Immunoliposomes as Enzyme-Carriers (Immuno-Enzymosomes) for Antibody-Directed Enzyme Prodrug Therapy (ADEPT): Optimization of Prodrug Activating Capacity

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Received September 25, 1995; accepted January 15, 1996

Purpose. Immuno-enzymosomes are tumor-specific immunoliposomes bearing enzymes on their surface. These enzymes are capable of converting relatively nontoxic prodrugs into active cytostatic agents. The enzyme β-glucuronidase (GUS)[4] was coupled to the external surface of immunoliposomes directed against ovarian carcinoma cells. This study aimed at optimization of the prodrug-activating capacity of these immuno-enzymosomes by increasing the enzyme density on the immunoliposomal surface.

Methods. To achieve coupling of GUS to the liposomes, introduction of extra thiol groups was required. Two thiolating agents were examined: iminothiolane and SATA.

Results. When iminothiolane was used, aggregation of enzymosomes was observed above enzyme densities of 10 μg GUS/μmol lipid (TL). An increased electrostatic repulsion of the enzymosomes, created by inclusion of additional negatively charged lipids and by lowering the ionic strength of the external aqueous medium resulted in enzyme densities ≥ 20 μg GUS/μmol TL without aggregation. Utilizing SATA, ≥ 30 μg GUS/μmol TL could be coupled without aggregation, even at physiological ionic strength. It was shown that the enzyme density on immuno-enzymosomes, and thus on the tumor cell surface, strongly influences the antitumor effect of the prodrug daunorubicin-glucuronide against in vitro cultured ovarian cancer cells. The antitumor effect of immuno-enzymosomes with enzyme densities of about 20 μg GUS/μmol TL was similar to that of the parent drug daunorubicin.

Conclusions. SATA-mediated thiolation of GUS-molecules enabled the preparation of immuno-enzymosomes with high enzyme densities while avoiding spontaneous aggregation. In vitro antitumor activity experiments showed that the improved immuno-enzymosome system is able to completely convert the prodrug daunorubicin-glucuronide into its parent compound.

KEY WORDS: monoclonal antibody; liposomes; antibody-directed enzyme prodrug therapy; ADEPT; targeted drug delivery.

INTRODUCTION

A new indirect drug-targeting strategy in cancer chemotherapy is the use of antibodies to carry enzymes to tumor cells, referred to as antibody-directed enzyme prodrug therapy (ADEPT; for recent reviews 1, 2). In ADEPT, an enzyme is linked to an antibody (antibody-enzyme conjugate) that binds to an antigen preferentially expressed on tumor cells. After binding of the antibody-enzyme conjugates to tumor cells, a relatively nontoxic prodrug, which is matched with the enzyme, is administered and the active drug is generated in close proximity of the tumor cell. It was shown by several groups that selective conversion of prodrug into active drug at the tumor site can be obtained by these antibody-enzyme conjugates (3, 4). The first pilot-scale clinical trial of the ADEPT approach for cancer treatment has recently been reported by Bagshawe et al. (5).

To target prodrug-activating enzymes to the tumor site, we proposed the use of so-called immuno-enzymosomes (antibody-targeted liposomes (immunoliposomes) bearing enzymes on their surface (6)). A theoretical advantage of the immuno-enzymosome approach, as compared to the use of antibody-enzyme conjugates, is that many more than one enzyme molecule can be delivered to the tumor site by a single targeted carrier unit. This offers the possibility to substantially increase the enzyme density at the target cell surface, which is in particular valuable for tumors with little antigen expression, and thereby to induce a higher efficiency of specific prodrug activation.

We showed earlier using the enzyme β-glucuronidase (GUS) and the prodrug epirubicin-glucuronide that immuno-enzymosomes directed against ovarian cancer cells were as efficient in the induction of an antitumor effect in vitro as antibody-enzyme conjugates (6). However, the antitumor effect was less than that of the parent drug epirubicin. To increase the antitumor effect with immuno-enzymosomes, attempts were made to couple more enzyme molecules to the liposomes. However, with the technology used at that time, the liposomes showed a strong tendency to aggregate at enzyme densities ≥ about 10 μg GUS/μmol lipid (TL; i.e. when more than about 45 enzyme molecules were bound per liposome particle).

This study aimed at optimization of the prodrug-activating capacity of immuno-enzymosomes by increasing the enzyme density on the immunoliposomal surface while avoiding aggregation. We have attempted to avoid the problem of aggregation by employing a higher negative zetapotential of the immunoliposomes and varying the thiolation procedure of the enzyme. Now enzyme densities over 30 μg GUS/μmol TL (i.e. >125 enzyme molecule per liposome) were obtained without the occurrence aggregation. It is shown that with increasing enzyme density on the immuno-enzymosomes, the antitumor effect of the prodrug daunorubicin-glucuronide (dauno-glu) towards in

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[4] **ABBREVIATIONS:** ADEPT, antibody-directed enzyme prodrug therapy; CHOL, cholesterol; Dauno-glu, daunorubicin-glucuronide; DMEM, Dulbecco's Modified Eagle's Medium; DMF, dimethylformamide; DTT, dithiothreitol; EPC, egg-phosphatidylcholine; EPG, egg-phosphatidylglycerol; FCS, fetal calf serum; GUS, β-glucuronidase; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; MEM, minimal essential medium; MPB-PE, N-[4-(p-maleimidophenyl)butyryl] phosphatidylethanolamine; NEM, N-ethylmaleimide; PBS, phosphate buffered saline; PE, phosphatidylethanolamine; SATA, N-succinimidyl S-acetylthioacetate; SMPB, succinimidyl 4-(p-maleimidophenyl)butyrate; SRB, sulforhodamine-B; TCA, trichloroacetic acid; TL, total lipid (phospholipid + cholesterol).

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vitro cultured OVCAR-3 cells pre-exposed to immuno-enzymosomes is enhanced. The antitumor effect of dauno-glu after pre-exposure to immuno-enzymosomes with high enzyme densities was similar to that of the parent drug daunorubicin, indicating a complete conversion of the prodrug to its parent compound.

MATERIALS AND METHODS

Materials

Fetal calf serum (FCS) was obtained from Bocknek Laboratories (Canada). DMEM was obtained from Flow Laboratories (Irving, Scotland, UK). Daunorubicin-glucuronide (Dauno-glu) was donated by Dr. R.G.G. Leenders (University of Nijmegen, The Netherlands). F(ab')$_2$ fragments of 323/A3 were 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). Iminothiolane and succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB) were obtained from Pierce (Oud-Beijerland, The Netherlands). Egg-phosphatidylcholine (EPC), 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), trichloroacetic acid (TCA), dithiothreitol (DTT), and sulforhodamine-B (SRB) were obtained from Sigma Chemical Co. (St. Louis, USA). All other reagents were of analytical grade.

Preparation of Fab'-fragments

The mouse monoclonal antibody 323/A3 (IgG1 type) recognizes a Mr 43 kDa membrane glycoprotein which is expressed on a variety of carcinomas (e.g. 4). F(ab')$_2$ fragments of 323/A3 were incubated 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 (7). DTT was removed by applying the incubation mixture onto a Sephadex G-25M column (PD-10; Pharmacia, Woerden, The Netherlands). Elution occurred with acetate buffer pH 6.5 (100 mM sodium acetate, 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).

Enzyme Thiolation Using Iminothiolane

GUS was first purified by gel filtration on a Sephadex G-25M column (PD-10) with phosphate buffered saline (PBS containing 1 mM EDTA; pH 7.2). To introduce extra thiol groups, GUS was incubated with iminothiolane at a ratio of ±50 μg iminothiolane per mg GUS (i.e. about 100-fold molar excess of iminothiolane) in PBS (pH 7.2) containing 1 mM EDTA for 45 min at room temperature (4, 6). Iminothiolane and PBS were removed by applying the incubation mixture onto a Sephadex G-25M column. Pre-equilibration and elution occurred with acetate buffer pH 6.5. Modified GUS appearing in the void volume was used immediately for covalent attachment to freshly prepared liposomes (see below).

Enzyme Thiolation Using SATA

GUS was first purified by gel filtration on a Sephadex G-25M column (PD-10) with phosphate buffered saline (PBS containing 1 mM EDTA; pH 7.2). Then GUS was incubated with SATA (dissolved in dimethylformamide (DMF)) at a molar ratio of 1:8 (GUS:SATA) for 20 min at room temperature in nitrogen atmosphere (8). SATA, DMF and PBS were exchanged for acetate buffer pH 6.5 using gel filtration (Sephadex G-25M). Modified GUS (GUS-ATA) appearing in the void volume is stable for at least one month at 4°C. GUS-ATA can be used for covalent attachment to freshly prepared liposomes after deprotection of the SH-group by the addition of hydroxylamine (NH_2OH; see below).

Coupling of Fab' Fragments and Thiolated GUS to Liposomes

N-[4-(p-maleimidophenyl)butyryl]phosphatidylethanolamine (MPB-PE) was synthesized from SMPB and PE, purified and analyzed as described before (7). MPB-PE was incorporated into the liposomal bilayers to allow covalent coupling of thiolated GUS and Fab' fragments to the liposomal surface. The liposomes were composed of EPC:EPG:CHOL at a molar ratio of 10:1:4 or 10:3:4 with 2.5 mol% MPB-PE. A mixture of the appropriate amounts of lipids in chloroform was evaporated to dryness by rotary-evaporation under reduced pressure. After flushing with nitrogen for at least 20 minutes, the lipid film was hydrated in Hepes/NaCl buffer (20 mM Hepes, 149 mM NaCl, 1 mM EDTA, pH 7.4). The resulting liposome dispersion (10 μmol lipid (TL)/ml) was sequentially extruded through polycarbonate membrane filters with 0.6 μm and 0.2 μm pore size under nitrogen pressure, yielding a mean size of about 0.20–0.25 μm. After extrusion the Hepes/NaCl buffer outside the liposomes was replaced by acetate buffer pH 6.5 using ultracentrifugation (200,000 × g, 60 min). The freshly prepared liposomes were mixed with thiolated GUS and freshly prepared Fab' fragments (concentrations during incubation ranged from 4–6 μmol TL/ml, about 0.2–1 mg thiolated GUS/ml and/or about 0.3 mg Fab'/ml). When GUS thiolated with SATA (GUS-ATA) was used, 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 (100 μl hydroxylamine/ml incubation mixture). The coupling reaction was carried out overnight at 4°C under constant rotation in nitrogen atmosphere. Finally, the immunoliposomes were separated from unconjugated GUS-ATA and Fab' fragments by ultracentrifugal sedimentation at 200,000 × g during 30 minutes. The pellet was resuspended and washed twice with Hepes/NaCl buffer (20 mM Hepes, 149 mM NaCl, 1 mM EDTA, pH 7.4) or Hepes/glycerol buffer (20 mM Hepes, 261 mM glycerol, 1 mM EDTA, pH 7.4). Liposomes to which GUS alone is coupled are further referred to as enzymosomes. Liposomes to which both GUS and Fab' are coupled are referred to as immuno-enzymosomes. Liposome dispersions were stored at 4°C.

Liposome Characterization

Lipid phosphate was determined by the colorimetric method of Fiske and Subbarow (9). The enzyme activity was measured with p-nitrophenyl-β-D-glucuronide (10 mM in PBS/0.1% BSA). Samples (10 μl) were incubated with this substrate (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. The amount of protein coupled to the liposomes was determined by the method of Wessel and Flügge (10), with bovine serum albumin as standard. The total amount of monoclonal antibody and/or enzyme coupled to the liposomes was expressed as μg of protein/μmol of 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 GUS is coupled) with the protein density and enzymatic activity of immuno-enzymosomes. It was assumed that the presence of Fab' does not interfere with enzymatic activity. Mean particle size was determined by dynamic light scattering with a Malvern 4700 system using a 25 mW helium-neon laser and the Automeasure version 3.2 software (Malvern, Ltd., Malvern, UK). For viscosity and refractive index the values of pure water were used. As a measure of the particle size distribution of the dispersion the system reports a polydispersity index. This index ranges from 0.0 for an entirely monodisperse up to 1.0 for a polydisperse dispersion.

In Vitro Cytotoxicity

The human ovarian cancer cell line NIH:OVCAR-3 (11) 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 prodrug daunorubicin-glucuronide (dauno-glu) was donated by Leenders et al. (synthesis see 12). A suspension of OVCAR-3 cells in PBS was incubated with immuno-enzymosomes or PBS for 60 min at 4°C (10^7 cells/ml). Unbound liposomes were removed by centrifugation (5 min, 300 × g) and discarding the supernatant. The cell pellet was washed twice with PBS containing 0.1% BSA and resuspended in MEM (2×10^6 cells/ml). Then, 2×10^4 cells were seeded in a U-bottom 96-well plate. The prodrug was added at a final concentration between 0–20 μM dauno-glu. After incubation for 24 h, fresh medium (DMEM) was added and the cells were grown for another 72 h. The cytotoxic effect of dauno-glu was determined using the SRB-assay as described earlier (6). The cultures were fixed with 5% trichloroacetic acid (TCA) at 4°C for 1–2 h, washed with water and stained with 0.4% sulforhodamine B solution (SRB) dissolved in 1% acetic acid. After 15 min, the plates were washed with 1% acetic acid and air-dried. The bound dye was dissolved with 10 mM Tris and the optical density was measured at 490 nm using a Biorad microplate reader (Biorad Laboratories BV, Veenendaal, The Netherlands). The cytotoxic activity is expressed as the degree of cell proliferation relative to that of cells not incubated with dauno-glu.

RESULTS

Optimization of Coupling GUS Thiolated with Iminothiolane

GUS was thiolated using the thiolating agent iminothiolane and added to liposomes containing the anchor molecule MPB-PE, which enables the formation of a stable thio-ether linkage. Firstly, the same liposome composition as used earlier (EPC:EPG:CHOL at a molar ratio of 10:1:4) was chosen to prepare enzymosomes (6). As shown in Fig. 1A, dynamic light scattering analysis revealed aggregation of the liposomes at enzyme densities above about 10 μg GUS/μmol TL (i.e. when more than about 40–45 enzyme molecules are coupled per liposome). The aggregation process did not start during incubation of the MPB-PE liposomes with the thiolated enzyme, but after removal of unbound GUS by ultracentrifugation.

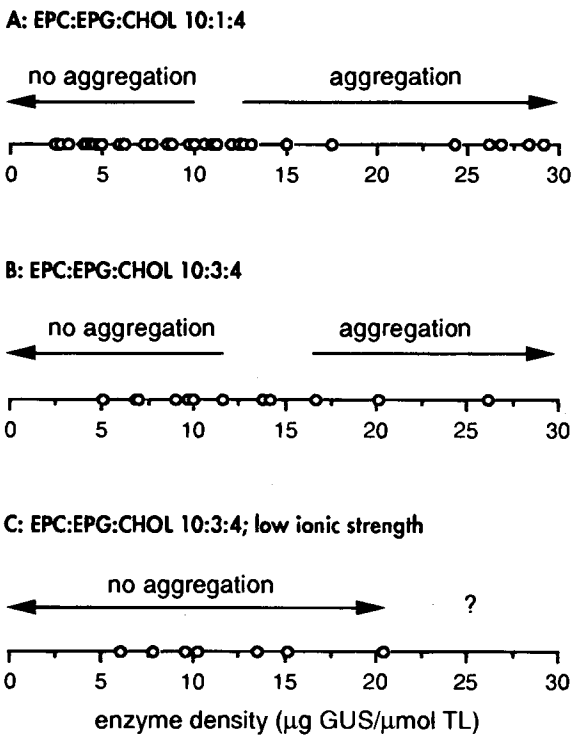


Fig. 1. Effect of liposome composition and ionic strength of the external aqueous medium on aggregation of enzymosomes prepared with iminothiolane-thiolated GUS. Enzymosomes were prepared by incubation of MPB-PE liposomes with GUS thiolated with iminothiolane. A and B: liposomes in Hepes/NaCl buffer, C: liposomes in Hepes/glycerol buffer, i.e. low ionic strength. The enzyme density and particle size were determined as described under Material and Methods. Aggregation was defined by a measured particle size >0.35 μm and polydispersity index >0.3. Each circle indicates a liposome dispersion.

Increasing the electrostatic repulsion between enzymosome particles was considered as a possible method to overcome the spontaneous aggregation of enzymosomes at enzyme densities ≥ 10 μg GUS/μmol TL. The negative charge of the liposomes was increased by incorporation of additional amounts of the negatively charged EPG (3-fold more) in the liposomal bilayers. As shown in Fig. 1B, these enzymosomes prepared (EPC:EPG:CHOL, 10:3:4) aggregated at enzyme densities higher than 12–15 μg GUS/μmol TL. Because of this slight improvement, this liposome composition was used in further experiments. To enhance the electrostatic repulsion effect even more, the ionic strength of the external aqueous medium in which enzymosomes were dispersed, was substantially reduced by replacing NaCl by glycerol (Hepes/glycerol buffer, i.e. buffer with reduced ionic strength). Using these conditions, we were able to increase the level of GUS-conjugation to more than 20 μg GUS/μmol TL (i.e. ≥80 enzyme molecules per liposome) without inducing liposome aggregation (Fig. 1C).

Optimization of Coupling GUS Thiolated with SATA

The disadvantage of using iminothiolane as thiolating agent was the poor reproducibility of the degree of GUS-coupling. It was not possible to predict the exact coupling efficiency of GUS to the liposomes. Therefore, another thiolating agent, SATA, was also tested. GUS was incubated with SATA (at a GUS:SATA molar ratio of 1:8), leading to GUS-ATA, in which thiol groups are protected with an acetyl group. GUS-ATA was incubated with the anchor-containing liposomes in the presence of hydroxylamine to deprotect the SH-group. Rather unexpectedly, much higher enzyme densities (≥33 μg GUS/μmol TL, i.e. over 125 GUS molecules per liposome) could be achieved without aggregation of the liposomes (Fig. 2B). It did not appear necessary to use buffer with low ionic strength to obtain these results.

Enzymatic Activity

The enzymatic activity of GUS was monitored during all steps involved in the enzymosome preparation. No influence of the thiolating agents on the enzymatic activity of GUS was observed. However, the enzymatic activity of thiolated GUS was reduced to about 30–40% of the original activity after coupling to the liposomes. The use of the low ionic strength buffer seemed to further reduce the enzymatic activity to less than 20%. In the initial experiments employing SATA as thiolating agent, the coupling reaction of GUS-ATA with the liposomes was stopped using N-ethylmaleimide (NEM). Upon addition of NEM, a complete loss of the enzymatic activity occurred. Therefore, in further experiments the use of NEM was avoided; the reaction was terminated by removal of the unbound enzyme by ultracentrifugation.

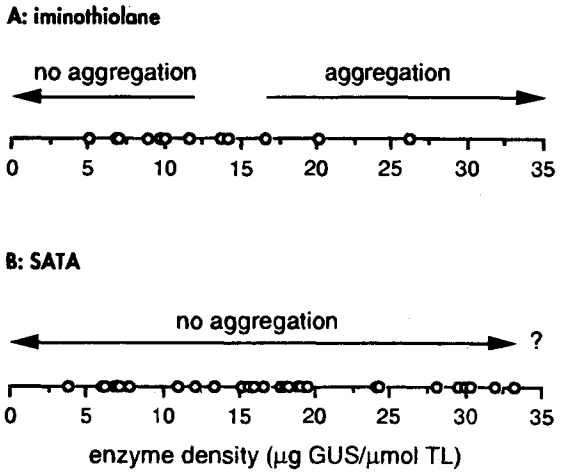


Fig. 2. Influence of the thiolation procedure on aggregation of enzymosomes. Enzymosomes (EPC:EPG:CHOL, 10:3:4 containing 2.5 mol% MPB-PE) were prepared by incubation of the liposomes with GUS, thiolated with iminothiolane (A) or SATA (B). Liposomes were stored in buffer with normal ionic strength (Hepes/NaCl buffer). The enzyme density and particle size were determined as described under Material and Methods. Aggregation was defined by a measured particle size >0.35 μm and polydispersity index >0.3. Each circle indicates a liposome dispersion.

In Vitro Cytotoxicity

The specific activation of the prodrug daunorubicin-glucuronide (dauno-glu) was examined by first incubating OVCAR-3 cells with immuno-enzymosomes, washing the cells to remove unbound immuno-enzymosomes and then exposing the cells to dauno-glu. Fig. 3 shows that the higher the enzyme density on the immuno-enzymosomes, the stronger the antitumor effects after addition of dauno-glu. Immuno-enzymosomes with a low average enzyme density (7 μg GUS/μmol TL; IC50 > 10 μM) were as ineffective as the prodrug alone (IC50 > 10 μM). Immuno-enzymosomes with a high average enzyme density (21 μg GUS/μmol TL; IC50 5 μM) were nearly as effective as completely converted prodrug achieved by incubating dauno-glu in combination with excess amounts of free GUS (IC50 2 μM). No difference in antitumor activity was observed between prodrug with excess GUS and corresponding molar concentrations of the parent drug daunorubicin HCl (not shown), indicating that all prodrug is converted by the enzyme into the daunorubicin parent drug.

DISCUSSION

ADEPT is a new experimental approach in cancer treatment aiming for the generation of cytotoxic molecules selectively in the close proximity of tumor cell membranes (1–4). Usually, it involves the administration of an antibody-enzyme conjugate, followed by the injection of a relatively nontoxic prodrug. Nearby the tumor cell membrane, the prodrug is converted into the active parent compound by the targeted enzyme. Previously we have presented a modification of this approach utilizing immunoliposomes as targeted carriers for the prodrug activating enzymes (referred to as 'immuno-enzymosomes'; 6). A theoretical advantage of the use of immuno-enzymosomes over the use of antibody-enzyme conjugates is that many more than one enzyme molecule can be coupled to one targeted

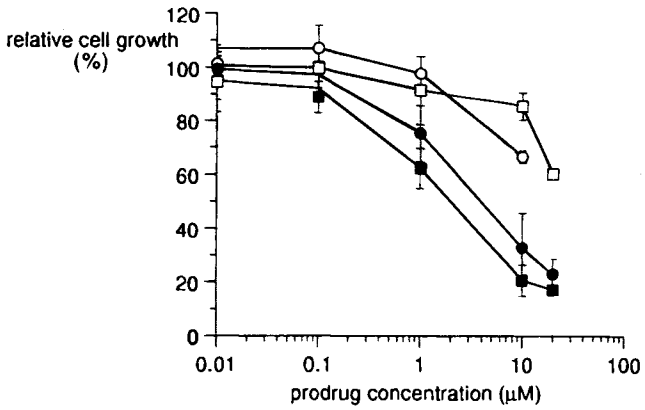


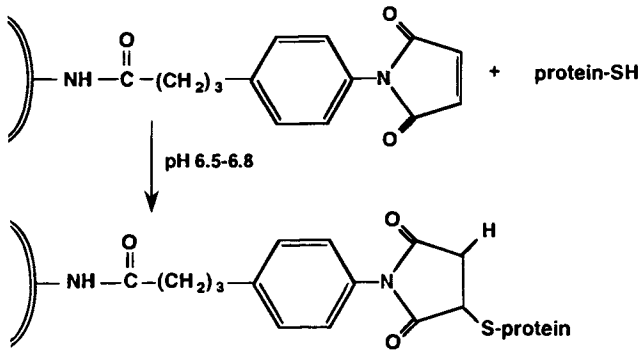
Fig. 3. In vitro antitumor activity of the prodrug dauno-glu to OVCAR-3 cells pretreated with immuno-enzymosomes or excess GUS. NIH:OVCAR-3 cells were pre-incubated with PBS (□,■) or with immuno-enzymosomes with a low density (○; about 7 μg GUS/μmol TL and 6 μg 323/A3/μmol TL) and a high enzyme density (●; about 21 μg GUS/μmol TL and 7 μg 323/A3/μmol TL) for 60 minutes at 4°C. To cells pretreated with PBS, excess of GUS was added (■). Cells were exposed to dauno-glu for 24 hours. Growth was measured with sulforhodamine B after another 72 hours. Relative cell growth is defined as cell growth relative to that of cells without dauno-glu (= 100%). The mean cell growth of 2–4 separate experiments is shown.

carrier unit. This creates the opportunity to increase the enzyme density at the tumor cell surface, and thereby to obtain more efficient conversion of the prodrug into the active drug (i.e. larger amounts of the active drug are formed), with consequently improved antitumor effect. In addition, immunoliposomes tend to interact with target cells through a multivalent binding principle resulting in a strong avidity for the target cell (13). Utilizing immuno-enzymosomes for ADEPT will be most useful when low antigen densities on the tumor cell surface restrict the effect of antibody-enzyme conjugates.

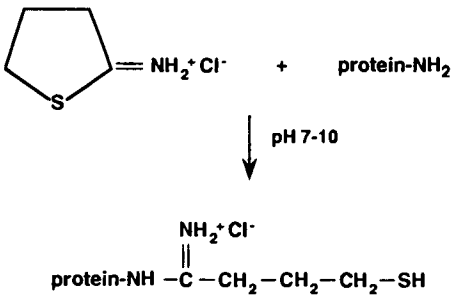
Earlier (6) we showed that the prodrug activating potential of immuno-enzymosomes was limited by their strong tendency to aggregate above enzyme densities of about 10 μg GUS per μmol TL, i.e., when more than 40 to 45 enzyme molecules are present on the outer surface of one immunoliposome particle. Here we show that it is possible to improve the immuno-enzymosome-mediated antitumor activity through enhancing enzyme surface density on the immuno-enzymosomes while avoiding the problem of liposome aggregation.

Many different techniques for the coupling of proteins to liposomes have been described (reviewed by e.g. 14). We have used one of the most popular conjugation methods involving the use of MPB-PE (Scheme 1). The sulfhydryl group of thiolated proteins reacts with the double bond of the maleimide moiety on the surface of the MPB-PE liposomes, and as a result a stable thio-ether linkage is formed. Incubation of liposomes with nonthiolated GUS, resulted in low enzyme densities (less than 1 μg GUS/μmol TL). Therefore, extra thiol groups were introduced in GUS to achieve a more efficient coupling process.

Initially we used iminothiolane for the introduction of additional SH-groups in GUS (Scheme 2). Enzymosomes prepared with iminothiolane-thiolated GUS (EPC:EPG:CHOL 10:1:4) were shown to aggregate at enzyme densities above 10–12 μg GUS/μmol TL (Fig. 1A). As a possible approach to overcome the spontaneous aggregation of the enzymosomes we considered the possibility of increasing the electrostatic repulsion between the enzymosome particles. Incorporation of additional amounts of the negatively charged phospholipid EPG (EPC:EPG:CHOL 10:3:4) allowed the coupling of somewhat higher amounts of enzyme molecules per particle (12–15 μg GUS/μmol TL, Fig. 1B). Upon lowering the ionic strength of the external aqueous medium and thereby increasing the negative zetapotential of the particle even further, it was possible



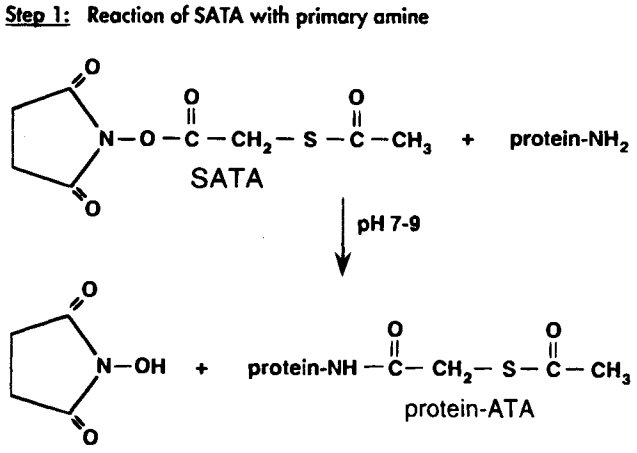
Scheme 1: Coupling of thiolated proteins to liposomes. Thiolated proteins are incubated with MPB-PE containing liposomes. The sulfhydryl group reacts with the double-bound of the maleimide moiety and as a result a stable thioether cross-linkage is formed.



Scheme 2: Thiolation of proteins with iminothiolane. Proteins are thiolated by incubation with iminothiolane. After reaction of iminothiolane with lysine side chain amine groups present in the protein, thiol groups are introduced.

to couple over 20 μg GUS/μmol TL without aggregation (Fig. 1C). Thus, using these conditions, we were able to obtain enhanced levels of GUS-conjugation to liposomes without occurrence of aggregation. It is noteworthy that when enzymosome-aggregation occurred, the aggregation process started after removal of unbound GUS, i.e. not in the incubation mixture of the liposomes with GUS. This spontaneous aggregation could be prevented by the addition of cystein, which 'caps' the nonreacted maleimide groups, at the end of the incubation period (not shown). This observation points to the involvement of cross-linking in the observed spontaneous aggregation of the particles, which, in addition, may have been promoted by the high shear stress developed during the ultracentrifugation process.

The coupling of GUS thiolated with iminothiolane to liposomes led to variable results in terms of reproducibility of the level of GUS-conjugation, therefore we also tested an alternative thiolating agent for proteins, SATA (Scheme 3; 8). An anticipated advantage of using SATA, is that the thiolated protein (with protected SH-groups, GUS-ATA), can be stored for long periods of time (at least one month) at 4°C, whereas protein thiolated with iminothiolane has to be used directly for coupling to the liposomes. Enzymosomes prepared with GUS-ATA did not show a strong aggregation tendency: high enzyme densities (over 30 μg GUS/μmol TL) were obtained without aggregation problems, even at physiological ionic strength (Fig. 2B). The exact reason why thiolation with SATA produces more favorable results than thiolation with iminothiolane is unclear at present. One explanation might be that thiolation with SATA introduces less extra SH-groups in GUS, thereby reducing the involvement of cross-linking in the aggregation process of enzymosomes. The most likely explanation, however, for the differences between iminothiolane and SATA in the observed aggregation relates to the involvement of electrostatic interactions. Thiolation with SATA results, in contrast to thiolation with iminothiolane, in the loss of one positive charge per thiolated amine group in GUS. This reduced positive charge of GUS may result in an increased negative zetapotential and therefore in a more pronounced electrostatic repulsion of the negatively charged enzymosomes. SATA-mediated thiolation of GUS-molecules solved the problem of spontaneous aggregation of liposomes after GUS-conjugation: immuno-enzymosomes with enzyme densities higher than 30 μg GUS/μmol TL can be prepared without aggregation. Hereby we eliminated one major impediment to the success of the immuno-enzymosome approach for ADEPT. Moreover immuno-enzymo-



Scheme 3: Thiolation of proteins with SATA. Proteins are thiolated by a two-step procedure using SATA. Step 1 involves the introduction of acetylated thiol groups after reaction of SATA with lysine side chain amine groups present in the protein. By the addition of hydroxylamine in step 2, the thiol groups are deacetylated.

somes prepared according to the SATA protocol do not noticeably aggregate during storage for at least 4 weeks at 4°C.

An important issue in the conjugation of enzymes to antibodies or liposomes, is preservation of the enzymatic activity. When NEM was used to terminate the coupling reaction of GUS-ATA with the MPB-PE liposomes, the enzymatic activity was completely lost. NEM binds to free SH-groups, and may have interacted with SH-groups present in the enzymatic active centre of GUS. In cases where the reaction was not stopped with NEM, it was observed that the enzymatic activity was reduced to about 30–40% as compared to the situation before coupling. Taking into account the intact enzymatic activity after thiolation, it seems that the covalent binding of GUS to the liposomes has a negative effect on the enzymatic activity. One explanation is that GUS, when attached to the liposomal surface, might have become less flexible in exposing the enzymatic active centre to the substrate. Alternatively, some SH-groups necessary for enzymatic activity might be involved in the coupling of GUS to the liposome.

The impetus for our attempts to increase the enzyme density on the immuno-enzymosomes comes from the expectation that more efficient prodrug activation at the tumor cell membranes could increase the killing of cancer cells. Earlier, we presented the concept of immuno-enzymosomes using the prodrug epirubicin-glucuronide (epi-glu). In this report we describe our findings utilizing another anthracycline prodrug, daunorubicin-glucuronide (dauno-glu), with a more favorable enzymatic hydrolysis rate than epi-glu (12, 15). The antitumor activity against human ovarian cancer cells (NIH:OVCAR-3 cells) induced by the combination of cell bound immuno-enzymosomes and the prodrug dauno-glu was studied in vitro. Our data indeed confirm that an increased enzyme density on the immuno-enzymosomes results in a corresponding enhancement of the antitumor effect (Fig. 3). Enzymosomes, which do not bind to the tumor cells and therefore are removed before addition of the prodrug, were not studied, as it was observed earlier (6) that enzymosomes were ineffective in prodrug conversion. The antiproliferative effect of dauno-glu against cells pre-exposed to immuno-enzymosomes (average enzyme density of 21 μg GUS/μmol TL), was similar to that of dauno-glu incubated with the cells when excess of GUS is present, or to corresponding molar concentrations of the parent drug daunorubicin itself. These results indicate that the cell bound immuno-enzymosomes were able to convert almost all prodrug added to its cytotoxic parent compound.

In summary, we have demonstrated that it is possible to substantially increase the enzyme density on immuno-enzymosomes while avoiding aggregation of the liposomes. It was confirmed in vitro that the higher the enzyme density, the better the prodrug activation capability of the immuno-enzymosomes. Comparative studies of the present optimized immuno-enzymosome system and the relevant antibody-enzyme conjugates regarding their selective prodrug activation properties in vitro and in vivo are in progress.

ACKNOWLEDGMENTS

We wish to thank Ir. P.H.J. Houba for assisting in the in vitro antitumor activity experiments, Dr. R.G.G. Leenders for the synthesis of the prodrug daunorubicin-glucuronide and Dr. R.M.J. Liskamp for critical reading the manuscript. The gifts of the monoclonal antibody 323/A3 by Prof. Dr. S.O. Warnaar (Centocor Europe BV) and phospholipids by Lipoid GmbH were greatly appreciated. This work was supported by the Dutch Cancer Society, project no. IKMN 90-17.

REFERENCES

1. P. D. Senter, P. M. Wallace, H. P. Svensson, V. M. Vrudhula, D. E. Kerr, I. Hellström and K. E. Hellström. Generation of cytotoxic agents by targeted enzymes. *Bioconj. Chem.* **4**:3–9 (1993).
2. M. P. Deonarain and A. A. Epenetos. Targeting enzymes for cancer therapy: old enzymes in new roles. *Br. J. Cancer* **70**:786–794 (1994).
3. K. D. Bagshawe, C. J. Springer, F. Searle, P. Antoniw, S. K. Sharma, R. G. Melton and R. F. Sherwood. A cytotoxic agent can be generated selectively at cancer sites. *Br. J. Cancer* **58**:700–703 (1988).
4. H. J. Haisma, E. Boven, M. van Muijen, J. de Jong, W. J. F. van der Vijgh and H. M. Pinedo. A monoclonal antibody-β-glucuronidase conjugate as activator of the prodrug epirubicin-glucuronide for specific treatment of cancer. *Br. J. Cancer* **66**:474–478 (1992).
5. K. D. Bagshawe, S. K. Sharma, C. J. Springer and P. Antoniw. Antibody directed enzyme prodrug therapy: a pilot-scale clinical trial. *Tumor Targeting* **1**:17–29 (1995).
6. M. H. Vingerhoeds, H. J. Haisma, M. van Muijen, R. B. J. van de Rijt, D. J. A. Crommelin and G. Storm. A new application for liposomes in cancer therapy: immunoliposomes bearing enzymes (immuno-enzymosomes) for site-specific activation of prodrugs. *FEBS Lett.* **336**:485–490 (1993).

7. 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 and G. Storm. Design of immunoliposomes directed against human ovarian carcinoma. *Biochim. Biophys. Acta* **1235**:126–139 (1995).
8. R. J. S. Duncan, P. D. Weston and R. Wrigglesworth. A new reagent which may be used to introduce sulfhydryl groups into proteins, and its use in the preparation of conjugates for immunoassay. *Anal. Biochem.* **132**:68–73 (1983).
9. C. H. Fiske and Y. Subbarow. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**:375–400 (1925).
10. D. Wessel and U. I. Flügge. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* **138**:141–143 (1984).
11. 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 and R. F. Ozols. Characterization of a human ovarian carcinoma cell line (NIH:OVCAR-3) with androgen and estrogen receptors. *Cancer Res.* **43**:5379–5389 (1983).
12. R. G. G. Leenders, K. A. A. Gerrits, R. Ruijtenbeek, H. W. Scheeren, H. J. Haisma and E. Boven. β-Glucuronyl carbamate based pro-moieties designed for prodrugs in ADEPT. *Tetrahedon Lett.* **36**:1701–1704 (1995).
13. J. L. M. Heeremans, R. Prevost, P. Los, C. Kluft and D. J. A. Crommelin. Fibrin binding glu-plasminogen coated liposomes in vitro. *Thromb. Haemostas:* **75:**1 134–139 (1996).
14. J. Connor, S. Sullivan and L. Huang. Monoclonal antibody and liposomes. *Pharmacol. Ther.* **28**:341–365 (1985).
15. H. J. Haisma, M. van Muijen, H. M. Pinedo, and E. Boven. Comparison of two anthracycline-based prodrugs for activation by a monoclonal antibody-β-glucuronidase conjugate in the specific treatment of cancer. *Cell Biophys.* **24/25**:185–192 (1994).