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Preparation of immunoliposomes bearing poly(ethylene glycol)-coupled monoclonal antibody linked via a cleavable disulfide bond for ex vivo applications

M. Mercadal [a], J.C. Domingo [a,*], J. Petriz [b], J. Garcia [b], M.A. de Madariaga [a]

[a] *Department of Biochemistry and Molecular Biology, Faculty of Chemistry, University of Barcelona, Martí i Franqués, 1, 08028 Barcelona, Spain*

[b] *Department of Cryobiology and Cellular Therapy, Oncologic Research Institute, Autovía de Castelldefells, Km. 2,7, 08907-L'Hospitalet Barcelona, Spain*

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Abstract

Several methods for the preparation of sterically stabilized immunoliposomes (SIL) have recently been described. This report examines an established method for coupling anti-CD34 My10 mAb to poly(ethylene glycol)-liposomes (PEG-liposomes) containing the anchor pyridyldithiopropionylamino-PEG-phosphatidylethanolamine (PDP-PEG-PE) via a cleavable disulfide bond. Efficient attachment of pyridyldithio-derivatized mAb took place (equivalent to coupling ca. 70% of total input protein) at 2 mol percent of the functionalized PEG-lipid. The My10-SIL bound specifically to CD34+ cells (human leukemic KG-1a and hematopoietic progenitor cells) and the extent of binding was a function of liposomal lipid concentration, the mAb density in the liposome surface and the CD34 cell expression. In mixtures with CD34- cells (CHO or Jurkat), CD34+KG-1a cells were determined by flow cytometry at percentages (1–4%) similar to those reported in clinical samples (such as cord blood, mobilized peripheral blood and bone marrow) using a direct immunostaining with My10-SIL. The disulfide bond was stable in cell culture medium (10% of fetal calf serum) during 8 h and cell-bound SIL can be released from cells by treatment with dithiothreitol as reducing agent under mild conditions (1 h of incubation with 50 mM DTT at 20°C). SIL binding and subsequent dithiothreitol treatment did not influence the cell viability. Our approach should contribute to the development of targetable liposomal vehicles to CD34+ cells for use in ex vivo conditions as sorting of hematopoietic stem cells.

Keywords: Sterically stabilized immunoliposome; Poly(ethylene glycol); Disulfide bond; CD34 antigen; Stem cell

1. Introduction

The description of sterically stabilized liposomes (SL) containing lipid derivatives of poly(ethylene glycol) (PEG) has meant increased opportunities for specific, ligand-mediated, targeting of liposomes to

Abbreviations: mAb, monoclonal antibody; SIL, sterically stabilized immunoliposome; SL, sterically stabilized liposome; PEG, poly(ethylene glycol); mPEG-PE, methoxy poly(ethylene glycol)-phosphatidylethanolamine; PDP-PEG-PE, pyridyldithiopropionylamino-poly(ethylene glycol)-phospahtidylethanolamine; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; P, polydispersity index; DTT, dithiothreitol; CF, carboxyfluorescein; MFI, mean fluorescence intensity; GAM, $F(ab)_2$ fragment goat-anti-mouse IgG; TL, total lipid

* Corresponding author. Fax: +34-93-4021219; E-mail: domingo@sun.bq.ub.es

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specific sites [1]. The in vivo properties of SL, which include low RES uptake, prolonged circulation lifetimes and also accumulation at pathological sites, have been widely reviewed [1]. Also in in vitro studies, the presence of PEG on the liposome surface was shown to provide an effective method to improve liposome stability in a biological milieu [2], related to the inhibition of serum protein adsorption [3,4] and cell adhesion [3].

The attachment of monoclonal antibody (mAb) to the surface of SL is one of the most effective ways of targeting antigen expressing cells [5,6] and, sometimes, specific uptake by target cells is observed [7]. This approach is being applied principally to improve the therapeutic efficacy of anticancer drugs against neoplastic diseases [5,8], although the approach is not limited to the treatment of cancers [2]. Several approaches to the preparation of sterically stabilized immunoliposomes (SIL) have recently been developed [4,5,9,10]. The interference of liposome-grafted mPEG chains, observed with both coupling of ligands to the lipid bilayer and with the interaction of these ligands with the intended biological targets, could be minimized by attaching the protein to the distal end of the PEG chains [4,5,9,10]. Moreover, the presence of PEG coat improves immunospecific binding to target cells in vitro, probably by reducing nonspecific binding of SIL to irrelevant cells [11]. Experiments in vitro have shown that the specific binding of SIL to target cells increased both with the lipid concentration and the mAb density on the liposome surface [4,5, 9,10].

Today, the covalent methods described for coupling mAb to the PEG terminus use linkage chemistry to obtain in vivo stable antibody-PEG linkage, e.g., thioether [4,5], amide [10] or hydrazone [8,9] attachments. For ex vivo applications such as immunomagnetic cell sorting [12], the ability to remove attached liposomes to cells after treatment might be useful. This can be achieved by introducing a cleavable linkage between the polymer chain and the mAb.

In the present study, we describe the preparation of SIL containing a thiolytically cleavable bond between the mAb and the PEG based on the use of the heterobifunctional *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) reagent developed originally for the preparation of classical immunoliposomes [13]. The My10 mAb, specific to the human CD34 antigen, was used in this study and KG-1a cells were used as target cells as they are well characterized for their CD34 expression [4,13]. The CD34 antigen was the target of selection because it is selectively expressed by only 1–4% of normal bone marrow cells, including the earliest assayable hematopoietic progenitors [14]. Moreover, the CD34 lack of expression by mature hematopoietic cells makes the antigen a suitable candidate target molecule for isolating hematopoietic stem cells as a part of autotransplantation procedures [15]. The effect of experimental variables on the SIL preparation and on the targeting ability in vitro was examined. On the basis of these studies, we present a modified design of SIL for ex vivo applications that exhibits high levels of selective targeting and that allows the rapid chemical release of the liposomes from the target cells while guaranteeing cell viability [16].

2. Experimental procedures

2.1. Materials

Egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE) and methoxy PEG (2000) phosphatidylethanolamine (mPEG-PE) were obtained from Avanti Polar Lipids Inc. Cholesterol (Chol), fluorescein isothiocyanate, isomer I (FITC), SPDP and dithiothreitol (DTT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). tBoc-NH-PEG(3400)-CO_2-NHS was obtained from Shearwater Polymers Inc. 5(6)-Carboxyfluorescein (CF), obtained from Eastman-Kodak, was purified as described elsewhere [17].

My-10 clone, human leukemic KG-1a, human T-lymphoma Jurkat and Chinese hamster ovary cells (CHO) were obtained from the ATCC (American Type Culture Collection, Rockville, MD, USA). Cells were grown as a suspension culture in RPMI-1640 medium (Gibco), supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units of penicillin and 100 μg/ml streptomycin (Biological Industries, Kibbutz Beth Haemek, Israel), at 37°C in a humidified atmosphere of 5% CO_2 and split 1:2 every 2 days.

Mouse mAb HPCA2 (anti CD34 FITC-IgG1, clone 8G12) and isotype-matched control IgG1 (clone MOPC-21) were obtained from Becton Dickinson (San Jose, CA, USA).

2.2. Synthesis of pyridyldithiopropionylamino-poly(ethylene glycol)-phospahtidylethanolamine (PDP-PEG-PE)

PDP-PEG-PE was prepared as described in [4]. Briefly, t-Boc-NH-PEG(3400)-CO_2-NHS (70 mg) dissolved in $CHCl_3$ (0.5 ml) was reacted with PE (15.3 mg), in the presence of TEA (16 μl), for 2 h at room temperature. Afterwards, the excess of PE was removed by precipitation in acetonitrile. The resulting product was incubated for 20 min at room temperature with TFA (40% v/v) to eliminate the t-Boc group. Next, the amino-PEG-PE obtained was coupled to SPDP (SPDP/lipid = 1.2) by incubation for 5 h at room temperature in the presence of TEA. The final product was crystallized in ether and obtained with a weight yield of 70%. The purity of the product was confirmed by TLC, obtaining a single negative ninhydrin and phosphate-positive spot. The purity determined by the PDP/lipid ratio was $>90\%$. PDP groups were determined by the release of 2-thiopyridone upon the addition of excess DTT [18] and phospholipid was measured by phosphorus assay [19].

2.3. Preparation of antibodies

The mAb (2 mg/ml) was labelled with FITC as described elsewhere [13]. Briefly 50 μg FITC were incubated with 1 mg mAb in 0.25 M carbonate/bicarbonate buffer (NaCl 0.1 M, pH = 9.1) overnight at 4°C, with gentle stirring. The reaction mixture was light-protected. The unreacted dye was removed by gel filtration over Sephadex G-50 spin columns. The FITC-mAb was eluted in the void volume. The conjugation ratio (number of FITC molecules per mAb) was determined by measuring the optical density at 280 and 495 nm, obtaining six FITC molecules/mAb.

In order to bind the mAb or FITC-mAb to the liposomes, the mAb (2 mg/ml in PBS) was conjugated with the heterobifunctional reagent, SPDP (SPDP/mAb 15/1 molar ratio). Excess SPDP was removed by Sephadex G-50 spin column. As estimated spectrophotometrically [18], there were four PDP residues per mAb molecule.

2.4. Liposome preparation

Liposomes composed of PC/Chol 2/1 and 5 mol percent of PEG derivatives were prepared by hydrating dry lipid films in PBS pH = 7.4 or CF 50 mM in PBS. When indicated, rhodamine-PE (Rho-PE) was incorporated at an amount of 0.1 mol percent of total lipid (TL). The resulting multilamellar preparations were then passed through polycarbonate membrane filters with 0.4, 0.2 and 0.1 μm pore size (manufactured by Nucleopore Corp.) in an extrusion device (Lipex Biomembranes Corp.) to obtain unilamellar vesicles of approximately 100 nm in diameter [4]. The prepared vesicles were sized by dynamic laser light scattering using a PCS41 optic unit (Malvern Autosizer IIC). The system reports a polydispersity index (P) as a measure of particle size distribution. The complete incorporation of PDP-PEG-PE at a concentration up to 5 mol percent was shown by the 100% release of 2-thiopyridone from liposomes following reduction of PDP with DTT as determined spectrophotometrically [4,13].

2.5. Preparation of SIL

Two methods were used to bind the mAb to the terminus of PEG on SL:

2.5.1. Method 1

Liposomes containing PDP-PEG-PE were thiolated by incubation with DTT 50 mM for 30 min at 4°C. DTT was separated by passing the liposomes over a Sephadex G-50 column eluted with PBS pH = 7.4. The thiolated liposomes (1.2 μmol) were incubated for 24 h at room temperature with varying amounts of FITC-mAb-PDP or mAb-PDP at a final TL concentration around 3.6 mM. To block free thiols on the liposome surface, the suspension was incubated with iodoacetamide (4 mM) for 2 h at room temperature [4], since these groups can increase nonspecific interactions [5,6]. Excess iodoacetamide was removed with unbound mAb.

2.5.2. Method 2

Thiolated mAb (mAb-SH) was obtained reducing

the mAb-PDP with DTT 50 mM at pH = 4.5. Excess DTT was removed using a Sephadex G-50 spin column. The protein with reduced thiols must be kept in a N_2 atmosphere and used immediately. Freshly prepared mAb-SH were mixed with liposomes containing the coupling lipid, PDP-PEG-PE, at similar concentrations to those in method 1, and were incubated for 24 h at room temperature, with stirring.

In both methods, the free mAb was removed by chromatography on Sepharose 4B. SIL were eluted in the first fractions as measured by phospholipid [20] and FITC fluorescence determinations [4,13].

2.6. SIL characterization

The amount of mAb coupled to liposomes was calculated using FITC-mAb-SIL. The lipid concentration was quantified by phospholipid determination [20]. The protein was determined by FITC fluorescence in comparison to a standard curve [13]. The number of vesicles was calculated as previously described [21] and was estimated at 2.74×10^{12} vesicles/μmol TL.

The stability of SIL was determined by measuring the release of an entrapped dye (CF). Fluorescence intensity ($\lambda_{ex} = 492$; $\lambda_{em} = 520$), before and after liposome lysis with 5% Triton X-100, was measured with a Kontron SFM25 spectrofluorimeter [22].

2.7. In vitro cell binding studies

The binding of SIL entrapping CF to cells was analyzed by flow cytometry using a direct immunostaining assay [4,13]. As CD34+ cells, we used the KG-1a cell line and human hematopoietic progenitor cells purified by magnetic cell sorting from apheresis of a donor patient. In both experiments, exponentially growing cells (10^6 cell/ml), resuspended in PBS supplemented with 1% bovine serum albumin (PBA), were incubated with SIL for 30 min at 4°C. Afterwards, they were rinsed twice in PBA and resuspended at the final concentration of 10^6 cells/ml. As a control, cells incubated with SL entrapping CF were used. In the assay with cell mixtures, the percentage of CD34+ cells was determined by direct immunostaining assay using SIL entrapping CF (100–200 μM) and the mAb HPCA-2 labelled with FITC (2 μg/ml), and by indirect immunoassay using the My10 mAb (2 μg/ml) and a secondary Ab $F(ab)_2$ fragment goat-anti-mouse IgG (GAM-FITC) (5 μg/ml). The analyses were performed using an EPICS XL-MCL flow cytometer (IZASA-Coulter, Spain), equipped with an argon ion laser tuned at 488 nm as excitation source. The green fluorescence (CF) was measured through a BP 525 nm filter. All fluorescence measurements were collected as a logarithmic signal. Sample acquisition was stopped when the 90LS-fluorescence histogram 10 000-gated events were achieved. All data were stored as listmode hardcopies and analyzed with the EPICS XL-MCL Workstation Software version 2.0.

The binding of SIL to KG-1a cells was also corroborated by spectrofluorimetry. SIL (200 μM) were incubated with cells (10^6 cells/ml) for 30 min at 4°C. The cells were washed three times with PBA and the cell-associated fluorescence was measured in the presence of a 5% Triton X-100 on a Kontron SF25 fluorimeter with excitation wavelength set at 492 nm and emission at 520 nm.

2.8. Cleavage of SIL bound to cells

The SIL bound to the cells were removed by addition of 20 or 50 mM DTT (from 1 M stock solution) and the suspension incubated for 30 min or 1 h at different temperatures. After treatment, the cells were rinsed three times in cold PBA to remove the DTT and then analyzed.

2.9. Viability assay

The viable cells were enumerated in a hemocytometer on the basis of trypan blue dye exclusion. Untreated cells, maintained in the same conditions, were taken as a control. The viability was also determined by flow cytometry using double labelling with propidium iodide (PI) and fluorescein diacetate (FDA). The cells were washed and resuspended in 50 μl of PBS. Afterwards, l0 μl of PI (20 μg/ml) and 10 μl of FDA (1 μg/ml) were added to the cell suspension, which was incubated for 30 min at 4°C. Following incubation, the green (FDA) and red fluorescence (PI) cells were analyzed at 488 nm excitation and fluorescence signals were collected at 525 nm and 620 nm, respectively.

Cell cycle analysis was also performed using flow

Table 1
Effect of the coupling method on mAb binding to liposomes

Method	PDP/mAb molar ratio	mAb density (mAb/liposome)	Coupling efficiency (%)
SPDP-1	70	10 ± 1	45 ± 6
SPDP-2	70	11 ± 2	50 ± 10

Liposomes were composed of a PC/Chol 2/1 molar ratio and contained 4 and 1 mol percent of PEG-PE and PDP-PEG-PE, respectively. The coupling procedures are described in detail in the text. The coupling efficiency is expressed as the percent of initial mAb attached to the liposomes. Means ± S.D., $n = 3$.

cytometry. After 24 h of incubation at 37°C, the cells were rinsed three times with cold PBA and permeabilized with MeOH (70%) at −20°C for 15 min. Cells were washed with cold PBA and resuspended in a PBS solution containing 0.5% RNase, 20 μg/ml PI and incubated for 30 min at room temperature prior to analysis.

3. Results and discussion

3.1. Conjugation of My10 mAb to SL

We have previously described the preparation of anti-CD34 SIL with a combination of PEG-PE derivatives with short and long PEG chains, which improve SIL preparation efficiencies and targeting [4]. Sterically stabilized unilamellar liposomes, composed of PC/Chol 2/1 and 5 mol percent of PEG-lipid derivatives (PDP-PEG-PE and mPEG-PE), were prepared by the extrusion technique through 100 nm filters and had an average size of 130 ± 5 nm with a P of 0.084 ± 0.004, in accordance with previous results [4].

In order to prepare SIL, two different methods were used to conjugate My10 mAb to the PEG chain terminus through a cleavable disulfide bridge as described in Section 2 (SPDP method). In Method 1, SPDP-activated mAb was coupled to thiolated liposomes, similar to the method described previously when using SMPB reagent (SMPB method) [4,5]. Method 2, as in conventional immunoliposome preparation [13], involved the coupling of thiolated mAb to pyridyldithio groups linked to the end of the PEG chains. Both conjugation strategies were equally effective and resulted in a comparable number of My10 mAb molecules per vesicle (Table 1).

The amount of mAbs bound per vesicle depends mainly on three factors, as is the case for other covalent coupling methods used to prepare SIL: (1) the

Table 2
Coupling efficiency and mAb density in liposomes containing PDP-PEG-PE

Mol percent PDP-PEG-PE	PDP/mAb molar ratio	mAb density (mAb/liposome)	Coupling efficiency (%)
1	15	12 ± 3	10 ± 3
1	30	12 ± 2	21 ± 2
1	70	10 ± 1	45 ± 10
1	140	5 ± 1	41 ± 6
2	15	70 ± 7	33 ± 3
2	30	56 ± 4	50 ± 5
2	70	30 ± 3	69 ± 4
2	140	13 ± 2	60 ± 2
5	30	96 ± 9	34 ± 6
5	70	65 ± 11	56 ± 6
5	140	32 ± 5	55 ± 1
0[a]	0	2 ± 1	1 ± 1

Liposomes were composed of a PC/Chol 2/1 molar ratio and contained 5 mol percent total PEG derivatives, consisting of a combination of mPEG(2000)-PE and PDP-PEG(3400)-PE as indicated. The coupling procedure used was the SPDP-1 and it was described in detail in the text. The coupling efficiency is expressed as the percent of initial mAb attached to the liposomes. Means ± S.D., $n = 3$.
[a]It was calculated using the largest amount of mAb added

mol percent of the linker lipid incorporated into the liposomes, (2) the mAb concentration and (3) the PDP/mAb concentration ratio in the incubation mixture [4–6,13,23]. Table 2 shows the optimization of coupling efficiency and liposomal mAb density obtained in varying incubation conditions. As described previously [4,5], the number of mAb bound to liposome increases with the increase in reactive groups on the liposome surface and with the increase in the amount of mAb added. Different mAb surface densities can be obtained from 5 to 100 mAb/vesicle and nonspecific binding of protein to the liposomes was not observed (Table 2).

The coupling efficiency increases as the mAb/PDP molar ratio increases, reaching a maximum efficiency (70%) at ratios of 70 which represents a 70-fold excess of PDP groups. A similar behavior has been obtained when using the SMPB method [4], but the coupling of My10 mAb to liposomes by the latter method was more efficient due to the higher reactivity of the maleimido group (coupling efficiencies were near 100%), in line with those described in the bibliography [6]. Moreover, in contrast to the SMPB method [4,5], the coupling efficiency obtained by SPDP method was dependent on the percentage of linker lipid in the liposomes (Table 2), obtaining a maximum efficiency at 2 mol percent of PDP-PEG-PE incorporated. On the other hand, the decrease in the coupling efficiency observed in the case of the 5 mol percent of PDP-PEG-PE supports the previously suggested hypothesis [4] that the interference of PEG chains with the access of mAb to its coupling site could be decreased by using a functionalized PEG derivative longer than an inert PEG derivative in the brush conformation.

The presence of My10 mAb on the liposome surface did not significantly change their physicochemical parameters as vesicle size and liposome permeability. The size of SIL (13, 30 and 70 mAb/vesicle) was found to be 135 ± 12, 133 ± 10 nm and 140 ± 15 ($P < 0.2$), respectively. The CF leakage of prepared SIL at 37°C for 2 h was less than 3% (data not shown).

3.2. Antigen binding activity

The preservation of antigen-binding activity of the My10-SIL was corroborated by flow cytometry (Fig. 1) and by fluorescence confocal microscopy (data not shown). The binding was evaluated by using My10-

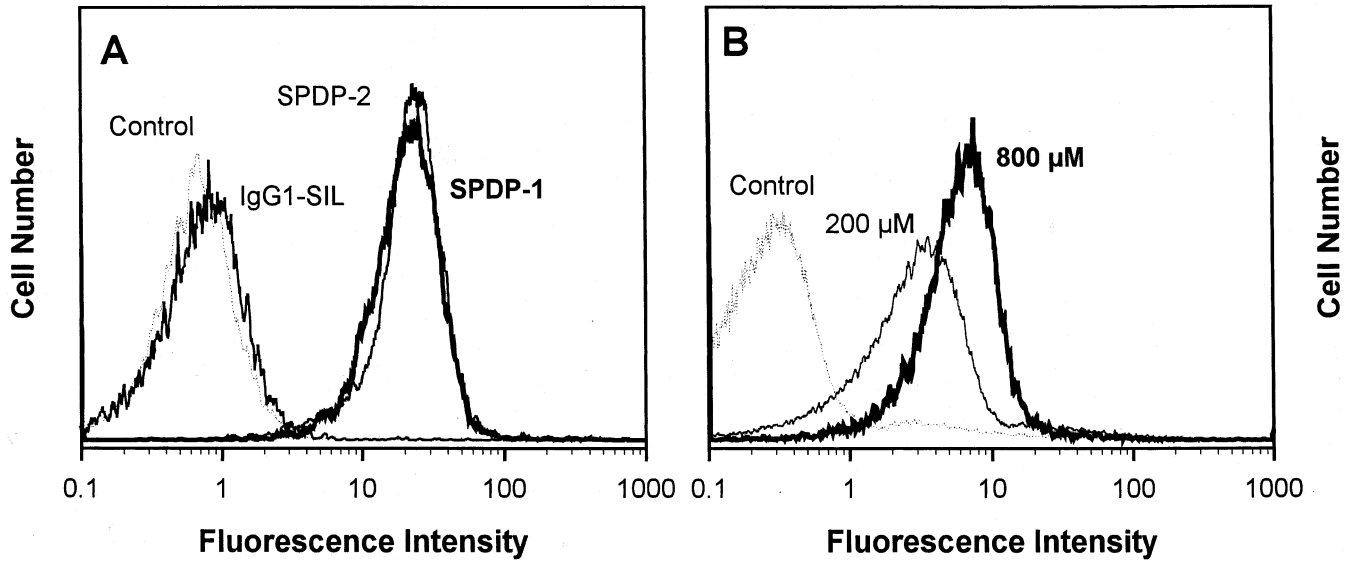

Fig. 1. (A) Cytofluorimetric determination of My10-SIL (200 μM TL, 10 mAb/liposome) binding to KG-1a cells prepared by methods SPDP-1 and SPDP-2 from the experiments shown in Table 1. (B) Cytofluorimetric determination of My10-SIL (13 mAb/liposome) binding to hematopoietic CD34+ cells at two lipid concentrations. SIL entrapping CF and prepared by SPDP-1 method were incubated with cells (10^6 cell/ ml) for 30 min at 4°C and rinsed twice with PBA. The non-specific fluorescence binding was measured incubating cells with 200 μM TL of bare SL or IgG1-SIL (12 mAb/liposome) entrapping CF. The *x*-axis represents the logarithm of green fluorescence signal, and the *y*-axis represents cell count.

SIL containing CF as an aqueous marker after incubation with cells at 4°C, in order to inhibit the endocytosis process. Both methods of My10-SIL preparation produced similar results. The SIL prepared by Method 1 presented a similar binding to CD34+KG-1a cells, expressed as mean fluorescence intensity (MFI), as that presented by the SIL prepared by Method 2, with the same number of mAb/vesicle (Fig. 1A). In these conditions, it was estimated that an average of 4450 ± 500 liposomes were bound per cell, calculated on the basis of the Rho-PE fluorescence associated to cells. Also, these results indicated that the blockage process of the free thiol groups with acetamide did not affect the antigen binding activity of SIL. The degree of SIL binding per cell was related to the degree of antigen expression on the cells. As can be seen in Fig. 1B, the My10-SIL were bound to purified CD34+ hematopoietic progenitor cells (> 95% of CD34+ cells), but this binding was minor to KG-1a cells due to their low level of antigen expression.

To determine the specificity of the My10-SIL toward CD34+ cells, negative control binding experiments were performed using isotype-matched control liposomes (IgG1-SIL) (Fig. 1A) and liposomes without mAb (data not shown). These negative control incubations resulted in very low binding values to CD34+KG-1a cells and it was independent of the nature of the reactive group on the liposome surface (methoxy, PDP or acetamide), in accordance with previous results [4]. In addition, My10-SIL did not bind significantly to CHO or Jurkat cells, which do not express CD34 antigen as confirmed by flow cytometry (data not shown). The MFI obtained was in

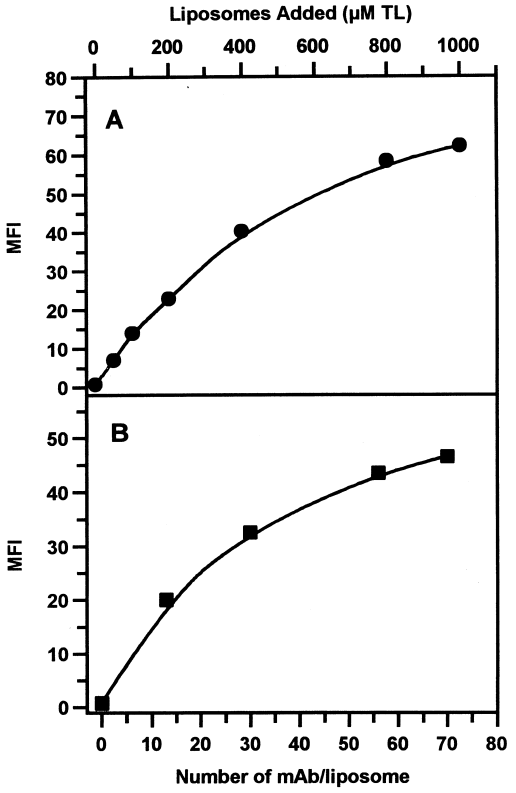

Fig. 2. (A) Effect of the lipid concentration on the degree of SIL binding to KG-1a. My10-SIL (13 mAb/vesicle) prepared by the SPDP-1 method and entrapping CF were incubated for 30 min at 4°C with KG-1a cells (10^6 cells/ml) and analyzed by flow cytometry. (B) Effect of the mAb density on the SIL binding. My10-SIL (200 μM TL) prepared by SPDP-1 method and containing different amounts of mAb/vesicle were incubated with cells (10^6 cells/ml) for 30 min at 4°C. Samples were analyzed by flow cytometry as described previously using bare SL entrapping CF as negative control. The experiments were repeated three times, with S.D. < 8%.

Table 3
MFI and percentage of the subpopulations of CD34+ KG-1a cells in mixtures with CD34- cells

CD34− cells	Initial percent of CD34+ cells	Lipid concentration (μM)	HPCA-2		My10+GAM-FITC		My10-SIL	
			MFI	(%)	MFI	(%)	MFI	(%)
Jurkat	2.5	100	18.5 ± 2.3	2.2 ± 0.2	n.d.	n.d.	5.8 ± 0.8	2.2 ± 0.1
CHO	2.5	100	17.6 ± 2.0	2.3 ± 0.1	57.6 ± 3.0	2.2 ± 0.1	6.0 ± 0.6	2.3 ± 0.3
CHO	2.5	200	17.6 ± 2.0	2.3 ± 0.1	57.6 ± 3.0	2.2 ± 0.1	42.5 ± 3.0	2.5 ± 0.2
CHO	4.5	200	17.8 ± 1.8	4.7 ± 0.2	47.3 ± 4.1	4.2 ± 0.2	38.5 ± 3.2	4.1 ± 0.3

Cell mixtures of KG-1a CD34+ cells and CD34- cells (CHO or Jurkat) were treated by direct immunostaining assay with My10-SIL (10 mAb/vesicle and prepared by SPDP-1 method) entrapping CF or with the HPCA-2 mAb labelled with FITC and by indirect immunoassay with the mAb My10 and the secondary Ab GAM-FITC. Samples were analyzed by flow cytometry and the results are expressed as the MFI and the percentage of CD34+ cells. Means ± S.D., $n = 3$.

Table 4
Stability of vesicle disulfide linkage in presence of culture medium (10% of FCS) at 37°C

Time (h)	0	2	6	9	24
PDP-PEG-PE (%)	4.8 ± 0.1	4.8 ± 0.2	4.8 ± 0.2	4.8 ± 0.1	4.2 ± 0.1

Liposomes composed of PC/Chol 2/1 molar ratio and containing 5 mol percent of PDP-PEG-PE were incubated at 37°C during different times in RPMI-1640 medium (10% of FCS). The intact disulfide linkage remaining at the liposome surface (pyridyldithio groups, PDP) was calculated by the 100% release of 2-thiopyridone from liposomes following reduction of PDP groups with DTT as determined spectrophotometrically following separation of vesicles on gel filtration over Sephadex G-50 spin columns. Means ± S.D., $n = 3$.

the same order as that obtained by liposomes without mAb.

The degree of SIL binding to cells at 4°C depends on the liposome concentration and on the mAb density at the liposome surface (Fig. 2). As the concentration of the added SIL was increased (at a constant cell concentration), the number of liposomes bound per cell increased, having a saturation tendency (Fig. 2A). Similar behavior was observed when increasing the liposomal mAb density to more than 80 mAb/vesicle (Fig. 2B). These results are in accordance with previous studies [4–6,24]. The modest increase in the amount of SIL binding obtained with hematopoietic progenitor cells (low level of CD34 expression), by increasing liposome concentration (Fig. 1B), demonstrates that the saturation level of SIL binding is dependent on the degree of CD34 expression.

3.3. Specificity of targeting in cell mixtures

The use of immunoliposomes to increase the sensitivity of the analysis and the sorting of cells by immunofluorescence according to specific marker molecules has been proposed elsewhere [12,25]. Theoretically, antibodies conjugated to fluorochrome-filled liposomes have a much higher sensitivity of

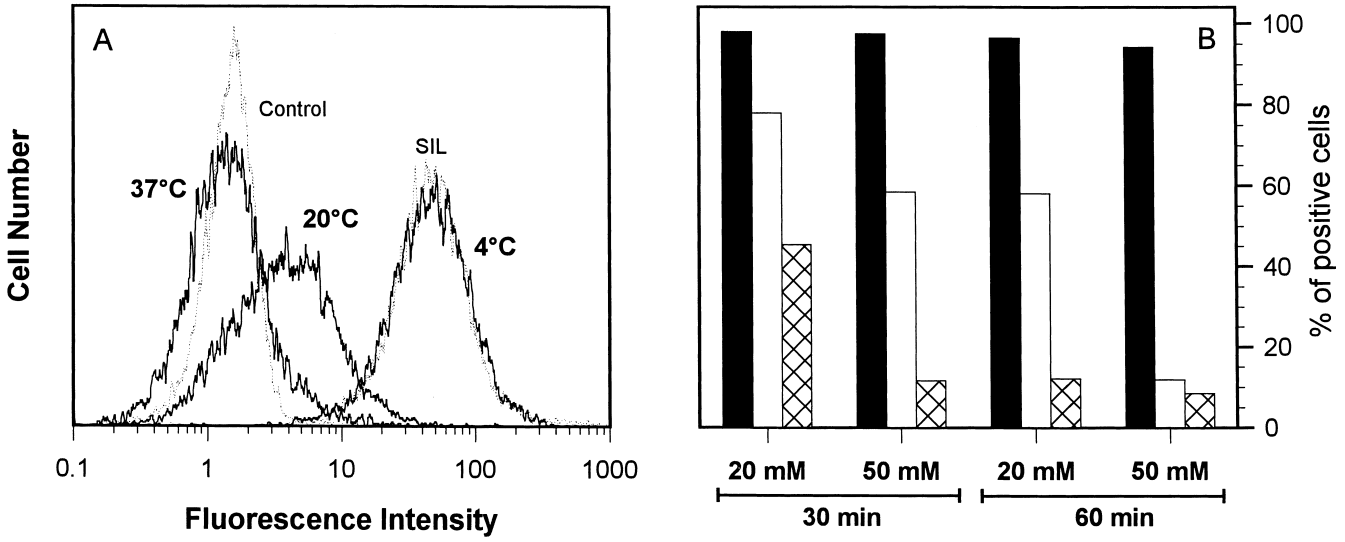

Fig. 3. Release of bound SIL from CD34+KG-1a cells. (A) Fluorescence histograms of KG-1a cells with My10-SIL bound after treatment with 50 mM DTT for 30 min at three different temperatures (solid lines). SIL (200 μM TL, 13 mAb/liposome) prepared by the SPDP-1 method and entrapping CF were incubated with cells (10^6 cells/ ml) for 30 min at 4°C and rinsed twice with PBA (dotted line). The non-specific fluorescence binding was measured incubating cells with bare SL entrapping CF (dotted line). The *x*-axis represents the logarithm of green fluorescence signal, and the *y*-axis represents cell count. (B) Percentage of positive cells after different treatments with DTT at 4°C (filled bars), at 20°C (open bars) and at 37°C (dashed bars). As a negative control, cells incubated with bare SL entrapping CF under the same conditions were used. The experiments were repeated three times, with S.D. < 10%.

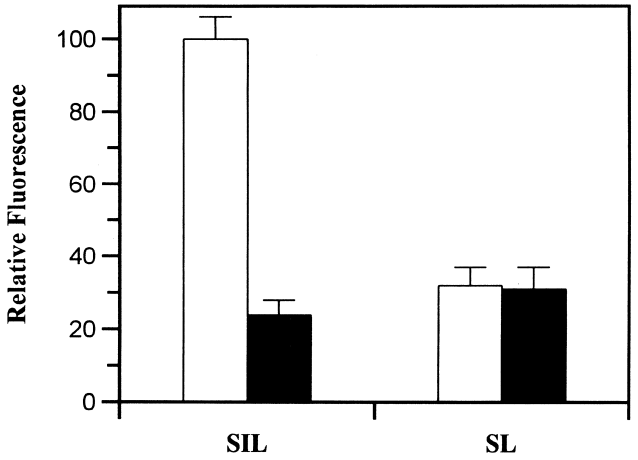

Fig. 4. Release of the KG-1a cell-associated fluorescence by the treatment of the cells with DTT. The cells (10^6 cells/ml) were incubated with 200 μM of TL of SL or SIL (13 mAb/liposome and prepared by the SPDP-1 method) entrapping CF, 30 min at 4°C and rinsed twice with PBA (open bars). Afterwards, the cells were treated with 50 mM DTT during 1 h at 20°C (filled bars). The cell-associated fluorescence was measured on a Kontron SF25 spectrofluorimeter at excitation of 492 nm and emission of 520 nm wavelength. Means ± S.D., $n = 3$.

detection than single labelled antibodies since one liposome can contain a large number of fluorochrome molecules. Given that CD34+ cells are only present at low percentage (1–4%) in clinical samples (as cord blood, mobilized peripheral blood and bone marrow) and in order to develop criteria for the possible future clinical applications of anti-CD34 SIL, we tested the determination by flow cytometry of the percentage of CD34+KG-1a cells in mixtures with CHO or Jurkat CD34- cells by direct labelling using My10-SIL entrapping CF. Direct labelling with the anti-CD34 HPCA2-FITC mAb or by indirect labelling with My10 mAb and a secondary antibody GAM-FITC served as an internal control for specificity and sensitivity. MFI values and percentage of the subpopulations of CD34+KG-1a cells are summarized in Table 3. Direct immunostaining with My10-SIL (100 μM TL) is sufficient to separate populations of positive and negative cells optically according to green fluorescence (data not shown), although the MFI of the positive cells was smaller than that obtained with HPCA2 mAb (Table 3).

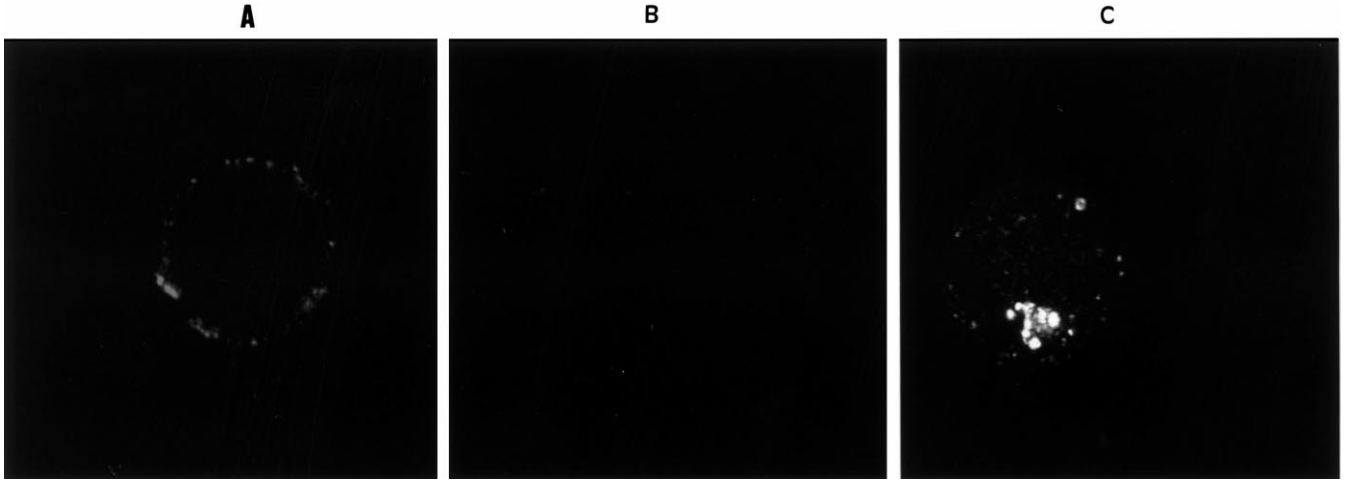

Fig. 5. Analysis by confocal microscopy of KG-1a cells incubated with labelled anti-CD34-SIL. (A) Cross sections through the middle of the cells incubated with CF-SIL (200 μM) during 1 h at 20°C. (B) Cross-sections through the middle of the cells as in (A) after treatment with 50 mM DTT during 1 h at 20°C and (C) bright field image of the cells as in (B). SIL entrapping CF (30 mAb/vesicle) were incubated with KG-1a cells (5×10^5 cells) during 1 h at 20°C. Unbound immunoliposomes were separated from cells by centrifugation (1500 rpm, 5 min). The pellet cells were washed three times with cold PBA. Confocal microscopy was performed on a Leica TCS 4D (Leica Lasertechnik, Heidelberg, GmbH, Germany) confocal scanning laser microscope adapted to an inverted Leitz DMIRBE microscope. The light source was an Argon krypton laser. Green fluorescence was excited at 488 nm. The conditions included a PL Fluotar 100×/1.30 inversion oil objective. Images of 512×512 pixels were stored on a erasable magneto optical disk and then printed on a Mitsubishi CP2000E high resolution color printer. The photographs revealed that the SIL were present only on the cell surface, which is in accordance with the examination of optical cross-section through the middle of the cells (A). Subsequent incubation with 50 mM DTT for 1 h at 20°C, which reduces the disulfide bridges, removed the cell-associated fluorescence (B).

Moreover, as the binding of SIL to cells increases with the liposomal concentration added (Fig. 2A), the sensitivity of this system can be raised by increasing lipidic concentration. Independently of the initial percentage of CD34+ cells and increasing the liposomal concentration to 200 μM TL, we obtained two populations of cells well defined with a threefold increase in the MFI of positive cells compared to direct immunostaining with HPCA2 mAb and with a similar MFI to that obtained by indirect immunostaining with My10 mAb and GAM-FITC (Table 3). Furthermore, in the latter case, quantitative separation of positive from negative cells using My10-SIL was obtained with a lower concentration of My10 mAb (1.3 μg/ml with My10-SIL versus 2 μg/ml with My10 mAb and GAM-FITC) besides a minor manipulation of the sample.

3.4. Cleavage of cell bound SIL and CD34+ cell viability

The disulfide exchange reaction used here for mAb–liposome conjugation is not stable under in vivo conditions because of the susceptibility of the disulfide linkage to reduction by serum components such as glutathione [26]. For ex vivo conditions, the mAb–vesicle disulfide linkage was quite stable (Table 4). For example, a percentage higher than 87.5% of the original linkage remained stable during 24 h of incubation in the cell culture medium (10% of FCS), as described elsewhere [26].

Previous studies have shown that cells can be released from synthetic supports by simple exposure to DTT under mild conditions and also this process does not affect the cell capacity to form colonies [16]. In our case, one would expect the My10-SIL entrapping CF to be released during the incubation with DTT as reducing agent. Fig. 3A shows the results of the experiment with 50 mM DTT at three temperatures during 30 min of incubation. The reduction in cell fluorescence is not due to the natural dissociation of the SIL related to antigen–antibody equilibrium as shown by the retention of fluorescence in the absence of DTT at the three temperatures assayed (data not shown). Neither is the decrease in the cell-fluorescence related to the leakage of entrapped dye, since incubation of SIL with DTT does not damage the vesicle membrane (data not shown), in line with previous results [26]. Analyses of a range of conditions for the release of SIL from the cells are shown in Fig. 3B. SIL are released at 37°C after 30 min of incubation with 50 mM DTT, whereas 1 h was needed with 20 mM DTT. The release is negligible at 4°C while at 20°C, 1 h of incubation was needed with 50 mM DTT.

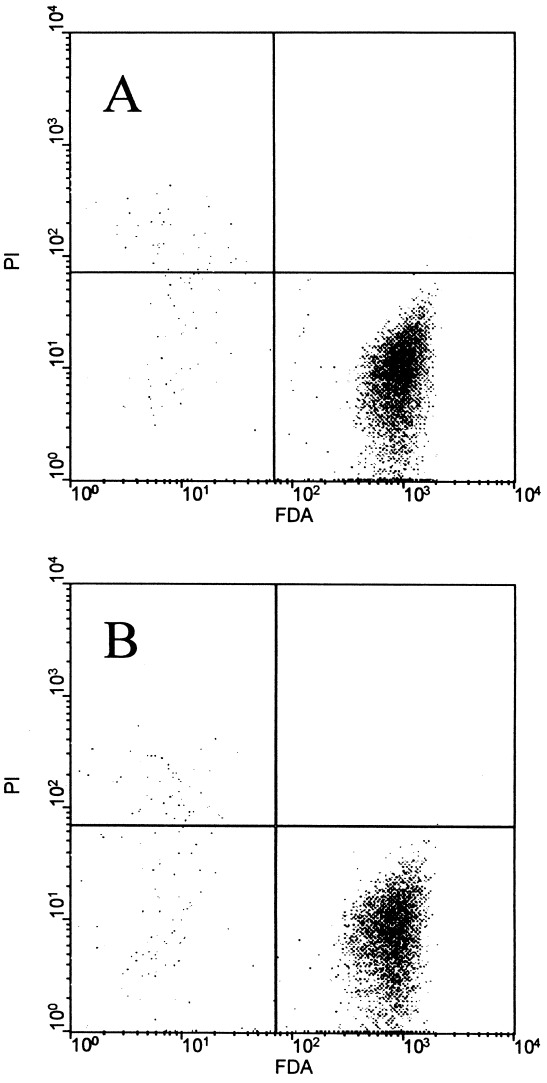

Fig. 6. Flow cytometry biparametric dot plots showing viable KG-1a cells based on FDA positive cells (green fluorescence) and PI negative cells (red fluorescence) after 24 h of incubation at 37°C. (A) Untreated KG-1a cells (control). (B) Cells incubated with SIL (13 mAb/liposome and prepared by SPDP-1 method) for 1 h at 20°C and, after this time, the SIL bound to cells were released by treatment with 50 mM DTT at 20°C during 1 h. Regarding FDA+/PI- staining, neither, untreated KG-1a cells nor SIL+DTT treated cells showed a decreased viability.

The results obtained were confirmed by spectrofluorimetry and by confocal fluorescence microscopy. The cell-associated fluorescence, after incubation with SIL, was removed by treatment with 50 mM DTT at 20°C for 1 h, reaching the same fluorescence levels as cells incubated with bare SL (Fig. 4). The photographs of confocal microscopy revealed that the fluorescence associated with cell surface by incubation with SIL at 4 or 20°C during 1 h could be eliminated by incubation with DTT (Fig. 5). The loss of fluorescence was not associated with a CF leakage, due to the treatment with the reducing reagent, because when SIL, with a thioether linkage between mAb and liposome were used, no loss of fluorescence was observed [4].

In order to use the SIL as cell markers for practical purposes, it was first necessary to demonstrate that the interaction between liposomal and cellular membranes did not influence the cell viability. KG-1a cells were incubated with SIL for 1 h at 20°C and, after this time, the SIL bound to cells were released by treatment with 50 mM DTT at 20°C for 1 h. Cell viability was determined by flow cytometry after 24 h of incubation in culture medium. The cell mortality for treated cells was equal to that for control cells, but always less than 3% (Fig. 6).

4. Concluding remarks

Over the last few years a number of advances have been made in the development of methodologies for the preparation of SIL. Now, with a number of useful reagents in our repertoire, it is possible to achieve reasonable mAb densities on liposomal surfaces, while sacrificing very few of the beneficial properties of PEG-liposomes. In order to exploit the potential of immunoliposomal systems to the full, this report examines an established method for coupling anti-CD34 My10 mAb to PEG-liposomes containing the anchor PDP-PEG-PE via a cleavable disulfide bond. The amount of protein bound to the vesicles can be controlled by varying the mAb and/or varying lipid concentrations in the coupling mixture. Different mAb surface densities can be obtained from 5 to 100 mAb/vesicle with a maximum coupling efficiency of 70% working at mAb/PDP molar ratios of 70 and at 2 mol percent of PDP-PEG-PE conditions. The antigen-binding activity of the conjugates was well preserved and the My10-SIL bound specifically to CD34+ (KG-1a and hematopoietic progenitor cells). The degree of SIL binding to CD34+ cells depends on the liposome concentration, on the mAb density in the liposome surface and on the CD34 cell expression. Direct immunostaining with My10-SIL allowed determination by flow cytometry CD34+ KG-1a cells at percentages similar to those found in clinical samples in mixtures of KG-1a cells with CD34- cells (CHO or Jurkat). Quantitative separation of positive from negative cells using My10-SIL was obtained for at lower concentration of mAb compared to indirect immunostaining and with a threefold increase in the MFI of positive cells compared to direct immunostaining with HPCA2 mAb. For ex vivo conditions, the mAb–vesicle disulfide linkage was quite stable and the liposomes can be released from cells by simple exposure to DTT as reducing agent under mild conditions (1 h of incubation with 50 mM DTT at 20°C). The ability to remove attached vesicles represents an advantage in in vitro experiments designed to study the cellular uptake of liposomes, as it was pointed out previously with conventional immunoliposomes [26]. After SIL binding and subsequent DTT treatment, the cell viability was guaranteed. One future objective would be the characterization and optimization of My10-SIL binding to CD34+ hematopoietic progenitor cells in order to develop criteria for the possible clinical applications of anti-CD34 SIL. With this aim, the preparation of My10-SIL loaded with CF and magnetic beads could be used as staining reagent for analysis and sorting cells with low expression of target antigen (such as CD34 antigen in hematopoietic stem cells), as described in other systems [12]. Moreover, the phospholipid coating of SIL makes the magnetic separation vehicles more biological than other immunomagnetic systems as magnetic microspheres or dextran-magnetite particles.

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