Design of immunoliposomes directed against human ovarian carcinoma

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Abstract

Factors (protein/lipid ratio, pH of incubation medium, incubation time, anchor molecule density in the bilayer) affecting the covalent binding of anti-ovarian carcinoma Fab' to liposomes containing the anchor molecule MPB-PE (N-(4-(p-maleimidophenyl)butyryl)phosphatidylethanolamine) were explored. Standard experimental conditions were chosen and information on the relevant physicochemical parameters of the liposome dispersions was collected (mean particle diameter, size distribution, charge). The reproducibility of standard immunoliposomes prepared in subsequent batches in terms of Fab' binding, particle size and charge was established. In addition, preservation of immunoreactivity, no marker loss, and no aggregation/fusion was found for the standard immunoliposomes over a period of at least 3 weeks at 4°C. In vitro up to 35000 immunoliposomes were estimated to bind per human ovarian carcinoma cell. Internalization of the immunoliposomes could not be demonstrated. Electron micrographs showed binding of specific immunoliposomes to human ovarian carcinoma cells growing intraperitoneally in athymic nude mice.

1. Introduction

In principle, the conjugation of cell-specific antibodies to liposomes containing chemotherapeutic agents provides the possibility for selective drug delivery and cell type specific cytotoxicity [1–4]. Indeed, such liposomes, referred to as immunoliposomes, have been shown to increase cytotoxicity selectively in vitro in several murine tumor cell lines [5–10]. Only a limited number of studies investigated the possibility to direct immunoliposomes to specific cells or tissues in vivo [11–18]. From these studies the anatomical and physiological constraints interfering with successful site-specific delivery of drugs can be derived. Maximum benefit of active targeting with immunoliposomes in vivo can be expected when the immunoliposomes are injected into the compartment where the target tissue or cells are localized.

Intraperitoneal (i.p.) administration of immunoliposomes containing tumoricidal agents has a strong pharmacological and biological rationale for the treatment of ovarian cancer. Ovarian tumors usually spread over the serosal surfaces of the peritoneal cavity; dissemination via the blood circulation occurs later and less frequently. A major cause of death in ovarian cancer patients is the inability to control the disease within the peritoneal cavity. While the primary tumor and larger tumor nodules may be removed surgically, micronodular disease and floating tumor colonies which are confined to the peritoneal cavity, cannot be treated adequately by surgery [19,20].

Immunoliposomes specifically directed against human ovarian carcinoma cells were prepared, characterized and tested for their binding capacity to human ovarian carcinoma cells grown in vitro and in vivo. The effect of experimental variables on the liposome-cell interaction was quantified. These immunoliposomes were developed

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to interact in vivo after i.p. administration with ovarian carcinoma cells localized in the peritoneal cavity and to deliver antitumor drugs in or in close proximity of these target cells [39]. In this study electron microscopic evidence was established for a specific interaction between immunoliposomes and the i.p. located target cells in athymic nude mice.

2. Materials and methods

2.1. Materials

Egg-L-α-phosphatidylcholine type V-E (PC), cholesterol (Chol), 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (Hepes), dithiothreitol (DTT) and N-ethylmaleimide (NEM) were obtained from Sigma (St. Louis, MO, USA). Egg-phosphatidylglycerol (PG) was supplied by Nattermann (Cologne, Germany). Carboxyfluorescein (CF) was purchased from Eastman Kodak (Rochester, NY, USA) and was purified by the method described by Ralston et al. [21]. $[1\alpha,2\alpha(n)\text{-}^3H]$Cholesteryloleoylether (spec. act. 1.71 TBq/mmol) was supplied by Amersham (Buckinghamshire, UK). Soluene-350 and Hionic Fluor was purchased from Packard Instrument (Downers Grove, IL, USA). All other reagents were of analytical grade.

2.2. Monoclonal antibodies

Hybridomas producing the monoclonal antibodies OV-TL3 and RIV1000 (both of mouse IgG1 type) were grown in BALB/c athymic nude mice [33,34]. The antibodies were purified and Fab' fragments were isolated as follows: mouse ascites was mixed with an equal volume of glycine/NaCl buffer (1.5 M glycine, 3.0 M NaCl, pH 8.9) and filtered through 0.2 μm Minisart NML filters (Sartorius, Göttingen, Germany) before application onto a Protein A-Sepharose CL-6B column (Pharmacia AB, Uppsala, Sweden). The unbound material was removed from the column by washing with glycine/NaCl buffer until no further change in absorption at 280 nm was seen. The monoclonal antibodies were eluted in 100 mM citrate buffer pH 5.0. The purified antibodies were pooled, neutralized with 2 M Tris base solution, concentrated with a Centriprep 30 concentrator (Amicon, Danvers, MA, USA) and dialyzed overnight against acetate buffer (100 mM NaAc, pH 7.4) at 4° C. F(ab')₂ fragments were produced by pepsin digestion. The pH of the purified IgG solution was adjusted to 4.2 immediately before digestion. Pepsin was added at a ratio of 1 mg per 30 mg of IgG. The mixture was incubated in a water bath at 37° C overnight. After adjusting the pH to 7.4 with 2 M Tris base solution the protein solution was mixed with glycine/NaCl buffer, filtered through a 0.2 μm filter and applied onto a Protein A-Sepharose CL-6B column. The unbound fractions, mainly representing F(ab')₂ fragments and pepsin, were

collected and concentrated. Finally, the concentrated protein solution was applied to a Superdex 200 pg (26/60) column (Pharmacia) eluted with acetate buffer (100 mM NaAc, 88 mM NaCl, pH 7.4) in order to separate the F(ab')₂ fragments from pepsin and residual amounts of Fc fragments and IgG. The F(ab')₂ fragments were stored at −20° C prior to use.

Purity of IgG and F(ab')₂ was assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions with a PhastSystem and PhastGel Homogeneous 12.5 gels (Pharmacia). To determine the apparent molecular weights low weight standards (Bio-Rad Laboratories, Richmond, CA, USA) were used.

Isoelectric focusing (IEF) was performed with the same system using PhastGel IEF 3-9 gels (Pharmacia). The samples were routinely applied at the anodic side of the gel. The pH gradient over the gels was monitored with an IEF calibration kit (Pharmacia). Electrophoresis and silver staining were carried out according to manufacturer instructions.

The immunoreactivity was tested by flow cytometry. Cultured monolayers of OVCAR-4 cells (see below) were treated with trypsin/EDTA (0.25%/0.02%) and washed with Dulbecco's phosphate-buffered saline (DPBS, Gibco, Paisley, UK). OV-TL3 or RIV1000 (IgG or F(ab')₂) antibodies were diluted in DPBS containing 0.1% NaN₃ as indicated. Cells (5 · 10⁵) were incubated at 4° C for 45 min in 50 μl antibody solution. After incubation, cells were washed twice with DPBS by centrifugation (5 min, 800 × g) and incubated at 4° C for 45 min with 50 μl fluorescein-conjugated goat anti-(mouse IgG) (Capel Labs, Cooper Biomedical, Malvern, PA, USA) at a dilution of 1:80. The cells were washed twice and fixed with 200 μl 0.1% paraformaldehyde and kept on ice until fluorescence was measured. The percentage of fluorescent cells is presented after subtraction of the percentage of fluorescent cells stained after incubation with only fluorescein-conjugate. Fluorescence analysis was performed in a fluorescence-activated cell sorter (Becton & Dickinson Immunocytometry Systems, Mountain View, CA, USA). Analysis of the data was performed with Consort 30 software (Becton & Dickinson) on a Hewlett Packard 9920 S Computer. Data were calculated for 10⁴ cells after gating the dot plots of the volume-sidescatter diagram.

2.3. Preparation of Fab' fragments

F(ab')₂ fragments of OV-TL3 and RIV1000 (3–4 mg) in 1–1.5 ml of acetate buffer (100 mM NaAc, 88 mM NaCl, pH 5.5), were routinely reduced with 20 mM DTT at pH 5.5 for 90 min at room temperature under nitrogen atmosphere [22]. DTT was removed by gel chromatography on a Sephadex G-25M column (PD-10, Pharmacia). Pre-equilibration and elution occurred with deoxygenated acetate buffer (100 mM NaAc, 88 mM NaCl, pH 6.5)

under nitrogen atmosphere. Fab' fragments appearing in the void volume were used immediately for covalent attachment to freshly prepared MPB-PE liposomes. Completion of the reduction process was checked by HPLC (TSK-3000 SW column, length 60 cm, LKB, Bromma, Sweden), after adding an excess of NEM to an aliquot of the resulting protein solution. The HPLC equipment consisted of the following components: a solvent delivery system (Waters, model 6000A), a manual injector (Rheodyne, model 7125), a UV/VIS variable wavelength detector (Waters, model 450) operating at 280 nm. After injection of 25 μl of the standards or the sample, the column was eluted isocratically with phosphate buffer (0.1 M, 0.1 M KCl, pH 7.0) using a flow of 1 ml/min. The following standards were used for calibration: bovine serum albumin and its dimer ovalbumin, carbonic anhydrase, lactalbumin and insulin.

2.4. Preparation of (immuno)liposomes

N-(4-(p-Maleimidophenyl)butyryl)phosphatidylethanolamine (MPB-PE) was synthesized, purified and analyzed as described before [22,23]. MPB-PE was incorporated into the liposomal bilayer to couple Fab' fragments covalently to liposomes. Lipid mixtures composed of PC/PG/Chol/MPB-PE of varying molar ratios were dissolved in chloroform and dried to a thin film by using a rotary evaporator at 40° C under reduced pressure. Radioactive (immuno)liposomes were prepared by addition of [^{3}H]cholesteryloleoylether to the chloroform mixture. After evacuation for at least 1 h the lipid film was hydrated in Hepes buffer (20 mM Hepes, 135 mM NaCl, pH 7.4) or alternatively in CF solution (100 mM CF, 10 mM Tris-HCl, pH 7.4). The resulting multilamellar liposome dispersion was sequentially extruded through 0.6 μm and 0.2 μm polycarbonate membranes (Uni-pore, Bio-Rad) under nitrogen pressure [24]. Vesicles formed by this procedure had on the average 1.5 bilayers as reported by Jousma et al. [25]. After extrusion, the outside buffer was exchanged by applying ultracentrifugation (80 000 $\times$ g, 45 min). The pellet was redispersed in acetate buffer (100 mM NaAc, 88 mM NaCl, pH 6.5) followed by flushing with N_2 (g). Routinely (unless otherwise stated), freshly prepared liposomes (8–12 μmol TL/ml) were incubated with freshly prepared Fab' fragments (0.3–0.4 mg/ml) in nitrogen atmosphere. Samples were taken to determine the Fab' and the lipid concentration during incubation. The coupling reaction was carried out at 4° C under constant rotation overnight. Finally, the immunoliposomes were separated from unconjugated Fab' fragments by ultracentrifugation at 80 000 $\times$ g during 45 min. The pellet was resuspended and washed twice with Hepes buffer. MPB-PE liposomes, not incubated with Fab' fragments and further referred to as unconjugated liposomes, were exposed to the same treatment. Liposome dispersions were stored at 4° C under nitrogen atmosphere.

2.5. Liposome characterization

Lipid phosphate was determined by the colorimetric method of Fiske and SubbaRow [26]. Protein was determined by the method of Wessel [27], with bovine serum albumin (BSA) as standard. The amount of monoclonal antibody coupled to the liposomes was expressed as μg of protein per μmol of total lipid (TL). At the concentration used (100 mM), CF fluorescence is fully quenched. Leaked CF will attain in the medium a concentration which allows the dye to fluoresce. Before and after destruction of the liposomes by addition of 0.5% Triton X-100 and subsequent heating (70° C, 30 min), CF fluorescence was assayed at 518 nm emission wavelength and 489 nm excitation wavelength in a Kontron Instruments spectrofluorimeter, Model SFM 25 (Watford/Herts, UK) [28]. Radioactivity was measured in Hionic Fluor as scintillation mixture in a Tri-Carb 1500 liquid scintillation counter (Packard Instruments, Downers Grove, IL, USA). Mean particle size was determined by dynamic light scattering (DLS) with a Malvern 4700 system using a 25 mW He-Ne laser and the automeasure vsn 3.2 soft-ware (Malvern, UK). For viscosity and refractive index the values of pure water were used. As a measure of the particle size distribution of the dispersion the system reports a polydispersity index. This index ranges from 0.0 for an entirely monodisperse up to 1.0 for a completely polydisperse dispersion. Zeta-potentials were measured in a PC-3 cell with a Malvern zeta-sizer IIC (Malvern).

2.6. Release kinetics in buffer and serum

The release characteristics of CF-containing MPB-PE liposomes and OV-TL3 immunoliposomes were determined after incubation for 5, 24 and 48 h in Hepes buffer (20 mM Hepes, 135 mM NaCl, pH 7.4) and fetal calf serum (FCS) (Gibco). (Immuno)liposomes at a concentration of approx. 10 μmol TL/ml were mixed (1:1) with buffer or serum. The incubations were performed in duplicate at 37° C under continuous agitation. CF release was assayed as described above.

2.7. Tumor model

The human ovarian cancer cell line OVCAR-4 originated from Dr. Hamilton (National Cancer Institute, Bethesda, MD, USA) [29,30] and was maintained in Dulbecco's modified Eagle's medium (DMEM) (Flow Laboratories, Irving, Scotland, UK) supplemented with fetal calf serum (10%) and glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml) and Fungizone (0.26 μg/ml). An in vivo OVCAR-4 tumor model was developed in the NMRI athymic nude mice [31] (bred at Harlan CPB, Zeist, The Netherlands) by i.p. inoculation of OVCAR-4 cells. After establishing the tumor model only i.p. ascitic tumor cells were used for serial transfer. The ani-

mals developed gross ascites 4–5 weeks after i.p. inoculation with solid tumor growth localized on the diaphragm, mesentery and the abdominal wall.

2.8. Cell binding assay

Cell binding assays were performed on in vitro growing OVCAR-4 cells or on cells present in ascites collected from OVCAR-4 tumor bearing athymic nude mice. Cultured monolayers of OVCAR-4 cells were treated with trypsin/EDTA (0.25%/0.02%). A suspension of cells in PBS was mixed with an equal volume of CF containing (immuno)liposomes. Incubations were performed in duplicate or triplicate under continuous agitation. Unbound (immuno)liposomes were separated from the cells by centrifugation (800 × g, 5 min). The cell pellet was washed twice with PBS and resuspended in 0.5 ml PBS containing 0.5% Triton X-100 (v/v), followed by heating for 30 min at 70° C to lyse cell-bound (immuno)liposomes. After centrifugation at 1500 × g during 10 min, supernatants were analyzed for CF as described above. In case of radioactive (immuno)liposomes the washed cell pellet was transferred into a vial and digested by the addition of 1 ml Soluene-350 at 40° C for one night, yielding a clear solution. Radioactivity was measured in Hionic Fluor as described above.

2.9. Electron microscopy

OV-TL3 immunoliposomes (coupling ratio: 6 μg Fab'/μmol TL) and MPB-PE liposomes were administered i.p. in OVCAR-4 tumor-bearing NMRI athymic nude mice. Solid i.p. tumors (0.5–0.6 g) and ascites (1–2 ml) were present at the time of injection. Each mouse received a liposome dose of 8 μmol TL in 0.5 ml of Hepes buffer pH 7.4. After 5 h, the mice were killed and the ascites was collected and washed twice with PBS by centrifugation (800 × g, 5 min). The final cell pellet was double-fixed in glutaraldehyde-tricomplex fixative (glutaraldehyde-potassium ferrocyanide-calcium chloride-cacodylate buffer), followed by a postfixation in an osmium-tricomplex solution (osmic acid-potassium ferrocyanide-calcium chloride-cacodylate buffer) [32]. Dehydration was performed in an ascending series of ethanols and the cells were embedded in Epon 812. Diamond-cut ultrathin sections with a Reichert OM U3 ultramicrotome were examined either unstained or uranyl acetate contrasted in a Philips EM 300/301 electron microscope.

3. Results and discussion

Factors which should be taken into account when considering the preparation of immunoliposomes are: (1) a sufficient quantity of antibodies must be bound to the liposomal surface in a reproducible way; (2) the integrity of the liposomes should be preserved during the coupling

process; (3) the liposome-antibody complex must be sufficiently stable on storage and after administration in vivo; (4) the homing capacity of the antibodies should be preserved after binding to the liposomes.

3.1. Preparation and characterization of antibody (fragment) preparations

The mouse monoclonal antibody OV-TL3 is a promising candidate for immunotargeting of ovarian cancer. It is directed against cell surface antigenic determinants present on more than 90% of human ovarian carcinomas of different histological types and shows very little affinity for nonovarian carcinoma cells [33]. The monoclonal antibody RIV1000, in the present studies used as an irrelevant monoclonal antibody, is directed against human lymphocytes [34].

The purity of the purified antibody preparations (IgG and F(ab')$_2$) was confirmed by SDS-PAGE. The pI values of the intact antibodies as well as the F(ab')$_2$ fragments, produced by pepsin digestion of the IgG molecules, were determined by IEF. For the OV-TL3 antibody the pI values of the bands ranged from 6.8 to 7.1 for intact IgG and from 8.2 to 8.7 for F(ab')$_2$ fragments. The pI values of the RIV1000 antibody ranged from 5.8 to 6.6 for intact IgG and 5.5 to 8.0 for F(ab')$_2$ fragments. This indicates that the F(ab')$_2$ and therefore also the Fab' fragments of the OV-TL3 antibody were positively charged at neutral pH.

The immunoreactivity of purified IgG and F(ab')$_2$ fragments of OV-TL3 and RIV1000 to OVCAR-4 cells was determined using flow cytometry (Fig. 1) The data indicate that at the protein concentrations tested the reactivity of F(ab')$_2$-OV-TL3 was maintained. In addition, the results
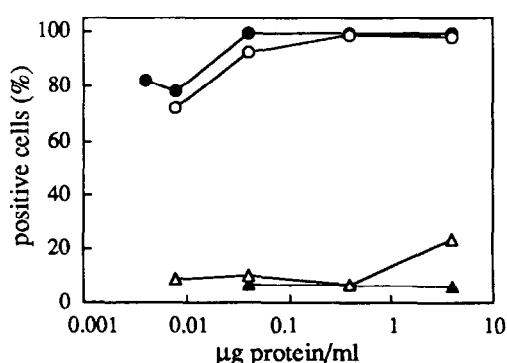

Fig. 1. Flow cytometric analysis of OV-TL3 and RIV1000 binding to OVCAR-4 cells. Percentage positive OVCAR-4 cells as a function of the concentration of (●) OV-TL3 IgG, (○) OV-TL3 F(ab')$_2$, (▲) RIV1000 IgG and (△) RIV1000 F(ab')$_2$. Cells were examined for cell-associated fluorescence by flow cytometry as described in Materials and methods. The mean percentage of fluorescent cells determined by flow cytometry is presented after subtraction of the percentage of fluorescent cells stained after incubation with only fluorescein-conjugate. The S.D. varies between 1–10% of the mean value. Each point represents at least three experiments.

illustrate the suitability of the RIV1000 antibody to be used as an irrelevant antibody in our studies concerning targeting of immunoliposomes to ovarian carcinoma. OV-TL3 did not react with nonrelevant target cells (the murine squamous cell carcinoma 5D04 [35] and human bladder carcinoma T24) in flow cytometric studies (results not shown).

For immunospecific targeting Fab' fragments of OV-TL3 were covalently linked to MPB-PE liposomes (see below). The Fab' fragments were generated from F(ab')$_2$ fragments by dithiothreitol (DTT) incubation. The experimental conditions used (incubation for 90 min at pH 5.5 at room temperature) were taken from Martin and Kung [36]. Addition of excess N-ethylmaleimide to the reaction mixture was used to stop the reduction process. The DTT concentration was assessed for a complete reduction of F(ab')$_2$ dimers to Fab' monomers. The HPLC profiles presented in Fig. 2 show that this is achieved at a DDT concentration of 20 mM. The same result was obtained for RIV1000 F(ab')$_2$ (results not shown). Therefore, it was decided to use routinely 20 mM DDT for the preparation of Fab' fragments.

3.2. Choice of preparation method

We prepared routinely multilamellar liposomes (MLV) consisting of PC, PG, Chol, and the sulfhydryl-reactive phospholipid derivative MPB-PE (2.5 mol%). Bangham and co-workers reported on the preparation of MLV for the first time [37], and their method has proven to be very popular. MLV are suitable for the encapsulation of a variety of substances and can be made with a wide variety of lipid compositions [38]. A difficulty with Bangham's method is the lack of control over the vesicle size distribution. Therefore, the liposomes were extruded through polycarbonate membranes with pore sizes of 0.6 and 0.2 μm to overcome this problem. Interestingly, instead of having a multilamellar nature, such extruded liposomes have been reported to be uni- or oligolamellar (one to three bilayers) [25].

Many different techniques for the coupling of antibodies to liposomes have been described [1,40]. We have used one of the most popular conjugation methods involving the use of the bifunctional agent MPB-PE [22] (Fig. 3). This technique was selected from a number of options for the following reasons: (1) mild conditions are maintained during coupling, avoiding a decrease in the immunoactivity of the coupled antibody fragment; (2) Fab' fragments are covalently linked to the MPB-PE anchor present in the outer lipid bilayers of preformed liposomes. This method presumably results in an adequate orientation of the Fab' fragments on the liposomes: the antigen binding sites are outward directed and fully accessible to antigen binding. Moreover, the thio-ether bridge has proven to be stable in vivo, contrary to the alternatively used disulfide bridge; (3) the Fc part of the IgG molecule is removed. This can be of
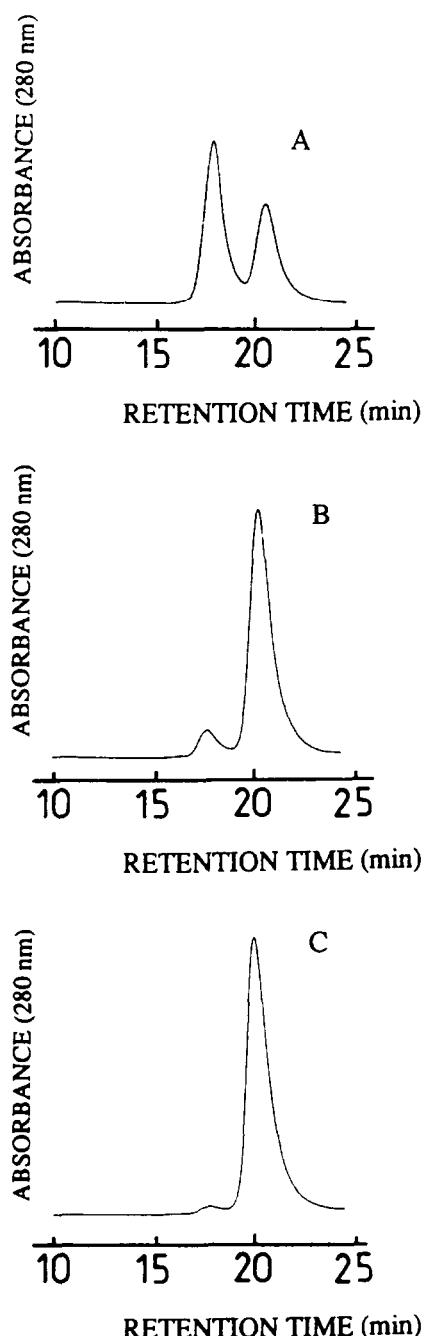

Fig. 2. Effect of DTT concentration on the reduction of OV-TL3 F(ab')$_2$ dimers to Fab' monomers. HPLC-analysis on a TSK 3000 SW column after reduction of OV-TL3 F(ab')$_2$ at various DTT concentrations: (A) 2 mM, (B) 10 mM, and (C) 20 mM. Incubations were performed in acetate buffer (100 mM NaAc, 88 mM NaCl, pH 5.5) for 90 min. The reduction process was stopped after addition of excess NEM (see Materials and methods). The molecular weights were estimated by plotting the molecular weights of the injected standards against their retention times. The first peak corresponded to a molecular mass of 90 kDa (OV-TL3 F(ab')$_2$) and the second with 43 kDa (OV-TL3 Fab').

great importance for in vivo use of Fab' vesicles. In this way the Fc receptor of macrophages is not activated and elimination of the liposome, by the mononuclear phago-

cyte system (MPS), might be reduced and slowed down [14].

3.3. Optimization of the coupling of Fab' to MLV

Coupling of a (sulfhydryl-group exposing) protein to liposomes containing maleimide residues merely requires mixing of the liposomes and the protein, which has been reduced and separated from DTT. The coupling efficiency was investigated under various reaction conditions. The liposomes used were composed of PC, PG, Chol and 2.5 mol% MPB-PE (38.5:4:16:1.5 molar ratio).

The stability of the maleimide group of MPB-PE is known to be sensitive to pH [41]. Especially at pH values greater than 7.0 rapid degradation of the maleimide residue has been reported [42]. A pH of 6.5 was recommended for use in the coupling reaction [22,43]. This pH was also used in our studies. However, as the marker carboxyfluorescein (CF), occasionally entrapped to monitor cell binding, becomes protonized and loses its charge at low pH [28,44], the preformed MPB-PE liposomes were prepared and stored at a pH of 7.4. The MPB-PE vesicles were used as freshly as possible; the maleimide function was exposed to pH 7.4 for maximally 8 h. Nevertheless, it was investigated whether the relatively short exposure to pH 7.4 may cause a significant loss of maleimide reactivity which might then be expected to result in a drop in coupling efficiency. Liposomes containing 2.5 mol% MPB-PE were prepared at pH 5.5 as well as at pH 7.4 under standard experimental conditions (described in Materials and methods). Fab' fragments were coupled to the freshly prepared vesicles at pH 6.5. The protein to lipid ratios (coupling ratios) were similar and amounted to 9.5 μg Fab'/μmol TL and 10.7 μg Fab'/μmol TL for the 'pH 5.5 dispersion' and the 'pH 7.4 dispersion', respectively. These results indicate that we could use a pH of 7.4 during our standard preparation procedure prior to Fab' coupling. Results of Peeters et al.

Table 1
Coupling efficiency and incubation time

Incubation time (h)	Incubation temperature (°C)	Coupling ratio (μg Fab'/μmol TL)	Coupling efficiency [a] (%)
0.5	20	2.8	8
1.5	20	6.0	17
14	4	11	31

Freshly prepared OV-TL3 Fab'-fragments (0.37 mg/ml) were incubated with MPB-PE liposomes (PC/PG/Chol/MPB-PE; 38.5:4:16:1.5) (10.4 μmol TL/ml) under N_2 atmosphere. At the time indicated the reaction was stopped by addition of excess NEM. The coupling ratio at each time point was determined following separation of Fab'-liposomes from unreacted Fab'-fragments by ultracentrifugation as described in Materials and methods. A typical experiment out of two performed is shown.

[a] Coupling efficiency (%) was calculated by dividing the final coupling ratio by the ratio of protein to lipid present during the coupling reaction × 100%.

[45] and Loughrey et al. [42] indicate that the exposure to pH 7.4 for prolonged periods (> 8 h) results in a decrease of coupling efficiency. Therefore, in line with their results, the time span between the start and the end of exposure to this pH never exceeded 8 h.

It has been reported that an incubation time of 6 h is sufficient for protein conjugation to MPB-PE liposomes [43]. For reasons of practical convenience it was examined whether the coupling reaction could proceed overnight Table 1 presents results of the effect of incubation time/temperature on the coupling ratio of the reaction product. It was found that under typical reaction conditions as described in Materials and methods much more of the added OV-TL3 Fab' was coupled to the liposomes overnight (14 h) than in the shorter incubation time periods. Bredehorst et al. [46] studied the time course of the coupling reaction over a period of 20 h. They observed that the coupling ratio reached a plateau value in about 5 h after start of incubation. Based on their and our results, it
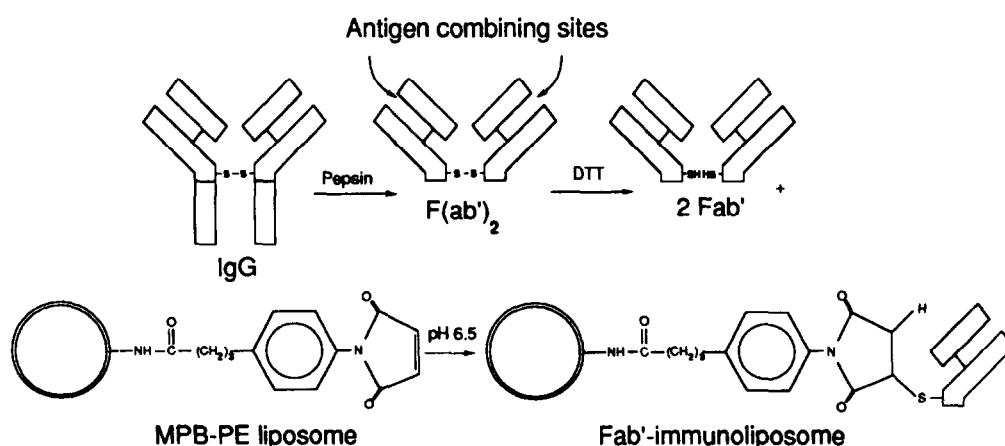

Fig. 3. Schematic representation of the coupling of Fab' fragments to MPB-PE liposomes. F(ab')₂ fragments are prepared by pepsin digestion of IgG. Fab' fragments are generated by reduction of F(ab')₂ with DTT at pH 5.5. After removal of DTT, Fab' fragments are immediately mixed with MPB-PE liposomes at pH 6.5 and allowed to react under N₂ atmosphere overnight. The sulfhydryl group of the Fab' fragment forms a stable thioether cross-linkage with the double bond of the maleimide moiety on the surface of the MPB-PE liposomes (from Ref. [22]).

Table 2
Effect of mol fraction of liposomal MPB-PE on the coupling efficiency

MPB-PE (mol %)	Incubation ratio [a] (μg Fab'/μmol TL)	Coupling ratio (μg Fab'/μmol TL)	Coupling efficiency [b] (%)
0	47	0.04	0.09
1	40	9.1	23
2.5	36	11	31
5	37	17	46

Freshly reduced OV-TL3 Fab'-fragments (0.37 mg/ml) were mixed with different MPB-PE-liposome dispersions varying in mol% MPB-PE (PC/PG/Chol/MPB-PE; 40 − x:4:16:x) (7.8–10.4 μmol TL/ml) and allowed to react under N_2 atmosphere at 4° C for 14 h. The coupling ratio was determined following separation of Fab'-liposomes from unreacted Fab'-fragments by ultracentrifugation as described in Materials and methods. A typical experiment out of two performed is shown.

[a] Ratio of protein and lipid present during the coupling reaction.

[b] Coupling efficiency (%) was calculated by dividing the coupling ratio by the ratio of protein to lipid present during the coupling reaction × 100%.

was decided to use overnight incubation for Fab' coupling at 4° C.

An important factor influencing the ultimate coupling ratio (expressed as μg Fab'/μmol TL) is the mol fraction of MPB-PE in the liposomes. Table 2 shows the increase in the amount of coupled Fab' with an increasing mol fraction of MPB-PE. No nonspecific binding of Fab' to the liposomes was observed (Table 2). Coupling efficiencies of up to 30 to 50% were readily achieved under the standard experimental conditions employed (as described in Materials and methods). The efficient coupling of protein to liposomes containing lower levels of MPB-PE is of particular importance as higher concentrations of this anchor molecule (> 2.5 mol%) dramatically affected liposome stability [46]. For this reason we have chosen, as other investigators [22,47], 2.5 mol% MPB-PE (on a total lipid basis) as the standard maleimide content of the liposomes.

The amount of coupled Fab' molecules to 2.5 mol% MPB-PE liposomes could be varied by changing the initial Fab' concentration or by changing the liposome concentration in the coupling reaction mixture (Fig. 4). The relation-

ship between coupling ratio and Fab' concentration appeared to be linear over the concentration range from 0.1 to 0.7 mg/ml of Fab' fragments. In addition, the coupling ratio appears to be inversely correlated with the total liposome concentration in the reaction mixture. Interestingly, by increasing the Fab' concentration or decreasing the lipid concentration in the incubation mixture the coupling efficiency increased and leveled off at 30%. Flexibility of the method for adjusting desired coupling ratios is illustrated. Routinely, we used approx. 0.3–0.4 μg Fab'/ml and approx. 8–12 μmol TL/ml for the preparation of OV-TL3 (and RIV1000) immunoliposomes.

3.4. Characterization

A proper physicochemical characterization of immunoliposomes is not always included in the literature reports. The physicochemical properties of immunoliposomes will strongly influence their disposition in vivo and their behavior in vitro [48,49]. Therefore, it is important to prepare immunoliposomes well characterized in terms of Fab' coupling ratio, particle size and surface charge, with accept-
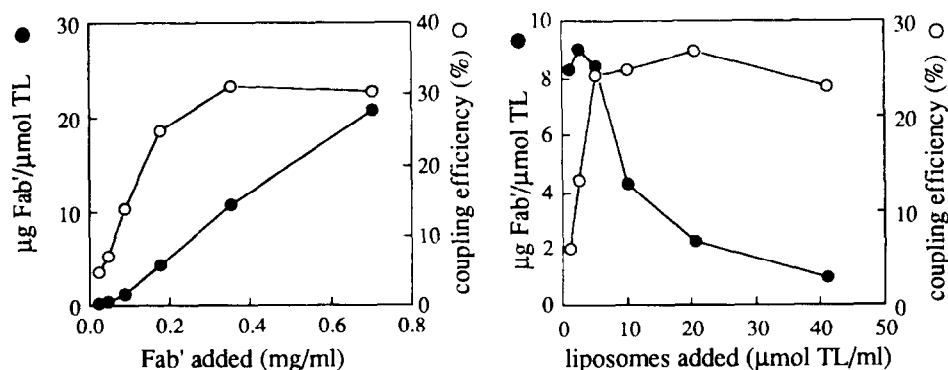

Fig. 4. Coupling of Fab' to liposomes: Fab' concentration (left) and lipid concentration (right) dependence. PC/PG/Chol/MPB-PE (38.5:4:16:1.5, molar ratio) liposomes (left panel: 10.2 μmol TL/ml; right panel: various concentrations) were incubated under N_2 atmosphere at 4° C in acetate buffer (100 mM NaAc, 88 mM NaCl, pH 6.5) overnight with freshly prepared OV-TL3 Fab' fragments (left panel: various concentrations; right panel 0.18 mg Fab'/ml). The coupling ratio (μg Fab'/μmol TL) was determined following separation of Fab' liposomes from unreacted Fab' fragments by ultracentrifugation as described in Materials and methods. Coupling efficiency (%) was calculated by dividing the coupling ratio by the ratio of protein to lipid present during the coupling reaction × 100%. Results of a typical experiment out of three performed are shown.

able long term stability and preservation of immuno-reactivity.

Characteristics of standard preparations of the OV-TL3 and the RIV1000 immunoliposomes are presented in Table 3. The protein to lipid (coupling ratio) varied only slightly between the different immunoliposome preparations. It was estimated that at a coupling ratio of 10 μg Fab$'$/μmol TL approx. 240 Fab$'$ molecules were present on one liposome particle. By covalent attachment of OV-TL3 Fab$'$ fragments to the MPB-PE liposomes the negative zeta-potential was altered from -24 ± 2 mV to -17 ± 1 mV for the MPB-PE liposomes and the OV-TL3 immunolipo-somes, respectively. The mean diameter was found to be approx. 0.25 μm with low polydispersity indices for both MPB-PE liposomes and immunoliposomes on the day of preparation. Bredehorst et al. [46] observed that coupling of Fab$'$ fragments to liposomes containing 5 mol% MPB-PE caused a Fab$'$ concentration-dependent increase in size and polydispersity of the liposomes directly upon prepara-tion. Under the experimental conditions used in this study (see legend Table 3) these effects were of minor impor-tance.

3.5. Stability

The following aspects concerning the stability of the OV-TL3 immunoliposomes were examined: (1) the occur-rence of changes in vesicle size; (2) retention of entrapped contents; (3) influence of serum on the retention of en-trapped contents; (4) preservation of immunoreactivity on storage. The liposomes contained entrapped CF as a fluo-rescent marker for monitoring liposomal stability.
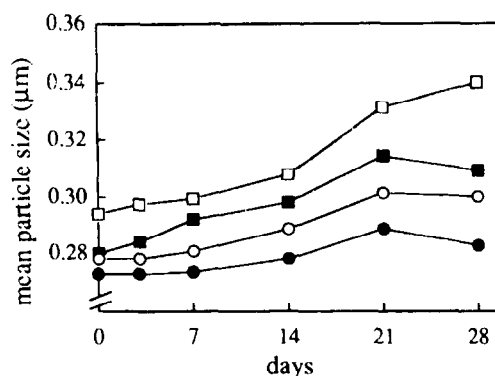

Fig. 5. Particle size stability of (immuno)liposomes as a function of Fab$'$ density. The mean particle diameter of liposomes with and without Fab$'$ OV-TL3 coupled was measured as described in Materials and methods at various days after storage at 4° C under N_2 atmosphere. Results derived from four preparations out of eleven preparations are shown. The S.D. varies between 0.2 and 2% of the mean value. Coupling ratios amounted to: ($\square$) 21 μg Fab$'$/μmol TL, ($\blacksquare$) 11 μg Fab$'$/μmol TL, ($\bigcirc$) 4 μg Fab$'$/μmol TL, and ($\bullet$) 0 μg Fab$'$/μmol TL (unconjugated MPB-PE liposomes).

The mean particle size of immunoliposomes with differ-ent coupling ratios (up to 21 μg Fab$'$/μmol TL) changed slightly during storage of the dispersion at 4° C for a period of 4 weeks (Fig. 5). No change in polydispersity index was found for the MPB-PE liposomes (pd 0.15) during 4 weeks of storage and a moderate increase in polydispersity from 0.14 to 0.22 was measured for OV-TL3 immunoliposomes with a coupling ratio of 21 μg Fab$'$/μmol TL (results not shown). A slight increase in mean particle size was observed with increasing number of Fab$'$ fragments coupled per liposome (Fig. 5). The loss of

Table 3

Characterization of standard preparations of MPB-PE liposomes, OV-TL3 immunoliposomes and RIV1000 immunoliposomes

Characteristics	MPB-PE liposomes	OV-TL3 immunoliposomes	RIV1000 immunoliposomes
Coupling ratio (μg Fab$'$/μmol TL) [a]	–	10.2 ± 1.5 ($n = 8$)	9.1 ± 0.9 ($n = 5$)
Coupling efficiency (%) [b]	–	32 ± 8 ($n = 7$)	30 ± 6 ($n = 4$)
Estimated number of Fab$'$ per liposome [c]	–	240	240
Zeta-potential (mV)	-24 ± 2 ($n = 5$)	-17 ± 1 ($n = 4$)	–
Mean particle diameter (μm)	0.26 ± 0.03 ($n = 5$)	0.25 ± 0.02 ($n = 8$)	0.26 ± 0.02 ($n = 5$)
Polydispersity index [d]	0.16 ± 0.04 ($n = 5$)	0.15 ± 0.03 ($n = 8$)	0.15 ± 0.4 ($n = 5$)

MPB-PE liposomes, OV-TL3 and RIV1000 immunoliposomes were prepared according to the standard method described in Materials and methods. Freshly reduced OV-TL3 and RIV1000 Fab$'$-fragments (concentrations varying between 0.29 and 0.37 mg/ml) were mixed with MPB-PE-liposomes (PC/PG/Chol/MPB-PE; 38.5:4:16:1.5) (concentrations varying between 8.0–11.6 μmol TL/ml) and allowed to react under N_2 atmosphere at 4° C overnight.

[a] The coupling ratio (μg Fab$'$/μmol TL) was determined following separation of Fab$'$-liposomes from unreacted Fab$'$-fragments by ultracentrifugation as described in Materials and methods.

[b] Coupling efficiency (%) was calculated by dividing the coupling ratio by the ratio of protein to lipid present during the coupling reaction $\times$ 100%.

[c] The number of Fab$'$ molecules coupled per liposome, at a coupling ratio of 10 μg Fab$'$/μmol TL, was estimated using the following assumptions: a molecular weight of Fab$'$ of 50 kDa; surface area of $29 \cdot 10^{10}$ μm^2/μmol TL; an average number of 1.5 bilayers as determined for almost identically prepared PC/PS/Chol (10:1:4) liposomes (25); mean particle diameter of 0.25 μm. A total liposomal surface area of 0.59 μm^2 per liposome and a number of $5 \cdot 10^{11}$ liposomes per μmol TL were calculated.

[d] Polydispersity index is a measure of the particle size distribution of the dispersion. This index ranges from 0 for an entirely monodisperse up to 1 for a completely polydisperse dispersion.
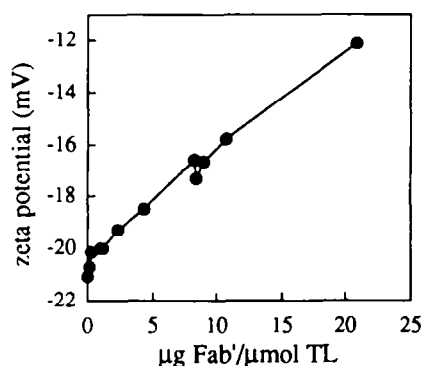
Dashes indicate not determined.

Fig. 6. Effect of the coupled amount Fab' on the zeta-potential of OV-TL3 immunoliposomes. Zeta-potentials of OV-TL3 immunoliposomes with varying coupling ratios are presented. The data shown refer to the preparations used for the experiment presented in Fig. 5.

negatively charged molecules in the bilayers contributes to prevention of aggregation (and/or fusion). The zeta-potential of the immunoliposomes and the Fab' coupling ratio appear to be linearly related: the higher the coupling ratio, the lower the negative zeta-potential (Fig. 6). The decrease in negative zeta-potential as a function of coupling ratio is most likely related to our observation that the OV-TL3 Fab' fragments are positively charged at neutral pH (see above). The neutralizing capacity of increasing amounts of coupled Fab' leading to reduced electrostatic repulsive forces may induce a tendency of immunoliposomes to aggregate. Interestingly, literature reports on increases in size and polydispersity are based on studies employing immunoliposomes with coupling ratios by far exceeding those used in the present study [36,46].

CF over a 3-week period storage was minimal (< 5%; results not shown). The permeability of liposomes was also studied in the presence of fetal calf serum. The MPB-PE liposomes and the OV-TL3 immunoliposomes showed only minor CF leakage during incubation in 50% serum at 37° C for 2 days (results not shown). With respect to preservation of immunoreactivity, the capacity of the immunoliposomes to bind to in vitro cultured ovarian carcinoma cells was maintained after storage for 1 month at 4° C (results not shown).

Various coupling procedures have been reported with detailed analysis of coupling efficiency, preservation of antibody activity, and stability of the cross-link between antibody and liposome [3]. However, very few studies have addressed the stability of immunoliposomes on storage. Bredehorst et al. [46] observed that covalent coupling of Fab' fragments to preformed MPB-PE liposomes can have major effects on liposomal integrity, size, and polydispersity. Such effects were not observed in this study. In general, inclusion of sufficient amounts of cholesterol is important to obtain liposomes with low permeability characteristics [50]. In addition, it is likely that the presence of

3.6. Binding to ovarian carcinoma cells

As specific association of immunoliposomes with their target cells is a necessary requirement for target-specific drug delivery, we evaluated the binding of OV-TL3 immunoliposomes containing CF as an aqueous marker to the OVCAR-4 human ovarian tumor cell line. Measurements of cell binding after incubation with cells at 37° C may include both binding and uptake; therefore, cell binding was studied at 4° C since endocytosis does not occur at this temperature [51,52]. To show specificity of the OV-TL3 immunoliposomes towards the OVCAR-4 cells, negative control binding experiments were performed. OV-TL3 immunoliposomes were incubated with nonovarian carcinoma cells, the murine gastric squamous cell carcinoma 5DO4 was used [35]. In addition, the degree of cell binding towards OVCAR-4 was determined for nonspecific immunoliposomes (bearing the irrelevant antibody RIV1000), MPB-PE liposomes, liposomes without incorporated MPB-PE and liposomes without incorporated MPB-PE preincubated with OV-TL3 Fab' fragments. All these negative control incubations resulted in very low
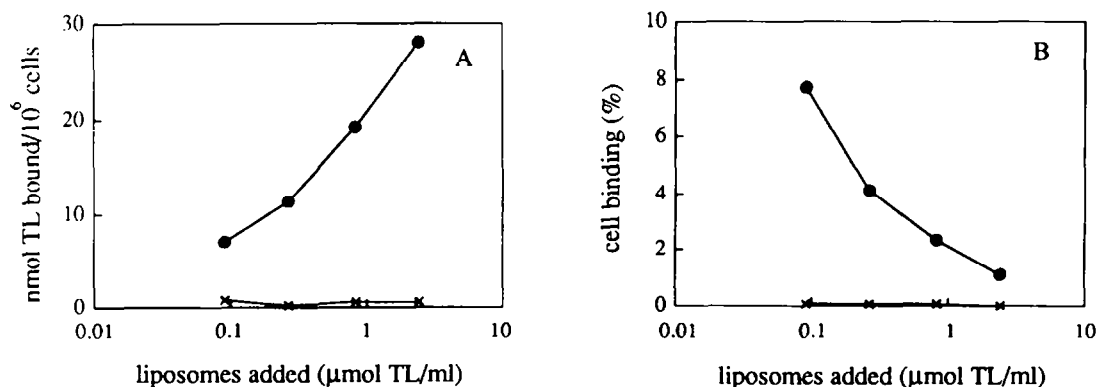

Fig. 7. Cell binding: lipid concentration dependence. CF-containing (●) OV-TL3 immunoliposomes (11 μg Fab'/μmol TL) and (×) MPB-PE liposomes were incubated at varying liposome concentrations with in vitro growing OVCAR-4 cells (10⁶ cells/ml) for 90 min at 4° C. Results are presented as: (A) the absolute amount of (immuno)liposomes bound and (B) % of added liposomes bound. Results of a typical experiment out of three performed are shown.
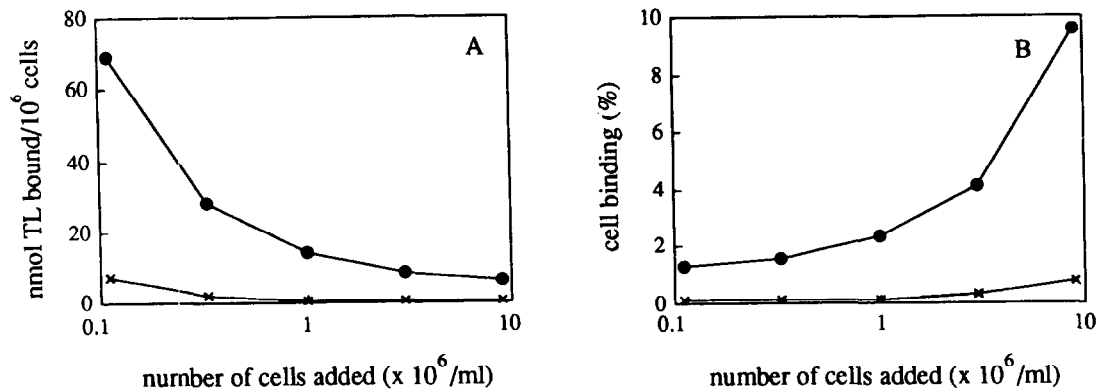
Fig. 8. Cell binding: cell concentration dependence. CF-containing (●) OV-TL3 immunoliposomes (11 μg Fab'/μmol TL) and (×) MPB-PE liposomes were incubated at a lipid concentration of 0.82 μmol TL/ml with in vitro growing OVCAR-4 cells at varying concentrations for 90 min at 4° C. Results are presented as: (A) the absolute amount of (immuno)liposomes bound and (B) % of added liposomes bound. Results of a typical experiment out of three performed are shown.

binding values. In Figs. 7–9 only the control data obtained with MPB-PE liposomes and RIV1000 immunoliposomes are presented.

Fig. 7 shows the degree of (immuno)liposome binding to OVCAR-4 cells after 1.5 h of incubation at 4° C. As the concentration of added OV-TL3 immunoliposomes was increased (at a constant cell concentration), the absolute amount of bound immunoliposomes increased (A), while the fraction bound decreased (B). The opposite was the case when the tumor cell concentration was increased (at a constant liposome concentration) (Fig. 8). It was estimated that approx. $3.5 \cdot 10^4$ liposomes were bound per cell at a binding ratio of 70 nmol TL/10^6 cells (Fig. 8). Fig. 9 shows the dependency of the degree of cell binding on the incubation time. Under the chosen conditions, cell binding was maximal within 0.5 h after start of the incubation, demonstrating that an incubation time of 1.5 h was sufficient.

All in vitro incubations presented so far were performed at 4° C. In view of the in vivo situation, it was of interest
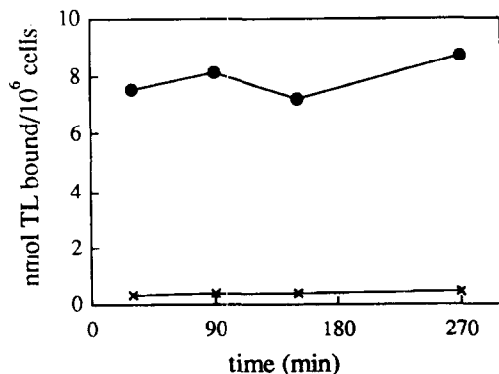
to compare the binding capacity of the OV-TL3 immunoliposomes at 4° C and at 37° C. As we observed cell-mediated leakage of liposomal CF during incubation at 37° C [8,53,54], we did not use CF as liposomal marker. Instead [³H]cholesteryloleoylether was incorporated in the liposomal bilayers as this lipid has proven to be a reliable marker for monitoring liposomes in vivo [55,56]. Fig. 10 shows that the cell binding profiles obtained at both temperatures are comparable. This finding suggests that, if internalization of bound liposomes occurs, this process is relatively slow. Recent experiments are in agreement with this suggestion as they did not provide evidence for efficient cellular internalization of the relatively large (about 0.25 μm), cell-bound immunoliposomes [58]. Internalization of liposomes usually requires a high rate of internalization of the surface receptor (e.g., the transferrin receptor will usually internalize its ligand [59]) in combination with a
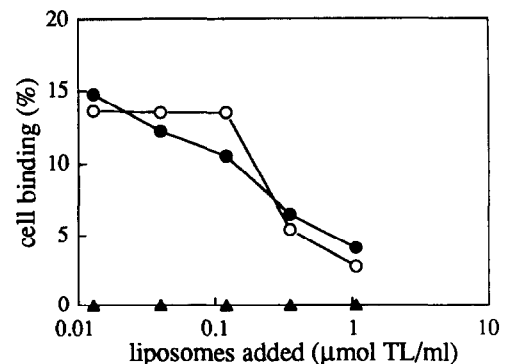

Fig. 9. Cell binding: incubation time dependence. CF-containing (●) OV-TL3 immunoliposomes (9.7 μg Fab'/μmol TL) and (×) MPB-PE liposomes were incubated at a lipid concentration of 0.27 μmol TL/ml with in vitro growing OVCAR-4 cells (10^6 cells/ml) at 4° C. Results are presented as the absolute amount of (immuno)liposomes bound. Results of a typical experiment out of two performed are shown.

Fig. 10. Cell binding: incubation temperature dependence. Radiolabeled [³H]cholesteryloleoylether-containing OV-TL3 immunoliposomes (6.5 μg Fab'/μmol TL) and RIV1000 immunoliposomes (4.0 μg Fab'/μmol TL) were incubated at varying lipid concentration with in vitro growing OVCAR-4 cells (10^6 cells/ml) for 90 min at 4° C and 37° C. Results are presented as % of added liposomes bound. OV-TL3 immunoliposomes incubated at (○) 4° C and (●) 37° C. RIV1000 immunoliposomes incubated at (▲) 4° C and 37° C. Results of a typical experiment are shown.

small size (i.e., less than about 0.1 μm) of the bound liposomes.

Specific binding was additionally demonstrated on in vivo propagated OVCAR-4 cells harvested in ascites form. As these cells grew in large cell clusters it was not possible to determine the cell concentration. Therefore, no comparison can be made with the in vitro propagated OVCAR-4 cells with regard to cell binding capacity. The incubations were performed by mixing (1:1) freshly harvested ascites with CF-containing (immuno)liposomes (16 μmol TL/ml). Under the experimental conditions cell

binding percentages amounted to $34 \pm 1\%$ (mean ± S.D. of three incubations) for the OV-TL3 immunoliposomes (6 μg Fab'/μmol TL) and 1.0% (mean of two incubations, individual values deviated 0.1% from the mean) for MPB-PE liposomes.

Finally, Fig. 11 shows electron micrographs of in vivo growing OVCAR-4 cells 5 h after i.p. administration of (immuno)liposomes. These EM micrographs revealed immunoliposomes associated with exposed tumor cells confirming the ability of OV-TL3 immunoliposomes to bind to ovarian carcinoma cells in vivo. Negative control exper-
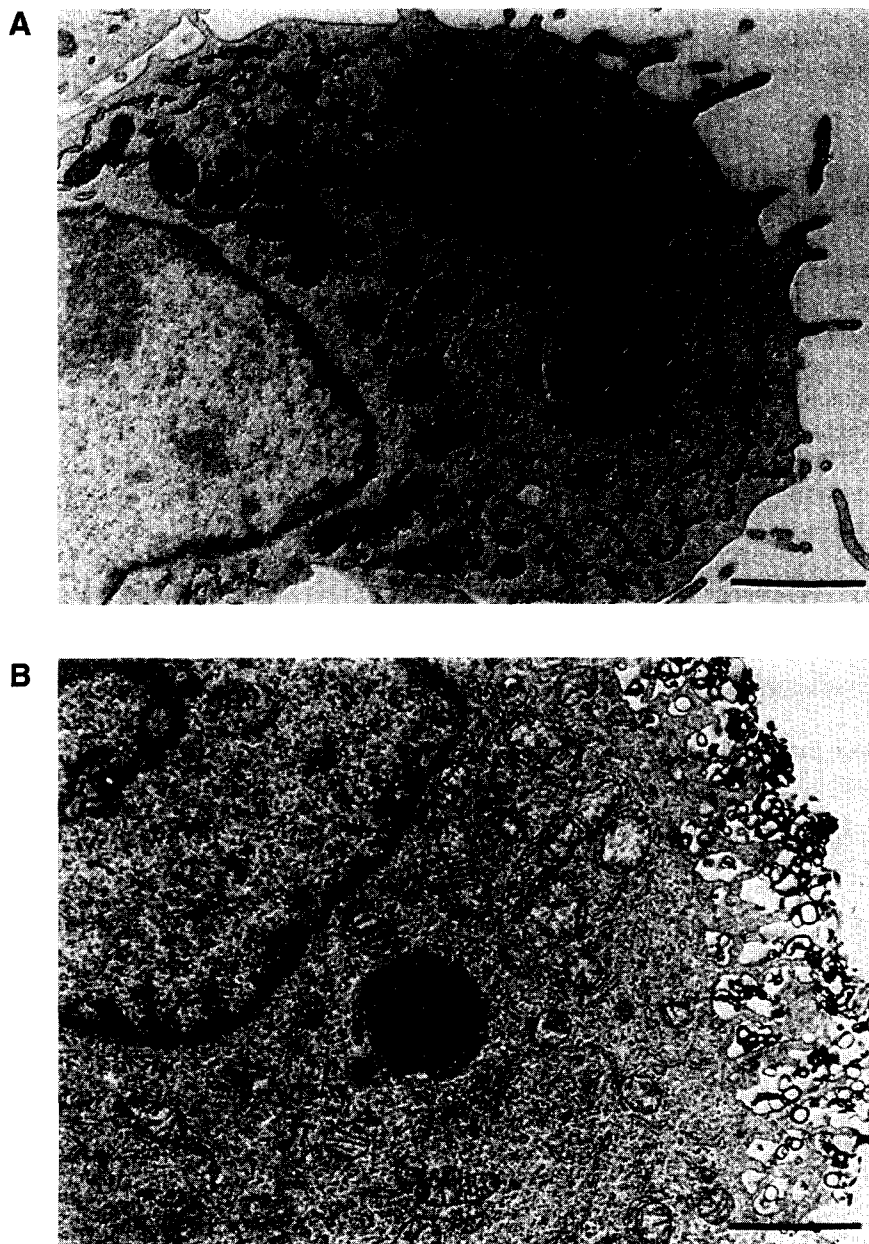

Fig. 11. Electron micrographs of i.p. growing OVCAR-4 cells 5 h after (immuno)liposome administration. OV-TL3 immunoliposomes (6 μg Fab'/μmol TL) or unconjugated MPB-PE liposomes were administered i.p. (dose: 8 μmol TL/mouse) in OVCAR-4 tumor-bearing NMRI athymic nude mice. 5 h after injection ascites was collected and treated as described in Materials and methods. Electron micrographs showing OVCAR-4 ascitic cells after treatment with: (A) unconjugated MPB-PE liposomes, (B–D) OV-TL3 immunoliposomes. Bar indicates in: (A) 1.34 μm, (B) 1.74 μm, (C) 0.87 μm, and (D) 0.2 μm.
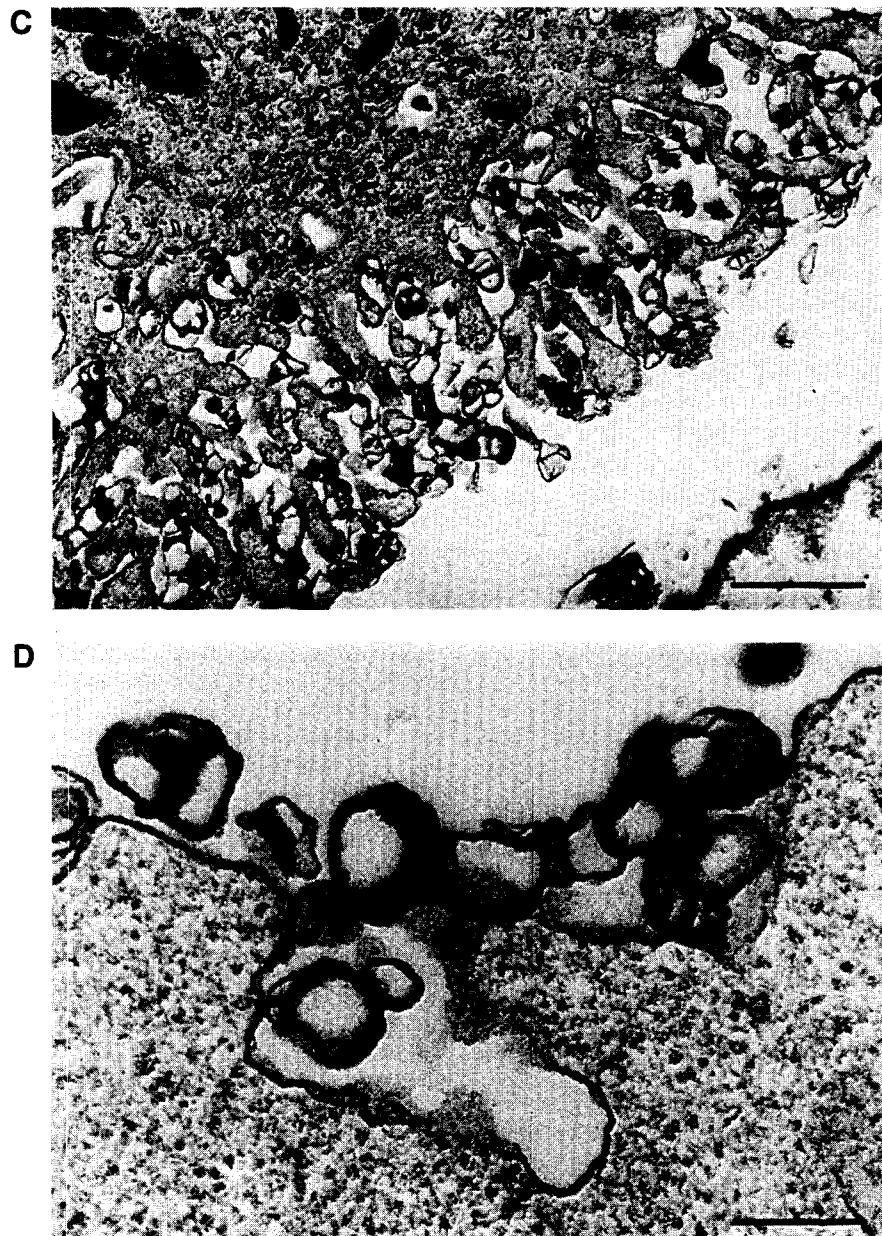
Fig. 11 (continued).

iments with MPB-PE liposomes did not reveal any interaction upon inspection.

3.7. Concluding remarks

Immunoliposomes bearing antibodies which are directed against cell surface antigens such as those associated with transformed cells, may have therapeutic potential. However, at the present time, such targeted liposomal systems have mainly been used for in vitro applications such as diagnostic assays [57]. In order to exploit the full potential of antibody-targeted carrier systems a versatile and reliable methodology for coupling is required. This report addresses an established method for the binding of

Fab' fragments of OV-TL3 (monoclonal anti-ovarian carcinoma IgG) to liposomes (extrusion MLV) containing the anchor MPB-PE. The amount of protein bound to the vesicles can be controlled within limits by varying the protein and/or lipid concentration in the coupling reaction mixture. The product was stable over at least 3 weeks in terms of leakage of encapsulated CF, particle size and antigen binding capacity. OV-TL3 immunoliposomes were shown to bind specifically to OVCAR-4 target cells in vitro and in vivo. The subsequent events which follow cell binding are currently under investigation since liposome binding may not necessarily be followed by the delivery of the encapsulated drug into the target cell. Uptake of immunoliposomes by target cells has been shown to occur

via receptor-mediated endocytosis, which varies considerably from one surface receptor to another and from one cell type to another [60]. Recent results from our laboratory did not provide evidence that internalization of OV-TL3 immunoliposomes by the OVCAR-4 tumor cells is of quantitative importance [58]. One future objective is to use other homing ligands directed to surface receptors known to be internalized by cells at a high rate, and conjugate them with target cell specific small-sized immunoliposomes. Several other options for drug entry into the target cell which do not depend on internalization of the carrier are also under investigation. One option is that the encapsulated drug simply leaks out of the cell-bound immunoliposomes in the close proximity of the tumor cell. A sufficiently high concentration gradient over the cell membrane may result in considerable cellular drug uptake. Special attention is additionally focused on the use of 'special function' immunoliposomes from which release of entrapped agents is triggered by environmental manipulations (e.g., by a slight change of the temperature or pH) after their binding to the tumor cells [3,4].

Acknowledgements

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