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Rapid report

Preparation of immunoliposomes directed against CD34 antigen as target

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Abstract

The My-10 monoclonal antibody has facilitated the search of haematopoietic stem cells by recognizing selectively the human CD34 antigen. In the present work, My-10 immunoliposomes directed specifically against CD34 + cells were prepared, characterized and tested in vitro. Binding to target cells at 4°C of immunoliposomes containing carboxyfluorescein as aqueous marker was evaluated by flow cytometry and fluorescence microscopy. These immunoliposomes demonstrated their capacity to bind specifically to CD34 + cells. Studies have shown that 9 antibodies/vesicle were sufficient to obtain a good binding efficiency. The product was stable over one month at 4°C in terms of leakage of encapsulated carboxyfluorescein, particle size and antigen binding capacity. © 1998 Elsevier Science B.V.

Keywords: Immunoliposome; Monoclonal antibody; CD34 antigen; Stem cell

CD34 antibodies recognise a cell surface sialomucin-like molecule that is selectively expressed by only 1–4% of normal bone marrow cells, including the earliest assayable haematopoietic progenitors [1,2], and by vascular endothelial cells [3]. Its lack of expression by mature haematopoietic cells [4] makes the CD34 antigen a logical candidate target molecule to exploit for isolating haematopoietic stem cells as a part of autotransplantation procedures [5], possible gene therapy strategies, and the study of the functions and regulation of blood forming cells [6]. Furthermore, although the function of CD34 molecule is currently unknown, its engagement by antibodies has no significant adverse effects on in vitro colony-forming cells [7]. Anti-CD34 monoclonal antibodies (mAbs) have also been used for the antigenic analysis of lymphohaematopoietic progenitor cells [8], for the enrichment of CD34 + cells [9] as well as for leukaemic blast characterization. The capillary endothelium also contains CD34 + cells and therefore it has been used in angiogenesis studies [3].

For a therapeutic intervention, the conjugation of cell-specific mAbs to liposomes, referred to as immunoliposomes (IL), provides the possibility for selective drug delivery [10,11]. The advantages of IL as delivery system is now well established [12,13]. Many methods for the covalent coupling of mAbs to lipo-

Abbreviations: mAb, monoclonal antibody; IL, immunoliposome; CF, carboxyfluorescein; MFI, mean fluorescence intensity; GAM, $F(ab)_2$ fragment goat-anti-mouse IgG; TL, total lipid

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somes are available [10,14], the coupled mAbs maintain their specificity for target antigens and the IL can bind to the target cells by multivalent interactions [15].

In the present work, IL directed specifically against CD34 + cells were prepared using the My-10 mAb which facilitates the search of haematopoietic stem cells by recognizing selectively the human CD34 antigen [16]. This mAb stains immature haematopoietic precursor cells and all haematopoietic colony forming cells from bone marrow [17], cord blood and adult peripheral blood [18]. My-10-IL, prepared by the established SPDP coupling method, were characterized and evaluated for their in vitro binding capacity to KG-1a cells expressing the human CD34 antigen. The effect of experimental variables on the IL-cell interactions was quantified. The use of antibodies to target liposomes to human CD34 antigen expressing cells has not been reported previously.

Egg phosphatidylcholine (PC) and *N*-(3-(2-pyridyldithio)-propionate)-phosphatidylethanolamine (PDP-PE) were obtained from Avanti Polar Lipids Cholesterol (Chol), fluorescein isothiocyanate, isomer I (FITC), *N*-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP) and dithiothreitol (DTT) were from Sigma (St. Louis, MO). 5(6)-carboxyfluorescein (CF), obtained from Eastman-Kodak, was purified as described elsewhere [19].

Anti My-10 clone and KG-1a cell line were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown as a suspension culture in RPMI-1640 medium (GIBCO), supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units 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 two days.

FITC labelling of the My-10 mAb (2 mg/ml) was obtained by incubation with 50 or 100 μg FITC/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 6 or 12 FITC molecules/mAb, respectively.

The liposomes were prepared as described previously [20]. Briefly, a lipid mixture of PC/Chol/PDP-PE in a molar ratio 12/6/1 in chloroform was dried, vacuum desiccated, and resuspended in PBS pH = 7.4 or CF 50 mM in PBS. The multilamellar vesicles were sequentially extruded through polycarbonate membrane filters with 0.8, 0.4 and 0.1 μm pore size (manufactured by Nucleopore) in an extrusion device (Lipex Biomembranes) in order to obtain unilamellar vesicles. A solution of My-10 or FITC-My-10 in PBS (2 mg/ml) was thiolated with SPDP (SPDP/mAb 15/1 molar ratio). The excess of SPDP was removed by Sephadex G-50 spin column and the product was reduced with DTT 50 mM at pH = 4.5. As estimated spectrophotometrically [21], there were 4 PDP residues per mAb molecule. The excess of DTT was removed from the mAb using a Sephadex G-50. The protein with reduced thiols was kept in a N_2 atmosphere and used immediately. Effective coupling was performed by incubating liposomes (1.2 μmol) with different amounts of previously reduced mAb (0.2 to 1.6 mg/ml), for 24 h at 4°C under stirring, to avoid precipitation of the former. Free mAb was removed by chromatography on Sepharose 4B. IL were eluted in the first fractions as measured by phospholipid and FITC fluorescence determinations.

The amount of monoclonal antibody coupled to liposomes was calculated using FITC-My10-IL. The lipid concentration was quantified by phosphorus analysis [22]. The protein was determined by FITC fluorescence in comparison to a standard curve. The number of vesicles was calculated as previously described [23]. 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 and immunoliposomes 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 [20].

Cell binding assay was performed with exponentially growing cells, rinsed twice in PBS supplemented with 1% bovine serum albumin (BSA) and

resuspended at the final concentration of 10^6 cells/ml. For direct immunofluorescence assay, the cell suspension was mixed with different amounts of free My10, CF-entrapped My-10-IL or FITC-My10-IL. All incubations were carried out during 30 min at 4°C. Indirect immunofluorescence assay was performed by further incubation with the FITC-conjugated $F(ab)_2$ fragment goat-anti-mouse IgG (GAM-FITC) (Kallestad, Austin, TX). Finally, cells were rinsed twice with PBS prior to analysis. Cell binding analysis was performed using an EPICS Elite flow cytometer (IZASA-Coulter, Spain), equipped with an argon ion laser tuned at 488 nm as excitation source. Green fluorescence (FITC, 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 10000 gated events were achieved. All data were stored as listmode hardcopies and analyzed with the EPICS Elite Workstation Software v. 3.0.

The use of IL for target-specific drug delivery requires a specific association with their target cells. In order to achieve this goal, different factors should be taken into account such as a sufficient quantity of antibodies that must be bound to the liposomal surface with their homing capacity preserved. Besides, the integrity of the liposomes should be preserved during the coupling process and the liposome-antibody complex must be stable on storage.

As it has been demonstrated previously [24], the amount of mAbs bound per vesicle depends on the mAb/liposomes concentration ratio in the incubation mixture. Incubation of liposomes (1.2 μmol) with different initial mAb concentrations (0.2 to 1.6 mg/ml), allows to obtain IL with 9 to 72 mAb molecules/vesicle, as demonstrated by analysis of the fluorescence of mAb FITC-My-10 bound to the liposomes (data not shown). The relationship between the number of mAb/vesicle coupled and mAb concentration appeared to be linear ($r = 0.9913$) over a concentration range from 0.2 to 1.6 mg/ml (Fig. 1). Previous works demonstrated that 1–2 Ab/liposome could be a sufficient antibody density to bind to target cells [25], therefore, IL bearing 9 mAb/vesicle were used to assay cell binding.

The physicochemical properties of IL influence notably their behaviour in vitro [24], therefore they

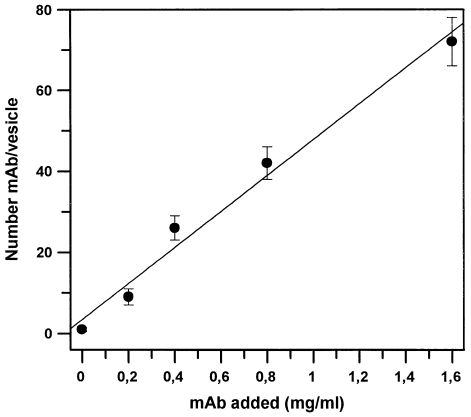

Fig. 1. The number of mAb molecules/vesicle dependence on mAb concentration in the incubation mixture (means ± S.D., $n = 3$). PC/Chol/PDP-PE (12/6/1, molar ratio) liposomes (1.2 μmol) were incubated under N_2 atmosphere for 24 h at 4°C under stirring, with a fresh solution of thiolated FITC-IgG (0.2 to 1.6 mg/ml) in PBS. Free mAb was removed by chromatography on a Sepharose 4B.

were characterized prior to its immunoreactivity assay. The size of liposomes was found to be approximately 130 nm with a polydispersity index of 0.14 (data not shown). The presence of antibody on the liposome surface did not change significantly the liposome size nor the permeability of liposomes using CF as aqueous marker (data not shown). The stability of the prepared IL was demonstrated by controlling changes in vesicle size and CF retention. IL could be stored at 4°C in PBS during one month without change in their size (data not shown) and CF release (5–6%).

The immunoreactivity of My-10 mAb to KG-1a cells was demonstrated by flow cytometry using a second antibody (GAM-FITC) (Fig. 2A). In order to analyze immunospecific targeting of My-10-IL by flow cytometry, these were incubated with KG-1a cells using bare liposomes as a control. Incubations were performed at 4°C in order to inhibit uptake processes. Direct immunofluorescence assay using FITC-My10-IL (6 FITC molecules/mAb) did not permit to demonstrate specific association of IL with their target cells. Moreover, the further use of a secondary GAM-FITC mAb showed fluorescence histograms overlapping with negative control (Fig. 2A). The results are not related to a loss of antigen specificity of the labelled My-10 as showed by flow cytometry (Fig. 3). They should rather be related to

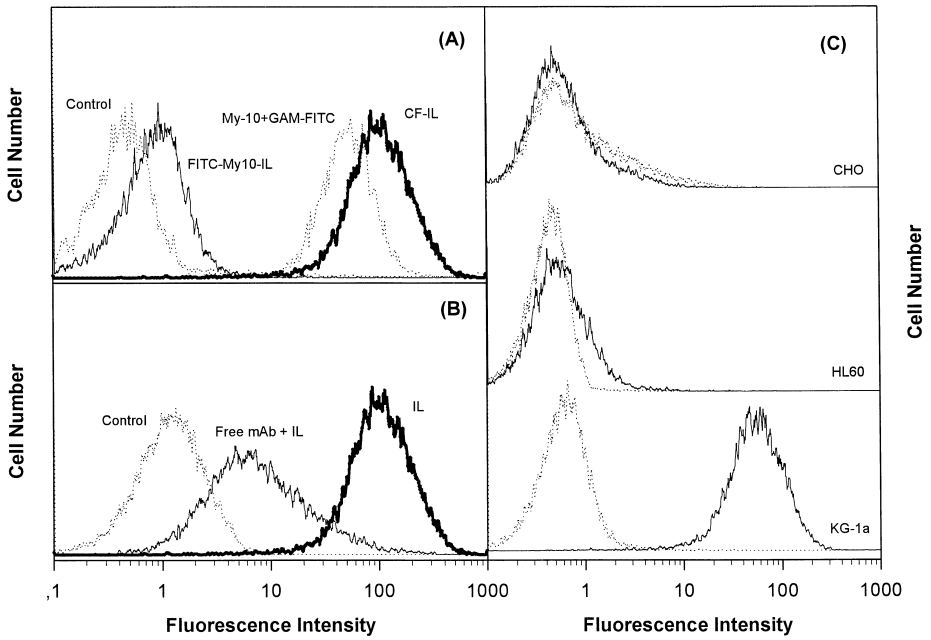

Fig. 2. (A) Cytofluorimetric determination of My-10 IL, CF-IL and free My-10 mAb-FITC binding to KG-1a cells. Monoclonal antibody My-10 FITC-conjugated (0.5 μg/ml) and My-10 FITC-IL (200 μM total lipid) were incubated with cells (10^6 cell/ml) 30 min at 4°C, after washing twice with PBA cells were incubated with a secondary antibody FITC-conjugated $F(ab)_2$ fragment, goat-anti-mouse IgG 30 min at 4°C. Cells were direct immunostained CF-IL (200 μM total lipid) 30 min at 4°C and rinsed twice in PBA. Non-specific fluorescence binding was measured incubating cells with bare CF-liposomes. *X*-axis represents the logarithm of green fluorescence signal, and *Y*-axis represents cell count. (B) Specificity of the union immunoliposome-cell CD34 + . Decrease of CF-IL-cell binding by preincubation of cells with free My-10 mAb. KG-1a (10^6 cell/ml) were incubated 30 min at 4°C with 200 μM CF-IL (right plot) or liposomes as negative control (left plot) and rinsed twice in PBA. An aliquot of KG-1a cells was preincubated with free antibody 30 min at 4°C (5 μg), washed with PBA to eliminate unbound antibody and incubated with 200 μM CF-IL (medium plot). The fluorescence associated with cells was analyzed by flow cytometry. (C) Specificity of the union immunoliposome-cell CD34 + . Binding of CF-IL on various cell lines. HL60, CHO (CD34 − cells) and KG-1a (CD34 + cells) (10^6 cell/ml) were incubated with CF-IL or bare liposomes (200 μM) as negative control. Fluorescence associated to cell was measured by flow cytometry. First plot of each uniparametric histogram represents the negative control. *X*-axis represents the logarithm of green fluorescence signal, and *Y*-axis represents cell count.

the low amounts of antibody bound. An increase in the My-10 mAb labelling (12 FITC molecules/mAb) produced a loss of the antigen specificity (data not shown), which is in accord with previous works [26].

Fluorescent dyes can be entrapped into IL allowing a signal enhancement in direct immunofluorescence assays [26]. In this way, My-10-IL entrapping CF (CF-IL) were prepared using the above coupling conditions. Firstly, the stability of CF-IL in the presence of cells was determined. Cells did not mediate leakage of liposomal CF during incubation at 4°C (data not shown). The specific labelling of cells stained with CF-IL showed several fold increased resolution above control (Fig. 2A). The mean fluorescence intensity (MFI) obtained by direct label of cells with CF-IL was similar to that obtained with the system My-10 + GAM-FITC (Fig. 2A). IL-cell interaction was also confirmed by confocal microscopy (data not shown). The photographs revealed that the IL were present only on the cell surface, which was confirmed by examination of optical cross-section through the middle of the cells.

In order to show the specificity of the IL towards the CD34 expressing cells, negative control binding experiments were performed. CF-IL were incubated with HL60 (a human promyelocytic leukaemia cell line) and CHO (Chinese hamster ovary cells), which did not express the CD34 antigen (Fig. 2C). CHO cells showed no significant fluorescence histograms displacement and the non specific binding was inferior to 5%. Unspecific binding to HL60 cells was slightly higher (ca. 20%), probably due to a possible

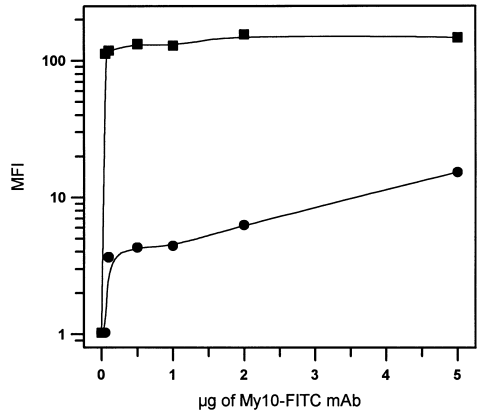

Fig. 3. Cytofluorometric determination of the binding of FITC-My-10 mAb (6 FITC/mAb) to KG-1a cells (5×10^5 cells). Mean fluorescence intensity (MFI) is plotted against FITC-My10 mAb amount added. By direct immunofluorescence, using FITC-labelled My-10 mAb (6 FITC/mAb) (●) or indirect immunofluorescence with a PE-conjugated $F(ab)_2$ fragment goat-anti-mouse IgG (GAM-PE) secondary mAb (5 μg) (■).

phagocytic activity [27]. Furthermore, specific IL-cell interactions were demonstrated by blocking the binding site by preincubation of the target cells with free My-10 mAb. An inhibition of 80% of specific My-10-IL-binding was obtained by previous incubation of cells with 5 μg of free mAb (Fig. 2B). A steric hindrance might be an explanation for the inhibition of IL binding to KG-1a cells.

In order to optimize the binding assay, some parameters such as the antibody density in the liposome surface and the liposomal lipid concentration in the incubation mixture were studied [12]. KG-1a cells were incubated with different amounts of CF-IL (9 mAb/vesicle). The IL bound to KG-1a cell line in a dose-dependent manner (Fig. 4A). Saturation of IL binding was achieved at 400 μM TL. Controversial results on the dependence of cell-binding to mAb density at the liposome surface have been reported [12,25]. The degree of IL binding is dependent on the number of mAb molecules present on the liposome surface (Fig. 4B). We found that an increase of the mAb density from 9 to 72 mAb/vesicle arises the binding efficiency, even though it shows a saturation behaviour. Similar results were previously described [28].

In conclusion, we have prepared immunoliposomes bearing My10 mAb which are directed against human CD34 antigen expressing cells, as haematopoietic stem cells. These immunoliposomes have been characterized and their capacity to bind specifically to target cells in vitro has been demonstrated. The product was stable over one month at 4°C in terms of leakage of encapsulated CF, particle size and antigen binding capacity. Besides, our studies shown that 9 mAb per vesicle were sufficient to obtain a good binding efficiency. Further studies will be addressed to analyze the subsequent events which follow IL-cell binding via the CD34-mAb interaction that largely dictates the therapeutic potential of immunoliposomes as drug delivery system to haematopoietic stem cells. Moreover, for in vivo applications, the preparation of immunoliposomes sterically stabilized by conjugation of mAb to the PEG terminal polymer will be explored [28].

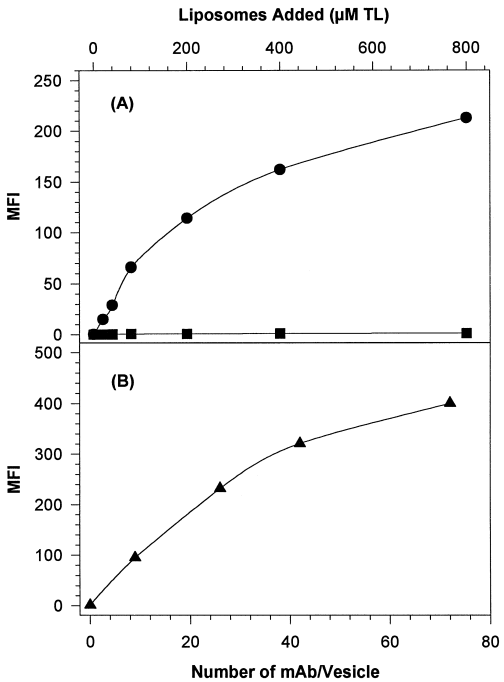

Fig. 4. (A) Effect of the lipid concentration on the degree of immunoliposomes binding to KG1-a. CF-IL (9 Ab/vesicle) (●) or CF-liposomes (■) were incubated 30 min at 4°C with KG-1a cells (10^6 cells/ml) and analyzed by flow cytometry. (B) Effect of the antibody density on the immunoliposome binding. Immunoliposomes containing different amount of mAb/vesicle or liposomes (200 μM) were incubated with cells (10^6 cells/ml) during 30 min at 4°C. Percentage of cell bound CF fluorescence was determined by flow cytometry.

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