Volume 336, number 3, 485–490 FEBS 13466 December 1993
© 1993 Federation of European Biochemical Societies 00145793/93/$6.00

A new application for liposomes in cancer therapy

Immunoliposomes bearing enzymes (immuno-enzymosomes) for site-specific activation of prodrugs

M.H. Vingerhoeds[a,*], H.J. Haisma[b], M. van Muijen[b], R.B.J. van de Rijt[a], D.J.A. Crommelin[a], G. Storm[a]

[a]*Department of Pharmaceutics, Faculty of Pharmacy, Utrecht University, PO Box 80.082, 3508 TB Utrecht, The Netherlands*
[b]*Department of Medical Oncology, Free University Hospital, Amsterdam, The Netherlands*

Received 15 November 1993

We have tested a new type of immunoliposomes which may effectively mediate the targeting of enzymes to be used for site-specific prodrug activation (immuno-enzymosomes). The enzyme β-glucuronidase, capable of activating the prodrug epirubicin-glucuronide (epi-glu), was coupled to the external surface of immunoliposomes directed towards ovarian cancer cells. A significant increase in cytotoxicity of the prodrug epi-glu was shown when the in vitro cultured cancer cells were pretreated with these immuno-enzymosomes.

Monoclonal antibody; Liposome; Antibody-directed enzyme prodrug therapy; ADEPT; Targeted drug delivery

1. INTRODUCTION

The use of current antitumor drugs is strongly limited by the lack of selectivity for cancer cells. During the last decade immunoliposomes (antibody-directed liposomes) have been studied to establish their potential in cancer therapy as a tool to achieve efficient site- specific drug delivery [1–7]. Despite progress made in targeting drug-containing immunoliposomes to cancer cells in vitro and in vivo, considerable problems still remain with respect to antitumor efficacy. In order to deliver the encapsulated drug effectively to the target cell the drug must remain within the vesicle during the transport to the target site. It has to exert its action after binding of the immunoliposomes to the receptors on the target cell surface. However, even though immunoliposomes may be tightly bound to the target cell surface, this is not a guarantee for intracellular delivery of the liposome contents. In principle, two pathways for drug entry into the target cell can be envisaged. Firstly, uptake of the immunoliposomes by the cells or fusion with the cells, followed by intracellular release of the drug may occur [8,9]. Unfortunately, however, many tumor cells are not capable of internalizing liposomes efficiently. Secondly, release of the encapsulated drug in the close proximity of the target cells may result in a considerable cellular drug uptake. In this case it is necessary that the released drug is rapidly taken up by the tumor cells, which depends on its physicochemical nature (e.g. lipophilicity). Methods to construct immunoliposomes to release entrapped agents through environmental manipulations (e.g. in response to changes in pH or temperature) are being investigated [10–12].

Another approach to generate sufficient and site-specific antitumor activity concerns enzymes, capable of locally converting relatively non-toxic prodrugs into active cytotoxic agents. These enzymes can be coupled to the external surface of immunoliposomes directed towards cancer cells. After binding of these immuno-enzymosomes to the target cells, a prodrug is administered and the active drug is generated in the close proximity of the tumor cell (Fig. 1). The use of enzymes for selective prodrug activation at the tumor site has been described earlier as antibody-directed enzyme prodrug therapy (ADEPT). In this approach, enzyme is linked to an antibody that binds to an antigen preferentially expressed on tumor cells. The few studies carried out with antibody-enzyme conjugates in in vitro and in vivo models showed that selective conversion of prodrug into active drug at the tumor site can be obtained [13–16].

In this report, we describe the coupling of the enzyme β-glucuronidase (GUS) to the external surface of immunoliposomes, specifically directed against human ovarian carcinoma cells. These immuno-enzymosomes were

*Corresponding author.

Abbreviations ADEPT, antibody-directed enzyme prodrug therapy; CHOL, cholesterol; DMEM, Dulbecco's Modified Eagle's Medium; DTT, dithiothreitol; Epi-glu, epirubicin-glucuronide; GUS, β-glucuronidase; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; MEM, minimal essential medium; MPB-PE, *N*-[4-(*p*-maleimidophenyl)butyryl] phosphatidyl ethanolamine; PBS, phosphate-buffered saline; PC, egg L-α-phosphatidylcholine; PG, egg-phosphatidylglycerol; SRB, sulforhodamine-B; TCA, trichloroacetic acid; TL, total lipid (phospholipid + cholesterol).

tested for their enzymatic activity, stability and capability to bind to the tumor cells. Recently, it has been reported that the relatively non-toxic glucuronide prodrug epirubicin-glucuronide (epi-glu) can be converted to the parent drug epirubicin by GUS [15]. We demonstrate that epi-glu can preferentially kill human ovarian cancer cells that were previously exposed to the immuno-enzymosomes.

2. MATERIALS AND METHODS

2.1. *Materials*

DMEM and fetal calf serum were obtained by Flow Laboratories (Irving, Scotland, UK). $F(ab')_2$ fragments of OV-TL3 were donated by Centocor Europe BV (Leiden, The Netherlands). GUS from *E. coli* K12 was purchased from Boehringer (Mannheim, Germany). Iminothiolane was purchased from Pierce (Oud-Beijerland, The Netherlands). EggPC and eggPG were a gift from Lipoid KG (Ludwigshafen, Germany) and Nattermann GmbH (Cologne, Germany), respectively. Cholesterol, SRB, DTT, TCA and *N*-ethylmaleimide were obtained from Sigma Chemical Co. (St. Louis, USA).

2.2. *Cancer cell line and cell culture*

The human ovarian cancer cell lines NIH:OVCAR-3 [17] and A2780 [18] were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml).

2.3. *Preparation of Fab'-fragments*

The monoclonal antibody OV-TL3 is directed against human ovarian carcinoma [19]. $F(ab')_2$ fragments of OV-TL3 were incubated with 20 mM DTT in acetate buffer at pH 5.5 (100 mM sodium acetate, 63 mM sodium chloride, 1 mM EDTA) for at least 90 min at room temperature [2]. 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 sodium chloride, 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 MPB-PE liposomes (see section 2.4).

2.4. *Preparation of immunoliposomes*

N-[4-(*p*-Maleimidophenyl)butyryl]phosphatidylethan olamine (MPB-PE) was synthesized, purified and analyzed as described before [2,20]. MPB-PE was incorporated into the liposomal bilayers to allow covalent coupling of Fab' fragments to the liposomal surface. The composition of the bilayer of the liposomes used was eggPC:eggPG:CHOL:MPB-PE at a molar ratio of 38.5:4:16:1.5. A mixture of the appropriate amounts of lipids in chloroform was evaporated to dryness by rotary-evaporation at 35°C under reduced pressure. After flushing with nitrogen for at least 20 min, the lipid film was hydrated in HEPES buffer (20 mM HEPES, 149 mM NaCl, 1 mM EDTA, pH 7.4). The resulting liposome dispersion was sequentially extruded through polycarbonate membrane filters with 0.6 μm and 0.2 μm pore size (Unipore, Biorad, Richmond, CA) under nitrogen pressures up to 0.8 MPa. After extrusion the HEPES buffer outside the liposomes was replaced by acetate buffer, pH 6.5, using ultracentrifugation (100,000 × *g*; 45 min). The freshly prepared liposomes were mixed with freshly prepared Fab' fragments (concentrations during incubation ranged from 6–12 μmol total lipid (TL)/ml and 0.25–0.35 mg Fab'/ml, respectively). The coupling reaction was carried out overnight at 4°C under constant rotation in nitrogen atmosphere. Finally, the immunoliposomes were separated from unconjugated Fab' fragments by ultracentrifugal sedimentation at 100,000 × *g* during 30 min. The pellet was resuspended and washed twice with HEPES buffer. MPB-PE-containing liposomes not incubated with Fab' fragments are referred to as MPB-PE liposomes. Liposome dispersions were stored at 4°C.

2.5. *Enzyme thiolation*

GUS was first purified by gel filtration on a Sephadex G-25M column (PD-10) with phosphate buffered saline (PBS). To introduce extra thiol groups, GUS was incubated with iminothiolane at a ratio of ± 50 μg iminothiolane per mg GUS in PBS (pH 7.2) containing 1 mM EDTA for 45 min at room temperature [15]. This results in the addition of, on the average, approximately 4 thiol groups per GUS molecule without a significant effect on the enzyme activity. 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 MPB-PE liposomes.

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 stopped by the addition of 50 μl of 1 M glycine (pH 10.6) and absorbance was read at 405 nm.

2.6. *Coupling of thiolated GUS to liposomes*

The coupling procedure of GUS to MPB-PE liposomes is similar to the coupling procedure of Fab' to liposomes. The freshly prepared MPB-PE liposomes were mixed with freshly thiolated GUS (concentrations during incubation ranged from 6–12 μmol TL/ml and 0.25–0.35 mg GUS/ml, respectively, resulting in an incubation ratio of about 30 μg GUS/μmol TL). The coupling reaction and separation of unconjugated thiolated GUS was carried out as described under section 2.4. GUS-liposomes are further referred to as enzymosomes. Liposomes to which both enzyme and Fab' are coupled are referred to as immuno-enzymosomes. For the preparation of the immuno-enzymosomes, MPB-PE liposomes were incubated with Fab' fragments and GUS present in the same incubation mixture under the conditions mentioned in section 2.4.

2.7. *Liposome characterization*

Lipid phosphate was determined by the colorimetric method of Fiske and Subbarow [21]. The enzyme activity was measured with *p*-nitrophenyl-β-D-glucuronide as described above in section 2.5. 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 of protein per μmol of TL. The amount of enzyme protein coupled to immuno-enzymosomes was estimated by comparison with enzymosomes, to which only enzyme is coupled.

Mean particle size was determined by dynamic light scattering with a Malvern 4700 system using a 25 mW helium-neon laser and the AUTOMEASURE vsn. 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 completely polydisperse dispersion.

2.8. *In vitro cell binding*

Specific cell binding of immuno-enzymosomes in vitro was studied with OVCAR-3 cells. The cell line A2780 served as a control cell group. Cells were harvested with PBS/EDTA (0.02%), washed and diluted with PBS to a suspension containing 1×10^7 cells/ml. Immuno-enzymosomes, enzymosomes and immunoliposomes were diluted to a lipid concentration of about 1 μmol/ml. The various liposome types were incubated with an equal volume of the cell suspension in PBS during 1 h at room temperature. Unbound liposomes were separated from the cells by centrifugation (300 × *g*, 3 min). The cell pellet was washed twice with 4 ml of PBS containing 1% BSA. The degree of cell binding was assessed by measuring the enzyme activity in the redispersed cell pellet.

2.9. *In vitro cytotoxicity*

The prodrug epi-glu was isolated from urine from patients treated with epirubicin as described before [15]. The in vitro cytotoxicity of epi-glu was determined with and without pretreatment of the tumor cells with the various types of liposomes. A suspension of OVCAR-3 cells in PBS was incubated with immuno-enzymosomes, enzymosomes, immunoliposomes or PBS for 60 min at 4°C (10^7 cells/ml; 5–10 μmol TL/ml). Unbound liposomes were removed by centrifugation (5 min, 300 × *g*). The cell pellet was washed twice with PBS 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 to provide a final concentration of 10 μM. After incubation for 24 h fresh medium (DMEM) was added and the cells were grown for another 72 h. The cytotoxic effects of epi-glu were determined using the SRB-assay [23]. The cultures were fixed with 5% ice-cold trichloric acetic acid (TCA) at 4°C for 1–2 h, washed with water and stained with 0.4% sulforhodamine-B solution (SRB). Fifteen minutes later, 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 540 nm using a Titertek multiscan (Flow laboratories, Helsinki, Finland).

3. RESULTS AND DISCUSSION

Fig. 1 illustrates the strategy of converting a prodrug to the active drug by cell-bound immuno-enzymosomes. The impetus for examining this concept comes from our observations that efficient binding of cytostatic-containing immunoliposomes to ovarian carcinoma cells in vivo is not inducing a corresponding increase in antitumor response [2,3]. We have addressed three basic questions. (1) Is it possible to couple GUS to the outer surface of immunoliposomes with preservation of its enzymatic activity? (2) Does the presence of GUS (MW 280 kDa) on the outer surface of the immunoliposomes prevent the Fab′ (MW 50 kDa) -target cell interaction? (3) Are tumor cell-associated immuno-enzymosomes able to activate the glucuronide prodrug epi-glu?

Immuno-enzymosomes were prepared by coupling GUS and Fab′-fragments of the monoclonal antibody OV-TL3 in one incubation step to liposomes containing the anchor molecule MPB-PE. The maleimide group of this anchor molecule reacts with a thiol group, forming a stable thio-ether bond [20]. Incubation of non-modified GUS with MPB-PE liposomes resulted in a low coupling efficiency (results not shown). Therefore, before coupling of GUS to the liposomes, extra SH-groups were introduced in the molecule by incubation with iminothiolane. Results on the coupling of Fab′ and GUS to MPB-PE liposomes are presented in Table I. Fab′ (immunoliposomes: 13.0 ± 2.5 μg/μmol TL; $n = 5$) was coupled to a higher extent to the MPB-PE liposomes than GUS (enzymosomes: 4.7 ± 1.4 μg/μmol TL; $n = 5$). The respective coupling ratios were not significantly different when both proteins were coupled to the liposomes simultaneously (immuno-enzymosomes: Fab′ 13.5 ± 4.1 μg/μmol TL and GUS 4.0 ± 1.6 μg/μmol TL; $n = 4$). It was estimated that around 400 Fab′ and 20 GUS molecules were present on one immuno-enzymosome particle. The coupling procedure had only a minor effect on the enzyme activity (results not shown). On the day of preparation, the mean diameter was found to be about 0.29 μm and 0.30 μm with acceptable polydispersity indices for enzymosomes and immuno-enzymosomes, respectively. The mean particle size and enzyme activity did not change significantly during storage at 4°C for a period of 4 weeks (results not shown). A sharp increase in particle size and polydispersity index, however, was observed when the GUS coupling ratio of the (immuno)enzymosomes exceeded 10 μg/μmol TL. This implies that not more than about 50 GUS molecules can be covalently linked to the liposomes without occurrence of aggregation. Therefore, the cell binding and cytotoxicity experiments were performed with liposomes with a GUS coupling ratio lower than 10 μg GUS/μmol TL.

The potential problem of GUS-induced hindrance of Fab′-mediated target cell association was studied by measuring the binding of the immuno-enzymosomes to human ovarian cancer cells in vitro. The liposomes were incubated with the cells in suspension during 1 h at room temperature. After removal of the unbound liposomes, the enzyme activity was determined. This method does not allow to measure the degree of cell

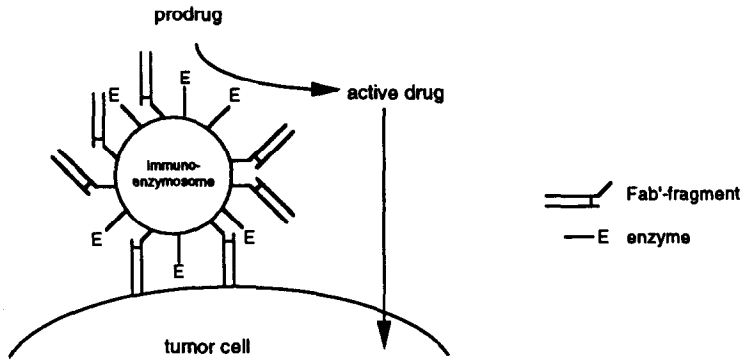


Fig. 1. Schematic representation of the concept of antibody-directed enzyme prodrug therapy with immuno-enzymosomes. The immuno-enzymosomes are first allowed to bind to the target cells. Then a prodrug is given, which is activated by the immuno-enzymosomes in close proximity of the target cell. The active drug can subsequently kill the cell.

binding of immunoliposomes as they do not contain enzymes. As shown in Fig. 2, the immuno-enzymosomes showed a higher binding affinity for OVCAR-3 cells than for A2780 cells which lack the antigen for OV-TL3 ($P < 0.05$). Enzymosomes showed only negligible binding to both OVCAR-3 and A2780 cells. These results illustrate the importance of the presence of the specific antibody on the outside of the liposomes for obtaining specific cell binding and indicate that the target binding capability of the Fab'-fragment is preserved upon coupling to the immuno-enzymosomes. This is not surprising as we estimate that around 400 Fab' and only 20 GUS molecules are present on the external surface of one immuno-enzymosome particle. Thus, even if the GUS molecules (MW 280 kDa) would hamper the target recognition of the neighbouring, much smaller, Fab'-residues (MW 50 kDa), more than enough Fab'-fragments should be available for binding to the target cells.

The specific activation of the prodrug epi-glu at the target cells was investigated by first incubating OVCAR-3 cells with the various liposome types for 1 h, washing the cells to remove unbound liposomes, and then exposing the cells to prodrug (final concentration 10 μM). The presence of cell-associated liposomes did not inhibit the formation of a cell monolayer. As shown in Fig. 3, only pre-incubation of the target cells with immuno-enzymosomes resulted in an enhancement of the cytotoxicity of the added prodrug (epi-glu). Pretreatment with enzymosomes or immunoliposomes was clearly ineffective. This demonstrates the ability of the cell-bound immuno-enzymosomes to convert the prodrug to an agent with increased antineoplastic activity. These findings point to a potential usefulness of immuno-enzymosomes in prodrug activation therapy.

Several enzymes conjugated to monoclonal antibodies are currently being evaluated for selective activation of prodrugs at the tumor site. A theoretical advantage

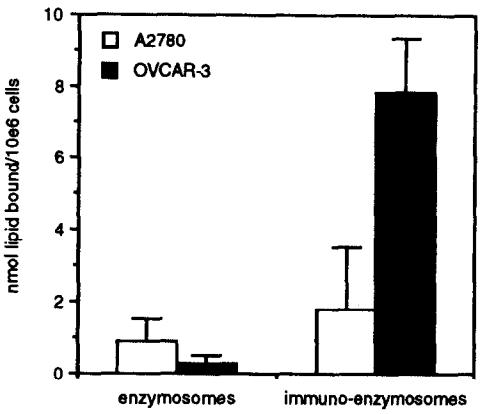


Fig. 2. In vitro cell binding of enzymosomes and immuno-enzymosomes. NIH:OVCAR-3 cells (target cells; grey bars) or A2780 cells (control cells; white bars) were incubated with enzymosomes and immuno-enzymosomes for 60 min at room temperature. After washing the cells with PBS containing 1% BSA to remove unbound liposomes, the enzymatic activity was determined. The cell binding is expressed as the absolute amount of (immuno)enzymosomes bound/10^6 cells.

of the present immuno-enzymosome approach, as compared to the use of antibody-enzyme conjugates, is that much more than one enzyme molecule can be delivered to the tumor site by a single targeted liposome. This offers the possibility to increase the enzyme density at the target cell surface, and thereby to induce a higher efficiency of specific prodrug activation. To test this hypothesis, OV-TL3-enzyme conjugates were prepared as described previously [15]. The degree of cell binding and prodrug activation of the antibody-enzyme conjugates appeared to be similar to that of the immuno-enzymosomes (results not shown). It should be realized, however, that the prodrug activation potential of immuno-enzymosomes still can be considerably improved by increasing the amount of coupled enzyme per liposome. However, one major recognized limitation of the immuno-enzymosome formulation used in the pres-

Table I

Characterization of liposome preparations

Characteristics	MPB-PE liposomes	Enzymosomes	Immuno-lipozomes	Immuno-enzymosomes
Coupling ratio Fab' (μg Fab'/μmol TL)[a]	–	–	13.0 ± 2.5	13.5 ± 4.1
Coupling ratio GUS (μg GUS/μmol TL)[a]	–	4.7 ± 1.4	–	4.0 ± 1.6
Estimated number of Fab' molecules per liposome[b]		–	322 ± 82	439 ± 127
Estimated number of GUS molecules per liposome		26 ± 8	–	23 ± 10
Mean particle diameter (μm)	0.22 ± 0.01	0.29 ± 0.02	0.25 ± 0.01	0.30 ± 0.01
Polydispersity index	0.14 ± 0.01	0.25 ± 0.05	0.17 ± 0.03	0.25 ± 0.03

Mean values ± standard deviation; the number of dispersions was at least 4 and in most cases 5. Liposomes were prepared and characterized according to the method described in section 2.

[a]The coupling ratio (μg Fab'/μmol TL and μg GUS/μmol TL) was determined using a protein determination assay, an enzyme-activity assay and a phosphate determination method as described in section 2.7.

[b]The number of Fab' and GUS molecules coupled per liposome was estimated using the following assumptions: molecular weights 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 [30]; a surface area of 1×10^{11} μm^2/μmol TL.

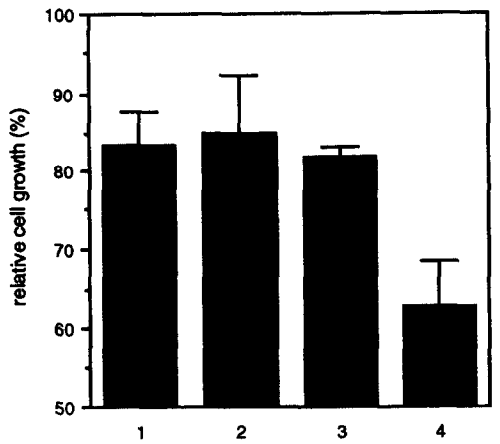


Fig. 3. In vitro antitumor activity of the prodrug epi-glu to OVCAR-3 cells pretreated with liposomes. NIH:OVCAR-3 cells were pre-incubated with (1) PBS, (2) immunoliposomes, (3) enzymosomes and (4) immuno-enzymosomes for 60 min at 4°C. Cells were exposed to epi-glu (10 μM) for 24 h. Growth was measured with sulforhodamine-B after another 72 h. Relative cell growth is defined as cell growth relative to that of untreated cells (=100%). The mean cell growth of three separate experiments is shown.

ent study is their tendency to aggregate above a coupling ratio of about 10 μg GUS/μmol TL. Currently, we attempt to optimize the immuno-enzymosome-mediated antitumor activity through enhancing enzyme surface density while avoiding the problem of liposome aggregation. Another point to be taken into account is that in this study over 400 Fab′ fragments and only about 20 GUS molecules were coupled to the liposomes. Less Fab′ fragments may already be sufficient for efficient cell binding. Therefore, attempts are presently being made to increase the amount of enzyme and decrease the amount of Fab′ coupled on the surface of the immuno-enzymosomes.

The enzyme β-glucuronidase occurs in both prokaryotic and eukaryotic organisms. In mammalian tissue the enzyme is present in lysosomes and microsomes and blood levels are low [24,25]. To investigate the immuno-enzymosome concept we used in this study GUS from *E. coli.* However, to prevent potential problems with the immunogenicity of bacterial GUS, in patients the human GUS enzyme should be used. It is generally accepted that the liposomal carrier in itself is not causing serious immunogenic adverse reactions [26,27]. However, the association of a protein to the outside of the liposomes may induce an immune response. When immunoliposomes are administered only once, the immune response is not likely to affect the targeting efficiency because the targeting process will generally be faster than the establishment of an immune response [28]. However, in many therapeutic applications a multiple injection schedule is needed. Immunogenic adverse reactions might by diminished by the administration of immunosuppressors [29]. In addition, the use of human or humanized monoclonal antibodies might reduce the risk for an immunogenic response. Also, it cannot be excluded that the combination of the enzyme and the monoclonal antibody on a liposome results in an even more increased immunogenicity.

In summary, we have introduced and tested a new type of immunoliposomes which can effectively mediate the targeting of enzymes to be used for specific prodrug activation. Further studies will include the validation of the concept in tumor-bearing animals and will mainly focus on comparing the merit of immuno-enzymosomes with that of antibody–enzyme conjugates as well as of drug-containing immunoliposomes.

REFERENCES

[1] Nässander, U.K., Storm, G., Steerenberg, P.A., De Jong, W.H., de Groot, G., Poels, L.G., van Hoesel, Q.G.C.M. and Crommelin, D.J.A. (1990) in: From Clone to Clinic (D.J.A. Crommelin and H. Schellekens eds.) Developments in Biotherapy, vol. 1, pp. 357–365, Kluwer Academic Publishers, Dordrecht/Boston/London.
[2] Nässander, U.K. et al. (1991) Liposomes, Immunoliposomes and Ovarian Carcinoma, Ph.D. Thesis, Utrecht.
[3] Nässander, U.K., Steerenberg, P.A., Poppe, H., Storm, G., Poels, L.G., De Jong, W.H. and Crommelin, D.J.A. (1992) Cancer Res. 52, 646–653.
[4] Peeters, P.A.M., Storm, G. and Crommelin, D.J.A. (1987) Adv. Drug Del. Rev. 1, 249–266.
[5] Storm, G., Peeters, P.A.M., Nässander, U.K. and Crommelin, D.J.A. (1990) in: Trends in drug research (V. Claasen ed.) Pharmacochemistry Library, vol. 13, pp. 333–349, Elsevier, Amsterdam/Oxford/New York/Tokyo.
[6] Straubinger, R.M., Lopez, N.G., Debs, R.J., Hong, K. and Papahadjopoulos, D. (1988) Cancer Res. 48, 5237–5245.
[7] Wright, S. and Huang, L. (1989) Adv. Drug Del. Rev. 3, 343–389.
[8] Connor, J. and Huang, L. (1986) Cancer Res. 46, 3431–3435.
[9] Litzinger, D.C. and Huang, L. (1992) Biochim. Biophys. Acta 1113, 201–227.
[10] Ho, R.J.Y., Rouse, B.T. and Huang, L. (1986) Biochemistry 25, 5500–5506.
[11] Magin, R.L. and Niesman, M.R. (1984) Cancer Drug Deliv. 1, 109–117.
[12] Merlin, J. (1991) Eur. J. Cancer 27 (8), 1026–1030.
[13] Bagshawe, K.D., Springer, C.J., Searle, F., Antoniw, P., Sharma, S.K., Melton, R.G. and Sherwood, R.F. (1988) Br. J. Cancer 58, 700–703.
[14] Senter, P.D., Saulnier, M.G., Schreiber, G.J., Hirschberg, D.L., Brown, J.P., Hellström, I. and Hellström, K.E. (1988) Proc. Natl. Acad. Sci. USA 85, 4842–4846.
[15] Haisma, H.J., Boven, E., van Muijen, M., de Jong, J., van der Vijgh, W.J.F. and Pinedo, H.M. (1992) Br. J. Cancer 66, 474–478.
[16] Wang, S., Chern, J., Yeh, M., Ng, J.C., Tung, E. and Roffler, S.R. (1992) Cancer Res. 52, 4484–4491.
[17] Hamilton, T.C., Young, R.C., McKoy, W.M., Grotzinger, K.R., Green, J.A., Chu, E.W., Whang-Penn, J., Rogan, A.M., Green, W.R. and Ozols, R.F. (1983) Cancer Res. 43, 5379–5389.
[18] Eva, A., Robbins, K.C., Andersen, D.R., Scinivasan, A., Tronick, S.R., Reddy, E.P., Ellmore, N.W., Galen, A.T., Lautenberger, J.A., Papas, T.S., Westin, E.H., Wong-staal, F., Gallo, R.C. and Aaronson, S.A. (1982) Nature 295, 116–118.
[19] Poels, L.G., Peters, D., Van Megen, Y., Vooijs, G.P., Verheyen, R.N.M., Willemen, A., Van Niekerk, C.C., Jap, P.H.K., Mungyer, G. and Kenemans, P. (1986) J. Natl. Cancer Inst. 76, 781–791.

[20] Martin, F.J. and Papahadjopoulos, D. (1982) J. Biol. Chem. 257, 286–288.
[21] Fiske, C.H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375–400.
[22] Wessel, D. and Flügge, U.I. (1984) Anal. Biochem. 138, 141–143.
[23] Skehan, P., Storeng, R., Scudiero, D.A., Monks, A., McMahon, J., Vistica, D., Warren, J.T., Bokesh, H., Kenney, S. and Boyd, M.R. (1990) J. Natl. Cancer Inst. 82(13), 1107–1112.
[24] Dutton, G.J. (1966) Glucuronic Acid Free and Combined, Academic Press, New York/London.
[25] Fishman, W.H. (1970) Metabolic Conjugation and Metabolic Hydrolysis, Academic Press, New York/London.
[26] Alving, C.R. (1993) Biochim. Biophys. Acta 1113, 307–322.
[27] Storm, G., Oussoren, C., Peeters, P.A.M. and Barenholz, Y. (1993) in: Liposome Technology, vol. III, 2nd edn. (G. Gregoriadis ed.) pp. 345–383, CRC Press, Boca Raton.
[28] Maruyama, K., Holmberg, E., Kennel, S.J., Klibanov, A., Torchilin, V.P. and Huang, L. (1990) J. Pharm. Sci. 79, 978–984.
[29] Ledermann, L.A., Begent, R.H.J., Bagshawe, K.D., Riggs, S.J., Searle, F., Glaser, M.G., Green, A.J. and Dale, R.G. (1988) Br. J. Cancer 58, 654–657.
[30] Jousma, H., Talsma, H., Spies, F., Joosten, J.G.H., Junginger, H.E. and Crommelin, D.J.A. (1987) Int. J. Pharm. 35, 263–274.