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Comparative studies of the preparation of immunoliposomes with the use of two bifunctional coupling agents and investigation of in vitro immunoliposome-target cell binding by cytofluorometry and electron microscopy

R.A. Schwendener¹, T. Trüb¹, H. Schott², H. Langhals³, R.F. Barth⁴, P. Groscurth⁵
and H. Hengartner¹

¹ Institute of Pathology, Department of Experimental Pathology, University Hospital, Zurich (Switzerland), ² Institute of Organic Chemistry, University of Tübingen, Tübingen (F R G), ³ Institute of Organic Chemistry, University of Munich, München (F R G), ⁴ Department of Pathology, The Ohio State University, Columbus, OH (U S A) and ⁵ Institute of Anatomy, Division of Cell Biology, University of Zurich, Zurich (Switzerland)

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The two coupling agents SPDP (*N*-succinimidyl-3-(2-pyridyldithio)propionate) and SATA (*N*-succinimidyl-*S*-acetylthioacetate) were compared in their efficiency and feasibility to couple monoclonal antibodies (Abs) via thioether linkage to liposomes functionalized by various lipophilic maleimide compounds like *N*-(3-maleimidopropionyl)-*N*²-palmitoyl-L-lysine methyl ester (MP-PL), *N*-(3-maleimidopropionyl)phosphatidylethanolamide (MP-PE), *N*⁶-(6-maleimidocaproyl)-*N*²-palmitoyl-L-lysine methyl ester (EMC-PL), and *N*-(6-maleimidocaproyl)phosphatidylethanolamine (EMC-PE). The composition of the liposomes was soy phosphatidylcholine (SPC), cholesterol, maleimide compounds and α -tocopherol (1:0.2:0.02:0.01, mol parts), plus *N*⁴-oleylcytosine arabinoside (NOAC) as cytostatic prodrug (0.2 mol parts) and a new, lipophilic and highly fluorescent dye *N,N'*-bis(1-hexylheptyl)-3,4:9,10-perylenebis(dicarboximide) (BHPD, 0.006 mol parts). From the maleimide derivatives MP-PL was the most effective in terms of preservation of the coupling activity in dependence of liposome storage. The coupling of the monoclonal A B8-24.3 (mouse IgG2b, MHC class I, anti H-2k^b) and IB16-6 (rat IgG2a, anti B16 mouse melanoma) to the drug carrying liposomes was more effective and easier to accomplish with SATA as compared to SPDP. Coupling rates of 60–65% were obtained with SATA at molar ratios of 12 SATA:1 Ab:40 maleimide spacer groups on the surface of one liposome. The highest coupling rates with SPDP were obtained at the ratio of 24 SPDP:1 Ab:40 liposomal maleimide groups, with an Ab binding efficiency of only 20–25%. The optimal in vitro binding conditions to specific target cells (EL4 for B8-24.3-liposomes and B16-F10 for IB16-6-liposomes) were determined by cytofluorometric measurement of the liposomal BHPD fluorescence with SATA linked Abs. Optimal immunoliposome binding to specific epitopes on the target cells was achieved with 1–2 Ab molecules coupled to one liposome, with immunoliposome concentrations of 20–130 nM and with a small incubation volume of 0.3–0.4 ml. The specificity of the binding of B8-24.3-liposomes to EL4 target cells was visualized by scanning electron microscopy. Antibody mediated endocytic uptake of immunoliposomes could be demonstrated by transmission electron microscopy.

Abbreviations Ab(s), antibody(ies), Ab-ATA, SATA-modified Ab, Ab-A-SH, activated SATA-modified Ab, Ab-PDP, SPDP-modified Ab, Ab-P-SH, activated SPDP-modified Ab, BHPD, *N,N'*-bis(1-hexylheptyl)-3,4:9,10-perylenebis(dicarboximide), FITC, fluorescein isothiocyanate, DTNB, 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), DTPA-SA, diethylenetriaminopentaacetic acid monostearate, EMCS, *N*-(6-maleimidocaproyloxy)succinimide, EMC-PE, *N*-(6-maleimidocaproyloxy)phosphatidylethanolamine, EMC-PL, *N*⁶-(6-maleimidocaproyloxy)-*N*²-palmitoyl-L-lysine methyl ester, IMDM, Iscove's modified Dulbecco medium, ³H-NSP, *N*-succinimidyl[2,3-³H]propionate, ³H-PC, 1,2-dipalmitoyl-L-3-phosphatidyl[*N*-methyl-³H]choline, MP-PL, *N*⁶-(3-maleimidopropionyloxy)-*N*²-palmitoyl-L-lysine methyl ester, MPS, *N*-(3-maleimidopropionyloxy)succinimide, NOAC, *N*⁴-oleylcytosine arabinoside, PE, phosphatidylethanolamine, PL, *N*²-palmitoyl-L-lysine methyl ester HCl, SATA, *N*-succinimidyl-*S*-thioacetate, SEM, scanning electron microscopy, SPC, soy phosphatidylcholine, SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate, TEM, transmission electron microscopy, Tris, 2-amino-2-hydroxymethylpropane-1,3-diol

Correspondence R.A. Schwendener, Institute of Pathology, Department of Experimental Pathology, University Hospital, CH-8091 Zurich, Switzerland

Introduction

The targeted delivery of drug-carrying liposomes to specific sites in the organism requires the attachment of molecules like Abs or their fragments to the surface of the liposomes. Such immunoliposomes recognize specific epitopes on the target cell. The concept of immunoliposome-mediated drug targeting involves the combination of liposome preparation techniques, Ab coupling methods, the detection of specific binding to the target and the demonstration of a pharmacological effect on the target cell. The stepwise improvement of such complex systems remains the subject of ongoing and future investigations [1,2].

Most of the concepts of preparation of immunoliposomes which contain cytostatic drugs remain at the level of basic development. In view of a therapeutic application we intend to establish methods which allow the preparation of stable, non-toxic unilamellar immunoliposomes on a preparative scale. Bearing these aims in mind, we devised in this study effective antibody coupling methods.

The covalent coupling of Abs, Ab fragments or peptides to ligands which are located on the surface of liposomes can be achieved by numerous chemical methods [3–16].

Bifunctional ligands have become useful tools for the coupling of proteins to liposomes. Among a variety of such molecules, *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) is currently one of the most used linkers, although it has a number of disadvantages like its susceptibility to traces of reducing agents and the short half-life of the reactive sulfhydryl groups [16]. *N*-Succinimidyl-*S*-acetylthioacetate (SATA) is an alternative coupling agent [16].

In this work we present the results that were obtained by the direct comparison of Ab coupling methods using SPDP and SATA as linkers to liposomes which contained *N*⁴-oleylcytosine arabinoside (NOAC), a lipophilic derivative of arabinosylcytosine, as cytostatic prodrug [17,18]. In particular, the coupling of the two Abs, B8-24.3 specific for the mouse MHC class I H-2k^b surface antigen on EL4 mouse T lymphoma cells and IB16-6, specific for a surface antigen on B16 mouse melanoma cells, to small unilamellar drug carrying liposomes was investigated. In addition, the new highly fluorescent lipophilic dye *N,N'*-bis(1-hexylheptyl)-3,4,9,10-perylenebis(dicarboximide) (BHPD) was used as fluorescence label for the liposomes.

Materials and Methods

Materials

The lipid soy phosphatidylcholine (SPC, Epikuron 200) was obtained from L. Meyer, Hamburg, F.R.G.

Phosphatidylethanolamine (PE) was from Lipid Products, S. Nutley, Surrey, U.K. Cholesterol, 3-maleimidopropionic acid, 6-maleimidocaproic acid, *N*-(3-maleimidopropionyloxy)succinimide, *N*-(6-maleimidocaproyloxy)succinimide, Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid), DTNB), dithiothreitol and *N*-ethylmaleimide were from Fluka, Buchs, Switzerland. *N*-(Succinimidyl-3-(2-pyridylthio)propionate (SPDP) and Sephadex G-50 medium were from Pharmacia. Metrizamide was obtained from Sigma, St. Louis, MO, U.S.A. All analytical grade buffer compounds, sodium cholate, DL- α -tocopherol, silica gel 60 and the organic solvents were from Merck, Darmstadt, F.R.G.

The radioactive compounds *N*-succinimidyl[2,3-³H]propionate (³H-NSP, 2.8–4.1 TBq/mmol), 1,2-dipalmitoyl-L-3-phosphatidyl[*N*-methyl-³H]choline, (³H-PC, 1.5–3 Bq/mmol) and manganese chloride (⁵⁴MnCl₂) were from Amersham Int., Amersham, U.K.

*N*²-Palmitoyl-L-lysine methyl ester HCl (PL) was synthesized as described [15]. The lipophilic maleimide derivatives *N*⁶-(3-maleimidopropionyl)phosphatidylethanolamide (MP-PE) and *N*⁶-(6-maleimidocaproyl)phosphatidylethanolamine (EMC-PE) were prepared in analogy to the published synthesis of *N*-(4-(*p*-maleimidophenyl)butyryl)phosphatidylethanolamine [4]. *N*⁴-Oleyl-ara-C (NOAC) was prepared as described before [17] and the lipophilic metal complexing agent DTPA-stearate as described in Ref. 19.

The highly fluorescent lipophilic perylene dye *N,N'*-bis(1-hexylheptyl)-3,4,9,10-perylenebis(dicarboximide) (BHPD) was obtained from 7-tridecanamine as described before [20]. Before use BHPD was purified on a silica-gel column with CHCl₃ as eluent.

N-Succinimidyl-*S*-acetylthioacetate (SATA) was prepared according to Ref. 21.

Methods

Synthesis of N⁶-(6-maleimidocaproyl)-N²-palmitoyl-L-lysine methyl ester (EMP-PL)

6-Maleimidocaproic acid (0.96 g, 4.5 mmol) dissolved in 4 ml dry CH₂Cl₂ were adjusted to pH 7–8 with *N*-methylmorpholine. After addition of 1.02 g (4.95 mmol) DCCl and stirring for 10 min 1.96 g (4.5 mmol) *N*²-palmitoyl-L-lysine methyl ester HCl (PL) were added. The reaction mixture was stirred at room temperature for 18 h in the dark. The precipitate was filtered off and washed with 10 ml CH₂Cl₂ and the combined filtrates evaporated to dryness. The residue was dissolved in 20 ml CH₂Cl₂ and chromatographed on a silica-gel column (21.5 × 5 cm). The column was first eluted with 2.5 litre CH₂Cl₂, followed by 2.5 litre CH₂Cl₂/MeOH (97.3, v/v), 1 litre CH₂Cl₂/MeOH (95.5, v/v) and 2 litre CH₂Cl₂/MeOH (90.10, v/v). The required product was eluted within the fourth elution step. Fractions were pooled and evaporated to

dryness yielding 1.7 g (67%) of analytically pure product which was homogeneous ($R_f = 0.7$) by TLC using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (95:5, v/v) as eluent. The functional groups of the ultraviolet absorbing compound (254 nm) were identified on TLC by means of spray reagents [15]. The identification of the product was confirmed by elementary analysis and mass spectroscopy ($m/z = 592$). Accordingly, the lipophilic maleimide derivative N^6 -(3-maleimidopropionyl)- N^2 -palmitoyl-L-lysine methyl ester (MP-PL) was prepared.

Preparation of N^4 -oleyl-ara-C liposomes containing various lipophilic maleimide derivatives

Small unilamellar liposomes were prepared by detergent dialysis as described before [17]. The matrix lipid composition used throughout all liposome preparations was soy phosphatidylcholine (SPC)/cholesterol/DL- α -tocopherol at 1:0.2:0.01 mol parts with 20 mg SPC/ml (26 $\mu\text{mol}/\text{ml}$) initial lipid concentration. The maleimide derivatives MP-PL, MP-PE, EMC-PL and EMC-PE were added at 0.02 or 0.05 mol parts. N^4 -oleyl-ara-C (NOAC) concentration was 0.2 mol parts. The lipophilic fluorescent label BHPD was incorporated at 0.006 mol parts into the liposome membranes.

All lipids including the corresponding maleimide derivative plus NOAC and BHPD were dissolved in $\text{MeOH}/\text{CHCl}_3$ (1:1, v/v). Sodium cholate as detergent was added at a ratio of 0.6–0.7 moles referred to the sum of the concentrations of all membrane-forming lipids. In some preparations the liposomes were labeled by addition of trace amounts of ^3H -PC or by the incorporation of 0.003 mol parts of the lipophilic chelating agent DTPA-SA to which radioactive manganese ($^{54}\text{Mn}^{2+}$) ions were complexed after liposome formation [19]. After the removal of the organic solvents on a rotatory evaporator (40°C, 60 min) the lipid/detergent mixtures were solubilized by addition of phosphate buffer (67 mM disodium hydrogen phosphate dihydrate/67 mM potassium dihydrogen phosphate (pH 7.4)). The resulting micellar solutions (10–20 ml) were dialyzed against 5–10 l of the same buffer at 40°C for the first 2 h, followed by dialysis at 25°C for another 24–36 h. The liposome preparations were filtered through 0.45 μm sterile filters (Sartorius) and stored at 4°C. Liposome sizes and homogeneity were determined by laser light scattering and negative stain freeze-fracture electron microscopy [17].

The mean number of membrane-forming molecules, which comprised all lipophilic compounds constituting the membrane bilayer of one liposome and the total number of liposomes formed by the lipid concentration unit of 1 mg SPC/ml, were calculated from the mean vesicle diameters obtained from the laser light scattering data and from the assumptions on vesicle geometry parameters as made by Huang and Mason [23]. For example, the membrane of a spherical liposome with a

diameter of 50 nm contains 20 000 phosphatidylcholine molecules, based on the volume of one PC molecule of 1.253 nm^3 . Assuming the same volume for all other membrane constituting molecules, approximately 400 maleimide molecules (MP-PL or MP-PE) are distributed over the bilayer of one liposome.

Determination of liposome concentrations by BHPD fluorescence

The fluorescent dye BHPD was used to measure liposome concentrations and as a fluorescent marker for cytofluorometric analysis of liposome-cell binding (cf Binding experiments). Liposome concentrations were determined by the measurement of BHPD absorption at 526 nm in $\text{MeOH}/\text{CHCl}_3$ (1:1, v/v) or at 522 nm in aqueous media (67 mM phosphate buffer (pH 7.4)) with a Shimadzu UV 240 instrument. BHPD concentrations below $4 \cdot 10^{-7}$ M were measured with a SPF-500 Aminco spectrofluorometer at 489 nm excitation and 533 nm emission wavelength.

The stability of BHPD incorporation into the liposome membrane was monitored by dialysis of 0.5 ml liposome aliquots in Spectrapor 2 tubings against 200 ml 67 mM phosphate buffer (pH 7.4) at 4°C during 30 days. The BHPD concentrations were determined in the liposomes at intervals of 48 h.

Reactivity of the liposomal maleimide functions

The concentrations of reactive maleimide groups situated on the outer liposome surface was determined by the reaction with an excess of cysteine followed by the determination of unreacted cysteine with Ellman's reagent [23].

To 100 μl liposomes containing a calculated amount of $1 \cdot 10^{-5}$ mol maleimide groups that are distributed over the inner and outer layers of the lipid bilayer, $2.5 \cdot 10^{-5}$ mol cysteine was added in 67 mM phosphate buffer (pH 6.0). The mixtures were kept under nitrogen and stirred during 30 min. Then $3.2 \cdot 10^{-5}$ mol Ellman's reagent (DTNB) in Tris buffer (0.2 M 2-amino-2-hydroxymethylpropane-1,3-diol/0.2 M HCl (pH 8.2)) were added to a final volume of 1 ml. The reaction product of excess cysteine with DTNB was measured after 10 min at 412 nm and compared to the value obtained with reference liposomes containing no maleimide derivatives.

Antibodies

The monoclonal Ab B8-24.3 (mouse IgG2b, MHC class I, anti H-2k^b) [24] was obtained from ascites of BALB/c mice, as previously described [25]. The Ab was precipitated with 40% ammonium sulfate and further purified by HPLC on a hydroxyapatite column (100 \times 7.8 mm, BioGel HPHTTM, Bio-Rad) using a phosphate buffer gradient (10–300 mM (pH 6.8)) with a flow of 0.5 ml per min.

The rat monoclonal Ab IB16-6, an IgG2a, was produced as previously described [26]

Antibody labeling with N-succinimidyl[2,3-³H]propionate (³H-NSP)

The Abs were trace labeled with ³H-NSP according to the method of Kummer [27]. Antibody solutions (1 mg/ml) in 0.1 M sodium borate/0.1 M HCl plus 0.5 M sodium chloride (pH 0.5) were reacted with ³H-NSP at a 50% molar excess during 1 h at 0°C. Unreacted ³H-NSP was removed by dialysis against two 1000-fold volumes of 67 mM phosphate buffer.

Antibody-liposome coupling with SPDP

Modification of antibodies with SPDP

SPDP was linked to the Abs according to the method described by Carlsson [28] with minor modifications.

To 10–40 mg/ml Ab in 0.1 M phosphate buffer/0.1 M sodium chloride (pH 7.5) a solution of SPDP (150 mM in ethanol) was slowly added at room temperature until the molar ratio of Ab to SPDP was either 1:24, 1:12 or 1:6. The reaction lasted 60 min, followed by dialysis at 4°C over 24 h against two 1000-fold volumes of phosphate buffer to remove unreacted SPDP. The dialyzed Ab-PDP was stored at 4°C. The amount of linked SPDP molecules was determined from aliquots after reduction of the disulfide bonds with a 100 mM dithiothreitol solution by measurement of the absorption of 2-thiopyridone at 343 nm.

Reduction of Ab-PDP and coupling to maleimide liposomes

The Ab-PDP solutions were dialyzed against acetate buffer (0.1 M sodium acetate/0.1 M acetic acid plus 0.1 M NaCl (pH 4.5)) at 4°C over 24 h. Dithiothreitol was added to a final concentration of 25 mM. After 60 min incubation at 25°C the reaction mixture was fractionated at room temperature on a Sephadex G-50 column (30 × 1 cm) with 67 mM phosphate buffer (pH 6.0). With a flow of 0.5–1 ml/min the activated Ab (Ab-P-SH) was separated from dithiothreitol and 2-thiopyridone. The coupling to maleimide liposomes was performed at 25°C immediately after column separation of the activated Ab. Molar ratios of Ab-P-SH to liposomal maleimide of 1:10, 1:20 and 1:40 were used. Antibody concentrations varied between 0.15 and 0.6 mg/ml and the lipid concentration was 1–5 mg SPC/ml. The coupling was performed under nitrogen with moderate stirring during variable time periods (cf Results) in volumes of 2–12 ml, depending on the corresponding ratios. *N*-Ethylmaleimide dissolved in a minimal volume of 67 mM phosphate buffer (pH 7.4) was added at a 24-fold excess referred to the Ab concentration to stop the reaction.

Antibody-liposome coupling with SATA

Modification of antibodies with SATA

The Ab modification with SATA was performed following the protocols as described [16,21]. The Ab dissolved in 50 mM phosphate buffer/1 mM EDTA (pH 7.5) at concentrations of 4–10 mg/ml (0.25 – $1 \cdot 10^{-9}$ M) were reacted during 60 min at 25°C under nitrogen and gentle stirring with SATA (150 mM) which was dissolved in a minimal volume of dimethylformamide. The molar ratios of Ab to SATA were 1:24, 1:12 and 1:6. Unreacted SATA was removed by dialysis at 4°C against 1000 volumes of the same buffer over 24 h.

To measure the amounts of Ab-linked SATA, aliquots of Ab-ATA were deacetylated with hydroxylamine, followed by the determination of free sulfhydryl groups with Ellman's reagent as described above.

Deacetylation of Ab-ATA and coupling to maleimide liposomes

1 ml of the Ab-ATA solution was deacetylated by the addition of 0.1 ml hydroxylamine HCl (0.5 M in 50 mM phosphate buffer/25 mM EDTA (pH 7.5)) during 60 min at 25°C under nitrogen and stirring. Coupling of activated Ab (Ab-A-SH) to maleimide liposomes was carried out immediately thereafter at pH 6.0 and at molar ratios of Ab-A-SH to liposomal maleimide molecules of 1:10, 1:20 and 1:40 using an Ab-A-SH concentration of 0.3 mg/ml. The reaction was stopped at various time points (0.2 to 20 h) after incubation at 25°C by the addition of *N*-ethylmaleimide at a molar ratio of 1:24.

Separation of immunoliposomes from free antibody

Uncoupled SPDP- or SATA-modified Abs were separated from immunoliposomes using two different methods. To follow the coupling reaction between activated Ab and maleimide-liposomes aliquots of 100 µl were withdrawn from the reaction mixture at different time intervals (cf Fig 1). Free Ab was separated from immunoliposomes by HPLC using a Bio-Gel TSK 40 (300 × 7.5 mm) column and a flow of 67 mM phosphate buffer (pH 7.4) of 0.75 ml/min. Liposomal BHPD absorption was monitored at 522 nm and fractions of free Ab and immunoliposomes were collected and pooled separately. The Ab concentrations were determined by measurement of ³H-NSP activity by scintillation counting.

The second method was used to separate larger volumes (5–10 ml) of the immunoliposome mixtures. Uncoupled, activated Abs were separated from the immunoliposomes by flotation on a discontinuous metrizamide gradient [3,8,29]. In a 5 ml nitrocellulose ultracentrifuge tube (Beckman, Ultraclear) the immunoliposome coupling solution was mixed with 60% metrizamide to yield a final concentration of 20%.

metrizamide, overlaid by 2 ml of 10% metrizamide and followed by 0.5 ml 67 mM phosphate buffer (pH 7.4) as top layer. The density gradient was centrifuged during 12–16 h at $95\,000 \times g$ at 4°C in a L8-9M ultracentrifuge (Beckman). The floating immunoliposomes, which were visible as a pink fluorescent band, were carefully removed from the underlying metrizamide phases, pooled and dialyzed twice against 400 ml of 67 mM phosphate buffer (pH 7.4) to remove reversibly bound metrizamide.

Kinetics and efficiency of the binding of SPDP- and SATA-modified antibodies to maleimide liposomes

Binding curves of SPDP- or SATA-modified Abs to maleimide liposomes were obtained by removing aliquots from the incubation mixtures at various sampling timepoints and stopping the coupling reaction with *N*-ethylmaleimide. Excess of free activated Ab (Ab-P-SH, Ab-A-SH) was separated by HPLC on a TSK40 column. Fractions containing immunoliposomes were collected and pooled (pool 1) and the lipid concentration determined by BHPD fluorescence. The concentrations of coupled Abs in pool 1 and of free activated Abs (Ab-P-SH, Ab-A-SH) collected in pool 2 were determined by scintillation counting of the ^3H -NSP labeled Abs.

In vitro binding of immunoliposomes to target cells

Cell culture

The T cell lymphoma cell line EL4 of H-2k^b haplotype detectable with the B8-24.3 Ab, the hepatoma cell line B16-F10 specifically recognized by the IB16-6 Ab and the thymoma cell line BW5147 (H-2k^k) as a negative control were cultured in Iscove's modified Dulbecco medium (IMDM, KC Biological, Lenexa, KS, USA) supplemented with 5% heat inactivated fetal calf serum (Gibco, Grand Island, NY, USA), 10 mM L-glutamine (KC Biological), 100 IU/l penicillin/streptomycin (KC Biological) and 0.1 mM 2-mercaptoethanol (Merck, Darmstadt, FRG). The cells were grown in plastic culture flasks (Falcon, Oxnard, CA, USA) at 37°C , 5% CO_2 in a humidified incubator. Adherent cells were detached by brief treatment with trypsin/EDTA (Gibco).

Binding experiments

Immunofluorescence and cytofluorometric analysis

Binding activity and specificity of native and activated Ab-P-SH and Ab-A-SH were routinely tested *in vitro*. Antibody-cell binding was monitored by immunofluorescence as previously described [30] using goat-anti-mouse FITC-IgG (TAGO, Burlingame, CA,

USA) for the labeling of B8-24.3 and goat-anti-rat FITC-IgG (EY Laboratories, San Mateo, CA, USA) for IB16-6, respectively for second Ab labeling. Specific immunoliposome cell binding was determined by cytofluorometric analysis based on the fluorescence of BHPD incorporated into the liposome membranes of the cell bound liposomes (cf. liposome preparation).

Target cells (1.5×10^6) suspended in phosphate buffer were mixed with immunoliposomes (1–130 nM, corresponding to 2.5×10^6 liposomes per cell) in a volume of 300–400 μl in glass test tubes during 60 min at 4°C . The cells were then washed three times with 1 ml of 67 mM phosphate buffer (pH 7.4) containing 1% bovine serum albumin and 0.02% sodium azide and resuspended in 0.5–1 ml of the same buffer. The cell bound BHPD fluorescence intensity was measured on an Epics Profile Fluoro Cytometer (Coulter Corp.) at 470 nm excitation and 530 nm emission wavelength.

Binding of BHPD-labeled liposomes was determined by the measurement of the fluorescence intensity in gated detection windows which were set individually for chosen populations of viable EL4 and B16 cells, respectively. Non-specific Ab-liposome binding was determined by incubating B8-24.3-liposomes with B16-F10 cells and IB16-6-liposomes with EL4 cells, respectively (cf. Table II). 10 000 gated cells were analyzed at flow rates of 500–1000 cells/s. For Ab-specific liposome binding the window was set in a manner that natural cell fluorescence (Fig. 2, peak A) and fluorescence from the non-specifically adsorbed, Ab-free liposomes (Fig. 2, peak B) was excluded. The fluorescence intensity of bound immunoliposomes measured in the preset window (Fig. 2, peak C) is given in percent of the total fluorescence intensity from all cells analyzed and measured in the widest window.

Competition of immunoliposome cell binding by free antibody

Prior to the addition of immunoliposomes to the EL4 target cells free Ab was added as binding competitor. 1.5×10^6 EL4 cells were incubated during 60 min at 4°C either with B8-24.3 Ab (0.1 nM), corresponding to 4×10^8 molecules per target cell, or with an undiluted B8-24.3 hybridoma cell culture supernatant and at 1:2, 1:20, 1:200, 1:2000 and 1:20000 dilutions thereof.

Accordingly, B16-F10 cells were incubated with equally diluted IB16-6 Ab solutions starting from 0.025 nM.

After three washings with 67 mM phosphate buffer (pH 7.4), which contained 1% bovine serum albumin and 0.02% sodium azide, the cells were incubated at 4°C during 1 h in a volume of 0.36 ml with 2.5×10^6 (26 nM) immunoliposomes per target cell. An average of 1.3 Ab was coupled per vesicle and binding to the cells was determined by cytofluorometric analysis as described above.

Morphology of B8-24 3-liposome binding to EL4 cells

Ab-liposomes and MP-PL-liposomes were incubated with EL4 cells for specific binding using the same conditions as above. BW5147 cells were used as negative controls. After incubation with liposomes the cells were washed twice with 0.1 M sodium cacodylate buffer (pH 7.2), fixed with 2% glutaraldehyde for scanning electron microscopy (SEM) and seeded on glass coverslips precoated with poly-L-lysine (M_r 60 000, Sigma, St Louis, MO, U.S.A.). The dehydrated and dried samples were coated with gold (10 nm) and examined in a SEM 505 (Philips).

For transmission electron microscopy (TEM) the cells were incubated during 60 min at 4°C, followed by an additional incubation at 37°C during 120 min. The cells were then prefixed with 2% glutaraldehyde plus 0.8% paraformaldehyde and postfixed with an aqueous solution of 1% OsO_4 containing 1.5% $\text{K}_4(\text{FeCN})_6$. Fixed and pelleted cells were transferred into 2% agar, dehydrated in an alcohol series and embedded into epon. Ultrathin sections contrasted with uranyl acetate and lead citrate were studied in a TEM 420 (Philips).

Results

Properties of NOAC-liposomes containing various maleimide derivatives

NOAC-liposomes containing 0.02 mol parts of the maleimide derivatives MP-PL, MP-PE, EMC-PL and EMC-PE were of 50 nm mean vesicle diameter and physically stable for more than 60 days when stored at 4°C. At 0.05 mol parts maleimide derivative incorporated, the liposomes were comparably stable with the exception of the MP-PL liposomes that formed precipitates soon after preparation. With all types of functionalized liposomes a reduction of their binding activity during storage was observed. We chose to use MP-PL as linking agent because of its simple synthesis and satisfactory liposomal incorporation stability.

For the calculation of the molar ratios of Ab to coupling agent (SATA, SPDP) to reactive maleimide groups on the surface of one liposome, the liposomes containing the initial concentration of $5.3 \cdot 10^{-4}$ M (0.02 mol parts as referred to SPC) maleimide derivative were diluted to obtain molar ratios of Ab to maleimide of 1:10, 1:20 and 1:40. The fluorescent dye BHPD is very stably incorporated within the liposome membranes. Due to its high fluorescence quantum yield, very low BHPD concentrations of 10^{-7} to 10^{-9} M are sufficient to obtain highly fluorescent liposomes. The dye does not interfere with other membrane components, and because of its high lipophilicity no measurable leakage of BHPD from the liposomes occurred (data not shown). These properties render BHPD particularly suitable for the labeling of liposomes and the detection of their binding to cells by fluorometric methods.

Antibody-liposome coupling

Antibody activation with SPDP and SATA

The most important advantage of using SATA over SPDP is that no reducing agent like dithiothreitol is required for the activation of the thiol groups. Remaining traces of the reducing agent diminish the efficiency of the coupling reaction [28]. The activation of SATA is accomplished with hydroxylamine which has the additional advantage to increase the stability of free thiol groups [16,21].

The deacetylation reaction yielding thiolated Ab should last 30–60 min to guarantee quantitative de-blocking of the thiol-groups.

The SPDP coupling method was more time consuming with the necessity of two dialysis and one column chromatography steps to remove unreacted small molecules. The SATA method afforded only one dialysis step after its coupling to the Ab.

Both Abs (B8-24 3 and IB16-8) which were derivatized with SPDP formed precipitates during the reduction step with dithiothreitol at a pH of 4.5. The amount of the precipitate formed was dependent on the molar SPDP to Ab ratio. At a ratio of 12 SPDP to 1 Ab, 6–7 (50–60%) SPDP molecules were linked to one Ab molecule as determined by the yield of 2-thiopyridone.

SATA derivatized Ab did not precipitate during activation. The exact number of SATA molecules linked per Ab could not be determined by spectroscopic methods. Therefore, the differing numbers of effectively linked ^3H -NSP labeled Ab to the maleimide functionalized liposomes were taken to compare the efficiency of the two coupling agents.

In Fig. 1, the reaction time dependence of the coupling of either SPDP (filled symbols) or SATA (open

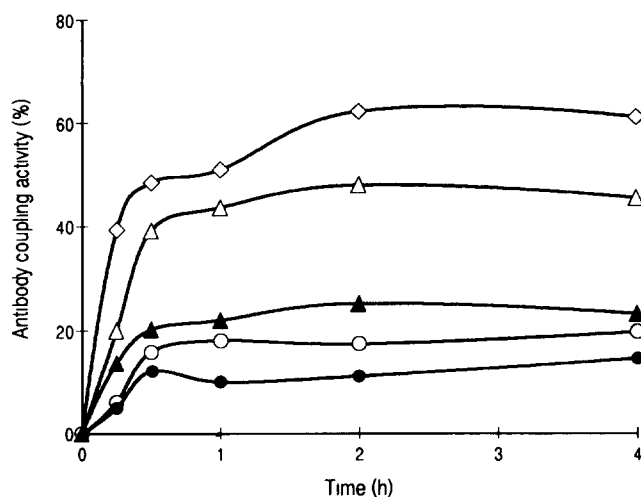


Fig. 1 Coupling of SPDP- and SATA-modified B8-24 3 Ab to MP-PL liposomes at molar ratios of SPDP to Ab of 1:12 (●) and 1:24 (▲) and of SATA at 1:6 (○), 1:12 (◇), and 1:24 (△) with a 1:40 molar ratio of Ab to maleimide groups on the liposomes. The initial concentration of B8-24 3 was $2 \cdot 10^{-6}$ M (=100%). The coupling reaction was made at room temperature under nitrogen and at pH 6.0.

symbols) modified B8-24.3 Ab to MP-PL liposomes is shown. The coupling via SPDP was most efficient at the ratio of 24 SPDP to 1 Ab with a rate of 20–25% after 1–4 h incubation time

Using SATA as coupling agent, higher activities were obtained at lower SATA:Ab ratios. At a ratio of 6:1 a binding rate of 20% was obtained. Maximal and reproducible binding resulted with the 12:1 ratio with rates of 60–65% Ab bound at incubation times of 2–4 h. Generally, incubations lasting longer than 4 h did not increase the coupling efficiency.

The SATA modified Ab B8-24.3 had an optimal coupling efficiency at a pH of 6 with 62% bound after 2 h incubation time. Binding efficiency was reduced to 48% and 34% at pH values of 5 and 4, respectively.

The number of reactive maleimide groups on the liposome surface influenced the yield of coupled Ab. At molar Ab to liposomal maleimide ratios of 1:10 and 1:20 the binding efficiency of SATA activated B8-24.3 Ab was reduced by 50% for each ratio as compared to the usual ratio of 1:40 (data not shown). At the molar ratios of 1:6, 1:12, and 1:24 of SPDP and SATA used to modify the Abs no loss of their specific binding activity was observed.

Cytofluorometric determination of immunoliposome-cell binding

The specific binding of immunoliposomes to target cells was examined using MP-PL-immunoliposomes that were coupled with SATA. The specific binding activity of the Abs was checked at all stages of their modification by immunofluorescence. The following results summarize the effects of the variation of different experimental parameters. Flow cytometric analysis was made by the use of the liposomal BHPD fluorescence to detect immunoliposome cell binding. The fluorescence intensities of the liposomes were measured in preset and constant windows as shown in Fig. 2 (cf. Methods).

Incubation conditions and competition experiments

To determine optimal immunoliposome binding to the target cells, the incubation conditions were modified as follows: (a) varying the number of linked Ab per liposome (cf. Table I), (b) varying the concentration of immunoliposomes incubated per target cell and, (c) changing the incubation volume. At least 0.3–2 Abs must be linked per liposome in order to obtain specific binding to the target cell. With 0.3 Ab per liposome we found a binding rate of 26% (cf. Table I) which indicates that about two thirds of the liposomes did not contain a linked antibody.

The cross-control incubations of Ab-liposomes with nonspecific target cells demonstrate the specificity of the Ab-liposomes (cf. Table II). In contrast to the results of other investigations, we found that the linkage

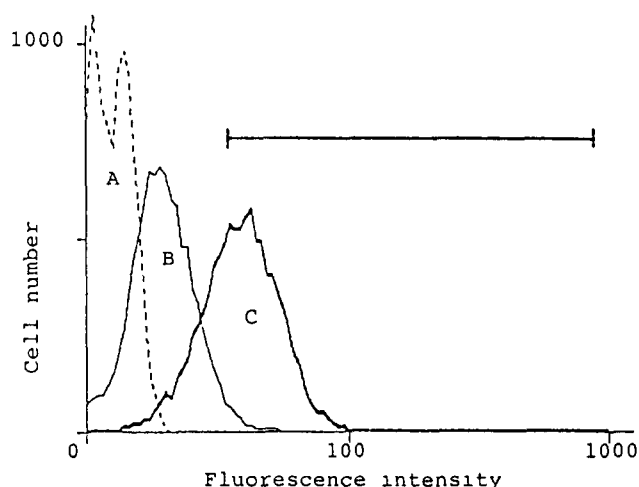


Fig. 2 Cytofluorometric determination of liposome-cell binding. Fluorescence intensities of BHPD containing liposomes bound to B16-F10 cells: (A) self fluorescence of B16-F10 cells, (B) non-specific binding of MP-PL liposomes (controls) and (C) specific binding of IB16-6 modified liposomes. Bar, preset measuring window for the determination of specific Ab-liposome binding. Similar window settings were used for the measurement of B8-24.3 modified liposomes to EL4 cells.

of more than two Ab molecules per liposome does not significantly increase binding efficiencies [3–5,10,16].

In Table II the binding of immunoliposomes, depending on the liposome concentration is shown. Significant Ab mediated binding of liposomes was achieved at concentrations of immunoliposomes in the range of 20–130 nM. Control liposomes lacking linked Ab showed binding rates of only 0.1–4.2% compared to 43–78% of the liposomal BHPD fluorescence detected on the cells that were incubated with variable concentrations of immunoliposomes (cf. also Fig. 4).

The incubation volume in which the immunoliposomes were mixed with the target cells strongly influenced the binding rates. Adherent B16-F10 cells (1.5×10^6) were incubated during 60 min at 4°C with 26 nM

TABLE I

Liposome-cell binding in dependence of numbers of SATA-coupled Ab molecules per liposome

Immunoliposomes (26 pM or 4×10^{12} vesicles) or MP-PL liposomes were incubated with 1.5×10^6 cells during 60 min at 4°C in a volume of 0.36 ml. Percentage of cell bound BHPD fluorescence was determined by cytofluorometry.

Antibodies coupled per liposome	Percent cell bound BHPD fluorescence		
	immuno-liposomes	MP-PL liposomes	cells alone
EL4 cells			
0.3	26	1.5	0
1.3	87	2	0
B16-F10 cells			
0.3	13	0.3	0
1.2	66	0.3	0
1.9	78	0.3	0

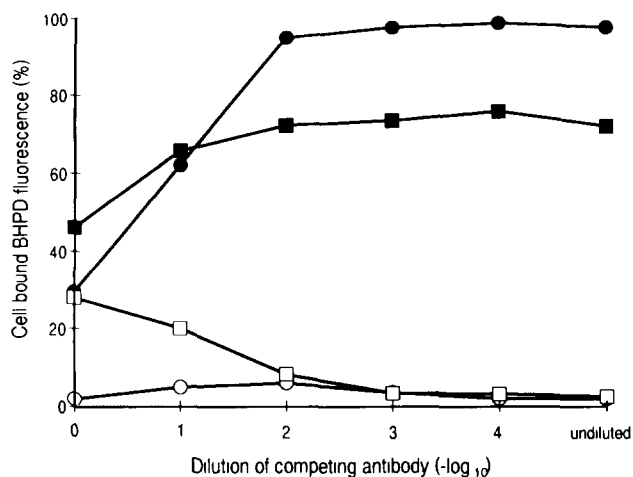


Fig 3 Liposome binding competition with free B8-243 and IB16-6 Ab added before incubation of the EL4 and B16F10 target cells with liposomes ●, B8-243 coupled liposomes, ■, IB16-6 coupled liposomes, ○, MP-PL liposomes (non-specific binding to EL4 cells), □, MP-PL liposomes (non-specific binding to B16-F10 cells) The initial concentration of free B8-243 Ab was 0.1 nM and that of IB16-6 0.025 nM Free Ab was incubated during 60 min at 4°C with the cells After three washings with 67 mM phosphate buffer (pH 7.4) containing 1% bovine serum albumin and 0.02% sodium azide liposomes (26 nM, 2.5×10^6 vesicles per cell) were added to give a final incubation volume of 360 μ l Cell bound BHPD fluorescence was determined after incubation for 60 min at 4°C

IB16-6 modified liposomes (1.3 Ab/liposome) in volumes of 3.6 ml, 1.2 ml and 0.36 ml Specific immunoliposome binding was highest with 57% in the smallest volume, whereas at 1.2 ml and 3.6 ml only 3.3% and 0.5% immunoliposomes, respectively, were specifically bound The competitive action of unmodified Ab added to target cells ahead of immunoliposomes is shown for B8-243 and IB16-6 in Fig 3 At excess concentrations of $(1-4) \times 10^8$ free Abs per cell corresponding to $(0.6-2.4) \times 10^{-8}$ M per 1.5×10^6 tumor cells the subsequent binding of immunoliposomes was reduced to 30% in the case of B8-243 (Fig 3, filled circles) and to 47% for IB16-6 (Fig 3, filled squares) The dilution of the competing free Ab increased immunoliposome binding

The 20–30% of non-specifically bound control MP-PL liposomes to the B16-F10 cells was probably caused by weak non-specific binding to adsorbed Ab molecules on the cell surface The specific binding of immunoliposomes to EL4 cells was visualized by scanning electron microscopy (SEM) and transmission electron microscopy (TEM), as shown in Fig 4, a–c and Fig 5, a–c

A total number of 4×10^{12} liposomes (26 nM) functionalized with an average of 1.3 B8-243 Abs per vesicle

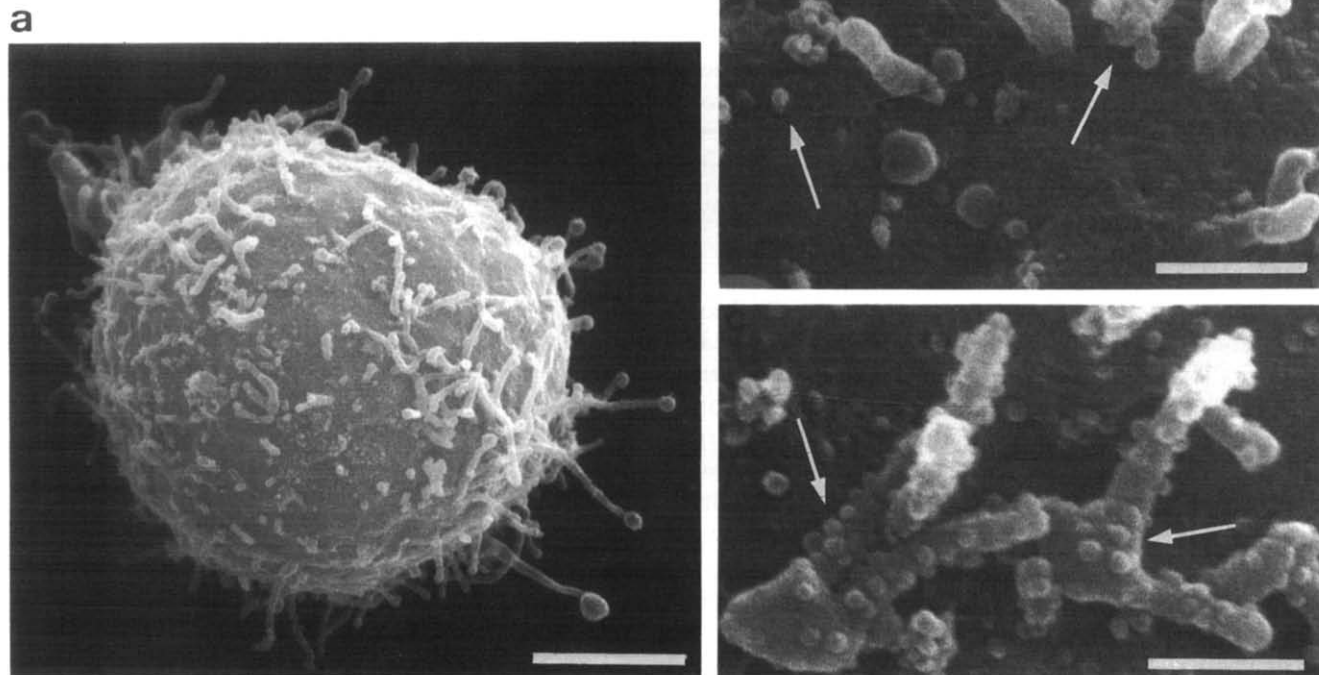


Fig 4 (a) SEM appearance of an EL4 cell, 12000 \times Bar 1.6 μ m (b) Detail of the cell surface of an EL4 cell which was incubated with Ab-free control liposomes The arrows show adsorbed single liposomes and liposome aggregates 64000 \times Bar 0.3 μ m (c) Specific binding of B8-243-liposomes with single liposomes predominantly bound to the cell's microvilli (arrows), 64000 \times Bar 0.3 μ m

TABLE II

Liposome-cell binding in dependence of immunoliposome concentration

SATA coupled immunoliposomes containing an average of 1.3 Ab per liposome or MP-PL liposomes were incubated as described in Table I. Percentage of cell bound BHPD fluorescence was determined by cytofluorometry. Non-specific Ab-liposome binding was determined by cross-incubations (2.5×10^6 liposomes/cell) with EL4 and B16-F10 cells, respectively.

Liposome concentration (nM)	Percent cell bound BHPD fluorescence		
	Ab-liposomes	MP-PL liposomes	cells alone
EL4 cells			
1	0.3	0	0
5	17	0.2	0
26	84	1.0	0
B16-F10 cells			
4	0.4	0.1	0
22	43	1.3	0
26	66	2.5	0
130	79	4.2	0
Controls			
B8-24.3 on B16-F10	1.2	—	0
IB16-6 on EL4	0.2	—	0

were incubated with 1.5×10^6 EL4 cells, as described in Methods. Fig. 4a shows an EL4 cell with typical surface structures. Unspecific binding of MP-PL liposomes is

shown in Fig. 4b as single liposomes or liposome clusters adsorbed on the cell surface (arrows). Antibody mediated specific binding of liposomes to the cell surface is demonstrated in Fig. 4c. High numbers of single liposomes are bound predominantly to the microvilli and also to a lesser extent to the smooth surface.

On the cell surface sector shown in Fig. 4c which has a calculated surface area of about $1 \times 10^{-12} \text{ m}^2$, an average of 100 liposomes were specifically bound. Based on a total surface of one EL4 cell of $1 \times 10^{-10} \text{ m}^2$, about 10,000 immunoliposomes would be specifically bound to the cell. The number of non-specifically bound liposomes (Fig. 4b) was not determined but the visual comparison shows that a smaller amount of the liposomes were non-specifically adsorbed.

The receptor mediated endocytic uptake of immunoliposomes is demonstrated in Fig. 5, which, in Fig. 5a shows an EL4 cell at low magnification with several attached liposomes (arrows). The Figs. 5b and 5c display the uptake process of a single liposome at different stages, showing the electron dense liposomes as black dots. The sizes of the OsO_4 -stained liposomes correspond to their original diameters. On control micrographs these electron-dense particles were not present (not shown). The binding to the cell membrane (Fig. 5b) is followed by the formation of a coated pit (Fig. 5c).

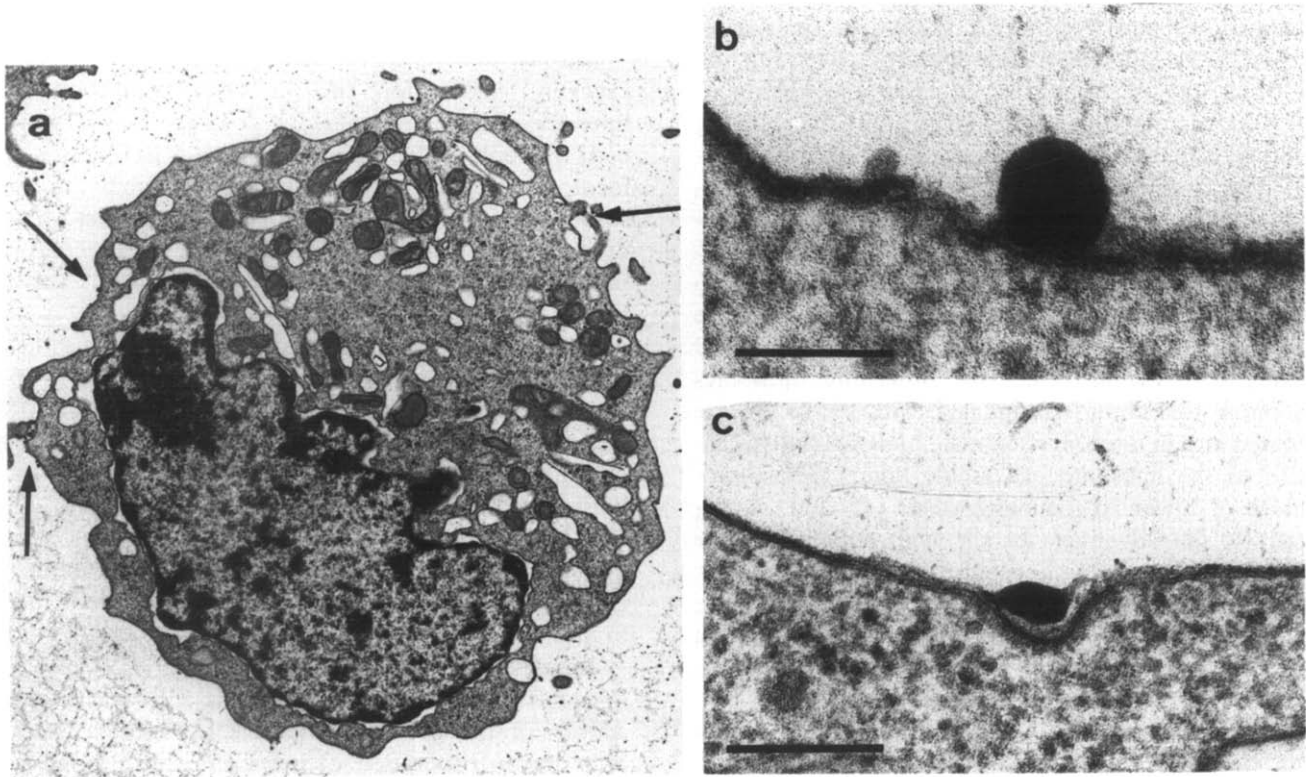


Fig. 5 (a) TEM morphology of an EL4 cell which was incubated with B8-24.3-liposomes. Bound liposomes can be recognized on the cell membrane (arrows). $7600\times$. Bar $2.5 \mu\text{m}$. (b) Detail of an EL4 cell showing a single liposome bound to the cell membrane. $230,000\times$. Bar $0.09 \mu\text{m}$. (c) Formation of a coated pit indicating the receptor mediated uptake of a single liposome. $100,800\times$. Bar $0.2 \mu\text{m}$.

and further internalization via coated vesicles (not shown)

Similar micrographs were obtained with the specific binding of IB16-6 modified liposomes to B16-F10 cells

Discussion

With the aim of preparing Ab modified liposomes that carried *N*⁴-oleyl-ara-C as cytostatic drug, and that bind specifically to target tumor cells, we compared in this study the coupling properties of two monoclonal IgG Abs to the surface of such liposomes using the two bifunctional coupling molecules SPDP and SATA. The liposomes were functionalized with various lipophilic maleimide derivatives and labelled with a highly fluorescent lipophilic fluorescent dye. In spite of the very complex membrane composition we obtained unilamellar liposomes of a mean diameter of 50 nm of which the Ab coupling capability was preserved for more than 14 days. Among the lipophilic maleimide derivatives MP-PL was the most effective in terms of preservation of the coupling activity in dependence of liposome storage. This is a favourable result because the synthesis of this maleimide derivative is simpler, cheaper and less equivocal than the PE derivatives. Moreover, it can be expected that the lysine derivative does not take part in lipid exchange as much as the corresponding PE derivatives.

As described for a comparable system of Ab coupling to liposomes [16], SATA showed to be a more efficient linker than SPDP, especially concerning the following aspects compared to SPDP the linking of SATA to amino groups of Abs followed by the coupling of the activated Abs to liposomes is methodically simpler, safer and faster. Due to these advantages, significantly higher yields of coupled Ab molecules are obtained.

The coupling yield of 60–65% shows that the two-step strategy of thioether linkage described here is more economic compared to the three step method proposed recently [15]. In the three step procedure liposomes containing free amino groups, e.g. with PE or PL are prepared and in a second step maleimide derivatives are linked to the liposomes via amide bonds. The disadvantage of lower Ab coupling yields (15–20%) is opposed by the advantage of the high stability of the amino group containing liposomes which are functionalized with maleimide derivatives immediately before the coupling of the Ab. Moreover, the modification of the liposomes with maleimide groups is restricted to the outer surface of the liposome membrane.

Using the optimized Ab-liposome coupling conditions, *in vitro* immunoliposome-target cell binding experiments were performed to investigate the feasibility of an *in vivo* approach with the aim to target drug carrying liposomes to a specific site of the organism.

Irrespective of the bifunctional linker used or the particular chemical method applied to couple Abs to liposomes our findings allow to draw the following conclusions.

(a) An average of one to two Ab molecules linked to the surface of one liposome is sufficient for specific binding of the immunoliposome to the target cell. Higher numbers of Ab molecules linked to one liposome do not increase the binding efficiency and might rather cause liposome aggregation and destabilization [6,7].

(b) To obtain specific binding to the target cells, immunoliposome concentrations in the range of 20–130 nM were necessary. The complete saturation of the Ab binding epitopes may not be necessary for the killing of the target cell by action of the liposomally incorporated cytotoxic drug. As suggested by other investigators [31,32], receptor mediated endocytic uptake of a liposome is increasing the toxic effect of the drug delivered into the cell.

(c) Controversial results have been reported on the competitive action of free Abs that occupy binding epitopes on the target cells. We found a competitive effect of free specific Abs incubated with the target cells before the addition of the immunoliposomes (cf. Fig. 3), using a 10–100-fold excess of competing free Abs. Similar results were described by Wolff and Gregoriadis [33], whereas in other studies like the ones by Heath and co-workers [34,35], soluble Ab coinubated with Ab-liposomes did not decrease the binding efficacy of the liposomes.

(d) The new lipophilic fluorescent dye BHPD is an excellent agent for the *in vitro* monitoring of liposome–cell interactions. In contrast to the widely used hydrophilic dye carboxyfluorescein BHPD remains incorporated in a very stable fashion within the liposome membrane and as compared to more water soluble dyes, no leakage occurs in the course of the coupling procedures and during liposome storage [6]. A further advantage of BHPD is that its fluorescence is not quenched after the incorporation into liposome membranes and due to its very high quantum yield, concentrations in the range of nanomoles are sufficient to detect liposome–cell binding. BHPD contains two hexylheptyl chains which are located symmetrically at both sides of the perylene ring structure. Due to these alkyl-chains it is conceivable that the dye is firmly intercalated between the fatty acid chains of both bilayer moieties, preventing its transfer into other membranes.

Besides that, the incorporation of BHPD into the liposomal membrane is not inducing non-specific interactions of the liposomes. This is remarkable because after the incorporation of other lipophilic fluorescent dyes strong nonspecific interactions between the liposomes and cells or biopolymers occurred. We made such observations for example with lipophilic derivatives of

fluoresceinamine, dansylcadaverine, salicylic acid and 4-chloro-7-nitrobenzofurazane which were synthesized by us (data not shown)

Our results support the findings that immunoliposome targeting potentially may be more effective than non-specifically adsorbed liposomes as drug delivery systems. However, optimal *in vivo* targeting of drugs by means of immunoliposomes can only be achieved by further refinement of these complex systems.

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