In Vivo Targeting of OV-TL 3 Immunoliposomes to Ascitic Ovarian Carcinoma Cells (OVCAR-3) in Athymic Nude Mice

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ABSTRACT

Specific binding of immunoliposomes to target tumor cells was investigated in a xenograft model (athymic nude mice) of i.p. growing human ovarian carcinoma (OVCAR-3). For the first time, quantitative evidence is presented that attachment of a tumor-specific antibody (OV-TL 3) dramatically enhances the association of liposomes with i.p. growing OVCAR-3 cells. The OV-TL 3-mediated binding of liposomes to the OVCAR-3 cells was rapid; 30 min after i.p. injection approximately 70% of the injected dose of OV-TL 3 immunoliposomes was associated with the OVCAR-3 cells while for unconjugated liposomes a value of only approximately 3% was obtained. At 2 h after injection, a maximal binding level of 84% was achieved in case of the OV-TL 3 immunoliposomes whereas the binding level of unconjugated liposomes was still about 3%. Twenty-four h after injection about 83% of the injected dose of OV-TL 3 immunoliposomes still was associated with the OVCAR-3 cells, compared to about 10% of the injected dose of unconjugated liposomes. Accordingly, unconjugated liposomes disappeared from the peritoneal cavity much faster than the OV-TL 3 immunoliposomes. By comparison with immunoliposomes bearing irrelevant antibody, the specificity of the binding of the OV-TL 3 immunoliposomes to the OVCAR-3 cells was demonstrated. In addition, it was observed that the sustained high OV-TL 3 immunoliposome levels found in the peritoneal cavity are a result of both reduced particle clearance from the peritoneal cavity and the tenacious binding of the immunoliposomes to the tumor cells. Finally, data are presented showing that the degree of binding of OV-TL 3 immunoliposomes to OVCAR-3 cells in vitro and in vivo correlates positively with the antibody (Fab') density on the liposomes.

INTRODUCTION

Ovarian cancer is one of the most common fatal gynecological malignancies. The OVCAR-3 human ovarian carcinoma cell line grown i.p. in nude mice provides a model system suitable for studying ovarian cancer (1-8). The progression of the disease in this model closely parallels the human disease with animals developing intraabdominal tumors and ascites (2). Since ovarian cancer remains confined to the peritoneal cavity throughout most of its lifetime, it has been suggested that i.p. therapy with immunoliposomes may be a useful treatment of this disease (9, 10).

During the last decade immunoliposomes (antibody-directed liposomes) have been studied to establish their potential as a tool to achieve efficient site-specific drug delivery (11-15). While several groups have demonstrated that antibodies can be used to increase the specificity of liposomal drug delivery to tumors in vitro (9, 16-21), the attempts to show similar effects with immunoliposomes against solid tumors in vivo have proved to be far more difficult (11-13). Two major problems limit the successful application of i.v. injected immunoliposomes in the treatment of solid tumors: poor ability of immunoliposomes to gain access to the tumor because of restricted possibilities for extravasation; and rapid clearance of i.v. injected liposomes by the mononuclear phagocyte system (12, 22). These potential limitations are less critical in the case of i.p. administration of immunoliposomes to treat peritoneally confined target tissue like ovarian carcinoma, inasmuch as the immunoliposomes and the target tumor cells are present in the same compartment. Because these immunoliposomes are retained in the peritoneal cavity for longer periods than most free drugs (23-28), i.p. administered immunoliposomes bearing appropriate ligands are likely to be able to make contact and adhere to i.p. residing ovarian carcinoma cells. It was earlier reported by Straubinger et al. (9) that immunoliposomes bearing the monoclonal antibody OC-125 were able to bind specifically to OVCAR-3 cells in vitro. In addition, qualitative evidence of the binding of these immunoliposomes to OVCAR-3 ascitic cells in vivo was presented by showing fluorescence images. However, quantitative evidence of the in vivo interaction is still lacking.

In the present study the mouse monoclonal antibody OV-TL 3 was used. 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. OV-TL 3 has proved to be a promising candidate for immunotargeting of ovarian cancer (4, 7, 8). The binding of OV-TL 3 immunoliposomes to human ovarian carcinoma cells (OVCAR-3) in vitro was investigated. The binding of OV-TL 3 immunoliposomes to ascitic OVCAR-3 cells present in the peritoneal cavity of athymic nude mice was studied using a (double) radiolabeling technique.

MATERIALS AND METHODS

Materials. The human ovarian cancer cell line NIH:OVCAR-3 originated from the American Type Culture Collection (Rockville, MD). Fetal calf serum and RPMI 1640 supplemented with 25 mm Hapes3 buffer and l-glutamine were obtained from Gibco, Ltd. (Paisley, United Kingdom). The monoclonal antibodies OV-TL 3 and RIVI000 were purchased from Eastman Kodak Company (Rochester, NY) and was supplied by Dr. L. G. Poels (University of Nijmegen, the Netherlands) and by M. F. Leerung (National Institute of Public Health and Environmental Protection, Bilthoven, the Netherlands), respectively. [U-14C]Sucrose (specific activity, 20 GBq/mmol) and [1α,2α-3H]cholesteryl oleoyl ether (specific activity, 1.71 TBq/mmol) were supplied by Amersham (Amersham, Buckinghamshire, United Kingdom). CF was purchased from Eastman Kodak Company (Rochester, NY) and was purified by the method described by Ralston et al. (32). PC, Chol, Hepes, DTT, bovine serum albumin, and N-ethylmaleimide were obtained from Sigma Chemical Co. (St. Louis, MO). Egg phosphatidylglycerol was supplied by Nattermann GmbH (Cologne, Federal Republic.

3 The abbreviations used are: TL, total lipid (phospholipid + cholesterol); Hapes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CF, carboxyfluorescein; PC, egg l-α-phosphatidylcholine type V-E; Chol, cholesterol; DTT, dithiothreitol; MFP-PE, N, N-[N-(p-maleimidophenyl)butyryl]phosphatidylethanolamine; PBS, phosphate-buffered saline.
lic of Germany), Hionic Fluar, Soluene-350, and Plasmosal were purchased from Packard Instrument Co., Inc. (Downers Grove, IL). All other reagents were of analytical grade.

Monoclonal Antibodies. Hybridomas producing the monoclonal antibodies OV-TL 3 and RIV1000 (both of mouse IgG1 type) were grown in athymic nude BALB/c mice. The monoclonal antibody OV-TL 3 is directed against human ovarian carcinoma (29), and RIV1000, in the present studies used as an irrelevant monoclonal antibody, is directed against human lymphocytes (33, 34). The antibodies were purified, fragmented, and characterized as earlier described (35). F(ab')2 fragments of OV-TL 3 and RIV1000 were incubated in 20 mM DTT at pH 5.5 for at least 90 min (35). DTT was removed by applying the incubation mixture onto a Sephadex G-25 m column (PD-10; Pharmacia). Pre-equilibration and elution occurred with deionized acetate buffer (100 mM sodium acetate-88 mM NaCl, pH 6.5) under nitrogen atmosphere. Fab' fragments appearing in the void volume were used immediately for covalent attachment to preformed liposomes. In order to check the completeness of the reduction of F(ab')2 to Fab' by high performance liquid chromatography (TSK-3000 SW column; LKB, Bromma, Sweden), an excess of N-ethylmaleimide was added to an aliquot of the resulting protein solution (35). In all cases analysis showed that the protein solution consisted of Fab' fragments for more than 90%.

Preparation of (Immuno)liposomes. MPB-PE was synthesized, purified, and analyzed as described before (28, 36). MPB-PE is incorporated into the liposomal bilayers to allow a covalent coupling of Fab' fragments to the liposomal surface. The composition of the liposomal bilayer was PC:egg phosphatidylglycerol:Chol:MPB-PE with a molar ratio of 38.5:16:1:5. A mixture of appropriate amounts of lipids and [1^H]cholesterol oleyl ether in chloroform was evaporated to dryness in a rotary evaporator at 40°C under reduced pressure. After evaporation for at least 1 h, the lipid film was hydrated in Hepes buffer (20 mM Hepes-135 mM NaCl-1 mM sucrose, pH 7.4) containing 6 Mg/ml [3^C]Sucrose. At this stage of preparation, the lipid and ^H label concentrations were 110 nM/ml and 3 Mg/ml [1^H]cholesterol oleyl ether. The resulting liposome dispersion was sequentially extruded through polycarbonate membranes filters with 0.6 and 0.2 pm pore size (Uni-pore; Bio-Rad, Richmond, CA) under nitrogen pressures up to 0.8 MPa (37). After extrusion, the radiolabeled MPB-PE liposomes were separated from free (nonentrapped) radiolabel by ultracentrifugal sedimentation at 80,000 X g during 45 min. The pellet was resuspended in acetate buffer (100 mM sodium acetate-88 mM NaCl, pH 6.5) followed by flushing with nitrogen. Freshly prepared MPB-PE-liposomes in deionized buffer (concentrations during incubation ranging from 6 to 10 Mg/ml TL/ml) were mixed with freshly prepared Fab' fragments in phosphate buffered saline (PBS) at a concentration of 400 Mg/ml (35). The coupling reaction was carried out in a nitrogen atmosphere at 4°C under constant end over end rotation overnight. Finally, the immunoliposomes were separated from unconjugated Fab' fragments by ultracentrifugal sedimentation at 80,000 X g during 45 min. The pellet was resuspended and washed twice with Hepes buffer (20 mM Hepes-135 mM NaCl, pH 7.4). MPB-PE-liposomes not being incubated with Fab' fragments, further referred to as un conjugated liposomes, were exposed to the same treatment. Liposome dispersions were stored at 4°C under nitrogen atmosphere and used for in vivo experiments within 1 week after preparation. Nonradioactive CF containing immunoliposomes were prepared as described earlier (35).

Liposome Characterization. Lipid phosphate was determined by the colorimetric method of Fiske and SubbaRow (1925) (38). Protein was determined by the method of Wessel and Flugge (39), with bovine serum albumin as standard.

The amount of monoclonal antibody coupled to the liposomes was expressed as Mg of protein per Mg of TL. The protein/lipid ratios (Mg Fab'/Mg TL) of the immunoliposomes used in a specific experiment are indicated in the legends of the figures. The number of OV-TL 3 Fab' coupled per liposome was estimated using the following assumptions: a molecular weight for Fab' of 50 kDa; an average number of 1.5 bilayers; and an estimated surface area of 29 X 10^18 Mg^2/micrometer^2 TL as determined for PC:phosphatidylserine:Chol (10:1:4) liposomes prepared by a method almost identical to ours (40). A total liposomal surface area of 0.59 mm^2 and a number of 5 X 10^11 liposomes/micrometer^2 TL were calculated at a mean diameter of 0.25 mm.

At the concentration used for the preparation of CF liposomes (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 489 nm excitation wave length and 518 nm emission wave length in a Kontron Instruments spectrofluorometer, Model SFM 25 (Watford/ Hertfordshire, United Kingdom) (41).

Radioactivity of the liposomal dispersions was assayed with Hionic-Fluar as scintillation mixture and counted in a Tri-Carb 1500 liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL) in preset H/[3^C] channels using an external standard ratio method for quenching correction and correction for the contribution of [3^C] in the H channel. The incorporation efficiencies, corrected for lipid loss, were 106 ± 6% and 12 ± 2% (mean ± SD of 7 preparations) of [1^H]cholesterol oleyl ether and [1^C]Sucrose, respectively. On the basis of the amount encapsulated [3^C]Sucrose an internal aqueous volume of 1.1 ± 0.2 Ml/micrometer^2 TL was calculated.

Mean particle size was determined by dynamic light scattering with a Malvern 4700 system using a 25 Mw helium-neon laser and the auto measure vsn 3.2 software (Malvern, Ltd., Malvern, United Kingdom). 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. The mean particle size of the liposomes used was 0.25 ± 0.02 Mm with a polydispersity index of 0.15 ± 0.05 (mean ± SD of 7 preparations).

Cell Binding Assay. CF-containing immunoliposomes were incubated in vitro with human ovarian cancer cells NIH:OVCAR-3 (1). The cells were maintained in RPMI 1640 supplemented with fetal calf serum (10%), insulin (10 Mg/ml), penicillin (100 units/ml), streptomycin (100 Mg/ml), and Fungizone (0.26 Mg/ml). Cultured monolayers of NIH:OVCAR-3 cells were treated with trypsin/EDTA (0.25%/0.02%). A suspension of cells in PBS (4 X 10^6 cells/ml) was mixed with an equal volume of CF-containing immunoliposomes (lipid concentrations varying from 0.12 to 3.3 Ml/micrometer^2 TL). Incubations were performed in triplicate under continuous agitation for 90 min at 4°C. Unbound (immuno)liposomes were separated from the cells by centrifugation (800 X 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 X g during 10 min, supernatants were analyzed for CF as described above.

Animals. NMRI athymic nude mice (42) were bred at Harlan CPB (Zeist, the Netherlands). The animals were housed throughout the experiment under specified pathogen-free conditions in sterile filter top cages with sterile bedding. They received sterile standard food (SRM-A; Hope Farms, Woerden, the Netherlands) and acidified sterile water ad libitum. At the start of the experiment the animals were 5–8 weeks of age.

Tumor Model. Female NMRI athymic nude mice were inoculated s.c. with 5 X 10^6 NIH:OVCAR-3 cells in RPMI without supplements. Five months after inoculation the developed tumor was excised and mechanically dissociated and subsequently transplanted by inoculation of each mouse via both the s.c. and i.p. route. This passage procedure was repeated once, whereas after an ascitic growing tumor developed in the peritoneal cavity. For further serial transfer of tumor cells only i.p. ascitic tumor cells were used. Generally, the athymic nude mice developed a reproducibly growing ascitic tumor with only minor solid tumor growth. At 3–4-week intervals ascites was harvested from the donor animals by rinsing the peritoneal cavity with plain RPMI 1640 medium. Cells were centrifuged once (800 X g, 5 min) and resuspended in plain RPMI 1640 at a concentration of approximately 4 X 10^6 cells/ml; 0.5 ml of this cell suspension was injected i.p. into mice. The number of vital OVCAR-3 cells in the cell suspension was determined by trypan blue dye exclusion. The use of ascitic cells as inoculate leads to an inherent variability in cell counting, since OVCAR-3 cells grow in large numbers.
clusters making a viable cell count difficult; the mean number of cells present in one cluster was estimated to be 15. Mice were used 3–4 weeks after i.p. administration of the OVCAR-3 tumor cells, when abdominal ascites was visible.

**In Vivo Distribution.** To study the disposition and the integrity of (immuno)liposomes following i.p. administration both the lipid bilayer and the internal aqueous compartment were radiolabeled. \(^{3}H\)cholesteryl oleoyl ether was used as a marker of the lipid phase. Cholesteryl oleoyl ether does not exchange with proteins present in plasma (43) and is, in contrast to the ester analogue, not hydrolyzed in cells and therefore a useful marker for studying cellular uptake (43, 44). In order to obtain an impression of the integrity of the liposomes with respect to release of the encapsulated aqueous contents in the peritoneal cavity, \(^{[14}C\)sucrose was chosen as a label of the internal aqueous compartment. Leakage of \(^{[14}C\)sucrose from the (immuno)liposomes will result in lower \(^{14}C\) levels than expected on the basis of the \(^{3}H\) levels measured because leaked sucrose is cleared from the peritoneal cavity much faster than liposome-encapsulated sucrose.

Double-radiolabeled (immuno)liposomes were administered i.p. into mice bearing an ascitic OVCAR-3 tumor (2 µmol TL in a volume of 0.5 ml). In addition, also free \(^{[14}C\)sucrose (45 kBq) in 0.5 ml buffer (20 mM Hepes-135 mM NaCl-1 mM sucrose, pH 7.4) was injected. At different time points postinjection, radioactivity in peritoneal washings, blood, liver, and spleen was determined. Under light ether anesthesia blood samples were drawn from the retroorbital plexus into heparinized tubes. Then the mice were sacrificed and the peritoneal cavity was rinsed with 4 × 5 ml PBS. Liver and spleen were collected, weighed, and stored at −20°C until further analysis. The radioactivity in blood was determined as follows. Blood (100 µl) was mixed with 400 µl demineralized water and 3 drops of Plasmasol. The samples were mixed with 500 µl 27% hydrogen peroxide and decolorized overnight at 40°C. The radioactivity was assayed with Hionic Fluor as a scintillation mixture.

Immediately after the peritoneal cavity was rinsed, samples were drawn from the PBS washings for determination of the total amount of radioactivity present in the peritoneal cavity. Subsequently, the washings were centrifuged at 800 × g for 10 min in order to spin down the cells. The supernatant was collected and samples were drawn for the determination of the amount of radioactivity present in the peritoneal cavity, not associated with the cells. In addition, the weight of the cell pellet was determined. Samples of peritoneal washings (0.2 ml), supernatants (0.4 ml), and tissues (100 mg or less) were digested by the addition of 1 ml Soluene 350 and incubation at 40°C overnight, yielding clear solutions. Radioactivity was assayed with Hionic Fluor as a scintillation fluid. Prior to radioactivity measurements, the samples were stored overnight in the dark. All samples were counted in a Tri-Carb 1500 liquid scintillation counter as described above. Total radioactivity in peritoneal washings, blood, liver, and spleen was converted to percentage of administered i.p. dose. Blood volume was taken as 77.8 ml blood/kg body weight (45). The percentage of liposomes bound to OVCAR-3 cells in the peritoneal cavity was calculated by subtracting the amount of radioactivity present in the supernatants from the total amount of radioactivity present in the peritoneal washings prior to centrifugation.

Statistics. Differences in level of significance between group means were analyzed by Student’s t test (two-sided).

**RESULTS**

**In Vitro Cell Binding.** As a prelude to assessing the binding of OV-TL 3 immunoliposomes to in vivo growing OVCAR-3 cells, the binding capacity of these immunoliposomes was investigated in an in vitro binding assay. As shown in Fig. 1 it was found that the number of OV-TL 3 immunoliposomes bound to NIH-OVCAR-3 cells increased with the lipid concentration during incubation. In contrast, RIV1000 immunoliposomes showed only negligible binding. A correlation could be observed between the antibody density on the OV-TL 3 immunoliposomes and their binding capacity to the ovarian carcinoma cells.

**Clearance of Free Sucrose.** The amount of free \(^{[14}C\)sucrose present in the peritoneal washings, blood, liver, and spleen after i.p. administration in the OVCAR-3 ovarian carcinoma nude mouse model was measured. Relatively high \(^{[14}C\)sucrose recoveries were measured in the peritoneal cavity and the blood compartment 0.5 h after i.p. injection (23 ± 4% and 3.2 ± 0.4% respectively, n = 5), while 2 h postinjection \(^{[14}C\)sucrose was almost completely cleared from these compartments (0.59 ± 0.08 and 0.08 ± 0.02% respectively, n = 5). The uptake of \(^{[14}C\)sucrose by liver and spleen was very low (less than 0.6% of the injected dose 0.5 h after i.p. administration and less than 0.2% after 2 h).

**Comparison of OV-TL 3 Immunoliposomes and Unconjugated Liposomes.** Double-radiolabeled OV-TL 3 immunoliposomes and unconjugated liposomes (i.e., MPB-PE-liposomes without coupled Fab') were administered i.p. into OVCAR-3-bearing nude mice. Judging from the radioactivity values presented in Fig. 2, unconjugated liposomes disappeared from the peritoneal cavity much faster than the OV-TL 3 immunoliposomes. In fact, the fraction of OV-TL 3 liposomes recovered from the peritoneal cavity (indicated by the \(^{3}H\)-label) decreased only slightly during the 24-h observation period. At the end of the observation period, 87 ± 2% (mean ± SD) of the administered \(^{3}H\) dose of OV-TL 3 immunoliposomes was still present in the peritoneal cavity compared to 25 ± 8% in case of the unconjugated liposomes. The extent of liposome association with the peritoneal OVCAR-3 cells is presented in Fig. 3. The OV-TL 3 immunoliposomes associated rapidly and to a much greater extent with the OVCAR-3 cells than unconjugated liposomes. Thirty min and 2 h after injection, 70 ± 8% and 84 ± 5%, of the injected \(^{3}H\) dose, respectively, was associated with OVCAR-3 cells after immunoliposome injection compared to 3 ± 12% and 3 ± 4% in case of unconjugated liposomes. Twenty-four h after injection still 83 ± 2% of the injected \(^{3}H\)-dose of OV-TL 3 immunoliposomes was associated with the OVCAR-3 cells compared to 10 ± 3% after administration of unconjugated liposomes.

Levels of \(^{3}H\) label associated with liver and spleen are presented in Table 1. Only minor liposome uptake by these organs was observed over the 24-h observation period. Unconjugated liposomes associated with liver tissue to a higher extent than the OV-TL 3 immunoliposomes.

[Graph showing antibody density and lipid concentration on the binding capacity of OV-TL 3 immunoliposomes to NIH-OVCAR-3 cells in vitro.]

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Results are expressed as percentage of injected dose (mean ± SD with a tumor cell load of 4.5 ± 0.8 g/mouse (mean ± SD of 35 animals) were used. The protein/lipid ratio of the OV-TL 3 immunoliposomes was 21 µg Fab'/µmol TL. (Immuno)liposomes, double-radiolabeled with [3H]cholesteryl oleoyl ether and [14C]sucrose, were administered i.p. at a dose of 2 µmol TL in 0.5 ml. Mice with a tumor cell load of 4.5 ± 0.8 g/mouse (mean ± SD of 35 animals) were used. The protein/lipid ratio of the OV-TL 3 immunoliposomes was 21 µg Fab'/µmol TL. Results are expressed as percentage of injected dose [mean ± SD (bars) of 4–6 animals]. Difference between recovery of 3H-labels after administration in OV-TL 3 immunoliposomes and unconjugated liposomes: 0.5 h, P < 0.05; 2 and 24 h, P < 0.001; 5 h, P < 0.01. •—•, OV-TL 3 3H-immunoliposomes; ○—○, unconjugated 3H-liposomes; ○—■, OV-TL 3 14C-immunoliposomes; ○—○, unconjugated 14C-liposomes.

Comparison of OV-TL 3 Immunoliposomes and Immunoliposomes Bearing the Irrelevant Antibody RIV1000. To further establish the specificity of the binding of the OV-TL 3 immunoliposomes to the OVCAR-3 cells, immunoliposomes were prepared with the irrelevant antibody RIV1000, which recognizes an antigen present on human lymphocytes. OV-TL 3 immunoliposomes and RIV1000 immunoliposomes were administered i.p. to ascitic tumor-bearing nude mice. Fig. 4 shows the levels of radioactivity measured in peritoneal washings at different time points after injection. The OV-TL 3 immunoliposomes were eliminated from the peritoneal cavity at a slower rate than the RIV1000 immunoliposomes. Forty-eight h after administration 55 ± 6% of the injected 3H dose of OV-TL 3 immunoliposomes was still present in the peritoneal washings compared to 14 ± 4% in case of the RIV1000 immunoliposomes. Following a relatively short initial liposome-binding period, the fraction of OV-TL 3 immunoliposomes bound to the tumor cells remained nearly constant over the 48-h observation period (Fig. 5). Forty-eight h after injection the fraction of 3H label associated with the OVCAR-3 cells was 52 ± 6% and 8 ± 2% for the OV-TL 3 immunoliposomes and the RIV1000 immunoliposomes, respectively. Compared to the cell binding results presented in Fig. 3, the preparation of OV-TL 3 immunoliposomes used in this experiment showed less efficient binding to the OV-CAR-3 cells.

Levels of liposomal label recovered in liver and spleen were very low for both types of immunoliposomes (Table 2). The 3H levels recovered in the blood compartment were also very low for both immunoliposome types (less than 1% of the injected doses; results not shown).

Effect of OV-TL 3 Fab' Density on Liposomes on Degree of Cell Binding. Double-radiolabeled OV-TL 3 immunoliposomes dispersions with different antibody densities (0.33, 2.7, and 11 µg Fab'/µmol TL) were administered i.p. to athymic nude mice bearing OV-CAR-3 ascitic tumor cells. Fig. 6 depicts the recovery of 3H and 14C label in peritoneal washings at 0.5, 5, and 24 h after administration. Clearly, the reduction of the protein/lipid ratio from 11 µg Fab'/µmol TL to 2.7 µg Fab'/µmol TL resulted in an accelerated disappearance of the immunoliposomes from the peritoneal cavity. In addition, the cell binding results presented in Fig. 7 suggest that the ability of OV-TL 3 immunoliposomes to bind to OV-CAR-3 cells in vivo correlates positively with the antibody density on the liposomes.

Table 2

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<th>Time (h)</th>
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<th>Spleen</th>
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<td>Unconjugated liposomes</td>
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* Results are expressed as percentage of injected 3H dose (mean ± SD of 4–6 mice). For further data see legend to Fig. 2. **P < 0.05 (OV-TL 3 immunoliposomes vs. unconjugated liposomes).

Fig. 3. Degree of binding of OV-TL 3 immunoliposomes and unconjugated liposomes to OVCAR-3 cells present in the peritoneal cavity of athymic nude mice. For symbols see legend to Fig. 2. Difference between recoveries of 3H label after administration in OV-TL 3 immunoliposomes and unconjugated liposomes: all time points, P < 0.001.

Fig. 4. Recovery of OV-TL 3 immunoliposomes and RIV1000 immunoliposomes from the peritoneal cavity of athymic nude mice bearing an ascitic OVCAR-3 tumor. Immunoliposomes, double-radiolabeled with [3H]cholesteryl oleoyl ether and [14C]sucrose were administered i.p. at a dose of 2 µmol TL in 0.5 ml. Mice with a tumor cell load of 3.6 ± 0.6 g/mouse (mean ± SD of 39 animals) were used. The protein/lipid ratio of the OV-TL 3 immunoliposomes was 8.2 µg Fab'/µmol TL and that of the RIV1000 immunoliposomes was 7.9 µg Fab'/µmol TL. Results are expressed as % of the injected dose (mean ± SD of 4–6 mice). Difference between recoveries of 3H labels after administration in OV-TL 3 and RIV1000 immunoliposomes: 5 h, P < 0.01; 24 and 48 h, P < 0.001. •—•, OV-TL 3 3H-immunoliposomes; ○—○, RIV1000 3H-immunoliposomes; ○—○, RIV1000 14C-immunoliposomes.

Fig. 5. Degree of binding of OV-TL 3 immunoliposomes and RIV1000 immunoliposomes to OV-CAR-3 cells present in the peritoneal cavity of athymic nude mice. For symbols see legend to Fig. 4. Difference between recoveries of 3H labels after administration in OV-TL 3 and RIV1000 immunoliposomes: all time points, P < 0.001.
Table 2  Recovery of \(^{3}H\)cholesterol oleyl ether in liver and spleen after i.p. administration of OV-TL 3 immunoliposomes and RIV1000 immunoliposomes in athymic nude mice bearing an ascitic OVCAR-3 tumor

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<th>Time (h)</th>
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<th>Spleen*</th>
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* Results are expressed as percentage of injected \(^{3}H\) dose (mean ± SD of 4–6 mice). For further data see legend to Fig. 4.

Fig. 6. Effect of protein/lipid ratio on the recovery of OV-TL 3 immunoliposomes from the peritoneal cavity of athymic nude mice bearing an ascitic OVCAR-3 tumor. OV-TL 3 immunoliposomes, double-radiolabeled with \(^{3}H\)cholesterol oleyl ether and \(^{14}C\)glucos, were administered i.p. at a dose of 2 \(\mu\)mol TL in 0.5 ml. Mice with a tumor cell load of 3.6 ± 0.5 g/mouse (mean ± SD of 42 animals) were used. The protein/lipid ratios of the different OV-TL 3 immunoliposomes were: (•) 11 \(\mu\)g Fab'/\(\mu\)mol TL, (O) 2.7 \(\mu\)g Fab'/\(\mu\)mol TL and (A) 0.33 \(\mu\)g Fab'/\(\mu\)mol TL. --, \(^{3}H\) label; -- - --, \(^{14}C\) label. Results are expressed as percentage of injected dose (mean ± SD (bars) of 4–5 animals). Difference between recoveries of \(^{3}H\) labels after administration in OV-TL 3 immunoliposomes of different protein/lipid ratios: • versus O, 5 and 24 h, \(P < 0.001\); • versus A, 0.5, 5 h, \(P < 0.01\), 24 h \(P < 0.001\).

Again, the \(^{3}H\) levels recovered in liver and spleen were very low (Table 3). It can be concluded that the liver and spleen uptake was more pronounced at lower protein/lipid ratios and that the uptake increased with time. From the blood compartment very low \(^{3}H\) recoveries (less than 0.5% of the injected dose) were observed (results not shown).

DISCUSSION

The data in this paper show for the first time quantitatively that attachment of a tumor-specific antibody (OV-TL 3) dramatically enhances the association of liposomes with i.p. located tumor cells (OVCAR-3) in vivo (Figs. 3 and 5). Thirty min postinjection approximately 70% of the injected dose of OV-TL 3 immunoliposomes was associated with the OVCAR-3 cells in contrast with only 3% after administration of unconjugated liposomes (Fig. 3). Thus, at that time point a 23-fold increase in binding efficiency was achieved by using OV-TL 3 as a targeting ligand. After reaching a maximal binding level of 84% within 2 h after administration, the OV-TL 3 immunoliposomes showed a 28-fold binding advantage. No decrease in the percentage of bound OV-TL 3 immunoliposomes was seen during the remaining observation period (Fig. 3). Apparently, the OV-TL 3 immunoliposomes are able to form a strong complex with the OVCAR-3 cells. The percentage of cell binding never exceeded 10% of the injected dose after administration of unconjugated liposomes (Fig. 3) and RIV1000 immunoliposomes (Fig. 5). The method used to separate cell-bound from non-cell-bound (immuno)liposomes might lead to a slight overestimation of the actual percentage cell binding, because the possibility that nonbound (immuno)liposomes are present in between cells in the pellet after centrifugation cannot be totally excluded. It is also possible that a fraction of the "cell-bound" liposomes are associated with peritoneal macrophages. In earlier studies (28) it was demonstrated that minor fractions of platinum were associated with peritoneal macrophages after i.p. administration of cis-diaminedichloroplatinum(II) containing liposomes in non-tumor-bearing animals. The presence of a tumor in the peritoneal cavity can attract more macrophages and in this way cause a higher retention of the liposomes in the cavity compared to non-tumor-bearing animals. However, in ascites low amounts of peritoneal exudate cells were enumerated: approximately 1 × 10^6 peritoneal exudate cells/g tumor cell pellet. One g cell pellet contained about 1 × 10^8 OVCAR-3 cells.

Our observations concerning the slow absorption from the peritoneal cavity of the OV-TL 3 immunoliposomes (Fig. 2) and the high level of binding to the tumor cells (Fig. 3) strongly suggest a causal relationship. Nevertheless, the slow absorption rate of liposomes from the peritoneal cavity is not necessarily merely a function of the high extent of liposome binding to the tumor cells. This becomes clear when comparing the peritoneal retention time of the unconjugated liposomes (Fig. 2) and the RIV1000 immunoliposomes (Fig. 4) in OVCAR-3-bearing nude mice with that of unconjugated liposomes in non-tumor-bearing normal mice after i.p. injection. We have previously shown that 5 h after i.p. administration of double-radiolabeled unconjugated PC:phosphatidylerine:Chol (10:1:4)-liposomes in non-tumor-bearing BALB/c mice approximately 6% of the injected dose radioactivity was recovered in the peritoneal cavity (27). In the present study, 5 h postinjection approximately 40 and 60% of the injected dose of unconjugated liposomes and RIV1000 immunoliposomes, respectively, were recovered in the peritoneal cavity (Figs. 2 and 4). Because these (immuno)liposomes show only minor affinity for OVCAR-3 cells (Figs. 3 and 5), the relatively long retention time in the peritoneal cavity cannot be explained by an increased binding and consequently other factors must play a role. It is not likely that merely the difference in lipid composition explains the effect, because liposomes of varying lipid composition and size have shown similar elimination kinetics after i.p. administration (25, 26).

As a consequence of their particulate nature, (immuno)liposomes are not able to pass the vascular endothelium; instead they are absorbed from the peritoneal cavity through the lymphatics of the diaphragm (23, 25, 26, 46–48). Therefore, liposomes are likely to be retained in the peritoneal cavity for...
It has also been shown that virtually no i.p. injected liposomes accompanied by the formation of ascitic fluid might have resulted in longer periods than most free drugs (23-28). In our tumor model, the presence of a large ascitic tumor burden accompanied by liver and spleen does not by far reflect the recovery of radioactive liposomes (Table 1) and RIVI000 immunoliposomes within the peritoneal cavity, very low levels of radioactivity were measured in blood, liver, and spleen (Tables 1–3). However, unexpectedly, also the administration of unconjugated liposomes (Table 1) and RIV1000 immunoliposomes (Table 2) resulted in low levels in these tissues. The uptake of (immuno)liposomes by liver and spleen does not by far reflect the disappearance of liposomes from the peritoneal cavity. At present, the exact in vivo fate of the (immuno)liposomes leaving the peritoneal cavity is not clear.

In this study we have shown that the presence of OV-TL 3 immunoliposomes to OVCAR-3 cells is dependent on the number of Fab' molecules present on the surface of the liposomes. It was estimated that immunoliposomes with a protein/lipid ratio of 11 μg Fab'/μmol TL did couple 265 Fab' molecules per liposome using 0.25 μm as mean particle size. The effect of antibody density on the binding capacity for OVCAR-3 cells 5 h after administration is summarized in Fig. 8 (data taken from several experiments). The binding efficiency plateaus at protein/lipid ratios above 11 μg Fab'/μmol TL. Several authors have discussed the importance of multivalent interaction in antibody-mediated targeting to cells. Bragman et al. (55) showed that the binding capacity of intact anti-human glycophorin A antibody (IgG1) to erythrocytes was 10-fold higher than the binding of corresponding Fab fragments. This indicates that the divalent antibody is more effective than the monovalent Fab in binding to the cells due to a divalent interaction with the erythrocyte membrane. By increasing the antibody density of immunoliposomes, an increased degree of association with in vitro cultured target cells was obtained (Fig. 1). This phenomenon has also been reported by other investigators (56–58).

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In this study we have shown that the presence of OV-TL 3
monoclonal antibodies conjugated to the surface of liposomes substantially increases the lipidosome binding to ascitic OVCAR-3 cells after i.p. administration in nude mice (Figs. 3 and 5). A high fraction (maximally 84%) of the injected dose OV-TL 3 immunoliposomes was bound to the cells within 2 h after administration and this binding was maintained during the 24-h observation period. Sustained high immunoliposome levels were found in the peritoneal cavity as a result of both reduced particle clearance from the peritoneal cavity as well as the strong binding of the immunoliposomes to tumor cells. The OV-TL 3-mediated binding of liposomes to the OVCAR-3 cells is rapid and dependent on the protein/lipid ratio used.

The aim of current efforts is to evaluate whether the observed important pharmacokinetic effects in vivo will lead to a substantial increase in therapeutic index when these immunoliposomes, loaded with antitumor drugs, are tested for antitumor activity.

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REFERENCES


IN VIVO TARGETING OF OV-TL 3 IMMUNOLIPOSOMES TO OVCAR-3 CELLS

Fig. 8. Effect of the protein/lipid ratio on the binding of OV-TL 3 immunoliposomes to OVCAR-3 cells 5 h after i.p. administration. Data taken from Figs. 3, 5, and 7. Bars, SD.

Bar, SD.
IN VIVO TARGETING OF OV-TL 3 IMMUNOLIPOSOMES TO OVCA-3 CELLS

In Vivo Targeting of OV-TL 3 Immunoliposomes to Ascitic Ovarian Carcinoma Cells (OVCAR-3) in Athymic Nude Mice

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