Exploiting CD38-mediated endocytosis for immunoliposome internalization

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CD38 appears to be a promising candidate in antibody therapy; it is upregulated on cell surfaces in many lymphoid tumors and undergoes rapid internalization after interaction with antibodies. The receptor-mediated endocytosis allows conjugating toxins/drugs that promote suicide only of the malignant cells. Here, we describe the preparation of CD38-immunoliposomes and test their functionality by incubating them with CD38+/− cells. Liposomes were prepared by extrusion of a lipid mixture containing a biotinylated polyethylene glycol-phospholipid and loaded with 5(6)-carboxyfluorescein. The anti-CD38 antibody (IB4) was biotinylated and then linked to streptavidin molecules; streptavidin acts like a bridge between the antibody and the biotinylated lipid of the liposomes. CD38+/− cells were incubated either with liposomes or immunoliposomes and analyzed by fluorescence microscopy and cytofluorimetry. The results indicated a specific mechanism of internalization, owing to CD38-mediated endocytosis, where CD38+ cells incubated with immunoliposomes scored top fluorescence levels. This coupling strategy, based on the use of a streptavidin bridge to prepare immunoliposomes, does not interfere with the cellular functionality and its broad potential use represents a great advantage. Here IB4, a murine monoclonal anti-CD38 antibody, was used to simplify the experiments, but the coupling procedure may be suitable also with human antibodies, against CD38 or other human markers. *Anti-Cancer Drugs* **19:599–605**

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Introduction

In this study, we evaluate the potential of immunoliposomes containing a biotinylated polyethylene glycol (PEG)-phospholipid and a streptavidin-conjugated anti-CD38 antibody for use in antitumor-targeted therapy. Human CD38 antigen, a 42–45 kDa type II transmembrane glycoprotein, is expressed on the surface of monocytes, platelets, natural killer cells, and myeloid cells [1]. Interestingly, CD38 is expressed either in the earlier or late stages of B and T cell differentiation, but not in the intermediate stages [2]. This peculiar pattern of expression in lineage-committed hematopoietic progenitor cells does not change in their malignant counterparts [3]. CD38 acts as a cell surface receptor triggering a wide variety of cell functions, including cell proliferation, protection from apoptosis, and cytokine release after its internalization [4]. Like many other receptors, CD38 undergoes rapid internalization after interaction with CD31 or with antibodies (Abs) mimicking the natural ligand(s), forming a receptor–ligand complex [5]. CD38 internalization never involves the entire set of surface molecules; in fact, the internalized fraction represents an almost constant percentage (30–40%) of the total amount of surface molecules. This may indicate that the cell surface holds two pools of CD38 molecules, only one of which undergoes internalization after Ab binding. However, there is no evidence that these two subsets of CD38 perform different enzymatic tasks. In previous studies, we have shown that CD38 endocytosis is preceded by extensive rearrangement of the cell surface, with the formation of glycosphingolipid-rich and cholesterol-rich plasma membrane microdomains [6]. Receptor-mediated endocytosis is essential for monoclonal antibody (mAb) therapy, as it allows the conjugation of toxins to be internalized only by target cells. Another important consideration in antibody therapy is antigen expression: tumor cells may express higher levels of target antigen than normal cells. As CD38 displays both these features, it is a promising candidate for use in antibody therapy, although clinical experience with anti-CD38 antibody has provided little evidence so far [7]. One recent approach in antibody therapy involves the generation of engineered antibodies, such as CDR-grafted, humanized IgG_1 and a chimeric fragment antigen binding–fragment crystallizable (Fc) (mouse fragment antigen binding cross-linked to two human γ_1 Fc) [8] construct. However, there is a limit to the amount of toxin or chemotherapic agent that can be loaded with antibodies to promote suicide of the malignant cells without affecting normal cells or to generate an

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immunologic response to the toxin. In an attempt to overcome this constraint, liposomes can be linked to the antibodies, allowing greater amounts of chemotherapeutic agent to be loaded and carried to the target cells. As traditional immunoliposomes are rapidly eliminated from circulation [9], it would be of some advantage to use sterically stabilized liposomes containing PEG-derivatized lipids [10]. Moreover, the antibody may be bound directly to the liposome surface or to the distal tip of the PEG chains. In the first strategy, PEG chains may sterically hamper the interaction between the antibody and the receptor on the target cells [11]. To preserve the biologic activity, the antibody is attached at the terminus of the PEG chains [12]. Here, we test the use of immunoliposomes characterized by a coupling procedure relying on a biotinylated PEG-phospholipid and a streptavidin-conjugated anti-CD38 antibody.

Materials and methods

Cell cultures

The human Raji (Burkitt lymphoma, American Type Culture Collection, Manassas, Virginia, USA) and K562 (chronic myelogenous leukemia, American Type Culture Collection) cell lines were maintained in continuous culture in RPMI-1640 medium supplemented with 10% fetal bovine serum, 4 mmol/l L-glutamine, 100 mmol/l Na pyruvate, 25 mmol/l Hepes, and antibiotics. These two lines exhibit different immunophenotypes for CD38: Raji cells express high levels of surface CD38, whereas K562 cells are CD38 – .

Biotin labeling of anti-CD38 monoclonal antibodies

The anti-CD38 mAb (IB4) was biotinylated using Sulfo-NHS-LC-Biotin [sulfosuccinimidyl-6-(biotin-amido) hexanoate] reagent from Pierce (Rockford, Illinois, USA). NHS-activated biotins react efficiently with primary amino groups ($-NH_2$) in pH 7–9 buffers to form stable amide bonds. Antibodies have several primary amines in the side chain of lysine (K) residues. To perform the biotinylation, a 20-fold molar excess of biotin reagent was used to label 2 mg antibody, allowing incorporation of four to six biotin groups per antibody molecule. We added 27 µl of 10 mmol/l solution of the biotin reagent to 1 ml of 2 mg/ml IB4, according to the manufacturer's instructions.

The reaction mixture was incubated for 2 h at room temperature and then desalted using Zeba Desalt Spin Columns (Pierce) to remove nonreacted biotin.

Preparation of streptavidin-conjugated IB4 monoclonal antibody

Two milligrams (33 nmol, from Pierce) of streptavidin was dissolved in 0.1 mol/l phosphate-buffered saline (PBS) and gently shaken during overnight incubation with the biotinylated antibody at room temperature. The molar ratio of streptavidin : IB4 was 3 : 1. The reaction mixture was purified from unbound streptavidin by Sephacryl S-200 gel filtration chromatography (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) followed by measuring the absorbance at 280 nm. Aliquots of streptavidin-conjugated IB4 mAb were stored at – 20°C.

Preparation of liposome containing 5(6)-carboxyfluorescein

The following reagents from Avanti Polar Lipids (Alabaster, Alabama, USA) were used: dual specificity phosphatase catalytic domain (5.2 µmol), cholesterol (4.5 µmol), PEG–distearoylphosphatidylethanolamine (DSPE) (0.3 µmol), and biotinylated PEG-phospholipid–DSPE (bio-PEG–DSPE) (0.015 µmol) [12]. The lipid mixture was dissolved in chloroform, dried using a Speed Vac, and then rehydrated using 10 mmol/l PBS containing 0.1 mmol/l 5(6)-carboxyfluorescein (Sigma, Milano, Italy). After undergoing five freeze–thaw cycles, the lipids were processed in an extruder (Avestin, Ottawa, Canada). Extrusion was carried out five times at 20°C through a 100 nm-pore size polycarbonate membrane and then nine times using a 50 nm polycarbonate membrane. Mean particle size and zeta potential of liposome in PBS were determined by dynamic light scattering (DLS) method using a zetasizer (Zeta sizer Nano ZS, Malvern Instruments, Worcestershire, UK). The liposome suspension was then purified by Sephadex G-75 (GE Healthcare Bio-Sciences AB) gel-filtration chromatography followed by ultraviolet detection at 280 nm to collect fractions containing fluorescent biotinylated liposomes.

Streptavidin bridge for the synthesis of immunoliposomes

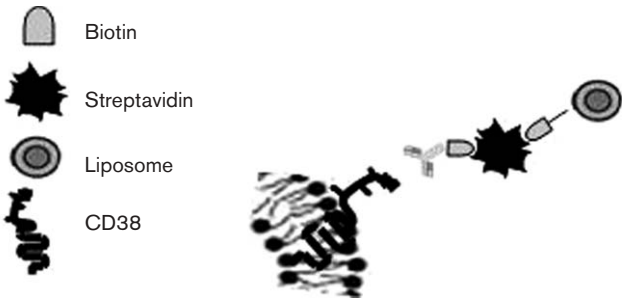
The lipid mixture used for the synthesis of the liposome contained bio-PEG–DSPE to act as the linker. This reagent is made up of a molecule of PEG of 2000 Da with a lipid (DSPE) at one end and a biotin molecule at the other. The biotin serves to link the mAb through a bridge of streptavidin (Fig. 1), whereas the PEG molecule works as a spacer to prevent any potential shielding effect that could block the interaction between the liposome and the mAb. Bio-PEG–DSPE was used at a molar ratio 1 : 1 with the streptavidin-conjugated mAb. Free biotin was mixed to streptavidin-IB4 to block excess biotin-binding sites potentially preventing cross-linking of the immunoliposomes. Fractions containing fluorescent biotinylated liposomes were incubated with streptavidin-IB4 overnight at room temperature.

Fluorescence microscopy and fluorescence-activated cell sorting analysis

Fluorescence-activated cell sorting (FACS) analysis was performed with a Becton Dickinson FACScan instrument (Mountain View, California, USA) equipped with an argon ion laser tuned at 488 nm. Data acquisition was done with CellQUEST software (Becton Dickinson). Cells were gated according to their scatter light and fluorescence intensity distribution was analyzed in histograms. Raji and

K562 cells were collected, washed twice with ice-cold PBS, resuspended in PBS + 2% bovine serum albumin to reduce unspecific binding, and incubated separately with PBS, fluorescent liposomes, or immunoliposomes (carrying 70 μg/ml of bound IB4 mAb) for 1 h at 37°C. Cells were also incubated with mock immunoliposomes as negative control, to exclude unspecific fluorescent signals. After incubations, cells were washed with PBS, observed under fluorescence microscopy, and then analyzed on a flow cytometer. The resulting histograms were displayed as a single overlay to evaluate the increment of fluorescence. Subsequently, Raji and K562 cells were treated with 100 nmol/l phorbol 12-myristate 13-acetate (PMA) (Sigma) to create conditions similar to that of natural inflammation. Treated cells were incubated with fluorescent immunoliposomes.

Fig. 1

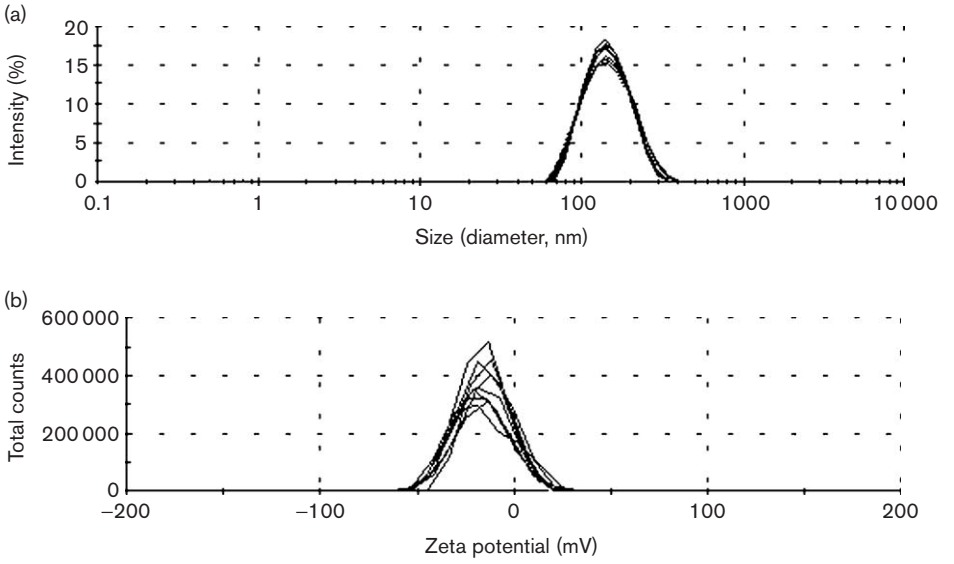


Schematic representation of coupling procedure. Streptavidin acts as a bridge between a biotinylated polyethylene glycol-phospholipid and a biotin-conjugated anti-CD38 antibody (Ab). Transmembrane CD38 binds Abs mimicking the natural ligand(s), forms a receptor–ligand, and undergoes rapid internalization.

Results

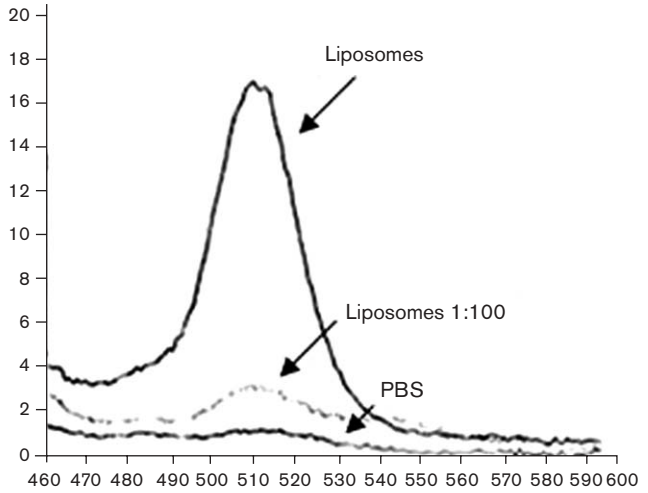
The present study evaluates the effectiveness of using a streptavidin bridge to synthesize an anti-CD38 immunoliposome. The lipid mixture for the liposomes contained Bio-PEG–DSPE, DSPC, and PEG–DSPE. The lipid film was rehydratated in PBS containing 0.1 mmol/l 5(6)-carboxyfluorescein, and the liposomes were prepared by using freeze–thaw cycles and the extrusion method. After extrusion, the liposome mixture was purified by gel-filtration chromatography and subjected to ultraviolet detection at 280 nm. The same aliquots were then analyzed by means of a Perkin–Elmer spectrofluorimeter (LS 50) (PerkinElmer Life And Analytical Sciences, Inc., Waltham, Massachusetts, USA), using excitation and emission wavelengths of 494 and 518 nm, respectively. Size and zeta potential determinations were performed by dynamic light scattering with a Malvern Zetasizer Nano ZS. All measurements were carried out at 25°C and repeated 10 times. Both the graphs referred to size and to zeta potential (Fig. 2a and b respectively) showed only one peak and the mean vesicle diameter was 148.7 nm [polydispersity index (PdI) = 0.093] whereas the average of the zeta potential was, −13.2 mV. To confirm the specificity of the observed fluorescence, we analyzed fluorescence emission intensity of 100-fold-diluted fluorescent-labeled liposomes (dilution in PBS) and fluorescence intensities of undiluted, fluorescent-labeled

Fig. 2



Graphs of size distribution by intensity (a) and zeta potential (b) performed by dynamic light scattering with a zetasizer (Zeta sizer Nano ZS, Malvern Instruments). In each graph, obtained by 10 repeat measurements, it is possible to detect only one peak, indicating that the liposome suspension was homogeneous. The mean diameter value was 148.7 nm and the mean zeta potential value was −13.2 mV.

Fig. 3



Graph of fluorescence emission intensity of PBS, 100-fold-diluted fluorescent-labeled liposomes (dilution in PBS) and undiluted fluorescent-labeled liposomes obtained by means of a Perkin–Elmer spectrofluorimeter (LS 50) excitation and emission wavelengths of 494 and 518 nm, respectively. PBS, phosphate-buffered saline.

liposomes. The graph in Fig. 3 shows a peak of the fluorescence intensity at 518 nm, corresponding to the concentration of liposome solution. The liposomes were also examined by fluorescence microscopy.

After verifying synthesis of the liposome, strepdavidin was used as a bridge in the preparation of immunoliposomes. In the first step, streptavidin was linked to biotinylated-IB4 and then coupled to liposomes through the bio-PEG–DSPE lipid. The final product was purified and then incubated with Raji and K562 cells. As described in the Materials and methods section, these cell lines display a different immunophenotype: Raji cells express high levels of CD38 on their cell membrane, whereas K562 cells do not express the antigen. To check for the actual receptor-mediated endocytosis, Raji and K562 cells were incubated with immunoliposomes as well as with liposomes.

The cells were first stained with liposomes to determine the ratio of internalization that occurred independent of the receptor. After incubation, the cells were analyzed by means of a flow cytometer; the histograms corresponding to the Raji and K562 cells (Fig. 4a) showed a comparable fluorescent profile, indicating unspecific fusion between the liposome and cell membrane.

In the second step, the cells were incubated with fluorescent and mock immunoliposomes. Samples stained with mock immunoliposomes did not give any fluorescent signals (data not shown) whereas incubation with fluorescent immunoliposomes provided different fluorescein isothiocyanate values for Raji and K562 cells in the histograms from FACS analysis (Fig. 4b). Raji cells incubated with immunoliposomes scored the highest levels of fluorescence. In K562 cells the histogram of fluorescence derived from incubation of cells with immunoliposomes has almost the same value of that referred to incubation with liposomes.

A significant fraction (35%) of the Raji cells internalized immunoliposomes through receptor-mediated endocytosis, as indicated by the lack of increased fluorescence after incubation of K562 with anti-CD38 immunoliposome. These observations were verified by fluorescence microscopy (Fig. 5a).

PMA was used to promote cellular activation, leading to an overexpression of CD38 on the surface of Raji and K562 cells. After incubation with immunoliposomes, the activated cells were analyzed by fluorescence microscopy and flow cytofluorimetry (Fig. 5b). The amount of fluorescent cells had increased in the Raji sample; instead, although they express CD38 membrane after PMA treatment, the percentage of fluorescent K562 cells did not increase (data not shown).

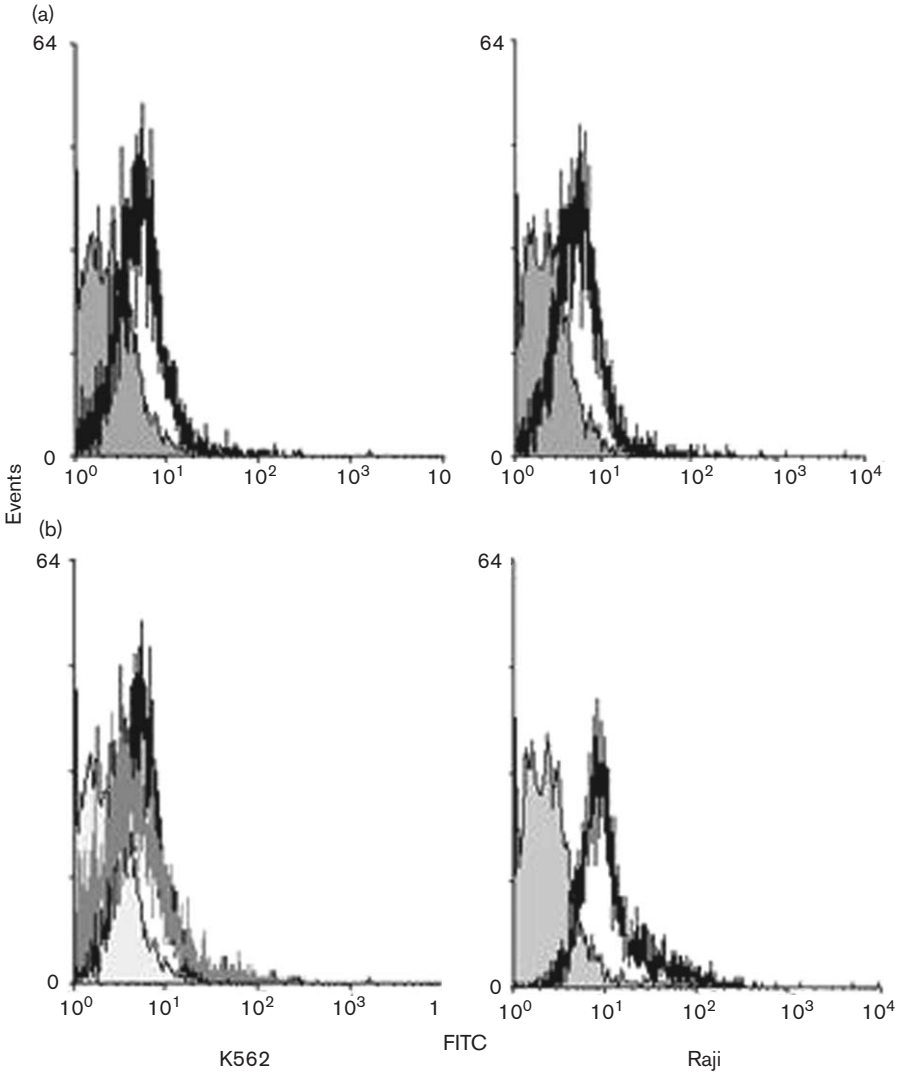
Discussion

This research describes the preliminary results obtained in attempting to assess the use of CD38-immunoliposomes as a tool in the antibody therapy.

Here, we prepared immunoliposomes by performing a coupling procedure by means of a streptavidin bridge between a biotinylated PEG-phospholipid and a biotin-conjugated anti-CD38 antibody. The liposomes were loaded with 5(6)-carboxyfluorescein to allow their visualization. CD38+ or CD38− cells were stained with liposomes or immunoliposomes and the fluorescence values obtained by FACS analysis showed a specific mechanism of internalization owing to CD38-mediated endocytosis.

CD38+ cells incubated with immunoliposomes scored the highest value of fluorescence, whereas the staining of liposomes with cells (CD38+/−) as well as the incubation of immunoliposomes with CD38− cells showed almost the same rate of internalization. This basal level depends on the fusion that normally occurs between the liposomes and cell membranes [13]. After PMA treatment, which promotes CD38 expression, Raji cells displayed an increase in immunoliposomes internalization, measured as higher value of fluorescence. PMA treatment on K562 cells, on the contrary, did not lead to any increase of fluorescence, despite the induced-CD38 expression on their cell membrane. We have already proved that internalization never involves the entire amount of surface molecules, indicating the presence of two distinct pools of CD38 [5,6]. In other works, we have, moreover,

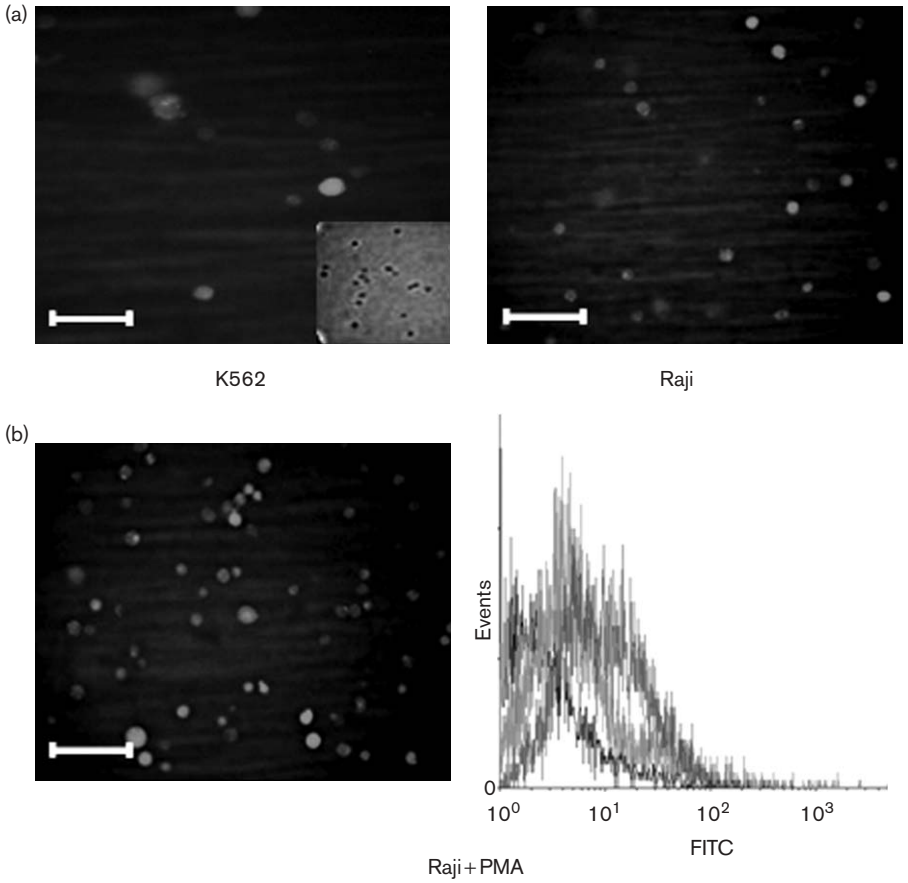
Fig. 4



Fluorescence-activated cell sorting analysis after incubation of K562 (left panel) and Raji (right panel) cells with fluorescent liposomes (a) and immunoliposomes (b). Solid grey histograms are referred to cells mixed with liposomes and immediately washed (background levels); black histograms correspond to the K562 and Raji cells incubated for 1 h with liposome and immunoliposomes. For K562, in panel b, are shown the histograms referred to liposomes (black) and also to immunoliposomes (grey), and they displayed the same value of fluorescence. FITC, fluorescein isothiocyanate.

demonstrated that CD38 on the surface of treated K562 cells is an active enzyme with cyclase activity [14]. Interplay between all of these observations implies that only a constant percentage of the total amount of surface CD38 makes internalization in immunophenotipically positive cells, and the induced-PMA CD38 in negative cells is enzymatically active, but it is not able to mediate endocytosis. These hypotheses may indicate that the induced CD38 belongs to a subset, which does not undergo internalization after Ab binding and has only an enzymatic role. In positive cells, the ability of some receptors such as CD38 to mediate endocytosis may be an important advantage, helping the internalization of toxins or chemotherapic agents conjugated to the antibody. Other prerequisites that CD38 needs to show for the use in therapy are not only the maximal efficacy in engaging human effector mechanisms, but also the acceptance to the host immune system and the tumor specificity. The attack on a tumor by an antibody offers the advantage of few side effects, but its efficacy seems to be limited [15]. The antibody usually attacks the tumor by the mechanism of antibody-dependent cellular cytotoxicity and this ability is linked to the recognition of the Fc region of mAb by the effector cells [16]. The use of murine antibodies shows two important limits: human natural killer cells are often not able to promote antibody-dependent cellular cytotoxicity for murine IgG1, IgG2a, and IgG2b subclasses [17]; and the immunogenicity in the patient is very high [18]. To avoid

Fig. 5



Fluorescence microscopy observations of K562 (left) and Raji (right) cells after incubation with immunoliposomes. The grey insert in K562 figure is obtained by light microscopy (a) to prove the presence of nonfluorescent cells. (b) fluorescence microscopy observation (left) and fluorescence-activated cell sorting analysis (right, black histogram) of phorbol 12-myristate 13-acetate (PMA)-activated Raji cells incubated with immunoliposomes. Dark grey histogram is referred to untreated Raji cells incubated with immunoliposomes, light grey histogram shows background level. Bars 1 cm = 45 μm.

these problems, chimeric antibodies have been developed; in this construct, there is a human Fc [19] or the majority of the Ig has been replaced by human sequences, leaving only the hypervariable regions from the original murine antibody [20]. These 'derivatives' offer the potential for reduced immunogenicity compared with murine monoclonals, and have a spectrum of effector functions determined by their isotype. In this study, we have used IB4, a murine mAb-anti-CD38, to simplify the experiments, but the coupling procedure used to prepare immunoliposomes may be suitable also with human antibodies. The advantage of this strategy is just the broad potential use, because it is possible to change the antibody and/or the chemotherapic agent loaded into liposomes by using the same coupling procedure. Another central consideration in the design of this immunotherapy is that of specificity. Even if CD38 is widely expressed on cells of the hematopoietic system, its expression is upregulated on cell surfaces, in many cases, of a variety of lymphoid tumors, notably multiple myeloma, AIDS-associated lymphomas, and posttransplant lymphoproliferations. Moreover, the most primitive pluripotent stem cells of the hemopoietic system are CD38-negative [21,22], and this guarantees their vitality after therapy and also the repopulation of normal cells of the blood.

CD38, characterized by receptor-mediated endocytosis and by tumor-specificity, seems to be a good candidate for antibody therapy; this feature may be enhanced by the use of liposome, to load drugs into target cells. Our strategy of coupling liposome and antibody does not interfere with CD38 functionality, and allows varying the antibody and the drug loaded into liposome without changing its structure.

The next aim is to prove this mechanism with humanized anti-CD38 antibodies, to evaluate the effective role of CD38 as a therapeutic tool.

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