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Rapid report

A novel strategy affords high-yield coupling of antibody to extremities of liposomal surface-grafted PEG chains

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Abstract

Several methodologies for the preparation of polyethylene glycol-grafted immunoliposomes have been developed by attaching antibodies to the terminus of the polymer. Unilamellar liposomes were prepared containing a combination of a functionalized polyethylene glycol(3400) and an inert polyethylene glycol(2000) phosphatidylethanolamine derivate up to 5 mol%. The greater length of the functionalized polyethylene glycol derivate did not alter the liposomal sterical stability or the remote loading of doxorubicin. Anti-CD34 immunoliposomes were prepared by the reaction of maleimide-derivatized My10 antibody with generated thiol groups at the periphery of the liposomes and efficiencies of nearly 100% were obtained. The greater accessibility of the reactive group makes this strategy more efficient than others described. The immunoliposomes prepared bound specifically to CD34+ cells.

Keywords: Immunoliposome; Sterically stabilized immunoliposome; CD34 antigen; Stem cell

The recent development of reticuloendothelial system (RES)-evading sterically stabilized liposomes (SL) prepared by coating the liposome surface with amphipatic polyethylene glycols (polyethylene glycol-phosphatidylethanolamine, PEG-PE, 4–8 mol% of the total lipid) holds a great promise for the liposomal drug delivery [1]. PEG-grafted liposomes exhibit dose-independent long circulating blood lifetimes, a reduced interaction with and uptake by liver and spleen macrophages and an enhanced accumulation in tumors [2–5]. PEG chains are believed to prevent or diminish the adsorption of opsonizing proteins, which direct the liposomes to macrophages [6,7], as a result of their conformational flexibility and their water-binding ability [5,8].

Conventional liposomes coated with monoclonal antibody (mAb) are quickly removed from the circulations by the RES and the immunotargeting efficiency depends on the mAb density on the surface, which limits their use for the systemic drug delivery system [5]. In the last few years, a number of advan-

Abbreviations: PEG, polyethylene glycol; PE, phosphatidylethanolamine; PDP, pyridyldithiopropionate; RES, reticuloendothelial system; SL, sterically stabilized liposome; mAb, monoclonal antibody; SIL, sterically stabilized immunoliposome; CF, carboxyfluorescein; MFI, mean fluorescence intensity; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)-propionate; SMPB, succinimidyl-4-(*p*-maleimidophenyl)butyrate; DTT, dithiothreitol; HPTS, 8-amino-naphthalene-1,2,3-trisulfonate; Dox, doxorubicin

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ces have been reported in methodologies for the preparation of sterically stabilized immunoliposomes (SIL) [9–11]. Attachment of proteins to the distal end of the PEG-terminus minimizes the interference of the polymer chains observed in antibody-antigen interactions and in conjugation reactions when mAbs are coupled to polar head groups of phospholipid components [9,12]. Various methodologies have been studied for coupling ligands to functionalized PEG-PE derivates incorporated into liposomes [9–11], which retained long survival times in the circulation and target recognition in vivo [13,14]. The parameters examined included the binding efficiency, antibody surface density, maintenance of liposomal steric stabilization, specific binding to target cells and drug remote loading efficiency.

In this report, we explore a new strategy to prepare SIL with a combination of both short methoxy-PEG(2000)-PE (mPEG-PE) and long functionalized PEG(3400)-PE. The mPEG chains attached to the liposome make these liposomes sterically stabilized. Coupling the mAb at the functionalized PEG-terminus on the same liposome may be to minimize interference of the polymers chains in conjugation reactions and in antibody-antigen interactions. We have used the coupling method developed by Allen et al., who used the PDP-PEG-PE derivate to attach the mAb to the liposomes via a thioether bond [10]. SILs directed specifically against CD34+ cells were prepared using the My10 mAb [15]. My10-SILs were characterized and evaluated for their in vitro binding capacity to KG-1a cells, a human acute myelogenous leukemia cell line expressing the human CD34 antigen. The effect of experimental variables on the SIL preparation and on the SIL-cell interactions was studied. To the best of our knowledge, this is the first study examining the use of PEG-PE derivates of different chain length to prepare SIL.

Egg phosphatidylcholine (PC), hydrogenated soy phosphatidylcholine (HPC), transphosphatidylated egg phosphatidylethanolamine (PE) and mPEG-PE were obtained from Avanti Polar Lipids. Cholesterol (Chol), *N*-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP), succinimidyl-4-(*p*-maleimidophenyl)butyrate (SMPB) and dithiothreitol (DTT) were from Sigma Chemical. t-Boc-NH-PEG(3400)-CO_2-NHS was from Shearwater Polymers. Doxorubicin (Dox) was obtained from Pharmacia Upjörn and 5(6)-carboxyfluorescein (CF) from Eastman-Kodak. TLC on silica gel 60G (Merck) (Cl_3CH/CH_3OH 80:18:2) was visualized with iodine vapor, ninhydrin and phospholipid phosphorus spray reagents.

The anti-My10 clone and cell lines were obtained from the American Type Culture Collection. A mouse IgG_1, an isotype non-specific Ab, was from Becton-Dickinson. KG-1a, CHO and Jurkat cells were grown as a suspension culture in RPMI 1640 medium and J774 cells were maintained in a monolayer culture in Dulbecco's modified Eagle's medium. For both cell lines, culture media were supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U penicillin and 100 μg/ml streptomycin (Biological Industries). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

We have synthesized a PE derivate of PEG(3400) with terminal pyridyldithiopropionate (PDP) groups (PDP-PEG-PE), as proposed by Allen et al. [10]. PE (15.3 mg) was added to a solution of t-Boc-NH-PEG(3400)-CO_2-NHS (70 mg) dissolved in $CHCl_3$ (0.5 ml), followed by TEA (16 μl). The solution was stirred for 2 h at room temperature. The solvent was evaporated and replaced with acetonitrile and the reaction mixture kept at 4°C overnight. Under these conditions, the excess of PE was precipitated and was eliminated by centrifugation. The purity of the product obtained was confirmed by TLC, obtaining a single negative ninhydrin and phosphate positive spot. To obtain the NH_2-PEG-PE, the t-Boc group was quantitatively eliminated by incubation, for 20 min at room temperature, with TFA (40% v/v). Full deprotection of the primary amino group was confirmed by TLC and visualized with ninhydrin spray reagent. Finally, NH_2-PEG-PE (60 mg), SPDP (5.8 mg) and 13 μl of TEA dissolved in $CHCl_3$ (0.5 ml) were reacted at room temperature for 5 h. The final product was crystallized in ether and obtained with a weight yield of 70%. The molar ratio PDP groups/phospholipid was higher than 0.9. PDP groups were determined by the release of 2-thiopyridinone upon the addition of an excess DTT [16] and phospholipid was measured by a phosphorus assay [17].

Liposomes composed of PC/Chol 2/1 and 5 mol% of PEG derivates were prepared by extrusion techniques, through polycarbonate filters with a 0.1 μm

pore size, as described elsewhere [15]. The vesicle size was characterized by dynamic laser light scattering using a PCS41 optic unit (Malvern Autosizer IIC). The system reports a polydispersity index as a measure of the particle size distribution. The stability of liposomes was determined by measuring the release of an entrapped dye, carboxyfluorescein (CF), in PBS buffer [18]. Dox loading was carried out by the ammonium sulfate gradient method proposed by Bolotin et al. [19].

The steric stabilization of the prepared liposomes was demonstrated by the following experiments. Firstly, the presence of a steric barrier in the liposome surface was demonstrated by the agglutination assay [3,18]. Secondly, the amount of serum protein adsorbed to liposomes of different compositions was determined as described previously [18]. Thirdly, the stability of liposomes in the presence of serum was analyzed by measuring the leakage of entrapped CF from the liposomes in the presence of 10% of human serum [18]. Finally, we determined the binding to and endocytosis of liposomes to a murine macrophage-like cell line, J774 [20]. Briefly, cells were plated at the density of 3×10^5 cells/9 cm^2 plastic culture dishes, 24 h before the experiments were carried out. Liposomes containing 30 mM pyranine (HPTS) were prepared as described above and incubated with supplemented culture medium for 10 min at 37°C before addition to the cells. Cells were incubated with the liposomes to a lipid concentration of 100 μM for different times. The non-associated liposomes in solution were washed away at the end of the incubation by rinsing three times with cold PBS. Cells were dislodged by treatment with PBS buffer containing 3 mM EDTA and after this, the associated fluorescence was measured immediately at λ_{ex} of 460 or 413 nm with a λ_{em} of 510 nm.

In order to prepare the immunoliposomes, a solution of My10 mAb (1 mg/ml) in PBS (pH = 7.4) was left to react for 2 h at room temperature with SMPB (1 mg/ml in dimethylformamide) in an SMPB/protein molar ratio of 20. The excess of SMPB was removed by a Sephadex G-50 spin column equilibrated with PBS at pH = 6.7. Simultaneously, a solution of liposomes containing PDP-PEG-PE was reduced by incubation with 50 mM DTT for 30 min at room temperature. DTT was removed and the thiolated liposomes were quickly mixed with the MPB mAb and left at room temperature overnight. Next, the liposome suspension was incubated with iodoacetamide (0.2 mM) for 2 h at room temperature in order to block the free thiols remaining on the liposome surface. Free mAb and the excess of iodoacetamide were removed by chromatography on Sepharose 4B [15]. The amount of mAb coupled to liposome was calculated fluorimetrically, using FITC-My10, as described previously [15].

The cell-binding assay was performed by direct immunofluorescence with My10-SIL entrapping CF [15]. Exponentially growing cells (10^6 cells/ml) were incubated with SIL for 30 min at 4°C. The analysis was performed in an EPICS Elite flow cytometer (IZASA-Coulter, Spain) using the 488 nm line of an argon ion laser as the excitation source. All fluorescence measurements were collected as a logarithmic signal. Sample acquisition was stopped when the 90LS-fluorescence histogram 10 000 gated events were achieved.

A SL of 100 nm diameter can be achieved by incorporation of 5 mol% of mPEG-PE [9,10]. In order to couple mAb at the terminal PEG chains, we incorporated in the lipid composition up to 2 mol% of the PDP-PEG-PE derivate. Their complete incorporation was shown by the 100% release of 2-thiopyridinone from liposomes following reduction of PDP groups with DTT as determined spectrophotometrically [16]. The substitution of mPEG-PE by PDP-PEG-PE did not alter their stability. The liposomes were stable at 37°C with a CF leakage below 5% in 3 h in PBS. They had an average size of 125 ± 5 nm and were stable for 2 months at 4°C without changes on their physicochemical parameters (data not shown). Dox could be loaded efficiently ($>92\%$) in the basis of the Dox/lipid ratio obtained after removing the non-encapsulated drug (Table 1). Similar results had been obtained with PEG chains of the same length [10]. The liposomal sterically stabilization was not altered by the replacement of mPEG-PE by PDP-PEG-PE. The agglutination assay shows that the presence of a hydrophilic barrier at the liposome surface inhibits avidin-biotin interactions (data not shown) [3]. Moreover, the leakage of CF entrapped in PEG liposomes in the presence of serum was 5-fold less than in conventional liposomes (data not shown), consistent with the lower amount of serum protein bound as a result of the hydrophilic barrier

Table 1
Serum protein adsoption, macrophage uptake and Dox loading of liposomes containing PDP-PEG-PE

Lipid Composition	Serum protein binding[a] (mg protein/mmol lipid)	Liposome-J774 cell interaction			Dox loading[d] (%)
		Binding[b] (mmol lipid/10^6 cells)	Total uptake[c] (mmol lipid/10^6 cells)	Endocytosis[c] (%)	
PC/Chol 2/1	628 ± 110	2.30 ± 1.61	4.32 ± 0.91	50	> 92
PC/Chol/mPEG-PE 12/6/1	185 ± 20	0.18 ± 0.16	0.51 ± 0.32	8	> 92
PC/Chol/mPEG-PE/PDP-PEG-PE 12/6/0.6/0.4	190 ± 13	0.18 ± 0.15	0.34 ± 0.11	8	> 92

[a]Binding of serum proteins to liposomes was determinated by incubation of liposomes with human serum for 1 h at 37°C. Unbound proteins were removed by a Ficoll gradient. Protein was determined by the BCA method.
[b]Binding of liposomes to the macrophagic cell line J774 was measured by incubation of HPTS liposomes with cells for 30 min at 4°C. After removing unbound liposomes, the associated cell fluorescence was mesured fluorimetrically at $\lambda_{ex} = 413$ nm and $\lambda_{em} = 510$ nm.
[c]Total uptake and endocytosis determinations were done by incubation of HPTS liposomes with cells for 6 h at 37°C. The amount of liposomes taken up was determinated fluorimetrically at $\lambda_{ex} = 413$ nm and $\lambda_{em} = 510$ nm. The ratio between excitation at 460 and 413 nm wavelength (460/413) was calculated and the percentage of endocytosed liposomes was estimated according to the method proposed by Daleke et al. [20].
[d]To the doxorubicin loading method, hydrogenated phosphatidylcholine was used instead of egg phosphatidylcholine.

(Table 1). These results are consistent with others published elsewhere [21,22].

The interaction with the macrophagic cell line J774 was also analyzed, since a low binding or uptake of liposomes with macrophage cells in vitro corresponds to a long time in vivo [23,24]. We entrapped in liposomes HPTS a fluorescence marker allowing the discrimination between the general uptake and the part of liposomes internalized into the low pH compartment [20]. Binding at 4°C, total uptake and the internalization at 37°C were lower for PEG liposomes than for plain liposomes, even if they had a combination of mPEG(2000) and PEG(3400) (Table 1). Sterical stabilization reduced the general uptake of liposomes in vitro by more than 8-fold and the internalization by about 6-fold in the conditions tested (6 h at 37°C), which is consistent with previous results [23].

SILs were prepared by coupling of maleimide activated mAb to the thiolated liposomes [10]. As demonstrated previously [10,12,15], the amount of mAb bound per vesicle depends on the number of reactive groups on the liposome surface and the PDP/mAb molar ratio in the incubation mixture. We tested various conditions, adjusting the PDP/mAb molar ratio up to 30 and the mol% of PDP-PEG-PE incorporated in the lipid composition from 0.5 to 2. The amount of antibody bound to liposome increased as the PDP/mAb ratio decreased and with the increase in PDP-PEG-PE percentage. On the other

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

mol% PDP-PEG-PE	PDP/mAb molar ratio	mAb density (mAb/liposome)	Coupling efficiency (%)
2	10	286 ± 10	98 ± 3
2	20	155 ± 12	100 ± 2
2	30	92 ± 6	96 ± 3
1	30	60 ± 6	92 ± 5
0.5	30	23 ± 3	89 ± 3
0[a]	0	2 ± 1	1 ± 1

Liposomes were composed of a PC/Chol 2/1 molar ratio and contained 5 mol% total PEG derivates, consisting of a combination of mPEG(2000)-PE and PDP-PEG(3400)-PE as indicated. The coupling procedure is described in detail in the text. The coupling efficiency is expressed as the % of initial mAb attached to the liposomes.
[a]Calculated using the largest amount of mAb added.

hand, the coupling efficiency, which was independent of the PDP-PEG-PE percentage, increased when the PDP/mAb ratio increased, showing a saturation for ratios above 10 (Table 2). These results are in accordance with those proposed by Allen et al. [10]. However, we obtained efficiencies near to 100% with PDP/mAb molar ratios >10, which were higher than others described elsewhere when PEG derivates of the same length were used [10]. In the absence of PDP-PEG-PE, very low amounts of mAb were associated with the liposomes (<2 mAb/vesicle), possibly through passive adsorption. One can estimate that, theoretically, up to 310 mAb can be bound to a 125 nm diameter liposome based on the assumption previously presented that the effective diameter of an IgG (150 000 Da) molecule is 142 Å [10]. The maximum number of mAb bound to liposome was achieved when we used a PDP/mAb molar ratio of 10 and liposomes that contain 2 mol% of PDP-PEG-PE. In these conditions, approximately 286 mAb bound to each vesicle, calculated in the basis of the fluorescence associated with liposomes [15], which coat 90% of the liposome surface. The high efficiency obtained entails a saving of mAb which production tends to be an expensive process.

The immunoreactivity of SIL to CD34+ KG-1a cells was demonstrated by flow cytometry using CF entrapped in liposomes as fluorescence marker. Incubations were performed at 4°C in order to inhibit uptake processes and, as control, cells were incubated with bare CF-SL. As can be seen in Fig. 1A, the attachment of the My10 mAb to SL resulted in an increased binding to KG-1a cells. The interaction was also confirmed by confocal microscopy (data not shown). The SILs were present only on the cell surface, this was confirmed by examination of optical cross-sections through the middle of the cells. Subsequent incubation with 50 mM of DTT, 30 min at 4°C, did not decrease the cell labelling, which suggests a stable linkage between the mAb and the liposome. To confirm that the cell-binding occurred through specific recognition of antibodies, SIL and SL were incubated with different CD34− cell lines, such as CHO or Jurkat (Fig. 1B). The SIL showed a

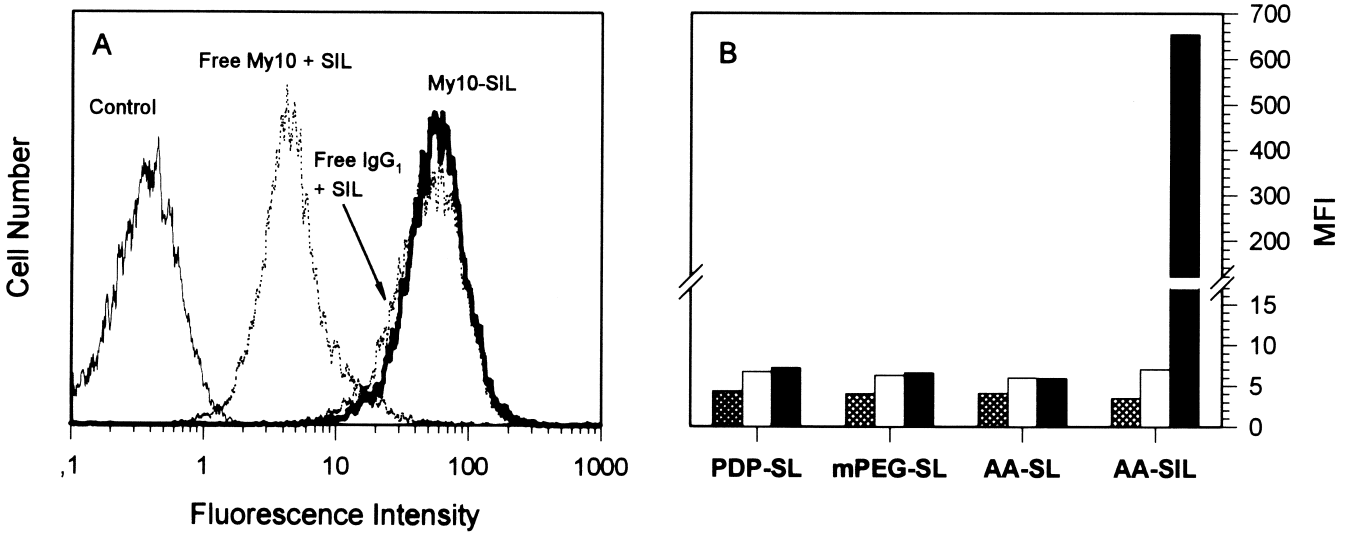

Fig. 1. (A) Cytofluorimetric determination of CF-SIL (23 mAb/liposome)-binding to KG-1a cells (solid line) and specific inhibition of binding by free My10 and non-relevant IgGl Ab (dotted line). SILs (200 μM total lipid) were incubated with cells (10^6 cell/ml), 30 min at 4°C and rinsed twice with PBA. An aliquot of Kg-1a cells was pre-incubated with free My10 or IgGl mAb (5 μg), 30 min at 4°C, washed in PBA to eliminate unbound antibody and incubated with 200 μM of SIL. Non-specific fluorescence-binding was measured by incubating cells with CF-SL. The X-axis represents the logarithm of the green fluorescence signal and the Y-axis represents the cell count. (B) Specificity of the SIL binding to CD34+ cells. Binding of CF-SIL or CF-SL to CD34− cells: CHO (dashed) and Jurkat (open), and to CD34+ cells: KG-1a (filled). Liposomes (200 μM) with PDP (PDP-SL), methoxy (mPEG-SL) or acetamide (AA-SL) groups at the liposome surface were incubated with cells (10^6 cell/ml), 30 min at 4°C. In the same conditions, SIL (23 mAb/vesicle) containing acetamide superficial groups (AA-SIL) were incubated with cells. Fluorescence associated to cells was measured by flow cytometry and represented as mean fluorescence intensity (MFI). Cells were used as negative control.

non-specific binding to CD34− cells similar to SL (Fig. 1B). The quenching of excess active thiol groups following the conjugation reaction seems to be important in achieving a good selectivity of the targeted liposome, since they could promote background binding to the cells lacking the target antigen possibly due to non-specific covalent attachment to cell surface molecules (thiol exchange) [10,12]. For this reason, the free thiol groups present on the liposome surface were quenched by incubation with iodoacetamide. The acetamide group did not affect liposome-cell binding, since the same non-specific binding was observed when a PDP group or an inert methoxy group was present on the liposome surface. Furthermore, an immuno-specific interaction between SIL and KG-1a cells was demonstrated by the partial inhibition of binding by free My10 mAb but not by an isotype-matched non-specific Ab (mouse IgG_1) (Fig. 1A). These results are in accordance with others [15] and, moreover, ruled out the possibility that the binding could be Fc-dependent, since KG-1a cells are FcR+ [25].

The amount of SIL bound to specific cells can be increased by modifying parameters such as the antibody density at the liposome surface and the liposomal lipid concentration in the incubation mixture, as it has been demonstrated previously [9,10,12,15]. Binding to KG-1a cells incubated with different amounts of SIL showed a dose-dependent manner having a tendency to saturation (Fig. 2A). The degree of SIL-binding is also dependent on the number of mAb molecules present on the liposome surface (Fig. 2B). We found that an increase in the mAb density raises the binding efficiency, even though it shows saturation behavior.

In conclusion, we have prepared SIL directed against CD34 antigen expressing cells with a thioether linkage between the mAb and the vesicle, which is stable in vivo. The use of a functionalized PEG derivate longer than a non-functionalized PEG derivate allows us to keep the liposomal steric stabilization and to obtain SIL with a wide range of achievable mAb densities at a high efficiency of coupling. These immunoliposomes show an efficient drug remote loading of Dox and effective binding to target cells in vitro. It is necessary to take into account that the linking of mAb to the distal ends of longer PEG chains could increase the immunogenic properties of

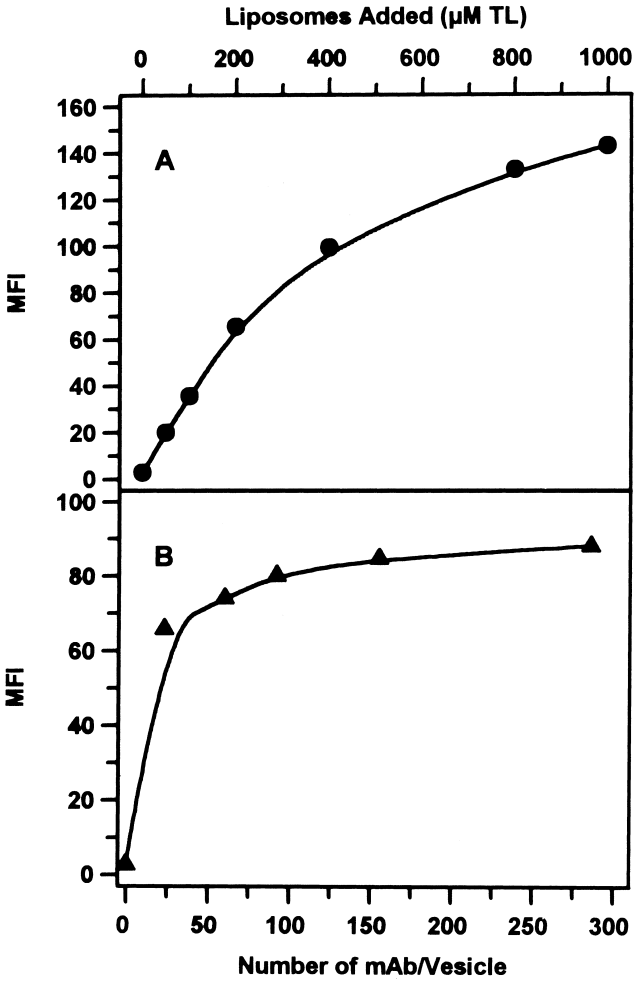

Fig. 2. (A) The effect of the lipid concentration on the degree of immunoliposomes binding to KG-1a cells. CF-SILs (23 mAb/vesicle) were incubated 30 min at 4°C with cells (10^6 cells/ml). (B) The effect of the antibody density on the immunoliposome-binding. Immunoliposomes containing different amounts of mAb/vesicle (200 μM) were incubated with KG-1a cells (10^6 cells/ml) during 30 min at 4°C. Samples were analyzed by flow cytometry as described previously using bare CF-SL as negative control.

SIL, as previously reported [11], related to a major exposure of the constant Fc region of mAb. Since the immunogenicity of SIL influences their longevity in the blood circulation and their ability to reach target sites, another strategie that includes the use of a minor immunogenic device like mAb fragments [11,14] or non-antibody ligands [26] could be used. Further studies will attempt to analyze the potential of My10 SIL as drug delivery system to CD34+ cells in vivo.

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