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Sterically stabilized liposomes bearing anti-HLA-DR antibodies for targeting the primary cellular reservoirs of HIV-1

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Abstract

The ability of liposomes bearing anti-HLA-DR Fab′ fragments at the end termini of polyethyleneglycol chains (sterically stabilized immunoliposomes) to target HLA-DR expressing cells and increase the accumulation of liposomes into lymphoid organs has been evaluated and compared to that of conventional liposomes, sterically stabilized liposomes and conventional immunoliposomes after a single subcutaneous injection to mice. The accumulation of sterically stabilized liposomes in lymph nodes was higher than that of conventional liposomes. Sterically stabilized immunoliposomes accumulated much better than conventional immunoliposomes in all tissues indicating that the presence of PEG has an important effect on the uptake of immunoliposomes by the lymphatic system. Fluorescence microscopy studies showed that sterically stabilized liposomes are mainly localized in macrophage-rich areas such as the subcapsular region of lymph nodes and in the red pulp and marginal zone of the spleen. In contrast, sterically stabilized immunoliposomes mostly accumulated in the cortex in which follicles are located and in the white pulp of the spleen. As the human HLA-DR determinant of the major histocompatibility complex class II is expressed on activated CD4+ T lymphocytes and antigen presenting cells such as monocyte/macrophages and dendritic cells, known as the cellular reservoirs of HIV-1, liposomes bearing anti-HLA-DR antibodies constitute an attractive approach to concentrate drugs in HIV-1 reservoirs and improve their therapeutic effect.

Keywords: Immunoliposome; Targeting; Lymph node; Tissue distribution

1. Introduction

It is well established that lymphoid tissues are the major reservoirs of human immunodeficiency virus type-1 (HIV-1) [1–5]. During primary infection, HIV-1 particles disseminate rapidly in the lymphoid organs where they replicate actively in CD4+ T-cells. In the period of clinical latency, HIV-1 becomes trapped onto the follicular dendritic cell (FDC) network, located in the germinal centers of the secondary lymphoid organs, and persistently infects activated CD4+ T-cells [6]. In the advanced stage of the disease, the microenvironment network within the germinal centers is destroyed, and the incapacity of FDC to retain HIV-1 particles has been postulated to contribute to the increased viral burden in the periphery [1]. As peripheral lymphoid tissues play a crucial role in effective immune response, it is im-

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portant to reduce or abrogate the production and the accumulation of HIV-1 particles in these tissues to preserve both their architecture and integrity.

Fortunately, highly active antiretroviral therapy (HAART), usually consisting in the combination of two nucleoside analogues and one protease inhibitor, has been shown to markedly diminish the number of HIV-1 RNA copies in secondary lymphoid tissues [7–9]. However, replication-competent HIV-1 was routinely isolated from resting CD4+ T-cells, even after 30 months of HAART, impairing the possibility to stop the treatment [8–14]. Increasing numbers of treatment failures resulting from toxicity, drug-resistant mutants and poor compliance of patients to drug regimen are emerging with long-term therapy [15]. Consequently, strategies aimed at reaching therapeutic levels of drugs into the lymphoid organs should be developed to improve the efficacy of antiretroviral agents and to prevent suboptimal concentrations of drugs within infected cells that could potentially lead to the development of resistance.

As liposomes are naturally taken up by cells of the mononuclear phagocyte system (MPS), liposome-based therapy represents a convenient approach to improve the delivery of drugs into lymphoid tissues [16–19]. Morphological observations on the fate of liposomes in regional lymph nodes suggest that liposomes remained stable within the lymphatic circulation and that they eventually enter into cells of the lymph nodes by phagocytosis to end up in the lysosomal apparatus [20]. On the other hand, the coupling of polyethyleneglycol (PEG) chains on the surface of liposomes (sterically stabilized liposomes) was shown to increase their ability to move through the lymph after subcutaneous injection and to decrease their rate of uptake by the MPS, increasing their residence time within plasma and/or lymph [21,22]. Consequently, attachment of antibodies to sterically stabilized liposomes represents an attractive strategy to combine prolonged circulation time and efficient delivery of therapeutic drugs to specific cell population and/or pathogen.

FDC, B lymphocytes, activated CD4+ T-cells and antigen presenting cells like macrophages are abundant in lymphoid tissues and all express substantial levels of the HLA-DR determinant of the major histocompatibility complex class II (MHC-II). In addition, it is well known that many enveloped viruses, including HIV-1, acquire host cell surface molecules during the budding process. It has been reported that the HLA-DR determinant is physically present on the virion surface and constitutes one of the most abundant host-derived molecules carried by HIV-1 [23,24]. Liposomes bearing surface-attached anti-HLA-DR antibodies thus constitute an interesting approach to target specifically HIV-1 reservoirs as well as free HIV-1 particles trapped in the FDC network. In the present study, the ability of liposomes bearing anti-HLA-DR Fab′ fragments at the end termini of PEG chains (sterically stabilized immunoliposomes) to target HLA-DR expressing cells and increase their accumulation in secondary lymphoid organs has been evaluated and compared to that of conventional liposomes, sterically stabilized liposomes and conventional immunoliposomes.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG) and distearoylphosphatidylethanolamine-*N*-[poly(ethyleneglycol)-2000] (DSPE-PEG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Dipalmitoylphosphatidylethanolamine-*N*-(4-(*p*-maleimidophenyl)butyryl) (DPPE-MPB) was purchased from Northern Lipids (Vancouver, BC, Canada) and distearoylphosphatidylethanolamine-[poly(ethyleneglycol)2000]-*N*-(4-(*p*-maleimidophenyl)butyryl) (DSPE-PEG-MPB) was custom-synthesized by Shearwater Polymers Inc. (Huntsville, AL, USA). [^{3}H]cholesterylhexadecylether was purchased from NEN Life Science Products (Boston, MA, USA). 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was purchased from Molecular Probes (Eugene, OR, USA). Lysyl endopeptidase obtained from *Achromobacter lyticus* was purchased from Wako Chemicals (Richmond, VA, USA).

2.2. Preparation of antibodies

Monoclonal antibodies IgG_{2b} (clone Y-17, clone OKT4, ATCC, Rockville, MD, USA) that react

with I-E subregion products (murine HLA-DR) or human CD4 was isolated from ascites fluids and purified using a protein G affinity column (Pharmacia, Baie d'Urfé, QC, Canada). Antibodies were sterilized on 0.22 μm low binding protein filters (Millipore, Bedford, MA, USA) and stored at −20°C in phosphate buffered saline (PBS, pH 7.4) until use. The immunoreactivity of antibodies was tested by flow cytometry on freshly prepared C3H mouse spleen cells and SUPT-1 cells. In brief, suspension of cells (10^6 cells/ml) was incubated with 1 μg of OKT4 or biotinylated Y-17 for 30 min at 4°C. Cells were washed with PBS and incubated with 1 μg R-phycoerythrin conjugated streptavidin (Jackson Immunoresearch Laboratories, West Grove, PA, USA) for Y-17 and with 1:50 fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG (Cederlane Laboratories Ltd, Hornby, ON, Canada) for OKT4 for 30 min at 4°C. Cells were then washed three times with PBS, fixed with 1% paraformaldehyde and kept on ice under darkness until assessment of fluorescence by flow cytometry (Coulter Electronics, Miami, FL, USA).

2.3. Preparation of F(ab′)$_2$ fragments

The F(ab′)$_2$ fragments were produced following incubation of the antibody (5 mg/ml) with lysyl endopeptidase (in 50 mM Tris–HCl, pH 8.5) in a 1:50 enzyme/substrate molar ratio for 2 h at 37°C. Lysyl endopeptidase cleaved IgG_{2b} at Lys-228E/Cys-229 without perturbing the disulfide bridges. The digestion products contained undigested IgG, F(ab′)$_2$ and Fc fragments. The enzyme was removed by gel chromatography on a Sephadex G-25M column (Pharmacia, Baie d'Urfé, QC, Canada). Fractions containing digestion products were determined using a BCA protein assay reagent kit (Pierce, Rockford, IL, USA) and pooled together. F(ab′)$_2$ fragments were separated with a protein A affinity chromatography column (Pharmacia, Baie d'Urfé, QC, Canada). The column retained Fc fragments and undigested IgG_{2b} whereas F(ab′)$_2$ fragments were collected. F(ab′)$_2$ fragments (110 kDa) were then concentrated using Centricon-50 (Amicon, Beverly, MA, USA) and resuspended in 1 ml phosphate EDTA buffer (100 mM sodium phosphate and 5 mM EDTA, pH 6.0).

2.4. Preparation of Fab′ fragments

F(ab′)$_2$ fragments were incubated with 6 mg of 2-mercaptoethylamine–HCl (0.05 M) for 90 min at 37°C. This product cleaved the disulfide bridges between the heavy chains but preserved the disulfide linkages between the heavy and light chains. The solution was eluted on a Sephadex G-25M column pre-equilibrated with acetate–EDTA buffer (100 mM anhydrous sodium acetate, 88 mM sodium chloride and 1 mM EDTA, pH 6.5) and Fab′ fragments were collected in 1 ml fractions. Fractions containing Fab′ fragments were determined using a BCA protein assay (Pierce, Rockford, IL, USA) and pooled together. The Fab′ fragments (55 kDa) were concentrated using Centricon-10, resuspended in acetate–EDTA buffer and kept under nitrogen atmosphere at 4°C until coupling to liposomes.

2.5. Preparation of (immuno)liposomes

Fig. 1 shows a schematic representation of the different liposomal formulations used in this study. Liposomes composed of DPPC/DPPG/DPPE-MPB (10:3:0.33 mol/mol) for immunoliposomes and DPPC/DPPG/DSPE-PEG-MPB (10:3:0.83 mol/mol) for sterically stabilized immunoliposomes were prepared according to the method of thin lipid film hydration. In brief, the lipid mixture was dissolved in chloroform in a round-bottomed flask and the organic solvent was evaporated to form a thin lipid film. In some experiments, [^{3}H]cholesterylhexadecylether (0.3 μCi/μmol lipid) was added as a radioactive tracer. The lipid film was then hydrated with acetate–EDTA buffer (pH 6.5). Multilamellar vesicles were sequentially extruded through 0.2 and 0.1 μm polycarbonate membranes (Nuclepore, Cambridge, MA, USA) using a stainless steel extrusion device (Lipex Biomembranes, Vancouver, BC, Canada). The mean vesicle size of the liposomes, as evaluated with a submicron particle analyzer (Coulter Electronics, Hialeah, FL, USA), was between 100 and 118 nm. The final concentration of liposomes was determined using an enzymatic colorimetric method (phospholipids B kit, Wako Chemicals, Richmond, VA, USA). Conventional liposomes were prepared as above except that no DPPE-MPB

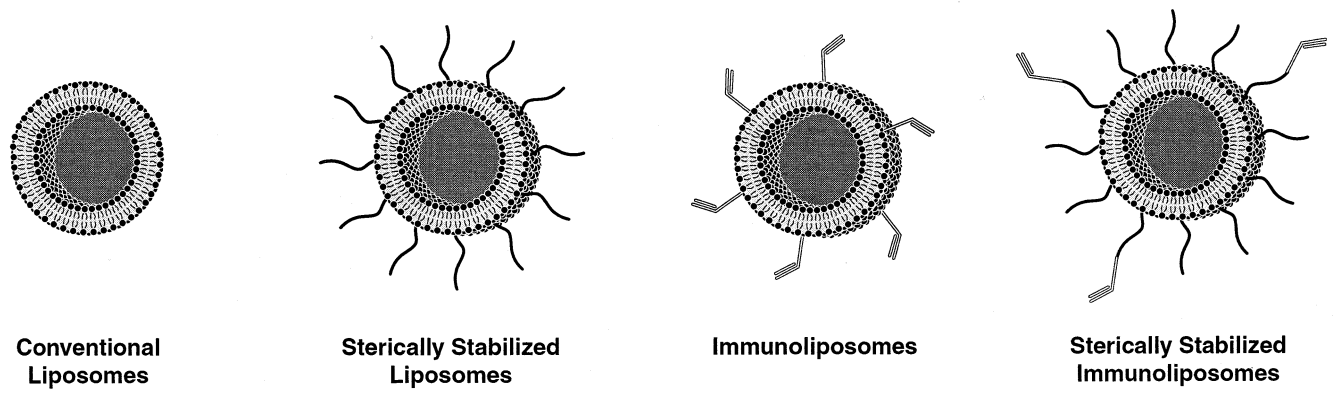

Fig. 1. Schematic representation of conventional liposomes, sterically stabilized liposomes (PEG-coated), conventional immunoliposomes bearing Fab′ fragments and sterically stabilized immunoliposomes bearing Fab′ fragments at the end termini of PEG chains.

was incorporated in the lipid composition. In the case of sterically stabilized liposomes, DSPE-PEG-MPB was replaced by DSPE-PEG.

2.6. Coupling reaction

Freshly prepared liposomes were incubated with freshly prepared Fab′ fragments (35 μg Fab′/μmol lipid) overnight at 4°C under continuous agitation and under nitrogen atmosphere. Liposomes bearing surface-attached antibodies (immunoliposomes) were separated from unconjugated Fab′ fragments by ultracentrifugation (100 000 × *g*, two times for 45 min at 4°C) and immunoliposomes were resuspended in PBS (pH 7.4). The total amount of Fab′ conjugated to liposomes (5–15 μg/μmol of lipids) was evaluated with the Coomassie protein assay reagent (Pierce, Rockford, IL, USA). The specificity and affinity of immunoliposomes toward the antigen has been previously demonstrated [25].

2.7. In vitro binding and specificity of immunoliposomes

The binding and specificity of sterically stabilized liposomes and immunoliposomes were evaluated on freshly prepared C3H mouse spleen cells by flow cytometry assay. In brief, suspension of cells (10^6 cells/ml) was incubated with 1.5 μmol of DiI-labeled conventional liposomes, immunoliposomes, sterically stabilized liposomes or immunoliposomes. Samples were washed and resuspended in PBS. The specificity of liposomes for cells was determined by flow cytometry from the fluorescence associated to DiI (fluorochrome incorporated into the lipid membrane). Controls consisted of a commercial biotinylated Y-17 antibody (Cederlane Laboratories Ltd, Hornby, ON, Canada) and a isotype-matching irrelevant murine monoclonal antibody (OKT4) or Fab′ fragment coupled to conventional liposomes or sterically stabilized liposomes. No decrease in specificity of immunoliposomes (conventional or sterically stabilized) was observed for up to 3 weeks as revealed by flow cytometry analysis.

2.8. Tissue distribution

A single bolus injection of conventional liposomes, sterically stabilized liposomes, conventional immunoliposomes or sterically stabilized immunoliposomes (2 μmol lipids/500 μl) was administered subcutaneously in the upper back below the neck of female C3H mouse (18–20 g; Charles River Breeding Laboratories, St-Constant, QC, Canada). At specific time points, animals were killed and blood was collected and the plasma was separated by centrifugation (6000 × *g* for 10 min at 4°C). At the same time, selected tissues were collected, washed in PBS and weighed. Tissues and plasma were then treated with Beckman tissue solubilizer (Beckman Instruments Inc., Irvine, CA, USA) and decolored with H_2O_2. Lipid levels in all samples were monitored by scintillation countings. Six animals per group were used for each time point.

2.9. Liposome fluorescent labeling

Liposomal lipids (2.5 mg/ml) were incubated with 10 μg/ml of DiI under darkness for 1 h at 60°C with agitation. Unbound DiI was removed by centrifugation (300×*g* for 15 min at 4°C) of 1 ml of the liposomal preparation through a 10 ml coarse Sephadex G-50 column.

2.10. Tissue preparation for optical microscopy

A single bolus injection of sterically stabilized liposomes and sterically stabilized immunoliposomes (2 μmol lipid/500 μl) was administered subcutaneously in the upper back below the neck of female C3H mice. At specific time intervals post-injection, animals were killed and tissues (spleen, brachial and cervical lymph nodes) were removed. Tissues were washed in PBS, embedded in OCT Tissues Tek (Bayer, Pointe-Claire, QC, Canada), frozen in liquid nitrogen and stored at −20°C. Tissue sections (10 μm thick) were cut using a Jung Frigocut 2800E (Leica Canada Inc., St-Laurent, QC, Canada) and deposited on slides pretreated with 2% aminoalkysilane. Coated slides were immediately observed with a Nikon Microflex HFX-DX fluorescence microscope. DiI fluorescence was observed with a rhodamine optic excitation filter (ex: 510–560 nm, dichroic mirror: DM 580 and barrier filter: BA 590). A minimum of three tissues from different animals was used for each time point.

2.11. Statistical analysis

All statistical analyses were performed using a computer package (Statview+SE Software, Abacus Concepts, Berkeley, CA, USA). The significance between groups was statistically evaluated using a one-way analysis of variance (ANOVA) test, followed by *t*-test with Fisher's corrections.

3. Results

3.1. In vitro binding of sterically stabilized immunoliposomes

Fig. 2 shows the levels of binding of sterically sta-

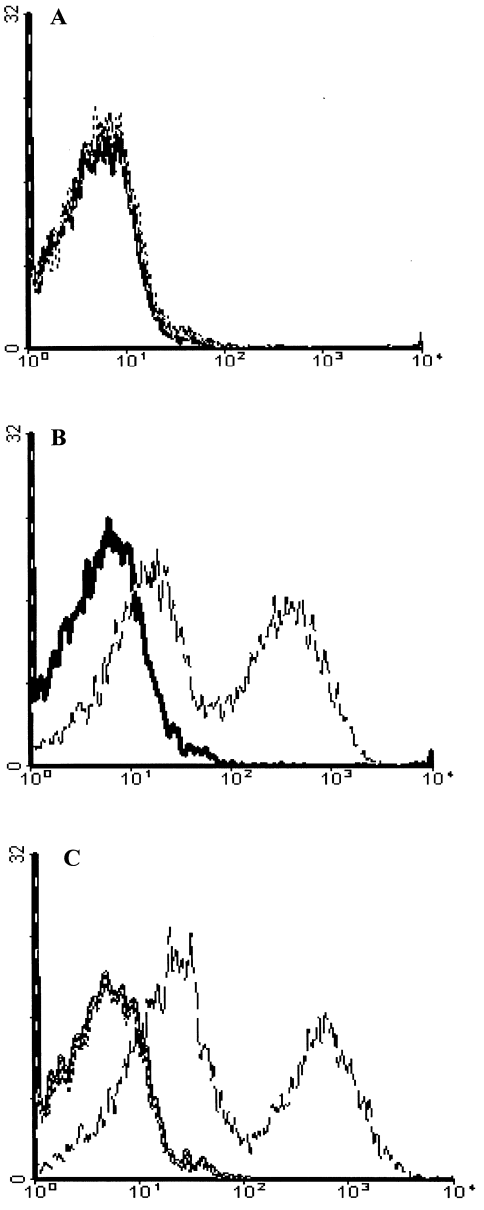

Fig. 2. Flow cytometry scans of mouse spleen cells incubated with (A) DiI-labeled sterically stabilized liposomes, (B) DiI-labeled sterically stabilized liposomes bearing anti-HLA-DR Fab′ fragments (IgG_{2b}, clone Y-17) and (C) commercial biotinylated Y-17 antibody revealed with R-phycoerythrin conjugated streptavidin. Cells alone (panel A, solid line); DiI-labeled sterically stabilized liposomes bearing isotype-matching irrelevant Fab′ antibody fragment (panel B, solid line); irrelevant biotinylated isotype-matching antibody (panel C, solid line); and streptavidin R-phycoerythrin incubated with cells (panel C, dotted line) were used as controls.

Table 1
Area under the curve of sterically stabilized and conventional liposomes in different tissues after the administration of a single subcutaneous dose (2 μmol) in C3H mice[a]

Tissues	Sterically stabilized liposomes	Conventional liposomes	Ratio sterically stabilized/ conventional liposomes
Cervical lymph nodes	922.6	810.5	1.1
Brachial lymph nodes	1172.7	823.2	1.4
Mesenteric lymph nodes	5.7	3.6	1.6
Inguinal lymph nodes	14.0	9.9	1.4
Popliteal lymph nodes	18.0	9.3	1.9
Liver	43.2	26.8	1.6
Spleen	38.3	20.5	1.9
Plasma	1.7	1.7	1.0

[a]Values, expressed in μmol lipids/g tissue (or ml of plasma)/h, were calculated from the mean values of the tissue distribution profile using the trapezoidal rule.

bilized liposomes and immunoliposomes on C3H mice cell spleen by flow cytometry. DiI-labeled liposomes were used to determine the specificity of immunoliposomes since we could not use a conjugated goat anti-mouse IgG against mouse spleen cells as non-specific binding would have occurred. As expected, results clearly demonstrated that sterically stabilized anti-HLA-DR immunoliposomes were specific to cells expressing the HLA-DR determinant of MHC-II being similar to that observed with a commercial anti-HLA-DR antibody control (33.5% positive cells, mean 41.0 and 31.8% positive cells, mean 57.0, respectively). Sterically stabilized liposomes did not bind to mouse cells as well as sterically stabilized liposomes bearing an irrelevant non-specific isotype-matching Fab′ antibody fragment.

3.2. Tissue distribution

In order to evaluate if the presence of PEG molecules on the surface of liposomes can prolong their circulation time and increase their rate of lymphatic uptake, we have first evaluated the tissue distribution of sterically stabilized and conventional liposomes following a single subcutaneous injection in the upper back of C3H mice. Table 1 shows the area under the curve of the concentration of sterically stabilized and conventional liposomes in different tissues after a period up to 240 h post-injection (time points 24, 48, 120 and 240 h). Results show that the accumulation of both liposome formulations was greater in lymph nodes near the injection site (e.g. cervical and brachial lymph nodes) when compared

Table 2
Area under the curve of sterically stabilized and conventional immunoliposomes in different tissues after the administration of a single subcutaneous dose (2 μmol) in C3H mice[a]

Tissues	Sterically stabilized immunoliposomes	Conventional immunoliposomes	Ratio sterically stabilized immuno/ conventional immuno
Cervical lymph nodes	1514.7	616.1	2.5
Brachial lymph nodes	1693.7	874.7	1.9
Mesenteric lymph nodes	16.0	5.5	2.9
Inguinal lymph nodes	34.8	15.8	2.2
Popliteal lymph nodes	70.8	26.3	2.7
Liver	61.5	25.5	2.4
Spleen	57.4	12.6	4.6
Plasma	3.6	1.7	2.2

[a]Values, expressed in μmol lipids/g tissue (or ml of plasma)/h, were calculated from the mean values of the tissue distribution profile using the trapezoidal rule.

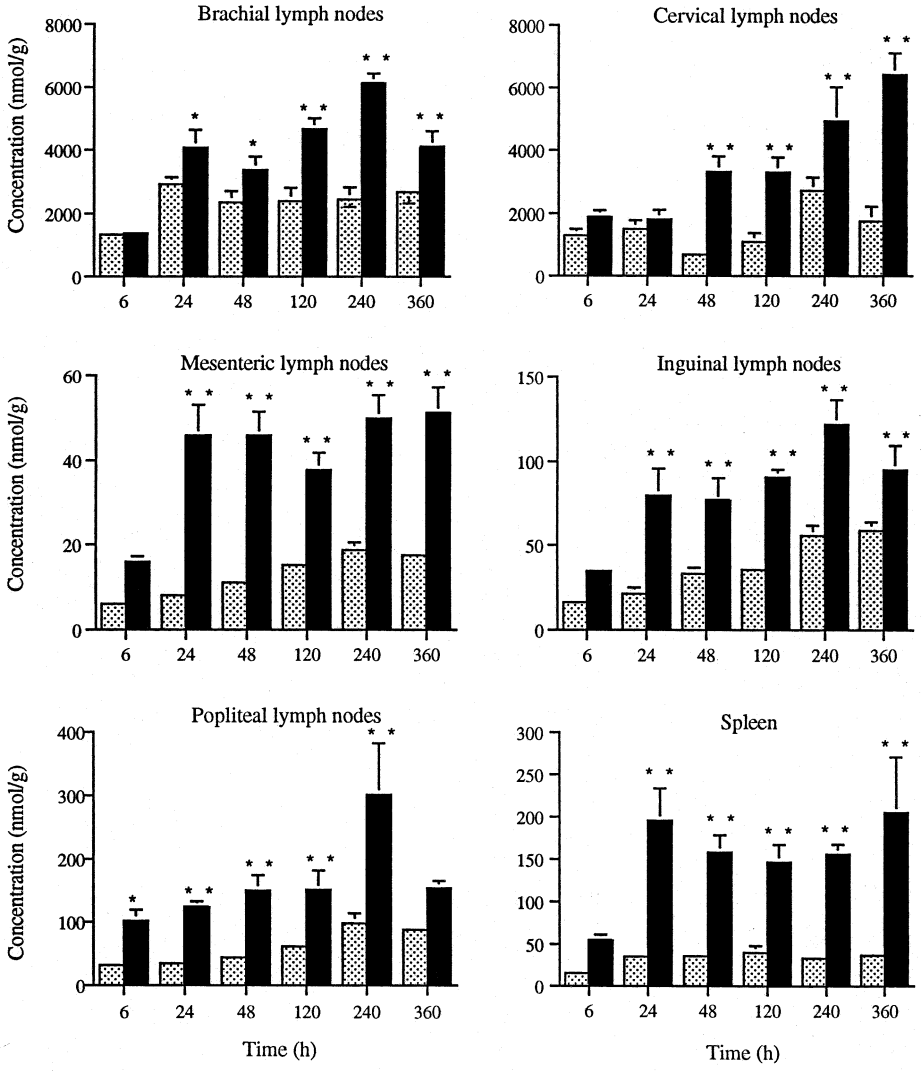

Fig. 3. Tissue distribution of conventional immunoliposomes (dotted bar) and sterically stabilized immunoliposomes (solid bar) in brachial, cervical, mesenteric, inguinal and popliteal lymph nodes, and spleen following a single subcutaneous injection to mice. Values represent the mean (±S.E.M.) obtained for six animals per group per time point. *, and **, significantly different ($P<0.05$) and ($P<0.01$), respectively when compared to conventional immunoliposomes.

to other nodes or tissues. Moreover, sterically stabilized liposomes accumulated more efficiently than conventional liposomes in all lymph nodes as well as in liver and spleen. Although very low, the accumulation of sterically stabilized liposomes in plasma was similar to that of conventional liposomes. It can be speculated that the enhanced accumulation of sterically stabilized liposomes in these tissues may be due to their reduced accumulation at the injection site and their prolonged circulation time in the lymphatic system which increase their uptake by lymphoid tissues.

We have next determined if the presence of anti-HLA-DR Fab′ fragments at the end termini of PEG-coated liposomes could also improve their tissue accumulation compared to PEG-free immunoliposomes. Once again, results show that the concentration of both immunoliposomal formulations was higher in brachial and cervical lymph nodes than in other tissues suggesting that liposomes administered subcutaneously accumulated preferentially in the regional lymph nodes (Fig. 3). In addition, sterically stabilized immunoliposomes targeted more efficiently all tissues, with a peak of accumulation at 240 h in

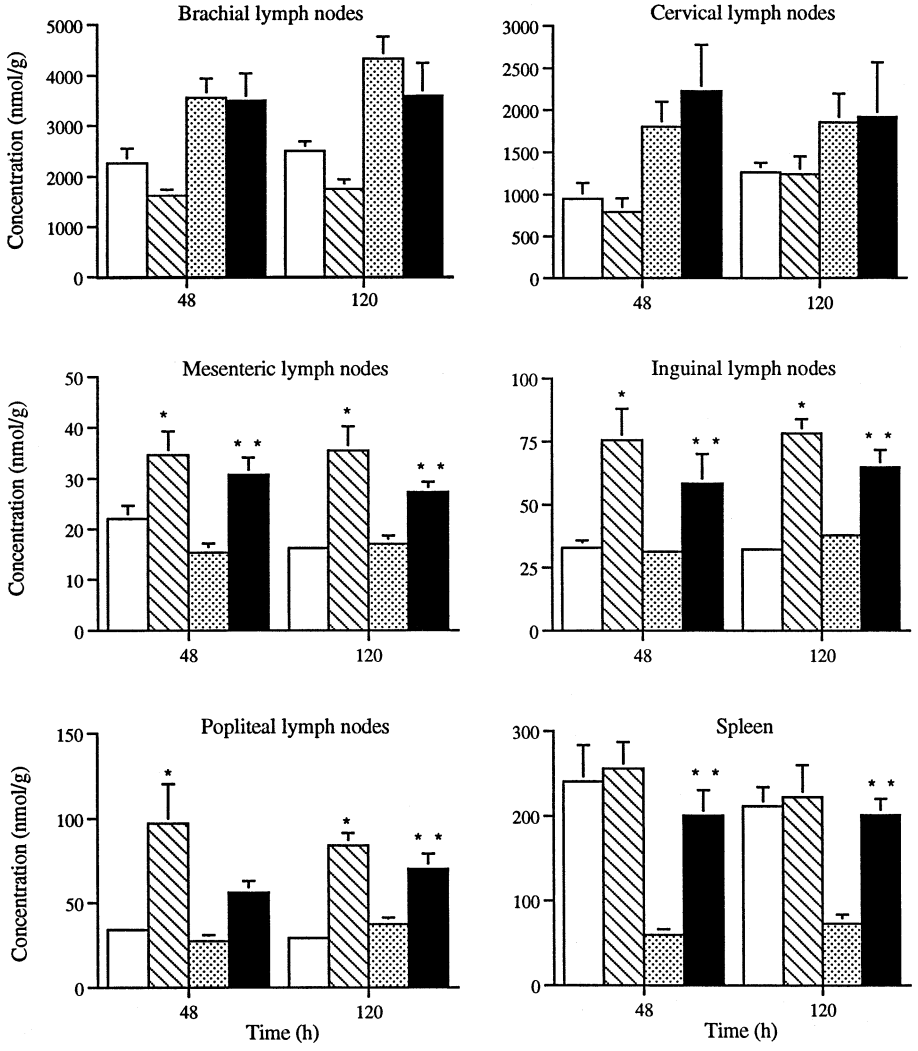

Fig. 4. Tissue distribution of conventional (empty bar), sterically stabilized (lined bar), conventional immunoliposomes (dotted bar) and sterically stabilized immunoliposomes (solid bar) in brachial, cervical, mesenteric, inguinal and popliteal lymph nodes, and spleen following a single subcutaneous injection to mice. Values represent the mean (±S.E.M.) obtained for six animals per group per time point. *, Significantly different ($P < 0.05$) when compared to conventional liposomes and **, significantly different ($P < 0.05$) when compared to conventional immunoliposomes.

brachial, inguinal and popliteal lymph nodes. The concentration of sterically stabilized immunoliposomes in mesenteric lymph nodes reached a plateau at 24 h post-injection and the tissue distribution profile was very similar to that observed in the spleen. There were no significant differences in the accumulation of sterically stabilized and conventional immunoliposomes at 6 h post-injection in lymph nodes and spleen, except for the popliteal lymph nodes, which are the farther lymph nodes from the injection site. The radioactive tracer in liposomes remained stable for the time period of the experiment (data not shown). Table 2 shows the area under the curve of sterically stabilized and conventional immunoliposomes in different tissues. Results clearly demonstrated that sterically stabilized immunoliposomes accumulated much better than conventional immunoliposomes in all tissues indicating that the presence of PEG has an important effect on the uptake of immunoliposomes by the lymphatic system.

In another set of experiments, the concentration of the four types of liposomes was determined at 48 and 120 h after their subcutaneous administration to C3H mice. Fig. 4 shows that the presence of PEG

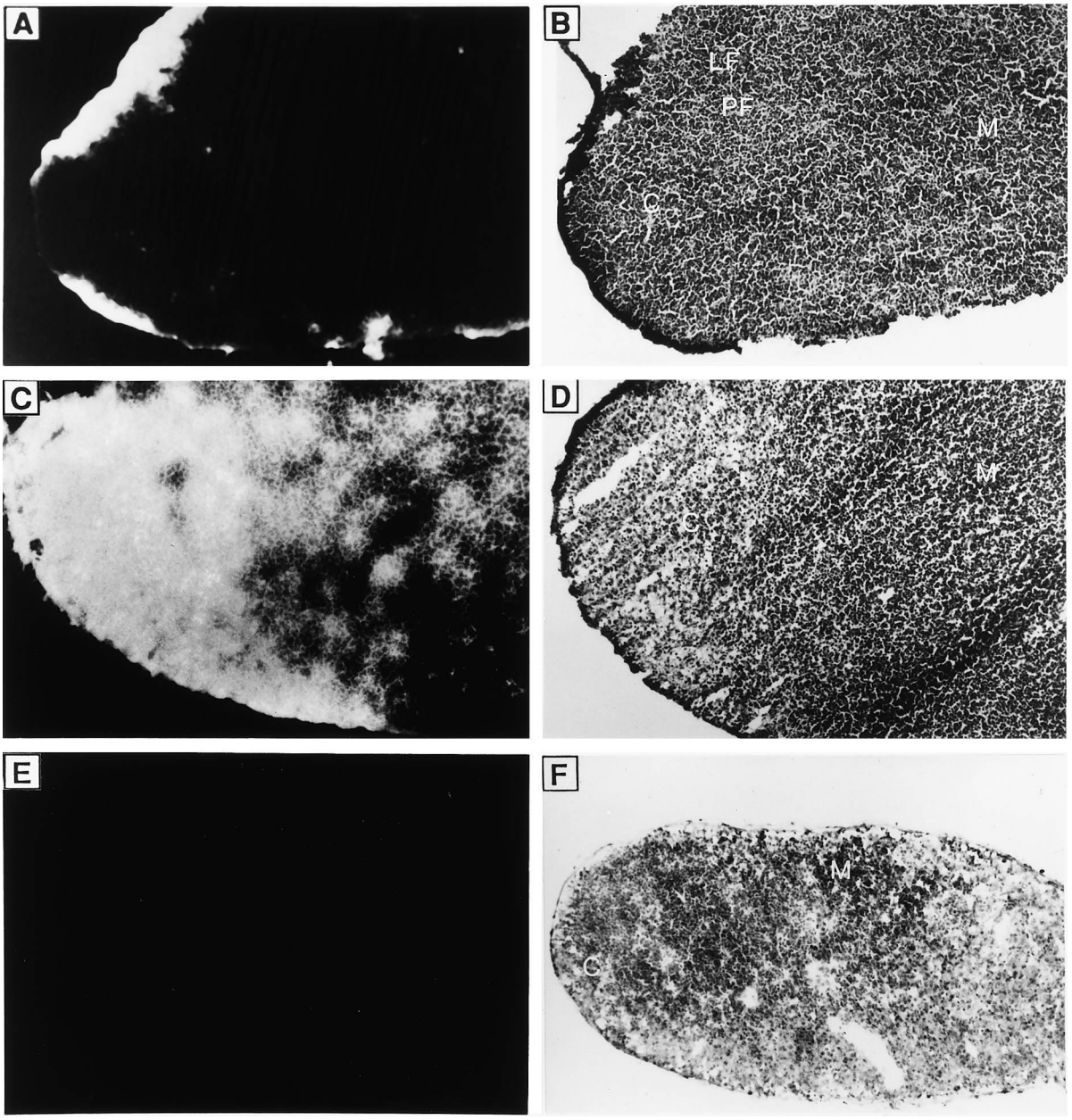

Fig. 5. Fluorescent micrographs of brachial lymph nodes of C3H mouse at 120 h following the administration of a single subcutaneous dose of DiI sterically stabilized liposomes (panel A), sterically stabilized immunoliposomes (panel C) and sterically stabilized liposomes bearing non-specific isotype-matching Fab′ fragments (panel E) to mice. Panels B, D and F represent the corresponding hematoxylin eosin coloration of tissues. Figure shows the cortex area (C), the parafollicular area (PF), the medulla (M) and the lymphoid follicle (LF). Magnification: 250×.

on the surface of liposomes or immunoliposomes had no significant effect on their accumulation in the regional lymph nodes (brachial and cervical) but significantly increased their uptake in other lymph nodes compared to conventional liposomes or immunoliposomes. On the other hand, there was no significant difference in the accumulation of sterically stabilized liposomes in the spleen when compared to conventional liposomes at these two time points. In contrast, the concentration of sterically stabilized immunoliposomes in this tissue was much higher when compared to conventional immunoliposomes.

In addition, the presence of anti-HLA-DR Fab′ fragments on both conventional and sterically stabilized liposomes greatly improved their accumulation in regional lymph nodes when compared to non-targeted liposomes. The presence of an irrelevant isotype-matching Fab′ fragment on sterically stabilized liposomes had no effect on the tissue distribution profile being similar to that of sterically stabilized liposomes (data not shown).

3.3. Tissue localization

Since sterically stabilized liposomes and sterically stabilized immunoliposomes represent the best formulation to target lymphoid tissues, we have next evaluated if the presence of anti-HLA-DR Fab′ fragments at the end termini of PEG chains affects the tissue localization of liposomes. Fig. 5 compared the localization of fluorescent sterically stabilized liposomes and sterically stabilized immunoliposomes in brachial lymph nodes at 120 h after their subcutaneous administration in mice. Results show that the localization of sterically stabilized immunoliposomes is very different from that of sterically stabilized liposomes. Sterically stabilized liposomes are mainly localized in the subcapsular area, probably in the afferent lymphatic vessel and around the afferent area. In contrast, sterically stabilized anti-HLA-DR immunoliposomes mostly accumulated in the cortex in which follicles (B-cells and FDCs) are located and in parafollicular areas in which T-cells, interdigitating dendritic cells and other accessory cells are abundant. No significant fluorescent signal was observed in the subcapsular area of the lymph nodes when non-specific isotype-matching Fab′ fragments were coupled to sterically stabilized liposomes.

Fig. 6 compared the localization of sterically stabilized liposomes and sterically stabilized immunoliposomes in the spleen at 48 h after their subcutaneous administration in mice. Results show that the accumulation of sterically stabilized immunoliposomes in this tissue is more important than that of sterically stabilized liposomes and that their localization is different. However, it should be noted that tissue localization studies using the fluorescent marker are qualitative and could not be used to evaluate the concentration of liposomes in these tissues. In the case of sterically stabilized liposomes, a weak fluorescence signal was observed in the marginal zone of the white pulp. In contrast, sterically stabilized immunoliposomes were largely concentrated in the lymphoid nodules of the white pulp and little in the marginal zone. The administration of free DiI did not result in any fluorescent signal in all studied tissues. Furthermore, the injection of DiI-labeled liposomes showed a disappearance of the fluorescent signal as a function of time indicating that the probe is eliminated with time. When non-specific immunoliposomes were injected to mice, a weak fluorescence signal was observed in the same area than that observed for sterically stabilized liposomes suggesting that the presence of irrelevant antibody had no effect on the tissue localization of liposomes.

4. Discussion

The introduction of triple drug therapy led to a lot of hope for the treatment of HIV-1 infection [26–29]. However, although this therapy can reduce the plasma viral load to undetectable levels, viral replication still occurs in the lymph nodes or other sanctuary sites of HIV-1 and this low viral level can lead to resistant mutations [10,11,26,29,30]. It was shown that initiation of HAART in infected individuals, as early as 10 days after the onset of symptoms of primary HIV-1 infection, did not prevent generation of latently infected resting CD4+ T-cells carrying integrated HIV-1 DNA despite the successful control of plasma viremia [31]. Since new anti-HIV-1 regimens do not eliminate the virus in the organism, a lifelong treatment is required. Problems associated with adherence to HIV-1 treatment and to side effects of triple combination therapy shed the light on the need of developing new efficient strategies [15,27].

One major advantage of liposomes as drug carriers is that their tissue distribution can be modulated through variations of their lipid compositions. It is thus possible to adapt the physicochemical properties of liposomal formulations according to the desired therapeutic objective. In the present study, the ability of different types of liposomes to target HLA-DR expressing cells and increase their accumulation in secondary lymphoid organs has been evaluated. The choice of the lipid compositions used in conventional liposomes and immunoliposomes was based

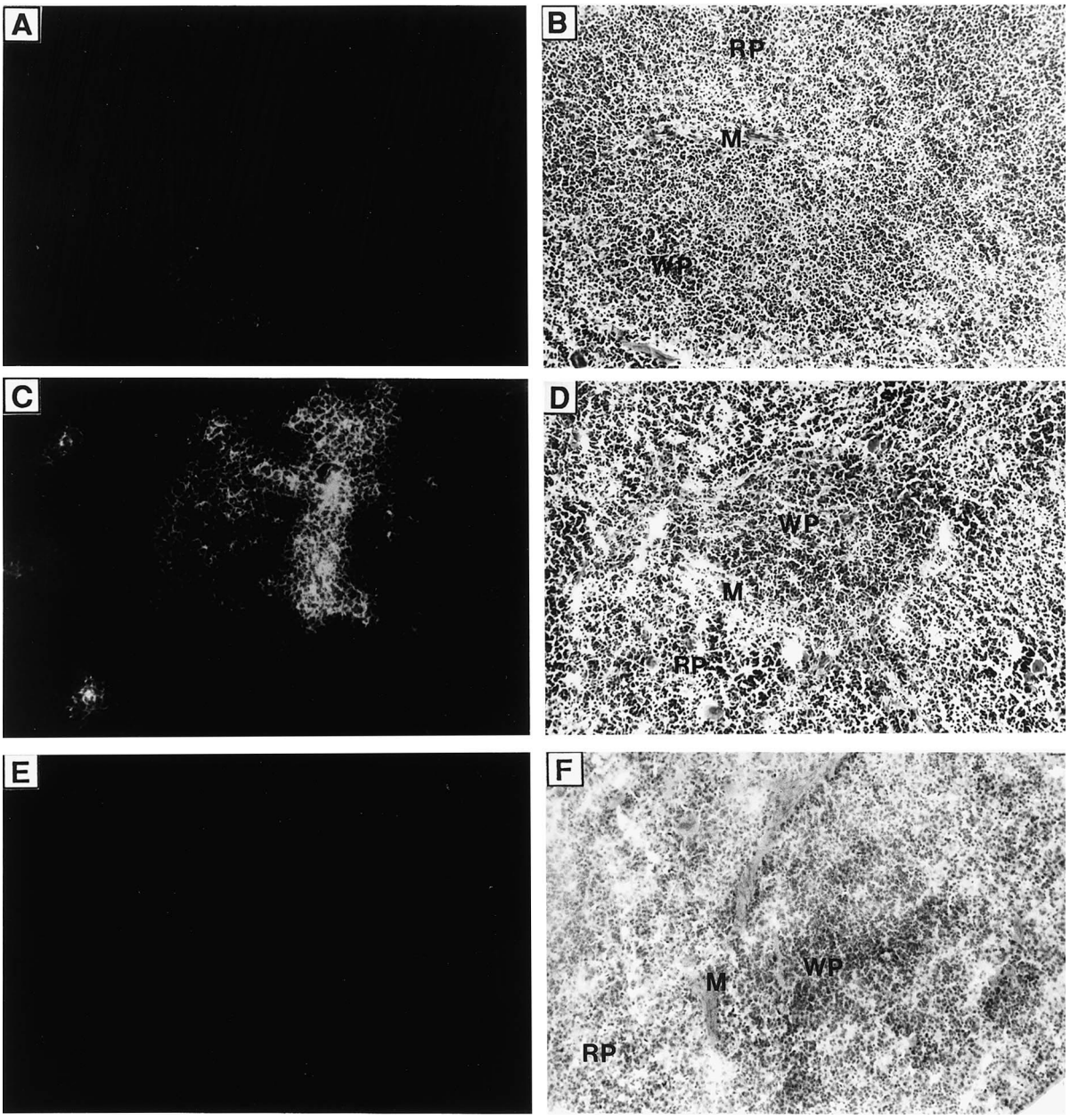

Fig. 6. Fluorescent micrographs of spleen of C3H mouse at 48 h after the administration of a single subcutaneous dose of DiI sterically stabilized liposomes (panel A), sterically stabilized immunoliposomes (panel C) and sterically stabilized liposomes bearing non-specific isotype-matching Fab′ fragments (panel E) to mice. Panels B, D and F represent the corresponding hematoxylin eosin coloration of tissues. Figure shows the red pulp (RP) and the marginal zone (M) surrounding lymphoid nodule of the white pulp (WP). Magnification: 250×.

on our previous studies showing that these formulations allowed efficient targeting of lymphoid tissues [25,32]. The use of 6% PEG in sterically stabilized liposomes was selected as it was demonstrated that a 5–10 mol% concentration of PEG results in a maximum circulation time [33]. Moreover, we have used DSPE rather than DPPE because it was shown that proteins linked to DSPE anchors leave the membrane slower than DPPE [34]. In the case of sterically stabilized immunoliposomes, a proper control would have been to use liposomes containing 3.5% DSPE-PEG and 2.5% DSPE-PEG-MAL. However, studies

performed in our laboratory showed that such formulation led to unfavorable coupling reaction, most likely because of the repulsive effect of PEG chains for proteins.

Our results demonstrated that the subcutaneous injection of sterically stabilized liposomes bearing anti-HLA-DR Fab′ fragments represents an attractive approach to specifically target the lymphoid tissues. The subcutaneous route of injection has been selected since it constitutes the most appropriate route for targeting lymph nodes [18,21,35]. In addition, it represents a simple route for patient self-administration and it might serve as a depot for the sustained release of drugs in vivo, reducing most likely the frequency of liposomal drug administration. However, mechanisms upon which liposomes reach lymph nodes are not well understood. It has been postulated that the sequestration of liposomes by these tissues could occur, either via a simple mechanical filtration or by phagocytosis by cells of the reticuloendothelial system [35]. In the case of mechanical filtration, it is important to use liposomes with a diameter less than 120 nm as it was shown that the fenestration by which filtration occurs is via pores through such a diameter [21].

The effect of PEG molecules on the surface of liposomes has been extensively studied after intravenous injection. It was shown that PEG prolonged the circulation time of liposomes, reduced their uptake by mononuclear phagocyte system and increased their ability to cross in vivo biological barriers [36]. However, the effect of steric stabilization of liposomes following subcutaneous administration is not well understood. It has been postulated that the presence of PEG could reduce the accumulation of liposomes at the site of injection and therefore increase their ability to move through the lymph [21,22]. Our results have shown that the presence of PEG did not substantially increase the accumulation of liposomes in the regional lymph nodes as previously observed [37]. However, it significantly increased the accumulation of liposomes in lymph nodes far from the injection site, suggesting that the presence of PEG may reduce phagocytosis by cells of MPS increasing their efficacy to target other lymph nodes among the lymphatic vessels. As expected, the incorporation of PEG did not reduce the accumulation of liposomes in the spleen and liver.

Many coupling procedures of antibodies to sterically stabilized liposomes have been proposed in the literature [22,38–41]. In this study, anti-HLA-DR Fab′ fragments were covalently attached to DSPE-PEG-MPB via the interaction of sulfhydryl (Fab′) and maleimide (MPB) groups. Thioether linkage is known to be one of the most useful and efficient reactions in bioconjugate chemistry. Moreover, we have used PEG 2000 because it allows efficient tissue targeting and high circulation half-lives [22]. Higher coupling efficiencies of Fab′ fragments to liposomes have been reported when Fab′ was attached at the terminal end of PEG chains [41,42]. In our experiments, the Fab′–liposomes coupling efficiency was in the range of 15–20% for both conventional and sterically stabilized liposomes, even if the concentration of MPB in sterically stabilized liposomes was higher than that of conventional liposomes (6% and 2.5%, respectively). These results suggest that the amount of MPB does not influence the coupling efficiency of Fab′ to the phospholipid vesicles. On the other hand, our results along with those of others [43] showed that the presence of free MPB on the surface of conventional or sterically stabilized liposomes does not influence attachment to cells in vitro or their tissue distribution.

Our data showed that anti-HLA-DR immunoliposomes accumulated better in brachial and cervical lymph nodes than that of conventional liposomes. However, the presence of anti-HLA-DR Fab′ fragments did not significantly enhance the liposomal accumulation in other lymph nodes. Although the coupling of Fab′ fragments rather than complete IgG is known to eliminate a range of Fc-mediated biological activities, like complement activation via the classical pathway and humoral responses of clearance via antibody–Fc receptor interactions, it is possible that conventional immunoliposomes are still rapidly taken up by cells of MPS present in lymph, impairing their possibility to target other lymphoid tissues far from the injection site. However, our results suggest that the attachment of Fab′ fragments at the end termini of PEG reduces the accumulation of liposomes at the injection site, diminishes their rapid uptake by cells of MPS and prolongs their circulation time improving their overall lymphatic uptake when compared to PEG-free immunoliposomes. This hypothesis is supported by

the fact that a higher concentration of sterically stabilized immunoliposomes was observed in all lymph nodes studied, especially at late time points, in lymph nodes far from the injection site and in plasma. The higher concentration of sterically stabilized immunoliposomes in plasma when compared to conventional immunoliposomes could be attributed to the presence of PEG which reduces the binding of plasma proteins prolonging thereby their circulation time.

Fluorescence microscopy studies demonstrated that the localization of sterically stabilized anti-HLA-DR immunoliposomes in lymphoid tissues was completely different than that of sterically stabilized liposomes. In fact, sterically stabilized liposomes accumulated only in macrophage-rich areas such as the subcapsular region of lymph nodes and in the red pulp and marginal zone of the spleen. Oussoren et al. have observed the presence of colloidal gold particles in macrophages of regional lymph nodes following the subcutaneous administration of PEG-gold liposomes to mice [19]. It was hypothesized that because of the slow progression of liposomes through the lymph nodes, enough time was available for efficient interactions of the PEG liposomal surface with phagocytic cell membranes. In contrast, sterically stabilized immunoliposomes highly accumulated in the deep cortex of lymph nodes which contains aggregates of B-cells and FDCs constituting the follicles. In HIV-1 infection, FDCs, which reside in germinal centers, display HIV-1 particles on their surface to selectively activate B-cells. CD4+ T-cells from the paracortical area migrate to the germinal centers in response to B-cell activation to be eventually infected. Our results showed that sterically stabilized immunoliposomes highly accumulate in these regions and could represent a convenient strategy to deliver drugs in this area of the nodes inhibiting more efficiently HIV-1 replication. Moreover, the injection of sterically stabilized immunoliposomes resulted in an intense fluorescence signal in the white pulp of the spleen, suggesting a selective targeting of B-cells in the follicles and of macrophages present in the marginal zone surrounding the lymphoid follicle.

The use of sterically stabilized immunoliposomes could represent a convenient strategy to concentrate anti-HIV-1 drugs at sites of infection, more particularly within lymphoid organs. Such targeted delivery system should reduce the dissemination of HIV-1 and preserve the FDC microenvironment that will likely protect the infected host from developing the characteristic immunodeficient state. The improved pharmacology of liposomal drugs could reduce the dose and frequency of administration of antivirals used in conventional therapy, facilitating drug compliance and limiting the occurrence of drug resistance. Given that HIV-1 particles acquire high amount of host-derived HLA-DR proteins, the encapsulation of destabilizing agents into sterically stabilized anti-HLA-DR immunoliposomes would be an interesting approach to target free viral particles trapped on FDC. The use of immunoliposomal drugs in HIV-1 therapy could hopefully offer new alternatives for the treatment of this retroviral infection.

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