plates (Costar) and left to adhere and grow for 2 days. One hour before the start of the experiment cells were washed with PBS (137.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.4) and 1.5 ml fresh medium with or without 10% FCS was added to the cells. Unless stated otherwise, all incubations were performed at 37°C.

2.7. Association, cellular uptake and degradation of liposomes

CC531 cells were incubated with [^{3}H]COE-labeled CC52 liposomes (100 nmol) for 4, 8, 24 and 48 h continuously or for 3 h followed by a liposome-free incubation period of 0, 3, 6, 24 or 48 h. Incubations were terminated by removing the incubation medium followed by thorough washing of the cells with ice-cold PBS. Cells were harvested by trypsinization with trypsin (0.05%) for 10 min at 37°C, centrifugation and a subsequent wash of the cell pellet with PBS. Label removable by the trypsin treatment was determined in the combined supernatants, representing cell-bound liposomes; non-removable label was determined in the cell pellet after lysing the cells with 10% sodium dodecyl sulfate (SDS). Total cell-associated label was determined by combining the radioactivity in the supernatant (removable label) and the cell pellet (non-removable label) and was normalized for the amount of cellular protein as determined by protein assay [25] using BSA as a standard.

For determining uptake and degradation, liposomes were double-labeled with [^{3}H]COE, a non-degradable marker and [^{14}C]COA, an intracellularly degradable marker, which after degradation results in release of [^{14}C]oleate into the medium and a con-

comitant increase in the $^{3}H/^{14}C$ ratio of the cell-associated radioactivity [26,27]. Cells were incubated in the presence of 10% FCS in order to extract the released [^{14}C]oleate from the cells. Cells were incubated and harvested as described above. The percentage of hydrolyzed [^{14}C]COA and thus the percentage of liposome degradation was calculated by normalizing the $^{3}H/^{14}C$ ratio of the cell-associated radioactivity at each time point for the initial $^{3}H/^{14}C$ ratio using the equation:

$$\%\ \text{hydrolysis of } [^{14}C]COA = ((^{3}H/^{14}C \text{ ratio at } t = x)/(^{3}H/^{14}C \text{ ratio at } t = 0) - 1) \times 100 \quad (1)$$

2.8. Uptake and metabolism of immunoliposome-incorporated [^{3}H]FUdR-dP by CC531 cells

CC531 cells were incubated with [^{3}H]FUdR-dP incorporated in CC52-MPB liposomes, CC52-MPB-PEG liposomes, CC52-Hz-PEG liposomes or control liposomes without antibody (50 or 100 nmol lipid) or with free [^{3}H]FUdR (2 nmol) as described above. Radioactivity was measured in the medium, in the supernatant after trypsin treatment and centrifugation (trypsin-removable), and in the trypsin-treated cell pellet (non-removable) after lysing the cells with 10% SDS.

To distinguish between hydrophobic FUdR-dP and hydrophilic FUdR or metabolites (hydrolyzed prodrug), chloroform/methanol extractions were performed on samples of all three fractions. Briefly, 1 ml of sample was mixed with 2.5 ml methanol and 1 ml chloroform. Hereafter 1.25 ml of chloroform and 1.25 ml of PBS were added to this mixture and the resulting biphasic system was centrifuged for 10 min at $1500 \times g$. The chloroform and methanol/water layers were isolated, dried under vacuum and redissolved in 200 μl of chloroform/methanol (9:1, v/v) or 400 μl PBS, respectively. ^{3}H-Label was measured by liquid scintillation counting. The distribution of the ^{3}H-label in the different fractions, i.e., medium, trypsin-removable, trypsin-non-removable, organic phase and water phase, was calculated as a percentage of the total amount of label retrieved.

The intra- and extracellular concentrations of the active drug FUdR were calculated using the amounts of label that were water-soluble in the chloroform/methanol extractions of the cell fraction and the medium fraction, respectively. The volume of the incubation medium was 1.5 ml, the volume of the tumor cells was calculated assuming a spherical cell shape with an average diameter of 14 μm and assuming that 2 days after plating 1×10^{5} cells the cell number was 2×10^{5} (adherence for 24 h, doubling time of the cell line after adherence is approximately 1 day). Because of the absence of FCS during the incubations, the cells did not proliferate as evidenced by a constant level of cellular protein determined using the protein assay as described above. During the trypsin treatment for the removal of cell-bound liposomes, water-soluble label was leaking from the intracellular pool. The amount of water-soluble label detected in the cells was corrected for this trypsin-induced leakage by adding the released amounts to the intracellular amounts of water-soluble label actually measured, together representing the total fraction of water-soluble FUdR intracellularly.

2.9. Inhibition of endocytosis and intralysosomal degradation

CC531 cells were incubated with CC52-MPB liposomes (50 nmol lipid) containing [^{3}H]FUdR-dP for 3 h followed by a liposome-free incubation period of 0, 3 or 6 h in the absence of FCS. Effects of the following inhibitors of endocytosis and intralysosomal degradation on the hydrolysis of liposomal FUdR-dP were measured: colchicine (50 mM), an inhibitor of microtubule polymerization, cytochalasin B and D (20 μg/ml), both inhibitors of actin polymerization, the ionophore monensin (20 mM) an inhibitor of vesicular transport, chloroquine (50 and 100 mM) and NH_4Cl (10 and 20 mM), both lysosomotropic amines known to increase intralysosomal pH. Inhibitors were added 5 min prior to the addition of the liposomes. Except for the control incubations, inhibitors were present during the 3-h incubation with liposomes and during the liposome-free incubation time. Incubations were stopped by removing the incubation medium and subsequent washing with PBS. Radioactivity was determined in the medium and in the cells after lysis with NaOH (0.4 M). The percentage of label in the medium is taken as the percentage of hydrolyzed FUdR-dP.

2.10. Electron microscopy

CC531 cells were incubated with colloidal gold-containing CC52-MPB liposomes for 3 or 24 h without FCS. Incubations were stopped by removing the liposome-containing medium and subsequent washing with ice-cold PBS. The cells were fixed in situ in 2% glutaraldehyde in 0.1 M cacodylate buffer (v/v) during 2 h at 4°C. The cells were processed for resin histology employing postfixation in a 1:1 mixture (v/v) of OsO_4, 2 g/100 ml in distilled water and $K_3Fe(CN)_6$, 3 g/100 ml in 0.2 M cacodylate buffer, with pH 7.4, ethanol dehydration and embedded in Epon 812 epoxy resin. After polymerization sections with a thickness of 70–90 nm were cut with an ultramicrotome (LKB III Ultratome, LKB Producter, Stockholm, Sweden). Sections were collected on copper G200 grids and stained with aqueous uranyl acetate and lead citrate (Reichert Ultrastainer Leica, Rijswijk, The Netherlands) and examined at an accelerating voltage of 60 kV in a Philips EM201 transmission electron microscope (Philips Analytical, Electron Optics, Eindhoven, The Netherlands).

2.11. Statistics

Statistical significance of differences was evaluated by a two-tailed unpaired Student's *t*-test.

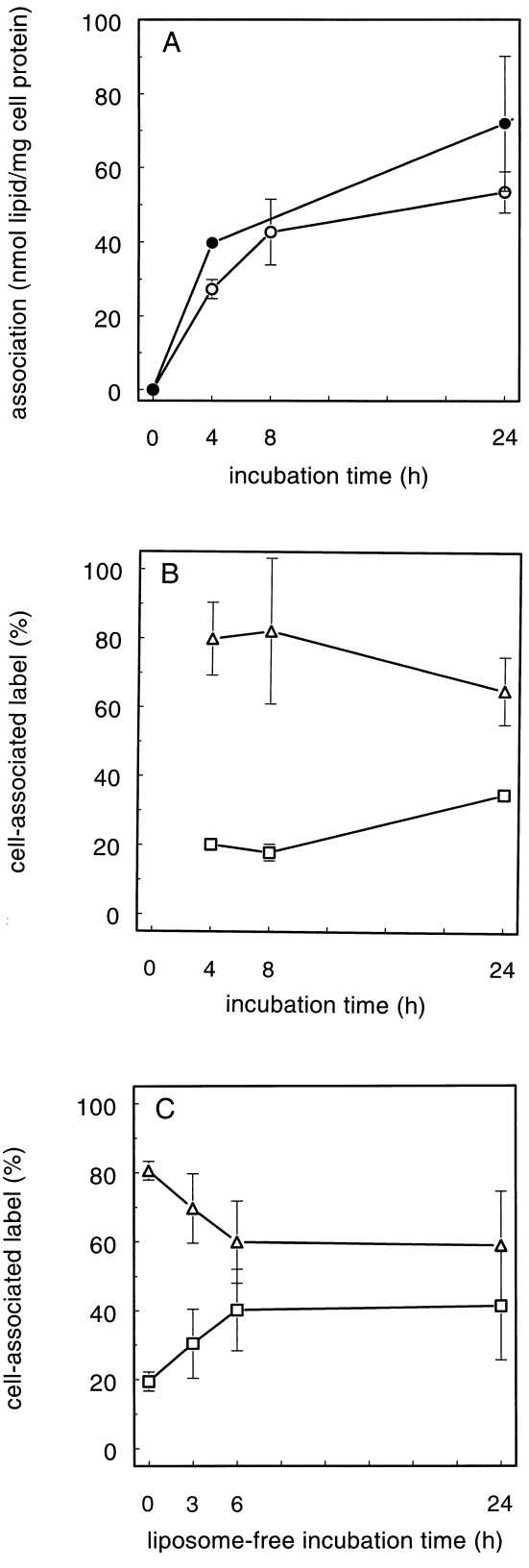


Fig. 1. Association of CC52-MPB liposomes with CC531 cells with time and the distribution in trypsin-removable or non-removable liposomes. (A) CC531 cells were incubated with CC52-MPB liposomes (100 nmol), labeled with [^{3}H]COE in the presence (●) or absence (○) of 10% FCS for the indicated periods ($n=3$). Cell-associated radioactivity was determined and normalized for the amount of cellular protein. (B,C) Distribution of cell-associated CC52-MPB liposomes in trypsin-removable (Δ) or non-removable (□) fractions. Cells were incubated with [^{3}H]COE-labeled immunoliposomes (B) continuous for 4, 8 or 24 h ($n=3$) or (C) for 3 h followed by a liposome-free incubation period of 0, 3 ($n=9$), 6 or 24 h ($n=6$), in the absence of FCS. At the end of the incubation, cells were treated for 10 min with trypsin at 37°C. Removable and non-removable fractions were separated by centrifugation as described in Section 2. Radioactivity was determined in both fractions and normalized for the amount of cellular protein.

3. Results

3.1. Association of [^{3}H]cholesterol ether-labeled immunoliposomes with CC531 cells

Fig. 1A presents the association of [^{3}H]COE-labeled immunoliposomes with CC531 cells in time in the presence or absence of 10% FCS. There was a steady increase in the amount of label associating with the cells over a period of up to 24 h, irrespective of the presence of FCS. In order to discriminate between bound and internalized liposomes, cells were incubated with trypsin. Fig. 1B shows that 4 to 8 h after incubation, around 20% of the label remained cell-associated after trypsin treatment and that this level slightly increased during 24 h. After a 3-h incubation with liposomes and a subsequent incubation without liposomes there was an increase of 20% in the amount of non-removable cell-associated liposomes and a concomitant decrease of immunoliposomes that could be removed by trypsin during the first 6 h (Fig. 1C). These observations may be explained by internalization of the liposomes or alternatively by an increase in the strength of the binding of immunoliposomes with the tumor cells in time, reducing the amount of liposomes removable by the trypsin treatment. Evidence compatible with the latter explanation is shown in Table 1. Incubation of CC531 cells with CC52 liposomes for 3 h at 4°C resulted in a significantly lower amount of immunoliposomes binding to the tumor cells, whereas a higher amount of the associated label was removable by trypsin than upon incubation at 37°C, suggesting a weaker interaction of immunoliposomes with tumor cells at 4°C resulting in turn in more effective removal by trypsin.

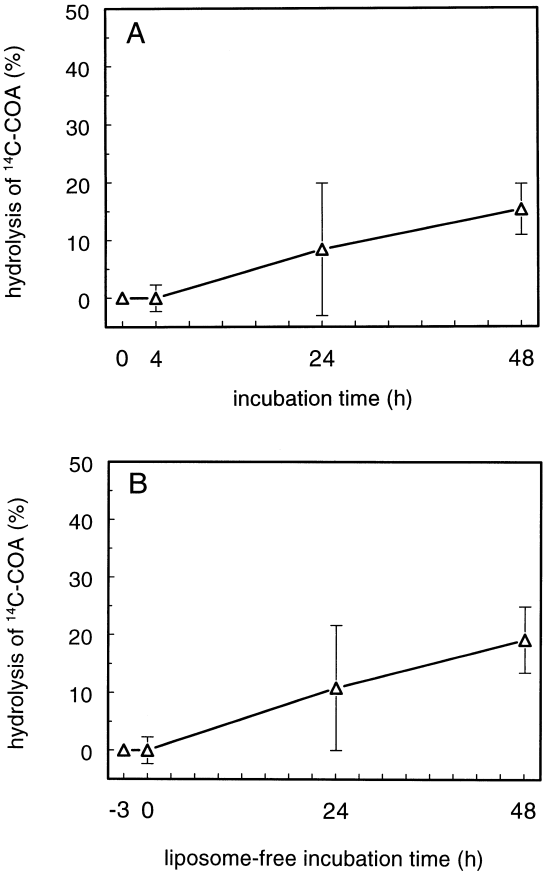


Fig. 2. Hydrolysis of immunoliposome-incorporated cholesteryl-[^{14}C]oleate by CC531 cells as a measure of liposome uptake and degradation. Tumor cells were incubated continuously (A) or for 3 h followed by a liposome-free incubation time (B) in the presence of 10% FCS with 100 nmol of immunoliposomes double-labeled with [^{3}H]COE, a non-degradable marker, and [^{14}C]COA, an intracellularly degradable marker, which after degradation results in a release of [^{14}C]oleic acid into the medium and a subsequent increase in the ^{3}H/^{14}C ratio of the cell-associated radioactivity. From the increase in isotope ratio the percentage of hydrolysis was calculated according to Eq. 1 from Section 2 ($n=5$).

Table 1
Influence of temperature on the interaction between [^{3}H]COE-labeled immunoliposomes and tumor cells

Liposome	Fraction	37°C	4°C	P value
CC52-MPB-PEG	Total cell-associated liposomes (nmol/mg)	3.72 ± 1.32	0.53 ± 0.19	0.0005
	% Removable	84.0 ± 4.3	94.2 ± 3.4	0.004
	% Non-removable	16.0 ± 4.3	5.8 ± 3.4	0.004

CC531 cells were incubated for 3 h at 4°C or 37°C with 100 nmol of immunoliposomes ($n=6$). The amount of cell-associated CC52-MPB-PEG liposomes was determined and normalized for the amount of cellular protein. The distribution in trypsin-removable or non-removable fractions is presented as a percentage of total label (± S.D.).

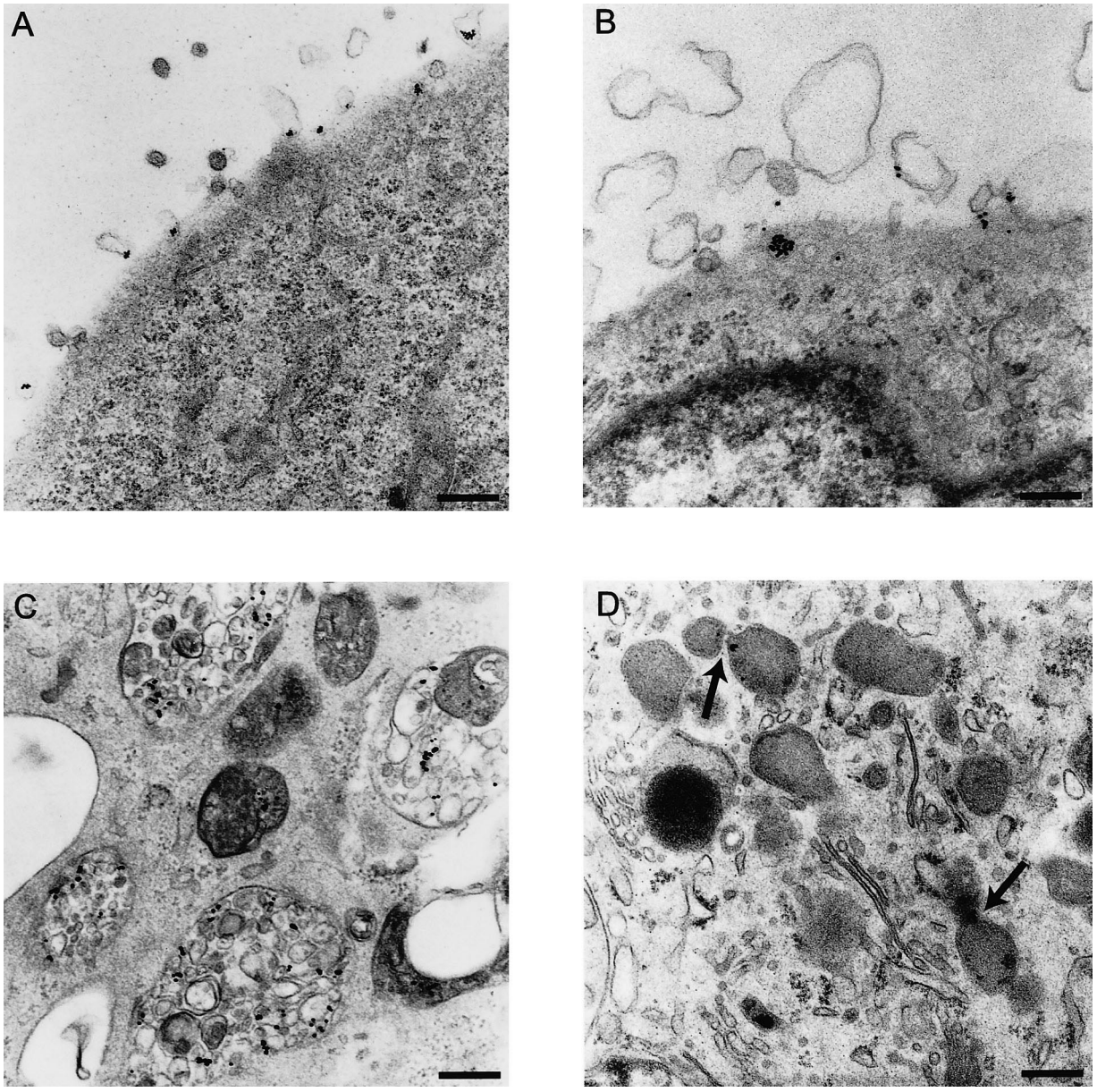


Fig. 3. Transmission electron micrographs of thin sections of cultured CC531 cells incubated with CC52-MPB-PEG liposomes containing colloidal gold as an encapsulated marker. (A) Binding of colloidal gold-containing CC52 immunoliposomes to the cell surface of a CC531 cell after a 3-h incubation, representing a typical example of the morphology after a 3-h incubation (bar = 300 nm). (B) CC531 cell with cell surface-bound gold-containing immunoliposomes and gold grains present in an early endosome (bar = 300 nm). (C) CC531 cell with internalized colloidal gold particles associated with liposome-like structures in an endosomal compartment of the cell (bar = 200 nm). (D) Colloidal gold present in lysosomes (indicated by the arrows). The latter two panels represent an only occasionally observed internalization of immunoliposomes 24 h after incubation with CC52 immunoliposomes (bar = 200 nm).

The presence of FUdR-dP in the bilayer of the immunoliposomes had no effects on the association of immunoliposomes with CC531 cells under any of the conditions applied (not shown).

3.2. Degradation of immunoliposomes

The degradation of CC52-PC/Chol liposomes by CC531 colon cancer cells was measured by using

double-labeled liposomes containing the metabolically inert cholesterol ether, [^{3}H]COE, and the metabolizable cholesterol ester, [^{14}C]COA. If the liposome encounters a cellular compartment containing hydrolytic enzymes, the cholesterol ester will be hydrolyzed. The ^{14}C-labeled oleate will be released and can leave the cell, thus causing an increase in the cellular ^{3}H/^{14}C ratio. From the increase in isotope ratio the percentage of hydrolysis of [^{14}C]COA and thus of the liposomes was calculated.

During a continuous incubation with immunoliposomes (Fig. 2A), as well as after a 3-h pre-incubation period with immunoliposomes followed by a liposome-free incubation period (Fig. 2B), only low levels of hydrolysis of the [^{14}C]COA were observed. During a 48-h continuous incubation or during 48 h following a 3-h preincubation period, hydrolysis of the cell-associated cholesterol ester amounted to less than 20%, i.e., a rate of hydrolysis of less than 0.5% per hour.

3.3. Interaction of colloidal gold-containing immunoliposomes with CC531 cells

Immunoliposomes, containing colloidal gold as an encapsulated marker, showed substantial binding to CC531 cells after a 3-h incubation as visualized by transmission electron microscopy (Fig. 3A). A vast majority of the cells were observed to have only cell surface-bound liposomes after a 3 or 24 h incubation with CC52-MPB liposomes. Only occasionally internalization of liposomal gold grains was observed; after a 3-h incubation this amounted to a few single gold particles in an early endosomal compartment (Fig. 3B) and after a 24-h incubation, in a few cells multiple gold particles were observed associated with membrane structures contained within endosomal compartments (Fig. 3C) or gold grains present in lysosomes (Fig. 3D). The internalization of immunoliposomes by only a fraction of the tumor cells in these morphological studies conceivably is reflected in low levels of hydrolysis of immunoliposomal cholesterol ester we observed in Fig. 2.

3.4. Immunoliposome-incorporated [^{3}H]FUdR-dP

The metabolic fate of the liposomal FUdR-dP was studied by monitoring the ^{3}H-label in the uracil moiety of FUdR-dP.

In contrast to our observations on the cholesterol ester, the FUdR-dP in CC52 immunoliposomes was rapidly hydrolyzed by the tumor cells (Fig. 4). Within 6 h more than 50% of the FUdR-dP associating with the tumor cells during a 3-h preincubation, was

Table 2
Fractionation of ^{3}H-label in chloroform- and water-soluble fractions after incubation of CC531 cells with [^{3}H]FUdR-dP-containing immunoliposomes

Fraction	Time after incubation (h)	Percentage of chloroform-soluble label	Percentage of water-soluble label
Medium	0	0	0
	3	17.0 ± 11.6	83.0 ± 11.6
	6	12.6 ± 9.2	87.4 ± 9.2
	24	17.2 ± 1.8	82.8 ± 1.8
Removable	0	86.9 ± 9.1	13.1 ± 9.1
	3	91.9 ± 2.0	8.1 ± 2.0
	6	89.9 ± 5.8	10.1 ± 5.8
	24	88.6 ± 4.4	11.4 ± 4.4
Non-removable	0	55.3 ± 8.2	44.7 ± 8.2
	3	55.2 ± 18.1	44.8 ± 18.1
	6	52.2 ± 4.9	47.8 ± 4.9
	24	42.3 ± 3.1	57.7 ± 3.1

After incubating CC531 cells for 3 h with [^{3}H]FUdR-dP-containing immunoliposomes followed by a liposome-free incubation time of 0, 3, 6 (n = 8) or 24 h (n = 3), medium and cell-associated (trypsin-removable or non-removable) samples were fractionated by chloroform/methanol extractions as described in Section 2. Data are expressed as percentage of retrieved label in either of the fractions (± S.D.)

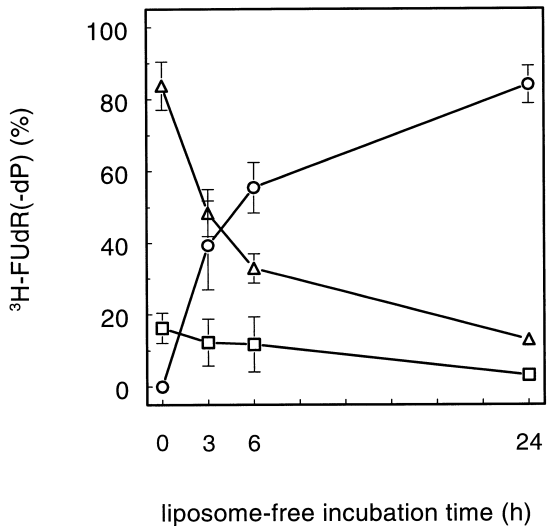


Fig. 4. Metabolic fate of [^{3}H]FUdR-dP incorporated in CC52-MPB liposomes during interaction with CC531 cells. Distribution of ^{3}H-label in medium (○), trypsin-removable (△) or non-removable (□) fractions after a 3-h incubation of CC531 cells with [^{3}H]FUdR-dP-containing CC52-MPB liposomes followed by a liposome-free incubation time, both in the absence of FCS ($n=8$).

hydrolyzed as shown by the release of ^{3}H-label in the medium, increasing to 80% in 24 h. As hydrolysis proceeded, the amount of trypsin-removable label decreased from more than 80% at the start of the incubation to less than 20% after 24 h. The non-removable label decreased in the same time from 20% to 7%.

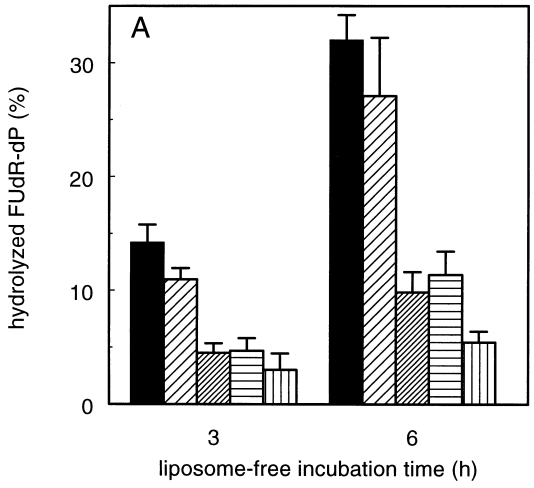
We further analyzed the nature of the cell-associated label, both trypsin-removable and non-removable, as well as the released radioactivity by fractionating these into chloroform- and water-soluble fractions so as to distinguish between the initial chloroform-soluble prodrug and its water-soluble metabolites (Table 2). Eighty to 90% of the label in the medium was water-soluble, representing completely deacylated prodrug; the remaining 10% to 20% was chloroform-soluble, representing intact prodrug. By contrast, as much as 90% of the cell-associated label that was trypsin-removable, was retrieved in the chloroform phase, representing liposomal FUdR-dP associated with cell-surface-bound liposomes. Only a small fraction of the trypsin-removable label (around 10%) was water-soluble, representing intracellularly formed FUdR, which was released from the cells due to the treatment with trypsin. Of the label that remains cell-associated after trypsin treatment, 42% to 55% was present in the organic phase, representing di- and/or mono-acylated FUdR, while 45% to 58% was detected in the water phase, representing intracellular FUdR or metabolites of FUdR. The amount of chloroform-soluble label tended to decrease in time, indicating the continuous hydrolysis of the prodrug. The presence of FCS had no effect on the results (not shown).

Within 24 h most of the immunoliposomal FUdR-dP which was associated with the cells during a 3-h incubation had been hydrolyzed to water-soluble FUdR (Fig. 4 and Table 2) of which the major part (76–82%) was detected outside the cells and a smaller amount (18–24%) inside the cells (Table 3). Taking into consideration the large difference between the intra- and extracellular volumes (approximately 5×10^4-fold) we can calculate that, depending on the incubation time, the intracellular FUdR-concentration was as much as 700–3000-fold higher than that in the medium (Table 3). The difference between the amounts of prodrug that were delivered to the tumor cells by immunoliposomes and by liposomes without antibody is presented in Table 4. With im-

Table 3
Distribution of hydrolyzed [^{3}H]FUdR-dP from CC52-MPB-liposomes between medium and cell fractions

Time after incubation (h)	Percentage of FUdR in medium	Percentage of FUdR intracellular	Ratio intracellular/extracellular FUdR-concentration
3	76.5 ± 2.9	23.5 ± 2.9	2979.9 ± 888.3
6	81.9 ± 9.4	18.1 ± 9.4	1747.3 ± 208.8
24	95.0 ± 1.2	5.0 ± 1.2	784.2 ± 163.3

Water-soluble ^{3}H-label in medium and cells was determined by chloroform/methanol extractions after incubating cells for 3 h with liposomes followed by a liposome-free incubation time of 3, 6 ($n=6$) or 24 h ($n=3$). Ratio of FUdR-concentration in cells vs. medium was calculated from the water-soluble ^{3}H-label determined in medium and cells, assuming the presence of 2×10^5 cells with an average diameter of 14 μm.



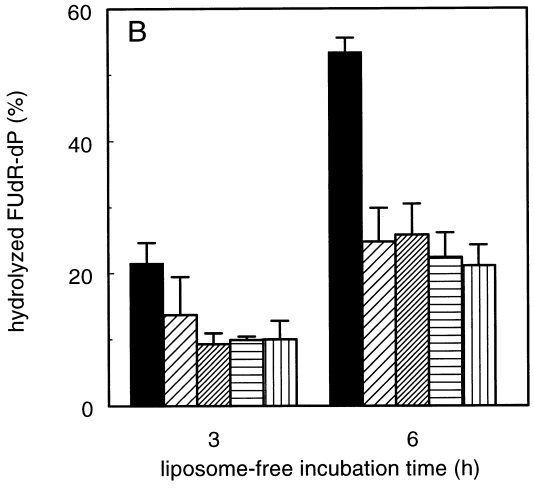


Fig. 5. Effect of inhibitors of endocytic uptake (A) or intralysosomal degradation (B) on the hydrolysis of [^{3}H]FUdR-dP from CC52-MPB liposomes by CC531 cells. CC531 cells were incubated for 3 h with 50 nmol liposomes containing [^{3}H]FUdR-dP followed by a liposome-free incubation time of 3 or 6 h, both in the absence of FCS. The percentage of label in the medium is taken as the percentage of hydrolyzed FUdR-dP. (A) Incubations (all $n=6$): control (filled bars), colchicine (wide cross-hatched bars), monensin (narrow cross-hatched bars), cytochalasin B (horizontally hatched bars), cytochalasin D (vertically hatched bars). (B) Incubations (all $n=6$): control (filled bars), NH_4Cl (10 mM) (wide cross-hatched bars), NH_4Cl (20 mM) (narrow cross-hatched bars), chloroquine (50 mM) (horizontally hatched bars) and chloroquine (100 mM) (vertically hatched bars).
←

munoliposomes, a 30-fold higher level of [^{3}H]FUdR-dP was associated with CC531 cells after 3 h incubation as compared to liposomes without antibody. This difference in the delivery of the prodrug was shown before to result in a much higher antiproliferative activity of immunoliposomal FUdR-dP than FUdR-dP in liposomes without antibody [13].

3.5. Effect of inhibitors

In order to gain further insight into the mechanism of interaction of the liposomal prodrug with the cells, we determined the effects of inhibitors of endocytosis and intralysosomal degradation on the hydrolysis of [^{3}H]FUdR-dP incorporated in immunoliposomes (Fig. 5). Except for colchicine, all inhibitors of endocytosis resulted in a substantial inhibition of the hydrolysis of FUdR-dP (Fig. 5A). Inhibition varied from about 65% for monensin to more than 80% for cytochalasin D. At 4°C there was no detectable hydrolysis (not shown). Inhibition of intralysosomal degradation with NH_4Cl or chloroquine also resulted in major inhibition of the hydrolysis of FUdR-dP (Fig. 5B).

Table 4
Efficiency of delivery of FUdR-dP to CC531 cells by immunoliposomes vs. control liposomes without antibody

Time after incubation (h)	Cell-associated FUdR(-dP) (pmol/mg cell protein)	
	MPB liposomes	CC52-MPB liposomes
0	3.6 ± 0.9	100.4 ± 5.7
3	3.1 ± 1.1	78.6 ± 3.0
6	4.0 ± 1.3	59.1 ± 3.0

Cell-associated ^{3}H-label was measured in time after a 3-h incubation with [^{3}H]FUdR-dP incorporated in CC52-MPB liposomes ($n=12$) or MPB liposomes ($n=6$) without antibody.

Fig. 6. Effect of attachment of PEG chains to the surface of immunoliposomes with antibody coupled either directly to the liposomal surface or to the distal end of the PEG chains on the metabolic fate of liposome incorporated [^{3}H]FUdR-dP. (A) Association of ^{3}H-labeled drug with CC531 cells after incubating cells with [^{3}H]FUdR-dP incorporated in CC52-MPB liposomes (159 μg CC52/μmol lipid) (○), CC52-MPB-PEG liposomes (32 μg CC52/μmol lipid) (△) or CC52-Hz-PEG liposomes (13 μg CC52/μmol lipid), or by adding free [^{3}H]FUdR (□). Cells were incubated with 50 nmol immunoliposomes containing FUdR-dP or the equivalent amount of free drug (2 nmol) for 3 h, followed by a drug-free incubation time of 3 or 6 h, both in the absence of 10% FCS ($n=6$). (B) Hydrolysis of [^{3}H]FUdR-dP from CC52-MPB liposomes (○), CC52-MPB-PEG liposomes (△) or CC52-Hz-PEG liposomes (▲). ^{3}H-Label in the medium was determined after a 3-h incubation with liposomes followed by liposome-free incubation period ($n=6$). (C) Rate of hydrolysis of cell-bound immunoliposomal [^{3}H]FUdR-dP from CC52-MPB liposomes (○), CC52-MPB-PEG liposomes (△) or CC52-Hz-PEG liposomes (▲) in time after a 3-h incubation period as revealed by the appearance of ^{3}H-label in the medium, plotted as a percentage of the total label retrieved ($n=6$).

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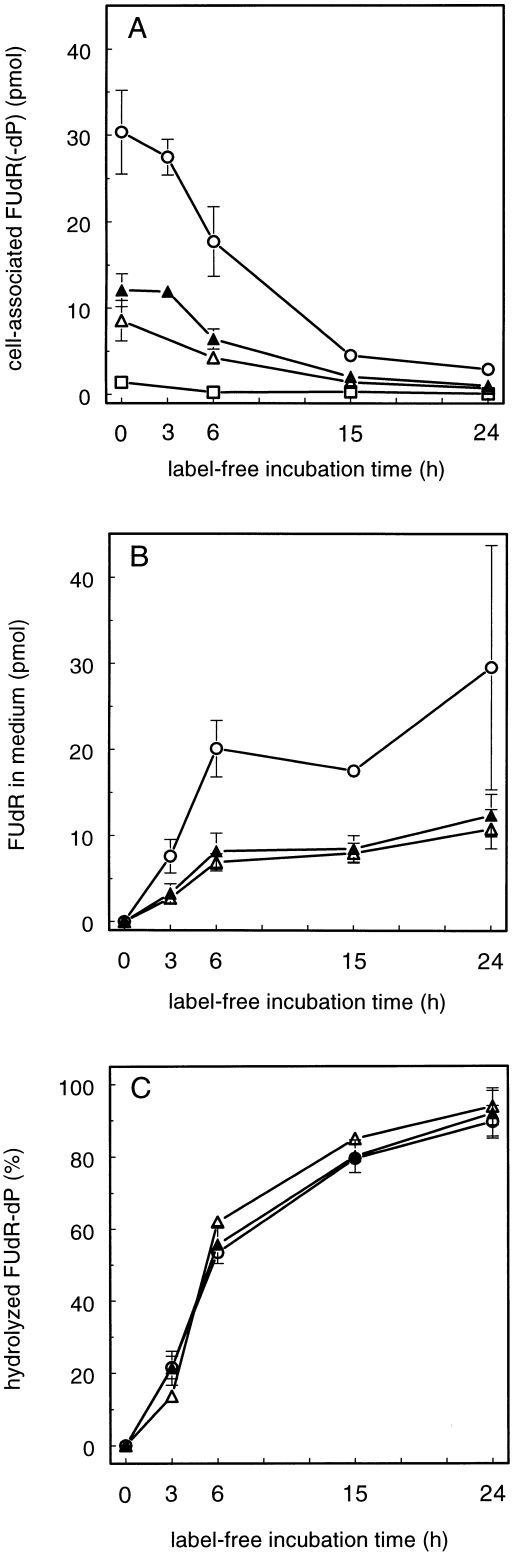

3.6. *Effects of PEG*

The efficiency of delivering the drug to the tumor cells by immunoliposomes with or without PEG or by adding free FUdR was followed in time after a 3-h preincubation period with liposomal [^{3}H]FUdR-dP or free [^{3}H]FUdR.

Fig. 6A shows that PEG, either in the case that antibody is coupled directly to the bilayer or when attached at the distal end of the PEG chain, has an inhibitory effect on the amount of prodrug associating with the cells. Nonetheless, the two PEG immunoliposomes are still far more efficient in delivering the drug than free FUdR or as compared to liposomes without antibody as is shown in Table 4. The decrease in the amount of cell-associated label in time was caused by the ongoing hydrolysis of FUdR-dP by the tumor cells and a subsequent release of drug in the medium (Fig. 6B), demonstrating that the FUdR-dP delivered to the tumor cells by PEG immunoliposomes was hydrolyzed. Fig. 6C shows that there was virtually no difference between immunoliposomes with and without PEG in the rate of intracellular hydrolysis of [^{3}H]FUdR-dP by the tumor cells, as revealed by the rates of appearance of label in the medium. These results indicate that there is no difference between the three immunolipo-

somal formulations in the way they deliver the drug to the tumor cells.

4. Discussion

In this report we address the mechanism by which an immunoliposomal drug delivery system for the anticancer drug FUdR is able to deliver FUdR intracellularly into colon cancer cells.

The interaction of CC52 immunoliposomes with CC531 cells resulted in degradation of less than 20% of the liposomes associating with the cells within 48 h, as shown by the hydrolysis of liposomal cholesterol ester (Fig. 2A,B). Morphological studies with colloidal gold-containing immunoliposomes revealed that, in a vast majority of the tumor cells, the immunoliposomes remain bound at the cell surface without internalization, although occasionally cells were observed with liposomal gold grains inside. Thus, the internalization of limited amounts of immunoliposomes by the CC531 cells, as revealed by the limited hydrolysis of cholesterol ester, is most likely due to heterogeneity in the cell population.

The low level of internalization of immunoliposomes may explain the fraction of immunoliposomes that cannot be removed by trypsin treatment (Fig. 1B,C). An alternative or complementary explanation for this effect is an increase in the strength or multivalency of the binding of the immunoliposomes to CC531 cells in time. Evidence pointing in this direction was obtained, indirectly, from incubations performed at 4°C. Immunoliposomes bound to CC531 cells at 4°C were removed by trypsin treatment more effectively than immunoliposomes bound at 37°C. Such difference in interaction between 4°C and 37°C may find its origin in the rigidifying effect of the low temperature on either the plasma membrane or the liposomal bilayer. This may restrict intrabilayer mobility of phospholipid-anchored antibodies and likely also of cell-surface antigens, resulting in fewer antibody molecules interacting with antigens and consequently in increased amounts of liposomes removed by trypsin at 4°C. Decreased intrabilayer movement of liposome-associated molecules in solid-type liposomes was proposed before to result in a decrease of the valency of interaction of liposomes with cells [28]. In view of the generally accepted non-exchangeability of cholesterol esters and ethers [29], we consider it unlikely that exchange of these labels between immunoliposomes and plasma membrane significantly contributes to the phenomena described.

Although internalization of immunoliposomes by tumor cells has been reported, quantities are mostly low [8,11,30]. Other reports in literature describe a complete lack of uptake of immunoliposomes [9,12]. The type of antigen that is targeted seems to play a minor role in determining whether or not the liposomes are taken up. For instance, immunoliposomes targeted to the HER-2 antigen were reported to be efficiently internalized by human breast cancer cells [9,30], less efficiently by MKN-7 human gastric or SKOV-3 ovarian carcinoma cells [30] and not at all by N-87 human gastric carcinoma cells [9]. Mechanisms triggering such uptake of immunoliposomes are still not fully understood.

In contrast to the liposomal cholesterol ester, the immunoliposome-incorporated lipophilic prodrug FUdR-dP is readily and almost completely hydrolyzed by the tumor cells, showing that uptake of the prodrug proceeds independent of internalization of the immunoliposomes as such. Within 24 h virtually all immunoliposomal FUdR-dP that was cell-associated after a 3-h incubation period, is hydrolyzed by the tumor cells (Fig. 4 and Table 2). FUdR from hydrolyzed FUdR-dP appears predominantly in the medium (80–90%), while 10–20% remains intracellular. However, when comparing the extra- and intracellular FUdR-concentrations, it appears that the intracellular concentration reaches 700- to 3000-fold higher levels than that in the medium. It remains to be seen how much of the intracellular FUdR is present as free drug, because FUdR or metabolites can be incorporated into DNA and RNA [31] or associate with thymidylate synthase [32]. In a previous study we have shown that the intracellular FUdR is effective in inhibiting CC531 cell growth [13].

The high intracellular concentration of hydrolyzed prodrug strongly suggests that the hydrolysis process occurs rapidly and is situated intracellularly. This is confirmed by the strong inhibition of hydrolysis of immunoliposomal FUdR-dP by a host of inhibitors of endocytosis or intralysosomal degradation. Colchicine had no significant inhibitory effect on the hydrolysis of FUdR-dP, indicating that microtubules

appear to have no function in this process. It has been shown before that colchicine and other microtubule-depolymerizing drugs have only minor effects on the uptake of immunoliposomes by Kupffer cells [33] and on the degradation of internalized proteins in a human carcinoma HEp-2 [34].

Taken together, these results lead us to postulate a selective transfer mechanism for immunoliposomal FUdR-dP. During the antibody–antigen-mediated interaction of CC52 liposomes and CC531 cells, FUdR-dP presumably is transferred from the liposomal bilayer to the plasma membrane of tumor cells, internalized by constitutive endocytic or pinocytic processes and hydrolyzed intralysosomally. Subsequently, the hydrolyzed prodrug diffuses into the cytoplasm from where it is either released into the extracellular compartment (medium) or utilized intracellularly for incorporation into nucleic acids. In both cases the drug can exert its cytotoxic effect: when remaining intracellular, it will have detrimental effects on DNA and RNA [31] and will inhibit DNA-synthesis by inhibiting thymidylate synthase [32]. Drug that is released from the tumor cell, in which it is hydrolyzed, can diffuse into surrounding tumor cells. This will constitute a favorable condition especially in case tumor cells are present that do not express the antigen on their surface, resulting in a so-called bystander effect. Hydrolyzed FUdR-dP released by Kupffer cells was shown before to display antitumor activity against C26 murine colon carcinoma [19].

The lipophilic prodrug FUdR-dP was proposed earlier to transfer from the bilayer of immunoliposomes to the plasma membrane of lung-endothelium cells as a result of the antibody–antigen-mediated interaction of the liposomes with the endothelial cells [15]. In a comparable way phosphatidylcholine transfer from liposomes containing the ganglioside GM_1 or lactosylceramide to hepatocytes was observed [35] and proposed to be mediated by an enhanced interaction between the liposomes and the hepatocytes [36]. Also, cellular uptake of a liposome-associated lipophilic derivative of methotrexate has been reported suggesting a mechanism of liposomal drug uptake without the need of internalization of the entire liposome [37,38].

Neither the rate of transfer of FUdR-dP nor the hydrolysis of cell-associated FUdR-dP is hampered by the presence of 4 mol% PEG_{2000} on the surface of CC52-MPB liposomes or by the coupling of antibody to the distal end of the PEG chains in the case of CC52-Hz-PEG liposomes (Fig. 6C). The lower levels of cell-associated FUdR-dP (Fig. 6A) and the concomitantly lower amounts of hydrolyzed prodrug in the medium (Fig. 6B) after incubations with PEG immunoliposomes can be explained by a lower amount of PEG immunoliposomes binding to the tumor cells. This, in turn, is most likely caused by the lower antibody density on the surface of PEG liposomes as mentioned in the method section. Still, PEG immunoliposomes much more efficiently delivered FUdR-dP to the tumor cells than liposomes without antibody (Table 4) or free FUdR (Fig. 6A). This is a relevant consideration in view of the preferred use in vivo of long-circulating PEG liposomes. Combined with the unaffected rate of hydrolysis of FUdR-dP in PEG immunoliposomes compared to non-PEG immunoliposomes, it illustrates that much higher intracellular levels of FUdR can be attained by PEG immunoliposomes than by liposomes without antibody or by free FUdR.

At present, we can only speculate on the mechanism of this transfer. Direct transfer from liposome bilayer to plasma membrane seems unlikely, since the presence of both the antigen–antibody bridge and the 7-nm PEG chains are expected to maintain a substantial distance between the two bilayers. Moreover, when the antibody is coupled at the distal end of the PEG chain, resulting in an even larger distance between the bilayers upon antibody–antigen interaction, we see no difference in prodrug transfer. Transfer through the aqueous phase would therefore seem to be more likely. The rate-limiting step in that case would be the release of the prodrug from the liposomal bilayer. The influence of serum factors on this process can be abolished as we showed previously; in non-immunoliposomes the prodrug remains tightly associated with the liposomes, even during prolonged incubation at 37°C in plasma [39]. These observations are in line with the lack of influence of serum on the results presented in Table 2 and Fig. 4 as emphasized in Section 3.4. Presumably, the antibody–antigen interaction on the cell surface results in conditions less favorable for the prodrug molecule to remain accommodated in the liposomal membrane.

The drug delivery mechanism described in this paper combines efficient delivery with the lack of need for internalization of the liposomes and provides an efficient system for the delivery of FUdR to colon cancer cells. It may also serve as a model system for the development of new (targetable) liposomal drug delivery systems for non-phagocytic cells by exploiting the liposomal bilayer for incorporation of lipophilic drugs or prodrugs.

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