Pharmaceutical Research, Vol. 20, No. 3, March 2003 (© 2003) *Research Paper*

Liposome-Mediated Targeting of Enzymes to Cancer Cells for Site-Specific Activation of Prodrugs: Comparison with the Corresponding Antibody–Enzyme Conjugate

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Received November 15, 2002; accepted December 4, 2002

Purpose. Immunoenzymosomes are tumor-targeted immunoliposomes bearing enzymes on their surface. These enzymes are capable of converting relatively nontoxic prodrugs into active cytostatic agents. The aims of this study were to compare the enzyme delivery capability of immunoenzymosomes with that of the corresponding antibody–enzyme conjugate and to evaluate whether immunoenzymosomes are able to mount a strong bystander effect.

Methods. Immunoenzymosomes exposing Fab′ fragments of the monoclonal antibody 323/A3 and the bacterial enzyme β-glucuronidase or the corresponding antibody–enzyme conjugate were incubated with OVCAR-3 cells (human ovarian carcinoma cells). Cell-associated enzymatic activity and the *in vitro* antiproliferative effect of a glucuronide prodrug of doxorubicin (DOX-GA3) were determined.

Results. At equal numbers of carrier units, the cell-associated enzymatic activity achieved by using immunoenzymosomes was 15-fold higher than that obtained after incubation with the corresponding antibody–enzyme conjugate. Increasing the amount of antibody–enzyme conjugate added to the cells could not compensate for their lower enzyme delivery capability. Immunoenzymosomes were able to induce inhibition of cell growth not only of tumor cells to which immunoenzymosomes were bound but also of a large number of neighboring cells.

Conclusions. Immunoenzymosomes are able (a) to target prodrug-converting enzymes more efficiently to tumor cells than the corresponding antibody–enzyme conjugate and (b) to mount a strong bystander effect.

KEY WORDS: (immuno)liposomes; enzymosomes; antibody–enzyme conjugate; antibody-directed enzyme prodrug therapy (ADEPT); enzyme targeting; prodrug activation.

INTRODUCTION

Conventional cancer chemotherapy is limited by lack of specificity toward the tumor cells. Antibody-directed enzyme prodrug therapy (ADEPT) addresses this problem and has been proposed as an alternative for conventional cancer therapy. In this approach an enzyme is linked to an antibody that binds to an antigen preferentially expressed on tumor cells. Subsequent administration of a prodrug results in selective conversion of the prodrug into the parent cytotoxic drug at the tumor site. For successful therapy, it is necessary that a bystander cytotoxic effect occurs: the converted prodrug should kill not only the tumor cells to which the conjugates have bound but also neighboring tumor cells.

As a modification of ADEPT, we have previously presented a liposome-based system (immunoenzymosomes) in which both an antibody and an enzyme are covalently coupled to the vesicle surface (1). As opposed to the antibody–enzyme conjugate, immunoenzymosomes can target more than one enzyme molecule per carrier unit. Therefore, we have previously hypothesized that the immunoenzymosome system should be able to provide a higher enzyme density at the tumor cell surfaces, thus being more efficient in specific prodrug activation. In this study, experimental support for this hypothesis is provided.

This paper aims to address the following two issues: (a) How does the enzyme delivery capability of the immunoenzymosome system compare with that of the corresponding antibody–enzyme conjugate? (b) Is the immunoenzymosome system able to mount a strong bystander effect? To this end, immunoenzymosomes were prepared exposing Fab′ fragments of the monoclonal antibody 323/A3 that bind selectively to OVCAR-3 cells (human ovarian carcinoma cells) and the enzyme β-glucoronidase (GUS), able to convert a glucoronide prodrug of doxorubicin (DOX-GA3). The enzyme delivery capability of the immunoenzymosomes was compared to that of the corresponding antibody–enzyme conjugate. In addition, their ability to convert DOX-GA3 into the antitumor drug doxorubicin after binding to the target cells was studied. The results point to a superior enzyme delivery capability of the immunoenzymosome system as compared to the corresponding antibody–enzyme conjugate. In addition, the immunoenzymosome/prodrug combination is able to produce a strong bystander effect.

MATERIALS AND METHODS

Reagents and Cells

The mouse monoclonal antibody 323/A3 (IgG type) was donated by Centocor Europe BV (Leiden, The Netherlands). β-Glucuronidase (GUS) from *E. coli* K12 and *p*-nitrophenyl-β-D-glucuronide were purchased from Boehringer (Mannheim, Germany). Succinimidyl 4-(*p*-maleimidophenyl)butyrate (SMPB) was obtained from Pierce (Oud-Beijerland, The Netherlands). Egg phosphatidylcholine (EPC) and egg phosphatidylglycerol (EPG) were donated by Lipoid GmbH (Ludwigshafen, Germany). Phosphatidylethanolamine (PE) was obtained from Nutfield Nurseries Lipid Products (Nutfield, UK). Cholesterol (CHOL), N-succinimidyl S-acetylthioacetate (SATA), bovine serum albumin (BSA), dithiothreitol (DTT), and FITC-conjugated goat antimouse IgG were obtained from Sigma Chemical Co. (St. Louis, Missouri). 4-Methylumbelliferyl-β-D-glucuronide tryhydrate was from Fluka (Buchs, Switzerland). All other reagents were of analytic grade.

Doxorubicin (Pharmachemie BV, Haarlem, The Netherlands) was purchased as a powder. The prodrug N-[4-doxorubicin-N-carbonyl (oxymethyl)phenyl] O-β-glucuronyl

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0724-8741/03/0300-0423/0 © 2003 Plenum Publishing Corporation

carbamate (DOX-GA3) was synthesized (purity > 99.9%) as described in Ref. 2. Chemical stability of the prodrug under the conditions relevant to the present experiments is described in Ref. 3.

The human ovarian cancer cell line NIH: OVCAR-3 (OVCAR-3) (4) was cultured as described in Ref. 5.

Preparation and Characterization of 323/A3-GUS Conjugate

Mab 323/A3 and GUS were conjugated using a thioether linkage as described (6). GUS was first purified by gel filtration on a Sephadex G-150 column (Pharmacia, Woerden, The Netherlands) (5). Subsequently, extra thiol groups were introduced by means of the thiolating agent N-succinimidyl S-acetylthioacetate (SATA) (5). Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC; Pierce, Oud-Bijerland, The Netherlands) was allowed to react with 323/A3 to produce a maleimide-activated antibody. The modified enzyme and antibody were combined at a 1:1 weight ratio, and the conjugation reaction was started by addition of hydroxylamine (Janssen, Geel, Belgium) at pH 7.0. After 60 min at room temperature, the reaction was stopped by gel filtration on Sephadex G-25M equilibrated with phosphate-buffered saline (PBS). The antibody–enzyme conjugate was concentrated by ultrafiltration using a 10PM30 filter (Amicon, Danvers, MA) and purified by gel filtration on a Superdex 200 preparative grade column (10 × 600 mm, Pharmacia) in PBS. The fractions containing conjugate with 1:1 antibody/enzyme ratio were pooled. BSA was added to a final concentration of 0.1% (w/v) to prevent loss of the enzyme activity in PBS. Enzyme activity was assessed with 4-nitrophenyl β-D-glucuronide as described (5). One unit (U) of enzyme activity is defined as the quantity of GUS needed for the conversion of 1 μmol of 4-nitrophenyl β-D-glucuronide to 4-nitrophenol. Protein concentration was determined by the method of Wessel and Flügge (7).

Preparation of 323/A3-GUS Immunoenzymosomes

GUS was purified and thiolated as described above. Fab′ fragments of the 323/A3 monoclonal antibody were produced by pepsin digestion (5). N-[4-(*p*-Maleimidophenyl)butyryl] phosphatidylethanolamine (MPB-PE) was synthesized from SMPB and PE, purified, and analyzed as described before (8). MPB-PE was incorporated into the liposome bilayers to allow covalent coupling of purified and thiolated GUS and Fab′ fragments to the liposome surface. Liposomes composed of EPC:EPG:CHOL at a molar ratio of 10:3:4 with 2.5 mol% MPB-PE were prepared by hydration of the lipid film in Hepes NaCl buffer (20 mM Hepes, 149 mM NaCl, 1 mM EDTA, pH 7.4). The resulting liposome dispersion was extruded through polycarbonate membrane filters with 0.2-μm pore size under nitrogen pressure, yielding a mean size of about 0.2 μm. After extrusion, the Hepes/NaCl buffer outside the liposomes was replaced by acetate buffer pH 6.5 using ultracentrifugation (200,000 *g*, 45 min.). Freshly prepared liposomes were mixed with purified and thiolated GUS and freshly prepared Fab′ fragments. Concentrations during incubation were 5 μmol total lipid/ml, 2 mg/ml of GUS, and 0.1 mg Fab′/ml. Freshly prepared hydroxylamine HCl (0.5 M hydroxylamine HCl, 0.5 M Hepes, 25 mM EDTA, pH 6.5) was added to the incubation mixture for deprotection of the enzyme thiol groups (100 μl hydroxylamine solution per milliliter of incubation mixture). The coupling reaction was carried out overnight at 4°C under constant rotation in a nitrogen atmosphere. Finally, the immunoenzymosomes were separated from unconjugated enzyme and Fab′ by ultracentrifugation (200,000 *g,* 45 min). The pellet was resuspended and washed twice with Hepes/NaCl buffer. Liposomes to which GUS alone is coupled are referred to as enzymosomes. Liposomes to which Fab′ alone is coupled are further referred to as immunoliposomes. Liposomes to which both GUS and Fab′ are coupled are referred to as immunoenzymosomes. Liposome dispersions were stored at 4°C.

Liposome Characterization

Lipid phosphate was determined by the colorimetric method of Rouser (9). The enzymatic activity was measured with *p*-nitrophenyl-β-D-glucuronide as described elsewhere (5). The amount of protein coupled to the liposomes was determined by the method of Wessel and Flügge (7) with bovine serum albumin as standard. The total amount of monoclonal antibody and/or enzyme coupled to the liposomes was expressed as micrograms of protein per micromole total lipid. The amount of enzyme coupled to immunoenzymosomes was estimated by comparison of the enzyme density and enzymatic activity of enzymosomes (to which only GUS is coupled) with the protein density and enzymatic activity of immunoenzymosomes. It was assumed that the presence of Fab′ does not interfere with the determination of the enzymatic activity. Mean particle size was determined by dynamic light scattering with a Malvern 4700 system using a 25-mW helium–neon laser. 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 solution up to 1.0 for a polydisperse solution.

Cell-Binding Assay

Adherent OVCAR-3 cells were detached with 0.05% trypsin, 0.02% EDTA in phosphate-buffered saline (PBS, pH 7.4) for 5 min at 37°C and were washed with cold PBS. Suspensions of OVCAR-3 cells in PBS containing 0.1% BSA were incubated with varying amounts of antibody–enzyme conjugate, immunoliposomes, or immunoenzymosomes at 4°C for 90 min. After incubation cells were washed twice with PBS containing 0.1% BSA by centrifugation (300 × *g,* 3 min) and incubated at 4°C for 30 min with FITC-conjugated goat anti-(mouse IgG) (Fab′ specific) at a dilution 1:200. After washing twice with ice-cold PBS, cell fluorescence was analyzed by a flow cytometer (FACScan, Becton & Dickinson Immunocytometry Systems, Mountain View, California), with excitation at 488 nm and emission at 515–545 nm. The fluorescence intensity of 10,000 viable cells was recorded. Mean fluorescence intensity was computed.

Enzyme Delivery Capacity of Immunoenzymosomes and Antibody–Enzyme Conjugate

Varying amounts of immunoenzymosomes or 323/A3-GUS conjugate were incubated with a suspension of OVCAR-3 cells in PBS containing 0.1% BSA (w/v) (10^6 cells/ml) for 90 min at 4°C. After the incubation period, immuno-

enzymosomes and conjugate not associated with the cells were removed by centrifugation (300 × *g*, 3 min). The cell pellet was washed twice with PBS containing 0.1% BSA. The cell-associated enzymatic activity was determined by incubation with 100 μl of 5 mM 4-methylumbelliferyl-β-D-glucuronide for 1 h at 37°C. The reaction was terminated by addition of 1 ml 0.1 M glycine (pH 10.6). Fluorescence was measured at excitation–emission wavelengths of 370–460 nm in a Perkin Elmer 3000 (Norwalk, Connecticut) spectrofluorimeter. Cells pretreated with an excess antibody (100 μg/ml) or with buffer alone served as controls.

In Vitro Antiproliferative Effect

The antiproliferative effect of the immunoenzymosome/prodrug combination was determined as follows. After trypsinization, a suspension of OVCAR-3 cells in PBS containing 0.1% BSA (w/v) were incubated with the immunoenzymosome preparation (0.3 μmol total lipid/ml) at 4°C for 60 min. Cells pretreated with an excess antibody (100 μg/ml) or with buffer alone served as controls. Thereafter, cells were washed in PBS, resuspended in culture medium (supplemented DMEM), and seeded in triplicate in 96-well culture plates (20,000 cells/well, 10 μl/well). Drug or prodrug was added (10 μl/well) to give final concentrations ranging from 1 nM to 50 μM. In separate wells, an excess of β-glucuronidase was present to determine the antiproliferative effects of the prodrug completely hydrolyzed by the enzyme. After 24 h, 200 μl of culture medium was added, and the cells were incubated for an additional 72 h. Cell proliferation was determined by use of the reagent WST-1 (Boehringer Mannheim), and the IC_{50} was calculated as the (pro)drug concentration that gives 50% growth inhibition when compared to control cell growth.

Bystander Effect

A suspension of OVCAR-3 cells in PBS containing 0.1% BSA (w/v) was incubated with immunoenzymosomes (final concentration 10 μmol total lipid/ml) for 1 h at 4°C (10^6 cells/ml). Immunoenzymosomes not associated with the cells were removed by centrifugation (300 × *g*, 3 min). The cell pellet was washed twice with PBS containing 0.1% BSA and resuspended in PBS containing 0.1% BSA (w/v) (50,000 cells/ml). Another cell suspension was prepared with nontreated OVCAR-3 cells at the same cell concentration. Both cell populations were mixed at varying ratios. The mixtures were added to 96-well plates (5000 cells/well). Then, DOX-GA3 (1 μM), doxorubicin (1 μM), or culture medium was added, and the cells were allowed to grow for 72 h. In separate wells, an excess of β-glucuronidase was present to determine the antiproliferative effects of the prodrug completely hydrolyzed by the enzyme. Cell growth was determined as described above. Cells pretreated with an excess antibody (100 μg/ml) or with buffer alone served as controls. The bystander effect is expressed relative to the antiproliferative effect of 1 μM of the parent drug doxorubicin ("relative cell growth").

RESULTS

Preparation of Immunoenzymosomes and the Corresponding Antibody–Enzyme Conjugate

Immunoenzymosomes were prepared by coupling Fab′ fragments of the monoclonal antibody 323/A3 and the enzyme β-glucuronidase (GUS) to liposomes containing the anchor molecule MPB-PE. Previously, we have reported that thiolation of GUS was required to achieve sufficient coupling to the liposomes (10,11). The preparation of the antibody–enzyme conjugate was carried out by coupling similarly thiolated GUS to the 323/A3 Mab (IgG). Table I summarizes the characteristics of the immunoenzymosome preparation and antibody–enzyme conjugate preparation used in this study.

Enzyme Delivery Capability: Immunoenzymosomes vs. Antibody–Enzyme Conjugate

Figure 1a shows that the immunoenzymosome system targets strikingly higher levels of enzymatic activity to the cells than the antibody–enzyme conjugate. The maximum level reached with the immunoenzymosomes was about 60 mU cell-associated enzymatic activity per 10^6 cells, whereas the maximum level reached with the antibody–enzyme conjugate was 4 mU/10^6 cells. This was the case not only when equal number of carrier units were used but also when the dose of conjugate was increased (100-fold) so that the enzymatic activity added to the cells during incubation was the same for both systems (Fig. 1b). The maximum enzyme-targeting level of the antibody–enzyme conjugate saturated at a cell-associated enzymatic activity of 8 mU/10^6 cells. Consequently, addition of more conjugate units did not result in a further increase in the amount of enzyme targeted to the cells. Cell binding of both immunoenzymosomes and immunoliposomes (liposomes bearing specific Fab′ fragments but no enzyme) proved to be specific, as preincubation of the cells with an excess of the 323/A3 monoclonal antibody resulted in more than a 10-fold decrease in the cell-associated enzymatic activity (data not shown). For immunoenzymosomes, two additional controls were performed: incubation of immunoenzymosomes with control cells (A2780 cells, which lack the antigen for 323/A3) and incubation of enzymosomes (lacking the specific Fab′ of 323/A3) with OVCAR-3. In both cases, no significant cell binding was observed (data not shown).

To ensure that the lower enzyme delivery capability of the antibody–enzyme conjugate was not a result of poor binding to the cells, the target cell binding efficiency of antibody–enzyme conjugate and immunoenzymosomes was studied by flow cytometry. At the same amount of Fab′ added to the cells, the cell-associated fluorescence (proportional to the cell-associated amount of Fab′) induced by the antibody–enzyme conjugate was 20-fold higher than that induced by the immunoenzymosomes and twofold higher than that obtained with immunoliposomes (Fig. 2a). Also, in the case of the same

Table I. Characteristics of Immunoenzymosomes and Antibody–Enzyme Conjugate Used in This Study

	Immunoenzymosomes	Antibody–enzyme conjugate
GUS (μg/μmol TL)	80	—
Number of GUS molecules/carrier unit	700	1
Fab′ (μg/μmol TL)	15	—
Number of Fab′ molecules/carrier unit	750	2
Enzymatic activity (U/carrier unit)	$6 \cdot 10^{-12}$	$6 \cdot 10^{-14}$

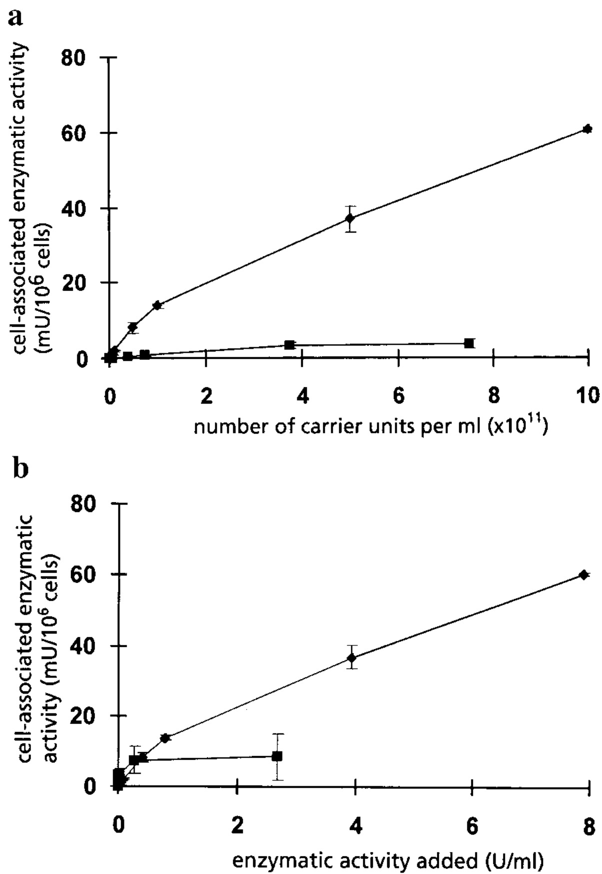

Fig. 1. Comparison of the enzyme-delivery capability of immunoenzymosomes (♦) and that of the corresponding antibody–enzyme conjugate (323/A3-GUS) (■) (a) as a function of number of carrier units added per milliliter and (b) as a function of the enzymatic activity added to the cells. Immunoenzymosomes and conjugate were incubated with the cells (10^6 cells/ml) for 90 min at 4°C. After washing of the cells with PBS containing 0.1% BSA to remove unbound liposomes or conjugate molecules, the enzymatic activity was determined. A typical experiment out of three performed is shown. Each point represents the mean ± SD of three determinations.

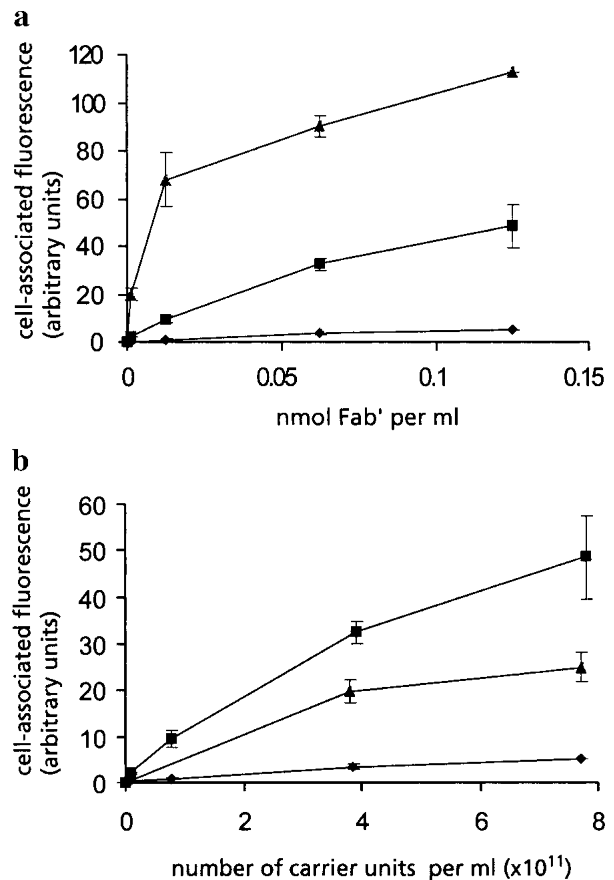

Fig. 2. Flow cytometric analysis of the binding of immunoenzymosomes (♦), immunoliposomes (■), or the corresponding antibody–enzyme conjugate (▲) to OVCAR-3 cells (a) as a function of the amount of Fab′ added to the cells and (b) as a function of the number of carrier units added per 10^6 cells. Cells were examined for cell-associated fluorescence by flow cytometry as described in Materials and Methods. The mean fluorescence value (± SD, $n = 3$) determined by flow cytometry is presented after substraction of that obtained for cells incubated with only FITC-conjugate. A typical experiment out of three performed is shown.

number of carrier units (conjugate or immunoenzymosomes), the resulting cell-associated fluorescence was much higher for the conjugate (Fig. 2b). This indicates that the antibody–enzyme conjugate binds to OVCAR-3 cells to a greater extent than immunoenzymosomes. It should be noted that one immunoenzymosome particle contains 750 Fab′ molecules, whereas the conjugate contains only two Fab′ molecules (Table I). Consequently, the same extent of cell binding for conjugate and immunoenzymosomes would result in a much higher cell-associated fluorescence in case of the immunoenzymosomes. Therefore, the data show that the antibody–enzyme conjugates are by far superior to immunoenzymosomes in terms of target cell binding efficiency.

In Vitro Antiproliferative Effect

The cytotoxic effect of the glucuronide prodrug of doxorubicin, DOX-GA3, after preincubation of OVCAR-3 cells with immunoenzymosomes was determined by measuring cell growth after drug exposure for 96 h. In the absence of GUS, the IC_{50} value of DOX-GA3 was 6 μM. The IC_{50} of DOX-GA3 to tumor cells preincubated with GUS-containing immunoenzymosomes was 10-fold lower and almost identical to that of the parent drug doxorubicin or to that of DOX-GA3 when the cells were coincubated with an excess of GUS (Fig. 3). The observation that incubation with immunoenzymosomes yielded OVCAR-3 cells equally sensitive to DOX-GA3 or to doxorubicin indicates a complete conversion of the prodrug to active drug.

Bystander Effect

We also studied whether prodrug activation can result in killing of neighboring cells to which no immunoenzymosomes were bound. Immunoenzymosomes were allowed to bind to OVCAR-3 cells for 30 min. After removal of unbound liposomes, the cells were cocultured at different ratios with non-pretreated OVCAR-3 cells. Subsequently, the cells were treated with 1μM DOX-GA3, which, under the conditions used in this assay, does not affect the growth of OVCAR-3 cells (12), and cell survival was determined. Figure 4 shows that the entire cell population could be killed when only 25% of the cells were preincubated with immunoenzymosomes.

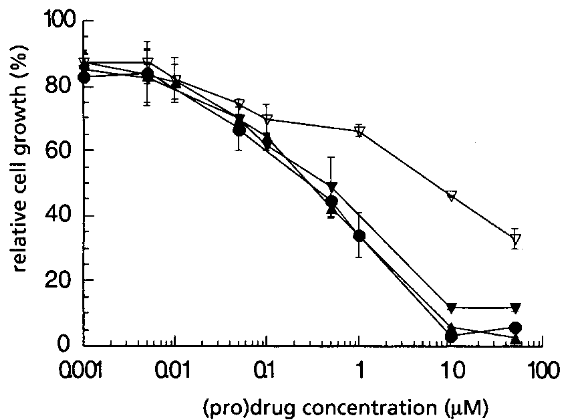

Fig. 3. Antiproliferative effect of the prodrug DOX-GA3 after preincubation at 4°C for 60 min with GUS-containing immunoenzymosomes (▼). DOX-GA3 was also added to nonpretreated cells (▽) or to cells that were cultured in the presence of an excess of GUS (▲). As a comparison, doxorubicin was added to nonpretreated OVCAR-3 cells (●). In all cases, cells were exposed to the drugs for 96h. Each individual point represents the average ± SD (*bars*) of three determinations.

When only 5% of the cells were preincubated with immunoenzymosomes, 75% of the cells died. This is indicative of a robust bystander effect mounted by cell-bound immunoenzymosomes. When the different cell populations were treated with 1 μM doxorubicin, 100% kill efficiency was observed in all cases. As a control, the different cell populations were cultured in DMEM in the absence of doxorubicin or DOX-GA3. In that case, 100% cell survival was found (data not shown). Furthermore, when, previous to the incubation with immunoenzymosomes, the cells were treated with excess 323/A3 antibody (100 μg/ml), a 10-fold decrease in sensitivity to DOX-GA3 was found, which indicates that the binding of immunoenzymosomes to the OVCAR-3 cells is specific (data not shown).

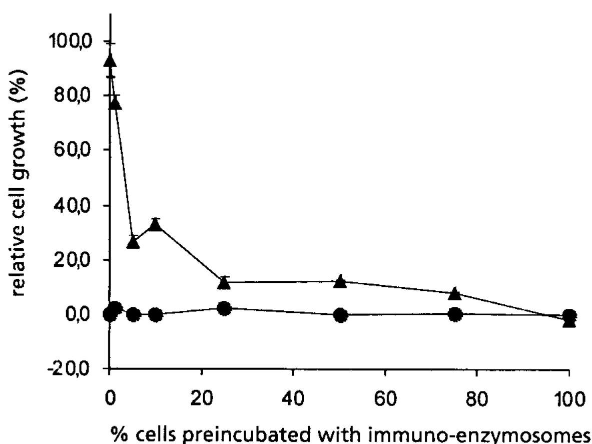

Fig. 4. Bystander effect. OVCAR-3 cells were preincubated with GUS-containing immunoenzymosomes (▲). The preincubated cells were cocultured in different ratios with nonpretreated OVCAR-3 cells and subsequently treated with DOX-GA3 (1 μM). Cell survival was determined. Each individual point represents the average ± SD (*bars*) of three individual determinations. The same cell populations were incubated with 1 μM of the parent drug doxorubicin (●). The results are expressed relative to the antiproliferative effect of 1 μM doxorubicin ("relative cell growth").

DISCUSSION

Antibody-directed enzyme prodrug therapy (ADEPT) is under evaluation as an indirect targeting approach for cancer chemotherapy (13–15). In our group, we concentrate on the development of a liposome-based system (immunoenzymosomes), which theoretically has several advantages over the antibody–enzyme conjugates currently proposed for ADEPT. Immunoenzymosomes are liposomes to which both antibody and enzyme molecules are covalently attached. The antibody is responsible for the specific binding to the tumor cells, and the enzyme for the conversion of an inactive prodrug into the parent cytotoxic drug at the site of the tumor. In previous papers we have focused our efforts on achieving a maximum enzyme density on the liposome surface. Our "first-generation" immunoenzymosomes contained fewer than 100 enzyme molecules per liposome particle (10,11). Recently, a "new generation" containing as many as 700 enzyme molecules per vesicle was developed (5).

Ovarian cancer is one of the most common fatal gynecologic malignancies. At the time of diagnosis, the disease is often at a progressed state and has already spread beyond the ovary within the peritoneal cavity. Metastases remain localized in the peritoneal cavity throughout most of the clinical course of ovarian cancer. Therefore, we consider that i.p. administration of immunoenzymosomes for ADEPT may be an attractive approach to improve the poor prognosis of ovarian cancer patients, as the intraperitoneally localized tumor cells can be expected to be directly accessible for the i.p. administered particles.

In this paper, immunoenzymosomes with maximized enzymatic activity were prepared. The surfaces of these particles present about 700 enzyme (GUS) molecules and 750 Fab′ (323/A3) molecules. In the first part of this paper, experimental support was collected for the hypothesis that more enzyme activity can be delivered to tumor cells by means of immunoenzymosomes than by using the corresponding antibody–enzyme conjugate. For that purpose, the enzyme delivery capability of the immunoenzymosomes was compared to that of the corresponding antibody–enzyme conjugate containing one GUS molecule per IgG (323/A3) molecule. When the same amount of both types of carrier units are incubated with OVCAR-3 cells under the same experimental conditions, immunoenzymosomes were superior enzyme-targeting vectors (Fig. 1a). In this case, immunoenzymosomes contained 100 times more enzymatic activity per carrier unit than the conjugate (Table I). Then the number of conjugate units was increased 100-fold, yielding a situation in which the quantity of enzymatic activity added to the cells during incubation was the same for both systems. Even in this case, the enzyme delivery capability of the immunoenzymosomes was clearly superior to that of the antibody–enzyme conjugate (Fig. 1b). The enzyme-targeting capacity of the conjugate was maximal at a cell-associated enzymatic activity of 8 mU/10^6 cells. It is likely that at that level all available receptor sites are occupied; therefore, increasing the amount of antibody–enzyme conjugate did not result in an increased cell-associated enzymatic activity. In contrast, with increasing amounts of immunoenzymosomes, a cell-associated enzymatic activity of at

least 60 mU/10^6 cells could be achieved, although saturation was not yet reached. This can be explained by the difference in enzyme "cargo": one immunoenzymosome particle is bearing 700 GUS molecules, whereas one antibody–enzyme conjugate contains only one GUS molecule. Therefore, even though immunoenzymosomes bind less efficiently to the target cells (Fig. 2), the enzyme delivery capability of immunoenzymosomes is superior to that of the corresponding antibody–enzyme conjugate. The fact that immunoenzymosomes bind to the target cells to a lower extent than immunoliposomes bearing a similar amount of Fab′ is probably related to a steric hindrance effect mediated by the presence of large amounts of bulky GUS molecules on the liposome surface. This observation has been discussed elsewhere (5). In that paper, evidence was also provided that the observed decrease in target cell binding of immunoenzymosomes as compared to immunoliposomes was not caused by an incomplete recognition of the cell-bound immunoenzymosomes by the secondary antibody.

In the second part of this paper the question was addressed whether immunoenzymosomes are able to exert a strong bystander effect. That is of great importance because it is unlikely that immunoenzymosomes will reach and bind to all the cells within a tumor. First, the ability of immunoenzymosomes, once bound to the target cells, to convert the prodrug DOX-GA3 into doxorubicin was confirmed. OVCAR-3 cells were 10-fold more sensitive to doxorubicin than to DOX-GA3, whereas OVCAR-3 cells that had been preincubated with immunoenzymosomes were equally sensitive to the prodrug or to the parent drug. This is in agreement with previously reported results (11) and demonstrates complete conversion of DOX-GA3 into doxorubicin mediated by the cell-associated enzyme molecules. Then, cells that had been preincubated with immunoenzymosomes were cocultured with untreated OVCAR-3 cells, and cell growth was evaluated. The results show that not only the proliferation of those cells that have bound immunoenzymosomes was affected but also the proliferation of neighboring cells was dramatically reduced. The strong bystander effect can occur because the conversion of DOX-GA3 into doxorubicin takes place extracellularly (16). Therefore, produced doxorubicin molecules can exert antitumor activity not only toward the cells bearing activating enzyme but also toward neighboring cells. A similar bystander effect has been previously reported for GUS-expressing OVCAR-3 cells, after being transfected with a plasmid encoding for GUS (12).

In summary, this study demonstrates that immunoenzymosomes can deliver enzyme molecules more efficiently to cancer cells than the corresponding antibody–enzyme conjugate *in vitro*. Once bound to the target cells, immunoenzymosomes are able to induce complete conversion of an inactive doxorubicin prodrug into the parent compound doxorubicin. The resulting doxorubicin molecules are able to mount a strong bystander effect. In future studies the application of immunoenzymosomes and their corresponding antibody–enzyme conjugate in ADEPT will be tested in tumor-bearing animals such as the clinically relevant mouse xenograft OVCAR-3 intraperitoneal tumor model.

ACKNOWLEDGMENT

María José Fonseca was supported by a grant from the Generalitat de Catalunya.

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