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Design of immuno-enzymosomes with maximum enzyme targeting capability: effect of the enzyme density on the enzyme targeting capability and cell binding properties

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Abstract

Immuno-enzymosomes have been proposed for the targeting of enzymes to cancer cells to achieve site specific activation of anticancer prodrugs. Previously, we reported that the enzyme β-glucuronidase (GUS), capable of activating anthracycline-glucuronide prodrugs, can be coupled to the surface of inmunoliposomes directed against human ovarian cancer cells (OVCAR-3). This study aimed at the design of an immuno-enzymosome formulation with maximum enzyme targeting capability. By purification of the commercially available enzyme β-glucuronidase (GUS), a 2-fold increase in the enzyme specific activity and a 4-fold increase in the enzymatic activity of immuno-enzymosomes was achieved. As a result, upon incubation with human ovarian cancer cells (OVCAR-3), cell-associated enzymatic activity increased correspondingly. The optimized immuno-enzymosomes were shown to bind to the target cells in a specific fashion. Above a GUS/Fab′ molar ratio of 0.5, impairment of the target cell binding ability of the immuno-enzymosomes was observed. This was likely due to a steric hindrance effect mediated by the presence of large amounts of bulky GUS molecules on the liposome surface. Nevertheless, increasing the GUS density on the surface of the immuno-enzymosomes to levels by far exceeding the GUS/Fab′ molar ratio of 0.5, yielded a considerably improved enzyme targeting capability.

Keywords: Liposome; Antibody-directed enzyme prodrug therapy; ADEPT; Enzyme targeting; Cell binding

1. Introduction

The efficacy of conventional anticancer drugs used in chemotherapy is limited by the lack of selectivity for cancer cells. In principle, the conjugation of cell specific antibodies to liposomes (immunoliposomes) containing chemotherapeutic agents provides the possibility for selective drug delivery and cell type specific cytotoxicity [1–4]. Another drug targeting strategy in cancer chemotherapy is the use of antibodies to carry enzymes to tumor cells. This concept is referred to as antibody-directed enzyme prodrug therapy (ADEPT) [5–8]. Usually it involves the administration of an antibody-enzyme conjugate, followed by the injection of a relatively non-toxic pro-

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drug after binding of the conjugate to the tumor cells and clearance from blood and other tissues. In close proximity of the tumor cell, the prodrug is converted into the parent cytotoxic agent by the targeted enzyme, which may result in selective tumor cell kill. Previously, we have presented a modification of this approach based on the use of immunoliposomes. In this case both antibody and enzyme are covalently coupled to the surface of liposomes, a system hereafter referred to as immuno-enzymosomes [9–11].

The enzyme chosen for our immuno-enzymosome system was β-glucuronidase (GUS), able to activate glucuronidated drugs. GUS has the advantage over other enzymes commonly used in ADEPT, such as alkaline phosphatase [12,13] that it is not present in blood at detectable levels, but localized only intracellularly [14]. Thus, untimely activation of prodrug is limited as hydrophilic glucuronide prodrugs do not readily diffuse into cells [15]. Another advantage of using GUS is that it is possible to use the human homologue. This may potentially reduce the immunogenicity problem induced by GUS attached to the liposomes. The selected targeting device, the monoclonal antibody 323/A3 (mouse IgG 1) [16], reacts with a pancarcinoma antigen epithelial transmembrane glycoprotein with a molecular mass of 40 kDa (referred to as EGP40 or epithelial cell adhesion molecule, Ep-CAM) [17]. This antigen is highly expressed on the surface of most carcinomas, including breast, ovary, lung and colon tumors. We used Fab′ fragments of the antibody for two reasons. Firstly, the method used for coupling the Fab′ fragments to the liposomes (covalent linkage to the MPB-PE anchor present in the outer lipid bilayers of preformed liposomes) ensures an adequate orientation of the Fab′ fragments on the liposomes, with the antigen binding sites directed outward and fully accessible to antigen binding. Secondly, the removal of the Fc part of the IgG molecule can be of great importance for in vivo use of immuno-enzymosomes. In this way the Fc receptor of macrophages is not activated and elimination of the liposome, by the mononuclear phagocyte system (MPS), might be reduced and slowed down [18].

A theoretical advantage of the so-called 'immuno-enzymosomes' over antibody-enzyme conjugates is that many more than one enzyme molecule can be included in one targeted carrier unit. Under the proper conditions, this may create the opportunity to increase the enzyme density at the tumor cell surface, which accordingly may lead to a more efficient conversion of the prodrug into the active cytotoxic parent compound. Earlier we have successfully prepared immuno-enzymosomes able to specifically bind in vitro to human ovarian carcinoma cells (OVCAR-3) and subsequently convert the prodrugs epirubicin-glucuronide [9] and daunorubicin-glucuronide [10] into their toxic parent compounds. The aim of this study was to design an immuno-enzymosome formulation with maximized enzyme activity. Since we considered it likely that steric hindrance by the bulky GUS molecule would negatively affect the interaction of the liposomal Fab′ fragments with the cells, we varied, in addition, the enzyme density on the immunoliposomes and investigated what influence this had on the amount of enzyme that can be delivered to the target cells.

2. Materials and methods

2.1. Chemicals

Fetal calf serum (FCS) was obtained from Bocknek Laboratories (Canada). DMEM was obtained by Flow Laboratories (Irving, UK). The mouse monoclonal antibody 323/A3 (IgG type) was donated by Centocor Europe BV (Leiden, The Netherlands). β-Glucuronidase (GUS) from *Escherichia coli* K12 and *p*-nitrophenyl-β-D-glucuronide were purchased from Boehringer (Mannheim, Germany). Cell culture flasks were from Falcon (Micronic BV, Lelystad, The Netherlands). Succinimidyl 4-(*p*-maleimidophenyl)butyrate (SMPB) was obtained from Pierce (Oud-Beijerland, The Netherlands). Egg phosphatidyl choline (EPC), egg-phosphatidylglycerol (EPG) were donated by Lipoid (Ludwigshafen, Germany). Phosphatidyl ethanolamine (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 anti-mouse IgG was obtained from Sigma Chemical Co. (St. Louis, MO, USA). 4-Methylumbelliferyl-β-D-glucuronide tryhydrate was from Fluka (Buchs, Switzerland). All other reagents were of analytical grade.

2.2. Purification of GUS

Commercially available GUS from *E. coli* K12 (Boehringer, Mannheim, Germany) was purified by gel filtration chromatography. GUS was applied onto a Sephadex G-150 column and eluted with phosphate buffered saline (PBS containing 1 mM EDTA, pH 7.2). The presence of protein in the collected fractions was checked by reaction with Coomassie protein reagent (absorbance was measured at 595 nm). Fractions containing protein were subsequently checked for enzyme activity. Diluted samples (10 μl) were incubated with *p*-nitrophenyl-β-D-glucuronide (10 mM in PBS containing 0.1% BSA) (190 μl) for 30 min at 37°C. The reaction was terminated by the addition of 50 μl of 1 M glycine (pH 10.6) and absorbance was read at 405 nm. Protein-rich fractions were pooled on the basis of the absorbance values. This enzyme solution was concentrated by means of a Macrosept concentrator (30K) (Centriprep, Amicon).

2.3. Enzyme thiolation

Thiolation of GUS was carried out by reaction with *N*-succinimidyl *S*-acetylthioacetate (SATA) as described before [10]. Purified GUS (*Pur*-GUS) was used directly. Unpurified commercial GUS (GUS) was first subjected to gel filtration chromatography on a Sephadex G-25M column (PD-10, Pharmacia, Woerden, The Netherlands) in order to exchange the medium (50% glycerol) for phosphate buffered saline (PBS). Either purified or non-purified GUS were incubated with SATA (dissolved in dimethylformamide (DMF)) at a molar ratio of 1:8 for 20 min at room temperature in a nitrogen atmosphere [19]. SATA, DMF and PBS were then exchanged for acetate buffer pH 6.5 using gel filtration chromatography (Sephadex G-25M). Modified GUS (GUS-ATA) was stable for at least one month at 4°C. For covalent attachment to freshly prepared liposomes, the new SH groups introduced to GUS were deprotected by addition of hydroxylamine (see below).

2.4. Preparation of Fab′ fragments

The mouse monoclonal antibody 323/A3 (IgG 1 type) recognizes an M_r 43 kDa membrane glycoprotein which is expressed on a variety of carcinomas [9,16]. $F(ab')_2$ fragments were produced by pepsin digestion. The pH of the IgG solution was adjusted to 4.5 immediately before digestion. Pepsin attached to 4% cross-linked beaded agarose was added at a ratio of 100 U per mg of IgG. The mixture was incubated under constant rotation at 37°C overnight. After adjusting the pH to 7.4 using 1.0 and 0.1 M NaOH solution, the mixture was centrifuged at 4000 rpm for 10 min to remove the pepsin-coated beads. The protein solution was filtered through a 0.2 μm filter, applied onto a Protein A-Sepharose CL-6B column (Pharmacia AB, Uppsala, Sweden) and eluted with 100 mM acetate buffer pH 7.4. Eluted fractions containing $F(ab')_2$ fragments were collected and concentrated by means of a Macrosept concentrator (30K). Purity of $F(ab')_2$ was assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions and by gel permeation chromatography using a BioSep-SEC-S2000 column (Phenomenex, CA, USA). Degassed phosphate buffered saline (PBS) was used as the mobile phase. Immediately before incubation with liposomes, Fab′ fragments were prepared by incubation of $F(ab')_2$ with 20 mM DTT in acetate buffer at pH 5.5 (100 mM sodium acetate, 63 mM NaCl, 1 mM EDTA) for at least 90 min at room temperature [20]. DTT was removed by applying the incubation mixture onto a Sephadex G-25M column (PD-10) and subsequent elution with acetate buffer pH 6.5 (100 mM NaAC, 40 mM NaCl, 1 mM EDTA, deoxygenated and flushed with nitrogen before use). Fab′ fragments appearing in the void volume were used immediately for covalent attachment to freshly prepared liposomes (see below).

2.5. Preparation of immunoliposomes, enzymosomes and immuno-enzymosomes

N-[4-(*p*-maleimidophenyl)butyryl]phosphatidylethanolamine (MPB-PE) was synthesized from SMPB and PE, purified and analyzed as described before [20]. MPB-PE was incorporated into the liposome bilayers to allow covalent coupling of 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). Occasionally, when immunoliposomes were prepared the lipid film was hydrated with a 40 mM calcein solution. 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 either GUS-ATA or *Pur*-GUS-ATA and freshly prepared Fab′ fragments. Concentrations during incubation were 5–10 μmol TL/ml, 1–2.5 mg/ml of thiolated GUS and 0.1–0.75 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 (*Pur*)-GUS-ATA SH groups (100 μl hydroxylamine solution per ml of incubation mixture). The coupling reaction was carried out overnight at 4°C under constant rotation in a nitrogen atmosphere. Finally, the immunoliposomes 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 only (*Pur*)-GUS is coupled (no Fab′) are further referred to as enzymosomes. Liposomes to which both (*Pur*)-GUS and Fab′ are coupled are referred to as immuno-enzymosomes. Liposome dispersions were stored at 4°C.

2.6. Liposome characterization

Lipid phosphate was determined by the colorimetric method of Rouser [21]. The enzymatic activity was measured with *p*-nitrophenyl-β-D-glucuronide as described above. The amount of protein coupled to the liposomes was determined by the method of Wessel and Flügge [22], with bovine serum albumin as standard. The total amount of monoclonal antibody and/or enzyme coupled to the liposomes was expressed as μg protein/μmol total lipid (TL). The amount of enzyme coupled to immuno-enzymosomes was estimated by comparison of the enzyme density and enzymatic activity of enzymosomes (to which only (*Pur*)-GUS is coupled) with the protein density and enzymatic activity of immuno-enzymosomes. 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 a monodisperse up to 1.0 for a polydisperse dispersion.

2.7. Cell binding assay

The human ovarian cancer cell line NIH:OVCAR-3 [23] was maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS, L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). The ovarian carcinoma cell line A2780, which lacks the antigen for 323/A3, was cultured under similar conditions and provided control cells. Adherent cells (OVCAR-3 or A2780) were detached with 0.05% trypsine, 0.02% EDTA in phosphate buffered saline (PBS, pH 7.4) for 5 min at 37°C, and were washed once with cold PBS. Cells were treated in triplicate with calcein-containing immunoliposomes, immuno-enzymosomes or enzymosomes for 90 min at 4°C with vortexing at 30 min intervals. Unless otherwise stated, the lipid concentration and cell concentration in the incubation medium were 1 μmol TL/ml and 10^6 cells/ml, respectively. Unbound liposomes were separated from the cells by centrifugation (300×*g*, 3 min). The cell pellet was washed twice with PBS containing 1% BSA. The degree of cell binding of immunoliposomes was assessed by fluorescence of pellets and supernatants (excitation-emission wavelength of 490–519 nm) in a Perkin Elmer LS-50 (Beaconsfield, Buckinghamshire, UK) spectrofluorimeter. Cell binding of enzymosomes and immuno-enzymosomes was determined by measuring the enzymatic activity in the redispersed cell pellet by reaction with the substrate *p*-nitrophenyl-β-D-glucuronide as described above. For direct comparison of the binding properties of immunoliposomes and immuno-enzymosomes flow cytometry was used. Suspensions of OVCAR-3 cells in PBS containing 1% BSA were incubated with varying amounts of immunoliposomes and immuno-enzymosomes at 4°C for 90 min. After incubation, cells were washed twice with PBS containing 1% BSA by centrifugation (300×*g*, 3 min) and incubated at 4°C for 30 min with FITC-conju-

Table 1
Enzyme activity of commercially available β-glucuronidase before (GUS) and after (*Pur*-GUS) purification and SATA reaction (*Pur*-GUS-ATA). Each value represents the mean ± S.D., $n = 5$

Enzyme	Enzyme activity (U/mg)
GUS	21 ± 3
Pur-GUS	42 ± 13
Pur-GUS-ATA	48 ± 11

gated 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 and Dickinson Immunocytometry Systems, Mountain View, CA, USA), 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.

2.8. Enzyme targeting capability of immuno-enzymosomes

Varying amounts of immuno-enzymosomes were incubated with OVCAR-3 cells (10^6 cells/ml) for 90 min at 4°C. Unbound liposomes were separated from the cells by centrifugation ($300 \times g$, 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 trihydrate 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, CT, USA) spectrofluorimeter.

3. Results

3.1. Effect of purification of commercially available β-glucuronidase on the enzymatic activity of immuno-enzymosomes

Purification of a commercial GUS preparation led to a 2-fold increase in specific activity (Table 1). As thiolation of GUS is necessary for efficient coupling to the liposomes [9,10], it was important to check whether the enzymatic activity was affected by reaction with the thiolating agent *N*-succinimidyl *S*-acetylthioacetate (SATA). Table 1 shows that purified GUS did not lose activity by reaction with SATA (*Pur*-GUS-ATA).

Immuno-enzymosomes and enzymosomes bearing unpurified or purified GUS were prepared by coupling the proteins (Fab′ and/or GUS) to liposomes containing the anchor molecule MPB-PE. Table 2 summarizes the characteristics of these liposome preparations. The size distribution of all preparations remained stable for at least 1 month of storage. Clearly, when purified enzyme was used the degree of GUS coupling to the liposomes was about 2-fold enhanced. As a result of the 2-fold increase in specific enzymatic activity upon purification (Table 1), and the 2-fold increase in the degree of GUS coupling (Table 2), the enzymatic activity of the liposomes

Table 2
Characterization of liposome preparations

Liposome Type	Mean size (μm)	GUS coupling ratio (μg GUS/μmol TL)	Fab′ coupling ratio (μg FAB′/μmol TL)	Enzymatic activity (U/μmol TL)
Ref-EL	0.28 ± 0.01	26 ± 2	–	0.17 ± 0.00
Ref-IEL	0.20 ± 0.00	20 ± 1	21 ± 4	0.12 ± 0.00
Pur-EL	0.25 ± 0.02	49 ± 3	–	0.65 ± 0.15
Pur-IEL	0.21 ± 0.02	41 ± 1	14 ± 3	0.48 ± 0.00

Enzymosomes were prepared by incubation of MPB-PE liposomes with purified (*Pur*-EL) or unpurified (*Ref*-EL) GUS-ATA.
Immuno-enzymosomes were prepared similarly (*Pur*-IEL and *Ref*-IEL, respectively), but in this case Fab′ fragments of the IgG 323/A3 were included in the incubation medium. Conditions in the incubation medium were 6 μmol TL/ml, 1 mg/ml of GUS-ATA and 0.5 mg/ml Fab′.
Results are expressed as mean values ± S.D.; the number of dispersions per group was at least three.
The coupling ratio (μg Fab′/μmol TL and μg GUS/μmol TL) was determined using a protein determination assay, an enzymatic activity assay and a phosphate determination assay as described in Section 2.

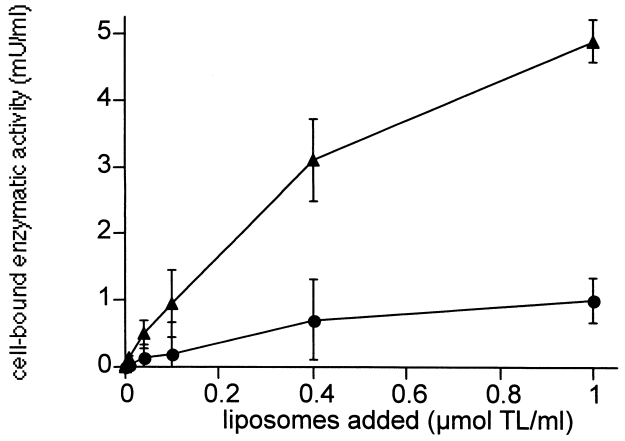

Fig. 1. Enzyme targeting capability of immuno-enzymosomes prepared with purified GUS (▲) as compared to that of immuno-enzymosomes prepared with non-purified GUS (●). Varying amounts of both liposome dispersions were incubated with cells (10^6 cells/ml) for 90 min at 4°C. After washing the cells with PBS containing 0.1% BSA to remove unbound liposomes, the enzymatic activity was determined. The cell binding is expressed as the cell-bound enzymatic activity. A typical experiment out of three performed is shown. Each point represents the mean ± S.D. of three determinations.

was 4-fold when purified GUS was used. Furthermore, the presence of the antibody had only a slight effect on the amount of enzyme that could be coupled to the liposomes. Accordingly, the enzyme activity bound to target cells after incubation with immuno-enzymosomes prepared with purified GUS (*Pur*-IEL) was expected to be higher than that obtained after incubation with immuno-enzymosomes prepared with non-purified GUS (*Ref*-IEL). To verify this, *Ref*-IEL and *Pur*-IEL were prepared under the same conditions and varying amounts of both preparations were incubated with OVCAR-3 cells. Fig. 1 shows that immuno-enzymosomes prepared with purified β-glucuronidase were able to deliver up to 5-fold higher enzymatic activity to the cells than those prepared with non-purified GUS. For the remainder of this study, purified GUS was used for the preparation of immuno-enzymosomes.

3.2. *In vitro targeting of 323/A3 immunoliposomes to ovarian carcinoma cells*

In order to determine the optimal conditions for further experiments, the effect of lipid concentration and number of cells present during the incubation on the degree of cell binding of immunoliposomes was determined. Fig. 2A shows that, at a constant cell concentration (10^6 cells/ml), the relative cell binding of the 323/A3 immunoliposomes decreased as a function of the concentration of added liposomes. For further experiments, a lipid concentration of 1 μmol TL/ml was chosen, since above that concentration the fraction of cell-bound immunoliposomes decreased substantially. Fig. 2B shows that the relative cell binding at 1 μmol TL/ml can be improved about 3-fold by a 10-fold increase in tumor cell concentration (from 10^6 to 10^7 cells/ml). For practical reasons,

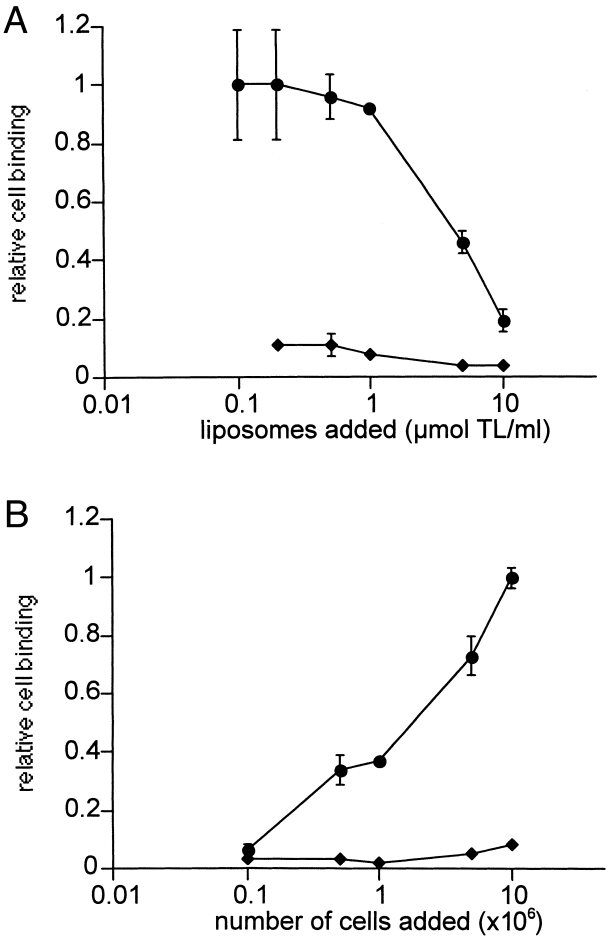

Fig. 2. Target cell binding of immunoliposomes. Calcein-containing (●) 323/A3 immunoliposomes (17 μg Fab′/μmol TL) and (♦) MPB-PE liposomes were incubated with in vitro growing OVCAR-3 cells for 90 min at 4°C. A: Cell concentration was fixed at 10^6 cells/ml and lipid concentration was variable. B: Lipid concentration was fixed at 1 μmol/ml and cell concentration was variable. Maximum cell binding was set at 1. A typical experiment out of three performed is shown. Each point represents the mean ± S.D. of three determinations.

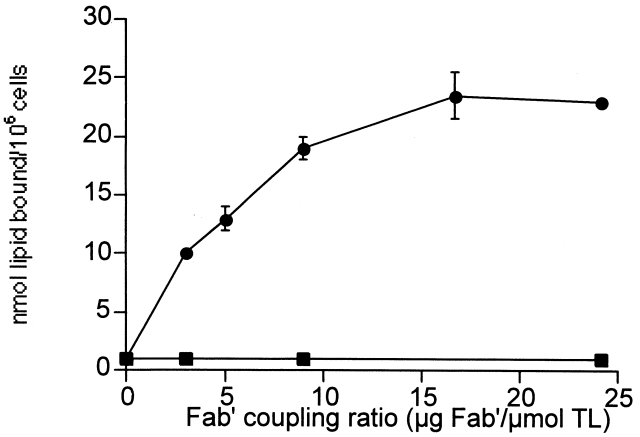

Fig. 3. Effect of the coupled amount of Fab′ on the cell (OVCAR-3) binding properties of calcein-containing 323/A3 immunoliposomes (●). Binding of immunoliposomes to OVCAR-3 cells pretreated with an excess of 323/A3 (■), served as a control. Liposomes (1 μmol TL/ml) were incubated with cells (10^6 cells/ml) for 90 min at 4°C. After washing the cells with PBS by centrifugation to remove unbound liposomes, fluorescence was determined. The cell binding is expressed as the absolute amount of immunoliposomes bound/10^6 cells. A typical experiment out of three performed is shown. Each point represents the mean ± S.D. of three determinations.

however, most of our experiments were performed at a tumor cell concentration of 10^6 cells/ml. Fig. 2A and B also show that the binding of MPB-PE liposomes (lacking the 323/A3 specific monoclonal antibody) to OVCAR-3 cells was negligible.

The effect of antibody density on the surface of the 323/A3 immunoliposomes on the degree of cell binding to OVCAR-3 cells is shown in Fig. 3. An increase in Fab′ density on the immunoliposomes resulted in an increase in cell binding. Under the conditions used in this study, cell binding was maximal at a density of around 15 μg Fab′/μmol TL. A further increase in Fab′ density did not result in a further improved cell binding. When the cells were pretreated with an excess of the specific 323/A3 monoclonal antibody (IgG), the binding of immunoliposomes was completely blocked.

3.3. *Effect of enzyme density on target cell affinity and enzyme targeting capability of immuno-enzymosomes*

Fig. 4 shows that immuno-enzymosomes are able to bind to OVCAR-3 cells in vitro in a specific manner. The degree of cell binding of the immuno-enzymosome preparation used (40 μg GUS/μmol TL and 14 μg Fab′/μmol TL) was comparable to that shown in Fig. 3 for immunoliposomes (without surface-attached GUS). To demonstrate the specific nature of the binding, several negative control binding experiments were performed: (1) immuno-enzymosomes did not bind to A2780 cells which lack the antigen for 323/A3; (2) the target cell binding of immuno-enzymosomes was strongly inhibited by pre-incubation of the cells with the specific monoclonal antibody 323/A3; and (3) enzymosomes (lacking the specific Fab′) showed negligible association to OVCAR-3 cells.

To investigate whether the surface-bound GUS had a negative effect on cell binding, the cell binding properties of both immunoliposomes and immuno-enzymosomes were studied by flow cytometric analysis. Fig. 5A shows that the binding affinity of immuno-enzymosomes bearing 25 μg GUS/μmol TL and 20 μg Fab′/μmol TL (GUS/Fab′ molar ratio 0.2) was comparable to that of the corresponding immunoliposomes. The same was observed for other immuno-enzymosome preparations with a GUS/Fab′

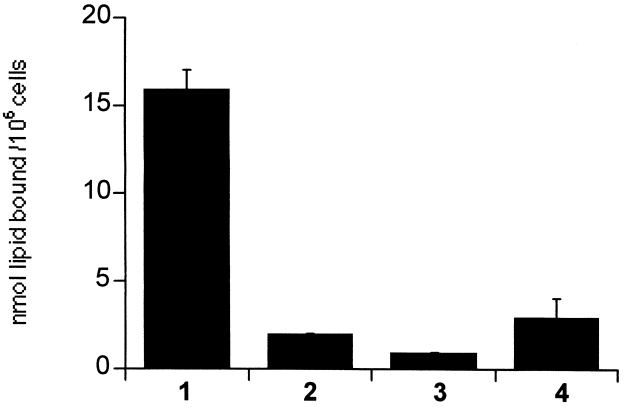

Fig. 4. In vitro cell binding of immuno-enzymosomes to OVCAR-3. (1) Binding of immuno-enzymosomes to OVCAR-3 cells, (2) binding of immuno-enzymosomes to OVCAR-3 cells pretreated with an excess of 323/A3, (3) binding of immuno-enzymosomes to control A2780 cells, and (4) binding of enzymosomes to OVCAR-3 cells. Liposomes (1 μmol TL/ml) were incubated with cells (10^6 cells/ml) for 90 min at 4°C. After washing the cells with PBS containing 1% BSA to remove unbound liposomes, the enzymatic activity was determined. The cell binding is expressed as the absolute amount of (immuno-)enzymosomes bound/10^6 cells. A typical experiment out of three performed is shown. Each point represents the mean ± S.D. of three determinations.

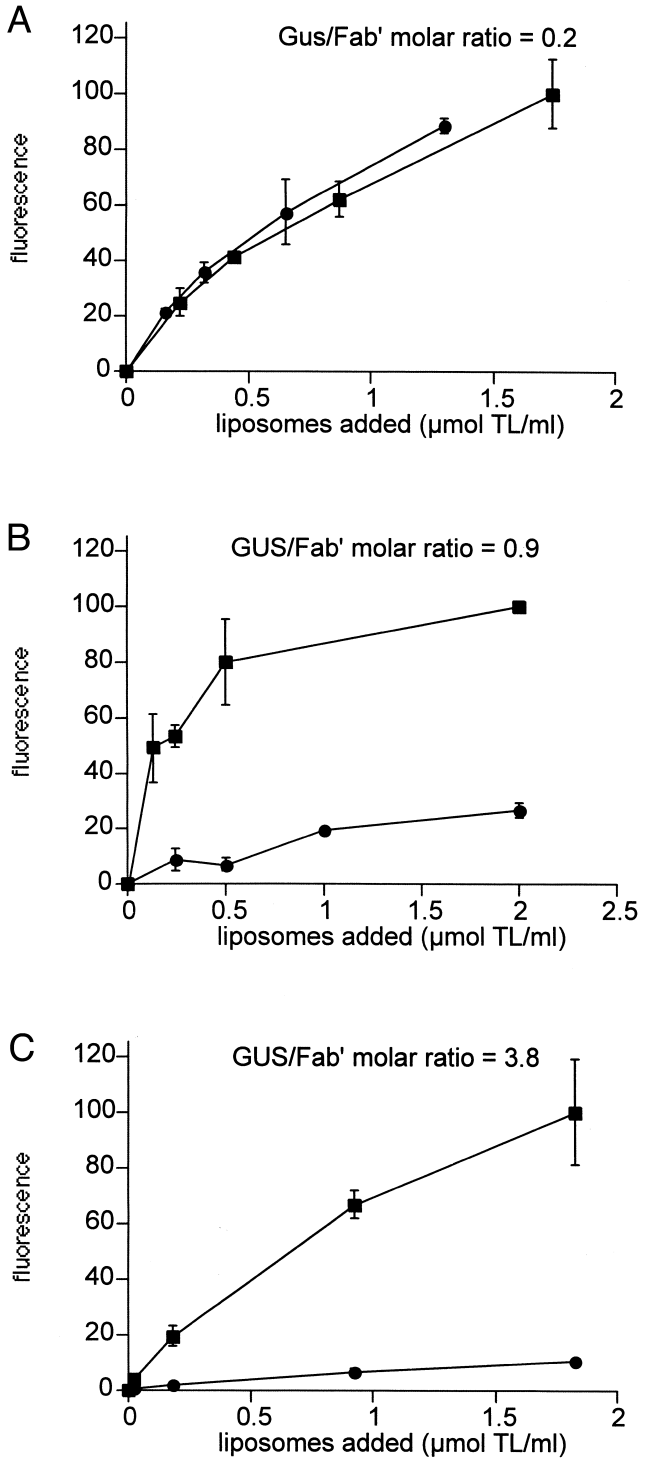

Fig. 5. Flow cytometric analysis of the binding of immuno-enzymosomes (●) to OVCAR-3 cells with varying GUS and Fab′ content as compared to immunoliposomes (■) bearing similar Fab′ densities. Cells were examined for cell-associated fluorescence by flow cytometry as described in Section 2. The mean fluorescence value (±S.D. $n=3$) determined by flow cytometry is presented after subtraction of that obtained for cells incubated with only FITC conjugate. A typical experiment out of three performed is shown. A: GUS coupling ratio: 25 μg GUS/μmol TL (170 molecules of GUS per liposome); Fab′ coupling ratio: 20 μg Fab′/μmol TL (700 molecules of Fab′ per liposome). GUS/Fab′ ratio = 0.2. B: GUS coupling ratio: 80 μg GUS/μmol TL (700 molecules of GUS per liposome); Fab′ coupling ratio: 15 μg Fab′/μmol TL (750 molecules of Fab′ per liposome). GUS/Fab′ ratio = 0.9. C: GUS coupling ratio: 70 μg GUS/μmol TL (380 molecules of GUS per liposome); Fab′ coupling ratio: 3 μg Fab′/μmol TL (100 molecules of Fab′ per liposome). GUS/Fab′ ratio = 3.8.
←

molar ratio lower than 0.5 (data not shown). However, the cell binding capability of immuno-enzymosomes with a higher content of GUS and/or a lower content of Fab′ (GUS/Fab′ molar ratio higher than 0.5) was notably reduced as compared to the corresponding immunoliposomes. The higher the GUS/Fab′ ratio, the more pronounced the hindrance on cell binding capability. Two illustrative examples with GUS/Fab′ molar ratios of 0.9 and 3.8 are shown in Fig. 5B,C, respectively. To ensure that the observed decrease in cell-associated fluorescence was due merely to GUS-mediated hindrance of the binding of immuno-enzymosomes to the cells and not to problems related to incomplete recognition of the cell-bound immuno-enzymosomes by the secondary antibody, the same set of experiments was performed with liposomes containing the fluorescent lipid label rhodamine-phosphatidyl ethanolamine. In all cases, similar results were obtained (data not shown).

The impact of the steric hindrance effect observed for immuno-enzymosomes with a GUS/Fab′ molar ratio >0.5 on their enzyme targeting capability was investigated. Immuno-enzymosomes bearing up to 80 μg GUS/μmol TL and varying contents of Fab′ were prepared. In all cases we observed that immuno-enzymosomes with the highest enzyme density were able to target the highest enzymatic activity to the cells, even in cases when cell binding was strongly reduced as a result of a strong GUS-mediated steric hindrance effect. This is illustrated in Fig. 6, which shows that an immuno-enzymosome with a GUS/Fab′ ratio of 3.8, displaying a strongly reduced cell binding (Fig. 5C) could nonetheless target far more

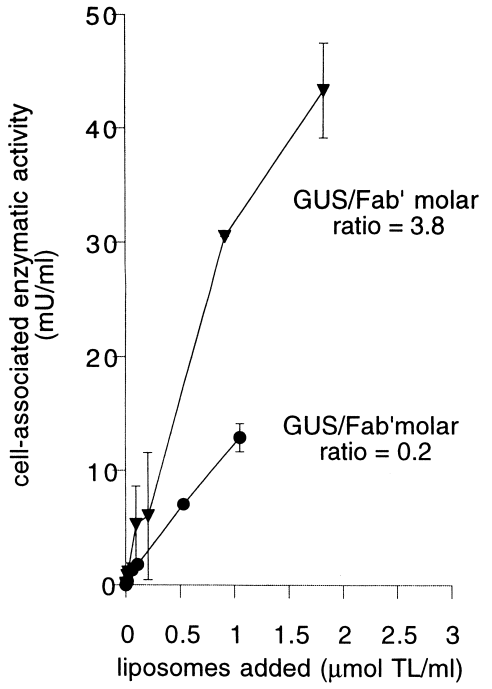

Fig. 6. Enzyme targeting capability of immuno-enzymosomes with varying amounts of GUS and Fab′: (▼) Immuno-enzymosomes with high GUS density and impaired cell binding properties (GUS coupling ratio: 70 μg GUS/μmol TL (380 molecules of GUS per liposome); Fab′ coupling ratio: 3 μg Fab′/μmol TL (100 molecules of Fab′ per liposome); GUS/Fab′ ratio = 3.8). (●) Immuno-enzymosomes with lower GUS density and preserved cell binding properties (GUS coupling ratio: 25 μg GUS/μmol TL (170 molecules of GUS per liposome); Fab′ coupling ratio: 20 μg Fab′/μmol TL (700 molecules of Fab′ per liposome); GUS/Fab′ ratio = 0.2). Varying amounts of the liposome dispersions were incubated with cells (10^6 cells/ml) for 90 min at 4°C. After washing the cells with PBS containing 0.1% BSA to remove unbound liposomes, the cell-bound enzymatic activity was determined. A typical experiment out of three performed is shown. Each point represents the mean ± S.D. of three determinations.

enzymes to the cells than a preparation with a GUS/Fab′ ratio of 0.2, displaying fully retained binding ability (Fig. 5A).

4. Discussion

In this paper we use the targetability of immunoliposomes to carry enzymes (immuno-enzymosomes), capable of activating anticancer prodrugs, to ovarian cancer cells. Ovarian cancer is one of the most common fatal gynaecological 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. The standard treatment of patients with advanced disease consists of surgical debulking of the tumor mass followed by combination chemotherapy. To improve the poor prognosis, complete elimination of the residual micrometastases is required. Metastases remain localized in the peritoneal cavity throughout most of the clinical course of ovarian cancer. Therefore, we consider that i.p. administration of immuno-enzymosomes for ADEPT might be an attractive approach for adjuvant therapy, as the intraperitoneally localized target cells can be expected to be directly accessible for the i.p. administered particles.

We have reported before that the introduction of additional thiol groups was necessary for efficient coupling of the enzyme β-glucuronidase (GUS) to the MPB-PE liposomes [9]. That led to our 'first generation' immuno-enzymosomes which contained 20 molecules GUS and about 400 Fab′ molecules per particle. In this paper we address the following questions: (1) To what extent is it possible to increase the amount of enzyme and/or enzymatic activity associated to the immuno-enzymosomes without occurrence of vesicle aggregation? (2) Are the cell binding properties of immuno-enzymosomes bearing maximal enzyme density preserved?

With regard to the first issue, an attempt was made to increase the enzymatic activity associated to the immuno-enzymosomes by purification of commercially available GUS. As a result of the removal of proteins other than GUS present in the commercial preparation, the specific activity of the enzyme (enzymatic activity per mg protein) was increased 2-fold (Table 1). This purification step also resulted in a 2-fold increase of the amount of GUS protein coupled to the liposomes (Table 2). The observation that more protein was coupled to the liposomes when purified GUS was used indicates that other non-protein components present in the commercial preparation and removed during the purification process competed with the thiol groups of the enzyme for the reaction with the liposome anchor molecule (MPB-PE).

We were able to achieve a further increase in GUS density by using a higher concentration of GUS during the incubation with the liposomes. Immuno-en-

zymosomes containing up to 80 μg GUS/μmol TL (700 GUS molecules per particle[1]) could be prepared without occurrence of aggregation. As a matter of fact, these immuno-enzymosomes had a 35-fold higher enzyme density and a 70-fold increased enzymatic activity than our 'first-generation' immuno-enzymosomes [9]. Previously, the preparation of such high density immuno-enzymosomes was not possible due to problems of vesicle aggregation. This improvement led us to investigate the effect of enzyme density on the cell binding properties of immuno-enzymosomes. It is well possible that the presence of large amounts of the bulky enzyme (280 kDa) interferes with the interaction between Fab′ (50 kDa) and the antigen expressed on the cancer cells, thus impairing the cell binding properties of immuno-enzymosomes. To address this problem, we first established that cell binding of immunoliposomes was maximal at a Fab′ density of approximately 15 μg/μmol TL (Fig. 3). Immuno-enzymosomes with a similar Fab′ content and relatively low GUS density (GUS/Fab′ molar ratio of 0.2), had fully preserved cell binding properties (Fig. 5A). However, immuno-enzymosomes with higher GUS density and/or lower Fab′ content (GUS/Fab′ molar ratio >0.5, see Fig. 5B, C) bound to a much lower extent to OVCAR-3 cells than immunoliposomes with a similar content of Fab′, thus suggesting that, indeed, the presence of the enzyme interferes with the binding properties of Fab′-bearing liposomes. Nevertheless, in spite of the occurrence of GUS-mediated hindrance of target cell binding, to achieve maximal enzyme targeting the most relevant parameter proved to be the absolute amount of liposome-associated enzymatic activity (Fig. 6). Immuno-enzymosomes bearing the highest enzymatic activity showed the best enzyme targeting capability even though their cell binding properties were notably impaired. Therefore, we conclude that increasing the enzyme density on the surface of immuno-enzymosomes, even up to levels that steric hindrance to the interaction with the target antigen is introduced, is an adequate strategy for developing immuno-enzymosomes with maximized enzyme targeting capability. In vivo studies addressing the applicability of immuno-enzymosomes carrying a high enzyme density for ADEPT in an i.p. xenograft of ovarian carcinoma are underway.

[1] The number of Fab′ and GUS molecules coupled per liposome were calculated according to the following assumptions: molecular weight of Fab′ 50 kDa and of GUS 280 kDa; an average number of 1.5 bilayers as determined for almost identically prepared PC:PS:CHOL (10:1:4) liposomes [24]; a surface area of 1×10^{11} μm^2/μmol TL.

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References

[1] V.P. Torchilin, Crit. Rev. Ther. Drug Carrier Syst. 2 (1985) 65–115.
[2] P.A.M. Peeters, G. Storm, D.J.A. Crommelin, Adv. Drug Deliv. Rev. 1 (1987) 249–266.
[3] S. Wright, L. Huang, Adv. Drug Deliv. Rev. 3 (1989) 343–389.
[4] M.H. Vingerhoeds, G. Storm, D.J.A. Crommelin, Immunomethods 4 (1994) 259–272.
[5] P. Senter, P.M. Wallace, H.P. Svensson, V.M. Vrudhula, D.E. Kerr, I. Hellström, K.E. Hellström, Bioconjug. Chem. 4 (1993) 3–9.
[6] M.P. Deonarain, A.A. Epenetos, Br. J. Cancer 70 (1994) 786–794.
[7] K.D. Bagshawe, C.J. Springer, F. Searle, P. Antoniw, S.K. Sharma, R.G. Melton, R.F. Sherwood, Br. J. Cancer 58 (1988) 700–703.
[8] H.J. Haisma, E. Boven, M. van Muijen, J. de Jong, W.J.F. van der Vijgh, H.M. Pinedo, Br. J. Cancer 66 (1992) 474–478.
[9] M.H. Vingerhoeds, H.J. Haisma, M. Van Muijen, R.B.J. Van de Rijt, D.J.A. Crommelin, G. Storm, FEBS Lett. 336 (1993) 485–490.
[10] M.H. Vingerhoeds, H.J. Haisma, S.O. Belliot, R.H.P. Smit, D.J.A. Crommelin, G. Storm, Pharm. Res. 13 (1996) 604–610.
[11] G. Storm, M.H. Vingerhoeds, D.J.A. Crommelin, H.J. Haisma, Adv. Drug Deliv. Rev. 24 (1997) 225–231.
[12] P.D. Senter, M.G. Saulnier, G.J. Schreiber, D.L. Hirschberg, J.P. Brown, I. Hellstrom, K.E. Hellstrom, Proc. Natl. Acad. Sci. USA 85 (1988) 4842–4846.
[13] H.J. Haisma, E. Boven, M. van Muijen, R. de Vries, H.M. Pinedo, Cancer Immunol. Immunother. 34 (1992) 343–348.
[14] H.H. Sedlacek, G. Seemann, D. Hoffman, J. Czech, P. Lorenz, C. Kolar, K. Bosslet, in: Antibodies as Carriers Of cytotoxicity, Darger, Basel, 1992.
[15] P.H.J. Houba, R.G.G. Leenders, E. Boven, J.W. Scheeren,

H.M. Pinedo, H.J. Haisma, Biochem. Pharm. 52 (1996) 455–463.
[16] D.P. Edwards, K.T. Grzyb, L.G. Dressler, R.E. Mansel, D.T. Zava, G.W. Sledge, W.L. McGuire, Cancer Res. 46 (1986) 1306–1317.
[17] S.V. Litvinov, M.P. Velders, H.A.M. Bakker, G.J. Fleuren, S.O. Warnaar, J. Cell Biol. 125 (1994) 437–446.
[18] R.J. Debs, T.D. Heath, D. Papahadjopoulos, Biochim. Biophys. Acta 901 (1987) 183–190.
[19] R.J.S. Duncan, P.D. Weston, R. Wrigglesworth, Anal. Biochem. 132 (1983) 68–73.
[20] U.K. Nässander, P.A. Steerenberg, W.H. de Jong, W.O.W.M. van Overveld, C.M.E. te Boekhorst, L.G. Poels, P.H.K. Jap, G. Storm, Biochim. Biophys. Acta 1235 (1995) 126–139.
[21] G. Rouser, S. Fluscher, A. Yamamoto, Lipids 5 (1970) 494–496.
[22] D. Wessel, U.I. Flügge, Anal. Biochem. 138 (1984) 141–143.
[23] T.C. Hamilton, R.C. Young, W.M. McKoy, K.R. Grotzinger, J.A. Green, E.W. Chu, J. Whang-Penn, A.M. Rogan, W.R. Green, R.F. Ozols, Cancer Res. 43 (1983) 5379–5389.
[24] H. Jousma, H. Talsma, F. Spies, J.G.H. Joosten, H.E. Junginger, D.J.A. Crommelin, Int. J. Pharm. 35 (1987) 263–274.