Targeted delivery of diphtheria toxin via immunoliposomes: efficient antitumor activity in the presence of inactivating anti-diphtheria toxin antibodies

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Abstract Diphtheria toxin (DT) has attracted considerable attention for anti-cancer therapy. However, its extensive use is prohibited by (i) its non-specific action which can result in substantial toxicity, (ii) most patients have low serum levels of anti-DT antibodies (AT antibodies) which can inactivate DT and (iii) its immunogenicity will boost the circulating AT antibody level, thereby further compromising the antitumor activity. To overcome these limitations, we have developed a new approach for targeted delivery of DT utilizing immunoliposomes. In this approach, protection against the non-specific action of DT is combined with efficient antitumor activity even in the presence of inactivating AT antibodies.

Key words: Diphtheria toxin; Immunoliposome; Targeted drug delivery; Monoclonal antibody; Ovarian cancer

1. Introduction

During the past two decades, diphtheria toxin (DT) has attracted considerable attention for its potential use in cancer therapy [1–3]. DT produced by *Corynebacterium diphtheriae* is toxic to most eukaryotic cells. It inhibits protein synthesis via ribosylation of elongation factor 2. An attractive feature of toxins like DT is that they are also able to kill non-dividing cells, which many conventional chemotherapeutic drugs do not [4]. Three major factors prohibit the extensive use of DT in cancer therapy. Firstly, its non-specific action can lead to severe side effects. Secondly, as in the industrialized countries most people are vaccinated against diphtheria, over 80% of the population have low serum levels of anti-diphtheria toxin antibodies (AT antibodies). These circulating AT antibodies can inactivate DT, thereby inhibiting the antitumor effect. Thirdly, because of its immunogenicity, the administration of DT will result in a drastic increase in the serum levels of AT antibodies within 2 weeks after administration, thereby further compromising the antitumor activity. Intravenous administration of DT on 3 consecutive days to 50 patients with advanced solid tumors resulted in an overall response rate of 48% [5]. However, when the responders were treated again with DT after regrowth of tumor lesions, no additional responses were observed, probably because of the presence of neutralizing AT antibodies.

To enhance its target cell specificity (e.g. towards tumor cells), complete DT or the enzymatic active part of DT (fragment A: DTA) has been coupled to monoclonal antibodies (immunotoxins; reviewed in e.g. [6]). For the same purpose, DTA has been encapsulated in pH-sensitive antibody-directed liposomes (immunoliposomes [7,8]). Knowledge of the structural and functional properties of several toxins, as well as the development of recombinant techniques have made it furthermore possible to construct fusion proteins in which the native receptor binding domain of DT is replaced by, e.g. growth factors [3,6,9].

It has been reported that circulating neutralizing AT antibodies strongly limit the therapeutic use of DT immunotoxins by early inactivation of the toxin and will particularly interfere in multiple injection schemes [10,11]. As an alternative to this approach, we have developed an entirely new concept for the targeted delivery of DT. Immunoliposomes are able to bind to tumor cells in vitro and in vivo if located at a body site that can be reached by the immunoliposomes [12–16]. Because it is difficult for immunoliposomes to pass from one body compartment (e.g. blood) to another, potential target sites must be selected carefully. Target cells in the bloodstream, lymph nodes, and body cavities such as the peritoneal cavity, pleural cavity, uterus, and bladder can be reached by immunoliposomes after intravenous or local administration. That immunoliposomes can be targeted efficiently to tumor cells was shown by, e.g. Nässander et al [13] and Ahmad et al. [14]. Immunoliposomes were able to bind rapidly and efficiently (i.e. more than 80% of the administered i.p. dose) to human ovarian carcinoma cells located in the peritoneal cavity of nude mice [13]. In lung carcinoma-bearing animals increased lung uptake of i.v. administered long-circulating immunoliposomes was observed compared to that in non-tumor-bearing animals [14].

We hypothesized that encapsulation into immunoliposomes may protect DT against inactivation by circulating neutralizing AT antibodies. After binding to the tumor cells, DT may

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Abbreviations: AT antibodies, anti-diphtheria toxin antibodies; AU, antibody unit; CHOL, cholesterol; DMEM, Dulbecco's modified Eagle's medium; DT, diphtheria toxin; DTA, diphtheria toxin fragment A; DTT, dithiothreitol; EPC, egg phosphatidylcholine; EPG, egg phosphatidylglycerol; FCS, fetal calf serum; Lf, limit of flocculation (quantity of DT that flocculates most rapidly when mixed with 1 unit of antitoxin); MPB-PE, *N*-[4-(*p*-maleimidophenyl)butyryl]phosphatidylethanolamine; PE, phosphatidylethanolamine; SMPB, succinimidyl 4-(*p*-maleimidophenyl)butyrate; SRB, sulforhodamine-B; TL, total lipid (phospholipid+cholesterol)

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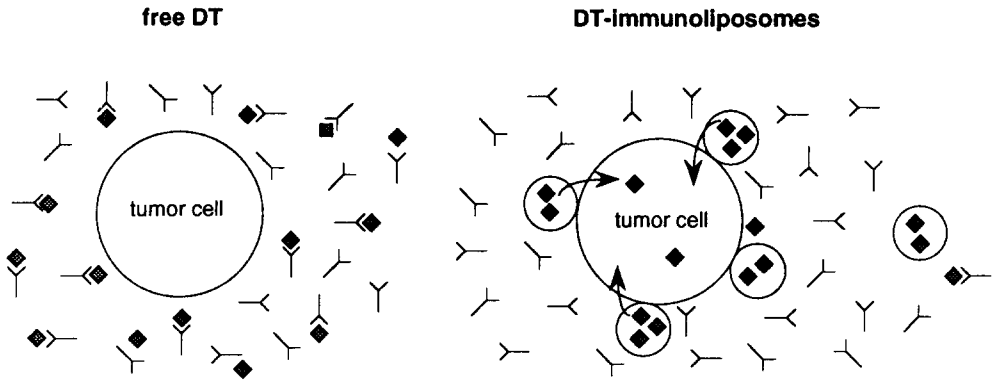

Fig. 1. Schematic representation of the proposed approach: protection against inactivating AT antibodies by incorporation of DT in tumor-specific immunoliposomes. DT (◆) administered as free drug and premature released DT from DT immunoliposomes is inactivated by circulating AT antibodies (—≺). By encapsulating DT in tumor-specific immunoliposomes, it is protected against the circulating AT antibodies and delivered in close proximity to the target cell, where it can exert its action upon leakage from cell-bound immunoliposomes.

leak out of the target cell-bound immunoliposomes in close proximity to the tumor cell (nanometer range), subsequently bind to its receptor, translocate into the cytoplasm and induce an antitumor effect. In addition to the protective effect of immunoliposome encapsulation, a second advantage of this system is that prematurely released DT, i.e. DT released prior to the actual binding of the immunoliposomes to the target cell and therefore potentially toxic, will be inactivated by the circulating AT antibodies. A schematic representation of the proposed therapeutical approach of DT exploiting the presence of AT antibodies is given in Fig. 1.

Here we provide evidence that the proposed hypothesis is realistic. DT encapsulated in tumor-specific immunoliposomes is highly active against in vitro cultured tumor cells when AT antibodies are present in the incubation medium. Under the same conditions, free DT and DT encapsulated in non-targeted liposomes do not display toxicity towards the tumor cells.

2. Materials and methods

2.1. Monoclonal antibody

All antibodies used in this study are mouse monoclonal antibodies of the IgG1 type. The monoclonal antibody OV-TL3 is directed against the OA3 antigen, present on over 90% of human ovarian carcinomas [17,18]. The antibody 323/A3 recognizes a 43 kDa membrane glycoprotein which is highly expressed on a variety of carcinomas [19,20]. The monoclonal antibody RIV1000, in the present study used as an irrelevant antibody, is directed against human lymphocytes [21,22].

$F(ab')_2$ fragments of the monoclonal antibodies (Centocor Europe BV, Leiden and RIVM, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands) were digested with 20 mM dithiothreitol as described earlier [12,23] and used immediately for covalent attachment to freshly prepared liposomes (see below).

2.2. Preparation of immunoliposomes

Egg phosphatidylcholine (EPC) and egg phosphatidylglycerol (EPG) were donated by Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (CHOL) was obtained from Sigma Chemical Co. (St. Louis, USA). *N*-[4-(*p*-Maleimidophenyl)butyryl]phosphatidylethanolamine (MPB-PE) was synthesized, purified and analyzed as described before [12,24]. MPB-PE was incorporated into the liposomal bilayers to allow covalent coupling of Fab' fragments to the liposomal surface. The bilayer composition of the liposomes used was EPC:EPG:CHOL:MPB-PE at a molar ratio 38.1:4:16:1.5. A mixture of the appropriate amounts of lipids in chloroform was evaporated to dryness in a rotary evaporator under reduced pressure. After flushing the lipid film with nitrogen (≥20 min), the lipid film was hydrated with a DT solution (RIVM DT79-1; 55 μmol total lipid (TL)/ml; 500 Lf/ml in 10 mM HEPES, 75 mM NaCl, 0.5 mM EDTA, pH 7.4; Lf denotes the limit of flocculation, i.e. the quantity of DT that flocculates most rapidly when mixed with 1 unit of antitoxin; 1 Lf roughly corresponds with about 2 μg pure DT and about 1/35 MLD (minimal lethal dose in guinea pigs)). The resulting liposome dispersion was sequentially extruded through polycarbonate membrane filters of 0.6 and 0.2 μm pore size which resulted in a mean particle size of approx. 0.25 μm. After extrusion the liposomes were centrifuged (100 000 × *g*; 30 min) and the pellet was redispersed in 100 mM acetate buffer pH 6.5 (with 40 mM NaCl and 1 mM EDTA). The freshly prepared liposomes were mixed with freshly prepared Fab' fragments (concentrations during incubation ranged from 6 to 12 μmol TL/ml and from 0.25 to 0.30 mg Fab'/ml, respectively). The coupling reaction was carried out overnight at 4°C with constant rotation under a nitrogen atmosphere. Finally, the immunoliposomes were separated from unconjugated Fab' fragments by ultracentrifugal sedimentation at 100 000 × *g* for 30 min. The pellet was resuspended and washed twice with HEPES buffer (20 mM HEPES, 149 mM NaCl, 1 mM EDTA, pH 7.4). MPB-PE-containing liposomes not incubated with Fab' fragments are referred to as 'unconjugated liposomes' throughout this paper. Liposome dispersions were stored at 4°C and used within 3 weeks.

2.3. Liposome characterization

Lipid phosphate was determined by the colorimetric method of Fiske and Subbarow [25]. The amount of antibody coupled to the liposomes was determined according to the method of Wessel and Flügge [26], with bovine serum albumin as standard and was expressed as μg of Fab' per μmol of TL. DT was determined fluorimetrically, after solubilization of the liposomes in Tween 80, using fluorescamine (Pierce, Rockford, USA; excitation wavelength, 390 nm; emission wavelength, 476 nm [27]). Mean particle size was determined by dynamic light scattering with a Malvern 4700 system (Malvern Ltd., Malvern, UK).

2.4. Antitumor activity in vitro

The human ovarian cancer cell line OVCAR-4 [28] was maintained in Dulbecco's modified Eagle's medium (Flow Laboratories, Irving, UK) supplemented with 10% fetal calf serum (Gibco Ltd., Paisley, UK), glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.26 μg/ml).

In vitro cell growth inhibition induced by DT and DT-(immuno)-liposomes was determined by the SRB assay based on the use of the dye sulforhodamine-B (SRB [29,30]). Briefly, monolayers of OVCAR-4 were treated with trypsin/EDTA (0.25%/0.02%) and washed with medium. To mimic a therapeutically relevant situation, incubations were performed with cells in suspension. Cells (1×10^6 cells/ml) were incubated (90 min, 37°C) with DT in free form or DT encapsulated in (immuno-)liposomes in the presence or absence of anti-diphtheria toxin antibodies (AT antibodies; Institute Pasteur Production, Paris,

France). After incubation, unbound liposomes, DT and AT antibodies were removed by centrifugation (500×g; 5 min). The cell pellet was washed twice and resuspended in culture medium with or without AT antibodies. Then, 5×10^4 cells per well were seeded in a flat-bottom 96-well plate and cultured for 72 h at 37°C and 5% CO_2. The cultures were fixed, stained and measured as described earlier [29,30]. The cytotoxic activity of DT was measured as the degree of cell proliferation relative to that of untreated cells (=100%). IC_{50} values indicate the DT concentration which results in 50% cell growth compared to untreated cells.

2.5. Statistics

The effect of different treatments was compared by a two-tailed Student's *t*-test assuming equal variances with 95% confidence interval. Differences were considered significant when the *p* value of comparison was less than 0.05.

3. Results

3.1. Antitumor activity of DT

DT was encapsulated in liposomes using the classical film method followed by extrusion (so-called extrusion MLV). The encapsulation efficiency was about 5% (on average about 0.44 Lf/μmol TL), suggesting that DT is located in the internal aqueous phase of the liposomes. We first evaluated the effect of immunoliposome encapsulation on the cytotoxic capacity of DT in the absence of AT antibodies. In line with expectations, DT encapsulated in tumor-specific OV-TL3 immunoliposomes was less active than free DT (Fig. 2). The IC_{50} values were about 0.002 and 0.02 Lf/ml for DT in free form and DT immunoliposomes, respectively. Immunoliposomes devoid of DT did not influence the cell growth at the lipid concentrations used (not shown). The antitumor activities of both free DT and DT immunoliposomes did not increase for DT concentrations ≥0.3 Lf/ml. At DT concentration of 0.3 Lf/ml, there was no difference in antitumor activity between free DT and DT encapsulated in targeted liposomes (i.e. 323/A3 and OV-TL3 immunoliposomes). Therefore, this DT concentration was used in the experiments designed to evaluate the effect of the presence of AT antibodies on the antitumor activity of DT immunoliposomes.

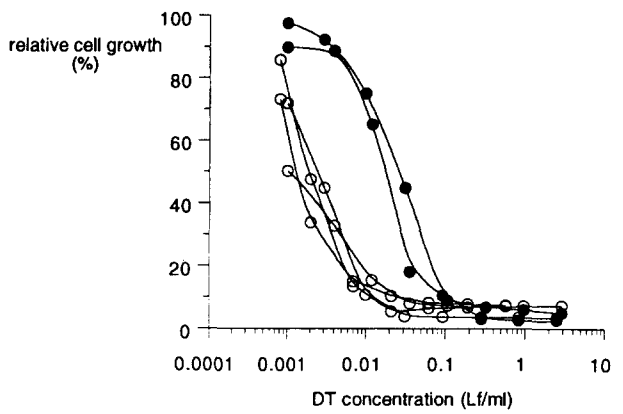

Fig. 2. In vitro antitumor activity of free DT and DT encapsulated in tumor-specific immunoliposomes. A suspension of OVCAR-4 cells was incubated with free DT (○) or DT encapsulated in OV-TL3 immunoliposomes (●) for 90 min at 37°C. After removal of unbound DT or DT immunoliposomes, cells were cultured for 72 h and relative cell growth was measured using the SRB assay. The results shown are derived from 2–4 separate experiments. The Fab' density on OV-TL3 immunoliposomes was about 10 μg Fab'/μmol TL.

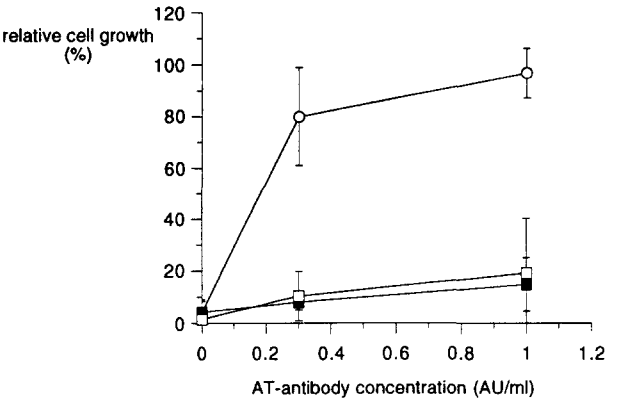

Fig. 3. Effect of the presence of AT antibodies on the in vitro antitumor activity of free DT and DT encapsulated in two types of specific immunoliposomes. A suspension of OVCAR-4 cells was incubated with 0.3 Lf/ml free DT (○) or DT encapsulated in 323/A3- (□) or OV-TL3 immunoliposomes (■) for 90 min at 37°C in the presence of AT antibodies (0, 0.3 or 1.0 AU/ml). After removal of AT antibodies, free DT and unbound immunoliposomes, cells were cultured for 72 h in AT antibody free medium and relative cell growth was measured using the SRB assay. Results are given as mean ± S.D. of 3–9 separate experiments. The Fab' densities of the 323/A3 and OV-TL3 immunoliposomes were 22 ± 12 and 21 ± 7 μg Fab'/μmol TL, respectively.

3.2. Effect of AT antibodies on the antitumor activity of DT

Fig. 3 shows that free DT can be completely inactivated by AT antibodies. A 3-fold excess of AT antibodies (1 antibody unit (AU)/ml) was sufficient to neutralize completely the antitumor effect of free DT at 0.3 Lf/ml (relative cell growth 97 ± 10%). In line with the proposed concept, at the AT antibody concentration of 1 AU/ml, 323/A3 and OV-TL3 DT immunoliposomes were still cytotoxic (relative cell growth 19 ± 21 and 15 ± 10%, respectively). In contrast, as shown in Fig. 4A, DT encapsulated in non-targeted liposomes (unconjugated liposomes, bar D; RIV1000 immunoliposomes, bar E) were by far much less effective in the presence of AT antibodies (1 AU/ml) as compared to DT in targeted liposomes (bars B,C, relative cell growth unconjugated liposomes and RIV1000 immunoliposomes was 85 ± 12 and 84 ± 15%, respectively). The AT antibodies themselves did not affect cell growth at the concentrations used (Fig. 4A, bar F).

In the experiments shown in Figs. 3 and 4A, AT antibodies were present during the 1.5 h incubation period of DT (liposomes) with the tumor cells. In the experimental set-up used, DT, AT antibodies and unbound liposomes were removed after the 1.5 h incubation period, and the cells were cultured for 72 h in AT antibody free medium, before the actual cell growth determination. To mimic a more therapeutically relevant situation, we also studied the cytotoxic effects when the AT antibodies were not removed during the 72 h culture period (Fig. 4B). Despite the resulting 3-fold decrease in cytotoxicity, tumor-specific DT immunoliposomes were still substantially more active than unconjugated DT-liposomes ($p < 0.003$) and free DT ($p < 0.002$).

In order to demonstrate that cell binding is a crucial requirement for achieving cytotoxic effects of DT immunoliposomes, we studied whether prolonged exposure of tumor cells to unconjugated DT-liposomes can confer some degree of cytotoxicity. Tumor cells were incubated for 1.5 h with unconjugated DT-liposomes (0.3 Lf/ml). After the removal of unbound liposomes and AT antibodies, the cells were seeded

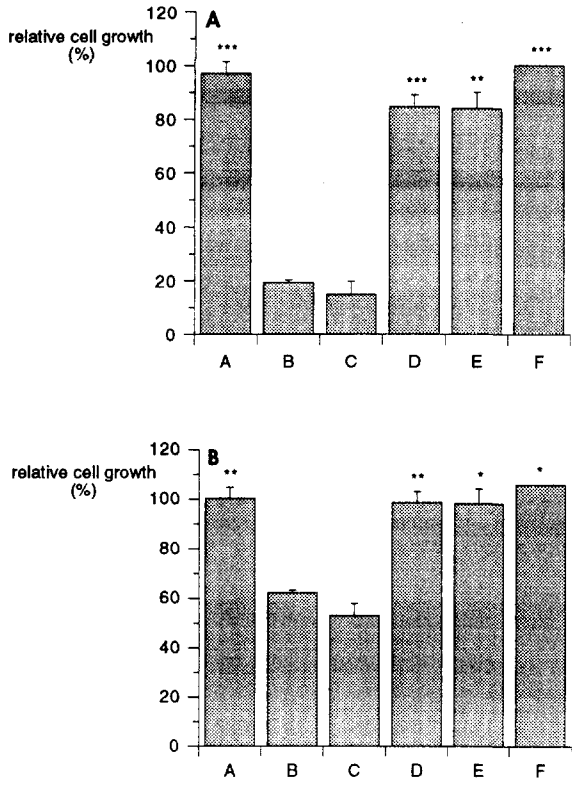

Fig. 4. Effect of the presence of AT antibodies on the in vitro antitumor activity of DT encapsulated in targeted and non-targeted liposomes. OVCAR-4 cells in suspension were incubated with 0.3 Lf/ml DT for 90 min at 37°C in the presence of AT antibodies (1.0 AU/ml). After removal of AT antibodies, free DT and unbound liposomes, cells were cultured for 72 h in AT antibody free (A) or AT antibody containing (B) medium and relative cell growth was measured using the SRB assay. Results are given as mean ± S.D. of 3–9 separate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (A, C–F vs B). Bars: A, free DT; B, DT in 323/A3 immunoliposomes (Fab′ density 22 ± 12 μg/μmol TL); C, DT in OV-TL3 immunoliposomes (Fab′ density 21 ± 7 μg/μmol TL); D, DT in unconjugated liposomes; E, DT in non-specific immunoliposomes (Fab′ density (RIV1000) 22 ± 9 μg/μmol TL); F, control (AT antibodies alone).

and cultured for 72 h in the presence of AT antibodies together with 0.03 Lf/ml unconjugated DT-liposomes (which roughly corresponds with the fraction of cell-bound immunoliposomes). No cytotoxicity was observed (results not shown), which indicates that cell binding of DT immunoliposomes is mandatory for antitumor action.

It was shown earlier by Nässander et al. [12,13] that the degree of cell binding of immunoliposomes depends on the Fab′ density on the liposomes (i.e. the amount of Fab′ coupled to the liposomes expressed as μg Fab′/μmol TL): the higher the Fab′ density, the greater the degree of cell binding. Fig. 5 shows that a higher Fab′ density of 323/A3 DT immunoliposomes is paralleled by an increase in the antitumor effect of these immunoliposomes in the presence of AT antibodies. These results again indicate that the immunoliposomes need to bind to the target cell in order to be able to exert antitumor activity in the presence of AT antibodies.

4. Discussion

DT is a potent inhibitor of protein synthesis in human cells. Its use for cancer treatment, however, is strongly limited by the lack of tumor cell specificity. An additional limiting factor is that, because of vaccination programs, most people have low levels of circulating AT antibodies. Furthermore, an increase in circulating AT antibody levels occurs within 2 weeks after DT administration [11]. These AT antibodies can inactivate DT prior to reaching the tumor cells. To overcome these problems, we have evaluated in vitro a new concept utilizing a tumor-specific liposomal delivery system for DT (Fig. 1). Liposomes can be targeted to tumor cells by coupling tumor-specific monoclonal antibodies to the surface (immunoliposomes), thereby increasing the specificity of the treatment and reducing the non-specific action of DT [16]. We demonstrate here that, simultaneously, the encapsulation of DT in immunoliposomes offers an escape from early inactivation by circulating AT antibodies. In addition to this protective effect provided by immunoliposomes, prematurely released DT, i.e. DT released from immunoliposomes which did not yet bind to the target cells and therefore potentially toxic, will be inactivated by the circulating AT antibodies.

As shown in Figs. 3 and 4A, DT encapsulated in tumor-specific immunoliposomes is highly active against in vitro tumor growth in the presence of AT antibodies. Free DT and

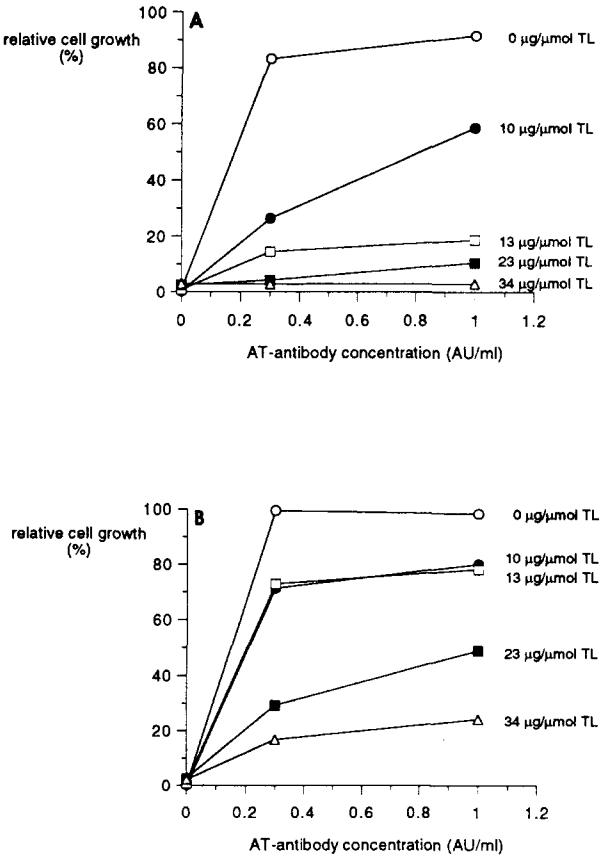

Fig. 5. Effect of the Fab′ density of 323/A3 DT immunoliposomes on the vitro antitumor activity in the presence of AT antibodies. A suspension of OVCAR-4 cells was incubated with 0.3 Lf/ml DT encapsulated in 323/A3 immunoliposomes with different Fab′ densities for 90 min at 37°C in the presence of AT (0, 0.3 or 1.0 AU/ml). After removal of AT, and unbound DT immunoliposomes, cells were cultured for 72 h in AT-free (A) or AT-containing (B) medium and relative cell growth was measured using the SRB assay. The Fab′ density of 323/A3 immunoliposomes was 0 (○), 10 (●), 13 (□), 23 (■), and 34 (△) μg Fab′/μmol TL.

DT encapsulated in non-targeted liposomes (unconjugated liposomes and irrelevant RIV1000 immunoliposomes) are not toxic for the cells under these conditions, due to inactivation by the co-incubated AT antibodies. Apparently, DT is cytotoxic only when incorporated in tumor-specific immunoliposomes. This suggests that cell binding of DT immunoliposomes is required for the induction of antitumor activity. The crucial importance of cell binding for achieving antitumor activity is underlined by our observations that DT-liposomes are not active when they lack a specific antibody and that the antitumor activity of specific immunoliposomes increases with increasing Fab' density on their surface (Fig. 5). In view of the requirement of cell binding for achieving cytotoxicity, no bystander effect of this treatment is to be expected. Therefore, multiple injection schemes are required to reach cells which will not be in contact with the immunoliposomes after the first injection. Such repeated dosage regimens will not be effective in the case of free DT due to its immunogenicity.

The mechanism behind the antitumor activity of tumor-specific DT immunoliposomes displayed in the presence of AT antibodies is not clear yet. We have reported earlier that cell-bound OV-TL3 immunoliposomes are hardly endocytosed by ovarian cancer cells [12]. The observation that the presence of AT antibodies during the 72 h culture period results in a reduced antitumor effect of cell-bound DT immunoliposomes (Fig. 4) would suggest that release of DT from cell-bound immunoliposomes rather than cellular internalization of the immunoliposomes is involved. In this respect, our approach differs from that of Huang and co-workers [7,8]. They encapsulated fragment A of DT (DTA) in pH-sensitive immunoliposomes. These immunoliposomes are supposed to deliver their contents to the cytoplasm of the target cell after endocytic uptake and subsequent fusion with the endosomal membrane as a result of the mildly acidic environment in the endosomes. By this route, an antitumor effect can be induced selectively against the target cells. However, in this approach endocytosis of immunoliposomes by tumor cells is essential in order to achieve delivery of DTA to the cytoplasm of the cell. As we use the whole DT molecule which is able to enter the cytoplasm by itself, our strategy does not necessarily depend on the endocytotic capacity of tumor cells and can therefore also be applied in the case of tumor cells which do not endocytose cell-bound immunoliposomes so easily. In principle, we show here that cell binding and subsequent DT release from the immunoliposomes in close proximity of the target cell are sufficient to induce an antitumor effect.

To increase the specificity of DT therapy, we incorporated DT in tumor specific immunoliposomes. Another approach is to couple DT directly to the monoclonal antibodies (immunotoxins, e.g. [6]). However, in multiple injection schemes, the application of immunotoxins is limited by their DT-related immunogenicity leading to inactivation of the DT-based immunotoxins by circulating AT antibodies prior to reaching the target site. In contrast, our approach in fact utilizes the circulating antibodies to reduce non-target site toxicity with preservation of antitumor activity. The antibody titer must be at a level where prematurely leaked DT molecules are neutralized by the circulating antibodies, thereby avoiding their toxicity. An antibody titer above 0.1 AU/ml (preferentially 0.1–2 AU/ml) is reported to be protective in preventing diphtheria in humans. Antibody titers $\geq$5 AU/ml can be observed shortly after (re)vaccination [31]. In our experiments the antibody concentrations were 0.3 and 1.0 AU/ml, which is comparable to the situation in patients. If necessary, it will be in principle possible to boost the level of circulating AT antibodies by (re)vaccination of the patients with diphtheria toxoid prior to therapy with DT immunoliposomes.

In conclusion, tumor-specific DT immunoliposomes can provide protection against the non-specific action of DT and display efficient antitumor activity even in the presence of AT antibodies. We expect that the proposed DT immunoliposome-based approach for the delivery of DT will not result in considerable toxicity for the patient: any DT molecules released prematurely from the immunoliposomes before tumor cell binding will be inactivated by circulating antibodies as most patients will be vaccinated against DT. Depending on the liposomes, site of administration, and tumor type, a large fraction of immunoliposomes can be bound to tumor cells. Unbound immunoliposomes are mainly cleared by cells of the mononuclear phagocyte system (MPS). In view of potential toxic effects, MPS uptake can be considered as advantageous, as the immunoliposomes will end up in the lysosomal compartment of these cells and most toxins are degraded by lysosomal enzymes [32].

This completely new concept might yield an effective and in principle low toxicity weapon to fight various forms of cancer that are accessible to immunoliposomes. Our future studies will particularly focus on the application of this concept for the treatment of peritoneal metastases of ovarian carcinoma as efficient target cell binding in vivo was observed in tumor bearing mice upon i.p. administration of immunoliposomes [13].

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